Kee-Yoeup Paek
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Production of Biomass and Bioactive Compounds Using Bioreactor Technology

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Preface

Plants are the major source of secondary metabolites which are used as pharmaceuticals, flavours, fragrances, colouring agents, food additives and agrochemicals. In recent decades, plant cell, tissue and organ cultures have emerged as an alternative over whole plant cultivation for the production of valuable secondary metabolites. Cells, adventitious roots, hairy roots, shoots and embryos have been successfully cultured *in vitro* for the large scale production of secondary metabolites. Strain improvement, selection of high-producing cell lines, optimization of medium and culture environment have led to the enhanced production of bioactive and value added products. In recent years, a couple of bioreactor configurations have been developed and successfully adopted for the *in vitro* cultivation of plant cells and organs. Bioreactors such as mechanically agitated, airlift and photo-bioreactors have been designed and used for large scale cultivation of algal, higher fungal and plant cells. Bioprocess engineering parameters such as mixing, oxygen supply and shear stress have been investigated towards successful commercial scale cultivation. Various bioprocess operation modes including batch, fed-batch, two-stage cultivation, and bioseparation of intracellular metabolites have been suggested for enhanced and sustainable recovery of secondary metabolite products. More recently, overexpression of regulatory genes in up-regulating a series of enzyme activities in the metabolic pathways is also being achieved through genetic and metabolic engineering approaches.

This book provides recent progresses and limitations of production of biomass and bioactive compounds using bioreactor technology as mentioned above. It contains six parts: Part I describes bioreactor designing advantages and limitations of bioreactor cultures; Part II deals with the production of biomass and bioactive compounds from cell suspension cultures; Part III contains chapters on production of secondary metabolites from suspension cultures of plant organs – shoots, adventitious roots and embryos; Part IV deals with the strategies for enhanced production of secondary products, large-scale cultures and metabolic engineering of selected metabolites; Part V contains bio-safety assessments of plant cell and organ culture products; and the final Part VI contains physiological disorders in plants cultured in bioreactors.

We thank all the authors and reviewers who have contributed to this project. Our special thanks to Sophie Lim, Associate Editor, and Peter Butler, Editorial Director, for their helpful editorial work. We also appreciate the staff of Springer Publishers for giving us the opportunity to present this book to our readers around the world.

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Part I
Bioreactor Design, Advantages and Limitations of Bioreactor Cultures

Chapter 1
Design of Bioreactors for Plant Cell and Organ Cultures

Milen I. Georgiev

Abstract Demands for sustainable supply of plant biomass and/or value added-molecules (incl. native and heterologous therapeutic proteins, specialty proteins and industrial enzymes) have been the driving efforts to develop alternative ways for their bioproduction. Plant cell and organ cultures have been demonstrated an efficient, cost effective and eco-friendly alternative to classical technologies (i.e. by harvest from wild) and chemical (semi)synthesis. The progress has resulted in development of several commercial processes for large-scale production of plant biomass and high value molecules, besides numerous proof-of-concept studies at laboratory- and pilot-scale. This chapter summarizes the bioreactor configurations for plant cell and organ cultures, and attempts to outline the immense potential of plant *in vitro* culture-based bioprocesses for sustainable supply of biomass and value-added molecules for various purposes along with the major challenges that remain.

Keywords Bioreactors • Cell cultures • Organ cultures • Mechanically driven systems • Pneumatically driven systems

1.1 Introduction: From Simple Carboy Systems to Large-Scale Bioreactors

Per definition bioreactor is any device or vessel that is used to carry out one or more biochemical reactions in order to convert any starting material (*inter alia* substrate) into product(s) [1]. Bioreactor cultivation and subsequent up-scaling represent the final steps in the development of bio-based processes. In general, the basic function of a bioreactor is to provide optimal conditions for effective cell growth and metabolism

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by strict regulation of various environmental (chemical and physical) key factors [2, 3]. Though it is difficult to dedicate the very first attempt to cultivate plant cells in bioreactors, the pioneering work of Routien and Nickell [4] is a benchmark. In 1956, authors were granted the first patent for the cultivation of plant cells *in vitro* in simple 20-L carboy systems. Soon after that, the National Aeronautics and Space Administration (NASA) started a research program on plant cell culture for regenerative life support systems. Plants and the relevant *in vitro* cultures were grown under various conditions of microgravity (space shuttles, parabolic flights, biosatellites, the orbital stations Salyut and Mir) along with ground studies using rotating clinostat vessels (reviewed in Sajc et al. [5]). In the 1970s, further attempts to develop bioreactor configurations suitable for plant cells resulted in the development of a conical glass V shaped reactor (as called by the authors V-shape fermenter) for plant cell suspension cultures. This V shaped reactor has proved useful for both biomass and metabolite production [6]. Later, the concept of high shear sensitivity of plant cells was developed and only air-lift reactors were considered suitable [7, 8]. For instance, Kurz and Constabel [9] wrote "The most suitable reactor developed so far is the airlift reactor.... However this design is only applicable to cultures with a cell dry weight lower than 20 g L^{-1}". Accordingly, *Nicotiana tabacum* cell suspension culture was up-scaled in 360-L and 1,500-L bubble aeration-type bioreactors [10]. However, several industrial-scale processes developed in early 1980s utilizing stirred-tank reactors (STRs) subsequently challenged these perceptions [2, 8]. Nowadays, ca. 60 years after first dedicated attempts to grow plant cells in bioreactors has become nearly impossible to select the "best" bioreactor configuration for different plant *in vitro* cultivations.

The selection and design of each bioreactor configuration and operational mode are unique, which however, underlying some basic principles, as low stress environment, adequate mixing, and oxygen and heat transfer [2]. In most cases, bioreactors available for microbial fermentation can be implemented for hosting plant cells with some slight modifications. In general, reactor design should ensure that nutrients are effectively provided to the cells. Cell growth and product formation kinetics should be assessed (by respective sampling) so that, the optimal environmental conditions can be defined and thus the most suitable operational mode to be determined. Transport phenomena, including mixing, shear forces, and oxygen transfer, should be continuously followed during the cultivation process in order to define the criteria for bioreactor design and up-scaling. Operating parameters, such as dissolved oxygen concentration (DO_2) and substrate concentration(s), temperature of cultivation, pH and agitation speed, among others, should be easy to monitor and set-up. In addition, the bioreactor configuration should be as simple and inexpensive as possible and it should be easily operated while ensuring long-term sterility [2].

1.2 High-Value Molecules Produced by Plants and Relevant *In Vitro* Culture

The vast chemical diversity of the plants has been exploited since time immemorial by humans to diminish and prevent pain, to produce pleasure, for use in religious ceremony and to cure various human disorders. The chemical entities responsible

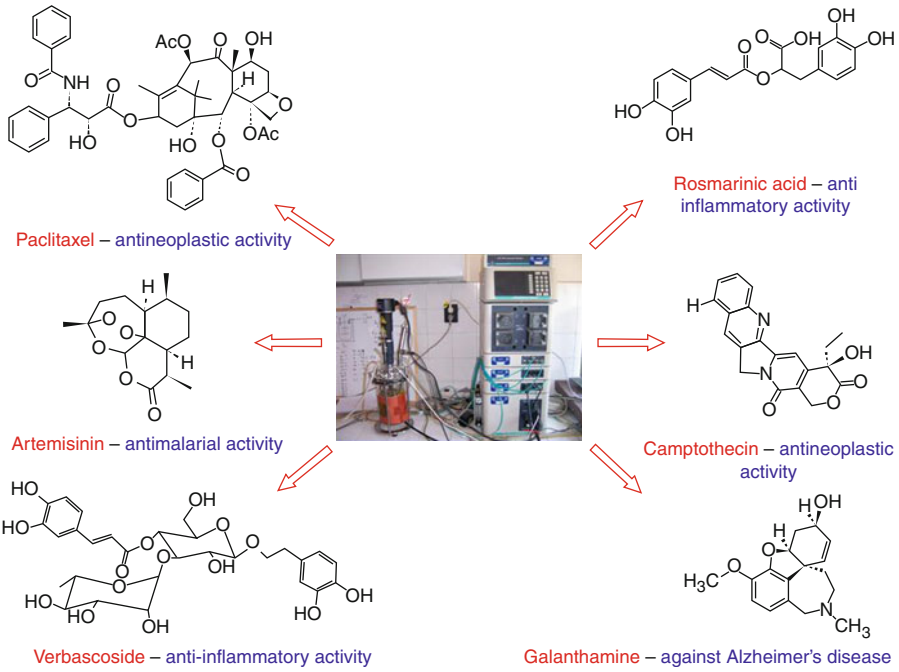


Fig. 1.1 Selected examples of high-value molecules produced by plant cell/organ culture, grown in bioreactors

for this biological activity are, in most cases, low-molecular weight compounds that are often accumulated at very low amounts in plants [11]. For instance, paclitaxel (Fig. 1.1) content in *Taxus* plants accounts on <0.02 % of the dry weight of the bark, where its levels are highest, therefore, commercial production of this complex diterpenoid by natural harvest is not economically feasible as *Taxus* plants grow very slow [12]. Recently, Wilson and Roberts [13] estimated that 340 tons of *Taxus* bark or 38,000 trees would be required to meet the 25 kg/year demand for the antineoplastic drug paclitaxel. In search of alternative solutions, two different routes for the total synthesis of paclitaxel were developed 20 years ago, however, the process involves 40+ reactions, utilizes harsh solvents, and has overall low product yields, which makes chemical synthesis of paclitaxel economically and environmentally unfavourable so far (reviewed in Wilson and Roberts [13]).

In addition, several plants accumulating metabolites of pharmaceutical interest are listed as endangered species (due to continuous overharvesting of natural populations), and therefore novel approaches have to be found in order to ensure the sustainable production of value-added molecules.

Today, over 25 % of modern medicines are derived either directly or indirectly from plants, especially in case of cancer therapy (60 %), infectious diseases (75 %), but also in metabolic syndrome and immuno-suppression therapy. Of course, these numbers also include microbial sources, but the significance of plants in the production of pharmaceuticals is undoubtful. To name a few, paclitaxel (Taxol®), galanthamine (Nivalin® and Reminyl®) and artemisinin (Fig. 1.1) are currently good

examples in this approach and amongst the blockbuster drugs worldwide [14]. The most important commercially relevant pharmaceuticals, derived from plants, are valued at over $ 25 billion per year in the USA alone [11]. In addition, World Health Organization (WHO) estimates, at least 80 % of the population in developing countries still relies exclusively on traditional medicine for their primary health care needs (Georgiev [14] and the literature cited therein). According to Food and Agriculture Organization of UN (FAO), world population is expected to grow by over a third, or 2.3 billion people, between 2009 and 2050 [15]. This purely means that in near future humans will face multiple challenges such as: (1) more food and fibre have to be produced to feed the growing population; (2) more feed stocks for a potentially huge bioenergy market and (3) more medicines (*inter alia* of natural origin) to cure human diseases.

Continuously increasing demands for plant biomass and therapeutic molecules, produced by ever greener processes, along with dramatic reduction in plant biodiversity, are the driving force to develop alternative ways to supply value-added molecules [8, 16]. Biotechnological production of secondary plant metabolites has been of interest for many decades. Nowadays, plant cell and organ cultures have become increasingly attractive and cost-effective alternatives to classical approaches (i.e. natural harvest and chemical synthesis) for the mass production of plant-derived metabolites ("green cell factories" concept), because of their several advantages. First, genetic modification in a contained system can readily be applied without the regulatory barriers associated with field grown crops. Second, a cell/organ culture system can be up-scaled in bioreactors with controllable production rates [16–18]. Furthermore, plant cell/organ culture is the only economically feasible way of producing some high-value molecules from rare and/or threatened plants. The progress in this field so far has resulted in the mass production of biomass and high-value molecules (*see below*) by different companies [2, 13, 19, 20].

1.3 Bioreactors for Dedifferentiated Plant Cell Culture

Stainless steel stirred tank reactors, bubble column reactors and air-lift reactors (Fig. 1.2a–c) directly derived from microbial bioprocesses are commonly used – with slight modifications – to grow plant cell suspension cultures up to 75 m^3 of culture volume [21]. Nowadays, stirred tank reactors are the most widely used reactor configurations for growing plant cells, because of their several advantages such as easy scale-up, good fluid mixing and oxygen transfer capacity, availability of numerous impellers types (reviewed in Georgiev et al. [2]) and compliance with current Good Manufacture Practices (cGMP) requirements [3]. Reasonably, most of the existing commercial processes with plant cell suspension cultures (discussed below) are based on STRs of m^3-scale. Towards minimization of production costs and validation efforts under cGMP regulations, several single-use bioreactor configurations for hosting plant cell culture have been developed recently, e.g. the wave-mixed reactors (Fig. 1.2d), slug bubble bioreactor (Fig. 1.2e), wave and

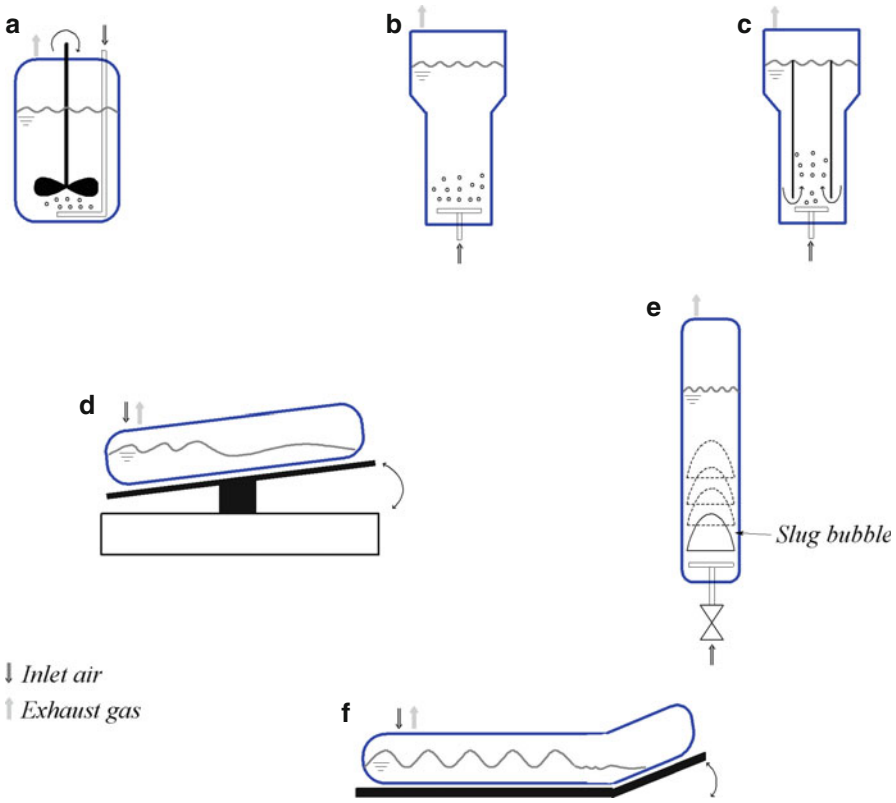


Fig. 1.2 Bioreactor configurations for plant cell culture. (**a**) Stirred tank reactor, (**b**) Bubble column reactor, (**c**) Air-lift reactor, (**d**) Wave bioreactor, (**e**) Slug bubble reactor, (**f**) Wave and undertow (Georgiev et al. [8] and the literature cited therein)

undertow bioreactor (Fig. 1.2f), CELL-Tainer, AppliFlex, Saltus Virbormix reactor, and OrbShake reactor [2, 8], among others. Single-use bioreactors are comprised of cultivation containers/bags, made of US Food and Drug Administration-approved plastics [21, 22]. Single-use bioreactors become increasingly accepted for biotechnological processes at small and medium size scale during the past decade. Numerous studies [23–25] have clearly shown their advantages, such as reduced contamination and cross-contamination rates, easy compliance with cGMP regulations, savings in time and costs, and reduced waste and thus clear environmental impact. Thus, single-use bioreactors have a wide range of applications for the production of therapeutic proteins [20, 26] and in the production of plant biomass and secondary metabolites for cosmetics purposes [2].

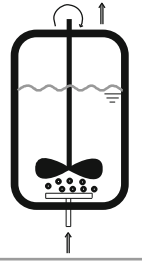
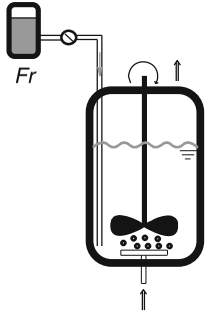
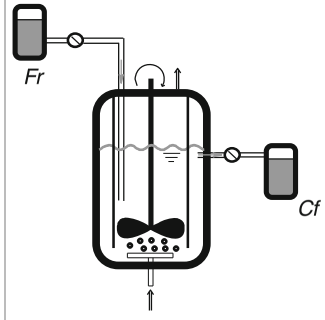
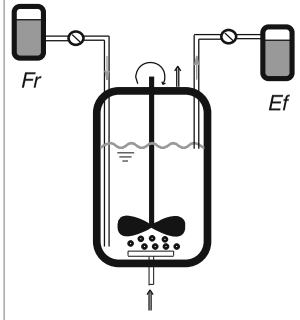
An effective bioreactor operational mode should provide adequate volumetric yield and overall high system productivity, which means more product(s) are formed per unit time per liter of bioreactor volume [2]. A major drawback of batch processes is that significant amount of time is taken up by the system and media sterilization,

filling and emptying, and cleaning the system. Thus, towards improving the cost-effectiveness of the plant cell culture-based bioprocesses, various operational modes have been developed, including multi-stage batch, fed-batch, single- or multi-stage continuous (chemostat), semi-continuous (draw-and-fill) and perfusion (continuous with cell retention) cultivation [2, 8]. A comparison of different cultivation modes and feeding regimes is summarized in Table 1.1. Among these, the most promising towards high productivity and thus most successfully applied ones for suspended plant cells are fed-batch and perfusion.

Towards the development of new bioreactor configurations, we designed a glass-column bioreactor, operated with pulsed aeration in the Laboratory of Applied Biotechnologies, Plovdiv, Bulgaria [27]. In bubble column reactors, the sole source of agitation is the pneumatic power input provided by isothermal expansion of the sparged gas from the bottom [1, 28]. The air balloon type of aeration (created *via* pulsed aeration) significantly reduces cell exposure to the local zones of high shear stress. Moreover, such type of aeration (also called slug bubble or Taylor-like) ensures both effective mass transfer of oxygen into the liquid medium and homogenization of the culture medium [3, 27]. The bioreactor was further used for cultivation of *Harpagophytum procumbens* (devil's claw) cell suspension and to study the production of pharmaceutically important verbascoside (a phenylethanoid glycoside, possessing desirable pharmacological activities for human health, such as antioxidant, antiinflammatory, antineoplastic, wound-healing and neuroprotective properties) [29]. As a result, both accumulated devil's claw biomass and the high-value verbascoside productivity in the column reactor with pulsed aeration were higher than the respective levels, reached in the shaken flasks and STRs. Consequently, the biomass [expressed as g biomass/(L day)] and verbascoside [expressed as mg verbascoside/(L day)] productivity were up to 30 % and threefold higher, respectively, in the pulse-sparged column bioreactor than other cultivation systems. The accelerated growth of the devil's claw cells and their high productivity imply that the pulse-aerated glass column bioreactor might be quite suitable system for hosting the plant cell suspension cultures. The construction of the bioreactor from glass also allows cultivation of phototrophic/photomixotrophic cultures [27]. A similar type of bioreactor, named the "slug bubble reactor", has been developed for the cultivation of *Nicotiana tabacum* cell suspension culture [30]. The slug bubble reactor is made of flexible gamma-sterilized biopharmaceutical grade polyethylene and can be operated in a single-use cultivation mode, as discussed above.

Nowadays, 30 years after the development of the first **industrial process** based on plant cells (shikonin production by *Lithospermum erythrorhizon* cell suspension culture), dozens of molecules are produced commercially using plant cell cultures. These include paclitaxel (Taxol®), berberine, ginseng biomass, *Echinacea* polysaccharides and several therapeutic and heterologous proteins, among others [2, 8, 13]. At present, in Ahrensburg (Germany), Phyton Biotech operates the world's largest cGMP plant cell culture facility with bioreactors specifically designed to meet the needs of plant cells in culture. The total production capacity of the taxanes train runs is up to 880,000 L/year. The Phyton Biotech is a global provider of chemotherapeutic agents including paclitaxel, docetaxel APIs (active pharmaceutical ingredients) and taxane

Table 1.1 Commonly used cultivation modes of feeding based on product yield and economical considerations

	Batch/ Repeated batch	Fed-batch	Perfusion	Continuous
				
Process manipulation	Low/medium	Medium	High	Medium
Cost (capital investment and labor)	Low/medium	Low	Medium	Low
Throughput	Low/medium	Low	High	Low
Product volumetric yield	Low	High	Medium	Low

Modified after Georgiev et al. [2]
Fr feeding reservoir, *Cf* cell-free spent medium, *Ef* effluent vessel

intermediates (www.phytonbiotech.com; accessed March 2014). The *Taxus* cell culture-based paclitaxel bioproduction was also commercialized by Samyang Genex Corporation (Taejon, South Korea) at m^3-scale [18]. In addition to relatively well established bioproduction of plant-derived molecules, in recent years, several biotech companies have been turning plants (and relevant cell suspension cultures) into drug factories in order to produce therapeutic proteins that could not be made otherwise or to make them cheaper [31]. Protalix BioTherapeutics (Israel) uses the next-generation recombinant protein expression system platform to produce a wide range of complex and biologically equivalent human proteins (e.g. ELELYSO, a plant cell-expressed form of the glucocerebrosidase enzyme for treatment of Gaucher's disease) in transgenic carrot and tobacco cell cultures in single-use plastic bag bioreactors (www.protalix.com; accessed March 2014). Another remarkable example includes the development of recombinant animal vaccine against Newcastle Disease virus, produced by transgenic tobacco cell cultures and marketed by Dow Agrosciences [20].

1.4 Bioreactors for Differentiated Plant Organ Culture

In general, the differentiated plant organ culture consist of plantlets, shoot culture, adventitious (=normal roots) and transformed root culture (=hairy roots; harbouring T-DNA of *Agrobacterium rhizogenes* pRi plasmid). In the past two decades, plant organ culture have become increasingly considered as an attractive platform for bioproduction of plant-derived metabolites and therapeutic proteins, because of their several advantages, such as genetic and biochemical stability, and capacity for organogenesis-associated synthesis of metabolites, to name a few [2, 7, 19, 32, 33].

Diverse bioreactor designs (Fig. 1.3) have been used for cultivation of differentiated plant organ culture, including mechanically driven reactors (e.g. STRs, wave-mixed and rotating drum reactors), pneumatically driven systems (e.g. air-lift reactors and bubble column reactors), besides abundant bed reactors (e.g. mist reactors and trickle-bed reactors) and temporary immersion systems [2, 34]. The morphology of differentiated plant organ culture demands a special consideration for the adequate bioreactor configuration, which should (1) provide low-shear environment for tissue growth and (2) ensure reduced mass transfer limitations. The formation of strong nutrient and oxygen gradients in the tissue is a major issue in densely packed plant tissue beds, e.g. non-homogeneous growth [2, 21, 34, 35].

Thus, the use of ordinary STRs is, in general, not highly recommended because of the high stress-sensitivity of plant organ culture. However, slight changes in the STR internal hardware configuration – e.g. separation of the plant tissue from the impeller (by using a mesh for instance) or just a simple reduction of the agitation speed – have resulted in successful cultivation of transformed root cultures of *Beta vulgaris* [36] and *H. procumbens* [37] and *Atropa belladonna* [38] in bioreactors of different scale (Table 1.2). Pneumatically driven air-lift and bubble column reactors are probably the most frequently used configurations for hosting differentiated plant organ culture [2]. Among others, a modification of air-lift reactor, named balloon type bubble bioreactor

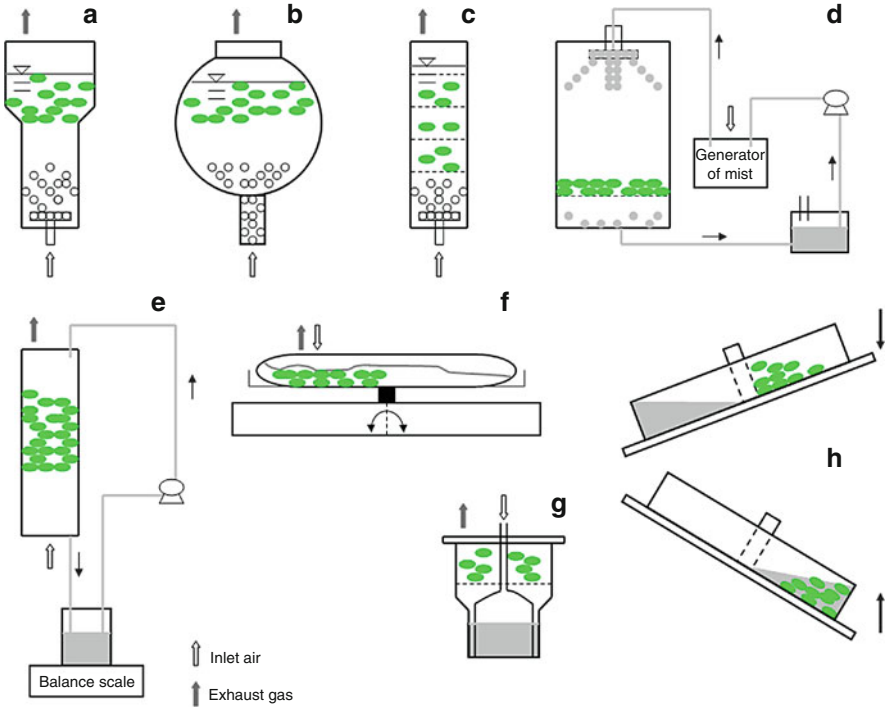


Fig. 1.3 Bioreactor configurations for plant organ culture. (**a**) Bubble-column bioreactor, (**b**) Balloon type bubble bioreactor, (**c**) Column photo-bioreactor with internal sections, (**d**) Mist bioreactor, (**e**) Trickle-bed bioreactor, (**f**) Wave bioreactor, (**g**) Temporary immersion system RITA®, (**h**) BioMINT reactor (Georgiev et al. [2] and the literature cited therein)

(Fig. 1.3b), appeared quite suitable for large-scale biomass and metabolite mass production by adventitious root culture of *Panax ginseng*, *Hypericum perforatum*, *Morinda citrifolia* and *Echinacea* (Baque et al. [19] and the literature cited therein). In a recent interesting study, Georgiev et al. [43] reported the successful cultivation of *Leucojum aestivum* shoot culture in modified glass-column bioreactor with internal sections (Fig. 1.3c) for production of galanthamine (naturally occurring alkaloid used in the treatment of mild-to-moderate Alzheimer's disease, marketed as Nivalin® and Reminyl®). The introduction of internal sections ensures submerged cultivation of the shoot culture and adequate mass and oxygen transfer, which resulted in high biomass accumulation (>20 g L^{-1}) and galanthamine production (1.7 mg L^{-1}).

Furthermore, mist bioreactor (Fig. 1.3d), trickle-bed reactor (Fig. 1.3e), wave induced bioreactor (Fig. 1.3f) as well as several temporary immersion systems (Fig. 1.3g, h) have been validated as suitable for cultivation of plant organ culture (Table 1.2) (recently reviewed in Georgiev et al. [2]). It should be, however, noted that most of the temporary immersion systems are still of laboratory-scale, therefore, further more detailed experiments in large-scale volumes are pending to prove their efficacy.

Table 1.2 Selected examples of bioreactor configurations (operated in batch mode) used for cultivating plant organ cultures

Bioreactor type[a]	Plant organ culture[b]	Product	Final density (g DW/L)/productivity [g DW/(L day)][c]	Reference
Mechanically driven				
Stirred tank reactor (5 L)	*Beta vulgaris* HR	Betalains	12.9/0.68	Georgiev et al. [36]
Stirred tank reactor (3 L)	*Harpagophytum procumbens* HR	Iridoids	8.98/0.64	Homova et al. [37]
Stirred tank reactor with separate impeller (25 L)	*Atropa belladonna* HR	Tropane alkaloids	6.02/0.20	Lee et al. [38]
Wave reactor (0.5 L)	*Panax ginseng* HR	Ginsenoside	11.6/0.41	Palazon et al. [39]
Pneumatically driven				
Bubble column reactor (2 L)	*Harpagophytum procumbens* HR	Iridoids	6.6/0.31	Ludwig-Müller et al. [40]
Bubble column reactor (2 L)	*Beta vulgaris* HR	Betalains	12.7/0.79	Pavlov et al. [41]
Air-lift reactor (2 L)	*Nicotiana tabacum* HR	Murine interleukin-12	4.8/0.34	Liu et al. [42]
Column photo-bioreactor with internal sections (1 L)	*Leucojum aestivum* SC	Galanthamine	20.8/0.59	Georgiev et al. [43]
Balloon type bubble bioreactor (1,000 L)	*Echinacea purpurea* AR	Caftaric, chlorogenic and cichoric acid	5.1/0.15	Baque et al. [19]
Bed reactors				
Mist reactor (4 L)	*Nicotiana tabacum* HR	Murine interleukin-12	5.2/0.37	Liu et al. [42]
Mist reactor (1.5 L)	*Artemisia annua* HR	Artemisinin	14.4/0.38	Kim et al. [44]
Mist trickle reactor (5 L)	*Centaurium erythraea* SC	Secoiridoids	17.76/0.63	Piatczak et al. [45]
Trickle bed (14 L)	*Hyoscyamus muticus* HR	No product	36.2/1.45	Ramakrishnan and Curtis [46]

Modified after Georgiev et al. [32]

[a]Bioreactor working volumes are given in parenthesis

[b]*HR* hairy root culture, *SC* shoot culture, *AR* adventitious root culture

[c]*DW* dry tissue weight

Industrialization of plant organ culture bioprocesses is not yet fully developed, mostly due to the morphological features of differentiated *in vitro* cultures and the resultant challenges [2, 34]. One such challenge is the way to transfer plant tissue inocula from seed reactor to large-scale reactor, which apparently cannot be preformed pneumatically (as usually done in suspended culture-based processes). The recently developed commercial system at 10 m^3-scale for biomass and bioactive ginsenoside production from *Panax ginseng* adventitious roots by CBN Biotech Company, South Korea [19] could contribute towards solving "inocula transfer" issues. Monitoring of the plant tissue growth in bioreactors during the cultivation process (up to several weeks) is another worth mentioning challenging issue [2, 34]. A number of methods are, therefore, developed for (indirect) estimation of the tissue growth (in *off-line* or in *on-line* mode), such as measuring conductivity, osmolarity and redox potential of the culture medium (thoroughly summarized in Georgiev et al. [47]). Nevertheless, more reliable and accurate methods for biomonitoring are continuously sought.

1.5 Conclusions and Perspectives

For ca. 60 years of research, we have witnessed profound changes in development of plant *in vitro* culture-based bioprocesses for mass production of biomass, and plant derived-molecules and therapeutic proteins, which clearly outlined their immense potential for commercialization. Dozens of commercial processes were then developed and several others are on the pipeline. Nowadays, the design and configuration of bioreactors used adequately reflect the physiological requirements of plant cell and organ culture. It increasingly appears likely that single-use bioreactors, originally developed for highly sensitive mammalian culture, will become more often used (e.g. 3D bioreactor-based systems) for growing plant cells that do not exhibit Newtonian fluid behaviour. Wider commercialization of plant *in vitro* culture-based processes implies the development of more reliable methods for bioprocess monitoring (for plant organ culture bioprocesses in particular) and improvement of overall process performance. Recent and emerging "omics" platforms (and metabolomics in particular) are likely to accelerate this process.

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Chapter 2
Disposable Bioreactors for Cultivation of Plant Cell Cultures

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Abstract The trend for using disposable bioreactors in modern biotechnological processes has also been adopted for plant cell cultivations. In fact, plant cell cultures are now being grown in disposable bioreactors with volumes up to 400 L. This trend has been witnessed for both the development and commercial manufacture of therapeutic proteins, secondary metabolite-based pharmaceuticals and cosmetic compounds. Prominent examples of commercial products are Protalix's ELELYSO and Mibelle Biochemistry's Phyto Cell Tech-derived bioactive compounds.

This chapter discusses the current state of disposable bioreactor technology for plant cell cultures. After a brief introduction to the general fundamentals of disposable bioreactors (relevant technical terms, advantages and limitations of disposable bioreactors) a current overview of disposable plant cell bioreactors and their instrumentation will be provided. We will describe the working principles and engineering characteristics of disposable bioreactor types that are scalable and successfully being used for the cultivation of plant cell suspension and hairy root cultures. In addition, we will provide selected application examples focusing on the cultivation of geraniol producing tobacco cells. The chapter will end with perspective on future developments of disposable bioreactor technology for plant cell cultures.

Keywords Bubble column • Disposable bioreactor technology • Hairy root culture • Instrumentation • Mist bioreactor • Orbitally shaken • Oscillating • Plant cell suspension culture • Recombinant protein • Secondary metabolite • Stirred • Tobacco

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Abbreviations

1 to 3D	1 to 3-dimensional
BY-2	Bright Yellow-2
CFD	Computational fluid dynamics
CHO	Chinese hamster ovary
DCO_2	Dissolved carbon dioxide
DO	Dissolved oxygen
EVA	Ethylene vinyl acetate
fw	Fresh weight
Glc	Glucose
GMP	Good manufacturing practice
hCTLA4Ig	Recombinant cytotoxic T-lymphocyte antigen 4 immunoglobulin
k_La	Oxygen mass transfer coefficient
Lac	Lactate
LED	Light emitting diode
PC	Polycarbonate
PE	Polyethylene
PP	Polypropylene
PS	Polystyrene
PU	Polyurethane
P/V	Specific power input
pcv	Packed cell volume
PVC	Polyvinylchloride
rpm	Rotations per minute
SBB	Slug bubble bioreactor
TI	Temporary immersion
vvm	Air volume per medium volume per minute
WUB	Wave and undertow bioreactor

2.1 Introduction

During the past 10 years, disposable bioreactors have increasingly replaced their reusable glass or stainless steel counterparts in modern biotechnological production processes. This trend is applied for research and commercial production processes up to medium volume scales [1, 2]. This is a logical consequence of the main advantages of disposable bioreactors when they are used and operated correctly, which include: reduced contamination rates, time and cost savings, lowered waste and environmental impact [3–5]. These advantages have been demonstrated in various

studies and can be attributed to the plastic materials (ethylene vinyl acetate, EVA; polycarbonate, PC; polyethylene, PE; polypropylene, PP; polystyrene, PS; polyurethane, PU and polyvinylchloride, PVC) that are used to make the cultivation containers for disposable bioreactors. The containers are either a free-standing, rigid vessels that are most frequently manufactured from PS or PC, or flexible two-dimensional (2-D), respectively three-dimensional (3-D) bags. Such culture bags are made from multilayered films which are typically composed of: (1) the contact layer that is in direct contact with the culture medium and cells, (2) the gas/vapor barrier layer that limits the diffusion of gases and vapor, (3) the external layer that provides the mechanical stability to the bag and (4) the tie layers which use physico-chemical interactions to bond the contact-, gas/vapor- and external layer together. PP, PE and EVA have proven to be suitable as contact layers [6–8]. In cases, where bags are used as cultivation containers, disposable bioreactors often require an additional bag holder. The holding device (e.g., stainless steel support container, tray or table), which fixes and shapes the bag, is often electrically heated or incorporates water-filled double jackets to control the temperature.

Regardless of whether the cultivation container is a rigid vessel or a flexible bag, if it is pre-sterilized by the vendor, by beta- or gamma-irradiation and discarded after a single bioprocess, it is deemed to be a disposable and single-use bioreactor. Not surprisingly, their application results in reduced cleaning and sterilization work. Disposable single-use bioreactors play a dominant role when high-value products such as therapeutic proteins have to be developed and manufactured within a short period of time. It is worth mentioning that the majority of commercially available disposable single-use bioreactors were originally designed to produce animal cell-based seed inocula and therapeutic proteins such as antibodies and vaccines [2, 9, 10].

However, there are disposable bioreactors, whose cultivation containers are used multiple times. Their plastic cultivation containers are either provided sterile or have to be steam- or gas-sterilized prior to use. By definition, these bioreactors are disposable multi-use bioreactors [11, 12]. Disposable single-use bioreactors are typically available as mid-level instrumented versions with volumes of up to 2 m^3 [13], whereas disposable multi-use bioreactors are less- or minimally instrumented plastic containers that are designed for cultivations at bench top and (more rarely) pilot scales (double digit L-range). In comparison to disposable single-use containers, disposable multi-usable versions are more complex and can be more time-consuming to operate. However, they are cheaper to manufacture and to purchase, and thus claimed as low-cost systems.

Both disposable multi-use and disposable single-use bioreactor types have been successfully used in *in vitro* cultivations of plant cell suspension cultures, hairy root cultures, embryogenic cultures, meristematic tissue and filamentous tissue cultures [12, 14, 15]. By introducing the Life Reactor (the first disposable bag bioreactor for plant cells) in the early 1990s, the company Osmotek paved the way for today's

cultivations aimed at the production of bioactive compounds for pharmacy and cosmetics. This bioreactor was the prototype of the Wilson's Plastic-lined Bioreactor [16] and, finally, the 400 L system [17, 18] used by the company Protalix to commercially manufacture the taliglucerase alpha, recombinant glucocerebrosidase, enzyme (commercially known as ELELYSO) under GMP (Good Manufacturing Practice) compliant conditions. The market launch of the first wave-mixed bag bioreactor, the Wave Bioreactor, in the late 1990s represented a further milestone in the development of disposable bioreactors [19]. It paved the way for further versions of wave-mixed disposable bioreactors such as the BIOSTAT Culti Bag RM, the AppliFlex or the Wave and Undertow Bioreactor (WUB) which will be discussed in detail in Sect. 2.3.1. These wave-mixed bag bioreactors are currently most widely used when plant cells are grown in disposable bioreactors. For example, Mibelle Biochemistry and Sederma has already developed and manufactured the bioactive compounds for cosmetics, such as Phyto Cell Tec Argan, Solar, *Vitis*, *Malus domestica*, Alp Rose and RESISTEM in the BIOSTAT Culti Bag RM [20]. Greenovation also uses the same bioreactor to produce different therapeutic proteins using *Physcomitrella patens* suspension cells.

Interestingly, there have been no reports of any negative influence on cell growth and expression of products caused by interactions between medium components and the inner contact layer of the plastic bag for plant cell cultivations. This limitation was described by Kadarusman et al. [21] and Altaras et al. [22] for cultivations of Chinese hamster ovary (CHO) cells propagated in chemically defined culture media in bags with a PE film as the contact layer. The strength of this effect was dependent on the sensitivity of the cell line, the composition of the culture medium, the initial cell density, the procedure for irradiating the bag and the bag storage procedure. The most-feared inhibitory substances which can migrate from the film material into the cell culture broth under processing conditions are called cytotoxic leachables [23, 24]. Recently Hammond et al. [25], identified bis (2.4-di-tert-butyl phenyl) phosphate as a leachable compound that decreases mitochondrial membrane potential and inhibits CHO cell growth at concentrations of just 0.1 mg L^{-1}. This substance is formed when PE bags are fabricated, subsequently irradiated (between 25 and 50 kGy) and when trisarylphosphite processing stabilizers were used. The risk of leachables being released from the disposable bags represents the most cited limitation of disposable bioreactors and the main reason for not using them in animal cell-based production processes.

2.2 Disposable Bioreactors Suitable for Growing Plant Cell Cultures

2.2.1 Instrumentation of Disposable Bioreactors

A second bottleneck of today's disposable bioreactors is their current instrumentation and automation. It is a fact that disposable bioreactors are not as highly

instrumented and automated as the reusable versions. The standard parameters for process control and automation parameters for plant cell cultivations in reusable bioreactors include temperature, pH-value, flow rate, dissolved oxygen (DO), agitation speed, filling level, foam level and pressure. In addition to these, advanced parameters such as vessel weight, conductivity, viscosity and substrate and metabolite concentrations can also be monitored.

As described by Glindkamp et al. [26] and Lindner et al. [27] disposable bioreactors are equipped with *in situ* and/or external sensors. Both *in situ* (in direct contact with the process fluid) and *external* (contacting the process fluid optically or *via* a sterile sample removal system) sensors are available as standard or disposable elements. Standard sensors have to be cleaned and sterilized separately and should be connected to the bioreactor *via* aseptic couplings before inoculating and starting the bioreactor. In contrast, disposable bioreactors with disposable sensors are shipped for ready to use. Nevertheless, the number of disposable sensor types and vendors that are available is considerably smaller than those for standard systems. Furthermore, the selection of the sensor is restricted to systems for which the manufacturer of the disposable bioreactor offers an implementation option. In other words, the compatibility of analytics is generally defined by the manufacturer of the disposable bioreactor.

Table 2.1 gives an overview of available disposable sensors, their measurement principles, measurement range and vendors. There is a wide range of disposable sensor types for temperature, pressure, pH-value and DO concentration. Constraints exist for process parameters such as flow rate, conductivity, dissolved carbon dioxide (DCO_2), biomass, substrate and metabolite concentrations since there are only a few systems available.

Due to the fact that all the disposable sensors shown in Table 2.1 were designed for animal cell cultivations, further limitations have to be considered when they are used for plant cell cultivations. These limitations are related in particular to optical pH and DO sensors and arise from differences in sensor measurement ranges, fluid flow behavior and the demand of light for phototrophic cultivation conditions. For example, optical pH sensors only deliver reliable measurements of values ranging between 5.5 and 8.5. But pH-values occurring in plant cell cultivations are normally between 4 and 6 [28, 29]. This means that measurements of a pH range between 4 and 5.5 (typical values within the first 48 h of cultivation that indicate early ammonium assimilation and late nitrate assimilation) are problematic while using optical sensors.

High viscosities of plant cell culture broths and changes from Newtonian to non-Newtonian fluid flow behavior [30–34] may complicate optical pH and DO measurements as a result of the background noise and incidental light intensity. However, the biggest issue of all optical sensors is their photobleaching, where phytochemical destruction of the dye being sensitive to the parameter which should be measured occurs. For this reason, disposable cultivation vessels containing disposable sensors have a restricted storage time and must be protected from light and as a result, their usage is not recommended for cultivations requiring light. For these kinds of cultivations, standard sensors are preferred, which have a further effect on the selection

Table 2.1 Selection of commercially available disposable sensors

Process parameter	Measurement principle	Measurement range	Vendor
Pressure	Semiconductors	0–5 bar	Finesse Solutions PendoTECH SciLog
Temperature	Semiconductors	−10–125 °C	BURNS Engineering GE Healthcare PendoTECH SciLog
pH	Optical and potentiometric	In the majority: 5.5–8.5 Max: 2–10	Finesse Solutions Metroglas Ocean optics PreSens Sartorius Stedim Biotech
DO	Optical	In the majority: 0–100 % Max: 0–250 %	Finesse Solutions Ocean Optics PreSens
DCO_2	Optical	1–25 %	PreSens
Flow rate	Infrared reflection, ultrasound Coriolis concept	0–20 L min^{-1} 5–24,000 g min^{-1}	Equflow, Levitronix PendoTECH
Conductivity	Semiconductors	1–200 µS cm^{-1}	PendoTECH SciLog
Biomass, cell density	Capacitance-based	10^5–10^9 cells mL^{-1}	Aber Instruments Fogale Nanotech
Glucose (Glc), lactate (Lac)	Enzymatic (ionselective electrodes)	Glc: 0.2–10 g L^{-1} Lac:0.1–5 g L^{-1}	C-CIT
Glutamine, glutamate	Enzymatic (ion-selective electrodes)	0.1–9 g L^{-1}	C-CIT

Remark: For a detailed explanation of the measurement principles the interested reader is referred to the homepages of the cited vendors [26] and [27]

of the type of plant cell bioreactor that can be used. Additionally not all disposable bioreactor types can be equipped with standard sensors.

2.2.2 Categorization Approach for Plant Cell Bioreactors

According to the technical literature, static bioreactors characterized by unenforced power input and used for screening studies at mL-scale are generally less relevant than the dynamic ones. Dynamic bioreactors being in the focus of our chapter

become important if the bioreactor volume rises and higher biomass respectively cell densities or product titers are required. A categorization approach for disposable plant cell bioreactors which takes their mixing and power input principle into account is shown in Fig. 2.1.

Three main classes can be distinguished: (1) mechanically driven disposable bioreactors, (2) hydraulically driven disposable bioreactors and (3) pneumatically driven disposable bioreactors. As summarized in Table 2.2, mechanically, hydraulically and pneumatically driven versions of disposable bioreactors with filling volumes up to 400 L have been operated to produce plant cell-based biomass, secondary metabolites and recombinant proteins such as antibodies.

Mechanically driven disposable bioreactors represent the largest group. Their mixing is either performed by rotating stirrers (Fig. 2.2a), tumbling stirrers (Fig. 2.2b), rocking platforms (Fig. 2.2c), raising platforms (Fig. 2.2d), vibrating perforated disks (Fig. 2.2e) or orbitally shaken platforms (Fig. 2.2f). In addition to the mechanically driven disposable bioreactor types listed in Table 2.2, disposable bubble columns (Fig. 2.2g) can be used when plant cell suspension cultures are being grown. Pneumatically driven bubble columns are simpler in design (immovable parts, no shaft seal). Mass and heat transfer is achieved by direct sparging of air or gas into the tall cultivation container. This results in the rising of bubbles which cause mixing and fluid circulation of the culture medium.

If the organ cultures (hairy root cultures, meristematic or filamentous tissue and embryogenic cultures) are being grown, continuous immersion of the cells (which is typical in mechanically and pneumatically driven bioreactors) may cause adverse effects. Process limitations, including limited gas exchange, cell hyperhydricity,

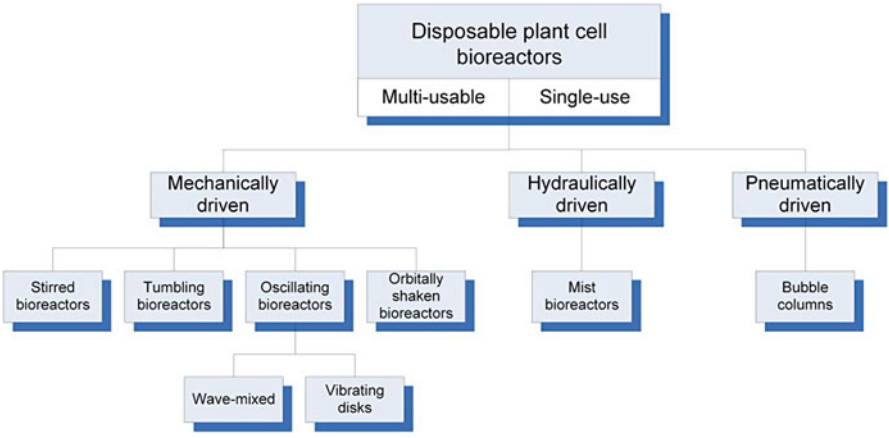

Fig. 2.1 Classes of disposable plant cell bioreactors according to their mixing and power input principles (only scalable dynamic versions are considered). This categorization approach is based on the general classification of disposable bioreactors which was recommended by DECHEMA's temporary working group "Single-use technology in biopharmaceutical manufacture"

Table 2.2 Disposable dynamic cell bioreactors that are successfully used for plant cell cultivations

Bioreactor type	Volume [L]	Culture type and plant species	Product	Reference
Stirred bioreactor	2–25[a]	Plant cell suspension culture (e.g. *Corylus avellana*, *Glycine max*, *Hordeum vulgare, Malus domestica, Nicotiana tabacum*, *Vitis vinifera*)	Secondary metabolites for pharmaceuticals, antibodies	[35, 36]
Bioreactor with tumbling stirrer	10[a]		Secondary metabolites for pharmaceuticals	See Sect. 2.4.2
Oscillating, wave-mixed bioreactor	1–100[a]		Secondary metabolites for pharmaceuticals, cosmetics and therapeutic proteins	[19, 37–45] See Sect. 2.4.2
	0.3–10[a]	Hairy root culture (e.g. *Harpagophytum procumbens*, *Hyoscyamus muticus*, *Panax ginseng*, *Nicotiana tabacum*)	Secondary metabolites for pharmaceuticals	[46, 47] See Sect. 2.4.3
	10–100[a]	Filamentous tissue *(Physcomitrella patens*)	Therapeutic proteins	[48]
	1–10[a]	Embryogenic culture (*Allium sativum*)	Secondary metabolites for pharmaceuticals and food	[46, 49]
Oscillating bioreactor with vibrating disks	100[a]	Plant cell suspension culture (*Malus domestica*)	Secondary metabolites for cosmetics	[11]
Orbitally shaken bioreactor	1–100[a]	Plant cell suspension culture (e.g. *Corylus avellana*, *Helianthus annuus*, *Nicotiana tabacum*, *Vitis vinifera*)	Secondary metabolites for pharmaceuticals, cosmetics, food and antibodies	[50–52]
	0.2–25[a]	Hairy root culture (*Nicotiana tabacum*)	Secondary metabolites for pharmaceuticals	[41]
Mist reactor	1–60[b]	Hairy root culture (e.g. *Ocimum basilicum*, *Panax ginseng*)	Secondary metabolites for pharmaceuticals and cosmetics, therapeutic proteins	[53, 54]
	1–4[c]	Embryogenic culture (*Daucus carota*)	Biomass for breeding experiments	[55]
Bubble column	1.5–400[b]	Plant cell suspension culture (e.g. *Glycine max, Hyoscyamus muticus, Oryza sativa, Nicotiana tabacum*)	Secondary metabolites for pharmaceuticals and food, therapeutic proteins	[16, 56–58]
	1.5–5[a]	Meristematic tissue, embryogenic culture (ornamental, vegetable and woody plant species)	Biomass for breeding	[59–62]

[a]Filling volume
[b]Total volume
[c]Related to the root bed

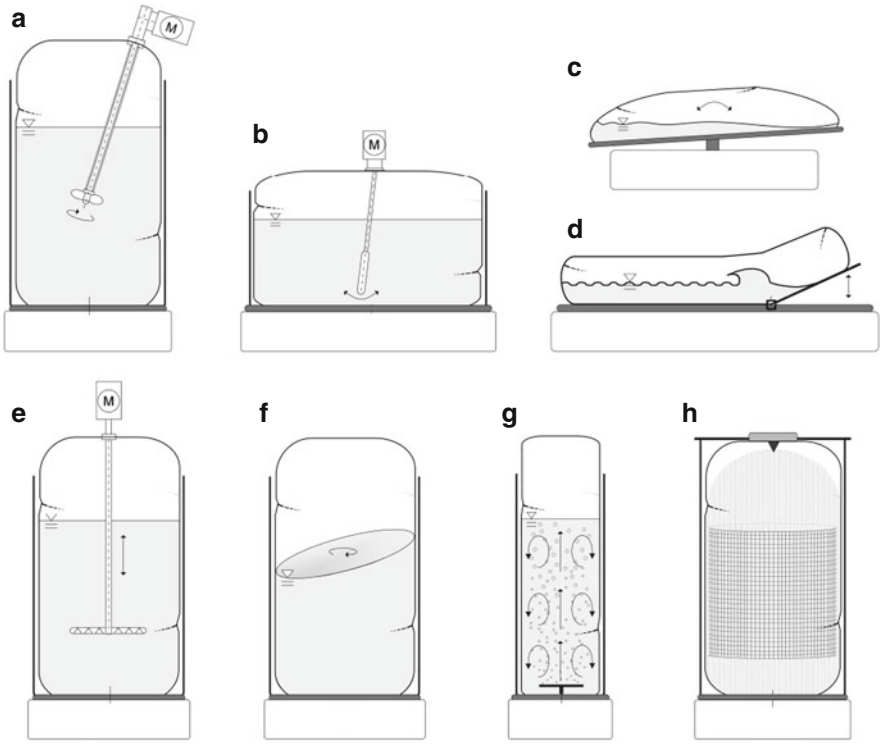

Fig. 2.2 Schematic diagrams of disposable plant cell bioreactor types. (**a**) Bioreactor with rotating stirrer, (**b**) Bioreactor with tumbling stirrer, (**c**) Bioreactor with rocking platform, (**d**) Bioreactor with raising platform, (**e**) Bioreactor with vibrating disk(s), (**f**) Bioreactors with orbitally shaken platform, (**g**) Bubble column, (**h**) Mist bioreactor

chlorophyll deficiency as well as changes in enzymatic activity and protein synthesis have been reported [63, 64]. Alternatively, temporary immersion (TI, also known as ebb-and-flow regime) was realized in specially designed disposable bioreactor types usually used for plant breeding purposes. Prominent examples of disposable TI bioreactors are Osmotek's Ebb-and-Flow Reactor [65] and Nestlé's Box-In-Bag Bioreactor [45, 65]. Using these bioreactors, a repetitive filling and draining of the bioreactor occurs. After temporarily wetting the entire organ culture with the culture medium, the excess medium is drained away by gravity. A similar effect can be achieved by operating a wave-mixed bioreactor bag with a low amount of culture medium (10–20 % of the total bag volume). As a result of the culture bag position and the filling level in the bag, there are two segments of the organ culture, the one which is immersed and the other which is not immersed. This cultivation technique [49] is beneficial for hairy root and embryogenic cultures for which disposable mist reactors were also developed.

In mist reactors (Fig. 2.2h) gas represents the continuous phase and power input is normally affected by pumps. The culture is exposed to air or other gas mixtures

and is periodically (seldom continuously) subjected to the nutrients from the culture medium. The cells are pumped from a medium storage vessel, and nutrients are delivered in the form of droplets whose diameters range between 0.01 and 10 µm [66]. Culture immobilization on a matrix is stringently required for mist reactors and is also shown to be advantageous for disposable wave-mixed bioreactors, but it is not required in the latter [49].

2.3 Scalable Disposable Bioreactors for Plant Cell Suspension and Hairy Root Cultures: Types and Their Engineering Characteristics

2.3.1 Disposable Wave-Mixed Systems

In disposable wave mixed bioreactors the complete platform and subsequently the sections of the platform on which the culture bag is fixed will move. The rocking movement induces a wave in the bag containing the medium and the cells. The wave movement introduces bubble-free oxygen into the fluid from the headspace of the bag and the surface of medium is continuously renewed. The generation and propagation of the wave within the bag influences the fluid flow, mixing time, oxygen mass transfer rate, shear stress acting on cells and, thus resulting in cell growth and product formation. As discussed by Löffelholz et al. [13] currently available wave-mixed bioreactors mainly differ in their oscillatory motion, which can be one-, two- or three-dimensional (1-D, 2-D or 3-D).

For cultivations, based on plant cell suspension and hairy root cultures only versions with 1-D oscillatory motion (BIOSTAT Cultibag RM, Wave Bioreactor, AppliFlex, WUB) have been used till date. The majority of engineering and biological data is described for the BIOSTAT Cultibag RM and its precursor, the BioWave. Its bag (filling volume up to 300 L) is fixed on the rocking tray that also regulates the temperature. In the BIOSTAT CultiBag RM, intensity of mixing and aeration can be controlled by the rocking rate, the rocking angle, the filling level of the bag (up to 50 % maximum) and the aeration rate [46]. Eibl et al. [38] and Werner et al. [19] summarized relevant engineering parameters for typical process conditions for animal and plant cell cultures in this bioreactor system. It was demonstrated that the fluid flow in the bag with 1-D oscillatory motion can be characterized by a modified Reynolds number. Furthermore, maximum oxygen mass transfer coefficients (k_La) of up to 10 h^{-1}, mixing times between 20 and 50 s, and specific power inputs (P/V) between 70 and 180 W m^{-3} were measured and calculated. These values are regarded as sufficient for growing both animal and plant cells. Using Computational Fluid Dynamic (CFD) simulations Werner et al. [19] discovered more homogeneous

energy dissipation and more homogeneous shear stress pattern in the BIOSTAT CultiBag RM than in stirred reusable cell culture bioreactors using Rushton turbines and paddle impellers. They assume that this is the reason for increased biomass respectively cell counts and product titers that have been shown in different comparative growth and production studies with Newtonian culture broths. The possibility of mass and gas transfer limitations was found for wave-mixed bags with 1-D oscillatory motion and fast growing tobacco Bright Yellow-2 (BY-2) suspension cells [38]. Cell growth (doubling times of 16 h) was accompanied by an increase in viscosity from 0.001 to 0.41 Pas during cultivation. Despite of doubling P/V by adjusting the maximum rocking rate (42 rpm) and the maximum rocking angle (10°) at maximum filling volume (2 L bag), no wave was generated. Indeed, the culture broth only moved slightly. Although wave-mixed bioreactors ensuring higher k_La values and power inputs (such as the CELL-tainer with its 2-D oscillatory motion bag [67] or the XRS Bioreactor System with its 3-D oscillatory motion bag [68]), there are currently no reports of their use for cultivation of either plant suspension cells or hairy roots.

A further disposable wave-mixed bioreactor with a 1-D oscillatory motion which has successfully been used to grow plant cell suspensions and hairy roots is the AppliFlex [36, 43]. This type is available up to a filling volume of 25 L and has a 3-D single-use bag. Furthermore, in contrast to the previously described BIOSTAT CultiBag RM which can only be operated with disposable sensors in the pillow-like (2-D) single-use bag, the AppliFlex can also be equipped with standard sensors. Studies revealed that it delivers comparable mass and gas transfer, growth and production results to those observed in the BIOSTAT CultiBag RM. Like the BIOSTAT CultiBag RM, an AppliFlex photobioreactor version operating with light emitting diodes (LEDs, white, blue and red light) can be supplied on request for light cultures (see also Sect. 2.4.2 and Fig. 2.3).

The main difference between the WUB, the AppliFlex and the BIOSTAT CultiBag RM is that the WUB has no rocker unit and the fact, that it was only designed for plant cell applications. In the WUB, the wave is induced by periodic upward movement of the flexible head and/or foot section of the horizontal table which displays the fixation platform on which the bag (20–250 L filling volume) is located. The subsequent undertow movement results from the platform(s) lowering. According to Terrier et al. [44] the k_La values are similar to those observed in the BIOSTAT CultiBag RM at comparable scales. The angle of the platform, the percentage of the filling volume located on and moved by the platform, the aeration rate and the time taken for the platform to perform one oscillation had the most impact on the k_La value.

Like disposable wave-mixed bioreactors with 1-D oscillatory motion, where foaming and flotation were found to be negligible in cultivations with plant and animal cells, the addition of an antifoam agent in disposable orbitally shaken systems is unnecessary.

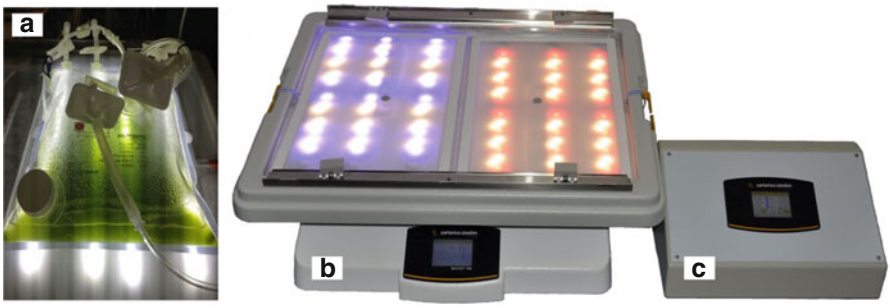

Fig. 2.3 Wave-mixed BIOSTAT CultiBag RM 20/50 with bottom-mounted LEDs for growing phototrophic cultures. (**a**) Suspension culture in a 2 L wave-mixed bag (**b**) Rocker with *blue* (455 nm), *red* (645 nm) and *white* (400–780 nm with a colour temperature of 3,000 K) LEDs (**c**) LED control unit

2.3.2 Disposable Orbitally Shaken Systems

Orbitally shaken erlenmeyers at mL-scale are widely used for inoculum productions and screening studies with plant and animal cell cultures. The results from engineering characterization of orbitally shaken erlenmeyers [69–74] provided the basis for increased acceptance of their use for process optimization. Determined mixing times, power consumptions, k_La values and energy dissipation rates indicated their suitability for growing animal and plant cell cultures without oxygen limitation and damages resulting from excessive shear stress. Furthermore, liquid distribution provoked by orbital shaking is well-defined and predictable [75]. These findings finally led to the scaling-up of orbital shaking technology for both animal and plant cells. The first successful scaling-up from shake flask to a 36 L orbitally shaken bioreactor was reported by Liu and Hong [76] for insect cells in 2001. For plant cell suspension cultures the possibility of scaling-up orbital shaking technology was first described 5 years later. Raval et al. [77] demonstrated that 20 and 50 L orbitally shaken bioreactors (rigid Nalgene vessels) could facilitate growth of BY-2 suspension cells. Encouraged by these results bag-based orbitally shaken bioreactor systems were developed.

A well-investigated disposable orbitally shaken bioreactor series is the OrbShake Bioreactor series which consists of a peripheral measurement and a control unit, and a support container made from stainless steel. There are commercial systems with 50 L (SB50-X) and 200 L (SB200-X) filling volumes that operate with 3-D cylindrical single-use bags in which single-use sensors are implemented. In the SB-50X (25 °C, 75–90 rpm, 0.12–0.3 vvm) *Corylus avellana* suspension cells were successfully propagated (data not shown). The feeding operation which ran for 22 days delivered a peak biomass concentration of 380 g fw L^{-1}. This means that the fresh weight of biomass increased more than fivefold, corresponding well to the biomass

increase found for cultivations with the same cell culture and in stirred (disposable single-use and reusable) bioreactors.

Meanwhile a 2,500 L prototype of the OrbShake Bioreactor (SB-2000X) was characterized and delivered identical maximum k_La values as the SB-200X (personal communication, Tibor Anderlei, Kühner AG, October 2013). Anderlei et al. [78] reported k_La values of up to 25 h^{-1} for the SB200-X with 100 L filling volume, and mixing times between 25 and 70 s depending on the shaking frequency which ranged between 50 and 70 rpm.

Klöckner et al. [79] established a scale- and volume-independent k_La correlation that was successfully verified for bioreactor volumes from 2 to 200 L for orbitally shaken bioreactors in general. This allows cultivation parameters (reactor diameter, shaking frequency, shaking diameter, filling volume, viscosity, k_La, gravitational acceleration) to be defined for different scales and ensures sufficient oxygen supply in growth studies of BY-2 suspension cells.

Further an orbitally shaken bag-based system that is suitable for growing plant cell suspensions and hairy roots is based on the Infors's Multitron Cell with shaker bag option which makes orbital shaking of 2-D culture bags (3 × 2 L bags or 2 × 10 L bags or 1 × 20 L bag) possible. Using the Infors' Multitron Cell with shaker bag option, the maximum filling volume per shaker unit is limited to 10 L. While generating a homogeneous distribution of the cell culture broth, the mechanical stress is low and the maximum gas transfer is comparable to those found in wave-mixed bioreactors with 1-D oscillatory motion [51]. Filling levels from 20 to 100 % (this relates to the maximum filling volume of the bag of 50 %) resulted in mixing time intervals between 8 and 50 s for shaking frequencies between 30 and 120 rpm and shaking diameters of 25 and 50 mm respectively. If the shaking diameter rose, the gas-liquid transfer increased and the mixing time decreased. Measurements of the k_La (values exceeded 15 h^{-1}) under typical process conditions confirmed that the oxygen supply is sufficient for unlimited plant cell culture growth. For tobacco BY-2, *Vitis vinifera* and *Helianthus annuus* suspension cells Werner et al. [51] and Greulich et al. [80] observed similar growth as seen in rocking (wave-mixed) bags. By realizing comparable fluid flow conditions, peak biomass concentrations between 380 and 480 g fw L^{-1} were measured after 7 days. It is also remarkable that the maximum filling volume, medium shaking frequency and maximum shaking diameter resulted in unexpectedly high k_La values. For example, in case of a 2 L culture bag (CultiBag RM 2 L operated at 1 L filling volume, 70 rpm and 50 mm) a k_La of 68 h^{-1} was achieved, whereas a k_La of 40 h^{-1} was determined for the 20 L culture bag (CultiBag RM 20 L running with 10 L filling volume at 60 rpm and 50 mm). Particularly in the 20 L bag, a range of operating parameters was identified, in which demixing occurred. This phenomenon has already been described for orbitally shaken flasks and is also known as out-of-phase operating condition [81, 82]. According to Büchs et al. [83] out-of-phase operation occurs when frictional forces exceed centrifugal forces during orbital shaking. Knowledge of this critical range helps to avoid inefficient feeding and process scale-up strategies.

2.3.3 Disposable Oscillating System with Vibrating Disk(s)

Compared to wave-mixed and orbitally shaken systems, disposable oscillating bioreactors with one or more vibrating disk(s) are of minor importance for cultivations involving plant cell cultures. The Saltus Vibromix Bioreactor, in which the power input is regulated *via* disk's amplitude and frequency, belongs to this disposable bioreactor family. Its core is the single-use bag (2 L up to 500 L filling volume), which is temperature controlled by a heating jacket incorporated into the stainless steel container. The bag contains a vertically oscillating hollow shaft on which one or more conically perforated disks are fixed. This results in an axial flow movement of the medium in the bag which mixes and aerates the cells. The conical shaped disk holes reduce vortex formation; induce the upward and downward flow, and influence the mixing and oxygen supply in the bag [84]. Due to high power inputs achieved in the Saltus Vibromix Bioreactor (a maximum value of 1,887 W m^{-3} was reported by Werner and Nägeli [85]), use of this disposable bioreactor for shear sensitive cell cultures is not recommended. At maximum power input, aeration rates between 0.05 and 1 vvm and 2 L filling volume k_La values between 26 and 82 h^{-1} were reported, in contrast to k_La values between 11 and 55 h^{-1} for a 10 L filling volume. In order to make the Vibromix Bioreactors more suitable for plant cell cultivation (and more efficient when operated with low power input), bags containing an additional sparger were developed. This design modification contributed to an increase in the plant cell biomass produced in a bag with apple suspension cells at 100 L filling volume (personal communication, Herbert Reichert, Meissner Filtration Products Inc., June 2011).

2.3.4 Disposable Stirred and Tumbling Systems

When carrying out a search for cultivations with disposable stirred bioreactors and plant cells only two references describing the cultivation in ThermoFisher's S.U.B. could be found at the time of writing this chapter [36, 38]. This is surprising because reusable stirred bioreactors are frequently used in plant cell suspension-based productions and they represent the largest plant cell bioreactors (with 75 m^3 in the paclitaxel production process).

The single-use bag of the S.U.B. (available from 50 L up to 2 m^3) is equipped with an angular stirrer (pitched blade stirrer that is off-center) and a sparger. The user can choose between an implemented micro- or ring sparger and standard- or single-use sensors. The 50 L reactor that was used in the studies of Raven et al. [36] and Eibl et al. [38] has been comprehensively characterized. CFD analysis revealed a downward pumping axial flow pattern of the stirrer and two different sized flow loops (Fig. 2.4) [86]. Assuming a steady fluid flow pattern, specific power inputs (P/V) of up to 19 Wm^{-3} were predicted by CFD for a 50 L filling volume and a tip

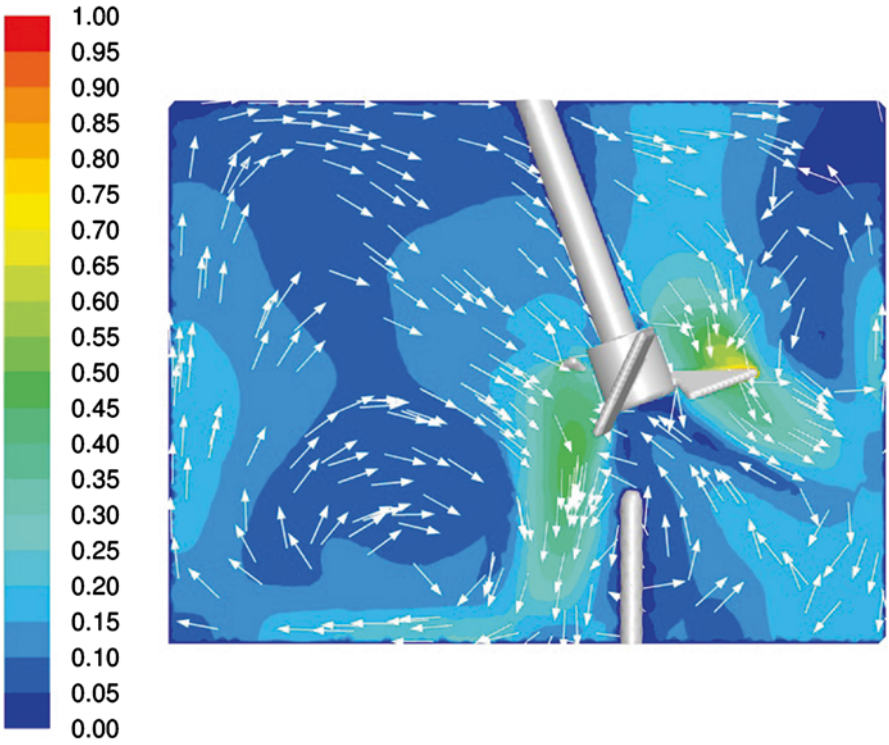

Fig. 2.4 CFD predicted flow pattern of the pitched blade impeller in the S.U.B. (25 L filling volume). The dimensionless velocity profile on the x-y plane is exemplarily depicted when the bioreactor is operated at 200 rpm, which corresponds to a tip speed of 1.23 ms^{-1}. In this case the P/V is 60 Wm^{-3} and the mixing time is 10 s. The arrows show the flow pattern, whereas the color contours illustrate the dimensionless velocity between 0 and 1

speed of 3.1 m s^{-1}. The mixing times (between 9 and 155 s) and k_La values (ranging from 2 to 25 h^{-1}) determined for typical cell culture conditions were comparable to reusable cell culture bioreactors at the same scale [87, 88]. Good correlation with the results which were experimentally determined or predicted by CFD was proven. Moreover, the results are in a similar range to those of other disposable stirred bag bioreactors such as the XDR, the Mobius CellReady and the BIOSTAT CultiBag STR (for more information about these bioreactors the reader is referred to [13, 86]). However, in experiments with the 50 L S.U.B. antibody expressing biomass delivered approximately 20 % lower levels of the packed cell volume (pcv), and a high flotation level of the grown BY-2 suspension cells was observed. This biomass flotation resulting in cell and product loss was attributed to the microsparger which was implemented in the bags used in the investigations [36].

Recent growth studies with *Corylus avellana* suspension cells have shown the efficiency of the UniVessel SU Bioreactor for growing plant cell suspension cultures

at bench top scale for the first time [35]. In growth experiments (batch mode) performed in the UniVessel SU Bioreactor and the BIOSTAT Bplus (this is the reusable counterpart of the UniVessel SU) comparable morphology, doubling times (3 days), growth courses and peak biomass concentrations (530 g fw L^{-1}) were observed. This was expected due to the comparability of the engineering data for both benchtop bioreactor versions, as proven by Kaiser et al. [89].

The UniVessel SU Bioreactor operated with a rigid single-use vessel (2 L filling volume) in which two-stage segment blade stirrers, an L-shaped macrosparger and a standard sensor were mounted. Previously realized CFD simulations confirm the presence of an axial flow pattern with downward pumping discharges from the stirrers at clockwise rotation [90]. Mixing times are dependent on P/V and filling volume. When the BIOSTAT UniVessel SU runs at tip speeds between 0.15 and 1.8 m s^{-1} (50 up to 625 rpm), P/V is between 0.4 and 435 W m^{-3}, and the mixing times range from 3 to 100 s [13].

A proof-of-concept-investigation of whether disposable tumbling systems can be applied to grow tobacco suspension cells has also been recently performed (see in detail in Sect. 2.4.2). It was carried out by using an Integrity Wand Mixer from ATMI (now a part of Pall Life Sciences) at bench top scale. The positive results can be taken as basis for further studies with corresponding bioreactor systems, such as the Nucleo Bioreactor and the Pad-drive Bioreactor. Both bioreactor systems differ only in the fact that their control units have a single-use cube-shaped bag with a paddle-shaped mixing which performs an elliptical rotating motion. The cultivation bag also includes a micro-sparger that is fixed at the mixing device and ensuring k_La values up to 200 h^{-1} [91]. As shown by Farouk and Moncaubeig [92], mixing in these bioreactors follows both radial and axial flow patterns, while the bag walls prevent vortex formation.

2.3.5 Disposable Bubble Columns

We have already mentioned that the Life Reactor (a bubble column bioreactor of 1 or 5 L filling volume) was the first disposable, scalable plant cell bioreactor cited in the literature. It had a conical shaped bag and was preferred for growing meristematic clusters and somatic embryos from ornamental, vegetable and woody plant species (e.g., lilies, orchids, potatoes, bananas) for plant breeding purposes. Ziv's results emphasized its superiority over reusable bubble columns [62]. Increased biomass growth was demonstrated, when foaming was reduced. Reduced foaming was also reported for the plastic-lined bioreactor in which *Hyoscamus muticus* suspension cells were propagated in bags of up to 100 L filling volume [56].

Scientists at Nestlé designed and characterized the Slug Bubble Bioreactor (SBB). At the bottom of the SBB (10–150 L filling volume) long bullet-shaped bubbles (slug bubbles) are intermittently generated by a solenoid valve. The slug bubbles are comparable to "Taylor bubbles" [93, 94] and rise to the top of the cylindrical bag. Air quantity is controlled by adjusting the air inlet pressure and the valve opening time and frequency in the SBB. Ducos et al. [45] hypothesized that mixing is

enhanced in the rear of every slug bubble. Maximum k_La values of 17 h^{-1} were measured. Increasing the valve opening time and/or the valve opening frequency resulted in an increase in an average gas flow rate and finally higher k_La values. Tobacco BY-2 cells grown in the SBB (10, 20, 500, 70 L filling volume) showed a similar growth behaviour to the WUB (10, 20, 30, 100 L filling volume, see Sect. 2.3.1) and a reusable stirred cell culture bioreactor (10 L filling volume). Doubling times between 38.4 and 60 h and peak biomass concentrations between 12.3 and 17.8 g dry weight L^{-1} were achieved. In contrast, biomass growth and isoflavone production were again more efficient in the SBB (20, 50 L filling volume) and the WUB (20 L filling volume) in comparison to the reusable stirred cell culture bioreactors. The reduced growth and isoflavone production in the reusable stirred bioreactor are ascribed to the higher shear stress that was assumed for this bioreactor type [45].

Kwon et al. [58] demonstrated the applicability of a disposable bag-based bubble column (3 L filling volume) for the production of recombinant cytotoxic T-lymphocyte antigen 4-immunoglobulin (hCTLA4Ig) using rice suspension cells. Oxygen transfer efficiency, mixing times, cell growth and protein expression were similar to a disposable wave-mixed bioreactor (3 L filling volume) and cell growth and hCTLA4 Ig productivity were slightly improved compared to a reusable stirred bioreactor (3 L filling volume). At this point, it should be mentioned that the pneumatically driven bench top bioreactor is referred to as an airlift bioreactor. As there is no visible mechanical separation created by a draught tube or baffles (dividing the flow in a riser and downcomer and being typical for airlift bioreactors) in the culture bag, we classified this disposable bioreactor as bubble column (and not as an airlift bioreactor type).

Interestingly, 400 L bag-based bubble columns are currently the largest disposable plant cell bioreactors in operation. They are based on the Life Reactor and are used in recombinant protein production (taliglucerase alpha, alpha galactosidase, alpha-1-anti-trypsin, anti-tumour necrosis factor fusion protein) by the company Protalix to grow genetically modified carrot cells. Details of this bioreactor design can be found in the respective patent [17].

2.3.6 Disposable Mist Bioreactor Systems

There are two disposable mist bioreactor designs being suitable for propagating plant organ cultures like embryogenic or hairy root cultures. These are the bench top systems developed by Pamela Weathers' group (1 up to 20 L root bed) and ROOTec company's 60 L mist bioreactor (Fig. 2.5). Both systems are based on a disposable bag in which a mesh matrix allows immobilization of cells and supports biomass growth [14, 53–55, 95]. The composition and preparation of Weathers' disposable mist bioreactor (versions with 1 and 4 L root bed) are comprehensively described by Liu et al. [54]. An ultrasonic nuzzle is applied in Weathers' disposable mist bioreactor for aerosol distribution in the headspace of the bag, whereas ROOTec's mist bioreactor uses a pneumatic distribution system (personal communication Jost Harr, ROOTec, February 2014). Mist reactors run either in batch or continuous mode. In batch mode (in which coalesced mist culture medium is collected) the

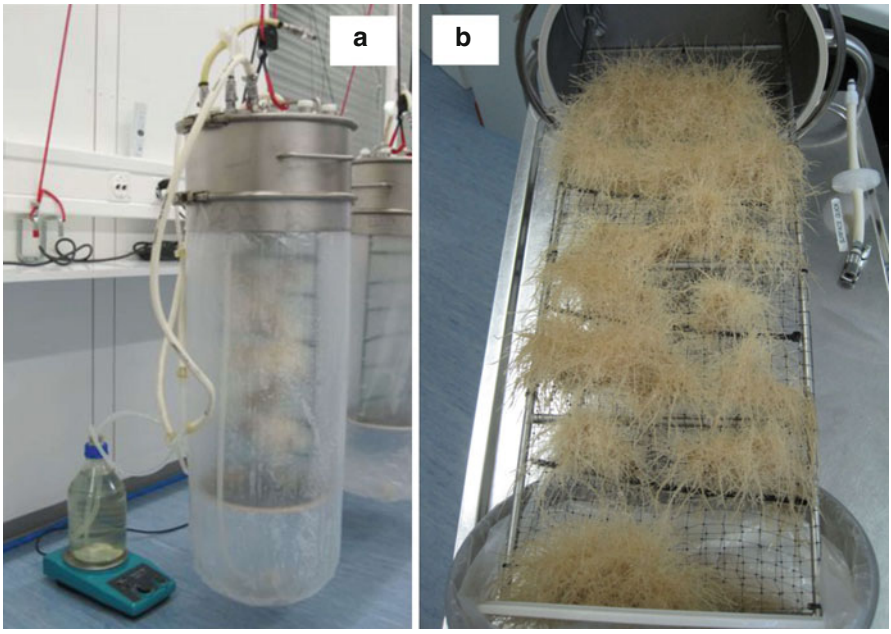

Fig. 2.5 ROOTec's disposable mist bioreactor. The mesh matrix supports root growth in long-term cultivation processes exceeding 3 or more months. This bioreactor was used in order to produce biomass from hairy roots of different plant species. Among others, hairy roots from *Atropa belladonna* (**a**), *Scutellaria baicalensis* (**b**), *Carlina acaulis*, *Cichorium intybus*, *Lepidium meyenii*, *Linaria alpina*, *Ocimum basilicum* and *Panax ginseng* have been grown. Photo and information were kindly provided by ROOTec (Switzerland)

medium can be recycled back into the bag after exciting it. In case of continuous cultivation, fresh medium is continuously fed into the bag and the mist culture medium is discarded after it has passed once over the organ cultures.

More reports have been published about cultivations in disposable Weathers' mist bioreactors than any other type of disposable mist bioreactor. In addition to embryogenic cultures from *Daucus carota* [55] mouse interleukin12 expressing tobacco hairy roots [54], *Artemisia annua* and *Arachis hypogaea* [95] hairy roots have also been successfully propagated.

2.4 Applications with Disposable Bioreactors

2.4.1 Tobacco Cell-Based Cultivations with Disposable Bioreactors

In this section, we present selected results of geraniol production using a transgenic tobacco cell suspension and a hairy root culture. Geraniol is an acyclic monoterpene alcohol that has a great potential for agriculture applications [96] as well as the

pharmaceutical and cosmetic industry [97–99]. Disposable mechanically driven bioreactors (wave-mixed BIOSTAT CultiBag RM, CultiBagRM orbitally shaken in the Multitron Cell and WandMixer with tumbling stirrer) were used for the studies at bench top scale (max. 25 L filling volume). The results obtained in batch and fed batch (feeding or feeding with medium exchange) experiments represent the basis for the scale-up approach targeting production of 1 g geraniol in disposable bioreactors that is described in Sect. 2.4.4.

Prior to the benchtop scale cultivation, investigations of growth and production behavior were carried out in non-instrumented disposable bioreactors (orbitally shaken erlenmeyers and TubeSpin Bioreactors) at mL-scale. The results indicated growth-coupled product accumulation for both transgenic cultures (the tobacco cell suspension culture of *Nicotiana tabacum* cv. Samsun NN and the hairy root culture of *N. tabacum* cv. Petit Havana SR1).

2.4.2 Cultivations with Geraniol Producing Tobacco Suspension Cells

Cell suspension cultures derived from transgenic tobacco (*N. tabacum* cv. Samsun NN) constitutively overexpressing *Valeriana officinalis* geraniol synthase (VoGES) [100] were maintained in Gamborg's B5 medium. The culture medium was supplemented with 0.1 mg L^{-1} kinetin, 1 mg L^{-1} naphthalene acetic acid and 20 g L^{-1} sucrose. The cells were subcultured once in a week. Growth conditions in the erlenmeyers were like the following: temperature 26 °C, illumination 16 h light, intensity ~95 µmols^{-1} m^{-2}, and 8 h darkness. The flasks were agitated on a shaker (25 mm shaking diameter) operating at 140 rpm.

Geraniol production was monitored in the wave-mixed BIOSTAT CultiBag RM with 2 L (1 L filling volume) or 20 L (10 L filling volume) culture bags and the 20 L Integrity Wand Mixer operated with 8.5 L filling volume. Production processes lasted between 16 and 23 days and were executed in batch mode with a light intensity of either 95 or 146 µmol s^{-1} m^{-2}. Illumination was ensured by internal LEDs in case of the BIOSTAT Culti Bag RM (Fig. 2.3), whereas illumination for the Integrity Wand Mixer was external (fluorescent tubes). Wave-mixed culture bags were inoculated with 10 % pcv (corresponding to 19.2 g fw L^{-1}) and operated at a rocking angle of 6° and an aeration rate of 0.1 vvm. The BIOSTAT Culti Bag RM's rocker rate was increased from 20 to 42 rpm during all cultivations. The Integrity Wand Mixer was operated at 40 rpm with an aeration rate of 0.3 vvm and the initial biomass concentration was 59 g fw L^{-1}. All cultivation conditions are summarized in Table 2.3.

Figure 2.6 shows the plots of biomass concentrations (Fig. 2.6a) and geraniol contents (Fig. 2.6b) achieved in wave-mixed culture bags. As expected, the conductivity (Fig. 2.6c) decreased during cell cultivations, ranging between 3.8 and 0.20 m Scm^{-1}. The pH-values (Fig. 2.6d) were in a typical range for plant cell cultivations (see Sect. 2.2.1). Assuming an identical initial biomass concentration for all experiments in 2 L wave-mixed bags, growth was similar and independent of the light intensity. Within 16 days we were able to generate peak biomass concentrations of

Table 2.3 Cultivation conditions for geraniol producing tobacco suspension cells

		BIOSTAT CultiBag RM with CultiBag RM 2 L		BIOSTAT CultiBag RM with CultiBag RM 20 L	ATMI Integrity WandMixer 20 L
Process mode		Batch	Batch	Batch	Batch
Filling volume	[L]	1	1	10	8.5
Cultivation time	[days]	16	16	18	16
Inoculation density	[%] pcv	10	10	10	5.9
Aeration rate	[vvm]	0.1	0.1	0.1	0.3
Rocking rate/ stirrer speed	[rpm]	20–38	20–42	22–37	40
Rocking angle	[°]	6	6	66	–
Illumination intensity	[µmol s^{-1} m^{-2}]	95	146	95	95
Light regime	[h]	16	16	16	16

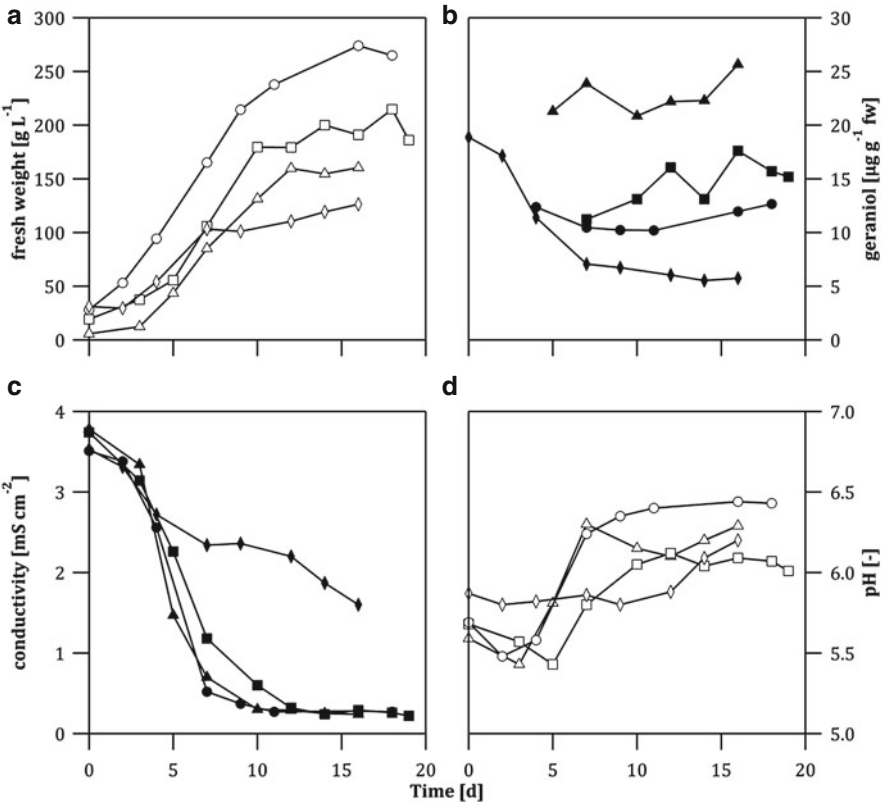

Fig. 2.6 Cultivation of geraniol producing suspension cells in the BIOSTAT Culti Bag RM and ATMI Integrity Wand Mixer under phototrophic conditions. (**a**) Biomass accumulation, (**b**) geraniol content, (**c**) conductivity, (**d**) pH-value. *Circle*: cultivation in a 20 L Culti Bag RM at 95 µmol s^{-1} m^{-2}. *Square*: cultivation in a 2 L Culti Bag RM at 146 µmol s^{-1} m^{-2}. *Triangle*: cultivation in a 2 L Culti Bag RM at 95 µmol s^{-1} m^{-2}. *Diamond*: cultivation in a 20 L ATMI Integrity Wand Mixer at 95 µmol s^{-1} m^{-2}

160 g fw L^{-1} of culture medium. Even a peak biomass concentration of 274 g fw L^{-1} was reached in the 20 L culture bag (containing 10 L culture broth) on the day 16. The geraniol containing biomass propagated in the wave-mixed 20 L bag was 21 % higher than that produced in the 20 L Wand Mixer (peak biomass concentration of 126.3 g fw L^{-1}). Geraniol accumulation in the produced biomass was determined according to the procedure described by Vasilev et al. [100] and in the medium it was determined in 5 mL medium aliquots with the addition of 0.5 mL buffer. Concentrations ranged between 12 to 26 µg g^{-1} fw in the wave-mixed bags and 5.7 µg g^{-1} fw in the Integrity Wand Mixer. Interestingly, up to 201.6 µg geraniol L^{-1} was detected in samples taken from the Integrity Wand Mixer medium. We determined that the 20 L Wand Mixer delivers 1.6 mg intracellular and 6.3 mg extracellular geraniol in comparison to 33 mg intracellular geraniol resulting from the biomass that can be produced in a 20 L wave-mixed bag. Consequently, it is more efficient to use a wave-mixed bioreactor for suspension cell-based terpenoid production and scaling-up processes.

2.4.3 Cultivations with Geraniol Producing Hairy Roots

Geraniol producing hairy root cultures have been established and maintained as described by Ritala et al. [41]. The roots were cultivated in Gamborg's B5 medium supplemented with 30 g L^{-1} sucrose. The inoculum for the experiments in the wave-mixed and orbitally shaken bags was generated either in petridishes or in orbitally shaken Tube Spin systems (Table 2.4). The Tube Spins are rigid centrifuge-like tubes which guarantee sterile gas exchange *via* the 0.22 µm membrane implemented in the cap. The Tube Spin 50 (10 mL filling volume) was inoculated with 10 g (fw) L^{-1} of hairy root biomass and was maintained at 26 °C, 240 rpm with a 25 mm shaking diameter for 10 days. Growth conditions were controlled in an INFORS HT Multitron shaker incubator. The Tube Spin 600 was operated with 60 mL filling volume at 120 rpm with the same INFORS HT control unit as the Tube Spin 50s.

All disposable bioreactors were inoculated with 5 g fw L^{-1} of hairy roots. However, the inoculation strategy was different (Table 2.4). The customized culture bags with screw caps were inoculated with whole roots, whereas the standard bags without screw caps were inoculated with root tips. The roots for these standard bags were cut and transferred into a 2 L medium storage bag prior to inoculation of the bioreactor culture bag and a so called "bag-to-bag" strategy was developed.

Experiments which were carried out under comparable process conditions in 2 and 20 L orbitally shaken (50 mm shaking diameter, 30 rpm, 0.2 vvm) and wave-mixed bioreactors (6°, 8 rpm, 0.2 vvm) revealed similar results for geraniol production, which were rather low (<10–11.8 µgg^{-1} fw). The biomass growth was similar in all bioreactor types, but was different in studied process modes applied in the cultivation (Fig. 2.7). The highest biomass growth was observed in the wave-mixed 50 L bag (Fig. 2.7 and Table 2.4). This approach culminated in a final biomass of 1.2 kg fresh weight, in which 7.9 µg intracellular geraniol g^{-1} fw was detected. Thus, the fresh root mass (in the 50 L wave-mixed bag) resulted in a total of 9.5 mg of intracellular geraniol in 57 days.

Table 2.4 Cultivation of geraniol producing hairy roots in various disposable bioreactors

Cultivation system/ bioreactor	Inoculum source	Duration [d]	Process mode	Filling volume [L]	Growth index[g]	Geraniol concentration [µg g^{-1} fw[h]]
2 L bag, BIOSTAT CultiBag RM	Petri dish	21	Batch	0.2[f]	27.8 29.8	8.8 8.7
		28	Fed batch[b]	0.2–0.3[f]	33.9 25.9	13.3 12.9
		29	Fed batch[c]		41.5	10.4
10 L bag, BIOSTAT CultiBag RM		28	Fed batch[b]	0.3–0.75[f]	92.6	8.8
20 L bag, BIOSTAT CultiBag RM	Petri dish[a]	29 28	Fed batch[c]	1–1.6[f]	17.5 37.1	6.3 11.8
50 L bag, BIOSTAT CultiBag RM	TubeSpin 600[a]	57	Fed batch[d]	2.5–4.5[f]	95.2	7.9
2 L bag, multitron cell	Petri dish	21	Batch	0.2[f]	26.6 24.2	9.2 9.2
20 L bag, multitron cell	TubeSpin 50[a]	29 57	Fed batch[c]	1–1.6[f] 0.5–2.6[f]	64.9 123.5	6.3 9.77
SBX-50	TubeSpin 600[a]	53	Fed batch[e]	5–10	20.5	9.63

[a]Single tips of hairy roots were inocultaed to the culture bag with a "bag-to-bag" strategy (see text)
[b]Two feeds
[c]Three feeds
[d]Two feeds and medium exchange on day 31, 38, 46 and 52
[e]Four feeds
[f]TI-like conditions
[g]Growth index represents the ratio of maximum biomass fresh weight to initial biomass fresh weight
[h]fw fresh weight

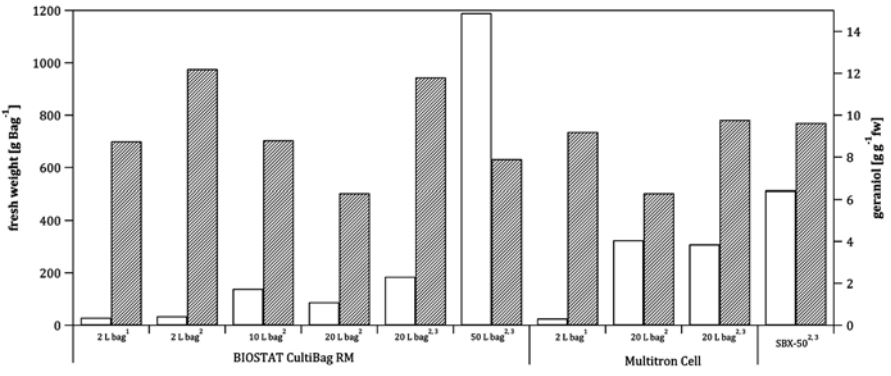

Fig. 2.7 Final biomass and geraniol contents in different disposable bioreactor systems. *White bar*: fresh weight; *Shaded bar*: geraniol content. [1] batch cultivation, [2] fed batch cultivation, [3] bag-to-bag and "single-tip" inoculation

In a SBX-50 production run (30–50 rpm, 0.4 vvm) 513.4 g fresh biomass delivering 9.6 µg geraniol g^{-1} fw was harvested after 53 days. This corresponds to a total amount of 4.9 mg of geraniol in a 50 L bag being roughly half of the amount produced in wave-mixed system within approximately same time period.

2.4.4 Generic Approach for Geraniol Production Process Up-Scaling

After comparing the results of the suspension cell- and hairy root-based geraniol production processes, we decided to work with the phototrophic suspension cell culture in order to develop a scale-up approach aimed at the generic production of 1 g geraniol in a wave-mixed bioreactor. According to our estimates this takes 41 days, using the three-step scale-up procedure depicted in Fig. 2.8. This includes (1) inoculum production in shake flasks, (2) inoculum-strain production in the BIOSTAT CultiBag RM 20/50 LED and (3) the geraniol production in the BIOSTAT CultiBag RM 600 LED.

The first step is based on the production of cells from the shake flask maintenance culture. It represents the generation of the inoculum for the initial production bioreactor, which is referred to as inoculum strain. Cells from the maintenance culture are used to inoculate five shake flasks (1,000 mL filling volume) with 400 mL culture volume in batch mode. After 1 week incubation (26 °C, 140 rpm, 25 mm shaking diameter, day 16 h – night 8 h rhythm, 95 µmol s^{-1} m^{-2}) sufficient cell material (pcv of 50 %) with high viability (>90 %) is available to start the inoculum strain production.

The second step requires the BIOSTAT CultiBag RM 20/50 equipped with a LED illumination platform. Pooled cells (biomass pcv of 10 %) from step (1) serve as inoculum for the 20 L culture bag running with 10 L filling volume at 26 °C, 0.2 vvm at 16 h light (95 µmol s^{-1} m^{-2}) and 8 h dark in batch mode. Driving the disposable bioreactor at a rocking angle of 6° and a rocking rate between 20 and

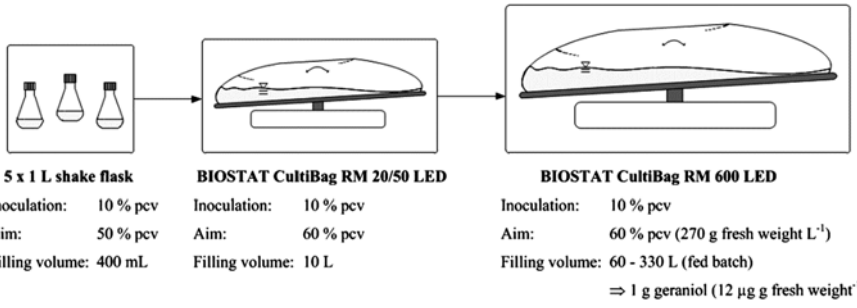

Fig. 2.8 Scheme of the generic approach for the manufacture of 1 g geraniol with suspension cells in wave-mixed bioreactors

38 rpm resulted in a biomass pcv of 60 % after 9 days. This is sufficient to inoculate the production bioreactor that should be operated in fed batch mode (two feeding steps).

The BIOSTAT CultiBag RM 600 equipped with LEDs was chosen for the geraniol production step (3) lasting for 25 days. The cell medium (10 L) from the 20 L culture bag has to be transferred into the 600 L culture bag and diluted with 50 L culture medium to be able to reach the initial biomass pcv of 10 %. After 5 days operation (rocking angle 6°, 14–20 rpm, 0.3 vvm) biomass pcv reaches 30 %, followed by a first feed with 120 L culture medium. By increasing the filling volume of the bag up to 180 L, biomass pcv drops to 10 %. Subsequently, the cultivation is continued for another 5 days until a biomass pcv of 30 % is ensured. Afterwards, second feeding is performed by adding 150 L fresh culture medium. This action involves a slight overfilling of the culture bag of about 10 % (as already mentioned, the maximum filling volume of wave-mixed bags is normally defined as 50 % of the total bag volume by the manufacturer). In addition, pcv drops to 16 %. After this final manipulation, cultivation runs 15 days until a biomass pcv of 60 % (corresponding to a final biomass concentration of 270 g fw L^{-1}) is achieved. In more specific terms, the 600 L culture bag provides a total amount of fresh biomass of 89.1 kg delivering 1.07 g geraniol (if we assume a similar geraniol concentration as detected in the experiments with 20 L culture bags).

2.5 Summary and Future Prospects

Disposable bioreactors are suitable for both cultivation of plant cell suspension and hairy root cultures. For the development and production of high-value products such as biopharmaceuticals e.g. therapeutic antibodies, disposable single-use versions are preferred, whereas disposable multi-use systems have advantages for low- and mid-value products due to the costs of the individual bags. Taking into account the physiological characteristics, growth, biosynthetic capacity of production cells and the required scale of the bioreactor, the user can choose a disposable bioreactor type that is mechanically, pneumatically or hydraulically driven. Today, the highest numbers of disposable bioreactors are available for suspension cells with a low to medium cell growth in Newtonian culture broths. Users have access to commercially available disposable bioreactors with up to 2 m^3 filling volume. The 400 L bag-based bubble columns from Protalix represent the largest disposable plant cell bioreactors available at the time of writing this article. Disposable bioreactors instrumented with standard sensors are recommended in order to monitor the process in case light is required for cultivation.

Mass transfer limitations may occur if the production cell line grows rapidly and there is a high increase of culture broth viscosity. Due to the possible limitations of power input in wave-mixed bioreactors with 1-D oscillatory motion, those with 2-D or 3-D oscillatory motion should be chosen in such cases. The use of disposable bubble columns and stirred bioreactors should also be investigated. However,

aeration with micro-spargers, which can result in strong flotation (and thus loss of cells and product) should be avoided.

Indeed, the most difficult types of cultivation to perform involve organ cultures, in particular hairy roots, whose root integrity must be maintained, since homogeneous root growth without mass transfer limitations must be guaranteed. In addition to wave-mixed bioreactors with 1-D oscillatory motion (that operate in a TI-like mode) disposable mist bioreactors are most suitable for hairy roots.

Promising results from cultivations with plant cell suspensions and hairy root cultures, and the growing interest in new plant cell-based products such as vaccines and bioactive substances for cosmetics will contribute to the increased application of disposable bioreactors in the future. It is undoubted that disposable bioreactors will allow more rapid and cheaper development and manufacture of products. If low- and mid-value products are the focus, there is the need for low-cost culture bags. The need for expensive, GMP approved culture bags that are currently used by the majority is questionable for these products.

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Chapter 3
Plant Derived Bioactive Molecules: Culture Vessels to Bioreactors

Poojadevi Sharma, Sonal Sharma, Sheetal Yadav, Anshu Srivastava, Indira Purohit, and Neeta Shrivastava

Abstract Bioactive compounds are the compounds having pharmacological or toxicological effects on humans and animals. At present, the bioactive compounds are categorized into two groups, secondary metabolites (originating from natural sources like plants, mammals, fungi, bacteria etc.) and therapeutic recombinant molecules (which are produced by using recombinant DNA technology in bacteria, mammals, plants etc.). Of the compounds produced from various sources, secondary metabolites produced from plants command highest market demand. Plants are also proved to be an ideal host system for the production of recombinant therapeutic molecules. Therefore, there has been a long pursuit for development of a technology which can provide high yielding plant based bioactive production system. An amalgam of plant cell culture and bioreactor technology was crucial in this direction. The plant bioreactor technology, so developed, has been put to the test many times for commercial scale production of plant bioactive molecules. There have been instances of success, but in general growth of plant bioreactor industry has been very slow. This chapter highlights various aspects of slow but successful growth of plant based bioactive production from culture vessel to bioreactor. We have evaluated the key drivers and accelerators which have made the journey of plant bioreactor industry successful. Speed breakers of this journey have also been discussed. Thorough and rigorous analysis of these parameters may help the industry/academia to speed up the growth of plant bioreactor industry for the production of bioactives.

Keywords Bioactives • Plant Bioreactors • Culture vessels

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3.1 Plant Bioreactor for Bioactives: Achievements vs Expectations

Bioactive compounds are the compounds having pharmacological or toxicological effects on humans and animals. Historic representatives of bioactives are secondary metabolites obtained from various natural sources like bacteria, fungi, mammals, plant etc. With the advent of rDNA technology therapeutic recombinant molecules are also included in this category. Amongst the various natural sources, bioactive molecules from plants command highest consumer demand. However, the commercial productivity of these molecules is influenced by low yield from natural sources (bioactive molecules are usually less than 1 % of the plant dry weight) and high cost of chemical synthesis. Therefore, plant cell culture technology was looked upon as an alternative production system for these high valued molecules. Subsequently, an amalgamation of plant cell culture and bioreactor technology has resulted in development of successful plant bioreactor technology for commercial scale production of plant bioactive molecules. Shikonin was the first bioactive to be produced at commercial level, Mitsui Petrochemical Industry Co. Ltd. (Japan) achieved this feat from plant cells of *Lithospermum erythrorhizon* in 750 L bioreactor in 1984. After a dry spell of about 20 years, another success was reported in the year 2002 for the production of Taxol by Bristol-Myers Squibb and Phyton Biotech, Inc. Till date, it is the largest commercial application of plant cell culture, utilizing the Chinese yew (*Taxus chinensis*) cultivated in 75,000 L bioreactors [1].

Realizing the potential of plant as an apt host system for recombinant therapeutic protein, the plant bioreactor industry took a leap in this area as well. Year 2012 was a hallmark year for plant bioreactor industry for recombinant protein production. The FDA (USA) gave its first ever clearance to a plant-made pharmaceutical product, Elelyso™ for treating Gaucher's disease [2]. A list of plant based bioactives produced commercially at bioreactor level and their manufacturers is given in Table 3.1. These examples clearly indicate that plant based bioactive production can be viably upscaled from culture vessels to bioreactors to achieve market scale productivities. However, the timelines of success do hint a fact that growth of plant bioreactor industry has been slow (Fig. 3.1). This chapter aims at discussion of various aspects of slow but successful growth of plant based bioactive production from culture vessel to bioreactor and also makes plant scientists to ponder over exploiting the potential of plant cell with measures to take care about its limitations (Fig. 3.2).

3.2 Key Drivers for Success in Plant Bioreactor Technology

Applied facet of plant tissue culture technique has provided a platform for mass production of plant derived bioactives using bioreactor technologies. Culture vessel phase of plant tissue culture technique helps in providing proof of concept which can further be translated into mass production strategies. Mass production in plant

Table 3.1 Plant based bioactives produced commercially at bioreactor level [10, 38]

Plant system	Bioactive	Manufacturer
Heterologous proteins		
Daucus carota	Elelyso™	Protalix (Israel)
Oryza sativa	Cell culture products (rh-lactoferrin, rh-albumin), (rh-lyzozyme, rh-transferrin)	Invitria (USA)
Secondary metabolites		
Catharanthus roseus	Arbtin	Mitsui Chemicals, Inc. (Japan)
Coptis japonica	Berberines	Mitsui Chemicals, Inc. (Japan)
Echinacea purpurea Echinacea angustifolia	Echinacea polysaccharides	Diversa (Germany)
Panax ginseng	Ginseng	Nitto Denko Corporation (Japan)
Taxus spp	Paclitaxel	Phyton Biotech, Inc. (USA/ Germany) Genexol® – Samyang Genex (Korea)
Podophyllum	Podophyllotoxin	Nippon oil (Japan)
Coleus blumei	Rosmarinic acid	A. Nattermann & Cie.GmbH (Germany)
Duboisia spp	Scopolamine	Sumitomo Chemical Co., Ltd. (Japan)
Lithospermum erythrorhizon	Shikonin	Mitsui Chemicals, Inc.(Japan)

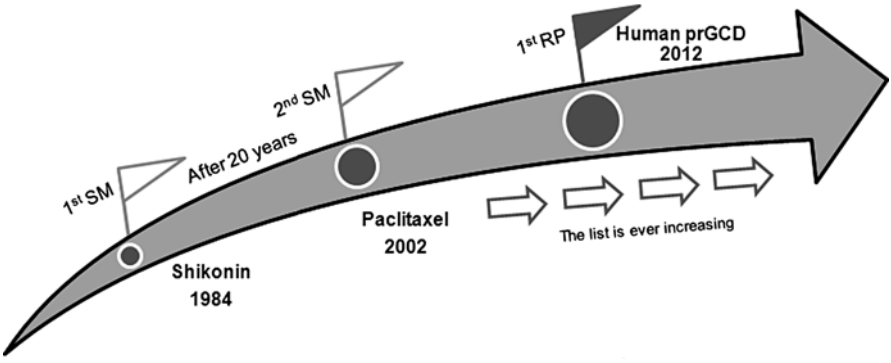

Fig. 3.1 Industrial bioreactor based bioactive production and market launch timeline. First marketed secondary metabolite (*SM*) was Shikonin by Mitsui Petrochemical Industry Co. Ltd in 1984 and second was Paclitaxel by Bristol-Myers Squibb in 2002. The gap was about 20 years. Following this, many secondary metabolites were launched in market using bioreactor technology. The only recombinant protein (*RP*) produced by bioreactor technology is human glucocerebrosidase (human prGCD) by Protalix BioTherapeutics which was launched recently in 2012

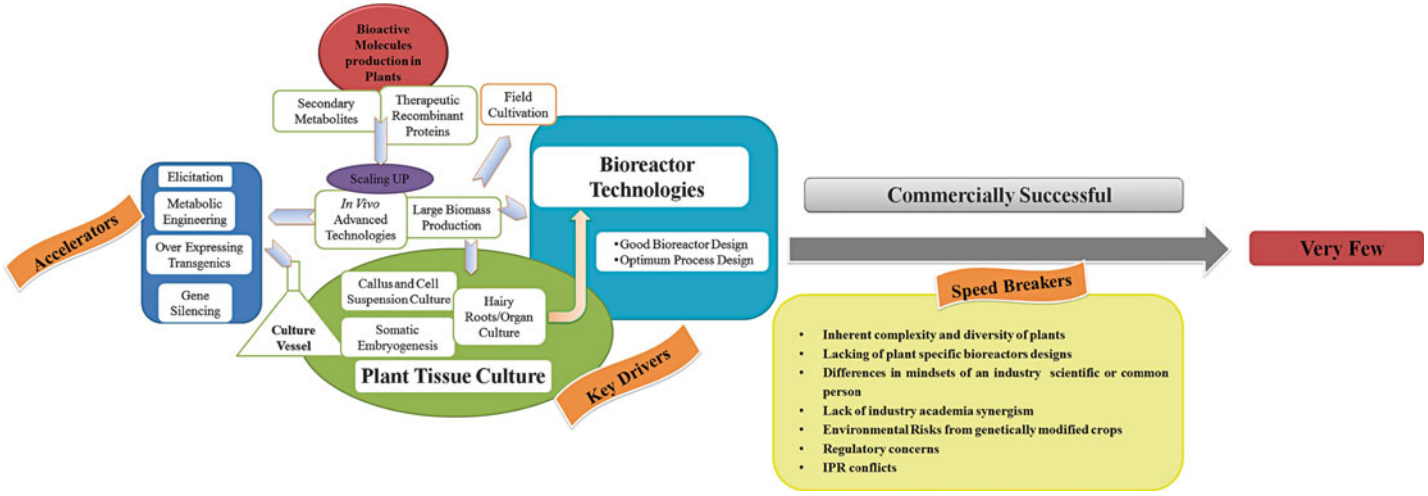

Fig. 3.2 Key drivers responsible for success stories of plant bioactives, accelerators to excel this success and different speed breakers which hamper the growth. Large scale production of natural as well as recombinant therapeutic molecules can be done by three ways- field cultivation, plant tissue culture and biomass production in bioreactors. Plant tissue culture can provide platform for production of bioactives for bioreactors. Process can be developed and optimized in culture vessels at small scale and then taken to fermenters. Some allied advance technologies can be applied *in vivo* to enhance the production

bioreactors provides stable yields in less space and controlled conditions. Following systems are recognized as key drivers for the success of plant bioreactor technology.

3.2.1 Callus and Cell Suspension Culture

Cell suspension culture provides higher biomass, fast growth and homogeneity in cells. Various secondary metabolites such as taxol, catharanthine have been produced using the cell suspension culture and callus culture at laboratory scale [3]. BY-2 and NT-1 cell lines of Tobacco are well characterized to support the production of recombinant bioactive molecules [4]. Many proteins including human anti-rabies monoclonal antibody and erythropoietin have been successfully produced using these cell lines [5]. Further, these cell lines have potentiality to secrete 0.6–80 KD protein molecules in the medium which helps in the reduction of down-stream processing cost [6]. Other plant cells such as rice, tomato and carrot have also been used for this purpose [7–9].

Suspension cultures have also proven their potential for their immediate application in bioreactors for industrial scale production. Elelyso (Taliglucerase alfa), a May 2012 USFDA approved recombinant drug for the treatment of Gaucher's disease and high paclitaxel producing *Taxus baccata* cells are some other examples for commercial scale production using cell suspension cultures in bioreactors [2, 10]. Suspension cultures of *Glycine max* and *Nicotiana tabacum* at capacity of 10–100 L, suspension culture of *Hyoscymus muticus* for the production of hyoscyamine in 100 L capacity bioreactor, production of anthraquinones form the cultures of *Frangula alnus,* production of azadirachtin from *Azadirachta indica* are some of the culture in the pipeline of commercialization [11–13].

In our laboratory, we have seen the potential of callus culture for the production of plant bioactive molecule vasicine. We found 0.011 % w/w vasicine concentration in leaf callus of *Adhatoda vasica* and 0.13 % w/w concentration of total sennosides in callus mass of *Cassia senna* [14].

3.2.2 Hairy Root Culture

Hairy root cultures being differentiated cultures are successful system in the production of bioactive molecules using bioreactors. Various secondary metabolites such as berberine, resveratrol and taxol as well as recombinant proteins such as murine lgG1 monoclonal antibody are successfully produced in this system [5, 15–17]. Hairy root cultures are genetically stable for long time and provide consistent expression of proteins in a relatively short time with higher biomass [18]. Hairy root culture of *Panax ginseng* is the present successful example for the commercialization of this technique for biomass production [3]. Reports have demonstrated the success of Atropin and Ginseng production through hairy roots which is more than the field

grown plants. ROOTec, a German based company produces camptothecin and podophyllotoxin from the hairy root cultures [19].

3.2.3 Bioreactor Design

Novel design concept has played a pivotal role in success stories of plant bioreactors. Different culture types require different optimized design considerations. Although many of the bioactives produced successfully in the conventional microbial bioreactors such as stirred tank reactor, bubble column, airlift etc. but the different prerequisites of plants such as higher sensitivity to shear stress due to a rigid cell wall, long generation time etc. raise the need of some modifications. Bioreactors with good mixing and lower shear stress are preferable for cell cultures [12, 20]. However, the most recent development in this field is the use of disposable bioreactors containing growth chamber made up of FDA approved biocompatible plastics [12]. These bioreactors use pre-sterilized bags which reduce the possibility of contamination and hence unnecessary efforts and costs required to maintain the production safety are negligible. US FDA approved Elelyso (Taliglucerase alfa) was produced in 400 L capacity disposable bioreactors by Protalix [10]. High paclitaxel production using *Taxus baccata* cells is another successful example of Big wave™ disposable bioreactor. Nestle R&D centre in France developed two disposable bioreactors of capacity 10–100 L (the wave and undertow bioreactors and slug bubble bioreactors) for culturing cell suspension of *Glycine max* and *Nicotiana tabacum*. Ebb and flow bioreactor is used to produce Hyoscyamine from the cell suspension cultures of *Hyoscymus muticus* of 100 L capacity [12, 13].

The bioreactors used for hairy root cultures are of three types: liquid phase, gas phase and hybrid [10]. Gas phase nutrient mist bioreactors and temporary immersion systems save the roots from hyperhydricity [21]. Introduction of meshes, cages and polyurathene foam for immobilization of roots give opportunity to culture roots in submerged conventional bioreactors [12]. Bubble column and airlift reactors are more successful conventional reactors than stirred tank at commercial level for roots due to their simplicity. However, hyperhydricity is one of the major concerns, so mist bioreactors or temporary immersion systems are preferred. The hairy root based company ROOTec developed their own mist bioreactors for the production of many secondary metabolites from the hairy root cultures of different plant species [19] (http://www.rootec.com/en/products/all-products). Many high capacity disposable reactors are also available commercially for the production from hairy roots [12].

3.2.4 Process Design

Operational considerations are also important factors for success of bioreactors based on cell cultures and hairy root cultures. Designing of bioreactor systems for suspension cultures is directed towards the reduction shear stress. Proper oxygen supply and

gaseous exchange are a very critical factor in case of cell suspension cultures. More gaseous flow than required can cause evaporation of some essential components. Improper aeration gives rise to foaming problem in cell culture during scale up. Temperature should be such which should not affect the bioprocess activities in the culture with the maintenance of the metabolite production. The optimum temperature should be 20–23 °C but can vary with the species as well as with the type of product [20, 22]. Many cell suspension cultures have tendency to form aggregates with different morphology. Deviation from the desired type of aggregation may affect the culture growth and cell-cell interaction which is necessary to maintain the productivity. Viscosity of culture medium changes due to formation of aggregates and high biomass. Accumulation of other metabolites can also change the rheology of the medium and hence growth. Consideration of shear stress is also necessary for the hairy roots as it can activate the wound response in hairy root cultures resulting in callus formation, thus causing reduction in productivity [20]. Roots can form a network due to which uniform supply of the medium may be obstructed. Mineral elements are the very important factors for the growth of roots and to increase biomass. Immersion time is a critical factor for root cultures and over lodging can cause hyperhydricity. Inoculum size also affects the productivity. More tissue mass can cause problems during scaling up.

3.3 Accelerators for the Plant Bioreactor Technology

Use of *in vitro* culture system coupled with genetic engineering has proved as an accelerator in the bioreactor technology. Prospects and skills of production of non-botanical products like expression of human genes in the plant system, transfer of non-botanical or trans-botanical metabolic pathway specific gene in the culture system have proved to be potential accelerators of the technology. Some of the examples like overexpression of genes encoding Limonene synthase in peppermint, overexpression of Chalcone isomerase resulting in increased flavonoids upto 78 % in tomato peel (*Lycopersicum esculentum*) [23], expression of a set of genes from marine sources encoding the fatty acid chain elongation and desaturation enzymes required for the synthesis of LC-PUFA from their C18 PUFA precursors in *Arabidopsis thaliana* seed, have helped the technology to be commercially more viable [24].

3.4 Speed Breakers in Plant Bioreactor Technology

As evident in Fig. 3.1 the pace of applied and commercialized growth of plant bioreactors is very slow in spite of having lot of potential and advantage over other bioreactor systems. Major reasons of this slow pace are associated with inherent complicated nature and rich diversity in the plant systems, and some are with the gap between academics and industries. Limited knowledge/acceptability of genetic engineering and transgenics are another eclipse in this growth. Following are some of the troughs which make the growth slow and which should be worked out to get maximum output by investing time and energies very smartly.

3.4.1 Plant Culture Systems

Many plants are non-amenable to grow in culture, those that could be easily grown under suitable conditions may lack desired biosynthetic activity or yield is too low to be commercially viable. Inherent features of plant cell system chosen for up scaling in plant bioreactor itself sometimes limit the process. Plant cell suspension cultures are heterogeneous in nature, therefore, bioactive yield from such cultures is variable. Probability of genetic instability makes the production inconsistent. In case of hairy root cultures, their growth creates a tight matrix within a culture, leading to nutrient transport limitations that result in areas of senescent cells [25]. These nutrient gradients also attribute to variability in root growth and productivity. Yield can also impede due to hyperhydricity which makes the roots very vulnerable. Further, both plant cell suspensions and hairy root cultures are sensitive to shear stress and mechanical agitation resulting in cell wounding [25]. Low protein production, contamination, high downstream processing cost, degradation of secreted protein in medium by proteolytic enzymes are the major limitations for the production of recombinant bioactive molecules in plant systems.

3.4.2 Post Translational Modifications

Being safer for humans, plants are considered as the most suitable host system for engineered biothearpeutics. Plants are easily approachable and can be utilized in raw form, this has given birth to the concept of edible vaccines. However, the highly modified and evolved internal physiological system of plants works as a barrier to produce foreign proteins in their original efficient form. There are many events such as proteolysis, misfolding, aggregation, oxidation of methionine, deamination of asparagine and glutamine, and glycosylation occurring in cells during the post translational modifications (PTMs) of recombinant bioactive molecules. All of these PTMs are important for the stability, solubility, bioactivity, efficacy and efficient secretion of the proteins. Glycosylation is one of the most crucial steps regarding the addition of correct glycans to the proteins in cells. Plants are highly competent and capable for the production of large and complex proteins and additionally plants provide higher biomass which is directly linked to the maximum production of bioactive molecules. However, during the post translational modifications plants add some unwanted β (1,2)-Xylose and core α (1,3)-Fucose residues instead of core α (1,6)-Fucose residues and terminal Neuraminic acid (NeuAc) resulting it to become immunogenic to humans [26, 27]. NeuAc, which is responsible for the activity and stability of the proteins, is not found in a plant. Till date, many bioactive molecules such as erythropoietin, human haemoglobin, human epidermal growth factors and human serum albumin etc. have been produced in plants [28]. Still, none of these products are available in market except glucocerebrosidase produced in the carrot suspension cells [8]. Therefore, protein glycosylation is one of

the major concern in the plant cells for the production of bioactive molecules which needs proper attention to resolve the problem of improper glycosylation in plants.

3.4.3 Bioreactor System Designs

The concept of bioreactors originated mainly for the microbial products and then extended to the higher eukaryotic systems. Earlier the microbial bioreactor designs were utilized for plants but different requirements of the systems raise the need of designs specifically developed for the plants like bubble column bioreactor, temporary immersion systems, continuous flow reactor, etc. An important critical step in general for all bioreactors is determination of inoculum size for differentiated plant *in vitro* systems [29]. Achieving uniform distribution of the cultures in the growth chamber is very difficult. Different techniques need to be used for inoculation in a bioreactor like using of a seed vessel to obtain inoculum roots that were transferred aseptically by means of helical screw in a 500-L hybrid reactor; homogenization and transfer of the biomass into bioreactors as slurry; change of the cultivation mode from batch to fed-batch [30]. Different bioreactor types can significantly affect the culture growth and product accumulation. Therefore, development of such processes needs optimization in direction to develop plant bioreactor based technologies.

3.4.4 Scientific Mindset

Scientists focus primarily on research rather than business or regulatory aspects. Basic researches are driven mainly by the curiosity and interest of a particular scientist. Research areas like development of *in vitro* cultures, elicitation, plant genetic engineering, bioreactor upscaling, etc. requires long period of time to get successfully translated into commercial value. Scientists are more comfortable with their slow pace of research and have patience to carry on research for years even with negative results because curiosity to solve the unresolved mystery. Such slow and long time frame for a research solution cannot cope up with the fast changing industry market interests. Academia should make to stand stable to cope up with fast changing technologies. Lack of proper resources and infrastructure also inhibit the scientists to take their efforts to commercially practical scale.

3.4.5 Industrial Mindset

Establishing plant fermentation systems involve large capital start-up costs. The batch times for plant cultures are very large, so maintenance and monitoring needs are also big. Industry thinks in terms of short range goals and wants result in short expected time frames. Industry cannot risk delays and loss of profits. While

considering money investment, industry prefers a low risk industry with proved profitable products. Mammalian cells are widely used by industry for the production of recombinant therapeutics which exhibit satisfactory glycosylation. Further industry is quite experienced with handling regulatory guidelines and cGMP issues for mammalian bioreactor industry as compared to plant bioreactor industry which is still emerging. On account of these factors, industry is still hesitant to venture into commercial plant bioreactor sector.

3.4.6 Lack of Academic and Industry Synergism

Differences in mindsets of an academician and a corporate person stop them to come together on a single platform. The working culture differences keep these two heads apart. An industrialist always thinks for profit and prefers to secure their researches in the form of patents or in the name of trade secrets. In contrast to institutes, leakage and sharing of knowledge is unacceptable in an industry. Demands of huge returns limit the institutes to work with industries. Mutually exclusive preferences, demands, visions and research achievements criteria weaken the faculty and firm collaboration. Slow pace of research in the academic institutes does not match with the higher expectations of industries of getting quick results. Examples are there which exhibited the power of crossing the lines to work together and enhances the surety of success. The successful production of shiknonin from the plant culture was a result of combined efforts of Kayoto University and Mitusi petrochemical in Japan. Another example of successful alliance is Kitasato University and Nitto Denko in Japan, resulting in the production of ginseng [31]. Existence of very few illustrations shows the necessity for more synergistic efforts with crystalline purity, sincere, honesty and immaculate transparency which are very essential to create and also to maintain the collaborations.

3.4.7 Public Mindset

The public i.e. consumers are the ultimate decision makers for a product to be commercially successful in market. At present, public has many issues with plant recombinant bioactive molecules.

Ethical Issues

Ethical issues of certain groups of public, including religious bodies have been major determinants in withdrawal of many such bioactive molecule producing plants from fields. They find it unethical or inhumane to introduce gene of animal or human origin into plants [32, 33].

Health Issues

General public is also worried about risk of allergenicity (usually glycoproteins) of these new recombinant bioactives from plants [34]. Transgenic technologies use sensitive genes such as, antibiotic marker genes and promoter sequences derived from viruses. During repetitive plant transformations, antibiotic resistance genes will accumulate and plant breeders will soon encounter difficulties in locating new, harmless antibiotic marker genes. The obvious fear is that antibiotic marker genes could be recruited into humans along with the gene for foreign proteins (and domestic animals) rendering antibiotics ineffective in curing bacterial infections. Plants producing insulin, growth hormones, plantibodies etc. are direct applications of genetic engineering for human health but for strict vegetarians it could pose an ethical issue.

Socio-economic Issues

Developing countries believe that genetically modified whole plant bioreactors are profit crops majorly for western developed countries and developing countries are only being exploited in the business for growing these plants at cheap rates. In midst of such anti-GM public perception, it is not easy to convince investors to fund plant bioreactor industry or even any related academic research.

3.4.8 Environmental Risks from Transgenics

Plants prove their potential to be a good choice for foreign protein production hence they are themselves considered as a natural bioreactors as genetically modified crops but these efficient bioreactors can harm ecosystem, food webs, biodiversity and germplasm. There are ample evidences that transgenic crops and their genes, through pollen dispersal, can spread even between species. There is also a low probability of chloroplast movement from transgenic oilseed rape into wild species [35]. The effects of transgene escape on the environment are uncertain, result into “genetic pollution”, for example tailoring herbicide resistance, poses threat that what and how much of the herbicide should be used, its persistence and residual effects and development of resistant target species or gene flow to non-target species. This can also threat ecosystems and biodiversity. Gene flow increases outcrossing that out competes in the ecosystem. Genetic transformation can harm biodiversity by reduction in insects that serve as food at higher trophic levels. For instance monarch butterflies feeding on GM corn leaves had deduced growth [36]. These risks may not be visible instantaneously but should be taken care of.

3.4.9 Regulatory Concerns/IPR Conflicts

The commercialization of plant biotechnology has advanced rapidly over the past 5 years. Intellectual property rights, mainly in the form of patents, have been fundamental to the commercial development of the technology. Several hundred patents on plant genes, techniques for genetic modification and transgenic plants have now been granted and many more have been filed. Although patenting in biotechnology generally is now widely practiced by public and private sector researchers alike, excessively broad claims and restrictive licensing remain a potential threat for innovation. Patenting and licensing in this area restricts competition and increases monopolies on key plant technologies. This may further restrict innovation, fair access and trade. The ultimate outcome in this direction could be decline in willingness to invest in research and development and share knowledge in public domain. Ownership of genes and the need for patents is a further area for ethical debate. Innumerable IPR court cases are filed among farmers and public sectors of developing countries, plant bioreactor industry, anti-GM NGOs, academicians who developed the technology etc.

Patents based on the natural therapeutic products are also a matter for concern. Most of the biodiversity is concentrated to some developing countries. They have ample resources to generate plant based natural products but are deprived in research resources. Most of the plant based therapeutics extract from the traditional knowledge acquired by the indigenous communities since time immemorial. Traditional knowledge of these countries is exploited by the multinational companies, modified, utilized and filed as patents. Resource limitations in developing countries restrict them to use their legacy for themselves and bound to buy their modified versions in high amount. Many examples of biopiracy are there like use of turmeric, neem, hoodia plant, banana extract; melon extract etc. for treating various diseases provokes the governments to raise voice to protect their traditional knowledge. Claiming rights related to improvements in plant traits (like enhanced yield of secondary metabolite) using advance technologies can also raise clashes because the basic genetic information used for transgenic plant bioreactor development is extracted from the ecosystems of developing nations [37]. These unnecessary IPR conflicts slow the research pace and reduce the productivity.

It is clearly evident that neither academia nor even industry would want to involve in such legal issues, therefore the industry would more likely feel comfortable in investing for a proposed plant bioreactor strategy only after thorough thought process which would require some considerable time investment.

3.5 Conclusion and Future Perspectives

Advances in the biotechnology particularly methods for culturing plant cell cultures has provided new means for the commercial production of even the rare medicinal plants and chemical they provide, so there has been a considerable interest in plant cell cultures as the potential alternative to the traditional agriculture for the

industrial production of the secondary metabolites. The objectives of many industries are to develop plant cell culture techniques to the stage where they yield secondary products more economically than the whole plant grown under natural conditions or synthesizing the product.

Design of a suitable bioreactor with low-shear impeller, and selection of an appropriate mode of cultivation is required for increased metabolite production. Optimization of medium ingredients by statistical techniques, application of appropriate mathematical models for optimized cell cultivation, feeding strategy of metabolic precursors, and extraction of intracellular metabolites by organic solvents can lead to significant enhancement in productivity of secondary metabolites.

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Part II
Production of Biomass and Bioactive Compounds from Cell Suspension Cultures

Chapter 4
Production of Carotenoids Using Microalgae Cultivated in Photobioreactors

Alexei Solovchenko and Konstantin Chekanov

Abstract Carotenoids comprise a diverse group of natural biomolecules with a plethora of beneficial effects. These compounds include potent bioantioxidants, provitamins, and safe colourants that are in high demand by pharmaceutical, cosmetic and food industries. A few species of unicellular algae (called carotenogenic microalgae) mainly the representatives of Chlorophyta, are among the richest biological source of carotenoids such as β-carotene and astaxanthin. This chapter covers the mass cultivation of the microalgae in closed systems (photobioreactors) for the production of value-added carotenoids. The biochemistry and regulation of the biosynthesis of secondary carotenoids are considered together with the biotechnology of most important carotenogenic microalgae species. Special attention is paid to the real-time optical monitoring of carotenoid accumulation in microalgal cultures.

Keywords Astaxanthin • β-carotene • Carotenoids • Dunaliella • Haematococcus • Microalgae • Photobioreactor • Stress

Abbreviations

Car	Carotenoid(s)
OB	Oil bodies
PAR	Photosynthetically active radiation
PBR	Photobioreactor(s)
PSA	Photosynthetic apparatus
PTOX	Plastidial terminal oxidase
ROS	Reactive oxygen species

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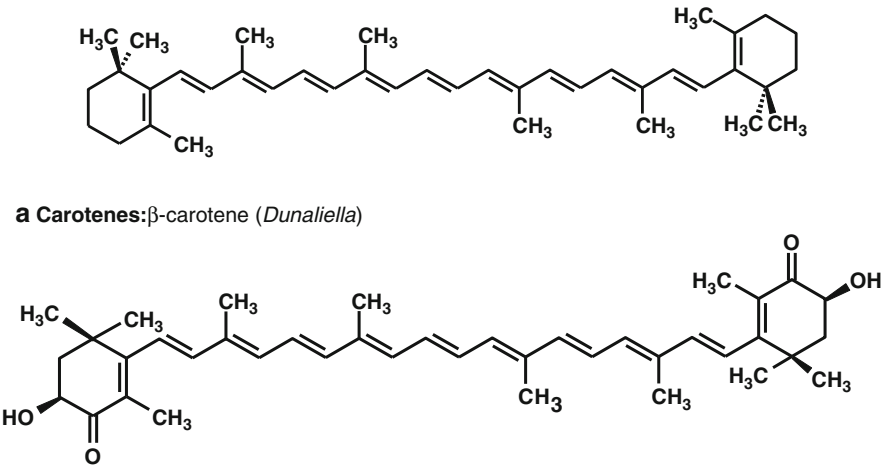

Fig. 4.1 Carotenoids include (**a**) carotenes and (**b**) xanthophylls native or non-native to photosynthetic apparatus;the latter could be accumulated as secondary (extrathylakoid) carotenoids [35, 44]

4.1 Introduction

"Carotenoids" is the name of a diverse (ca. 800 members) group of C_{40} coloured lipid-soluble molecules ubiquitous in photoautotrophic organisms [1–3]. In nature, carotenoids (Car) serve mainly as the accessory light-harvesting pigments and photoprotective compounds preventing photo-oxidative damage to photoautotrophic cells [4–6]. These compounds are also responsible for the red, orange and yellow colours of leaves, fruits, as well as many flowers. Numerous animal species contain carotenoids (e.g. astaxanthin in shrimp and salmon or rhodoxanthin in bird feathers) which are absorbed from the food, mainly unicellular algae [2]. The remarkable ability of Car to eliminate harmful reactive oxygen species (ROS) and other radical and non-radical harmful species in the cell [7, 8] is determined by the characteristic structure of these compounds featuring nine or more conjugated double bonds (Fig. 4.1).

It was found that Car exert a plethora of beneficial effects in animals and humans [9]; at the same time these organisms cannot synthesize Car pigments and the only source of Car for them is through the uptake of food. Probably the most studied is the role of vitamin A (retinol) produced from its precursor β-carotene. Being a potent scavenger of the free radicals, Car protect essential biological functions within the cell by terminating peroxidation of membrane lipids, preventing oxidative damage to proteins and DNA by UV and other pro-oxidant factors [10, 11]. Accordingly, Car could prevent or in some instances, cure various diseases such as cancers of various types, chronic inflammatory diseases, metabolic syndrome, diabetes, diabetic nephropathy, cardiovascular diseases, gastrointestinal diseases, liver diseases, neurodegenerative diseases, eye diseases, skin diseases, exercise-induced

fatigue, male infertility and renal failure [12–14]. The knowledge on the biological roles of carotenoids in humans and animals is advancing rapidly providing an impetus for research on the efficient ways of the production of Car.

Due to the important role of these pigments in pigmentation, growth and reproduction of commercially valuable animal species, certain Car found extensive use in the food and feed industry [15, 16]. Aquaculture which is currently the fastest growing sector of agriculture is another field supporting the rapid increase in the demand of Car, mainly astaxanthin, for the production of feed additives [2]. In particular, the farming of salmon and shrimp grew exponentially from 1980s till 2000s and the trend continued [17] so the market of Car feed, particularly of astaxanthin, tended to expand rapidly.

The traditional technique of industrial production of Car (particularly, β-carotene) is through the extraction from higher plant materials such as carrot roots, palm oil etc. [18, 19]. The market share of natural Car dwindled significantly upon the emergence of synthetic Car, since artificial synthesis is generally cheaper and offers a greater productivity. At the same time synthetic Car have their drawbacks such as lower bioavailability and increased toxicity when compared to their natural counterparts [20, 21] which leave alone the environmental hazards of the chemical synthesis [19].

The growing demand of Car stimulated the search for a robust industry-scale source of these compounds. It became clear that chemical synthesis of complex Car species that exist in nature as configurational isomers is greatly complicated by low yields and the necessity of the complex purification steps. On the other hand, microbial synthesis of Car appeared as an attractive alternative. Heterotrophic and photoautotrophic microbial processes employing respectively carotenogenic fungi or unicellular algae (microalgae) were developed for this purpose [2]. The latter process attracted a considerable attention over the last two decades as a result of the impressive progress in microalgal biotechnology. The advances in the fields of biochemistry and regulation of carotenogenesis in microalgae as well as design of highly efficient bioreactors and processes made it possible to produce Car from microalgae in a cost-effective manner. Indeed, a number of companies have established for the production of Car from the algal biomass at an industrial scale [22, 23].

An important choice for the production of Car from microalgae is that between cultivation in open ponds or in closed system—photobioreactors (PBR). Both cultivation methods have their advantages and drawbacks (Table 4.1) which are analyzed in detail elsewhere [22, 24]. Briefly, cultivation in PBR appears to be a more versatile method (although more expensive as well) specifically designed for cultivation of the species requiring precisely controlled conditions and susceptible to contamination. At the same time, outdoor cultivation in open ponds is feasible only in the regions characterized by a suitable climate and inexpensive supply of fresh water meaning that cultivation in PBR is an only option for the areas with temperate climate.

The present chapter is dedicated to the advantages and caveats of the production of secondary Car from microalgae cultivated in PBR. We will discuss the specifics of biosynthesis of Car in these organisms and its regulation. Special attention will be paid to PBR operation and maximizing Car output as well as for online monitoring of the microalgal culture conditions.

Table 4.1 The comparison of open and closed systems for cultivation of microalgae

Parameter	Open pond	Closed photobioreactor
Cultivation conditions control	Impossible	Easy achieved
Operational expenses	Low[a]	High
Capital expenses	Low[b]	High
Footprint	Large	Small
Maintenance	Simple	Complex
Contamination	Susceptible[c]	Easy to control
Productivity	Very low	Moderate to high
Light utilization efficiency	Low	High
Optimal cell density	Low	High to very high

[a]If a free water supply is available
[b]Provided that the land cost is negligibly low
[c]Unless extremophile algae resistant to contamination are cultivated

4.2 Biotechnologically Important Carotenoids and Carotenogenic Microalgae

More than 800 carotenoid species with linear or cyclic structure were discovered in plants, including microalgae so far [5, 25]. These pigments are divided according to their substituent composition into carotenes, the simple hydrocarbon compounds, and xanthophylls containing oxygen atoms within hydroxy-, epoxy- or keto-groups (Fig. 4.1). The carotenoids of the most of microalgal species are represented by carotenes and xanthophylls with characteristic three-headed absorption maxima in the blue part of the spectrum, 400–490 nm [3, 26, 27]. The carotenes, β-carotene, the xanthophylls, astaxanthin and lutein are the major carotenoids from the standpoint of commercial biotechnological production. The microalgal species and processes used for their commercial production are elucidated below.

4.2.1 Primary vs. Secondary Carotenoids

According to their role, Car could be divided into two major groups. Photosynthetic or primary Car are closely associated with photosynthetic apparatus (PSA) of the plant cells i.e. with thylakoid membranes where they participate in light-harvesting quench triplet Chl molecules, eliminate ROS and stabilize the pigment-protein complexes [28–31]. Primary carotenoids include β-carotene and a number of xanthophylls such as lutein, neoxanthin, violaxanthin, antheraxanthin, and zeaxanthin (in Chlorophyta); the structures of xanthophylls of other groups algae are much more diverse [4]. The composition of primary carotenoids is highly conserved

[26, 28]. Lutein is a biotechnologically important xanthophyll but it is accumulated within microalgal cells as a primary Car. So, the production of lutein is out of scope of the present review. The following discussion will be focused on the specific group of Car—secondary Car which are quite widespread in different taxa of plants [5, 32].

Under stressful conditions certain microalgal species accumulate Car outside the thylakoid membranes in dedicated structures such as plastoglobuli (inside the chloroplast) or cytoplasmic oil bodies (outside the chloroplast) similar to oleosomes of higher plants [33]. These extrathylakoid or extraplastidial secondary Car do not participate in photosynthesis and are represented by carotenoids of both native (e.g. β-carotene in *Dunaliella salina* [34, 35]) and non-native to PSA such as astaxanthin[36]. The secondary xanthophylls are often accumulated in the form of fatty acid esters [36]; the significance of xanthophyll esterification is covered in the Sect. 4.3.3. The proposed functions of secondary Car in the microalgae include blocking excess PAR, sink for the excess photo assimilates, suppression of ROS generation (mainly *via* consumption of O_2 in xanthophyll biosynthesis) and the detoxification of already produced ROS [33, 37–43].

The composition and stoichiometry of primary Car within PSA is under strict genetic and regulatory control, it means that these pigments cannot accumulate in very high amounts but only in certain proportion when compared to the other photosynthetic pigments, mainly chlorophylls [28]. On the contrary, secondary carotenoids can be accumulated in microalgae in the amounts by far superior to that of primary Car e.g. up to 6 % of dry weight in the case of astaxanthin in *Haematococcus pluvialis* [45].

Until recently, the functions of secondary Car in microalgae remained largely unclear. The studies carried out during the past decade suggest that the photoprotective function of secondary Car seems to be the most important. Evidently, secondary Car participate in the screening of excess PAR, prevent photooxidation of PSA components and storage lipids as recently reviewed by Solovchenko [33]. Understanding of the physiological significance of secondary Car accumulation is essential for the development of an efficient process for their production from microalgae. So, this topic is elaborated as below.

Optical screening. A large body of evidence points to the higher tolerance of microalgae with increased secondary Car content to high-light stress. The structural stability of cytoplasm-localized lipid droplets referred to as oil bodies (OB) containing secondary Car is important since it allows attaining a high local concentration of secondary Car. It is essential for the efficient attenuation of light before it reaches vulnerable structures in the cell [40, 42, 46].

Elimination of ROS. As noted above, Car are powerful scavengers of free radicals and quenchers of excited molecules. Evidently, secondary Car protect against peroxidation of storage lipids accumulated in OB. It is supposed that secondary Car-containing OB located around the nucleus form a barrier that protects DNA from oxidative damage [47]. The intensive synthesis of oxygenated Car in microalgae is also believed to reduce the O_2 concentration in the cell [48]. An important

factor controlling the formation of ROS under adverse environmental conditions is a sink of electrons from plastoquinone pool in the plastidic electron transport chain to plastidial terminal oxidase (PTOX) *via* the desaturase enzymes participating in the synthesis of Car [48, 49].

Sink for excessive photosynthates. Under unfavorable conditions, intense biosynthesis of secondary Car evidently provides a sink for the excess photo assimilates, thereby reducing the risk of photooxidative damage due to over-reduction of electron carriers in the chloroplast electron transport chain. Additionally, photoassimilates can be consumed for the biosynthesis of FA required for the esterification of secondary Car such as astaxanthin[49].

4.2.2 The Most Commercially Important Secondary Carotenoids

β-carotene

The β-carotene (Fig. 4.1a) is a pigment of increasing demand and a wide spectrum of commercial applications: as a safe food colorant (the most important application [22]); as vitamin A (retinol) precursor in food and animal feed; as an ingredient of cosmetics and multivitamin preparations; and as a health food product [2].

The main process employed for the production of natural β-carotene is the cultivation of the green, unicellular alga *Dunaliella salina* accumulating this Car to 12–14 % of cell dry weight under stressful conditions [50]. Chemically synthesized β-carotene is the all-trans isomer whereas β-carotene from *Dunaliella* is a mixture of two stereo isomers, all-trans and 9-cis in approximately equal proportions resulting in superior bioavailability and health-promoting properties substantiating the commercial interest to the algal carotenes [51]. The pioneering works on mass-cultivation of *Dunaliella* were carried out by Masyuk [52] and Semenenko [53] in USSR at 1965–1975 but essentially were not recognized at that time. Later, their vision was proved by vast experience of commercial growing of this microalga in other countries. State of art in *Dunaliella* production is extensively reviewed in [50, 54].

One of the greatest promises of microalgal sources of β-carotene is the mitigation of global deficiency of (pro) vitamin A. As emphasized by [55], the deficiency of this vitamin, especially in children, is one of the most noticeable nutritional problems in many parts of the world and affects an estimated 250 million children under 5 years of age (according to World Health Organization surveys, http://www.who.int/nutrition/topics/vad/en/index.html).

Market price of *Dunaliella* biomass could be as high as € 2,000 kg^{-1}; prices of β-carotene from *D. salina* are ranging from \$300–\$3,000 kg^{-1} depending on the purity and the form, crystals or 1–13 % oil solution; useful byproducts of β-carotene from *Dunaliella* are glycerol (up to 30 % of dry) and feed protein [54, 56].

Astaxanthin

Astaxanthin, the most powerful natural antioxidant [9] is synthesized by some microalgae [4], plants, fungi, and bacteria [2, 27]. Astaxanthin does not exert a pro-oxidant effect typical of other carotenoids [57]. Furthermore, astaxanthin is not a vitamin precursor hence it's overdose does not pose the threat of hypervitaminosis.

Molecule of astaxanthin has two asymmetric carbon atoms at the positions 3 and 3′ of the ionone rings at either ends of the molecule. Depending on the hydroxyl groups attached to these carbon atoms, different enantiomers of the molecule may appear. Generally, two configuration of an asymmetric atom are possible: R, when the hydroxyl group is above the plane of the molecule, and S, when the hydroxyl group is below the plane of the molecule. Hence the three possible enantiomers of astaxanthin are designated R, R, S,S and R,S (meso-form). The bulk of astaxanthin in *Haematococcus pluvialis*, the richest natural source of astaxanthin [58], is in the form of mono- and diesters of palmitic (16:0), oleic (18:1) orlinoleic (18:2) fatty acids. Fatty acids are esterified onto the 30 hydroxyl group(s) of astaxanthin after biosynthesis of the carotenoid, increasing its solubility and stability in the cellular lipid environment. The composition of astaxanthin esters in *H. pluvialis* is similar to that of crustaceans, the natural dietary source of salmonids: the astaxanthin pool of *H. pluvialis* red cysts is comprised of ca. 70 % monoesters, 25 % diesters and 5 % of the free xanthophyll [2].

Both free and fatty acid-esterified astaxanthin in *H. pluvialis* have optically pure (3S, 30S)-chirality [59]. The *Phaffia* yeast contains pure 3R, 30R astaxanthin, and synthetic astaxanthin is a mixture of all three isomers [60]. As reviewed by Johnson, Schroeder [2] and, more recently, by Lorenz, Cysewski [15], over 95 % of the feed market consumes synthetic astaxanthin, mainly from BASF (Germany) and Hoffman-La Roche (Switzerland) [56]. Synthetic astaxanthin contains only 25 % of the biologically active isomer. On the other hand, consumers demand natural products making the synthetic pigments, especially in feed additive, much less desirable extending hereby the opportunity for the production of natural astaxanthin by *Haematococcus*.

The production of synthetic astaxanthin for aquaculture is about 100 ton per year selling at $ 2,500 kg^{-1}. Estimated global production of microalgae for the same purpose is 1,000 ton per year. Accordingly, the production of *H. pluvialis* biomass should be increased to 10 000 ton per year to displace the synthetic pigment only in aquaculture [15, 56]. The main disadvantage of the natural astaxanthin from *H. pluvialis* is its high price ($ 7,000 kg^{-1}).

4.2.3 Carotenogenic Microalgae

Microalgal species, which display a characteristic accumulation of gross amounts of Car within the cells, are commonly referred to as carotenogenic microalgae. Accumulation of secondary Car is a typical of microalgae withstanding extreme

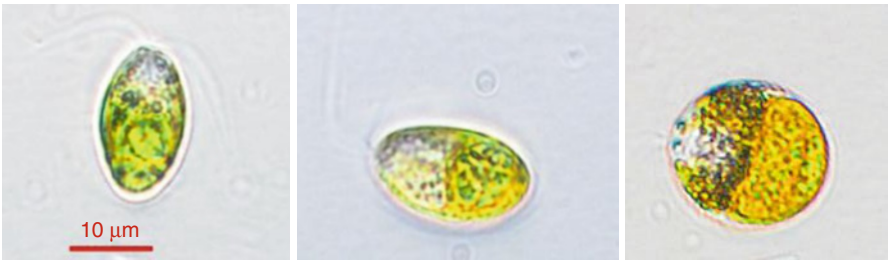

Fig. 4.2 Changes in the cell morphology of *Dunaliella salina* (from left to right) in the course of high-light and salinity stress induced accumulation of β-carotene (Courtesy of Dr. Elena Seliwanova (unpublished))

temperatures, irradiances, nutrient deficiencies, and salinities. Domination of carotenogenic microalgae in the ecosystems with extreme growth conditions is ascribed to their high tolerance to unfavorable environment, one of factors of which is the high content of secondary Car in their cells. Therefore, it is not surprising that the overwhelming majority of biotechnologically important microalgae are represented by extremophilic and stress-tolerant species.

Dunaliella salina

The genus *Dunaliella* (Chlorophyceae, Volvocales) includes a variety of biflagellated marine and fresh water microalgae (Fig. 4.2), detailed information about the taxonomy, the cell morphology and the ultrastructure of these species could be found elsewhere [61]. Algae from the genus *Dunaliella* lack a rigid polysaccharide cell wall, and are enclosed by a thin elastic plasma membrane covered by a mucous surface coat [50]. A few extremophilic representatives of *Dunaliella* (e.g. some strains of *D. salina*) are capable of accumulation of large amounts of β-carotene(up to 12 % of cell dry weight). This pigment is accumulated within the chloroplast but represents a typical secondary Car localized in the lipid droplets (so called carotene granules) surrounded by the dedicated proteins [62]. Accordingly, the induction of carotenogenesis in the *Dunaliella* occurs under severe stress.

The process of β-carotene accumulation is apparent as the change of color from green to orange or even red which often observed during season blooms of the algae in saline lakes and lagoons, the natural habitats of the carotenogenic *Dunaliella* species. Apart from high salt concentration, such habitats are often characterized by low availability of nitrogen, high temperatures and irradiance. Under such stressful conditions the carotenogenic representatives of *Dunaliella* not only grow well but thrive and often become the dominant microalgal species. Currently *Dunaliella* is mass cultivated predominantly in open ponds on saline and brackish or even sea water in desert areas. A few attempts to grow *Dunaliella* in PBR of different design (plastic tubes, plastic sleeves, shallow trays etc.) were not successful due to economical limitations [50].

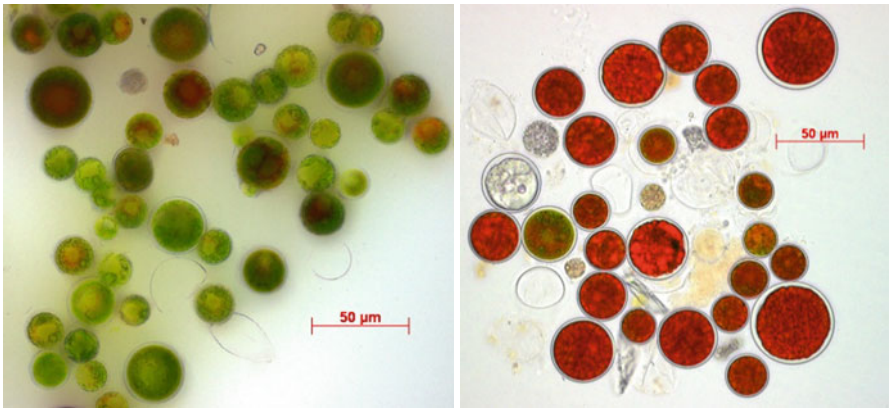

Fig. 4.3 Palmelloid cells (*left*) and (*right*) red cysts of *Hamatococcus pluvialis*. Note the beginning of astaxanthin accumulation in the green palmelloid cells. Astaxanthin content reaches the maximum in the cysts that almost lack chlorophyll (A. Solovchenko, unpublished)

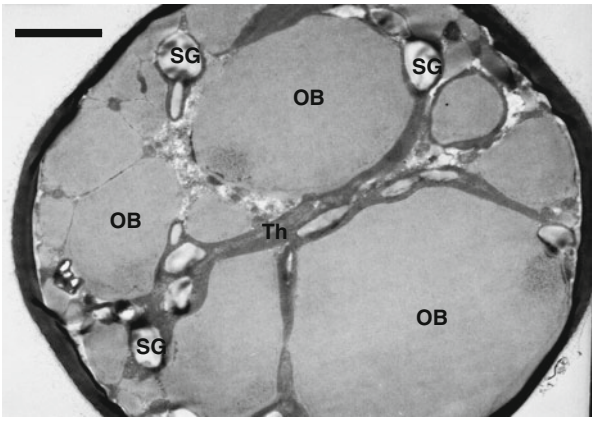

Fig. 4.4 Oil bodies in transmission electron micrograph of a red cyst of Hamatococcus sp. OB oilbodies, SG starch grains, Th thylakoids. Scale bar is 5µm (K. Chekanov, unpublished)

Haematococcus pluvialis

The green microalga *Haematococcus pluvialis* (Figs. 4.3 and 4.4) is the richest and therefore the most extensively studied [47, 63–65, 49] biological source of astaxanthin, the xanthophyll with a plethora of beneficial effects. Since the culture of green (vegetative) cells of *H. pluvialis* is very sensitive to contamination, this microalga is mass cultivated almost exclusively in PBR (Table 4.1). *H. pluvialis* accumulates

astaxanthin in cytoplasmic OB. Generally, accumulation of astaxanthin is induced under stressful conditions slowing down the cell division and enhanced by high irradiance [47].

4.3 The Biosynthesis of Secondary Carotenoid and Its Regulation

4.3.1 The Basic Steps of the Carotenoid Biosynthesis

The initial steps including the assembly of the carbon skeleton, desaturation, cyclization, and hydroxylation (all steps in case of secondary β-carotene) of the synthesis of primary and secondary Car are common and briefly described below; more elaborate reviews could be found elsewhere [33, 34, 48]. The specific stages of secondary Car biosynthesis are considered below; for additional detail see the recent review by Lemoine and Schoefs [49].

Assembly of the carbon skeleton. As in higher plants, the precursor of Car in green microalgae is isopentenyl pyrophosphate (IPP, C_5) originating from glycerophosphate-pyruvate or mevalonate pathway. The enzyme IPP isomerase reversibly converts IPP to its allyl isomer dimethyl allyl pyrophosphate (DMAPP), a primer for the isoprenoid chain synthesis. Successive attachment of three IPP molecules to a DMAPP molecule in the reaction catalyzed by GGPP synthase yields the molecule of geranyl geranyl pyrophosphate (GGPP, C_{20}). Two GGPP molecules add up to form the symmetric molecule of phytoene in the reaction catalyzed by phytoene synthase (PSY).

Desaturation and cyclization. Phytoene molecule, *via* four desaturation reactions catalyzed by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), is converted sequentially to phytofluene, ζ-carotene, neurosporin, and lycopene. The elongation of the conjugated double-bond system results in the transformation of the colorless Car precursors into colored compounds, starting from ζ-carotene. Plastid terminal oxidase (PTOX) supposedly serves as a co-factor of desaturases in microalgae contributing considerably to cell protection against oxidative stress (see above). Desaturation is also a rate-limiting stage of secondary Car biosynthesis.

The membrane-bound enzymes β-lycopene cyclase (CRTL-B) or ε-cyclase catalyzes the formation of cyclic Car from the symmetric linear molecule lycopene. Major Car of microalgae feature β- and ε-rings differing from each other by the position of the double bond (a typical example is lutein comprising one β- and one ε-ring). In spite of the high homology to β-cyclase, ε-cyclase, it produces only a single ε-ring with the formation of the monocyclic δ-carotene (ε-, ψ-carotene). It is assumed that cyclases play a key role in the control of cyclic secondary Car formation.

Oxygenation. The pathway of the biosynthesis of oxygenated secondary Car is relatively well studied [66–68]. The hydroxylation of α- and β-carotenes at the

position 3 yields, respectively, zeaxanthin and lutein. The addition of a ketogroup at the position 4 of one or both rings results in the formation of orange-red ketocarotenoids echinenone and canthaxanthin, the precursors of astaxanthin.

The reaction of β-carotene oxygenation is catalyzed by β-C-4-oxygenase (the ketolase designated as CRTO or BKT) encoded in *H. pluvialis* by *crtO* or *nkt* gene. The enzyme CRTO plays a key role in the accumulation of astaxanthin [67].

4.3.2 Stimuli Promoting Accumulation of Carotenoids in Microalgae

Primary or photosynthetic accumulated under conditions favorable for the cell division and culture growth i.e. under optimal irradiance, nutrient availability, and temperature. A certain increase in the primary Car yield could be achieved by application of mild stress conditions which promote accumulation of the Car over Chl but do not compromise the culture growth [22]. More severe stress leads to a decline in Chl and hence in primary Car due to highly conserved stoichiometry of these pigments within PSA.

The physiology of secondary Car accumulation appears to be more sophisticated. Generally, these pigments are accumulated under the influence of various stressors which slowdown or cease completely the algal cell division. A number of studies indicate the involvement of ROS in the induction of Car biosynthesis as secondary messengers. In particular, the addition to the culture of ROS generators, such as hydrogen peroxide, induces accumulation of secondary Car even in darkness, mimicking the action of environmental stressors; On the contrary, the addition of ROS scavengers suppresses carotenogenesis[69]. Overall, the plastoquinone pool which, under stressful conditions, stays in a reduced state most of the time serves as the redox sensor for the regulation of both primary and secondary Car biosynthesis [49].

Light intensity and quality. Many researchers concur that high irradiance efficiently induces the accumulation of secondary Car in carotenogenic microalgae, often in dose-dependent manner [47, 70, 71]. The data on the role of light in the induction of astaxanthin synthesis in *H. pluvialis* are more controversial. Although many findings confirm the stimulatory effect of high PAR irradiance on astaxanthin synthesis, the accumulation of this pigment occurs even in darkness in the presence of the organic source of carbon or a ROS generator [64, 72]. Remarkably, under unfavorable conditions the increased ROS concentrations triggering carotenogenesis could be reached even at a relatively low illumination intensity.

The spectral quality of light is also an important factor inducing carotenogenesis. However, little is known about the impact of UV on secondary Car biosynthesis in microalgae so far. Still, irradiation with UV-A (but not UV-B) in addition to PAR stimulated massive accumulation of β-carotene in *Dunaliella* [73, 74].

Lack of mineral nutrition. The deficiency or lack of nutrient elements, mainly nitrogen and phosphorus, is also a factor stimulating accumulation of secondary Car. Thus, nirogen and/or phosphorus starvation of *H. pluvialis*

resulted in the formation of cysts from vegetative cells accompanied by accumulation of astaxanthin [47]. *Parietochloris incisa* growing on nitrogen-free medium displayed an enhanced accumulation of β-carotene in OB [75]. Interestingly, herbicides impairing nitrogen assimilation by inhibiting glutamine synthase induced astaxanthin accumulation in *H. pluvialis*as efficiently as nitrogen starvation [76].

Osmotic stress. An abrupt increase in the concentrations of osmotics in the culture medium enhances Car accumulation in carotenogenic microalgae, usually after a certain lag. Thus, in *D. salina* treated with high NaCl concentration the duration of this lag depends on the initial salt concentration and the degree of its increase whereas the magnitude of carotenogenesis is determined by the final salt concentration [70].

Temperature. Suboptimal temperatures, especially in combination with high PAR also induce secondary carotenogenesis. In particular, in *D. salina* these stresses induced gross accumulation of β-carotene. Apparently, low temperatures are important for induction of carotenogenesis in snow algae [41].

Organic carbon feeding. Feeding with the organic carbon source such as organic acids or sugars often enhances accumulation of secondary Car by carotenogenic microalgae capable of heterotrophic of photoheterotrophic growth as reviewed by Solovchenko [33]. This effect was observed in *Trentepohlia aurea* after addition of peptone to medium and in *Chlorella protothecoides* at the addition of glucose and urea. The presence of mono- and disaccharides enhanced the synthesis of astaxanthinin *C. zofingiensis*. It is essential that the addition of the glucose analogs does not have the same effect. So, it cannot be ascribed to the osmotic action of the added sugars. Probably, the molecules sensing the presence of glucose such as hexokinase are involved in the induction of carotenogenesis.

4.3.3 Relationships Between Accumulation of Carotenoids and Lipids

It is important to realize that such hydrophobic molecules as Car, especially carotenes, cannot accumulate in an appreciable amount within the hydrophilic cytoplasmic or stromal compartments of the cell without a suitable depot. The primary Car are contained within the nonpolar 'pouches' of thylakoid membrane-bound pigment-protein complexes of PSA. So, they essentially do not interact with the hydrophilic environment [77]. By definition, secondary Car cannot share the same subcellular compartment with a primary Car. As noted above, they accumulated in the dedicated structures—plastoglobuli and OB. All the structures serving as the depot for secondary Car are formed with the participation of neutral lipids, mainly triacylglycerols (TAG) and dedicated amphiphilic proteins, oleoresins [78]. Furthermore, xanthophylls such as astaxanthin are esterified by fatty acids. Thus, in *H. pluvialis* and carotenogenic members of the genus *Chlorella* during the final steps of cyst formation, more than 95 % of secondary xanthophylls are converted to fatty acid

(predominantly from the C18 family) esters [58]. This makes possible the accumulation of large amounts of relatively polar xanthophylls in the hydrophobic environment of the OB.

The induction of secondary Car biosynthesis in *H. pluvialis* and other carotenogenic microalgae is accompanied by rapid TAG accumulation. Eventually, in this microalga TAG more than 95 % of cell lipids are represented by the TAG of secondary Car-containing OB. So, it is not surprising that a close connection exists between the secondary Car biosynthesis and lipid, in particular FA and TAG biosynthesis. Moreover, the formation of lipid inclusions, the potential depots for secondary Car, readily proceeds even when Car biosynthesis is inhibited but inhibition of TAG accumulation essentially abolishes the accumulation of the Car [79]. Therefore, it is clear that stress-induced accumulation of neutral lipids in the form of cytoplasmic or stromal inclusions is a prerequisite for the accumulation of secondary Car in microalgal cells. Accordingly, these events explain the similarity of the stimuli triggering the mass accumulation of lipids and those stimulating the biosynthesis of secondary Car in microalgal cells.

4.4 Production of Microalgal Carotenoids in Photobioreactors

A considerable effort is being invested into the development of cost-effective production of microalgal biomass which is difficult to achieve. Different cultivation systems have been designed for large-scale cultivation of microalgae [80, 81] which roughly fall into two distinct groups—open (ponds and their variations) and closed (photobioreactors, PBR; see e.g. Fig. 4.5). Each group has its advantages and drawbacks as summarized in Table 4.1.

Collectively, open systems are economically viable only if the cost of the land is very low, there is a free source of water, a contamination-resistant algal species (e.g. extreme halophile such as *Dunaliella salina*) is cultivated, and the climatic conditions are suitable. Mass cultivation of microalgae in open ponds is reviewed elsewhere [24, 82].

More recently developed and technologically advanced closed PBR are more versatile to support the growth of any microalga under precisely controlled conditions and without the risk of contamination. The major drawback of PBR is high construction and operation cost due to a technical complexity. At the same time PBR provide considerably higher productivity, higher quality of biomass and, what is more important, robust biomass composition due to more stable cultivation conditions.

The objective of the cultivation is to obtain maximum yield of biomass with certain minimal content of certain Car. The strategy to achieve this goal depends on the type of the Car. Generally, primary or photosynthetic Car such as lutein are accumulated along with biomass accumulation under conditions favorable for photosynthesis. The larger the photosynthetic antenna size, the higher the productivity of the microalgal culture in terms of primary Car content since lutein is predominantly

Fig. 4.5 Cultivation of *H. pluvialis* in tubular photobioreactor (Algatech, Israel). Note the tubular fences with green vegetative cell suspension (*right*) and those with red astaxanthin-rich cysts ready for harvest (*right*) (Photo: A. Solovchenko)

bound to light harvesting proteins comprising the antenna [29]. On the contrary, secondary Car such as β-carotene or astaxanthin are accumulated under stressful conditions retarding microalgal cell division [47, 62]. This makes apparent the importance of correct choice and careful maintenance of the cultivation conditions suitable for the algal growth and accumulation of the 'target' Car. In the following subsections, general considerations regarding the cultivation conditions of microalgae in PBR are given; the section concludes with the approaches for the production of secondary Car from the microalgae grown in PBR.

4.4.1 Illumination and Optimal Cell Density

Autotrophic cultivation of microalgae presumes adequate illumination of the culture in PBR. Illumination can be natural, artificial or combined. The productivity of cultivation under sunlight is less stable because of diurnal and seasonal fluctuations

in solar light. On the other hand, in PBR with artificial illumination a substantial part of cultivation costs is comprised by energy costs which are mitigated in part by increased productivity. Obviously, the optimal approach to illumination should combine the advantage of both natural and artificial illumination.

The conditions commonly referred to as 'intensive cultivation conditions' presume high cell density in PBR. Under such conditions, strong light absorption of pigments in the algal cells resulting in strong mutual shading. Therefore, at a cell density high enough (>1 $g \cdot L^{-1}$) only the cells within the thin (>10 mm) surface layer of the suspension are getting enough light for photosynthesis, other part of the PBR volume essentially resides in darkness [83]. At the same time, it is not feasible to increase the illuminated (photic) zone of the PBR by mere increase of the incident irradiance. The reason is that photo inhibition of the cells in the surface layers increases whereas the cells, those within more deep layers will not get enough light [83, 84].

There could be several ways to achieve a uniform illumination of microalgal cells in PBR at high cell densities. The first is to keep the light path (volume-to-surface ratio) in PBR to a minimum by choosing a flat panel design or a small-diameter tubular design. Essentially, an important goal of PBR design is to make the entire volume of a PBR its photic zone.

Another option is to mix the suspension at an optimal rate, so that each cell would reside in the photic zone just enough time to absorb the amount of light necessary for photosynthesis (see the Sect. 4.4.2 below). The ATP and NADPH molecules synthesized during the stay in the photic zone are supposed to support the CO_2 fixation when the cell travels through the dark volume of the PBR [85]. This allows the construction of PBR supporting ultra-high cell densities and to supply enough light without the risk of photo inhibition [86].

The third strategy is based on intermittent illumination [87]. The length and the intensity of light flashes as well as the lengths of dark intervals between the flashes are also selected to allow the cells to absorb enough light and to utilize the photosynthates during the dark periods. However, finding the optimal parameters for intermittent illumination appeared to be a complex task which has not been solved yet [85].

4.4.2 Mixing and Sparging

Mixing prevents the cell sedimentation and accelerates the mass transfer within PBR thereby assuring proper distribution of nutrients. Apart from a sufficient PAR irradiation, continuous CO_2 supply is also necessary for efficient photosynthesis. Moreover, photosynthetic O_2 evolution in a closed vessel of PBR would dramatically increase the dissolved O_2 to the level conductive for the photooxidative damage. So, it is clear that adequate gas (CO_2/O_2) exchange and nutrient distribution which is achieved through mixing is essential for attaining high growth rates and productivity of a microalgal culture in PBR [88].

The current PBR designs most frequently adopt mechanic or pneumatic mixing [89]. Mechanic mixing is usually accomplished by pumping the microalgal suspension

into a stirred tank fitted with some kind of stirring wheel. More practical is the pneumatic stirring with air or air-CO_2 mixture bubbled through the suspension in a vertical column or a flat panel (also called airlift). Additional benefits of pneumatic mixing include simultaneous CO_2 feeding and O_2 removal from the suspension.

Atmospheric CO_2 concentration is limiting photosynthesis in most photoautotrophic organisms. Accordingly, a certain enrichment of the gas mixture used for sparging PBR with CO_2 generally enhances the productivity [90–92]. The extent of the enrichment depends on the tolerance of the cultivated microalga to CO_2 which is species-specific [93]. Interactive effects of elevated CO_2 percentage in the bubbling gas mixture and other factors are so far largely unknown. Nevertheless this problem is expected to draw a considerable attention in view of the possible use of flue gases for CO_2 enrichment of microalgal cultures with the added value of the greenhouse gas sequestration [94].

Generally, higher the mixing rate, the higher will be the mass transfer efficiency. At the same time, one cannot increase the mixing rate above a certain threshold level because in a suspension flowing too rapidly microalgal cells encounter shear stress. This stress may result in the decline of the culture growth or even in cell disruption. Consequently, mixing rate will be limiting the growth of microalgae in PBR [95].

4.4.3 Temperature and pH

Together with illumination, mineral nutrition, and mixing temperature and pH (acidity) of the medium are among the most important factors determining the productivity of microalgal cultures [83]. A common approach to maintain the pH of the culture is CO_2 sparging on demand by means of a pH controller.

The temperature values close to the optimum promote the biomass accumulation and hence are favorable for the biosynthesis of primary Car such as lutein (see above). Extremely low temperatures induce the accumulation of secondary Car in so called snow algae many of which are carotenogenic species [41]. In spite of the fact that low temperatures efficiently induce carotenogenesis, it is impractical to apply in large-scale PBR.

Cultivation of microalgae in outdoor PBR poses the challenge of maintaining the optimum temperature which is mandatory e.g. during the first stage of cultivation (accumulation of biomass, see the Sect. 4.4.5 below). In particular, to prevent overheating of the culture in a cost-effective manner local resources are used such as cooling with deep ocean cold water *via* heat exchanger (in Hawaii) or evaporative cooling by mean of water sprinkling (in arid areas).

4.4.4 Medium Composition

Cultivation of algae can be photoautotrophic, heterotrophic and mixotrophic. Media with organic carbon sources such as acetate, glucose or molasses are used for mixo- and heterotrophic cultivation[96]. Such type of cultivation are not light-dependent

Table 4.2 The companies commercially producing astaxanthin-enriched biomass of *H. pluvialis*

Company	Country	Yield (ton/year)	PBR type
AstaReal (subsidiary of Fuji Chemical, www.bioreal.se, www.astareal.com)	Sweden	–[a]	Cylindric (indoor, mixotrophic)
Alga technologies (www.algatech.com)	Israel	–[a]	Tubular (outdoor)
Cyanotech (www.cyanotech.com)	USA (Hawaii)	13–15	Tubular + open pond
Mera pharmaceuticals (www.merapharma.com)	USA (Hawaii)	6.6	Tubular

Adapted from Del Campo et al. [22]
[a]No data

and lead to increase in the productivity in some cases. As a result, PBR for heterotrophic cultivation has a relatively small surface to volume ratio hence their design could be simpler in comparison with PBR for photoautotrophic cultivation. On the other hand, the use of organic carbon sources increases the risk of culture contamination, so, the sterility should be maintained more strictly in this case.

As shown by Kobayashi et al. [72], *H. pluvialis* can be grown mixotrophically or even heterotrophically (i.e. in the dark) employing an organic acid (e.g. acetate) or carbohydrate as the carbon source. Nevertheless, the current mainstream process of the production of astaxanthin from *H. pluvialis* relies on its photoautotrophic cultivation in PBR. The reason is higher productivity of the photoautotrophic process, let alone the high cost of organic medium constituents and the risk of contamination of the culture by heterotrophic bacteria. Still, examples exist for successful commercial implementation of the mixotrophic process (Table 4.2).

4.4.5 Cultivation of Carotenogenic Microalgae in Photobioreactors

Photobioreactors employed for cultivation of microalgae are evolved to a vast diversity, which has been reviewed extensively [97–100]. The most common designs include tubular, flat plate PBR, vertical and horizontal. An efficient PBR should provide uniform illumination of the culture, adequate mass transfer for nutrient delivery and gas (CO_2/O_2) exchange, easy maintenance and a precise control of cultivation parameters in a cost-effective manner. At the same time, there is no 'best reactor' performing equally well for different cultures and target products [100].

Generally, a PBR operates under conditions providing a rapid culture growth (biomass accumulation). At the same time, secondary Car are accumulated under stressful conditions that slow down the growth of microalgae. As a result, obtaining sufficient and sustainable biomass and Car productivities even in PBR is a non-trivial problem, especially under outdoor conditions, which are less controllable. Optimized productivity can be achieved by cultivation under conditions that allow at least partially circumventing the negative consequences of nutrient or light stress such as optimized cell densities, light path and geometry of the cultivation facility,

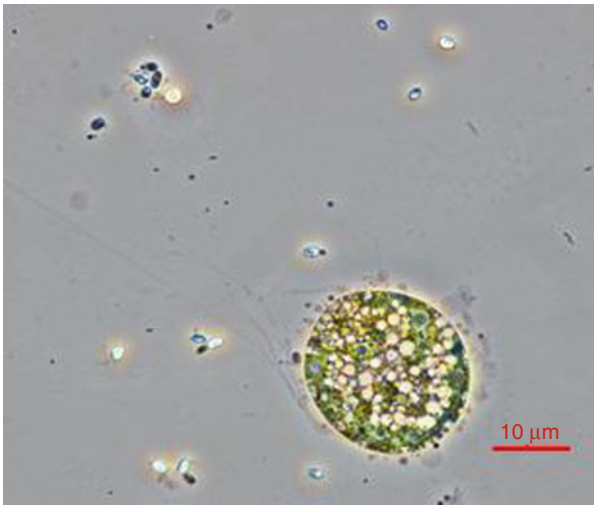

Fig. 4.6 A cell of *H. pluvialis* infested by a parasitic fungus (K. Chekanov, unpublished)

growth stage of the inoculum etc. Another option is to employ various types of the double-stage process, the form of batch cultivation where the culture is first grown under optimal conditions promoting accumulation of biomass with low Car content. After accumulation of sufficient amount of the green biomass the culture is subjected, at the second phase, to stressful conditions (e.g. nitrogen and/or phosphorus deprivation or an increase in salinity) causing cessation of cell division and promoting the accumulation of Car. For more efficient use of light energy, PBR designs were proposed combining the two stages where the cells cultivated in the central vessel (vegetative cells) are shaded by the culture grown in outer jacket. The surface of the outer jacket can be illuminated at high irradiances inducing carotenogenesis without the risk of photoinhibition of the cells grown in the inner shaded vessel.

The first stage (accumulation of the green biomass) in the case of *H. pluvialis* cultivation is almost exclusively carried out in PBR to prevent contamination and to strictly maintain near optimal growth conditions. The second stage (induction and accumulation of Car) occurs under stressful conditions making the contamination less likely hence this stage could be carried out in open ponds (Table 4.2). Still, contamination, especially by parasitic fungi (Fig. 4.6), is one of the most frequent reasons for the *H. pluvialis* culture crash [101] so, cultivation in PBR remains the preferred methods. Most methods for commercial production of astaxanthin-enriched *H. pluvialis* biomass provide the pigment content in the range 1.5–3 % DW [22].

The same time certain level of secondary Car accumulation could be achieved during vegetative cell growth as it was shown for *H. pluvialis* [22]. It is possible, by careful control of nutrient (nitrogen) content of the medium, to obtain a descent yield of astaxanthin under continuous culture. These findings made it possible to develop an alternative single-stage process for the astaxanthin-enriched biomass of *H. pluvialis*.

To the best of our knowledge, a commercially viable process for cultivation of *Dunaliella* in PBR does not yet exist but is arguably possible. The key drivers behind this effort are higher productivity and better quality of the biomass. As a result of optimization of cultivation conditions (irradiance and nitrogen availability), an average β-carotene content as high as 10 % DW is attainable [22].

4.4.6 Biomass Harvesting and Downstream Processing

One of the most challenging issues of developing commercially viable processes for microalgal bioproducts is biomass harvesting and dewatering. Sedimentation of the cells under the action of gravity is time-consuming and often incomplete. The most widespread techniques of microalgal cell harvesting are filtration, centrifugation, and chemical flocculation or bioflocculation and combinations of these methods [91, 92, 102]. For harvesting the Car-enriched biomass in large-scale cultivation in open ponds, a combination of flocculation and surface adsorption is used whereas the biomass grown in PBR is usually harvested by centrifugation. The astaxanthin-rich cysts of *H. pluvialis* feature higher density than the cultivation medium. Hence, they could be harvested by sedimentation under the action of gravity or a low-speed centrifugation.

Several attempts were made to employ vacuum or pressurized filtration for biomass harvesting. The diatomite and cellulose filters were efficient only for the separation of large cells whereas smaller cells such as that of *Chlorella* required membranous filters, which were clogged rapidly. Taking into account the need for frequent change or cleaning of the filters and high energy input, there was but small difference in the costs between filtration and centrifugation [92]. Centrifugation is a convenient, though energy intensive, and hence expensive method since it allows quick separation from the medium of more than 95 % of algal cells regardless of the species. As a result, the latter method remains preferred for obtaining the biomass for value-added product such as Car [103].

Flocculation is based on the use of reagent (such as $FeCl_3$ or $Al_2(SO_4)_3$) which compensates the negative charges at the surface of the cells which normally prevent the aggregation of microalgae [104]. Alternatively, alkali and polycations such as chitosan are used [105].

Some microalgae such as *Chlorella minutissma* are capable of bioflocculation i.e. begin to flocculate without addition of chemicals at the late stationary or under certain growth conditions. As a result of flocculation, cell aggregates are formed which are much easier to separate by filtration, low-speed centrifugation or even by gravity [104].

For large and heavy cells such as *H. pluvialis* red cysts, it turned feasible to use the combined process of sedimentation with subsequent centrifugation and drying of the paste in thin layer. Then, the biomass could be cracked e.g. by milling to fracture the thick and tough cell wall to increase the extractability or bioavailability of astaxanthin. However, the downstream processing of the biomass of carotenogenic

microalgae often constitutes the know-how of the corresponding companies which scarcely share any information about this step.

A considerable challenge is represented by extraction of Car from the microalgal cells. One of the main problems with extraction of pigments from microalgae is the presence of tough cell walls. A variety of organic solvents is used for pigments extraction [23]. The most common is extraction with a non-polar solvent, usually hexane. Selective extraction of astaxanthin from *H. pluvialis* is also achieved with dodecane and methanol. The extractability with this method could be as high as 95 % of the total pigments. Vegetable oils (so-called green solvent) such as olive oil allows the extraction up to extract 93.9 % of the pigments with the added benefit for environmental safety. Finally, extraction with super critical CO_2 is a promising alternative to the existing methods. This method provides shorter extraction time and saves toxic and expensive organic solvents. By contrast, CO_2 is relatively cheap, chemically inert, non-toxic and stable [23]. Major drawbacks of supercritical fluid extraction are contamination of the extract with chlorophylls and the need for expensive equipment.

4.5 Approaches for Optical Monitoring of Carotenogenic Algal Cultures

Mass cultivation of microalgae for value-added products such as Car requires fast and reliable techniques, preferably non-destructive, for on-line monitoring of the target product contents and the physiological condition of the algal culture. These techniques provide information, which is essential for timely and informed decisions on adjustment of illumination conditions, medium composition and for the choice of the time for biomass harvesting. Often, the decisions must be taken within hours and mistakes may lead to a significant reduction in productivity or in total culture loss [106]. Traditionally, the pigment content in microalgal cells is determined with the use of spectrophotometry and chromatography [79] which are time-consuming, expensive, and not readily available at mass cultivation facilities. These considerations make obvious the need for a reliable, rapid and preferably non-destructive technique for fast appraisal of the relative Car content in microalgal cultures.

Remarkably, the engagement of protective mechanisms based on the build-up of secondary Car within OB in the cells is accompanied by specific and directional changes in the optical properties of the algal suspensions [107]. Recent reports show that astaxanthin presence and subcellular distribution *in vivo* could be characterized [108] and even distinguished from β-carotene[44] using advanced spectral techniques such as Raman spectroscopy. Development of methods for non-destructive monitoring based on optical spectroscopy requires a deep understanding of the relationships between the changes in light absorption by algal cells and dynamics of their pigment and lipid contents.

In particular, the increase in the Car/Chl ratio under various stresses, including nitrogen starvation and high light, is characteristic of many microalgal species [33]. In particular, in *H. pluvialis* [47, 109] carotenogenesis occurs under stressful conditions in parallel with the degradation of Chl manifesting the reduction of photosynthetic

apparatus in order to avoid photooxidative damage [107, 110]. It was found that the Car/Chl ratio, but not the absolute amount of Chl or Car correlates directly with high light-stress tolerance in *H. pluvialis* [107] making Car/Chl an informative index of the cell physiological condition. As a result, the molar Car content exceeds that of Chl and the Car begin to exert the dominant contribution strongly to the absorption between 400 and 500 nm evident in many algal species [111]. This spectral feature was employed for the development of algorithms for estimation of Car/Chl ratio in the biomass of *Parietochloris incisa* [112, 113], *H. pluvialis* [107, 114], and *Nannochloropsis* sp. [115]. It should be noted that the correction for the contribution of light scattering into overall attenuation of light by the suspension prior to the normalization turned to be necessary for the precise estimation of the pigment ratio. The peculiarities of the spectra processing and the development of the algorithms are reviewed elsewhere [111].

Finding relationships between pigment content in *H. pluvialis* whole-cell suspension, especially cysts with high Car content (or Car/Chl ratio), turned to be a non-trivial problem, primarily due to the optical complexity of this system. In particular, *H. pluvialis* cells contain high amounts of pigments which are localized in specific structures (thylakoid membranes of chloroplast or cytoplasmic OB in case of Chl and primary Car or secondary Car, respectively) non-uniformly distributed within the cell volume [47, 78]. As a result, a number of serious obstacles for measurement of optical density spectra in *H. pluvialis* cell suspension arise including rapid cell sedimentation, significant influence of light scattering, strong pigment aggregation, and sieving effect.

The analysis of cell suspension spectra revealed that the amplitude of the raw absorbance spectra was not directly correlated to their pigment content. The normalization of the spectra to the red Chl maximum essentially equalized the contribution of Chl to light absorption making apparent the relative contribution of Car, which drastically increased in the course of carotenogenesis. Hence, the absorbance normalized to the Chl red maximum exhibited a tight relationship with Car/Chl in a broad range of its changes [114].

Collectively, there is a solid ground to believe that one could obtain a quantitative record of the development of stress-induced carotenogenesis in *H. pluvialis* and a number of other carotenogenic microalgae non-destructively *via* optical density measurements. In particular, the normalized optical density in the broad band around 500 nm, as well as the green edge features, could be employed in the development of models for rapid assay of Car/Chl in the algal cells suspensions. However, obtaining a calibration in the widest possible range of Car/Chl changes in any particular culture system and careful control of the biomass load per filter is crucial for the robustness of Car/Chl estimation.

4.6 Economic Aspects of Carotenoid Production with Microalgae

Global market of nutraceuticals and food supplements containing Car is characterized with confidence as vigorously growing. Thus, in 2010 it was estimated $1.2 billion and expected to grow to $1.4 billion by 2018 (BCC research;

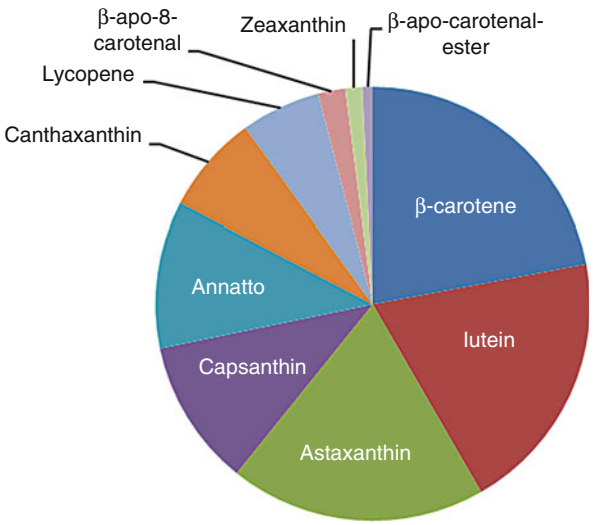

Fig. 4.7 The structure of global carotenoid market at 2010 (BCC research)

http://www.bccresearch.com/pressroom/fod/global-carotenoids-market-reach-$1.4-billion-2018). The market is dominated by β-carotene, lutein and astaxanthin (Fig. 4.7). The highest consumption of carotenoids is expected by the pharmaceutical industry [56].

Carotenoids from microalgae grown in PBR are, in main instances, unable to compete with their synthetic counterparts. This situation could be amended by optimization of the production processes. Thus, the basic steps of the commercial production of Car from microalgae include (i) algae cultivation, (ii) biomass harvesting, and (iii) extraction and purification of carotenoids [22]. The capital expenses for cultivation of microalgae include the cost of land, PBR, equipment for the harvesting and drying of biomass, communications and infrastructure. The operational expenses include salaries, nutrients (mineral components of the media and CO_2), energy, water, maintenance of machinery, taxes, insurance etc.[45]. The typical cost structure of *H. pluvialis* cultivation in PBR is as follows. Energy for illumination makes up ca. 40 %, the cost of culture medium is ca. 6 % [116]; biomass harvesting contributes up to 30 % to the total cost of product. The rest is comprised by the cost of biomass dehydration [92].

Generally, cost-efficiency of the system is determined by many factors including the design and the size of PBR. Thus, in case of a flat panel PBR, scaling up from 17 to 200 L significantly reduced the total costs of production per year from $ 394 kg^{-1} to $ 242 kg^{-1} dry biomass [116]. Still, the cultivation using raceway is 20 times cheaper than cultivation in PBR [45]. So, the target productivity in a PBR should be at least 20 times higher than in a raceway pond. On the other hand, open pond cultivation is not suitable for microalgal species susceptible for contamination such as vegetative cells of *H. pluvialis*. Hybrid technologies combining cultivation of vegetative cells in PBR with subsequent induction of carotenogenesis in open ponds (see Sect. 4.4.5 above) appear to be the cheapest solution. As shown by Li

et al. [45], the cost of astaxanthin in this case is estimated at \$ 718 kg^{-1}(\$ 18 kg^{-1} biomass containing about 2.5 % astaxanthin). This cost is much lower than the current cost of natural and even synthetic pigment. However, such a low expenses are achievable only if the labor cost and other costs are as low as they are in China [45].

4.7 Conclusions and Outlook

Carotenoid molecules are naturally designed for protection of vulnerable biomolecules in the cell from harmful effects of environmental stresses, ROS, and other aggressive chemical species. Certain Car such as β-carotene, astaxanthin, and lutein are of immense practical significance. These compounds are potent antioxidants with diverse beneficial effects on health. They are used as medicine preparations, widely consumed as nutraceuticals and safe food colorants and beauty product ingredients, functional food and feed additives.

Microalgae are the most efficient cellular factories of carotenoids though the examples of successful commercial cultivation of microalgae for Car are so far limited. The key reasons are expensive production of high-quality microalgal biomass and strong competition with cheap (though less efficient and environmentally safe) synthetic Car.

Several approaches could be proposed to make natural carotenoids from microalgae more competitive. First, we need to put an emphasis on the investigation of natural biodiversity to search for more efficient algal strains. Thus, Olaizola [117] estimated ca. twice more productive strains accumulating 10 % astaxanthin (vs. current 3–5 %) will make astaxanthin from *H. pluvials* competitive as feed additive. Taking into account that only 1–2 % of microalgal biodiversity currently estimated as several million species are known, the search for new efficient strains definitely holds promise.

Second, we need more thorough understanding of the physiology of secondary carotenogenesis in microalgae. The insight into these processes forms the foundation for the development of more energy-efficient and hence cost-efficient photobiotechnology for commercial production of Car from microalgal biomass. In particular, a considerable progress is being made in the techniques for on-line monitoring of physiological condition and biochemical composition of microalgal cultures *in situ* (in the PBR vessel) for timely, informed decisions for the culture management. Then, a considerable effort is invested in metabolic engineering of microalgae aimed to remove the major bottlenecks of the biosynthesis of Car.

Third, innovative methods for harvesting and preparation of the microalgal biomass and the extraction of Car are needed. The most important targets here are energy savings, completeness and selectivity of the extraction as well as avoidance of the expensive and toxic solvents.

Finally, there is a need to bridge the gap between the developed technology suitable for implementation at industrial scale and the latest scientific achievements in the field of Car production from microalgae.

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Chapter 5
Submerged Fermentation of Medicinal Fungus *Cordyceps sinensis* for Production of Biologically Active Mycelial Biomass and Exopolysaccharides

Jing-Kun Yan and Jian-Yong Wu

Abstract *Cordyceps* (*Ophiocordyceps*) *sinensis*, the Chinese caterpillar fungus or *Cordyceps* in brief, is an important medicinal fungus in Chinese herbal medicine with a wide range of health benefits and bioactivities. Because wild *C. sinensis* fungus (in the form of insect caterpillar-fungal fruiting body complex) is very expensive and rare in nature, mycelial fermentation has become the main source of *C. sinensis* fungal materials. Liquid or submerged fermentation of fungal mycelia has been widely exploited for large-scale production of *C. sinensis* mycelium biomass and exopolysaccharides (EPS). This chapter will give a brief introduction of the biological characteristics of the *C. sinensis* fungus and its medicinal functions and applications, and then mainly review the conditions and characteristics of *C. sinensis* mycelial culture for the production of mycelial biomass and EPS in shake-flasks and stirred-tank fermenters. In addition to the relevant studies reported by other research groups, this chapter will summarize the major findings from the studies by our own group with the Cs-HK1 fungus, including the fluid transport properties and the process parameters from small laboratory to large-scale industrial fermenters, the problems in separation and recovery of mycelial biomass and EPS from the viscous fermentation liquid, and the isolation, purification and molecular properties of polysaccharides.

Keywords *Cordyceps sinensis* • Cs-HK1 fungus • Mycelial culture • Stirred-tank fermenters • Exopolysaccharides • Product recovery

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Abbreviations

Cs	*Cordyceps sinensis*
DO	Dissolved oxygen
EPS	Exopolysaccharide
MW	Molecular weight
MWCO	Molecular weight cut-off
PE	Peptone
PS	Polysaccharide
PSP	Polysaccharide-protein complex
TKN	Total Kjeldahl nitrogen
YE	Yeast extract

5.1 Introduction

Edible and medicinal fungi (mushrooms) are widely applied to functional foods and nutraceutical products because of their proven nutritive and medicinal properties [1, 2]. Polysaccharides (PS) represent a major class of bioactive molecules from edible and medicinal fungi which have notable antitumor, immunomodulatory and other medicinal properties [3, 4]. PS-rich water extracts of mushrooms or mycelia have been applied to a wide range of functional food and cosmetic products, and some purified PS fractions such as β-glucans and PS-protein complexes (PSPs) from edible and medicinal fungi have found clinical applications for immunotherapy and cancer treatment, and as an adjuvant for chemotherapy/radiotherapy [5]. Because of the limited and unstable supply of wild mushrooms, cultivation of fungal mycelia or mushrooms (in fruiting body form) by solid and submerged fermentation has been a major source of fungal materials including the PS extracts. Liquid or submerged fermentation is a more favourable and efficient process than solid-state fermentation for the production of mycelial biomass and bioactive compounds, especially the exopolysaccharides (EPS) [6, 7].

Cordyceps (*Ophiocordyceps*) *sinensis*, the Chinese caterpillar fungus or Dongchong-xia-cao in Chinese, is a special mushroom with a fruiting body formed on caterpillars (Fig. 5.1). *C. sinensis* is one of the most famous and highly valued medicinal fungi in China [8], and has also attracted worldwide attention in recent years [9–12]. *C. sinensis* has been used traditionally in China mainly as a general tonic for a number of health benefits, e.g. strengthening the lung and kidney functions, restoring health after prolonged sickness, enhancing the physical performance, and improving the quality of life, while the recent studies have shown several pharmacological activities of *Cordyceps* including antitumor, antiaging, anti-fatigue, anti-inflammation, anti-atherosclerosis and antioxidant activities [13–15]. As the wild or natural caterpillar fungi as fungus fruiting body-caterpillar complexes are rare and cannot meet the increasing demand, mycelial fermentation has become a major source of *Cordyceps* materials. A number of studies have been reported over the last 10 years on mycelial fermentation of *C. sinensis* for the production of mycelial biomass and EPS [16, 17].

Fig. 5.1 *Cordyceps* (*Ophiocordyceps*) *sinensis* fruiting body-caterpillar complexes: morphology and natural habitat [18, 19]

This chapter presents a brief introduction of the, biological characteristics and medicinal properties of *C. sinensis*, and the recent studies on submerged fermentation of this fungus for the production of mycelial biomass and EPS. The studies by our group on the mycelial fermentation processes of a *C. sinensis* fungus, Cs-HK1, will be discussed in detail, including the medium composition and culture conditions, characteristics of mycelial morphology and broth rheology. This chapter it also points out on the problems and considerations in large-scale fermentation and product recovery, and the chemical properties, health effects and bioactivities of mycelium and polysaccharides.

5.2 *C. sinensis* species and value

Cordyceps sinensis (Berk.) Sacc. [≡ O*phiocordyceps sinensis* (Berk.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora] is an ascomycete fungus (instead of basidiomycetes for most of the edible mushrooms), which is a parasite on the caterpillars of the *Hepilus* spp. moths. As shown in Fig. 5.1, a mature *C. sinensis* caterpillar fungus forms a fruiting body on an insect larva. The infection of larva by the fungus usually starts in late autumn (with the colonization of the larva by fungal spores) and continues through the winter (underground), till the next spring or early summer, when a stalked

fruiting body protrudes from the head of the dead larva to appear above the ground. This fruiting body-caterpillar complex is called Dong-Chong-Xia-Cao 冬虫夏草 in Chinese which means "winter-worm and summer-grass", signifying its seasonal changes in morphology [8]. Natural *C. sinensis* are mainly distributed on the Qinghai-Tibetan plateaus at an altitude of 3,500–4,500 m above sea level, scattering over five provinces in western China, Tibet, Qinghai, Sichuan, Yunnan and Gansu [20].

In recent years, wild or natural *C. sinensis* has become increasingly scarce and classified as an endangered species because of reckless harvesting and unfavorable weather conditions for its proliferation [8]. Artificial cultivation of the caterpillar fungi is formidable owing to the complex fungal parasite-insect host relationship, a biological process and the special environmental conditions for the formation of natural caterpillar fungi. Cultivation of fungal mycelia by solid or liquid fermentation is the only viable alternative for mass production of *Cordyceps* material. Many fungal species have been isolated from natural *C. sinensis* caterpillar fungus such as *Paecilomyces sinensis*, *Paecilomyces hipeali*, *Cephalosporium sinensis*, *Tolypocladium sinensis*, and *Hirsutella sinensis* [21, 22]. Some of these species have been successfully applied to liquid and submerged fermentations for large-scale production of *C. sinensis* fungal mycelia. In recent years, there are many commercial health products made of *C. sinensis* fungal mycelia in capsules and various other formulations (Fig. 5.2). The Cs-4 fungus *Paecilomyces hepiali* Chen has been

Fig. 5.2 Commercial cordyceps health food products made of fungal mycelium

most widely applied to commercial production of *C. sinensis* mycelia by fermentation since the first Cs-4 mycelium product called "JinShuiBao Capsules金水宝胶囊" was made in 1982 by Jiangxi Jinshuibao Pharmaceutical Company Ltd. (Nanchang, Jiangxi, China) [13].

C. sinensis contains several classes of bioactive compounds, of which nucleoside analogues and polysaccharides are the two major classes. More than ten nucleosides and analogues have been isolated from *C. sinensis*, adenosine and 3′-deoxyadenosine or cordycepin are regarded as the marker constituents of *C. sinensis*. Polysaccharides are one of the most abundant classes, which account for 8–10 % of the biomass [23, 24]. Other bioactive compounds in *C. sinensis* include sterols, amino acids and peptides. It has been shown that the fungal mycelium biomass can have the similar chemical composition and pharmacological activities as that of natural *C. sinensis* fungus [25].

5.3 General Conditions for Submerged Fermentation of *Cordyceps*

5.3.1 Nutrient Requirements for Cordyceps *Mycelial Cultures*

The fungal cells in fermentation processes can utilize carbon, nitrogen and sulfur as the major nutrients for growth (biomass) and metabolite production (primary and secondary metabolites). Carbon is the major structural element of all the organic compounds in living organisms. Glucose and sucrose are most common carbon sources, and fructose and mannose are also used for certain species and production processes. Polysaccharides such as starch is often used as the less expensive carbon source, which needs to be hydrolyzed into its mono-sugar subunits by specific enzymes in the microorganisms before being utilized [26]. Nitrogen sources are divided into two classes, organic and inorganic. The most common organic nitrogen sources are those complex natural products such as yeast extract, peptone, corn steep liquor and casein hydrolysate. Nitrate (NO_3^-) and ammonium (NH_4^+) are most common inorganic nitrogen sources for many microorganisms including fungi. Most fungal cultures require at least one complex organic nitrogen source, and some cannot even utilize any form of inorganic nitrogen [27, 28]. Phosphorus and sulfur are another two major inorganic elements required for microbial and fungal growth. Inorganic salts such as KH_2PO_4 and K_2HPO_4 are most common phosphorus sources as well as the sources for potassium. Salts of SO_4^{2-}, e.g., $(NH_4)_2SO_4$ and $MgSO_4$, are common sulfur sources, and thiosulfate ($S_2O_3^{2-}$), sulfide or organic sulfur (such as the sulfur containing amino acids) are used as the sulfur sources for a some fungal species [27, 28].

As shown in Table 5.1, glucose and sucrose have been the common carbon sources, and yeast extract, peptone and corn steep powder have been the common nitrogen sources for various *C. sinensis* species in mycelial cultures. Inorganic salts KH_2PO_4, K_2HPO_4 and $MgSO_4$ are included as the K, P, S, and Mg sources in almost all the media; NH_4^+, NO_3^+ and Fe^{2+} are also added to a few. Most of the media have a simple composition containing altogether not more than five different components.

Table 5.1 Mycelial fermentation of *C. sinensis* fungi in liquid media and product yields

Fungal species	Medium composition	T (°C)	pH	Culture vessel	Period (d)	Biomass (g L^{-1})	EPS (g L^{-1})	References
C. sinensis CCRC36421	Sucrose 6.17 %, corn steep powder 0.5 %, $(NH_4)_2HPO_4$ 0.5 %, KH_2PO_4 0.15 % (w/v)	25	4.4	5-l jar fermentor: agitation 300 rpm	7		3.2	[25, 29]
C. sinensis	Sucrose 20, corn steep powder 25, $CaCl_2$ 0.78, $MgSO_4 \cdot 7H_2O$ 1.73 (g L^{-1})	20	4.0	5-l stirred-tank fermenter: aeration 2 vvm; agitation 150 rpm	16	20.9	4.1	[16]
C. sinensis 762	Sucrose 50, peptone 10, yeast extract 3 (g L^{-1})	18		Rotary shaker at 150 rpm	40	22.1		[30]
C. sinensis 16	Sucrose 2 %, yeast extract 0.9 %, K_2HPO_4 0.3 %,$CaCl_2$ 0.4 % (w/v)	25		Rotary shaker at 150 rpm	5	54.0	28.4	[17]
C. sinensis 1	Sucrose 3 %, corn steep powder 5 %, bean cake 4 %, KH_2PO_4 0.1, $MgSO_4 \cdot 7H2O$ 0.05 %, vitamin B1 0.01 %	22	6.5	Rotary shaker at 120 rpm	7		5.9	[31]
C. sinensis 383	Glucose 30, bean cake 20, $MgSO_4$ 2.0, KH_2PO_4 4.0 (g L^{-1})	24	7.0	Rotary shaker at 140 rpm	5		3.9	[32]
C. sinensis	Sucrose 20, yeast extract 2.0, KH_2PO_4 1.0, $MgSO_4 \cdot 7H_2O$ 0.6 (g L^{-1})	26	7.0	1.0-l shake flask: aeration 1 l/min; agitation 130 rpm	4	12.3	24.5	[33]
C. sinensis CCRC36421	Rice bran 1.5 %, molasses 0.5 %, CSL 3 %, KH_2PO_4 0.1 %, $MgSO_4$ 0.05 %	25	5.5	5-l jar fermenter: aeration 1.0 vvm; agitation, 150 rpm	5–6		48.9	[34]
C. sinensis CS001	Glucose 30, yeast extract 3, peptone 2, KH_2PO_4 0.6, $MgSO_4 \cdot 7H_2O$ 0.4, vitamin B_1 0.01, palmitic acid 1.0 (g L^{-1})	27	6.5	250-mL shake flask at 160 rpm	7		0.4	[35]

5.3.2 Oxygen Supply and Culture Conditions

C. sinensis fungi are aerobic organisms and their mycelial cultures require constant oxygen supply. In liquid fermentation, oxygen is usually supplied by air-sparging into the broth. The dissolved oxygen (DO) level in mycelial culture is a major factor which strongly affects the mycelial growth rate, and perhaps also affects the yield of bioactive products such as exopolysaccahrides (EPS) [36, 37]. On the other hand, the oxygen requirements may be different for cell growth and metabolite synthesis. In a previous study on *C. militaris* mycelial culture, for example, DO was controlled at 60 % air saturation during initial period or the first stage for mycelial growth and was lowered to 30 % in later period or the second stage for the cordycepin production, leading to 15 % higher cordycepin yield than with a single DO level throughout the entire culture period [38].

In addition to nutrients and oxygen, the medium pH and culture temperature are important factors for microbial cultures including fungal mycelial fermentation (Table 5.1). Although *C. sinensis* is a cold resistant species, its metabolism is inhibited when the cultivation temperature drops to 0 °C [39]. The fungus grows slowly at 1–4 °C, and most rapidly at 20–25 °C. Normally it grows well in slightly acidic pH range (pH 5–7). For submerged fermentation at 20–25 °C, the mycelial growth usually reaches the maximal concentration in 5–7 days of cultivation. The period of cultivation not only affects the yield but also the chemical composition and the medicinal property of the mycelial biomass.

5.3.3 Culture Vessels for Mycelial Fermentation and Yields of Biomass and EPS

Fermenters are the major pieces of equipment for large-scale production of mycelial biomass and fungal metabolites. Stirred-tank fermenters are dominantly used for liquid fermentation in both industry and laboratory, which provide efficient mixing and oxygen transfer conditions and also allow for monitoring and controlling the culture conditions [7, 40]. Disc turbine with vertical blades (Rushton) is the common and standard impellor found on industrial and laboratory fermenters, which pumps the fluid in a radial direction. Another type of impeller more suitable for mycelial broth of high viscosity is the axial flow hydrofoil design, which can pump liquid up or down and provide better bulk mixing than the Rushton type. Stirred-tank fermenters used in most previous studies on *C. sinensis* mycelial cultures were mostly agitated with the standard Rushton impellor [25, 37, 38, 41]. In mycelial culture fermenters, efficient mixing and oxygen transfer may become increasingly difficult with time because of the increase in broth viscosity and with the increase in biomass and EPS concentrations. The cell morphology and the rheological properties of mycelial broth have strongly affected by the hydrodynamic conditions. Consequently, these biological characteristics and physical properties are all major factors affecting the performance and productivity of the mycelial fermentation.

Table 5.1 provides a summary of the mycelial biomass and EPS yields of several *C. sinensis* species obtained in various conditions in shake-flaks and small

fermenters. The biomass and EPS yields varied in a wide range from 10 g L^{-1} to 54 g L^{-1}, and <1.0 g L^{-1} to >40 g L^{-1} with the fungal species and culture conditions, respectively. Most of these results were obtained in small volumes shake-flasks or stirred-tank fermenters.

5.4 Mycelial Culture of *C. sinensis* Fungus Cs-HK1

5.4.1 Fungal Species and Mycelial Culture

The Cs-HK1 fungus was originally isolated from the fruiting body of a natural *C. sinensis* in our lab and identified as a *Tolypocladium* sp. fungus (China General Microbiological Culture Collection Center: CGMCC6004) [42]. The Cs-HK1 mycelial culture was stored on solid potato-dextrose-agar (PDA) medium at 20 °C. The liquid culture medium was composed of 40 g L^{-1} glucose, 10 g L^{-1} yeast extract, 0.5 g L^{-1} $MgSO_4$ and 1 g L^{-1} KH_2PO_4. Liquid culture was usually propagated in 250 mL Erlenmeyer flasks each containing 50 mL of liquid medium on a shaking incubator operated at 150 rpm and 25 °C.

5.4.2 Carbon and Nitrogen Nutrients and Mycelial Growth in Liquid Culture

Various organic nitrogen sources including yeast extract (YE), peptone (PE), corn steep liquor (CSL) and urea, and inorganic nitrogen sources, such as KNO_3, NH_4Cl and NH_4NO_3 in medium containing 40 g L^{-1} glucose were compared. Among those organic nitrogen sources, YE and PE were most favorable for mycelial growth with a maximum mycelial dry weights of 16.5 and 13.8 g dw L^{-1}, respectively (Fig. 5.3a), and the highest biomass concentration was attained with 10 g L^{-1} yeast extract and 5 g L^{-1} peptone plus the inorganic salts. Therefore, the final liquid medium for Cs-HK1 mycelial culture was made of glucose (40 g L^{-1}), yeast extract (10 g L^{-1}), peptone (5 g L^{-1}), KH_2PO_4 (1 g L^{-1}) and $MgSO_4{\cdot}7H_2O$ (0.5 g L^{-1}). Fig. 5.3b shows the typical time courses of mycelial growth and sugar, and nitrogen consumption of the Cs-HK1 fungus in shake-flask cultures. The mycelium biomass showed a lag-phase in the first 1–2 days and exponential growth in the next 2–3 days (specific growth rate estimated at 1.1 d^{-1}). The biomass of mycelium reached a maximum concentration of 23.2 g dw L^{-1} on day 7 and changed a little during days 7–9, and then started to drop on day 10. The time courses of glucose and total nitrogen (TKN) appeared to be mirror images of the biomass growth curve, with both nutrient concentrations dropping slowly in the first 2 days, and rapidly from day 2 to day 6, and leveling off thereafter.

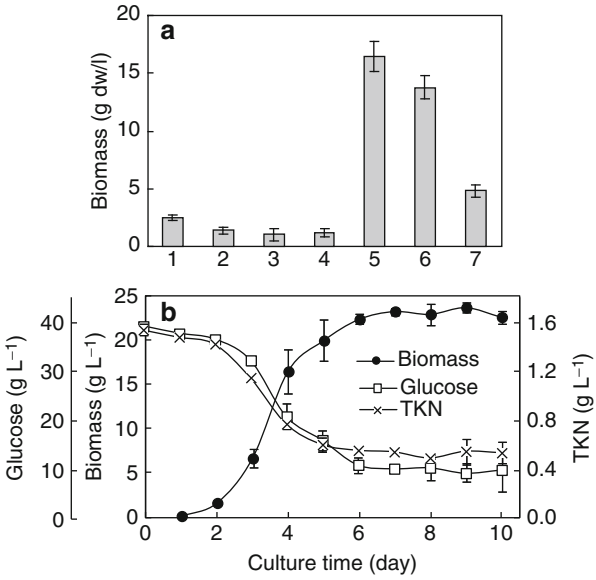

Fig. 5.3 (**a**) Effects of nitrogen sources on mycelial growth by Cs-HK1. *1* KNO_3; *2* NH_4Cl; *3* NH_4NO_3; *4* $CO(NH_2)_2$; *5* YE; *6* PE; *7* CSL; error bars for SD, n = 3; and (**b**) Time courses of mycelium biomass and sugar, and nitrogen concentrations in Cs-HK1 fungus liquid cultures (TKN for total Kjeldahl nitrogen nitrogen determined by the standard Kjeldahl method) [42]

Table 5.2 Contents of total protein, carbohydrate and polysaccharide and major nucleosides in natural *C. sinensis* (Cs) and Cs-HK1 fungal mycelium (cultured for 7 days)

Components	Cs-HK1 mycelium	Natural Cs
Protein (mg g^{-1})	11.7 ± 0.07	9.54 ± 1.11
Sugar (mg g^{-1})	654.6 ± 21.5	643.1 ± 18.0
Polysaccharide (mg g^{-1})	244.2 ± 11.6	129.5 ± 14.8
Nucleosides (μg/g dw)		
Uracil	13.3 ± 0.56	3.35 ± 0.30
Cytosine	5.03 ± 0.36	69.4 ± 7.15
Adenosine	1116.8 ± 8.25	264.6 ± 6.66
Cordycepin	65.7 ± 2.01	20.8 ± 2.56

Contents are expressed in per gram dry weight of mycelium and natural Cs; values represent means ± SE, n = 2

5.4.3 *Chemical Composition of Cs-HK Mycelium and Natural* C. sinensis

The protein and total carbohydrate contents of the Cs-HK1 mycelium were similar or slightly higher than in the natural species, and the PS content of mycelium was much higher than that of natural *C. sinensis* (Table 5.2). The contents of most nucleosides detected in the fungal mycelium were significantly higher than those in the natural *C. sinensis*. In particular, the contents of two marker constituents of *C. sinensis*, cordycepin and adenosine in the mycelium extract were about three and five times respectively of those in the natural species.

5.5 Cs-HK1 Mycelial Fermentation from Small to Large-Scale Fermenters

In previous studies, we have established the Cs-HK1 mycelial culture, derived the suitable medium and culture conditions, analyzed the chemical composition and bioactive components of Cs-HK1 mycelial biomass and detected the antitumor activities of mycelial extracts [42, 43]. The results showed that Cs-HK1 mycelial fermentation can produce a mycelial biomass with the similar medicinal properties as that of natural *C. sinensis*. The potential and capability of Cs-HK1 mycelial culture for commercial application needs to be evaluated in large-scale fermenters. Stirred-tank or mechanically-agitated fermenters are the most common culture vessels for liquid or submerged fermentation in the industry. Several factors are important for operation of stirred-tank fermenters, dissolved oxygen (DO), mycelial morphology and broth viscosity and rheological characteristics. In this part, we first studied the culture characteristics of Cs-HK1 fungus in small laboratory fermenters, and then evaluated the performance in pilot and large-scale industrial fermenters for mycelial biomass and EPS production, and finally examined the problems and alternatives for separation and recovery of mycelial biomass and EPS from fermentation broth. The mycelial fermentation experiments were performed in 1–15 L fermenters, and large-scale production in 2,500 L and 10,000 L fermenters.

5.5.1 Oxygen Uptake Rate

Specific oxygen uptake rate qO_2 was determined in a 1-L stirred-tank fermenter by the dynamic gassing-out method [44]. The fermenter used was a BIOSTAT® B Plus 1-L stirrer tank fermenter (Sartorius, Germany), having a total volume of 1.6 L and working volumes of 0.4–1 L. The agitator consisted of two 6-vertical blade disk impellers on a rotating shaft with an impeller/tank diameter ratio of 0.41. Wall baffles were removed during the experiments to avoid mycelial blockage and adhesion. The fermenter was assembled with probes or electrodes for dissolve oxygen (DO), pH, temperature, and antifoam detection, which were all connected to a controller panel and a computer. Air flow rate was kept at 1 vvm, and DO in the fermenter was controlled by adjusting the agitation speed. Another 15-L stirred-tank fermenter, BIOSTAT® B Plus (Sartorius, Germany) was also used for comparison of the mycelium and EPS production with those in 1-L. Antifoam 289 (Sigma, St Louis, MO, USA) was added to the medium at 0.1 % (v/v). The inoculum was taken from shake-flask culture of the Cs-HK1 incubated for 6–7 days at 20 °C, and added to the fermentation medium at 0.1 % (v/v). Culture pH was not controlled during the fermentation period.

The qO_2 measurement was conducted 24 h after inoculation when the mycelial culture was in the exponential growth phase. At this time, the air supply to the fermenter was abruptly stopped, so that, the DO concentration dropped with time approximately in a linear trend, and its slope (in mol $L^{-1}h^{-1}$) divided by the cell concentration (in kg L^{-1}) and equal to qO_2 (in mol O_2 kg^{-1} cell-h^{-1}). As shown in Fig. 5.4a, the DO in the liquid culture dropped slowly in the first day (day 0–1) after

inoculation, and then dropped steeply to nearly zero in the next 3 days (days 1–3) and remained at the low level thereafter. The rapid DO drop was in a parallel trend with the rapid mycelial growth. Between days 3 and 5, the DO was nearly depleted while the mycelial biomass was growing rapidly. The low DO level during this period was probably due to rapid oxygen consumption by the fungal cells and ineffective oxygen supply. The latter problem was caused by the inefficient mixing of the mycelial broth with the formation of a stagnant zone by the viscous mycelial broth near the wall and away from the impellers.

As shown in Fig. 5.4b, the specific oxygen uptake rate (qO_2) of Cs-HK1 in the 1-L mycelial fermenter, this exhibits the typical saturation kinetics of batch fermentation. qO_2 increased rapidly with DO from 0 to about 10 % air saturation and then remained relatively steady at higher DO levels. From this plot, we derived the critical DO as 10–20 % air saturation and the maximum qO_2 as 2 mol O_2 kg^{-1} cell-h^{-1} for the growth of Cs-HK1 mycelium in liquid culture.

According to the experimental results at different DO levels (5, 10 and 15 % air saturation) in the 1-L fermenters (Table 5.3), both biomass and EPS concentrations increased with the DO level. The small increase in the mycelial biomass (about 5 %)

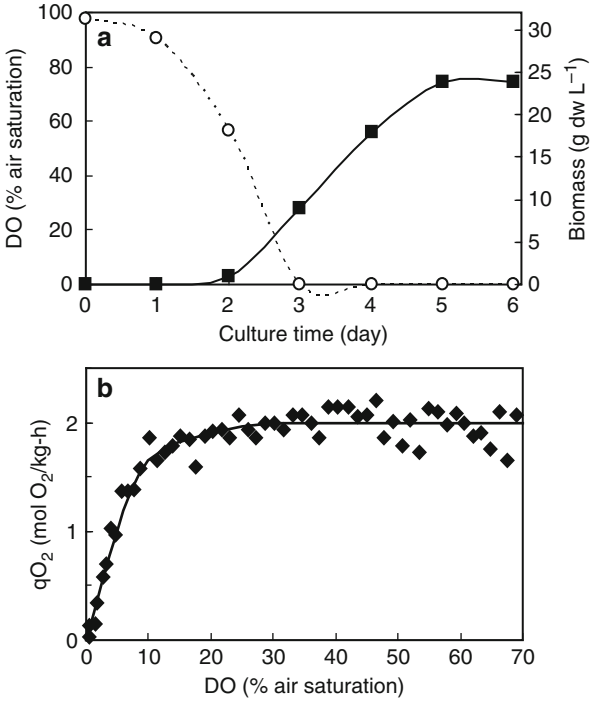

Fig. 5.4 Oxygen uptake rate of Cs-HK1 in a 1-L fermenter (agitated with two standard disk turbine impellers): (**a**) DO and biomass time courses (○ dissolved oxygen content; ■ biomass yield); (**b**) Specific oxygen uptake rate

Table 5.3 Mycelial biomass and EPS yields attained at different DO levels in 1-L fermenters (6-day period)

DO (% air saturation)	Biomass (g dw L^{-1})	EPS (g L^{-1})
5	22.5	3.2
10	24.2	4.1
15	25.4	4.7

with DO increased from 10 to 15 % was in agreement with the critical DO level around 10–20 % as suggested in Fig. 5.4b. However, the EPS production increased more dramatically with the DO level at that range, suggesting that a higher DO concentration was required for efficient EPS production.

5.5.2 *Mycelial Culture Characteristics of Cs-HK1 in Laboratory Fermenters*

Figure 5.5 shows the time courses of Cs-HK1 mycelial culture in 1-L fermenters at 10 % air saturation including biomass growth, nutrient consumption, medium pH change and EPS production. The mycelial biomass grew rapidly (exponentially) from day 2 to 5 and reached a maximum biomass 23.9 g dw L^{-1} on day 6; glucose concentration dropped to below 2 g L^{-1} on day 5 and to nearly zero on day 6; total nitrogen level dropped to 0.5 g L^{-1} which was about 17 % of the initial level. During the mycelial fermentation, the pH of culture medium dropped from 6.0 to 4.6–4.8 probably due to the organic acids produced by the fungal cells (Fig. 5.5b); the EPS production was nearly parallel to the mycelial growth, with the EPS concentration increasing most rapidly between 2 and 5 and reaching 4.6 g L^{-1} on day 6.

The Cs-HK1 mycelial culture in a 15-L fermenter followed the similar time course as that of biomass growth, oxygen consumption and medium pH change to those in the 1-L fermenters, exhibiting a very low DO level from day 3 to day 5 when the mycelial biomass was increasing rapidly. Table 5.4 presents a summary of

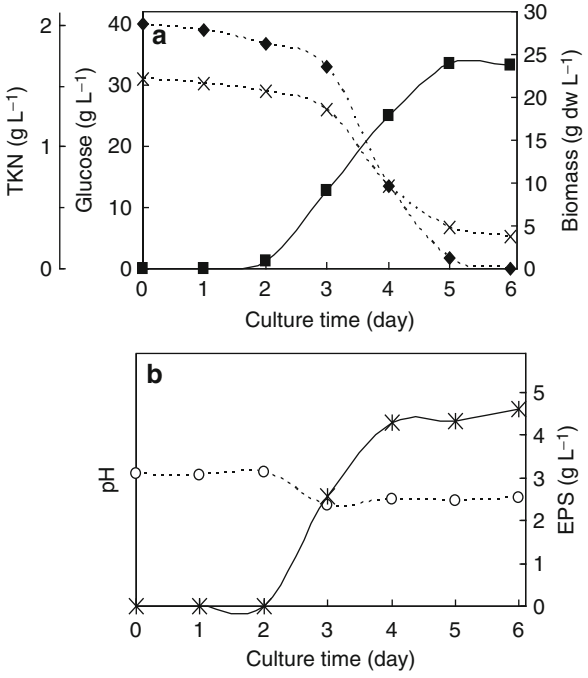

Fig. 5.5 Time profile of Cs-HK1 in 1-L fermenter at 10 % air saturation. (**a**) biomass yield and nutrients consumption; (**b**) culture pH and metabolite production (■ biomass yield; ◆ glucose; × TKN (total Kjeldahl nitrogen); ○ pH; ✳ EPS)

Table 5.4 Major culture parameters of Cs-HK1 mycelial culture in small lab fermenters and shake-flasks (all harvested on day 5)

	Shake-flask (50 mL)	Fermenters (liquid volume)	
		1 L	10 L
Maximum biomass, g dw L^{-1}	18.1 ± 1.88	23.9	21.5
Average growth rate, g dw day^{-1}	3.62 ± 0.37	4.78	3.58
Remaining glucose in medium, g L^{-1}	15.1 ± 2.1	1.8	7.4
Biomass yield on glucose, g dw g^{-1} glc	0.72	0.62	0.66
Cordycepin content, μg g^{-1} dw	20.5 ± 3.3	25.9	22.3
EPS concentration, g L^{-1}	2.01 ± 0.33	4.32	3.92
EPS yield on Glc, mg g^{-1} glc	80.6	113.0	120.2

the major growth parameters in the 1-L and 15-L fermenters and in shake-flasks. The biomass and EPS concentrations (on day 5) in both fermenters were higher than in shake-flasks, implying that the mycelial growth and EPS production rates were higher in the fermenters than in shake-flasks. This difference was probably attributed to more effective mixing and oxygen supply in the fermenters than the shake-flasks. In addition, the mycelial cultures in the fermenters had a lower biomass yield but higher EPS yield in glucose than in shake-flasks, suggesting that, at higher oxygen concentrations, more sugar can be converted to EPS biosynthesis than biomass. The similar trend has also been observed with other *Cordyceps* mycelial cultures in previous studies [36, 37, 41].

5.5.3 Morphological and Rheological Characteristics

As observed under a microscope (Fig. 5.6), the Cs-HK1 mycelia were in filamentous form in the early days of culture when the mycelial concentration was low, and gradually formed a center core (pellet) surrounded by long filaments. Such a highly filamentous mycelium resulted in a paste-like liquid which was extremely difficult to separate by filtration (as shown later).

Figure 5.7 shows the apparent viscosities of the fermentation liquid (with mycelia) and mycelium-free broth during the fermentation process. The broth viscosity started to increase from day 2 to day 3 and increased steeply from day 3 to 5, with a trend similar to the biomass time course shown in Fig. 5.5. As for the mycelium-free fermentation broth, the apparent viscosity did not show any significant increase in the first few days 0–3, and a sharp increase between days 4 and 5, exhibiting a similar trend to the EPS time course shown in Fig. 5.5. Apparently, the viscosity of broth can be mainly attributed to the mycelium, and that of medium to the EPS in the medium. The apparent viscosity of mycelial broth was 750 mPa-s and that of medium was 100 mPa-s at day 5 (at shear rate of 10.5 s^{-1}).

Figure 5.8 shows the rheological profiles of the mycelial culture broth and liquid medium on days 3–5, and Table 5.5 presents the corresponding parameters for the power-law model. Both culture broth (containing mycelia) and mycelium-free medium exhibited pseudoplastic rheology following the power-law ($\tau = K\gamma^n$) with

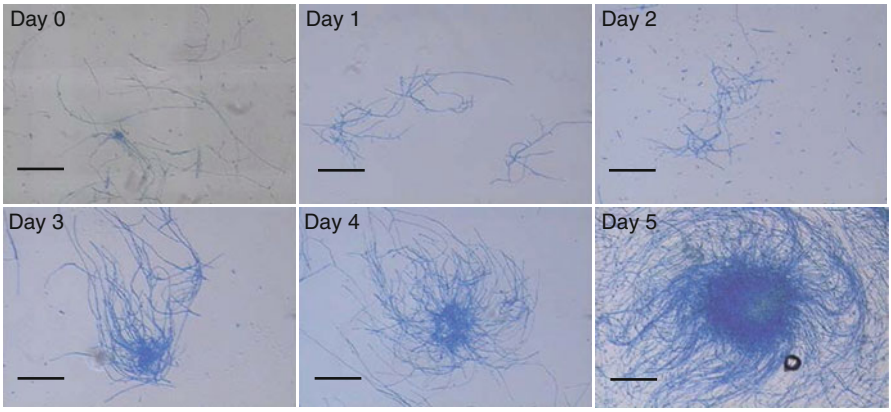

Fig. 5.6 Morphological changes in mycelia during the submerged culture of Cs-HK1 in a 15-L stirred-tank fermenter at 100× magnification (bar = 100 μm)

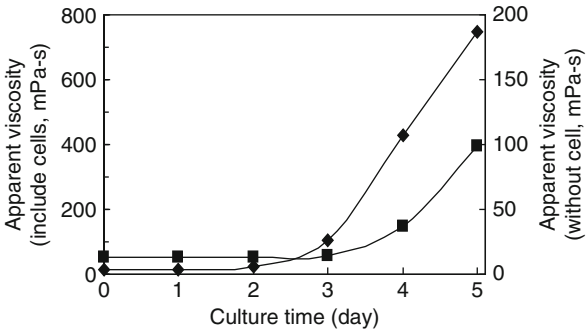

Fig. 5.7 The apparent viscosity of the fermentation broth of Cs-HK1 in a 15-L fermenter at the shear rate of 10.47 s^{-1} (◆: fermentation liquid with mycelia; ■: mycelium-free broth)

the flow behavior index $n < 1$, which is typical for mycelial broths and biopolymer solutions [7, 37, 45, 46]. The flow consistency index K increased steadily over the fermentation period in correlation with increase in the apparent viscosity of mycelial broth and liquid medium.

5.5.4 Mycelium and EPS Production in Large-Scale Industrial Fermenters

Figure 5.9 shows the major steps for the scaling up of Cs-HK1 mycelial culture from stock culture, shake-flask (starter) culture, seed fermenter and to the production fermenter, followed by the downstream processes for separation of biomass and PS. The large-scale fermentation was carried out in industrial stirred-tank fermenters of two different volumes, 2.5×10^3 L and 10×10^3 L. The air flow rate was kept at 1 vvm (same as in the small fermenters) and DO was controlled above the critical DO level (10 % air saturation) by adjusting the agitation speed. Antifoam

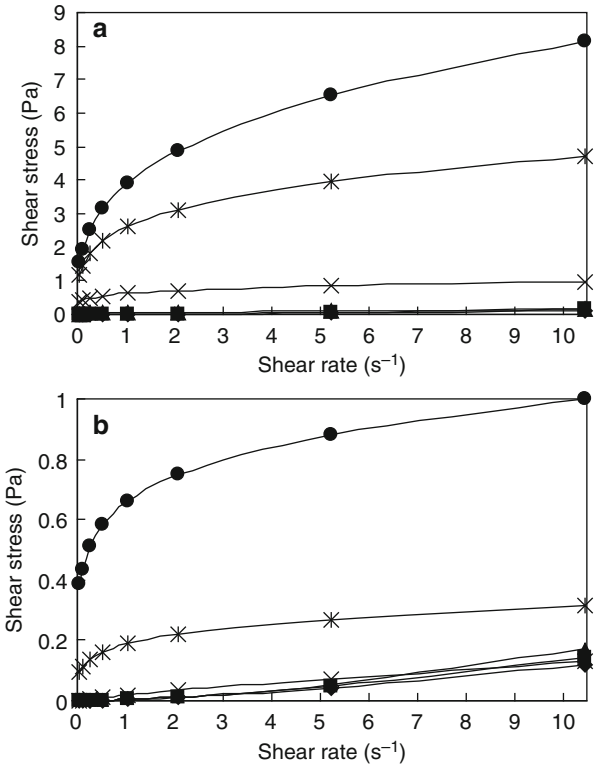

Fig. 5.8 Rheographs (shear stress vs. shear rate plots) of fermentation broth of Cs-HK1 in a 15-L fermenter, (**a**) fermentation liquid with mycelia; (**b**) mycelium-free broth (▲: day 2; ×:day 3; ✳: day 4; ●: day 5)

Table 5.5 Rheological parameters of Cs-HK1 mycelial culture broth according to the power-law

	Mycelial broth		Culture medium	
Culture time (day)	K (Pa-s^n)	n	K (Pa-s^n)	n
2	56.9	0.5	n.d.	n.d.
3	623.9	0.2	17.5	0.9
4	2572.2	0.3	185.8	0.2
5	3876.1	0.3	654.5	0.2

Note: Viscosity of mycelia broth and liquid medium on earlier days was too low and could not be measured by the same viscometer. Regression R^2 values >0.998 for all parameters
K flow consistency index, *n* flow behavior index

was added to the fermentation medium at 0.1 % (v/v). For inoculum preparation, the culture broth from shake-flasks (on day 7) was inoculated into a seed fermenter (100 L) at 0.1–0.3 % (v/v), which was operated for 3 days. The broth from the seed fermenter was pumped to the production fermenters to initiate the fermentation. After 5–6 days of operation, the fermentation liquid was sent to a plate-and-frame filter for separation of mycelium biomass from the liquid medium.

Figure 5.10 shows DO and pH changes in a 10×10^3 L fermenter (liquid volume ~5.0×10^3 L). Similar to that of the small fermenters (Fig. 5.4), the DO dropped rapidly to a low level from day 1 to 3. Table 5.6 presents the major production

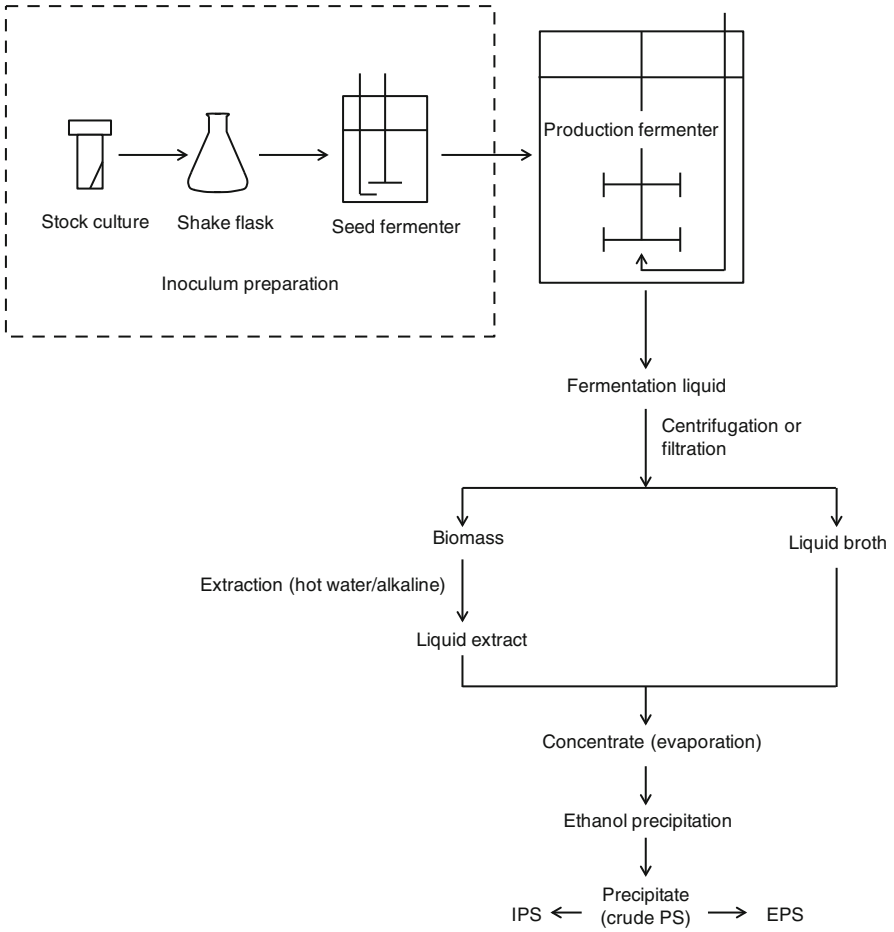

Fig. 5.9 Large-scale fermentation and downstream process for extraction and recovery of polysaccharides

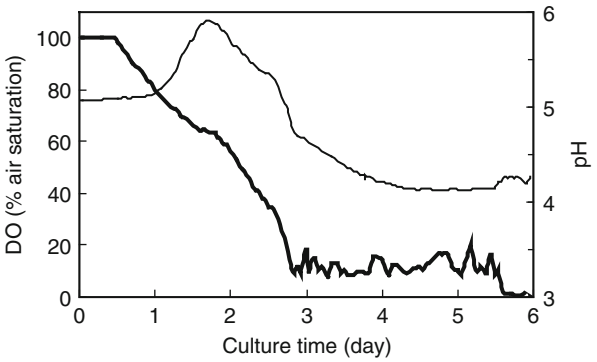

Fig. 5.10 DO and pH time courses of Cs-HK1 mycelial in 10×10^3 L fermenters (*bold line*: DO; *thin line*: pH)

Table 5.6 Production parameters of Cs-HK1 mycelial in large-scale fermenters and shake-flasks (6-day period)

	Shake-flask (50 mL)	Fermenter volume[a]	
		2.5×10^3 L	10×10^3 L
Maximum biomass, g dw L^{-1}	19.1 ± 0.66	22.1	23.5
Average growth rate, g dw day^{-1}	3.18 ± 0.06	3.68	3.91
Remaining glucose in medium, g L^{-1}	10.4 ± 1.1	2.5	3.2
Biomass yield on glucose, g dw g^{-1} glc	0.64	0.68	0.63
Cordycepin content, μg g^{-1} dw	35.4 ± 8.2	32.2	30.4
EPS concentration, g L^{-1}	2.12 ± 0.03	4.9	5.1
EPS yield on Glc, mg g^{-1} glc	71.6	130.7	138.6

[a]Actual liquid volume = 50–75 % fermenter volume

parameters of Cs-HK mycelial fermentation in 2.5×10^3 L and 10×10^3 L fermenters. Compared with the results in shake-flasks, the biomass and EPS yields (i.e. concentrations on day 6) were much higher, and the cordycepin contents (in mycelial biomass) were slightly lower. The higher growth rate and EPS yield in the large-scale fermenters may be attributed to more efficient mixing and oxygen supply than in shake-flasks. Comparative analysis of growth rate in various cultures revealed that performance was better in large fermenters than in shake-flasks and small laboratory fermenters. Thus, the scale-up process of Cs-HK1 mycelial culture in large fermenters was successful.

Moreover, the Cs-HK1 mycelial broth containing biomass and extracellular products harvested from the industrial fermenters has been tested for anti-fatigue activities in forced animal swimming experiments. The mycelium hot water extract showed the most significant effects, increasing the swimming endurance of mice up to 100 %, and also increasing the glycogen levels and reducing the lactic acid and blood urea nitrogen levels significantly [47].

5.5.5 Problems and Strategies for Recovery of Biomass and EPS from Viscous Broth

Filtration Resistance

Figure 5.11 shows the mycelial broth withdrawn from the industrial fermenters after 5–6 days of fermentation. The fermentation liquid was pumped to a plate-frame filter for separation of mycelial biomass from the spent medium. However, the paste-like mycelial broth caused great difficulty for filtration. With 200–800 mesh cloths, the filtration flux was negligible no matter how high was the filtration pressure; with a large-pore cloth (100 or lower mesh size), the filtrate contained large amount of mycelia. Centrifugation at relatively high speeds (e.g. 10,000 rpm) had to be applied to separate the biomass and the liquid medium.

Fig. 5.11 Cs-HK1 mycelial broth from industrial fermenters

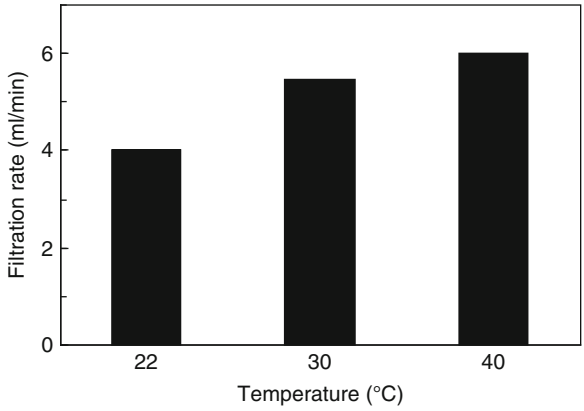

Fig. 5.12 Effect of temperature on filtration rate of mycelial broth

Liquid viscosity is a major factor contributing to filtration resistance. Since viscosity decreases with temperature, increasing the broth temperature may be effective to ease the filtration problem. Therefore, we tested the filtration of culture broth at elevated temperature. As shown in Fig. 5.12, the filtration rate was increased to 35 % with the temperature increase from 22 to 30 °C, and by 15 % from 30 to 40 °C. The results indicate that increase in the broth temperature can enhance the filtration process moderately.

Recovery of EPS from Fermentation Medium

Because of the large amount of ethanol required (four times the medium volume) for EPS isolation from the fermentation broth, ethanol precipitation is neither economical nor environment friendly. Ultrafiltration (UF) was exercised as an alternative for concentration and partial purification of EPS from the medium. With a 10 kDa MWCO UF membrane at 4 bar pressure and room temperature for 7 days, the EPS

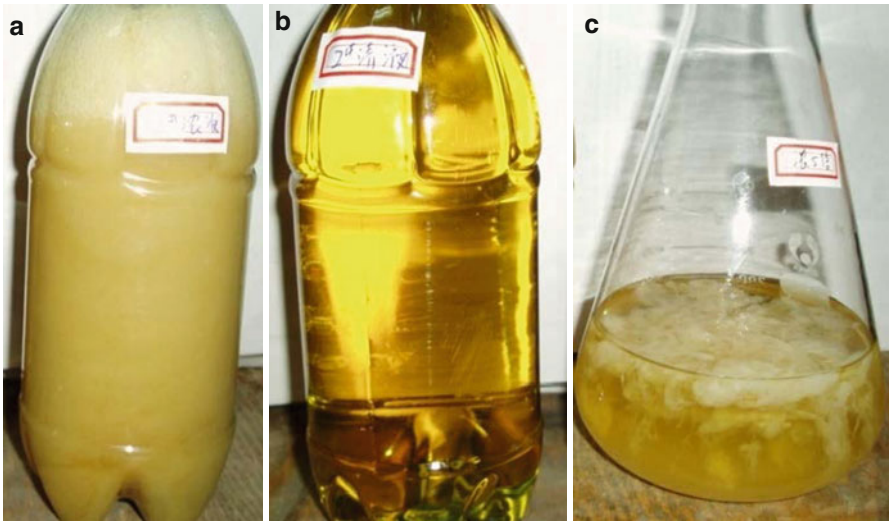

Fig. 5.13 Recovery of EPS from Cs-HK1 fermentation medium. (**a**) Concentrated EPS culture medium; (**b**) Permeate of UF; (**c**) EPS precipitated with ethanol

was concentrated by fourfold and the liquid volume was reduced by nearly 75 % (Fig. 5.13). Although the UF method was time-consuming, it could significantly reduce the ethanol volume required for EPS precipitation. The ultrafiltration rate may be increased with cross-flow operation and use of a higher MWCO membrane.

5.6 Molecular Structures and Properties of Polysaccharides

5.6.1 Extraction and Purification of Polysaccharides

Polysaccharides are the major bioactive constituents of *C. sinensis* species and other medicinal fungi [48–52]. As natural *C. sinensis* is very expensive, mycelium biomass produced by fermentation is a major source for the Cordyceps polysaccharides. In addition to the polysaccharides (IPS) extracted from the mycelium biomass, EPS can be produced by liquid fermentation of some *C. sinensis* fungal species, such as the Cs-HK1 mycelial culture in liquid media. As shown in Fig. 5.10, IPS is usually extracted from the mycelial biomass with hot water or aqueous alkaline. Organic solvent precipitation is a common method for the initial isolation of polysaccharides in aqueous solutions. Among various organic solvents, ethanol is the most favorable in laboratory and industry due to its relatively low cost, low toxicity to human and desirable physicochemical properties. The volume of ethanol required for the precipitation of a given PS depends mainly on its MW and the precipitation conditions and, in most cases, 3–5 volume ratios of ethanol to PS solution (70–80 % v/v) for complete precipitation of IPS in the aqueous extract solutions or EPS in liquid fermentation media.

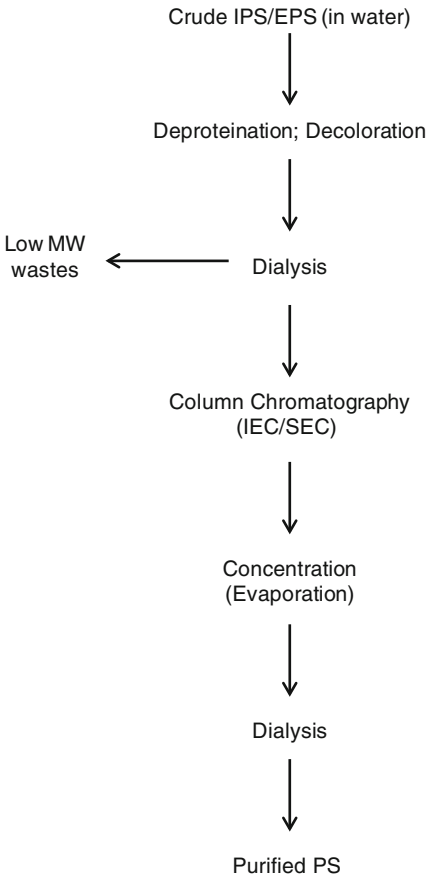

Fig. 5.14 Procedure of major steps for purification of polysaccharides isolated from mycelial extracts (IPS) or fermentation broth (EPS)

After the precipitation, the polysaccharide precipitate can be further purified, firstly by dialysis to remove salts and low-MW organic molecules, and then protein removal by chemical/enzymatic methods, and decoloration by physical and chemical methods. Further purification of the polysaccharides is performed through column chromatography, such as ion-exchange and gel filtration (Fig. 5.14).

However, the EPS isolated from the Cs-HK1 fermentation medium by a single-step precipitation with 4–5 volumes of ethanol was a crude mixture of PS, proteins and PSPs in a wide MW range. To precipitate the different MW fractions of EPS, we applied a step-wise or gradient precipitation procedure with ethanol volume ratios of 1/5, 1, 2, and 5 to the liquid medium (Fig. 5.15) [53]. Usually PS with a higher MW precipitates at a lower ethanol volume ratio or concentration in the solution. Table 5.7 shows the properties and compositions of EPS fractions attained from this experiment. The high-MW fraction $P_{1/5}$ attained at the lowest ethanol ratio was mainly composed of polysaccharides with negligible protein content, and vice versa. The gradient ethanol precipitation is a simple and workable for the initial fractionation of PS, proteins, and their complexes with different molecular sizes.

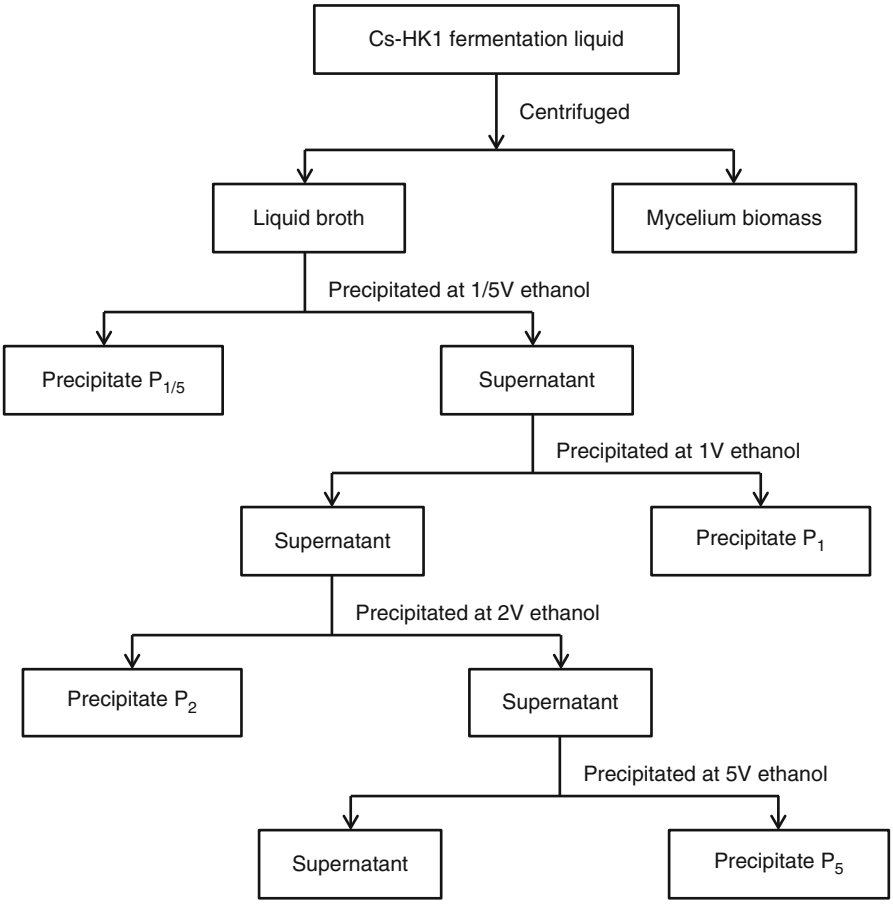

Fig. 5.15 Isolation of EPS fractions from fermentation broth of Cs-HK1 by gradient ethanol precipitation [53]

Table 5.7 Yields and chemical compositions of EPS fractions isolated from Cs-HK1 fermentation medium by gradient ethanol precipitation (Fig. 5.15) (in a 15 L fermenter filled with 8 L of liquid medium, run for 6 days at 20 °C, 1 vvm and DO above 20 % air saturation)

Frac	Yield (mg)	Sugar (wt %)	Protein (wt %)	MW (kDa)
$P_{1/5}$	2,854	83.6 ± 5.0	0.9 ± 0.2	47,400
P_1	375	31.2 ± 1.2	28.5 ± 1.4	2,870
P_2	1,822	21.0 ± 0.5	50.8 ± 0.1	630
P_5	1,815	6.3 ± 0.9	64.4 ± 0.4	16

5.6.2 Molecular Properties of Polysaccharides

As shown in Table 5.8, polysaccharides with different monosaccharide constituents, molecular weights (MWs) and chemical structures have been isolated from *C. sinensis* mycelia (IPS) and fermentation broth (EPS). Their average MW varied over a wide range from 10^3 to 10^7 Da. Their chemical structures have been characterized and elucidated based on established analytical methods involving infrared spectroscopy, liquid-state nuclear magnetic resonance (NMR), solid-state NMR, gas chromatography (GC), GC-mass spectroscopy (GC-MS), high-performance liquid chromatography (HPLC), acid hydrolysis, methylation analysis, periodate-oxidation, and Smith degradation [54–56]. These PS have shown antitumor, immunomodulatory, hypoglycemic and antioxidant activities.

IPS from cultured *C. sinensis* usually consist of glucose, mannose and galactose in various molar ratios with 1→4 (6)-glucopyranosyl (Glcp), 1→6-mannopyranosyl (Manp), and 1→4 (6)-galactopyranosyl (Galp) [49, 50, 52]. For example, our group isolated two water-soluble polysaccharides, WIPS and AIPS, from hot water and dilute alkaline extracts, respectively, of the mycelial biomass of a *C. sinensis* fungus Cs-HK1, which were characterized as α-D-glucans with a backbone of (1→4) linked α-D-glucopyranosyl (Glcp) (>60 %) (Fig. 5.16a, b) [57].

A few PS structures have also been purified from the EPS isolated from the culture broth of *C. sinensis*, which were mostly heteropolysaccharides. For instance, our group reported that EPS-1A which isolated from a fermentation broth of *C. sinensis* Cs-HK1 was found to be a slightly branched heterpolysaccharide with a backbone of (1→6)-α-D-glucose residues (~77 %) and (1→6)-α-D-mannose residues (~23 %). Branching occurs at the O-3 position of (1→6)-α-D-mannose residues of the backbone with (1→6)-α-D-mannose residues and (1→6)-α-D-glucose residues and terminated with β-D-galactose residues [58].

Some IPS and EPS also contained uronic acid, proteins and inorganic elements. These PS conjugates isolated from *C. sinensis* also represent a major class of bioactive compounds and may exert more important pharmacological effects than neutral polysaccharides. More recently, our group reported that an acidic polysaccharide AEPS-1, which has a linear backbone of (1→3)-linked α-D-Glcp residues with two branches, namely, α-D-Glcp and α-D-pyrano-glucuronic acid (GlcUp), attached to the main chain by (1→6) glycosidic bonds at every seventh α-D-Glcp unit (Fig. 5.16c) [59]. In addition, a novel poly-N-acetylhexosamine (polyhexNAc) of about 6 kDa with strong antioxidant activities has been purified from the low-MW fraction of EPS produced by the Cs-HK1 mycelial fermentation. The molecular structure was elucidated as a [-4-β-D-ManNAc-(1→3)-β-D-GalNAc-(1→] disaccharide repeating unit in the main chain with a Gal branch randomly occurring at the 3rd-position of ManNAc (Fig. 5.16d) [60].

Table 5.8 Polysaccharides from *C. sinensis* fermentation: molecular properties and bioactivities [48–52]

Fungal species	Molar composition	MW	Linkage/type	Bioactivities
IPS from mycelia (extracted by hot water if not stated otherwise)				
Paecilomyces sinensis (Cs-4)	Gal:Glc:Man =43:33:24	15 kDa	CS-F10 Galactoglucomann	Hypoglycemic activity
Cs-4	Glc:Man=9:1 (extracted with 0.05 M acetate)	7.7 kDa	Mannoglucan	Antitumor
Cs-4	Glc:Man:Gal=21:2:1	—	CS-Pp 1,3-β-D-glucan with 1,6-branched chain	Immunomodulatory
Cs-4	Glc:Man:Gal=2:1:1	460 kDa	→3-α-D-Glcp-1→3-β-D-Glcp-1→3-β-D-Galp-1→	Cholesterol esterase inhibitory
Cephalosporium sinense Chen	Man, Glc, Gal, Uronic acid	27 kDa	CAPS	Immunomodulatory
C. sinense Chen	Man:Glc: Gal=4:11:1	43.9 kDa	Galactoglucomanno-glycan (CPS-2)	Preventing chronic renal failure
C. sinense Chen	Glc:Man:Gal=1:0.6:0.75	210 kDa	CSP-1	Antioxidant; hypoglycemic
Tolypocladium sp. (Cs-HK1)	α-Glc (extracted with 1.25 M NaOH/0.04 % $NaBH_4$	1,150 kDa	AIPS α-D-(1→4)-glucan (86 %), (1→6) -α-D-glucose (14 %)	Antitumor, immunomodulatory
EPS from culture broth				
Cs-4	Man:Gal:Glc=10.3:3.6:1	43 kDa	CS-81002	Immunomodulatory
Cs-4	Glc:Man:Gal=2.4:2:1	82 kDa	Cordysinocan	Immunomodulatory
Cs-HK1	Man:Glc: Gal=23:1:2.6	104 kDa	EPS	Antitumor; immunomodulatory
Cs-HK1	Glcp:GlcUp=8:1	36 kDa	AEPS-1	Immunomodulatory
Cs-HK1	$ManNH_2$:$GalNH_2$:Gal=1.0:1.1:0.3	6 kDa	poly-N-acetylhexosamine	Antioxidant

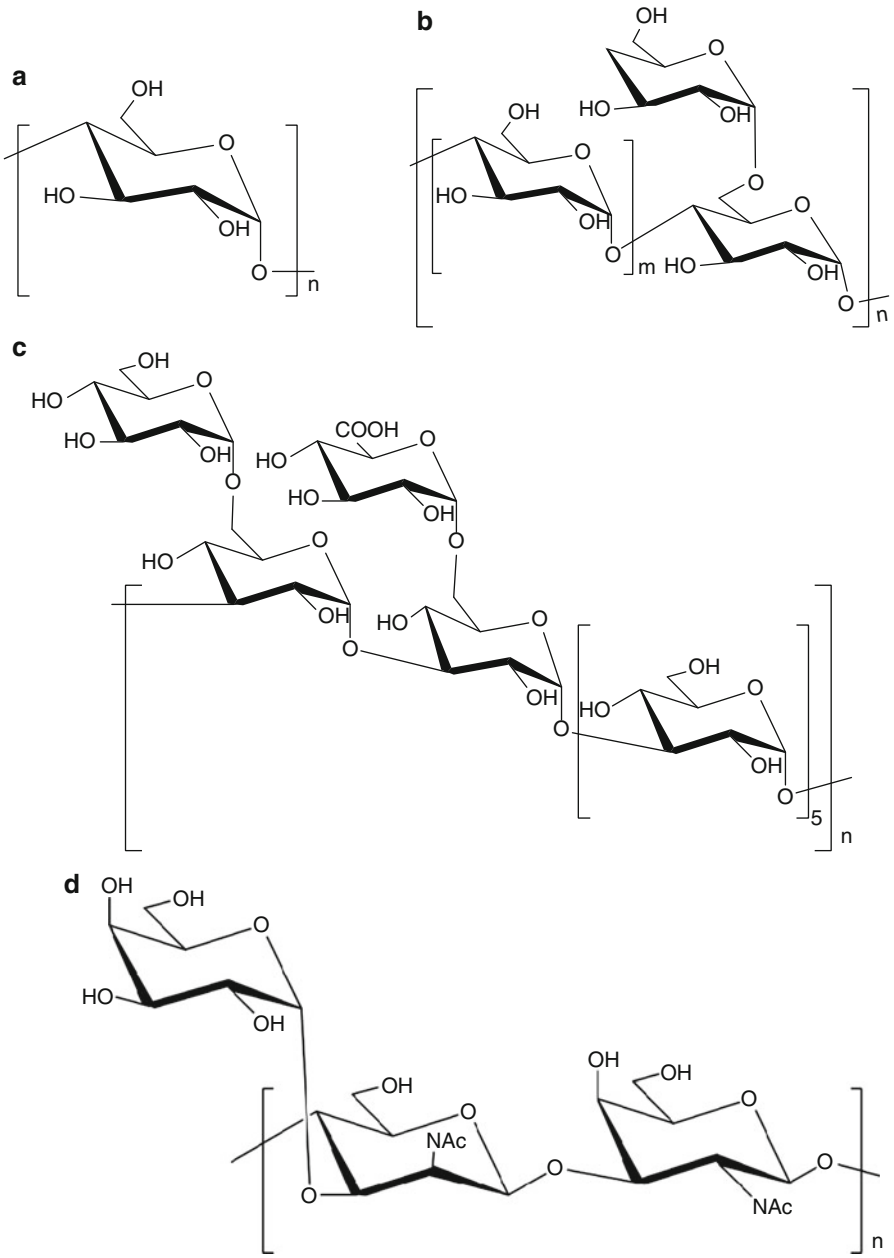

Fig. 5.16 The chemical structures of (**a**) AIPS: (1→4)-α-D-glucan and (**b**) WIPS: branched (1→4), (1→6) -α-D-glucan [57]; (**c**) AEPS-1 [59]; (**d**) polyhexNAc [60]

5.7 Concluding Remarks

Submerged fermentation of fungal mycelium is a relatively simple and cost-effective process, providing a renewable source of naturally-rare medicinal fungi. It also allows for convenient and fruitful manipulation of the culture conditions to control or enhance the accumulation of desired active ingredients. In addition to mycelium biomass, EPS in the fermentation broth are useful products with nutraceutical and therapeutic potential. There is a need to study further the relationship between the fermentation conditions and the contents of bioactive compounds in the mycelium biomass and to develop more effective downstream processes for the separation and recovery of mycelial biomass and EPS.

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Chapter 6
Ginseng Cell Culture for Production of Ginsenosides

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Abstract *Panax ginseng* C. A. Meyer (Araliaceae) is one of the most valuable oriental herbs and has been used as a healing drug and a health tonic in Korea, Japan and China since ancient times. Cultivation of ginseng in fields takes a long time, generally 4–6 years, and needs extensive efforts for quality control since plant growth is susceptible to many environmental factors including soil, shade, climate, pathogens and pests. On the other hand, the culturing of plant cells has been considered as a potential alternative for the efficient production of ginseng biomass and its active ingredients, such as ginseng saponins. In this chapter, the research work on cell suspension cultures of Korean ginseng (*P. ginseng*) using bioreactor technology, the various culture factors and the process variables such as growth regulators, sucrose concentration, types of bioreactors, inoculum density, aeration volume, gaseous composition such as oxygen, carbon dioxide and ethylene and elicitation on suspension cultures have been presented.

Keywords Bioreactor cultures • Ginseng • Ginsenosides • Panax ginseng • Suspension cultures

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Abbreviations

2 4-D	2, 4-Dichlorophenoxy acetic acid
DW	Dry weight
FW	Fresh weight
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MJ	Methyl jasmonate
MS medium	Murashige and Skoog medium
NAA	α-Naphthalene acetic acid
vvm	Air volume per medium volume per minute

6.1 Introduction

Panax ginseng C. A. Meyer (Araliaceae) has been widely used as a tonic and medicine since ancient times, particularly in oriental countries including Korea, Japan and China. It is effective for gastro-enteric disorders, diabetes, blood circulation, and has been used as an adjuvant to prevent various disorders. Thus, ginseng has been recognized as a miraculous medicine in preserving the health and attaining the longevity. The principal bioactive constituents of *P. ginseng* are the ginsenosides, a group of triterpene glycosides also known as saponins [1]. Currently, 30 ginsenosides have been recognized and they have been grouped under protopanaxadiol saponins (Rb group) and protopanaxatriol saponins (Rg group). Antiplatelet, hypocholesterolemic, antitumor, immunomodulatory functions, and their activity of improving the central nervous system have been attributed to the pharmacological importance of various ginsenosides [2, 3].

Plant cell suspension cultures are more advantageous for the for large scale production of secondary metabolites such as ginsenosides using bioreactor technologies [4]. The tissue culture of ginseng was first documented in 1964, after that numerous studies on ginseng were reported in the succeeding years [1]. Various successful efforts on *in vitro* culture of ginseng cells or tissues for the production of ginsenosides have been reported [5–8]. Murashige and Skoog (MS) [9] medium is commonly used for establishing cell and tissue cultures and other various physiological and physical factors have been investigated for the production of biomass and ginsenosides [1]. The addition of growth regulators is essential for cell growth, biomass accumulation and product formation. Choi [10] has investigated the *in vitro* culture of *P. ginseng* extensively and indicated that plant growth regulators such as 2, 4-D and kinetin in the medium affected the levels of saponins in callus and cell suspension cultures. For example, 3.62 % of total saponins were detected in the callus cultivated in MS medium containing 5 mg L^{-1} 2, 4-D and 1 mg L^{-1} kinetin, while 8.78 % was produced in 10 mg L^{-1} 2, 4-D and 1 mg L^{-1} kinetin medium. Zhong [11] found that a higher concentration of 7 mg L^{-1} kinetin inhibited the cell growth. The highest saponin content (13.9 %) was achieved in a medium containing 2.0 mg L^{-1} IAA and 0.07 mg L^{-1}

kinetin. Sucrose is a common carbon source used in ginseng cell cultures and the rate of biomass growth is usually directly correlated with the sugar consumption. Many researchers have worked out different types and concentration of sugars depending upon the cell lines used for cultures. Choi et al. [12, 13] found that the optimal concentration of sucrose for cell growth was between 30–50 g L^{-1}, and 70 g L^{-1} sucrose inhibited cell growth, while the saponin content showed a steady increase with the increase in sucrose concentration up to 60 g L^{-1}. In suspension cultures of *Panax notoginseng* cells, Zhang and Zhong [14] found that the constant or intermittent feeding of sucrose or other sugars was more effective than increasing the initial concentration to enhance the biomass and yield of secondary metabolites. Effects of nitrogen sources on the production of ginsenosides by cell cultures were investigated by Zhang et al. [15] and Ushiayama [16] and they have reported that a lower NH_4^+ to NO_3^- ratio is more favorable for saponin production. Zhang and Zhong [14] found that an increase in initial phosphate from 1.25 to 3.75 mM enhanced both cell growth and saponin yield in cell suspension cultures of *P. notoginseng*. The effect of K^+ and Cu^{2+} ions have also been investigated on cell growth and metabolite production in ginseng [17, 18].

Bioreactor cultures (stirred tank and airlift bioreactors) were established for large scale production of ginseng cell biomass and saponin production [19–21]. Impact of fed-batch cultures [14, 22], condition of the medium [23] and high-density cultures [14, 24–26] were experimented and the increased biomass and productivity of saponins and polysaccharides was achieved.

Recent reports show that saponins account for about 3–4 % in Korean ginseng, and more than 30 kinds of ginsenosides have been found in it, double the number of ginsenosides occurring in the ginsengs of other countries [2]. Considering that each of these ginsenosides has different pharmacological activities, it becomes apparent that Korean ginseng has a pharmacological effectiveness superior to those of other ginseng species [2]. Therefore, we were interested in establishing cell cultures of Korean ginseng and carried out a series of experiments for the production of ginsenosides in bioreactor cultures, and here we have summarized various aspects of ginseng cell cultures for the production of useful metabolites in a large scale.

6.2 Cell Suspension Cultures of Ginseng in Shake Flasks

6.2.1 Induction of Callus

Calli were induced from Korean ginseng (*P. ginseng* C.A. Meyer) root on MS semi-solid medium supplemented with 1.0 mg L^{-1} 2, 4-D and 3 % sucrose in the dark at 25 °C [27]. The callus proliferation was achieved on MS semi-solid medium supplemented with 2.0 mg L^{-1} NAA, 0.1 mg L^{-1} kinetin, and 30 g L^{-1} sucrose. Suspension cultures were established in 300 mL conical flasks containing 100 mL MS medium by adding 6 g callus and were maintained on rotary shaker at 105 rpm, in the dark at 25 °C. Cells were maintained by subculturing on to a fresh medium once every 15 days.

6.2.2 *Effects of Auxins on Cell Growth and Ginsenosides Production in Cell Suspension Culture of P. ginseng*

To understand the growth characteristics of cell suspension cultures of ginseng in shake flasks, the effects of plant growth regulators (2, 4-D, IBA, and NAA) on cell growth and saponin production, and nutrient utilization by the cultured cells were studied. The maximum biomass yield was obtained in medium containing 2, 4-D as compared to IBA or NAA. It was observed that a relatively lower concentration of IBA and NAA was unfavorable for cell growth and production of ginseng saponins (Table 6.1). With an increase in IBA or NAA concentration from 1 to 9 mg L^{-1} in the medium, the dry weight of cells increased and this phenomenon was reported from other cultures as well, in which high auxin levels were often good for cell growth [28]. In our experiments, the highest dry weight of cells was obtained with IBA (10.5 g L^{-1}) and NAA (9.7 g L^{-1}) at a concentration of 9 mg L^{-1}.

The effects of auxin (2, 4-D, IBA, and NAA) concentrations on saponin accumulation by *P. ginseng* cells were also studied. Total saponin production was significantly enhanced as the initial auxin level was raised from 1 to 7 mg L^{-1} of IBA and from 1 to 3 mg L^{-1} of NAA. However, a further increase in auxin concentration (up to 9 mg L^{-1}) led to a decrease in saponin accumulation (Table 6.1). The maximum saponin production of 7.29 ± 0.2 mg g^{-1} DW and 8.76 ± 0.1 mg g^{-1} DW was achieved at IBA concentration of 7 mg L^{-1} and NAA concentration of 3 mg L^{-1} respectively. These results are considered to be useful for exploration of the biosynthesis mechanism and in a large-scale bio-processing of the ginseng cell cultures.

Plant growth regulators are one of the key factors to influence the biomass accumulation and secondary metabolite production. In safflower cell cultures, high concentration of auxin was suitable for the cell growth, while high concentration of cytokinin was favorable for red and yellow pigment production in *Carthamus tinctorius* [29]. Son et al. [30, 31] found that 4 mg L^{-1} IBA was suitable for the mountain ginseng adventitious roots growth. So, the influence of growth regulators during *in vitro* culture is species specific. In this study, IBA (7 mg L^{-1}) was found to be

Table 6.1 Effect of auxins on cell growth and ginsenosides production in cell suspension culture

Auxin	Concentration (mg L^{-1})	Fresh wt. (g L^{-1})	Dry wt. (g L^{-1})	Ginsenosides (mg g^{-1} dry wt.)		
				Rg	Rb	Total
2,4 D	1	328 a[a]	11.9 a	1.81 ± 0.1	2.35 ± 0.2	4.16 ± 0.3
IBA	1	144 d	7.5 d	2.16 ± 0.3	3.05 ± 0.1	5.21 ± 0.3
	3	170 c	8.8 cd	2.09 ± 0.6	3.49 ± 0.2	5.58 ± 0.3
	5	178 c	9.1 c	2.43 ± 0.2	3.31 ± 0.4	5.74 ± 0.2
	7	216 b	10.1 b	2.6 ± 0.3	4.69 ± 0.2	7.29 ± 0.2
	9	226 b	10.5 b	1.42 ± 0.6	4.22 ± 0.2	5.64 ± 0.3
NAA	1	132 d	7.1 e	2.85 ± 0.4	5.33 ± 0.2	7.18 ± 0.2
	3	134 d	7.3 de	3.28 ± 0.3	5.48 ± 0.1	8.76 ± 0.1
	5	152 cd	7.8 d	2.61 ± 0.1	4.83 ± 0.2	7.44 ± 0.1
	7	164 c	7.6 cd	2.16 ± 0.1	4.08 ± 0.3	6.24 ± 0.3
	9	188 c	9.7 b	2.16 ± 0.5	2.45 ± 0.2	4.61 ± 0.3

[a]Mean separation by Duncan's multiple range test at $P \leq 0.05$

favorable auxin for the increase in cell mass as well as increase in total saponin yield (10.1 g L^{-1} DW and 7.29 ± 0.2 mg g^{-1} DW, respectively), while NAA at 3 mg L^{-1} was favorable for saponin accumulation but not effective for increasing cell biomass (7.3 g L^{-1} DW and total saponin 8.76 ± 0.1 mg g^{-1} DW). For this reason IBA (7 mg L^{-1}) was used during the cell suspension cultures of *P. ginseng*.

6.2.3 *Effect of IBA and Cytokinin Combinations on Cell Growth and Ginsenosides Production in Cell Suspension Culture of P. ginseng*

To determine the effect of types and concentrations of cytokinins on increase in cell biomass and ginsenoside production, kinetin and BA at 0.1, 0.5 and 1 mg L^{-1} were combined with 7 mg L^{-1} IBA. The results (Table 6.2) clearly showed that the addition of cytokinins (BA and kinetin) did not affect the proliferation of cells in culture.

The saponin productivity (particularly Rb group) was increased when the medium was supplemented with 0.1–0.5 mg L^{-1} BA or kinetin (Table 6.2). The highest saponin content was (7.08 ± 0.1 and 7.34 ± 0.2 mg g^{-1} DW) obtained with a combination of IBA at 0.5 mg L^{-1} of BA or kinetin. Further increase in cytokinin concentrations led to the decrease in ginsenoside content. A relatively high cytokinin level was not favorable to secondary metabolite synthesis. A similar phenomenon was also reported in other plant cell cultures. In *P. notoginseng* suspension cell cultures, Zhong [11] found that both the saponin content and cell biomass production were decreased with an increase in kinetin concentration.

Flow Cytometric Analysis of *Panax ginseng* Cells

To verify the genetic stability of regenerated cells, 2C DNA values of suspension cultures were analyzed by flow cytometry and compared with donor plant. The histograms obtained with *P. ginseng* cells of different inoculum densities cultured for 25 days in MS medium with 7 mg L^{-1} IBA and donor plant are shown in Fig. 6.2a.

Table 6.2 Effect of IBA and cytokinin combinations on cell growth and ginsenoside production in cell suspension culture of *P. ginseng*

Cytokinin	Concentration (mg L^{-1})	Fresh wt. (g L^{-1})	Dry wt. (g L^{-1})	Ginsenoside (mg g^{-1} dry wt.)		
				Rg	Rb	Total
	0	221 a[a]	11.0 ab	2.17 ± 0.1	4.36 ± 0.2	6.43 ± 0.2
BA	0.1	230 a	11.0 ab	1.81 ± 0.1	2.75 ± 0.2	4.56 ± 0.3
	0.5	252 a	11.5 a	1.75 ± 0.4	5.33 ± 0.1	7.08 ± 0.1
	1	242 a	11.0 a	1.79 ± 0.1	3.53 ± 0.2	5.33 ± 0.2
Kinetin	0.1	224 ab	11.1 ab	1.16 ± 0.1	4.61 ± 0.3	5.76 ± 0.3
	0.5	240 a	11.7 a	1.49 ± 0.3	5.85 ± 0.2	7.34 ± 0.2
	1	242 a	11.4 a	1.56 ± 0.2	3.51 ± 0.3	5.07 ± 0.1

[a]Mean separation by Duncan's multiple range test at $P \leq 0.05$

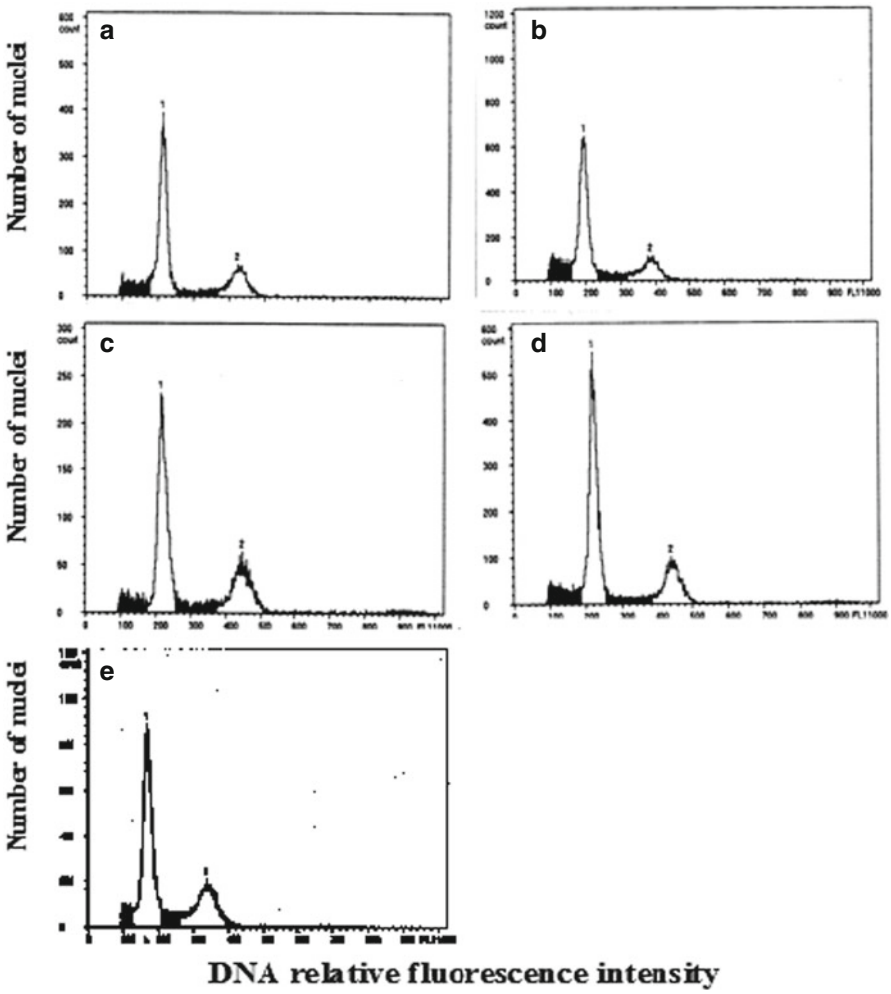

Fig. 6.1 Flow cytometric analyses of *P. ginseng* at different density cell. (**a**) 4 g, (**b**) 6 g, (**c**) 8 g, (**d**) 10 g/100 mL medium and (**e**) mother plant

In suspension culture, most of the cells were diploid (Fig. 6.1a–d), revealing a peak at nearly the same position as the standard diploid donor plant (Fig. 6.1e). However, in other plant species high chromosomal variability during *in vitro* cultures has been reported [32]. Nevertheless, *P. ginseng* callus line has mostly retained its ploidy level even after 4 years of culture on the callus induction medium containing relatively high levels of 2,4-D.

To examine more closely the cell division activity within a culture passage of 25 days, the percentage of cells in the three phases of the cell cycle, G1, S and G2 + M, were evaluated by flow cytometery in both donor plant and suspension culture cells with different inoculum densities (4, 6, 8, 10 g FW/100 mL MS medium). The

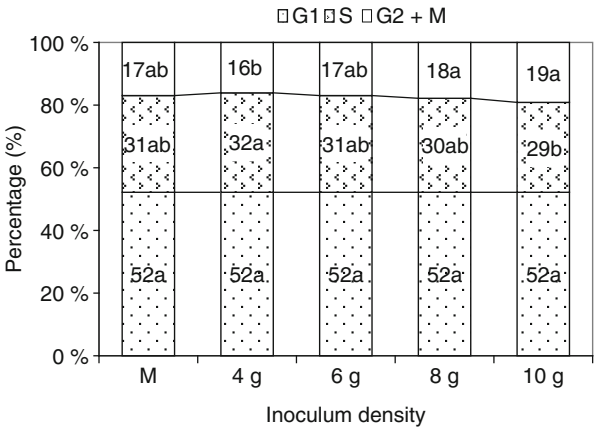

Fig. 6.2 Cell cycle analyses of *P. ginseng* cells

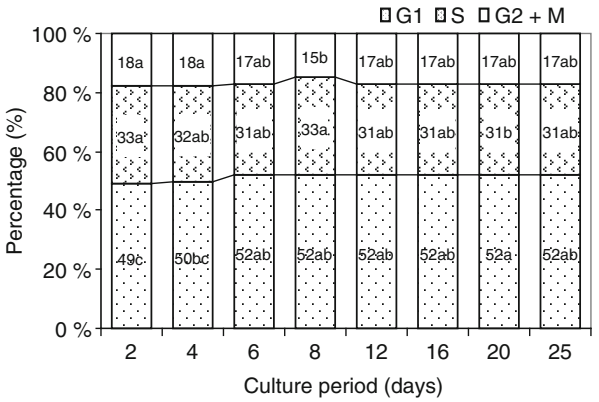

Fig. 6.3 Cell cycle analyses of *P. ginseng* cell cultured for 25 days

results are summarized in Figs. 6.2 and 6.3. The ratio of cells in S phase clearly show a parallel development at G1 (52 %), other phase variants show as S (29–33 %); G2 + M (15–18 %; Fig. 6.2).

The results of cell cycle analysis of cells at different phases of cell cycle over a period of 25 days also revealed the stability of cells without much variation (Fig. 6.3).

6.2.4 Effect of Sucrose Concentrations on Cell Growth and Ginsenoside Production in Cell Suspension Culture of P. ginseng

The effect of sucrose concentration at a range of 10–70 g L^{-1} were studied to find out the optimal sucrose concentration for cell growth and saponin production and the results are presented in Table 6.3. Dry cell weight increased with an increase in sucrose concentration from 10 to 30 g L^{-1}. Further, increase in sucrose

Table 6.3 Effect of sucrose concentrations on cell growth and ginsenoside production in cell suspension culture of *P. ginseng*

Sucrose concentration (g L^{-1})	Fresh wt. (g L^{-1})	Dry wt. (g L^{-1})	Ginsenoside (mg g^{-1} dry wt.)		
			Rg	Rb	Total
10	26.6 d[a]	2.9 d	0.32 ± 0.1	0.62 ± 0.2	0.92 ± 0.1
30	180.6 a	10.8 a	3.17 ± 0.1	4.19 ± 0.1	7.36 ± 0.2
50	98.2 b	8.4 b	1.06 ± 0.2	2.23 ± 0.1	3.29 ± 0.1
70	52.1 c	5.7 c	0.08 ± 0.1	1.56 ± 0.2	1.64 ± 0.2

[a]Mean separation by Duncan's multiple range test at $P \leq 0.05$

concentration of up to 50 or 70 g L^{-1} decreased the cell biomass. The highest biomass yield was obtained (180.6 g L^{-1} FW and 10.8 g L^{-1} DW) at 30 g L^{-1} of sucrose concentration.

In case of *Vitis vinifera* cell culture, both a lag phase and reduced cell concentration were observed under a relatively high sucrose concentration of 50 g L^{-1} [33]. For cell culture of *Coleus blumei*, a high initial sucrose concentration of 60 g L^{-1} led to a higher biomass accumulation without an obvious lag phase [34]. With suspension cultures of *Perilla frutescens*, the growth rate increased with an increase in initial sucrose level of up to 60 g L^{-1} in the medium [26]. Choi et al. [12, 13] found that the optimal concentration of sucrose for cell growth was between 30 and 50 g L^{-1} and 70 g L^{-1} sucrose inhibited cell growth, while the saponin content showed a steady increase with sucrose concentration of up to 60 g L^{-1} in *Panax ginseng*. It is clear that initial sucrose concentration is important for the proliferation of plant cells and its effect depends on a specific cell lines. Similarly, the saponin content of the cells was also dependent on initial sucrose concentration in the medium as that of cell mass (Table 6.3). Initial sucrose concentration of 30 g L^{-1}, significantly increased the saponin accumulation in the cells (7.36 ± 0.2 mg g^{-1} DW) and total saponin production decreased at a higher sucrose concentration of up to 70 g L^{-1}. In cell suspension cultures of *P. notoginseng* also, manipulation of medium sucrose could effectively enhance the saponin production [35].

6.3 Ginseng Cell Culture in Bioreactors

6.3.1 Effect of Bioreactor Types on Cell Growth and Saponin Production in Bioreactor Cultures of Panax ginseng

The effect of various types of airlift bioreactors such as cylinder, cone, balloon and bulb type bioreactors of 5 L capacity containing 4 L of optimized medium (MS medium with 7 mg L^{-1} of IBA, 0.5 mg L^{-1} kinetin and 30 g L^{-1} sucrose) was tested for biomass accumulation and metabolite production and results are presented in Table 6.4. Balloon type bioreactor was found suitable for biomass accumulation and

Table 6.4 Effect of bioreactor types on k_La coefficient and cell growth during cell culture of *P. ginseng*

Bioreactor type	Initial k_La (h^{-1})	Biomass			Growth rate[a]
		Fresh wt. (g L^{-1})	Dry wt. (g L^{-1})	% dry wt.	
Cylinder	5.25	240 b[b]	9.1 b	3.8	4.14
Balloon	6.98	255 a	10.6 a	4.0	4.82
Bulb	6.95	251 a	10.1 a	4.0	4.59
Cone	5.69	245 ab	9.8 ab	3.9	4.45

[a]Growth rate is the quotient of the dry weight after culture and the dry weight of the inoculum size
[b]Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

Table 6.5 Effect of bioreactor types on ginsenoside production

Bioreactor type	Ginsenoside (mg g^{-1} wt)			
	Rg	Rb	Total[a]	Rb: Rg[b]
Cylinder	1.45 ± 0.2	2.37 ± 0.3	3.82 ± 0.4	1.67 ± 0.4
Balloon	0.79 ± 0.1	3.38 ± 0.6	4.17 ± 0.6	4.27 ± 0.7
Bulb	1.09 ± 0.3	3.07 ± 0.5	4.16 ± 0.5	2.98 ± 1.2
Cone	1.35 ± 0.3	2.59 ± 0.1	3.95 ± 0.2	1.98 ± 05

[a]Total content = Rb + Rg
[b]Rb: Rg = (Rb1 + Rc + Rb2 + Rd)/(Rg1 + Re + Rf)

255.4 g L^{-1} FW, 10.6 g L^{-1} DW were recorded. The highest ginsenoside amount of 4.17 mg g^{-1} DW was also documented with balloon type bioreactors and this might be due to high initial k_La values (Table 6.5). Kim et al. [36] have also reported that balloon type bioreactors are suitable among the various configurations of bioreactors tested for ginseng adventitious root cultures.

6.3.2 *Effect of Aeration Volume on Cell Growth and Saponin Production in Bioreactor Cultures of Panax ginseng*

The cell multiplication, growth and accumulation of secondary metabolites in bioreactors were strongly influenced by aeration volume [37] and it is essential to investigated suitable aeration volume to achieve biomass and metabolite productivity. Constant aeration of 0.05, 0.1, 0.2 and 0.3 vvm as well as variable aeration volume of 0.5/0.1/0.2/0.3 (i.e., aeration volume was changed for every 6 days) were tested and the results of effect of aeration volume on cell growth and yield of ginsenosides are presented in Tables 6.6 and 6.7. Increment of aeration volume with the increasing time duration was found suitable for both biomass and ginsenoside productivity.

Table 6.6 Effect of aeration volumes on k_La and cell growth in bioreactor culture

Aeration volume (vvm)	Initial k_La (h^{-1})	Biomass			Growth rate[a]
		Fresh wt. ($g\ L^{-1}$)	Dry wt. ($g\ L^{-1}$)	% dry wt.	
0.05	4.95	165 d[b]	5.7 d	3.44	2.58
0.1	7.84	244 b	10.3 a	4.22	4.68
0.2	11.42	225 c	9.1 b	4.02	4.08
0.3	16.81	211 c	7.9 c	3.76	3.61
0.05/0.1/0.2/0.3[c]	5–16.58	263 a	10.6 a	4.04	4.82

[a]Growth rate is the quotient of the dry weight after culture and the dry weight of the inoculum size
[b]Mean separation within columns by Duncan's multiple range test at $P \leq 0.05$
[c]Aeration volume increased at 6-day intervals

Table 6.7 Effect of aeration volumes on ginsenoside production in bioreactor culture

Aeration volume (vvm)	Ginsenoside (mg g^{-1} wt)			
	Rg	Rb	Total[a]	Rb: Rg[b]
0.05	0.72 ± 0.1	1.82 ± 0.2	2.55 ± 0.3	2.52 ± 0.1
0.1	1.47 ± 0.3	2.54 ± 0.1	4.01 ± 0.4	1.76 ± 0.3
0.2	1.06 ± 0.6	2.65 ± 0.2	3.71 ± 0.3	3.58 ± 0.3
0.3	1.29 ± 0.2	2.32 ± 0.4	3.61 ± 0.2	1.86 ± 0.6
0.05/0.1/0.2/0.3[c]	0.98 ± 0.3	3.31 ± 0.2	4.28 ± 0.3	3.57 ± 0.8

[a]Rb: Rg = (Rb1 + Rc + Rb2 + Rd)/(Rg_1 + Re + Rf)
[b]Total content = Rb + Rg
[c]Aeration volume was increase at 6-day intervals

6.3.3 *Effect of Inoculum Density on Cell Growth and Saponin Production in Bioreactor Cultures of Panax ginseng*

The effect of inoculum density of the cultured cells on biomass and metabolite accumulation is well established by various studies [38–40]. For example, Jeong et al. [40] investigated the production of ginsenosides from adventitious root suspension cultures at an inoculum density of 2.5, 5.0, 7.5 and 10.0 g L^{-1} and reported 10 % increment in ginsenosides with an inoculum density of 5.0 g L^{-1}. The results of the effect of inoculum density on biomass and secondary metabolites accumulation in the present study are depicted in Fig. 6.4. Of the varied inoculums tested (40, 60, 80 and 100 g) 100 g L^{-1} fresh weight was good for fresh and dry biomass accumulation (Fig. 6.4a, b). However, metabolite accumulation was optimum with inoculum density of 60 g L^{-1} (Fig. 6.4c). The maximum saponin production of 4.4 mg g^{-1} DW was achieved and therefore, 60 g L^{-1} inoculum density was used for further experiments.

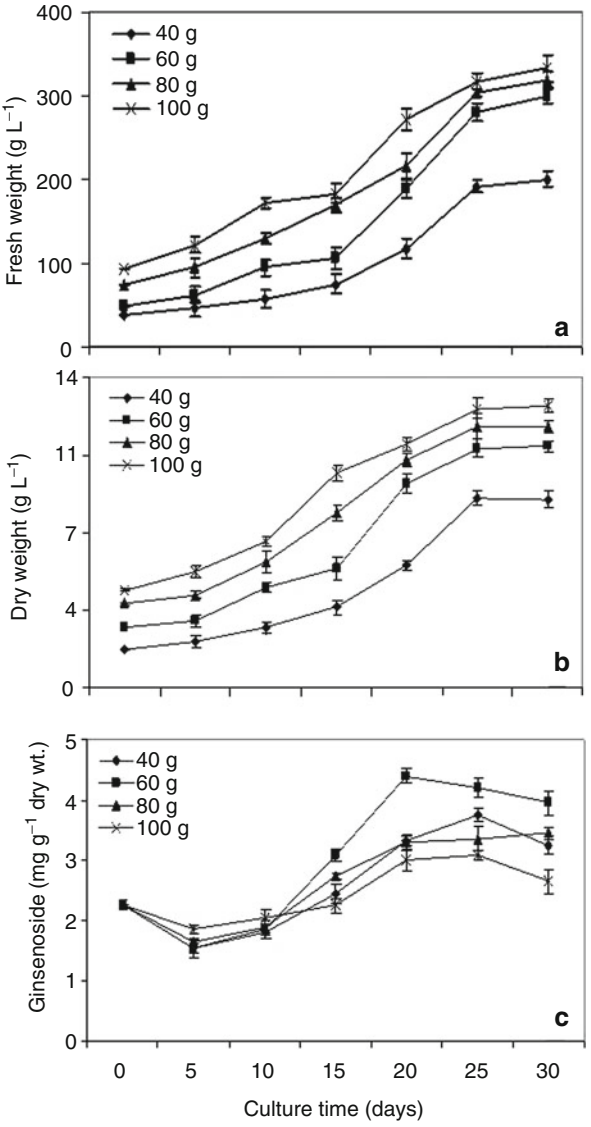

Fig. 6.4 Effect of inoculum density on biomass and ginsenosides accumulation of *P. ginseng* cells cultured in large-scale suspension cultures. Fresh weight (**a**), dry weight (**b**) and ginsenosides content (**c**)

6.3.4 Effect of Oxygen Supply on Cell Growth and Saponin Production in Bioreactor Cultures of Panax ginseng

Ginseng cells were cultured in 5 L capacity airlift bioreactors and the effect of oxygen levels was tested on accumulation of biomass and ginsenosides. The growth kinetics of *P. ginseng* cells cultivated in balloon type-bubble bioreactors at four

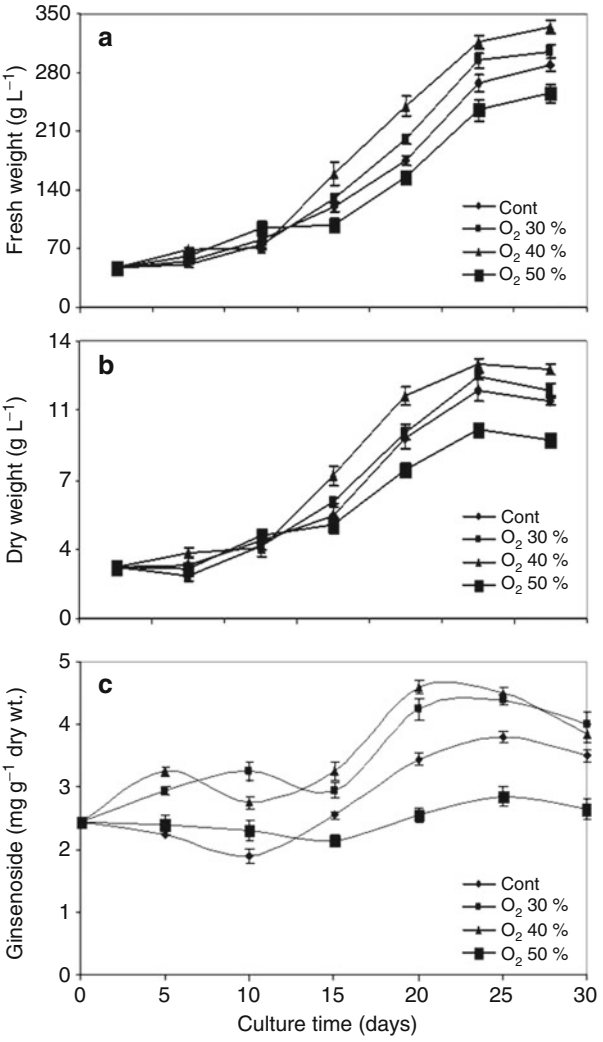

Fig. 6.5 Time profiles for (**a**) fresh cell weight, (**b**) dry cell weight and (**c**) saponin production in *P. ginseng* suspensions cultivated in 5 L balloon-type bubble reactors

different levels oxygen supply [20.8 % (control), 30 %, 40 %, and 50 %] are presented in Fig. 6.5 [41]. The cell growth and biomass accumulation increased gradually with the time duration and maximum biomass level was reached after 25 days. Similar growth patterns have been previously reported for *P. notoginseng* cell cultures [24]. The maximum FW (316 g L^{-1}) was achieved at 40 % oxygen; the corresponding DW was 12.8 g L^{-1} (Fig. 6.5a, b). Increase of 15 % in fresh cell biomass and 10 % in dry cell biomass were evident, compared to the control (20.8 % O_2 supply) cultures. Similar positive effects of oxygen supply on cell growth have been reported for tobacco suspension cultures [42].

Profiles of total saponin (ginsenoside) production are shown in Fig. 6.5c. Highest saponin accumulation was recorded on 20–25 days and declined thereafter. The maximum total saponin concentrations were 3.8, 4.4, 4.5 and 2.85 mg g^{-1} DW at 20.8, 30, 40, and 50 % O_2 supply respectively (Fig. 6.5c). Highest saponin levels

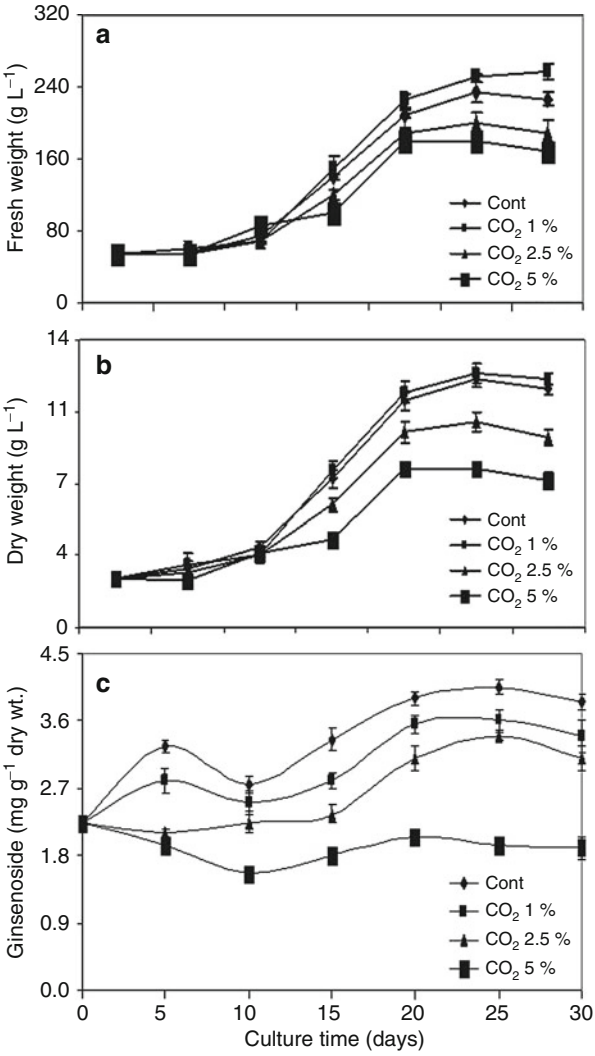

Fig. 6.6 Effect of CO_2 concentration on accumulation of cell fresh mass (**a**), dry mass (**b**), and production of saponins (**c**) of *Panax ginseng* cells cultivated in balloon type bubble bioreactors (rhomb – control, square – 1 % CO_2, triangle – 2.5 % CO_2, cross – 5 % CO_2)

were achieved at 40 % O_2 supply; the lowest levels were achieved at 50 % O_2 supply. The results indicate that oxygen supplementation to bioreactor-based ginseng cultures was beneficial for biomass accumulation and saponin production.

6.3.5 *Effect of Carbon Dioxide on Cell Growth and Saponin Production in Bioreactor Cultures of Panax ginseng*

Ginseng cell cultures were supplemented with various concentrations of carbon dioxide (1, 2.5 and 5 %) and their effect was tested on biomass accumulation and productivity of ginsenosides [43]. The fresh mass of cells with 0.03 % CO_2 (control) was 227 g L^{-1} and corresponding dry mass was 11.6 g L^{-1} (Fig. 6.6a, b). It was

found that optimum accumulation of fresh (258 g L^{-1}) and dry mass (12.1 g L^{-1}) was with the supply of 1 % CO_2 in the bioreactors. Thus 13.7 and 4.3 % increase in fresh and dry cell mass was evident with the supply of 1 % CO_2. This finding is in agreement with the earlier reported results that carbon dioxide is required for the growth of plant suspension cultures [44, 45]. The biomass accumulation declined with the further increase in CO_2 concentration. Fresh and dry cell mass was decreased by 30.1 and 38.3 %, respectively with the supply of 5 % CO_2.

The ginsenoside production increased with the lapse of time and significantly higher saponin content was observed in control condition (Fig. 6.6c). After 30 day of culture increased CO_2 supply 1, 2.5 and 5 % led to decrease in saponin accumulation up to 11.6, 19.5 and 50.6 %, respectively. However, the enhancement in secondary metabolites with an increase in CO_2 concentrations has been reported in the cell cultures of *Thalictrum minus* [46], *T. rugosum* [47], *Stizolobium hassjoo* [48] and *Catharanthus roseus* [49].

6.3.6 *Effect of Ethylene on Cell Growth and Saponin Production in Bioreactor Cultures of Panax ginseng*

The influence of ethylene supplementation at 5, 10 and 20 ppm levels were tested with ginseng cell cultures and results are depicted in Fig. 6.7a, b. Growth kinetics revealed similar trends as in case of O_2 and CO_2 supplementation. Increment of 16 and 8 % in dry biomass was recorded with supplementation of 5 and 10 ppm of ethylene respectively, whereas supplementation of 20 ppm decreased the biomass accumulation significantly when compared to control. This result suggests that ethylene had stimulatory or inhibitory effect on cell growth in bioreactor culture system. Similar results have been reported in cell culture of different *Taxus* species [50].

Figure 6.7c shows the profile of saponin content under different concentrations of ethylene in *P. ginseng*. The yield of ginsenoside production was decreased significantly in all the ethylene concentrations compared to control. Recently, Zhang and Wu [51] reported that ethylene inhibitors induce or stimulate the secondary metabolite production by inhibiting ethylene production endogenously or supplied concentration in the medium. Ethylene effects on growth and differentiation is highly variable and it is not yet clear why ethylene promotes growth, differentiation and secondary metabolite production in some case and inhibits them in others [52].

6.4 Elicitation

6.4.1 *The Effect of MJ on Cell Growth and Ginsenosides Production*

The growth and secondary metabolite accumulation in *Panax ginseng* cell culture are represented in Tables 6.8 and 6.9 [53]. Cell growth was significantly affected by the application of MJ. The fresh weight, dry weight and growth ratio of the cells, decreased with increasing MJ concentration, resulting in a cell growth ratio of 3.48 at 400 μM MJ.

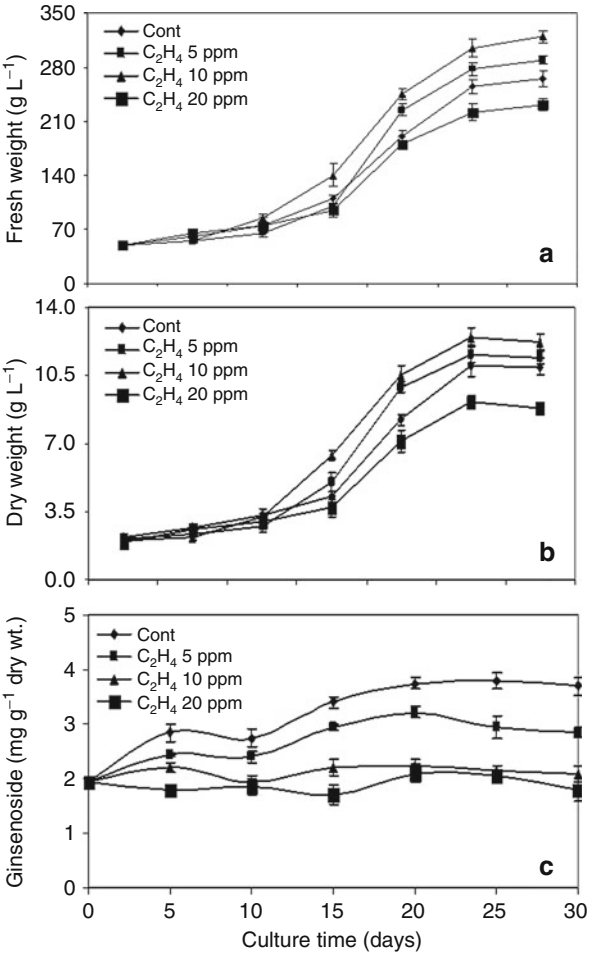

Fig. 6.7 Growth kinetics of cell fresh weight (**a**), dry weight (**b**) and ginsenoside accumulation (**c**) of *P. ginseng* in bioreactor cultures under different C_2H_4 concentrations

Table 6.8 The effect of methyl jasmonate (MJ) on ginseng cell growth after 25 days of bioreactor culture

MJ concentration (μM)	Biomass		Growth rate[a]
	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	
0	311.9 a[b]	12.6 a	4.62
50	304.2 ab	11.3 b	4.14
100	294.9 b	11.4 b	4.28
200	293.1 b	11.3 b	4.26
400	240.2 c	9.4 c	3.48

[a]Growth ratio is the quotient of the dry weight after cultivation and the dry weight of the inoculum
[b]Mean separation within column by Duncan's multiple range test at $P<0.05$

Table 6.9 Effect of MJ on biosynthesis of ginsenosides after 25 days of bioreactor culture

MJ concentration (μM)	Ginsenosides [mg g^{-1} DW]			
	Rg	Rb	Total[a]	(Rb: Rg)[b]
0	1.87 ± 0.1	2.09 ± 0.2	3.96 ± 0.3	1.12 ± 0.1
50	1.84 ± 0.5	2.80 ± 0.2	4.63 ± 0.4	1.59 ± 0.4
100	2.35 ± 0.1	4.84 ± 0.2	7.19 ± 0.3	2.06 ± 0.1
200	2.65 ± 0.1	6.17 ± 0.3	8.82 ± 0.3	2.32 ± 0.1
400	2.32 ± 0.3	4.62 ± 0.2	6.94 ± 0.3	1.97 ± 0.2

Values represent mean with standard error
[a]Total saponin content = Rb + Rg
[b]Rb: Rg = (Rb1 + Rc + Rb2 + Rd)/(Rg1 + Re + Rf)

On the other hand, ginsenosides content was significantly enhanced by the addition of MJ. Total ginsenosides content increased with increasing MJ concentration, and reached a maximum of 8.82 mg g^{-1} DW at 200 μM MJ, representing a 2.2-fold increase over the control (3.96 mg g^{-1} DW). Both Rb group and Rg group ginsenosides reached a maximum at 200 μM MJ but the content of Rb group ginsenosides increased more significantly than that of Rg group ginsenosides. There was 1.3-fold increment in Rg group ginsenosides, whereas threefold increment of Rb group ginsenosides was evident compared to the control. The Rb/Rg group ratio was 2.32 with an application of 200 μM MJ. Figure 6.8a shows the dynamic changes in the content of the Rb/Rg ratio with 200 μM MJ treatment over the control.

6.4.2 Accumulation of Ginsenosides After MJ Treatment in a Two-Stage Bioreactor Operation

Based on the results of the first experiment, bioreactor cell cultures were established and ginseng cells were cultured for 15 days without MJ treatment. Two hundred micrometer MJ was added to the cultures after 15 days for elicitation and accumulation of ginsenosides. Figure 6.8b–d shows ginsenosides accumulation in ginseng cell culture during 10 days of 200 μM MJ addition. The content of ginsenosides and Rb group ginsenosides gradually increased, reaching maximum values 8 days after treatment and showing a little change thereafter. Contents of total ginsenosides, Rb, and Rg group ginsenosides increased 2.9, 3.7, and 1.6 times, respectively. Among the Rb group ginsenosides, Rb1 content increased significantly by four times but the contents of Rb2, Rc and Rd increased only slightly. Among Rg group ginsenosides, Rg1 and Re showed 2.3-fold and 3.0-fold increments, whereas there was only a slight increment in Rf group ginsenosides. Similarly, jasmonates have been used to elicit higher accumulation of metabolites in cell cultures of *Taxus chinensis* [54] and *Panax notoginseng* [55].

This study indicates that MJ could increase the accumulation of individual ginsenosides and significantly modify the Rb/Rg group ratio. The strategy developed here,

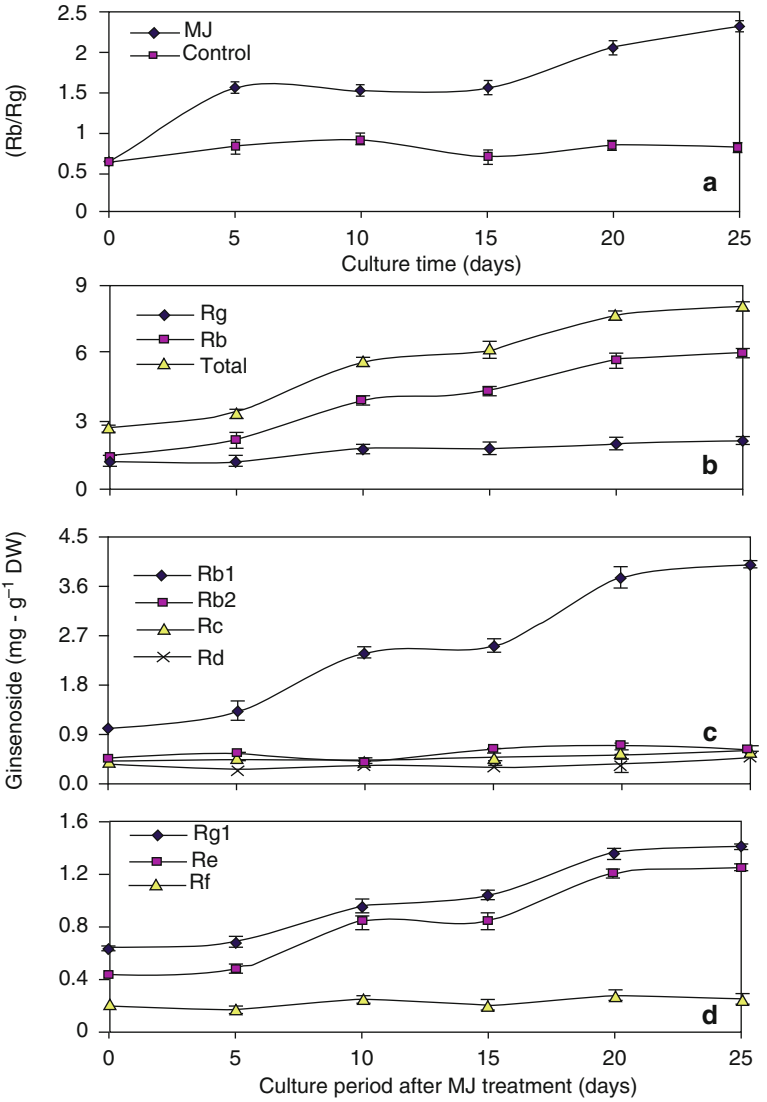

Fig. 6.8 Dynamic changes in Rb and Rg ginsenosides in ginseng cells grown for 25 days after methyl jasmonate (*MJ*) treatment (200 μM) in bioreactors (**a**). Accumulation of total (**b**), Rb (**c**) and Rg (**d**) group ginsenosides in ginseng cells grown for 10 days after MJ treatment (200 μM) in bioreactors. The cells were grown for 15 days without MJ

i.e., initial culturing of cells without elicitors and subsequent treatment of cell cultures with 200 μM MJ is useful for enhancing ginseng cell biomass as well as simultaneously enhancing ginsenoside production. Initial culturing of cells without elicitors and subsequent treatment of cell cultures with 200 μM MJ, is useful for enhancing ginseng cell biomass as well as the ginsenoside production simultaneously.

6.5 Scale up of Ginseng Production in Bioreactors

Based on the above results of 5 and 10 L bioreactor cultures (Fig. 6.9a, b), we cultivated pilot-scale cultures of ginseng cells in 500 and 1,000 L airlift bioreactors (Table 6.10 and Fig. 6.9c). The total biomass of 187 kg fresh weight and 6.2 kg dry weight with a total saponin production of 7.86 mg g^{-1} DW was obtained in 500 L drum bioreactor. Similarly, 400 kg fresh weight (Fig. 6.9d) and 13.2 kg dry weight with a total saponin production of 7.75 mg g^{-1} dry weight were also obtained in 1,000 L balloon type bioreactor. These results are comparable to that of ginseng cell cultures in

Fig. 6.9 *Panax ginseng* cell cultures in bioreactors. (**a**) Cell suspension cultures in 5 L balloon type airlift bioreactors. (**b**) 500 L drum type airlift bioreactor. (**c**) 1,000 L airlift bioreactor. (**d**) Biomass harvested from 1,000 L bioreactor

Table 6.10 Ginseng cell cultures in bioreactors

	Biomass				
Bioreactor type	FW	DW	DW (g L^{-1})	% dry wt.	Total saponin (mg g^{-1} DW)
5 L balloon	1.28 kg	54 g	13.1	4.22	8.82
500 L drum	187 kg	6.2 kg	12.4	3.32	7.86
1,000 L balloon	400 kg	13.3 kg	13.3	3.33	7.75

5 L bioreactor (Table 6.6). In the previous studies, stirred tank bioreactors have been used and taken to scale up process also [19]. Shamakov et al. [20] and Strogov et al. [21] determined the suitable agitation speed for efficient mixing and oxygen transfer. Asaka et al. [56] used airlift bioreactors and achieved higher biomass and ginsenoside productivity than stirred tank bioreactors. However, all these were batch cultures. In order to improve the productivity of biomass and secondary metabolites, fed-batch and high density cell cultures were used by Zhang and Zhong [14] and reported a cell concentration as high as 35 g dry cell L^{-1}. 2.8 and 3.4 fold increment saponin and polysaccharide productivity was revealed in high density fed-batch cultures. By adopting fed-batch, high density cell cultures in Korean ginseng it is possible to achieve improved productivity and research work is in progress in this direction.

6.6 Conclusions and Future Perspectives

Ginseng cell culture has been evolved as an alternative for field cultivation as it has the advantages of higher biomass accumulation and ginsenosides production. Extensive research work has been carried out on ginseng cell and tissue cultures, such as optimization of culture medium, physical conditions for the production of biomass and ginsenosides productivity [1]. In the current studies, cell cultures were established in Korean ginseng and various physiological and physical parameters which affect the biomass and metabolite accumulation have been optimized. Elicitation technology has been achieved for enhanced accumulation of ginsenosides. Cell cultures have been also established in large scale airlift bioreactors (500 and 1,000 L) to obtain voluminous biomass and metabolite productivity. Recently, various bioengineering parameters like fed-batch cultures [14], high density cell cultures [22, 23] have been adopted in *Panax notoginseng* cell cultures and obtained improved productivity of metabolites. Elicitation of ginseng cell cultures with vandate [57] and *N*, *N*'-dicyclohexylcarbodiimide [58] have also been reported for enhanced saponin productivity. There is scope for improvement of secondary metabolite production in Korean ginseng with the adoption of these techniques. Further, research in the improvement of the ginseng cell culture technologies may be useful for commercial production of ginseng raw material.

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Chapter 7
Production of Biomass and Bioactive Compounds from Cell Suspension Cultures of *Panax quinquefolium* L. and *Glycyrrhiza uralensis* Fisch.

Wen-Yuan Gao, Juan Wang, Jing Li, and Qin Wang

Abstract *Panax quinquefolium* L. and *Glycyrrhiza uralensis* Fisch. are important medicinal plants and health food that are used worldwide. Field cultivation of *Panax quinquefolium* and *Glycyrrhiza uralensis* is an extremely time consuming and labor-intensive process. Plant cell culture offers an alternative for obtaining valuable chemicals, especially plant-specific bioactive secondary metabolites. In this review, cell suspension cultures of *Panax quinquefolium* and *Glycyrrhiza uralensis* are described for the production of bioactive compounds.

Keywords Bioreactor • Cell suspension • *Glycyrrhiza uralensis* • *Panax quinquefolium*

Abbreviations

2, 4-D	2, 4-Dicholorphenoxy acetic acid
B5	Gamborg's medium
BA	Benzyl adenine
BTBB	Balloon-type bubble bioreactor
LH	Lactalbumin hydrolysate
MJ	Methyl jasmonate
MS	Murashige and Skoog medium

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NAA α-naphthalene acetic acid
PHE Phenylalanine
SOUR Specific oxygen uptake rate
vvm Air volume per culture volume per minute

7.1 Introduction

Panax quinquefolium (American ginseng) is a widely used herb belonging to the family *Araliaceae*. It has gained a tremendous global trade and is recognized as a health food supplement [1]. Dried root powder/extract of this plant is widely used as a health tonic for anti-stress, anti-fatigue, aphrodisiac and anti-aging properties [2, 3]. Ginseng saponins (ginsenosides), the secondary metabolites of this plant are its pharmacologically active components. The two major groups of ginsenosides are Rb and Rg groups derived respectively from 20 (S) protopanaxadiol to 20 (S) protopanaxatriol. Ginsenosides Rb_1, Rb_2, Rc and Rd are from the Rb group and ginsenosides Re, Rg_1 and Rg_2 are from the Rg group. Ginseng polysaccharide, a primary metabolite of the plant possesses antitumor and immunological activities [4].

Licorice roots and stolons of some *Glycyrrhiza* species, the oldest medicinal plants have been used by human beings. Glycyrrhizic acid, namely a kind of triterpenoid saponin, is an important pharmacologically active component in it [5]. Glycyrrhizic acid has significant effects similar to the adrenal cortical hormone and can be used in clinical trials as an anti-inflammatory, anti-ageing, decompression, immunity enhancer, and for improving the physiological functions and restraining cancer cells growth showing really a curative effect [5]. Flavonoids, the secondary metabolites of this plant are especially used for whitening and dispelling the freckle and as an anti-oxidative [5–8]. Polysaccharides, the primary metabolites in *Glycyrrhiza uralensis* have drawn the attention of researchers due to their physical and functional properties [9]. In recent years, licorice has been increasingly used as a healthy additive formulated into a variety of commercial products, including drugs, foods, drinks and cosmetics, which are marketed in Asia as well as in many other countries all over the world [5, 10].

Field cultivation of *P. quinquefolium* requires skillful land management techniques and an intensive pest control programme and also the plant has a prolonged growth period. Furthermore, intensive replanting of *P. quinquefolium* will lead to recurring diseases. So, a second planting made in the same field will often fail [11, 12]. Natural sources of wild licorice are very limited because of the low budding ratio of the seeds and destructive exploitation by people [13]. The current supply of licorice is mainly from field cultivation, which is an extremely time consuming and labour-intensive process. Plant cell culture offers an alternative for obtaining the valuable chemicals, especially plant-specific bioactive compounds. In this article, bioreactor culture of suspension cells in *P. quinquefolium* and *G. uralensis* are described.

7.2 The Cell Suspension Culture of *Panax quinquefolium*

7.2.1 Culture Medium and Conditions

The Murashige and Skoog (MS) medium and its modified form were mainly used for the cell suspension cultures of *P. quinquefolium*. Zhang et al. [14] have studied the effects of three various media formulation on the cell cultures of *P. quinquefolium* and the results showed that the biomass of *P. quinquefolium* cells and saponin content were optimum in Murashige and Skoog medium.

The changes in the components of the medium will affect the growth of cells and the synthesis of saponins. Reducing the concentration of KNO_3, $CaCl_2$ and $MgSO_4$ in the MS medium to 1/8, 1/6 and 1/4 of the original concentration will promote the growth of the *P. quinquefolium* cells. The reduction in the concentration of KNO_3 and $CaCl_2$ is not good for the saponin synthesis. However, reduction in the concentration of $CaCl_2$ appropriately will benefit the saponin synthesis. 0.65 mmol L^{-1} phosphate is the optimum concentration for the growth of *P. quinquefolium* cells and 1.25 mmol L^{-1} phosphate is optimum for the synthesis of saponin and polysaccharide [15]. These results suggested that inorganic ions have a regulative effect on the metabolism of saponins.

The consumption of sucrose is approximately 80 % in the growth cycle of *P. quinquefolium* suspension cells. It shows that 3 % sucrose was enough to provide the amount of the carbon that the cells need for the multiplication to produce new generation of cells [16]. In addition, the consumption of inorganic phosphate was highest (31 %), followed by the nitrogen source (22.5 %) and the calcium consumption was the lowest (9 %) among a large number of elements of the medium.

Among the various growth regulators supplemented to MS medium such as NAA, 2, 4-D, BA and Kinetin, it was 1.0 mg L^{-1} 2, 4-D and 0.25 mg L^{-1} kinetin supplemented medium showed optimum biomass growth and accumulation of ginsenosides.

The inoculum size was 4 % (fresh weight), culture temperature was 24 ± 1 °C and subculuting once in 20 days was found suitable for biomass accumulation. The cells grew slowly at lower temperatures (below 20 °C) as well as at higher temperatures (above 28 °C). In the experiments which were carried out to investigate the effect of pH, the higher (6.8–7.0) or lower (5.5) initial pH was not beneficial to the growth of cells and the synthesis of saponins. The higher fresh weight, dry weight and saponin content were obtained at the initial pH of 6.0–6.5 [17]. In addition, the agitation speed of 100–120 rotation per minute, aeration rate of 0.6–0.8 vvm, working volume range of 100–500 mL and the amplitude of 2.5 cm were suitable for accumulation of optimal biomass and saponins.

Effect of various elicitors were tested to facilitate hyper-accumulation of saponins in cell cultures of *P. quinquefolium*. The experimental results showed that 0.1 % mevalonate inhibited the growth of cells in suspension cultures and promoted the synthesis of saponin, which reached to 6.14 %; 100 mg L^{-1} leucine had no significant effect on the cell growth, but it promoted the synthesis of saponin; 444 mg L^{-1}

magnesium acetate, 160 mg L^{-1} ATP and 1.0 mg L^{-1} niacin promoted the growth of cultured cells and synthesis of saponin, respectively with the cobolt γ irradiation at 4,000 Gy ^{60}, the saponin content was higher than that of the control group, and the total saponin content reached to 10.40 % after subculturing for five times. In addition, both the osmotic pressure regulator mannitol and D – galactose promoted the synthesis of saponins [18].

7.2.2 Bioreactor Cultures

Application of bioreactors in the cultivation of plant cells as well as organ cultures for the production of biologically active metabolites has been reported for many plants [19–21]. The bioreactor culture system offers many advantages over classical tissue culture because the culture conditions in the bioreactor can be optimized by real-time manipulation of temperature, pH, oxygen, carbon dioxide and nutrients in the medium. Therefore, cell proliferation and regeneration rates can be increased. In addition, the production cost and time can be substantially reduced, product quality can be controlled and standardized, it can be free of pesticide contamination, and production can be conducted year-round without geographical constraints [22].

Dynamic Accumulation of Growth and Active Components

Cell suspensions were established in a 5 L stirred tank bioreactor containing 3 L MS liquid medium supplemented with 1.0 mg L^{-1} 2, 4-D and 0.25 mg L^{-1} Kinetin. Inoculum size was 75 g fresh weight and the cells were mixed using impeller (100 rpm). The airflow rate was adjusted 0.1 vvm during cultivation and pH of the medium was adjusted to 6.0 before autoclaving of the medium. The cell growth was initiated after 3 days of culture initiation, after 18 days the cells entered into a progressive deceleration phase and the maximum growth was achieved on day 21, after that, cells entered the stationary phase. The contents of ginsenoside Re increased slowly within first 6 days, and the highest contents of 0.58 mg g^{-1} was achieved on day 21. The contents of ginsenoside Rb1 and polysaccharide increased quickly during the earlier stage, and the highest contents were achieved on day 24 and day 18 respectively (Fig. 7.1) [23].

Because all primary and secondary metabolic events are governed by the active biomass, which is the total dry weight minus intracellular carbohydrate [24], the biosynthesis of both saponin and polysaccharide depend on active biomass. Therefore, the dry weight of cells and the contents of ginsenoside Re, Rb1 and polysaccharides reached the peak simultaneously and approximately on the 21st day. The polysaccharide contents reached to a maximum and then decreased when the cell growth entered the progressive deceleration phase. It may be attributed to the decrease in carbon source concentration in the medium, and in this case, the

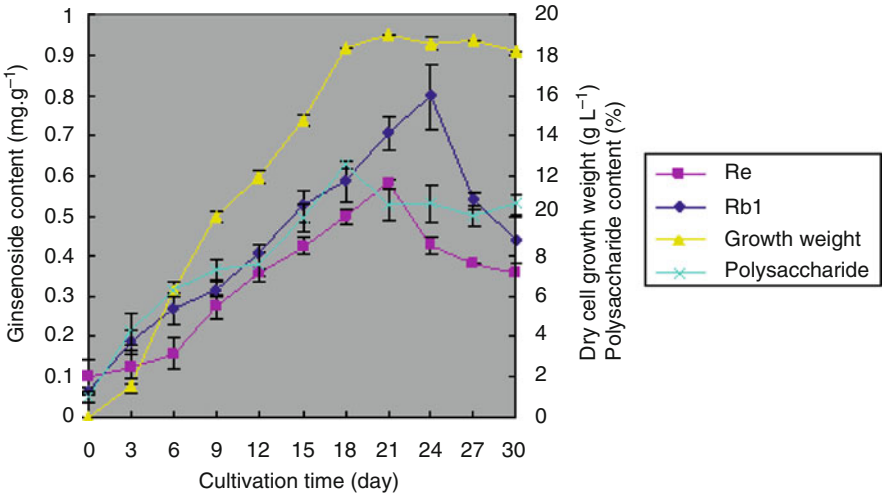


Fig. 7.1 Time profile of growth and active components in cell culture of *P. quinquefolium* in bioreactor. Each value represents mean ± standard error of three replicates

degradation rate of polysaccharide was higher than its synthetic rate, which affected the accumulation of polysaccharide. The degraded polysaccharide was used as a carbon source by the cells [23].

Effect of Sucrose

The sucrose concentration showed a sharp drop from 30 to 3.83 g L^{-1} within the first 3 days and slow consumption after 6 days (Fig. 7.2). However, the concentration of fructose and glucose increased quickly during the earlier stage, and the highest concentration was achieved on day 6 and 9 respectively. After this, the concentration of fructose and glucose decreased gradually and the glucose concentration was almost zero on the 18th day of culture, but a small amount of fructose remained and the fructose concentration decreased from 6.82 to 3.51 g L^{-1} within the final 10 days (Fig. 7.2) [23].

Glucose and fructose concentration in the medium increased significantly during the first stage and then decreased slowly. This indicates that extracellular hydrolysis of sucrose lead to the formation of glucose and fructose. This hydrolysis might be due to the acid invertase, which may have been secreted from cells into the medium. In the first stage of culture, with the consumption of sucrose, cell weight, Re, Rb1 and polysaccharide started to accumulate. Sugar concentration was getting low after 18 days of culture, at this time the cell entered the progressive deceleration phase and polysaccharide contents were decreased. However, the contents of ginsenoside continued to increase, which may be attributed to residual fructose [23].

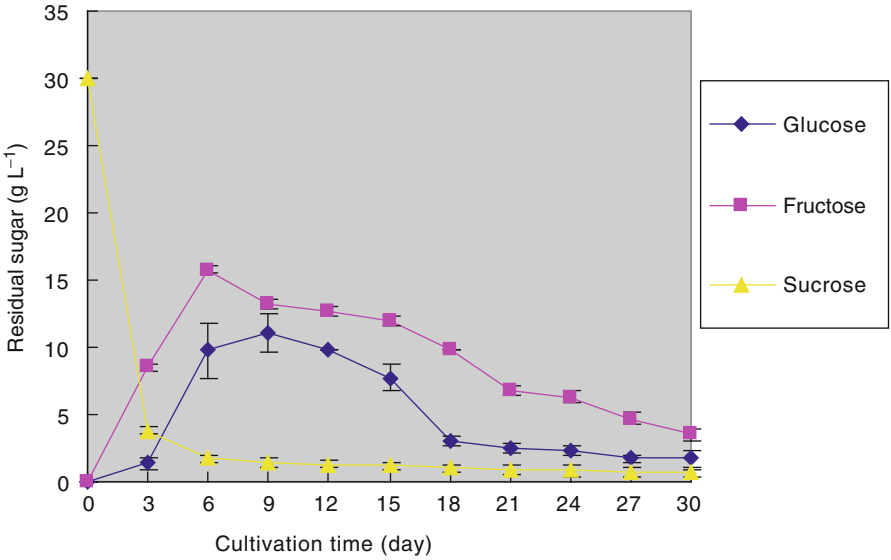


Fig. 7.2 Time profile of sugar consumption in cell culture of *P. quinquefolium* in bioreactor. Each value represents mean ± standard error of three replicates

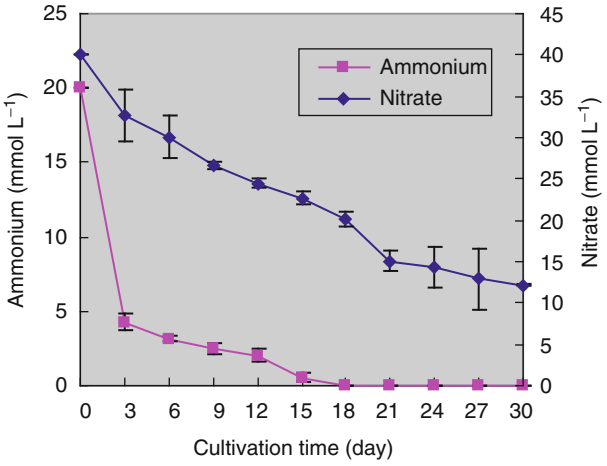


Fig. 7.3 Time profile of nitrogen consumption in cell culture of *P. quinquefolium* in bioreactors. Each value represents mean ± standard error of three replicates

Effect of Nitrogen

Ammonium concentration showed sharp drop from 20 to 4.27 mmol L^{-1} within first 3 days. Ammonium had not been detected after 18 days of cultivation. Nitrate was consumed gradually from culture imitation and had little change after 21 days of cultivation (Fig. 7.3) [23].

The preferential uptake of ammonium at the beginning of culture has been observed for many species, which is also associated with a fall in pH of the culture

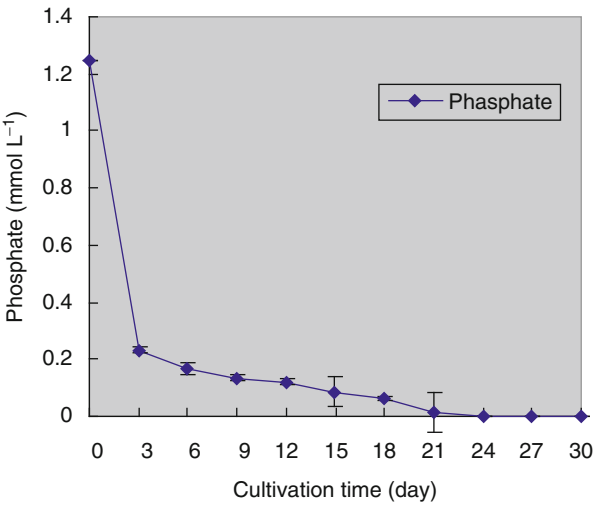


Fig. 7.4 Time profile of phosphate consumption in cell culture of *P. quinquefolium* in bioreactor. Each value represents mean ± standard error of three replicates

medium. Ammonium had been exhausted after 18 days of cultivation, at this time cells entered the progressive deceleration phase, and gradually stopped growing. The results indicated that ammonium was the crucial factor for cell growth. Besides, the synthesis of ginsenosides may be related to the residual nitrate. These results suggested that nitrate and ammonium have different effects on cell culture in *P. quinquefolium* [23].

Effect of Phosphate

Phosphate concentration showed a sharp drop in the first 3 days and its concentration could not be detected during day 24–30 (Fig. 7.4) [23]. Phosphate is another key nutrient for plant cell growth and metabolite formation. Phosphate participates in energy metabolism and biosynthesis. In this experiment, we found that phosphate was rapidly depleting in the medium during *P. quinquefolium* cell suspension culture and got exhausted after 24 days of cultivation, at this time, cells entered the stationary phase and the contents of Rb1 decreased gradually [23].

Elicitation

To stimulate the biosynthesis of saponins and polysaccharides *Panax quinquefolium* bioreactor cell cultures were treated with elicitors after 20 days of culture initiation. Combination of elicitors (100 mg L^{-1} LH and 2 mg L^{-1} MJ) and single elicitor (100 mg L^{-1}) were added and results are presented in Fig. 7.5. The combination of LH and MJ synergistically stimulated ginsenoside accumulation when compared to LH alone; however, there was no improvement in polysaccharide

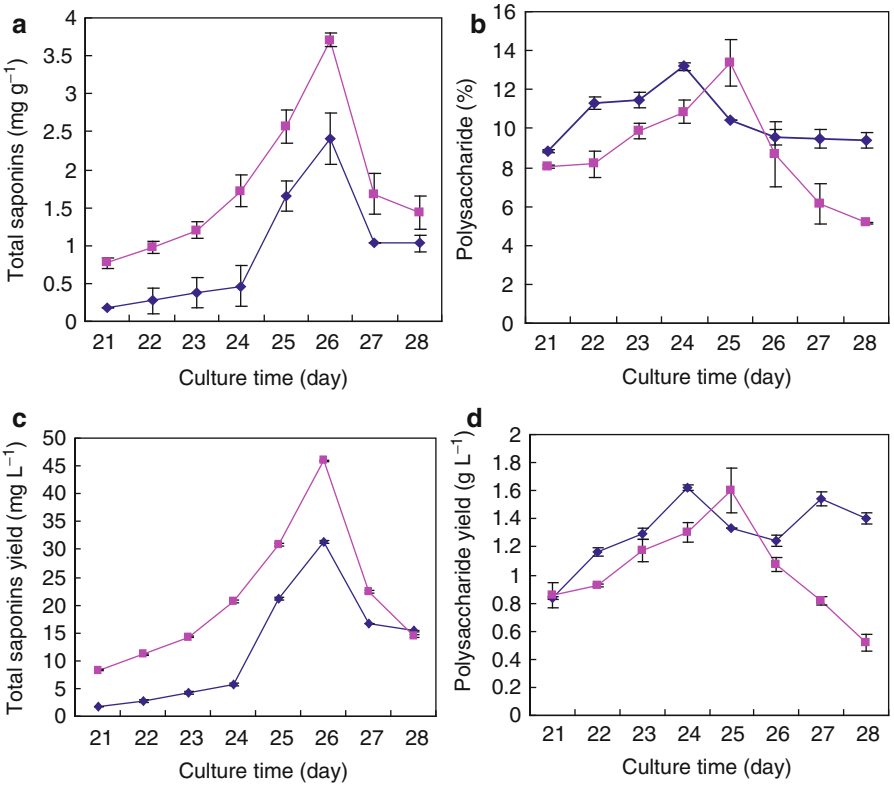


Fig. 7.5 Effects of LH and MJ combination on *P. quinquefolium* cells in 5 L bioreactor system. (**a**) Effects on saponin contents; (**b**) Effects on polysaccharide content; (**c**) Effects on saponin yield. (**d**) Effects on polysaccharide yield. Symbols: (◆) single 100 mg L^{-1} LH, (■) combination of 100 mg L^{-1} LH and 2 mg L^{-1} MJ

content. 31.37 mg L^{-1} saponins were recorded 100 mg L^{-1} treatment, whereas 45.93 mg L^{-1} saponins were recorded with combined treatment of LH and MJ (Fig. 7.5) [25].

Elicitor may induce the transcription and translation of specific genes in the secondary metabolic pathway, therefore, the content of active secondary metabolites in plant tissue cultures can be increased. At present, elicitors have received the widespread application in the studies on the production of active components in plant cell cultures. LH, which is an important nutrient in plant cell culture, is often used as an elicitor to enhance the contents of secondary metabolites [25]. Methyl jasmonate and its derivatives are considered to be involved in a part of the signal transduction pathway that induces particular enzymes catalyzing biochemical reactions to form defense compounds of low molecular weights in plants, such as polyphenols, alkaloids, quinones, terpenoids and polypeptides [25, 26].

Fed-Batch Cultures

When 30 g L^{-1} sucrose was fed to the cells which were cultured in bioreactors on day 16 at residual sugar level was below 15 g L^{-1}, dry cell growth rate and polysaccharide contents were higher than in batch cultivation. Higher polysaccharide productivity (1.608 g L^{-1}) was observed when compared with batch cultivation (0.819 g L^{-1}). Fed-batch cultivation did not significantly enhance the total saponin contents, while the total saponin contents showed a slow drop after 24 days when compared to that of batch cultivation. Because of an increase in biomass, the highest total saponin yield (7.828 mg L^{-1}) was obtained on day 24, and the value was about 36 % higher than that of the batch cultivation (Fig. 7.6) [27]. From this investigation, we found that fed-batch cultivation of *P. quinquefolium* cells significantly improved the production of ginsenoside and polysaccharides when compared to that of batch operation [27].

Two-Stage Cultivation

After 16 days of culture 30 g L^{-1} sucrose was added to the medium. In the second stage of culture, elicitors (100 mg L^{-1} LH and 2 mg L^{-1} MJ) were added to the medium on day 20. In the two-stage culture system, both the growth and polysaccharide content had a little change when compared to that obtained in fed-batch culture. However, the total saponin content was higher than that of fed-batch culture. Use of the treatment combining sucrose, LH and MJ caused a significant increase in total saponin yield (31.52 mg L^{-1}) in cell cultures after 27 days. Saponin yields were increased by 4.03- and 4.34-fold when compared to fed-batch cultivation and batch cultivation respectively (Fig. 7.7) [27].

Based on the results of carbon source consumption and elicitor effects, two-stage cultivation process was carried out in 5 L stirred tank bioreactor to enhance the cell density and metabolite production. In the second stage of culture, addition of LH and MJ significantly increased the total saponins in the cell cultures. The two-stage culture system was more effective in improving the contents of active components [27].

7.3 The Cell Suspension Culture of *Glycyrrhiza uralensis*

7.3.1 Culture Medium and Conditions

MS or B_5 medium are commonly used in the suspension cultures of *G. uralensis* cells. 2, 4-D has a significant influence on the biomass of the *G. uralensis* cells. In addition, the inoculum size was 6–10 %; the initial pH was 5.8–7.0; the concentration of the sucrose was 50 g L^{-1}; and the growth cycle was 21 days [28].

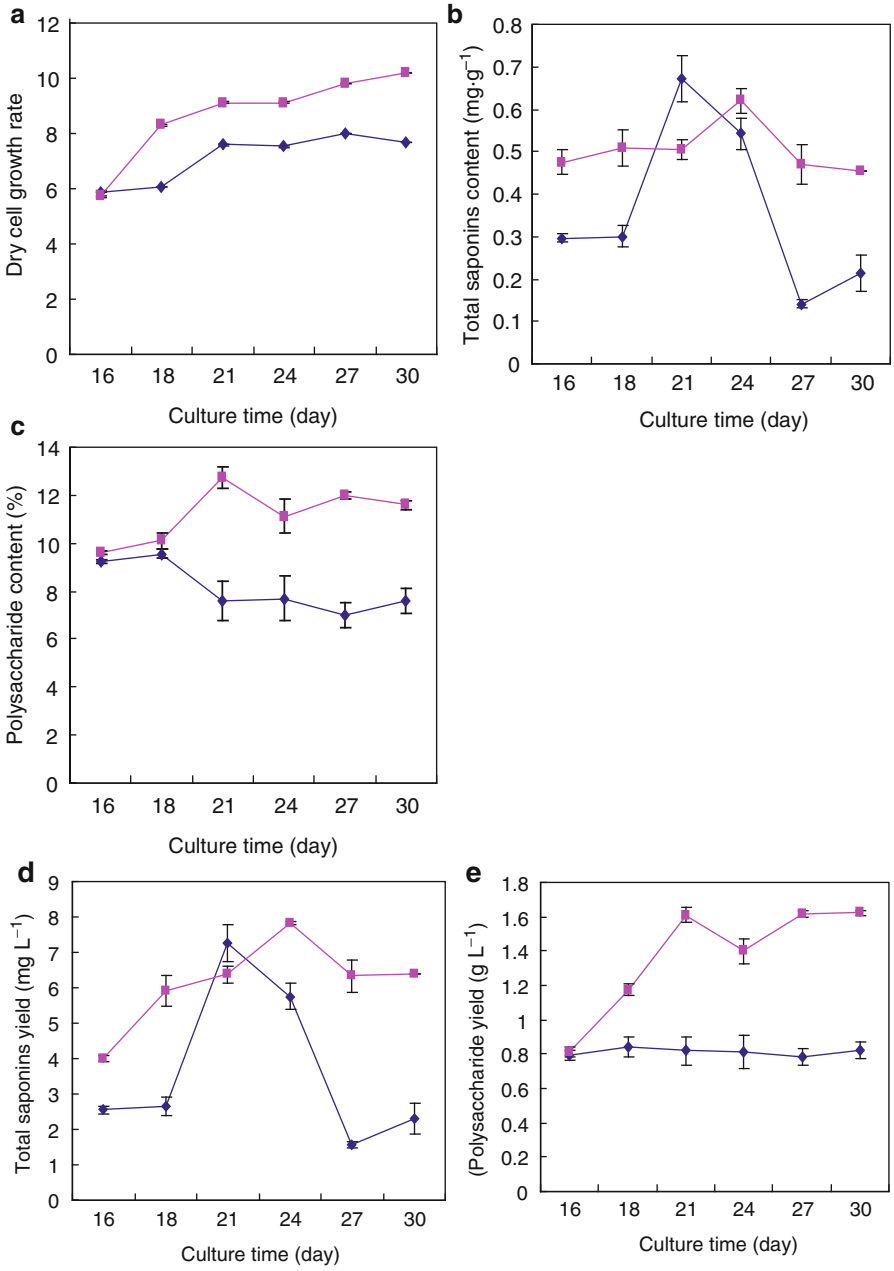


Fig. 7.6 Effects of fed-batch cultivation on *P. quinquefolium* cells in 5 L stirred tank bioreactor. (**a**) Effects on biomass; (**b**) Effects on saponin content; (**c**) Effects on polysaccharide content; (**d**) Effects on saponin yield. (**e**) Effects on polysaccharide yield. Symbols: (◆) batch cultivation, (■) fed-batch cultivation, add 30 g·L^{-1} sucrose on day 16

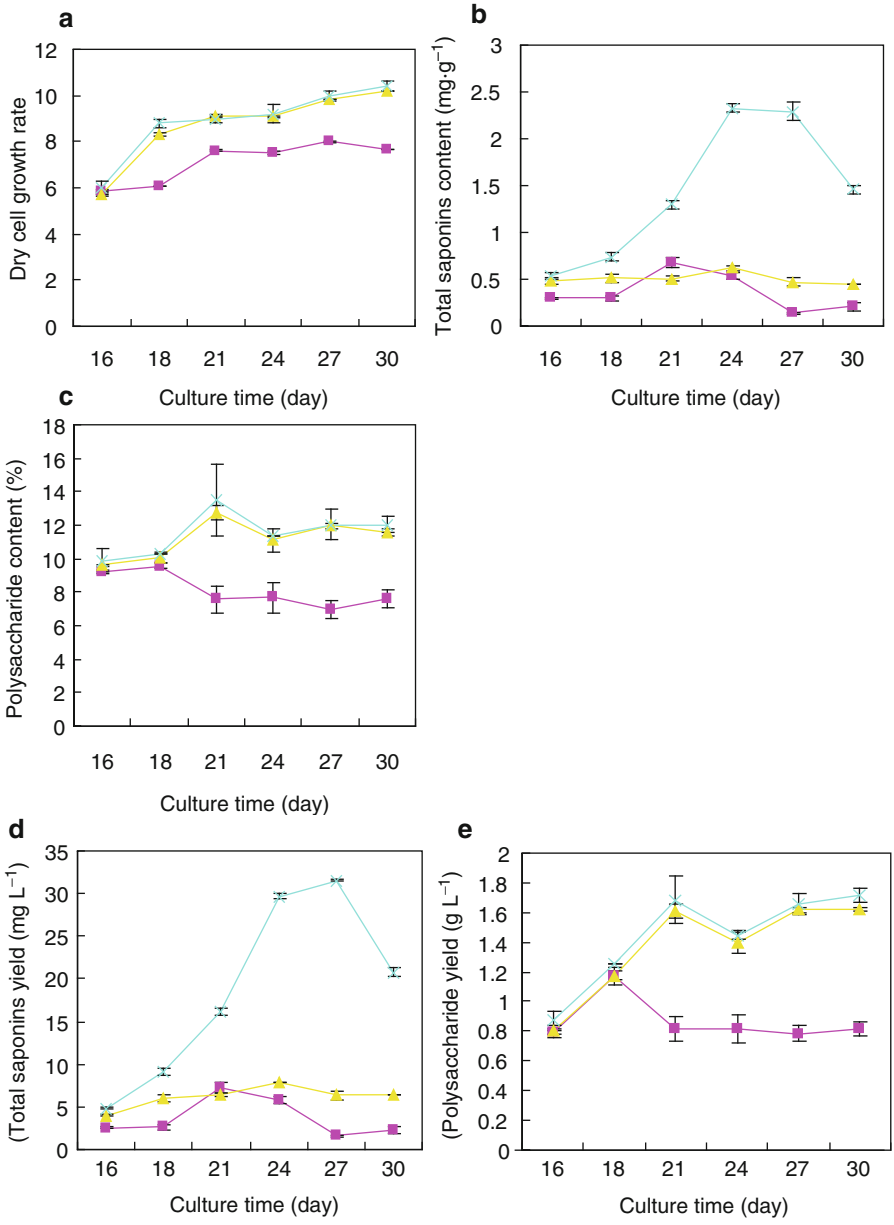


Fig. 7.7 Effects of two-stage culture on *P. quinquefolium* cells in 5 L bioreactor. (**a**) Effects on biomass; (**b**) Effects on saponin content; (**c**) Effects on polysaccharide content; (**d**) Effects on saponin yield; (**e**) Effects on polysaccharide yield. Symbols: (■) Batch culture; (▲) Fed-batch culture, add 30 g L^{-1} sucrose nod 16; (×) Two stage culture, add 30 g L^{-1} sucrose on day 16, add 100 mg L^{-1} LH and 2 mg L^{-1} MJ on day 20

In the shake flask culture, the concentration of nitrogen and phosphate were gradually reduced during the culture period. In the linear phase of the cell growth, the phosphate source was almost completely used. The sucrose in the culture medium of carbon sources converted to deoxidize sugar which can be directly used by the plant cells, eventually exhausted in 22 days.

A certain concentration of methyl jasmonic acid, salicylic acid and fungus polysaccharide can increase the yield of total flavonoids, increase the contents of H_2O_2 and malondialdehyde as well as the activity of phenylalanine lyase, catalase and peroxidase in the cells. The appropriate concentration of precursor phenylalanine, tyrosine, cinnamic acid and sodium acetate did not inhibit the growth of cells, and can promote the biosynthesis of total flavonoids. The accumulation of total flavonoids in *G. uralensis* cells can be promoted by the appropriate drought pressure and using the appropriate concentration of mannitol, KCl and sucrose [29].

7.3.2 Bioreactor Cultures

Optimization of the Aeration Volume

Glycyrrhiza uralensis cell suspension cultures were established in 5 L capacity balloon type bubble bioreactors (BTBB) containing 3 L MS medium supplemented with 1 mg L^{-1} 2, 4-D, 1.0 mg L^{-1} NAA, 0.2 mg L^{-1} BA and 30 g L^{-1} sucrose. Two-hundred gram cells (fresh weight) were cultured for 20 days at 23 ± 2 °C. The cultures were aerated at 0.2, 0.4, 0.6, 0.8 or 1.0 vvm. The highest accumulation of cell biomass was achieved at an aeration volume of 0.6 vvm. Similar to the findings for cell growth, the highest content of the triterpenoid saponins and flavonoids were obtained at an aeration volume of 0.6 vvm (Fig. 7.8) [30].

Optimizing the aeration volume is one conventional way of controlling the gaseous composition, which can affect the cell growth [31]. The air supplied into bioreactors play two important functions: one is supply of dissolved oxygen for metabolic activities, and the other one is agitation [32]. In general, a high aeration volume is beneficial in speeding up the transfer of oxygen into bioreactors, a process that improves both secondary metabolite accumulation and cell growth [33]. However, such a high volume is not always advantageous to the accumulation of biomass and compounds in plant cultures, probably because of physiological damage due to the excessive agitation and shear stress [31].

Dynamic Change of Growth and Specific Oxygen Uptake Rate (SOUR)

Cell growth was started rapidly 5 days after culture initiation, and the maximum dry weight was achieved on day 20. SOUR increased quickly within the first 5 days. After this, SOUR showed a sharp drop and was almost zero after 10 days (Fig. 7.9) [30].

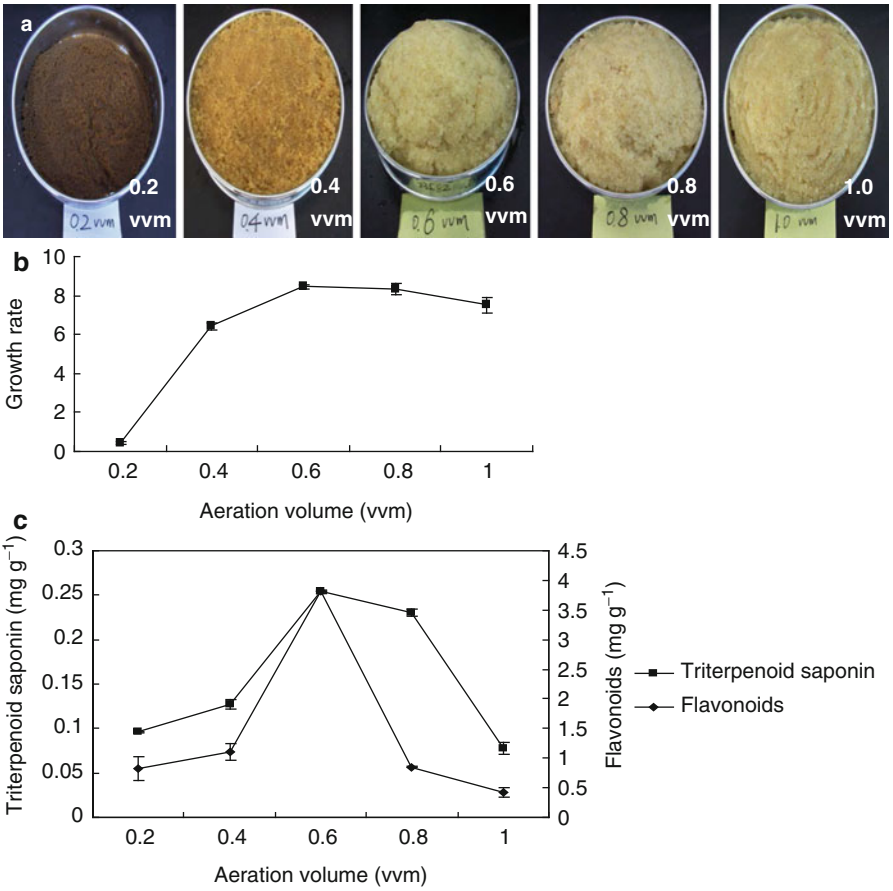


Fig. 7.8 Effects of aeration volume on *G. uralensis* cell cultures after 20 days of culture. (**a**) The *G. uralensis* cell harvested; (**b**) Effects on growth rate; (**c**) Effects on triterpenoid saponin and flavonoids content

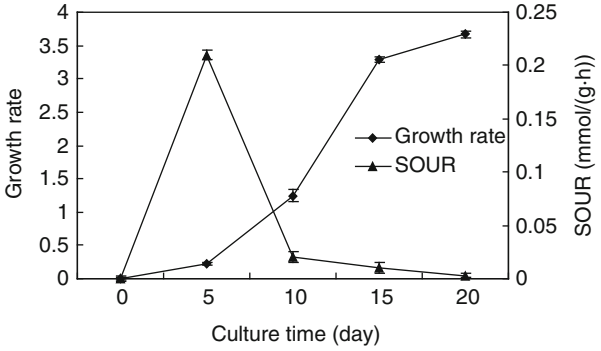


Fig. 7.9 Time profile of growth and SOUR in *G. uralensis* cell at 0.6 vvm. Each value represents mean ± standard error of three replicates

Step-Wise Aeration Treatment

The harvested cells of *G. uralensis* under three modes of aeration (0.6 vvm, 0.4–0.6–0.4 vvm and 0.6–0.4 vvm) after 20 days are presented in Fig. 7.10. The highest growth rate of cell biomass was achieved at an aeration volume of 0.6 vvm. The highest content of the triterpenoid saponins and flavonoids were obtained at an aeration volume of 0.6–0.4 vvm. Overall, the highest triterpenoid saponins yield (2.58 mg L^{-1}) and flavonoids yield (24.33 mg L^{-1}) was obtained at 0.6–0.4 vvm (Fig. 7.10) [30].

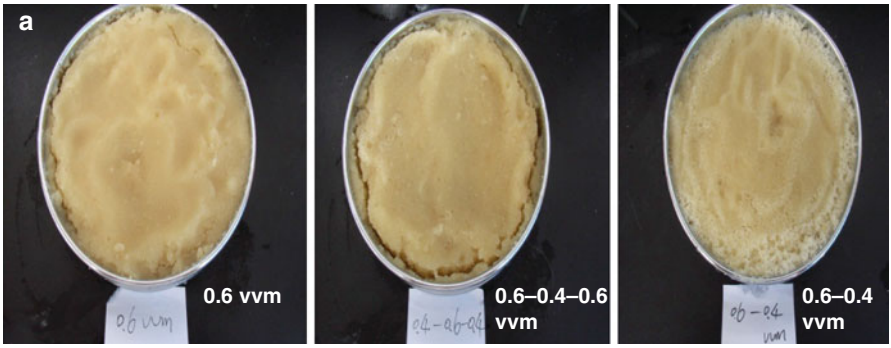
We have shown earlier that 5 days later, cells increase to grow rapidly and exhibit a stationary phase after 15 days. SOUR increases quickly within the first 5 days. After this, SOUR shows a sharp drop and will be almost zero after 10 days. For this reason, we designed a step-wise aeration treatment with 0.4–0.6–0.4 vvm and 0.6–0.4 vvm, respectively. Jeong et al. [34] and Lee et al. [35] reported that a gradual rise in aeration volume is favorable for growth of cells/organs in bioreactors because the high inflow of air agitates cells, thereby elevating the concentration of dissolved oxygen in the culture while accelerating the cell growth, whereas maintaining a constant, high aeration volume throughout the culture period inhibits their growth due to sheer stress [30].

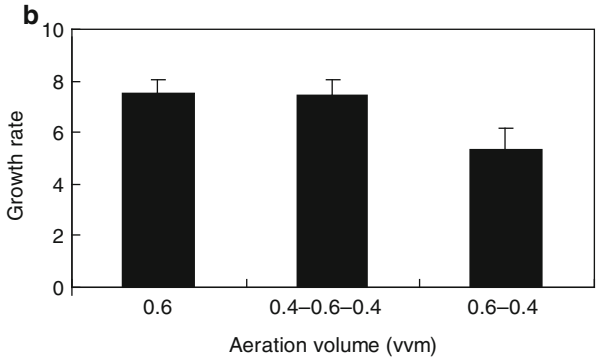
Growth Kinetics

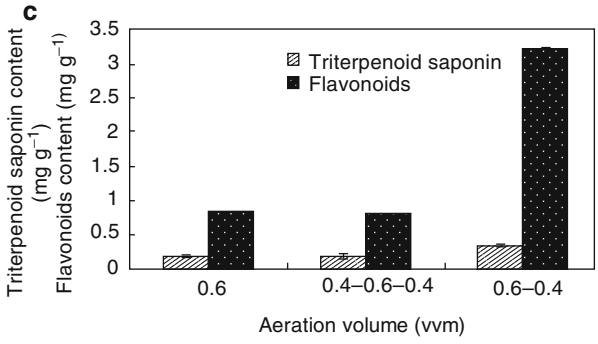
Bioreactor cultures were aerated with 0.6–0.4 vvm, growth kinetics was studied and results are presented in Fig. 7.11. The cell growth increased quickly from 0 to 15 days, after 20 days of cultivation, the dry cell growth rate reached its peak (8.78). SOUR increased quickly within the first 5 days. After this, SOUR showed a sharp drop and was almost zero after 10 days. The highest triterpenoid saponins and flavonoids contents were obtained on day 10 and day 15 respectively. Linear correlations and a high calculated correlation coefficients ($r^2 = 0.99$) were observed between cell growth and medium conductivity (Fig. 7.11) [31].

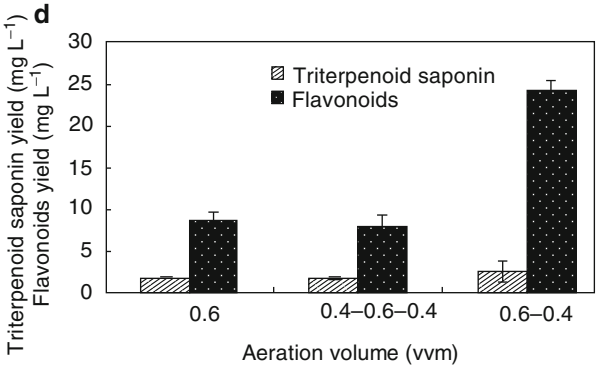
From our investigation, we found that SOUR of cells at an aeration volume of 0.6–0.4 vvm increased quickly within the first 5 days. After 5 days, SOUR showed a sharp drop and was almost zero after 10 days. This resembles the result of 0.6 vvm treatment [31]. It has been reported that conductivity values reflect nutrient uptake by the cells, therefore, electrical conductivity measurements have been used as an indirect method for biomass estimation [36]. A prompt measure can be obtained by exploiting the known linear relationship between changes in medium conductivity and cell growth [37].

Fig. 7.10 Effects of step-wise aeration on *G. uralensis* cells after 20 days of culture. (**a**) The *G. uralensis* cells harvested; (**b**) Effects on growth rate; (**c**) Effects on triterpenoid saponin and flavonoids content; (**d**) Effects on triterpenoid saponin and flavonoids yield


a
0.6 vvm
0.6–0.4–0.6 vvm
0.6–0.4 vvm


b
Growth rate
0
2
4
6
8
10
0.6
0.4–0.6–0.4
0.6–0.4
Aeration volume (vvm)


c
Triterpenoid saponin content (mg g−1)
Flavonoids content (mg g−1)
Triterpenoid saponin
Flavonoids
0
0.5
1
1.5
2
2.5
3
3.5
0.6
0.4–0.6–0.4
0.6–0.4
Aeration volume (vvm)


d
Triterpenoid saponin yield (mg L−1)
Flavonoids yield (mg L−1)
Triterpenoid saponin
Flavonoids
0
5
10
15
20
25
30
0.6
0.4–0.6–0.4
0.6–0.4
Aeration volume (vvm)

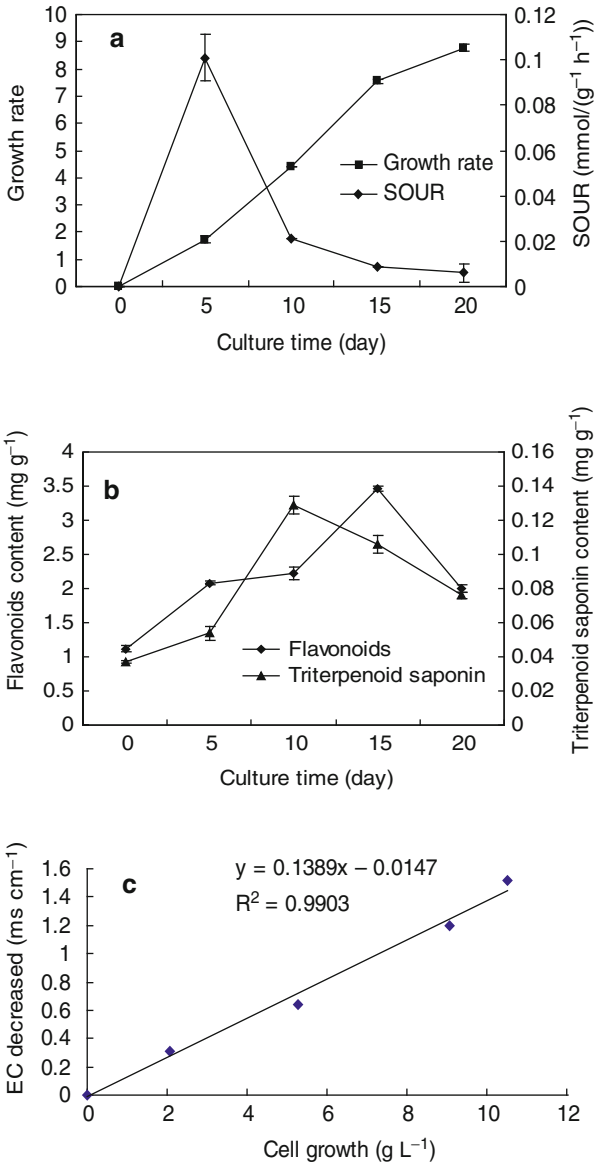


Fig. 7.11 Changes in growth rate and SOUR (**a**), changes in triterpenoid saponin and flavonoids content (**b**) of *G. uralensis* cells at 0.6–0.4 vvm. Relationship between changes in medium EC and cell growth (**c**) of *G. uralensis* cell at 0.6–0.4 vvm

Elicitation

Further, to increase the production of flavonoids and polysaccharides in cell culture, 2 mM PHE and 5 mg L^{-1} MJ were added to culture media after 10 days of culture. The combination of PHE and MJ synergistically stimulated flavonoids and

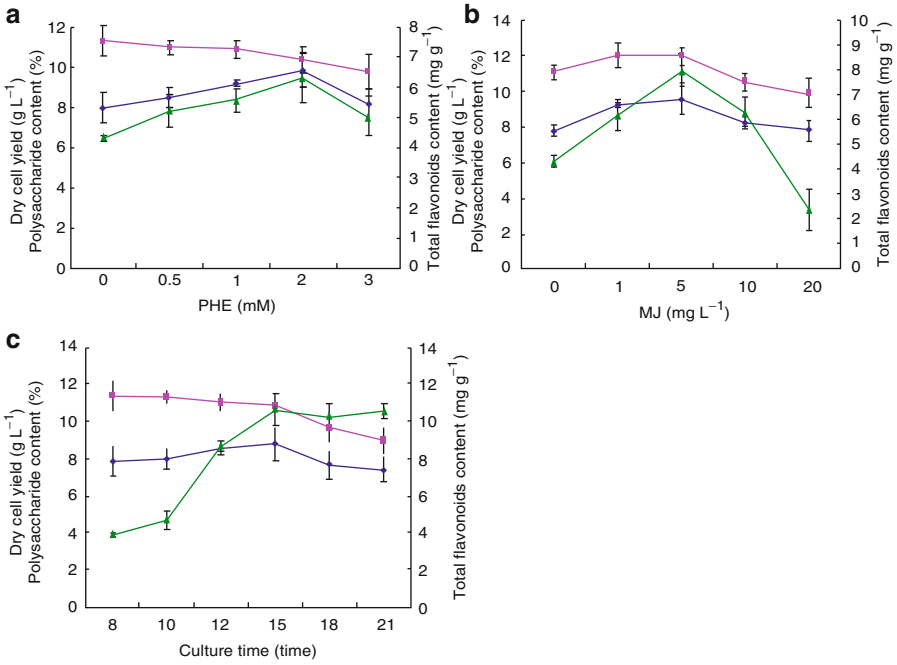


Fig. 7.12 Effect of PHE on cell growth and production of active components in 5-L BTBB (**a**). Effect of MJ on cell growth and production of active components in 5-L BTBB (**b**). Effect of MJ and PHE on cell growth and production of active components in 5-L BTBB (**c**). Dry cell yield (*red square*), polysaccharide content (*blue diamond*), total flavonoids content (*green triangle*). Each value represents mean ± standard error of three replicates

polysaccharide accumulation when compared with MJ alone, but did not significantly enhance the biomass content. Finally, these results led to higher flavonoids productivity (10.62 mg L^{-1}) than single treatment of 5 mg L^{-1} MJ (9.58 mg L^{-1}) (Fig. 7.12) [38].

Different Kinds of Cultivation Methods

Fed-batch cultivation of suspension cells was carried out in the BTBB, 30 g L^{-1} of sucrose was added on 8th day. Dry cell yield and polysaccharide content were both higher than that of the batch cultivation for 21 days culture. Dry cell yield reached from 10.73 g L^{-1} in batch cultivation to 12.08 g L^{-1} in fed-batch cultivation. Higher polysaccharide yield (1.19 g L^{-1}) was observed when compared with batch cultivation (0.56 g L^{-1}). Nevertheless, fed-batch cultivation did not significantly enhance the total flavonoids contents after 21 days culture when compared

with that of batch cultivation. A higher total flavonoids yield (55.42 mg L^{-1}) that was about 22 % higher than that of the batch cultivation was obtained on 21st day. In brief, fed-batch cultivation of *G. uralensis* cells improved the dry cell yield and accumulation of flavonoids and polysaccharides when compared with batch cultivation.

During the two-stage cultivation of suspension cells in BTBB, 30 g L^{-1} sucrose was added to the medium on 8th day and the elicitors (2 mM PHE and 5 mg L^{-1} MJ) were added to the medium on 10th day in the second stage of culture. Both the dry cell yield and the polysaccharide content in two-stage culture system slightly changed when compared with fed-batch culture, but were significantly higher than that in batch cultivation. Dry cell content was increased by 1.05- to 1.15-fold when compared with fed-batch cultivation and batch cultivation on 15th day. The polysaccharide yield was increased by 1.14- to 2.12-fold when compared with fed-batch cultivation and batch cultivation on 15th day. However, the total flavonoid content was obviously higher than that in fed-batch culture. The treatment of combined sucrose, PHE and MJ caused a significant increase in total flavonoids yield (132.36 mg L^{-1}) on 15th day, which was increased by 2.26- to 2.67-fold when compared with fed-batch cultivation and batch cultivation, respectively. In a word, two-stage cultivation system compared with fed-batch cultivation showed the unclear influence on the dry cell and polysaccharide contents, but obviously affected the total flavonoid production. Obviously, two-stage cultivation system had the significant impact on all of the dry cell, polysaccharides and total flavonoid contents when compared with the batch cultivation (Fig. 7.13) [38].

Sucrose, as a primary energy source, is the widespread carbon source for the plant cell culture. The rate of biomass growth is directly correlated with sucrose consumption. Therefore, we added the source on 8th day when carbon source reduced to a lower level, which significantly enhanced the *G. uralensis* cell growth. The carbon consumption was also consistent with the cell growth and might affect cell growth and flavonoid biosynthesis [38]. Depending on the effects of source, precursor and elicitor, two-stage cultivation process has been carried out in the 5 L BTBB to enhance the cell growth and secondary metabolite production. In addition, two-stage process with a high-density culture was practical for producing secondary metabolites. On the basis of dry cell yield and polysaccharide accumulation in cellular *Glycyrrhiza*, two-stage cultivation system was similar to the fed-batch cultivation, and was better than the batch cultivation. On the other hand, in view of flavonoids production, two-stage cultivation system was the best method among these three cultivation systems. These results provided the basis for commercial-scale cultivation in suspension cells of *G. uralensis* in bioreactor, which could provide a new way to produce biologically active substances for cosmetics and health food. Furthermore, the understanding of induction mechanism on secondary metabolic pathways should be explored and the scale of bioreactor should be further remarkably expanded after being treated with the combining elicitation of PHE and MJ [39].

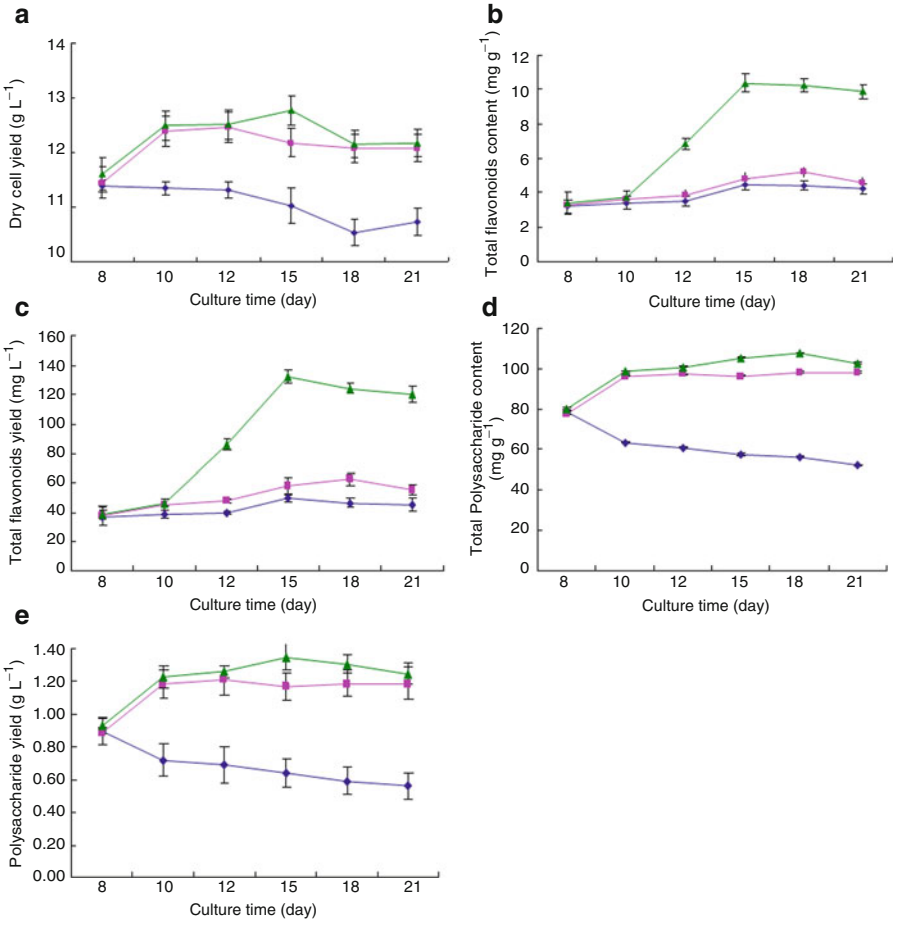


Fig. 7.13 Effects of two-stage culture on *G. uralensis* cell in a 5-L BTBB. Effects on biomass (**a**). Effects on total flavonoids content (**b**). Effects on total flavonoids yield (**c**). Effects on total polysaccharide content (**d**). Effects on total polysaccharide yield (**e**). Batch culture (*blue diamond*); fed-batch culture, 30 g L^{-1} sucrose added on day 8 (*red square*); two-stage culture, with the addition of 30 g L^{-1} sucrose on day 8 and 2 mM PHE and 5 mg L^{-1} MJ on day 10 (*green triangle*). Each value represents mean ± standard error of three replicates

Scale Up Process

Maximum growth rate of 3.18- and 10.86-fold were obtained in 5 L and 10 BTBBs, respectively after 20 days of inoculation, which were significantly higher than that in 0.5 L conical flask (1.33-fold). Bioreactor cultures of *G. uralensis* cells are shown in Fig. 7.14. Chen et al. [40] reported that maximum dry weight and growth rate of *G. uralensis* cells in 9 L airlift bioreactor were 16.25 g L^{-1} and 0.9 g L^{-1} day^{-1}, respectively, which were higher than that in conical flask. We also found that growth rate of *G. uralensis* cells in bioreactor was significantly higher than that of conical flask.

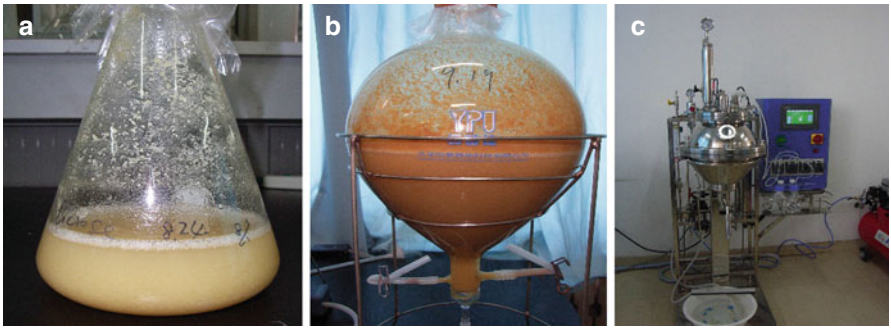


Fig. 7.14 Scale-up culture of *G. uralensis* cells (**a**) Culture in 0.5 L shake flask; (**b**) Culture in 5 L BTBB; (**c**) Culture in 10 L BTBB

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Part III
Production of Biomass and Bioactive Compounds from Organ Cultures: Shoot, Embryo and Adventitious Root Cultures

Chapter 8
Production of Caffeic Acid Derivatives from Adventitious Root Cultures of *Echinacea purpurea* (L.) Moench

Hosakatte Niranjana Murthy, Chun-Hua Wu, Yong-Yi Cui, and Kee-Yoeup Paek

Abstract *Echinacea purpurea* (L.) Moench, the purple cone flower is one of the world's most important medicinal herbs and active ingredients of purple cone flower are caffeic acid derivatives namely caftaric acid, chlorogenic acid, caffeic acid, cynarin, echinacoside and cichoric acid. Efforts have been made in the recent past for the production of caffeic acid derivatives from adventitious root cultures. Bioreactor cultures have been established for large-scale production of biomass and bioactive compounds and various physiological parameters affecting the biomass and accumulation of caffeic acid derivatives have been investigated. Advances in adventitious root cultures of *Echinacea purpurea* for the production of bioactive compounds have been summarized in this review.

Keywords Adventitious roots • Bioreactor cultures • Caffeic acid derivatives • Echinacea • Suspension cultures

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Abbreviations

DW	Dry weight
FW	Fresh weight
IBA	Indole-3-butyric acid
MS medium	Murashige and Skoog medium
SNP	Sodium nitroprusside
vvm	Air volume per medium volume per minute

8.1 Introduction

Echinacea purpurea (L.) Moench, the purple cone flower is one of the world's most important medicinal herbs which are widely grown in various parts of the world. Research shows that it has the ability to stimulate the immune response against the bacterial and viral infections. It is valuable in preventing or treating cold, flu and also skin infections such as acne and boils. *E. purpurea* is also a good wound healer [2]. Echinacea products are the most popular herbal immunostimulants in North America and Europe. Echinacea is the top selling single botanical product [10] and the market leader in volume of sale at 10 % of total medicinal herbs sales [18]. The potential compounds found in purple coneflower are caffeic acid derivatives namely caftaric acid, chlorogenic acid, caffeic acid, cynarin, echinacoside and cichoric acid (Fig. 8.1). Of these, cichoric acid has immunostimulatory properties and can promote phagocyte activity *in vitro* and *in vivo*. It has also been shown to have antiviral activity especially against HIV [1, 7].

Cell and organ cultures have emerged as a valuable route for biosynthesis of phytochemicals having medicinal importance. In this regard, *E. purpurea* cell and hairy root cultures were used to produce immunologically active caffeic acid derivatives and polysaccharides [6, 8, 9, 14, 16, 17], however, no further work on optimization of medium parameters, physical factors and large scale cultivation of cells and hairy roots were investigated. Nevertheless, successful adventitious root cultures were established by Jeong et al. [4] and Wu et al. [20–23] using bioreactor system for the production of potential bioactive compounds and various culture strategies have been worked out. In this review, the various bioprocessing methodologies which control the *E. purpurea* adventitious root biomass and bioactive compound production are highlighted.

8.1.1 Induction of Adventitious Roots and Establishment of Suspension Cultures

Calli masses were induced from the roots of *Echinacea purpurea* which were collected from the wild and cultured on Murashige and Skoog medium [11] supplemented with 2.0 mg L^{-1} IBA and 50 g L^{-1} sucrose in dark at 25 ± 2 °C [20].

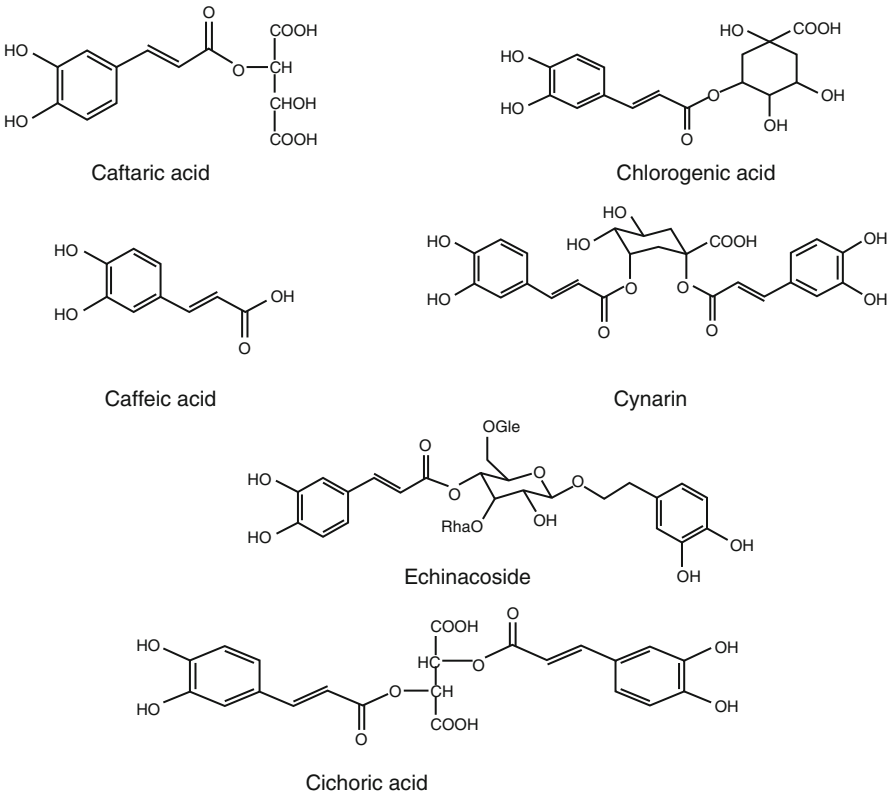

Fig. 8.1 Caffeic acid derivatives

Adventitious roots were induced from the calli masses upon subculturing to half strength MS medium supplemented with 2.0 mg L^{-1} IBA and 50 g L^{-1} sucrose.

Bioreactor cultures (balloon type bubble bioreactors with 5 L capacity) were initiated using 4 L half strength modified MS medium (ammonium and nitrate ratio was 5:25 mM) supplemented with 2 mg L^{-1} IBA and 50 g L^{-1} sucrose. Bioreactor cultures were established using 7 g L^{-1} inoculum and were maintained in dark at 25 ± 2 °C for 8 weeks. The growth pattern of adventitious roots typically showed a lag phase of 0–1 weeks, exponential phase of 2–7 weeks and then remained stationary after 7th week onwards (Fig. 8.2a) [4]. After 7 weeks of culture, the biomass reached its peak (10.5 g L^{-1} DW), which was 13 times higher than initial inoculum dry weight. The accumulation of dry adventitious root mass was found to be much higher in bioreactor cultures (10.5 g L^{-1} DW) than in the shake flask cultures (6.6 g L^{-1} DW) [4]. The electrical conductivity (EC) and hydrogen ion concentration (pH) of the medium decreased during the course of time (Fig. 8.2b) and this was due to depletion of nutrients in the culture medium and increased metabolite accumulation in the biomass. Similarly, there was decrease in sucrose concentration over the culture period and increase in fructose and glucose with the cultures (Fig. 8.3a). The kinetic changes in

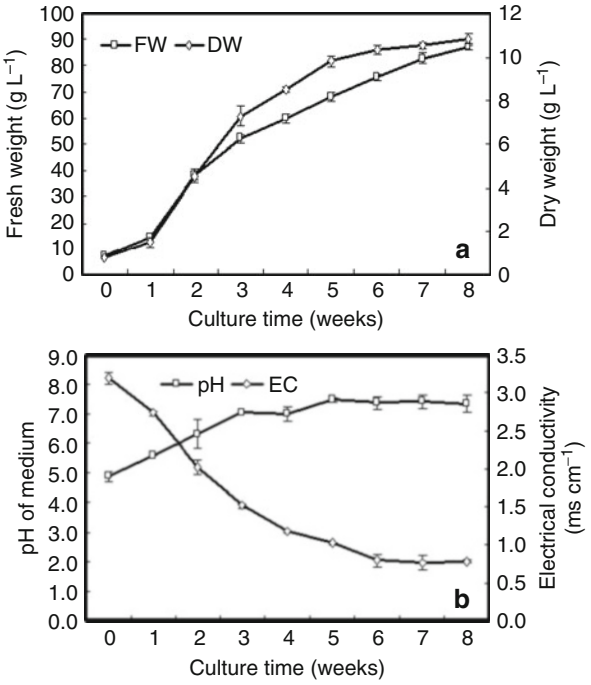

Fig. 8.2 (**a**) Time profiles for adventitious root growth (-○- fresh weight; -□- dry weight) of *Echinacea purpurea* cultured in 5 L balloon-type bubble bioreactors, (**b**) Changes in electrical conductivity (*EC*) and hydrogen ion concentration (pH) in adventitious root cultures of *E. purpurea* cultured in 5 L balloon type bubble bioreactor (-□- pH; -○- EC)

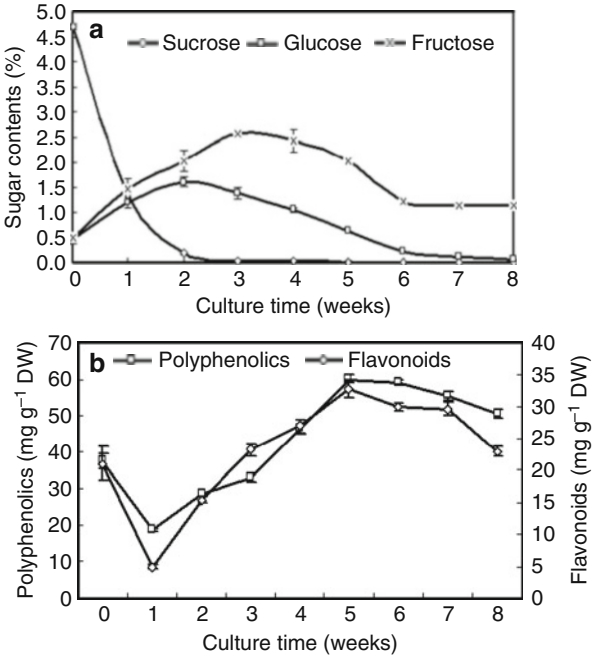

Fig. 8.3 (**a**) Time profiles for residual sugars in *E. purpurea* adventitious root suspensions cultured in 5 L balloon type bubble bioreactor (-◇- sucrose; -□- glucose; -x- fructose), (**b**) Kinetics of phenolic and flavonoid production by bioreactor cultured *E. purpurea* adventitious roots (-□- phenolics; -◇- flavonoids)

the concentrations of NH_4^+, Ca^{2+}, K^+, NO_3^-, Mg^{2+}, SO_4^{2-}, and HPO_4^- was reported in adventitious root cultures (Fig. 8.4) [4]. The uptake of these ions and hydrolysis of sucrose into simple sugars indicate the utilization of nutrients by the growing adventitious roots. These aspects observed during the culture also indicated that the bioreactor cultures adequately meet the nutritional needs of the *E. purpurea* adventitious root cultures (Fig. 8.4). The total phenolic and flavonoid contents were increased with the cultures over the period and their concentration was optimum after 5 weeks (60 and 32.8 mg g^{-1} DW respectively; Fig. 8.3b). The accumulation of 5.28 mg g^{-1} DW caftaric acid, 5.53 mg g^{-1} DW chlorogenic acid, and 27.51 mg g^{-1} DW cichoric acid in bioreactor cultures (Fig. 8.5) were higher than that of hairy roots cultivated in flask cultures (3.56 mg g^{-1} DW of caftaric acid, 0.93 mg g^{-1} DW chlorogenic acid, and 19.21 mg g^{-1} DW cichoric acid) [9]. Therefore, bioreactor cultures are suitable for the large scale production of caffeic acid derivatives.

8.1.2 *The Effect of Inoculum Density on Adventitious Root Growth and Metabolite Accumulation*

Table 8.1 illustrates the effect of inoculum density on the growth of adventitious roots and accumulation of phenolics and flavonoids. Among the various inoculum densities tested (2.5, 5.0, 7.0, 10.0 and 15.0 g L^{-1}) maximum biomass accumulation was obtained (79.0 g L^{-1} FW and 10.4 g L^{-1} DW) when 15 g L^{-1} of adventitious roots were used as inoculum. However, the maximum phenolic and flavonoid contents (58.5 mg g^{-1} DW and 38.6 mg g^{-1} DW respectively) were with the inoculum density of 7 g L^{-1}. Similarly, 4.1 mg g^{-1} DW caftaric acid, 5.1 mg g^{-1} DW chlorogenic acid and 28.1 mg g^{-1} DW cichoric acid were produced with the inoculum density of 7 g L^{-1} (Table 8.2) [4]. These results clearly demonstrate that inoculum density is also one of the factors which determine the accumulation of biomass and the productivity of bioactive compounds by *in vitro* cultures.

8.1.3 *The Effect of Different Aeration Rates on Biomass and Metabolite Accumulation*

Aeration is an important factor which influences the biomass growth and metabolite accumulation in bioreactors [3]. In airlift bioreactors, aeration fulfills three main functions: maintenance of aerobic conditions, desorption of volatile products, removal of metabolic heat. Additionally, aeration is also meant for agitation of biomass which promotes homogeneity of cultured cells and organs. The adventitious root cultures which were maintained with four modes of aeration, 0.05, 0.1, 0.2 and 0.3 vvm for initial 5 weeks showed a profound influence on biomass growth. Highest biomass growth of 70.1 g L^{-1} FW and 9.0 g L^{-1} DW was observed with the cultures

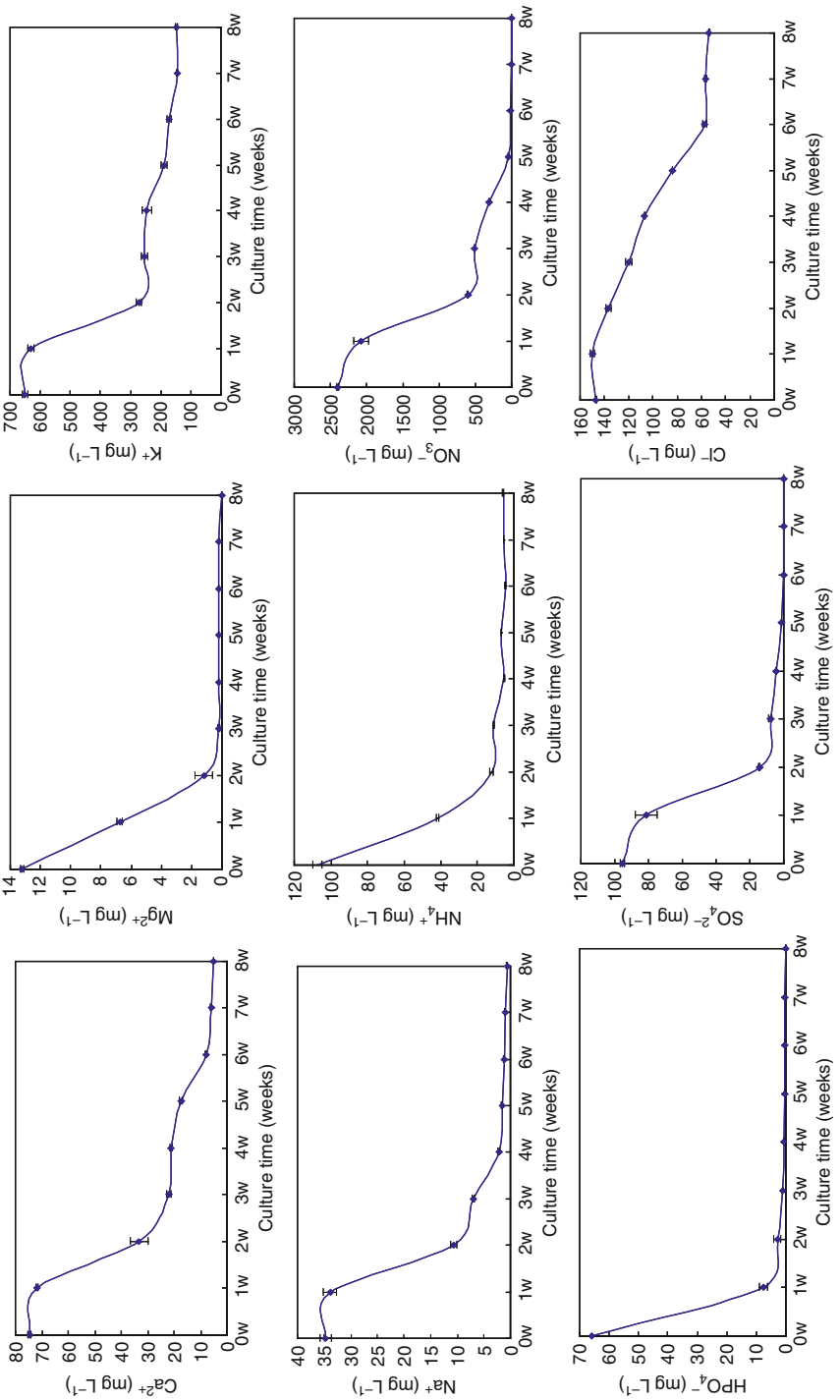

Fig. 8.4 Time profile of anion and cation contents during bioreactor culture of adventitious roots

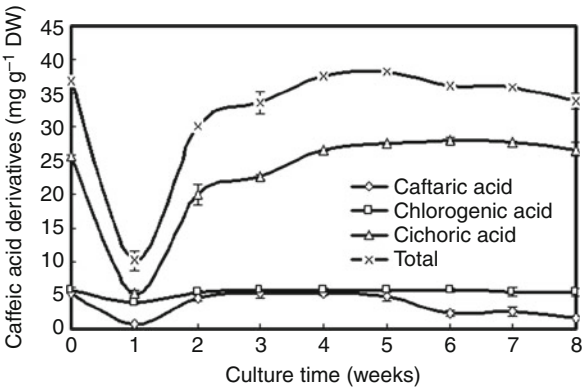

Fig. 8.5 Kinetics of production of caffeic acid derivatives from adventitious roots of *E. purpurea* cultured in bioreactor (-◇- caftaric acid; -▫- chlorogenic acid; -Δ-cichoric acid; -x- total caffeic acid derivatives)

Table 8.1 Effect of inoculum density on adventitious root growth of *Echinacea purpurea* and the productivity of phenolics and total flavonoids after 5 weeks of culture using 5 L balloon type bubble bioreactor containing 4 L MS half strength medium

Inoculum density (g L^{-1} FW)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio	Total phenolics (mg g^{-1} DW)	Total flavonoids (mg g^{-1} DW)
2.5	55.6 e[a]	4.5 e	15.4	34.2 e	25.8 d
5.0	59.2 d	6.3 d	10.5	55.2 b	33.8 b
7.0	67.2 c	9.0 c	10.8	58.5 a	38.6 a
10.0	74.8 b	10.1 b	8.2	45.4 c	27.6 c
15.0	79.0 a	10.4 a	5.4	40.6 d	23.4 e

[a]Mean separation within columns by Duncan's multiple range test at 5 % level

Table 8.2 Effect of inoculum density on the production of caffeic acid derivatives from adventitious roots of *E. purpurea* after 5 weeks of culture using 5 L balloon type bubble bioreactors containing 4 L MS half strength medium

	Caffeic acid derivatives (mg g^{-1} DW)[a]			
Inoculum density (g L^{-1} FW)	Caftaric acid	Chlorogenic acid	Cichoric acid	Total[b]
2.5	2.3 ± 0.1	4.2 ± 0.2	16.5 ± 0.2	23.0 ± 0.3
5.0	2.4 ± 0.1	4.5 ± 0.1	27.2 ± 0.1	34.1 ± 0.1
7.0	4.1 ± 0.1	5.1 ± 0.1	28.1 ± 0.4	37.3 ± 0.3
10.0	2.7 ± 0.1	4.5 ± 0.1	27.2 ± 0.1	34.4 ± 0.1
15.0	2.3 ± 0.1	2.7 ± 0.1	25.2 ± 0.1	30.2 ± 0.1

[a]Mean values of three replicates ± standard error
[b]Total caffeic acid derivatives = caftaric acid + chlorogenic acid + cichoric acid

aerated with 0.1 vvm (Table 8.3). The highest accumulation of phenolics and flavonoids (60.7 mg g^{-1} DW and 38.8 mg g^{-1} DW respectively), caftaric acid (4.7 mg g^{-1} DW), chlorogenic acid (5.6 mg g^{-1} DW) and cichoric acid (26.6 mg g^{-1} DW; Tables 8.3 and 8.4) [4] were also obtained when the aeration rate was 0.1 vvm.

Table 8.3 Effects of air supply on the growth of adventitious roots of *E. purpurea* and the productivity of phenolics, flavonoids after 5 weeks of culture using 5 L balloon type bubble bioreactors containing 4 L MS half strength medium

Air supply (wm)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio	Total phenolics (mg g^{-1} DW)	Total flavonoids (mg g^{-1} DW)
0.05	70.5 ab[a]	8.9 ab	10.6	60.9 a	37.8 a
0.1	70.1 ab	9.0 ab	10.8	60.7 a	38.8 a
0.2	63.1 de	8.2 c	9.7	61.1 a	38.3 a
0.3	61.4 e	8.1 c	9.5	58.7 b	35.6 b
0.05–0.1	66.1 cd	8.8 b	10.4	60.2 a	38.2 a
0.05–0.2	67.5 bc	8.8 b	10.5	61.1 a	38.9 a
0.05–0.3	71.9 a	9.2 a	10.9	61.1 a	38.7 a

[a]Mean separation within columns by Duncan's multiple range test at 5 % level

Table 8.4 Effects of air supply on the production of caffeic acid derivatives from adventitious roots of *E. purpurea* after 5 weeks of culture using 5 L balloon type bubble bioreactors containing 4 L MS half strength medium

	Caffeic acid derivatives (mg g^{-1} DW)[a]			
Air supply (wm)	Caftaric acid	Chlorogenic acid	Cichoric acid	Total[b]
0.05	4.7 ± 0.1	5.6 ± 0.1	25.0 ± 0.1	35.3 ± 0.1
0.1	4.7 ± 0.1	5.6 ± 0.1	26.6 ± 0.1	37.2 ± 0.2
0.2	3.8 ± 0.1	5.5 ± 0.1	24.1 ± 0.1	33.4 ± 0.1
0.3	3.6 ± 0.1	5.2 ± 0.1	22.5 ± 0.6	31.3 ± 0.5
0.05–0.1	4.8 ± 0.1	5.8 ± 0.1	27.6 ± 0.1	38.2 ± 0.1
0.05–0.2	4.8 ± 0.1	6.0 ± 0.1	28.3 ± 0.2	39.1 ± 0.3
0.05–0.3	4.9 ± 0.1	6.0 ± 0.3	28.1 ± 0.3	39.0 ± 0.4

[a]Values are means of three replicates ± standard error
[b]Total caffeic acid derivatives = caftaric acid + chlorogenic acid + cichoric acid

The higher aeration rates at 0.2 and 0.3 vvm were not suitable for biomass and metabolite accumulation.

8.1.4 The Effect of Incubation Temperature and Photoperiod

Productivity of secondary metabolites in cell and organ suspension cultures is also dependent on physical factors like temperature and light [13, 25]. Adventitious root cultures of *E. purpurea* were cultured under different temperatures including 10, 15, 20, 25 and 30 °C to verify the effect of temperature by Wu et al. [20] and results showed that adventitious root biomass accumulation was highest with the cultures incubated under 20 °C and optimum of 65.5 g L^{-1} fresh biomass and 10.4 g L^{-1} dry biomass was recorded, the growth ratio was 12.5 (Table 8.5). Accumulation of total polyphenolics and flavonoids was also optimal under 20 °C. Optimum of 4.7 mg g^{-1}

Table 8.5 Effect of incubation temperature on adventitious root growth of *E. purpurea* after 5 weeks of culture

Growth temperature (°C)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio
10	19.6 e[a]	2.8 e	2.6
15	39.3 d	6.5 d	7.4
20	65.5 a	10.4 a	12.5
25	60.3 b	9.5 b	11.4
30	56.1 c	8.3 c	9.8

[a]Mean separation within columns by Duncan's multiple range test at 5 % level

Table 8.6 Effect of incubation temperature on the production of caffeic acid derivatives from *E. purpurea* after 5 weeks of culture

	Caffeic acid derivatives (mg g^{-1} DW)[a]			
Growth temperature (°C)	Caftaric acid	Chlorogenic acid	Cichoric acid	Total[b]
10	1.9 ± 0.1	4.6 ± 0.1	13.4 ± 0.02	19.9 ± 0.1
15	4.4 ± 0.1	4.4 ± 0.1	22.0 ± 0.6	30.8 ± 0.5
20	4.7 ± 0.1	5.2 ± 0.1	28.4 ± 0.2	38.3 ± 0.1
25	4.1 ± 0.1	5.1 ± 0.1	25.1 ± 0.1	34.2 ± 0.1
30	4.1 ± 0.1	4.4 ± 0.1	25.3 ± 0.2	33.9 ± 0.2

[a]Mean values of three replicates ± standard error
[b]Total caffeic acid derivatives = caftaric acid + chlorogenic acid + cichoric acid

DW caftaric acid, 5.2 mg g^{-1} DW chlorogenic acid and 28.4 mg g^{-1} DW cichoric acid were recorded with the treatment of cultures at 20 °C (Table 8.6). Increase in incubation temperature from 20 to 30 °C reduced the accumulation of caffeic acid derivatives by 11 % (Table 8.6). *E. purpurea* adventitious root cultures were incubated in dark and different light irradiation conditions/photoperiod such as 0/24, 3/21, 6/18, and 12/12 h light and dark regimes and inhibition of adventitious root biomass accumulation under light regimes was recorded. Maximum biomass growth (67 g L^{-1} fresh biomass and 9.8 g L^{-1} dry mass) was observed in the dark grown cultures (Table 8.7) [20]. However, light favours the accumulation of caffeic acid derivatives and the amount of caftaric acid (6.5 mg g^{-1} DW), chlorogenic acid (5.2 mg g^{-1} DW) and cichoric acid (34.2 mg g^{-1} DW) were highest with the cultures treated with 3/21 h light and dark regime (Table 8.8). Light is an important physical factor, which influences the formation of primary and secondary metabolites.

8.1.5 Improvement of Metabolite Production by Medium Replenishment Strategy

Among the various strengths of MS medium tested for biomass growth and accumulation of metabolites, half strength MS medium was found suitable and in this medium optimum accumulation of fresh (73.6 g L^{-1}) and dry weights (10.03 g L^{-1})

Table 8.7 Effect of light irradiation on adventitious root growth of *E. purpurea* after 5 weeks of culture

Photoperiod light/dark (h)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio
0/24	67.0 a[a]	9.8 a	10.6
3/21	68.6 a	9.8 a	8.8
6/18	68.5 a	9.7 a	9.6
12/12	51.6 b	6.6 b	6.5

[a]Mean separation within columns by Duncan's multiple range test at 5 % level

Table 8.8 Effect of light irradiation on the production of caffeic acid derivatives after 5 weeks of culture of *E. purpurea*

	Caffeic acid derivatives (mg g^{-1} DW)[a]			
Photoperiod light/dark (h)	Caftaric acid	Chlorogenic acid	Cichoric acid	Total[b]
0/24	4.4 ± 0.3	5.0 ± 0.6	27.9 ± 0.7	38.5 ± 1.2
3/21	6.5 ± 0.3	5.2 ± 0.1	34.2 ± 0.6	47.3 ± 0.9
6/18	6.3 ± 0.3	4.5 ± 0.2	33.1 ± 0.5	45.2 ± 1.1
12/12	3.1 ± 0.1	3.2 ± 0.2	29.9 ± 0.1	37.0 ± 1.1

[a]Mean values of three replicates ± standard error
[b]Total caffeic acid derivatives = caftaric acid + chlorogenic acid + cichoric acid

Table 8.9 The effect of different strengths of MS medium on the adventitious root growth of *E. purpurea* and productivity of phenolics and flavonoids after 5 weeks of culture in 5 L balloon type bubble bioreactor containing 4 L of medium

MS medium strengths	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio	Total phenolics (mg g^{-1} DW)	Total Flavonoids (mg g^{-1} DW)
0.25	50.9 d	7.10 c	8.2	57.28 b	35.14 b
0.5	73.6 a	10.03 a	12.0	61.14 a	38.30 a
0.75	67.2 b	9.23 b	11.0	37.78 c	22.61 c
1.0	59.7 c	6.7 d	7.7	21.66 d	11.50 d

Mean separation within columns by Duncan's multiple range tests at 5 % level

and also growth ratio (12.0; Table 8.9) were reported [21]. The optimal phenolic and flavonoid contents were 61.14 mg g^{-1} DW and 38.40 mg g^{-1} DW respectively with 0.5 strength MS medium and this medium was also responsible for the highest accumulation of caftaric acid (4.35 mg g^{-1} DW), chlorogenic acid (4.87 mg g^{-1} DW) and cichoric acid (29.05 mg g^{-1} DW; Table 8.10). These results indicate that the adventitious roots of *E. purpurea* require only low nutrient concentrations, which is a critical determinant in controlling growth of adventitious roots and for the accumulation of secondary metabolites.

Fed-batch cultivation is frequently used in plant cell cultures for enhancing the biomass and production of secondary metabolites. Wu et al. [21] demonstrated the effects of feeding different strength MS medium on biomass growth and

Table 8.10 The effect of different strengths of MS medium on the productivity of caffeic acid derivatives from adventitious roots of *E. purpurea* after 5 weeks of culture in 5 L balloon type bubble bioreactors containing 4 L of medium[a]

MS medium strengths	Caffeic acid derivatives (mg g^{-1} DW)[a]			
	Caftaric acid	Chlorogenic acid	Cichoric acid	Total[b]
0.25 MS	2.19 ± 0.01	5.02 ± 0.01	28.06 ± 0.10	35.27 ± 0.08
0.5 MS	4.35 ± 0.01	4.87 ± 0.09	29.06 ± 0.01	38.28 ± 0.16
0.75 MS	3.03 ± 0.02	4.70 ± 0.01	19.93 ± 0.01	27.66 ± 0.01
1.0 MS	1.46 ± 0.01	2.86 ± 0.01	8.16 ± 0.01	12.48 ± 0.04

[a]Mean values of three replicates ± standard error
[b]Total caffeic acid derivatives = caftaric acid + chlorogenic acid + cichoric acid.

Table 8.11 Effects of media replenishment on the adventitious root growth of *E. purpurea* and productivity of phenolics and flavonoids after 5 weeks of culture in 5 L balloon type bubble bioreactor containing 4 L of half strength MS medium

Strength of medium and replenishment schedule	Medium strength	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio	Total phenolics (mg g^{-1} DW)	Total flavonoids (mg g^{-1} DW)[a]
Control		65.3 f	9.80 g	11.7	60.3 a	37.0 a
After 3 weeks initial culture	0.25 MS	78.8 c	12.65 c	15.4	59.2 a	34.4 b
	0.50 MS	83.1 a	14.76 a	18.2	59.2 a	34.2 b
	0.75 MS	82.4 a	14.78 a	18.2	56.8 b	31.9 cd
	1.00 Ms	83.6 a	14.94 a	18.4	54.8 b	30.6 e
After 3 weeks initial culture	0.25 MS	63.8 g	10.62 f	12.8	60.4 a	34.1 b
	0.50 MS	70.0 d	11.97 e	14.5	60.5 a	35.1 b
	0.75 MS	67.8 e	12.32 d	15.0	59.3 a	32.3 c
	1.00 MS	80.5 b	13.71 b	16.8	56.5 b	31.1 des

[a]Mean separation within columns by Duncan's multiple range test at 5 % level

metabolite production (Tables 8.11 and 8.12). Feeding of 0.5 MS medium at the end of 2nd week of culturing was found beneficial and was responsible for the highest biomass accumulation, i.e., the final fresh and dry biomasses were 83.1 and 14.76 g L^{-1}, respectively and growth ratio was 18.2 (Table 8.11). Medium feeding strategy was also helpful in the accumulation of caftaric acid (5.76 mg g^{-1} DW) and cichoric acid (26.12 mg g^{-1} DW). In *Panax notoginseng* cell cultures, carbon and nitrogen feeding enhanced cell growth, accumulation of ginseng saponin and polysaccharides [26]. The feeding of sucrose or sucrose combined with casein hydrolysate to basal medium during the growth phase (day 14) effectively sustained ginseng cell growth and increased the biomass growth index by 50–60 % over that of the control [24]. Therefore, the strategy of medium optimization and replenishment can be followed for improvement of biomass and metabolite accumulation.

Table 8.12 Effect of media replenishment on the productivity of caffeic acid derivatives from adventitious roots of *E. purpurea* after 5 weeks of culture in 5 L balloon type bubble bioreactors containing 4 L half strength MS medium

Strength of medium and replenishment schedule		Amount of caffeic acid derivatives (mg g^{-1} DW)[a]			
		Caftaric acid	Chlorogenic acid	Cichoric acid	Total[b]
Control		4.67 ± 0.08	5.73 ± 0.11	25.68 ± 0.06	36.07 ± 0.13
After 2 weeks initial culture	0.25 MS	5.88 ± 0.01	4.60 ± 0.23	27.24 ± 0.04	37.72 ± 0.18
	0.50 MS	5.76 ± 0.01	4.26 ± 0.06	26.12 ± 0.05	36.15 ± 0.02
	0.75 MS	5.59 ± 0.08	4.03 ± 0.05	25.99 ± 0.06	35.61 ± 0.07
	1.00 Ms	4.87 ± 0.08	3.83 ± 0.01	24.30 ± 0.11	33.04 ± 0.18
After 3 weeks initial culture	0.25 MS	6.98 ± 0.02	4.93 ± 0.01	26.11 ± 0.02	38.07 ± 0.02
	0.50 MS	6.14 ± 0.08	5.10 ± 0.10	25.18 ± 0.01	36.52 ± 0.18
	0.75 MS	5.43 ± 0.01	4.73 ± 0.26	24.78 ± 0.08	34.94 ± 0.10
	1.00 MS	5.16 ± 0.02	4.61 ± 0.04	19.08 ± 0.47	28.84 ± 0.45

[a]Mean values of three replicates ± standard error
[b]Total caffeic acid derivatives = caftaric acid + chlorogenic acid + cichoric acid

Table 8.13 Effect of elicitation with NO produced (SNP) on root growth, production of phenolics and flavonoids in the adventitious roots of *E. purpurea* after 5 weeks of culture in 5 L bioreactor containing 4 L half strength MS medium

SNP supply (μM)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio	Total phenolics (mg g^{-1} DW)	Total flavonoids (mg g^{-1} DW)
Control	70.1 a[a]	11.17 b	13.5	57.9 b	37.3 b
50	70.4 a	11.33 bc	13.7	57.4 b	37.4 b
100	69.8 a	11.31 b	13.7	61.1 a	39.9 c
250	69.4 a	11.99 a	14.6	53.6 c	35.6 a

[a]Mean separation within columns by Duncan's multiple range test at 5 % level

8.1.6 Elicitation

Wu et al. [22] investigated the involvement of nitric oxide elicitation in the synthesis of caffeic acid derivatives in the adventitious root cultures of *E. purpurea.* When roots were treated with (0, 50, 100, or 250 μM) sodium nitroprusside (SNP), an exogenous nitric oxide producer, the accumulation of phenolics, flavonoids and caffeic acid derivatives was enhanced (Tables 8.13 and 8.14). Elicitation with 100 μM was found suitable for production of metabolites, whereas higher concentration caused a decline in their accumulation. Wu et al. [22] have interpreted that boosting of metabolites due to 100 μM SNP treatment might be due to over production of phenylalanine ammonia-lyase (a key enzyme in secondary metabolite synthesis), whereas increased concentrations of SNP were responsible for severe stress that developed due to an over-accumulation of hydrogen peroxide (Fig. 8.6a). This was consequently manifested as damage to the membrane lipids (i.e. lipid peroxidation; Fig. 8.6b).

Table 8.14 Effect of elicitation with NO produced (SNP) on the accumulation of caffeic acid derivative in the adventitious roots of *E. purpurea* after 5 weeks of culture in 5 L bioreactor containing 4 L of half strength MS medium

	Caffeic acid derivatives (mg g^{-1} DW)[a]			
SNP supply (μM)	Caftaric acid	Chlorogenic acid	Cichoric acid	Total[b]
Control	3.22 ± 0.03 c	4.73 ± 0.12 ab	27.59 ± 0.16 c	35.53 ± 0.07 c
50	3.47 ± 0.02 b	4.53 ± 0.02 b	28.82 ± 0.54 c	36.82 ± 0.58 c
100	3.71 ± 0.03 a	4.95 ± 0.04 a	34.89 ± 0.32 a	43.55 ± 0.31 a
250	3.68 ± 0.04 a	4.60 ± 0.07 b	33.64 ± 0.44 b	41.93 ± 0.54 b

[a]Mean values of three replicates ± standard error
[b]Total caffeic acid derivatives = caftaric acid + chlorogenic acid + cichoric acid

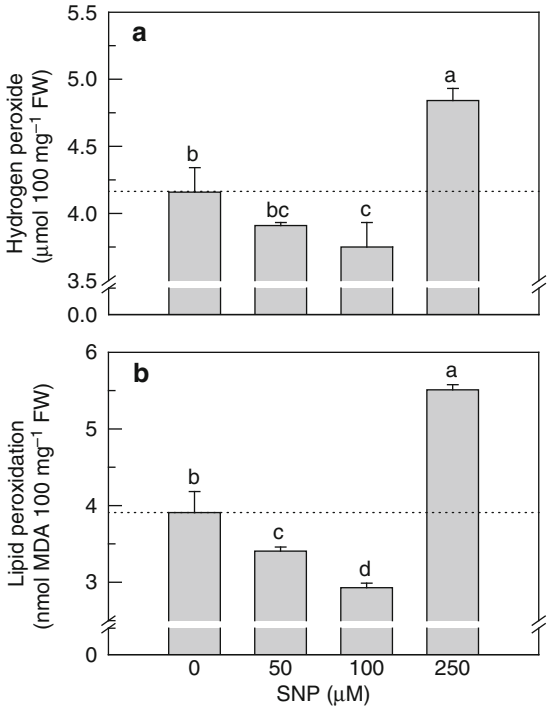

Fig. 8.6 Hydrogen peroxide (**a**) and lipid peroxidation (**b**) in the adventitious roots of *E. purpurea* as affected by NO elicitation after 5 weeks of culture

8.2 Scale-Up Cultures

8.2.1 Adventitious Root Culture in 20 L Bioreactors

Wu et al. [23] have established large-scale bioreactor cultures using 20 L balloon-type bioreactors for the production of *E. purpurea* adventitious roots and caffeic acid derivatives (Fig. 8.7a). They found that dry biomass of *E. purpurea* adventitious roots increased slowly from the day 10 and reached a peak of 11 g L^{-1} on the

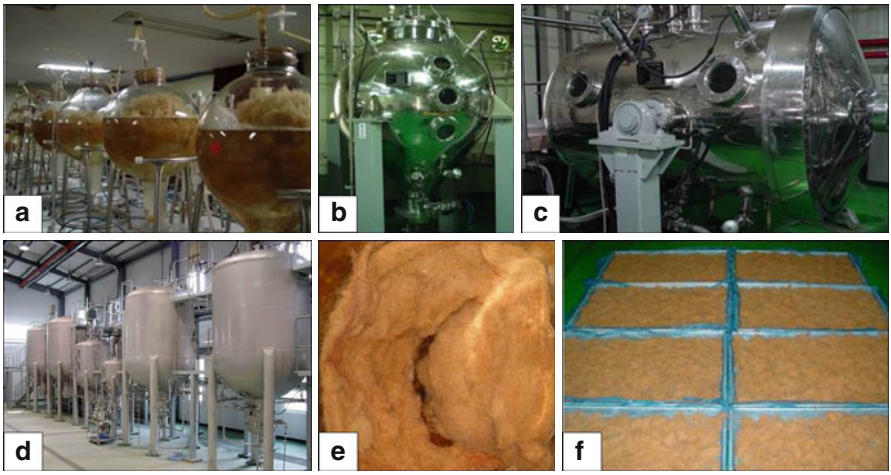

Fig. 8.7 Cultivation of adventitious roots in airlift bioreactors: Adventitious roots of *Echinacea purpurea* in 20 L airlift bioreactor (**a**), 500 L balloon type bubble bioreactor (**b**), 500 L horizontal drum type bioreactor (**c**), and 10,000 L vertical bioreactors (**d**). Adventitious root biomass harvested from bioreactor (**e** and **f**)

60th day. A 15-fold increment in total dry biomass was evident when compared with dry biomass (0.7 g L^{-1}) of initial inoculum. The total phenolics and flavonoids increased quickly from 10th day onwards and reached a maximum value of 57 and 34 mg g^{-1} DW respectively on the 50th day.

8.2.2 Pilot Scale Bioreactor Cultures

Pilot-scale cultivation of *E. purpurea* was evaluated by Wu et al. [23] and it was found that about 26 and 40 kg fresh and 3.6 and 5.1 kg dry biomass of adventitious roots could be achieved in 500 L and 1,000 L airlift bioreactors respectively after 50 days of culture (Table 8.15; Fig. 8.7b–d). Adventitious root biomass grown in pilot scale bioreactors (Fig. 8.7e–f) were also efficient in accumulation of caffeic acid derivatives and the contents of total caffeic acid were 27 mg g^{-1} DW and 31 mg L^{-1} DW respectively with adventitious roots grown in 500 L balloon type bubble bioreactor and 1,000 L drum bioreactor (Table 8.15). Among the different caffeic acid derivatives, cichoric acid content was higher (20.1 and 22.5 mg^{-1} DW) when compared to caftaric acid (2.8 and 3.9 mg^{-1} DW) and chlorogenic acid (4.4 and 4.9 mg^{-1} DW). During the scale up of plant cell and organ cultures, a decrease in the productivity may occur as confirmed by Kwok and Doron [5] and Scragg et al. [12], however, the scale-up of adventitious root cultures of *E. purpurea* did not show any decrease either in biomass production or in caffeic acid productivity.

Table 8.15 Growth and productivity of adventitious roots of *E. purpurea* in different capacity airlift bioreactors cultured in half strength MS medium supplemented with 2 mg L^{-1} IBA and 50 g L^{-1} sucrose

Bioreactor type and volume	Fresh weight (kg)	Dry weight (kg)	Caffeic acid derivatives (mg g^{-1} DW)[a]			
			Caftaric acid	Chlorogenic acid	Cichoric acid	Total[b]
20 L balloon	1.2 ± 0.1	0.2 ± 0.1	4.4 ± 0.5	5.4 ± 0.2	28.0 ± 1.5	37.9 ± 1.2
500 L balloon	26.3 ± 0.5	3.6 ± 0.1	2.8 ± 0.1	4.4 ± 0.4	20.1 ± 0.8	27.4 ± 0.5
1,000 L drum	40.5 ± 0.5	5.1 ± 0.1	3.9 ± 0.1	4.9 ± 0.1	22.5 ± 0.6	31.5 ± 0.6

[a]Mean values of three replicates ± standard error
[b]Total caffeic acid derivatives = caftaric acid + chlorogenic acid + cichoric acid

Table 8.16 Phenolics, flavonoids and polysaccharide contents in the dried adventitious root extracts of *Echinacea purpurea* from the various types and concentrations of solvents used for heat reflux extraction

Solvent concentration (%.v/v)		Total phenolics (mg g^{-1} DW)	Total flavonoids (mg g^{-1} DW)	Total polysaccharides[a] (mg g^{-1} DW)
Distilled water		25.20 f	12.5 e	52.9 b
Methanol	20	35.8 e	19.3 d	43.4 f
	40	36.4 e	22.9 cd	40.6 g
	60	47.1 b	32.6 a	53.8 a
	80	51.6 a	32.8 a	53.8 a
	100	14.1 g	3.3 f	50.3 c
Ethanol	20	33.7 ed	23.2 c	47.3 ed
	40	38.8 d	27.8 b	49.6 d
	60	52.3 a	32.4 a	49.6 d
	80	43.7 c	29.3 b	46.2 ef
	100	11.6 g	1.5 g	44. f

[a]Mean separation within columns by Duncan's multiple range test at 5 % level

8.3 Extraction of Caffeic Acid Derivatives from Adventitious Root Biomass

Extraction of bioactive compounds is the most important step in utilization of raw materials by the commercial sectors such as pharmaceutical, food and chemical industries. A heat reflux method was applied for the extraction of bioactive compounds including caffeic acid derivatives from powdered roots of *Echinacea purpurea* [19]. Three extraction variables, i.e., type, concentration of solvent (water, 20, 40, 60, 80 and 100 % methanol and ethanol each), extraction temperatures (40, 60, 80 °C) were compared. Results revealed that 60 % ethanol was found suitable for the extraction of the active ingredients (Tables 8.16 and 8.17). Optimum yields of phenolics (52.3 mg g^{-1} DW), flavonoids (32.4 mg g^{-1} DW), polysaccharides (49.6 mg g^{-1} DW), caftaric acid (4.9 mg g^{-1} DW), chlorogenic acid (5.4 mg g^{-1} DW) and cichoric acid (24.6 mg g^{-1} DW) could be achieved with use for 60 % ethanol.

Table 8.17 Contents of caffeic acid derivatives in the dried adventitious root extract of *Echinacea purpurea* as affected by the type and concentration of solvents used for heat reflux extraction

Solvent concentration (%.v/v)		Caffeic acid derivatives (mg g^{-1} DW)[a]		
		Caftaric acid	Chlorogenic acid	Cichoric acid
Distilled water		1.2 ± 0.1	0.7 ± 0.1	0.3 ± 0.1
Methanol	20	4.5 ± 0.1	1.5 ± 0.1	6.7 ± 0.1
	40	4.9 ± 0.1	1.6 ± 0.1	12.9 ± 0.1
	60	4.9 ± 0.1	5.0 ± 0.1	22.7 ± 0.2
	80	4.9 ± 0.1	5.0 ± 0.1	23.9 ± 0.1
	100	0.8 ± 0.1	0.2 ± 0.1	1.0 ± 0.1
Ethanol	20	3.9 ± 0.1	1.9 ± 0.1	6.1 ± 0.1
	40	4.7 ± 0.1	4.8 ± 0.1	21.3 ± 0.1
	60	4.9 ± 0.1	5.4 ± 0.1	24.6 ± 0.1
	80	1.2 ± 0.2	5.0 ± 0.1	16.2 ± 0.1
	100	0.2 ± 0.3	0.2 ± 0.1	0.33 ± 0.1

[a]Mean values of three replicates ± standard error

Table 8.18 Phenolics, flavonoids, and polysaccharides contents in the dried adventitious root extract of *Echinacea purpurea* as affected by the solvent temperature during heat reflux extraction for 2 h

Solvent temperature (°C)	Total phenolics (mg g^{-1} DW)	Total flavonoids (mg g^{-1} DW)	Total polysaccharides (mg g^{-1} DW)
40	52.9 ab[a]	32.3 ab	50.5 b
60	53.4 a	33.1 ab	56.6 a
80	53.1 ab	34.4 a	52.4 b

[a]Mean separation within columns by Duncan's multiple range test at 5 % level

Table 8.19 Content of caffeic acid derivatives in the dried adventitious root extract of *Echinacea purpurea* as affected by the solvent temperature during heat reflux extraction for 2 h

Solvent temperature (°C)	Caffeic acid derivatives (mg g^{-1} DW)[a]		
	Caftaric acid	Chlorogenic acid	Cichoric acid
40	3.4 ± 0.1	52.8 ± 0.4	21.6 ± 0.6
60	4.1 ± 0.1	3.6 ± 0.1	28.8 ± 0.5
80	4.4 ± 0.2	3.7 ± 0.1	0.4 ± 0.2

[a]Mean values of three replicates ± standard error

The extraction temperature is one of the important factors influencing the recovery of the bioactive compounds during heat reflux action [15]. The samples of *E. purpurea* adventitious roots were extracted with 60 % ethanol at 40, 60, and 80 °C for two hours and the results showed that treatment of samples at 60 °C was found suitable for obtaining optimum amounts of bioactive compounds (Tables 8.18 and 8.19).

8.4 Conclusion and Perspectives

Echinacea purpurea L. is one of the important and top selling medicinal plants widely used to alleviate colds, sore throats and other respiratory infection. The Echinacea products (e.g., infusions, tinctures and capsules) are used to stimulate immune system. The major immuno- stimulating properties are attributed to the bioactive compounds including caffeic acid derivatives. Efforts have been made by various groups of scientists to develop *in vitro* culture techniques for the production of caffeic acid derivatives through cell, adventitious and hairy root cultures [4, 6, 8, 9, 14, 16, 17, 20–23]. Successful, adventitious root cultures of *E. purpurea* have been developed and various techniques such as optimization of culture medium, physical parameters and strategies to improve bioactive compounds have been developed. For commercialization of *E. purpurea* adventitious roots, large-scale and pilot scale cultures have been achieved using airlift bioreactors. Efficient extraction methods using heat reflux method have also been developed.

Post-harvest storage of fresh *E. purpurea* adventitious roots and package and practice methods should be developed. Another major hindrance for commercialization of *E. purpurea* products is that the Korean Food drug and Administration (KFDA) considers Echinacea as drug rather than the functional food, however, United States Food Drug Administration (US FDA) recognized Echinacea products as herbal drug as well as functional food. Hence, various products of Echinacea are available in the markets of United States of America.

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Chapter 9
Adventitious Root Culture of *Morinda citrifolia* in Bioreactors for Production of Bioactive Compounds

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Abstract *Morinda citrifolia* (Noni) is one of the most famous oriental medicinal plants, which has been used in folk medicine by Polynesians owing to its anticancer, antibacterial, antiviral, antifungal, antitumor and antialergic effects. People are passionate about Noni because of its effectiveness against diabetes, high blood pressure and many other illnesses. Among diverse constituents of Noni, anthraquinone and its derivatives (rubiadin, alizarin and damnacanthal) have been found to be major components responsible for their biological and pharmacological actions. The increasing global demand for biomass of Noni reflects the issues and crisis created by diminishing renewable resources and increasing consumer populations. Moreover, continuous harvesting from its natural stands for diverse usages and reduced land for cultivation in the world accelerated the deficiency to the establishment of mother plants. As one of alternative approaches, cell and tissue culture has been widely explored for rapid and efficient production of biomass and bioactive compounds. Recently, adventitious root culture of *M. citrifolia* has been established in large-scale air-lift bioreactors in view of its commercial applications. In this chapter, various physiological, engineering parameters, and selection of proper cultivation strategy affecting biomass and bioactive compound production have been discussed. In addition, advances in adventitious root cultures including factors for process scale-up and recent research aiming at maximizing automation of the bioreactor production processes are also highlighted.

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Keywords Adventitious root • Anthraquinone • Bioreactor culture • Elicitation • Rubiadin • Scale-up process • Two-stage culture

Abbreviations

APX	Ascorbate peroxidase
AQ	Anthraquinone
BTBB	Bulb type bubble bioreactor
CAT	Catalase
DPPH	1, 1-diphenyl-2-picrylhydrazyl
G-POD	Guaiacol peroxidase
IBA	Indole-3-butyric acid
Kinetin	6 furfuryladenine
LEDs	Light emitting diodes
MeJa	Methyl jasmonate
MS	Murashige and Skoog
NAA	α-naphthalene acetic acid
PAL	Phenylalanine ammonia lyase
PGR	Plant growth regulator
PPF	Photosynthetic photon flux
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TDZ	Thiadiazuron
vvm	Air volume/culture volume/min
WP	Water potential

9.1 Introduction

Medicinal plants are the inexhaustible source of life saving drugs for majority of the world's population. Secondary metabolites accumulated in the medicinal plants are responsible for various bioactive properties. These compounds are isolated by solvent extraction from the naturally grown whole plants, and are used as pharmaceuticals, nutraceuticals, pigments, food staffs and cosmetics. Biosynthesis of secondary metabolites in plants grown in nature is often restricted to species or genus, or might be activated only during a particular growth and developmental stage, under specific season, or by nutrient availability and environmental stress. Moreover, for medicinal purpose, destruction of plants continuously from their natural stands has caused a major threat to the plant species for their existence. Clearly, the development of an alternative and complimentary method to whole plant cultivation for the stable production of biologically important secondary metabolites is an issue of considerable socioeconomic importance. For these reasons, in the past several decades, a lot of efforts have been made for plant cell culture as an alternative method to whole plant

cultivation for the production of pharmacologically important plant secondary metabolites [1]. However, the production of secondary metabolites by cell culture is not always satisfactory. Because, high water content in cells, foaming and wall growth in culture vessel and unstable production of metabolites are the main obstacles. Therefore, organ cultures such as adventitious root, hairy root, shoot and embryo cultures have gained popularity over the years [2–7].

Researches in plant biotechnological advances have shown that bioreactor cultivation of adventitious root is an attractive and alternative method to the whole plant, cell or hairy root culture for biomass and bioactive compound production. Adventitious roots induced under sterile condition in phytohormone supplemented medium have shown high rate of proliferation, tremendous potentialities of accumulation and stable production of valuable secondary metabolites [8, 9]. Therefore, to overcome the aforementioned problems, bioreactor technology is needed for the cosmic-scale cultivation of adventitious roots as a source of valuable biologically important plant-derived secondary metabolites. Bioreactor culture system provides better advantages than the traditional tissue culture system because the culture condition in a bioreactor can be controlled by online monitoring of important process parameters such as temperature, pH, and concentrations of oxygen and carbon dioxide inside the bioreactor vessel. The nutrient concentration can be optimized and nutrient uptake can also be enhanced by continuous medium agitation. Additionally, production cost and time can be reduced by enhancing cell proliferation and regeneration rates, quality of the product can be controlled, product can be freed from pesticide contamination, and the product can be harvested all year round to meet the increasing global demand [10, 11].

Morinda citrifolia (L.), commercially known as Noni, is a member of the Rubiaceae (coffee family) that has been used in folk remedies by Polynesians for over 2,000 years. It has a broad range of therapeutic effects [12], and contains several medicinally active compounds including polyphenolics, organic acids and alkaloids. Of the phenolic compounds, the most common are anthraquinones (AQ) [12].

The demand for Noni roots and extracts has increased in recent decades. For medicinal purposes, Noni root requires 2–5 years of field cultivation in regions with high temperature and humidity levels [13]. Additionally, Noni is susceptible to attack by a wide array of pests and diseases, and a continuous harvesting from its natural stands has posed a major threat to the established mother plants. Procurement of valuable secondary metabolites from cultivated plants is not always satisfactory [6]. Therefore, *in vitro* cell suspension culture was attempted for production biologically active secondary metabolites including AQ in *Morinda citrifolia* [13–17]. However, high water content, low AQ concentrations in cells, continuous foaming, and wall growth in the bioreactor are all obstacles for large-scale production of *M. citrifolia* in cell suspension cultures [6, 13]. As an alternative approach, we have established an adventitious root culture system of *M. citrifolia* in shake flasks and bioreactors [4, 18–22]. When culture systems are changed from shake flask to bioreactors and scaled up from pilot-scale to industrial levels, additional optimizations are needed. During scale-up, reduced productivity often results from any of several factors (shear stress, oxygen supply, nutrient requirements, gas composition, etc.) which can affect the performance of bioreactor cultures [5, 11, 23, 24]. Therefore,

it is essential to optimize these factors for each plant species and explant types (cell or root) to maximize the production of biomass and target metabolites. This review highlights the advances of adventitious root cultures, metabolite production and scope of commercialization of *M. citrifolia* in bioreactors.

9.1.1 Preparation of Explants

Adventitious roots were induced from 2-month-old young leaves of approximately 10×10 mm size collected from *in vitro*-grown plantlets of *Morinda citrifolia*. *In vitro* plantlets were raised from mature seeds after being sterilized with a 4 % sodium hypochlorite solution for 20 min then soaked in 2 % sodium hypochlorite for 10 min in a laminar hood. They were then washed in sterile distilled water and clipped using a sterilized clipper. Seeds were inoculated in test tubes containing 10 mL Murashige and Skoog (MS) medium without growth regulator [18, 19].

9.1.2 Induction and Proliferation of Adventitious Roots

Auxin Types and Their Concentrations for Induction of Adventitious Roots

The selected leaf explants collected from the *in vitro*-grown plantlets were placed on MS medium supplemented with different concentrations (0.5, 1.0, 2.0, 3.0 and 5.0 mg L^{-1}) of indole-3-butyric acid (IBA), 30 g L^{-1} sucrose, and 2.3 g L^{-1} gelrite in a Petri dish containing 25 mL medium for adventitious root induction. Cultures were maintained in 16-h photoperiod under a 20 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF) of fluorescent and darkness at 25 ± 2 °C for 5 weeks.

IBA and NAA showed differential effects on adventitious root induction from leaf explants of *M. citrifolia*. Under light conditions, numerous conspicuous adventitious roots were induced with lower concentrations of IBA (0.5 and 1.0 mg L^{-1}). With increasing IBA concentrations, complex structures of adventitious roots were made due to profound callusing (Fig. 9.1a). But the incidence of callusing increased with the increasing NAA concentrations compared to that of IBA with similar concentrations. Although numerous adventitious roots were induced by NAA compared to low concentrations of IBA, higher callus formation led to compact structures with adventitious roots. On the other hand, dark environment enhanced callusing instead of adventitious root formation in both IBA and NAA containing media (Fig. 9.1b). The pattern of callus formation showed similar trends with the increment of IBA and NAA concentrations. But the effect on callusing was more pronounced in NAA when compared to similar concentrations of IBA. The formation of adventitious roots was totally absent in the cultures containing higher concentration (5 mg L^{-1}) of NAA [18].

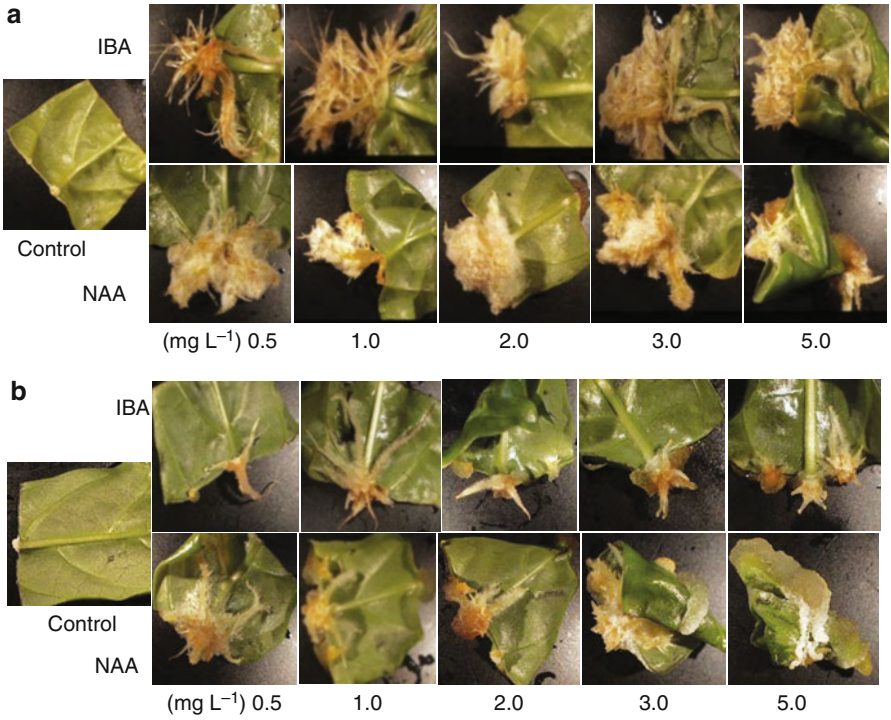

Fig. 9.1 Effects of IBA and NAA on the induction of adventitious root from leaf explants (cultured on MS medium supplemented with 30 g L^{-1} sucrose) of *Morinda citrifolia* under light (**a**) and dark (**b**) conditions after 5 weeks; Control (without hormone)

The study was focused on selecting a callus-free healthy root line that can maintain its sustainability to produce biomass and bioactive compounds. Considering this phenomenon, 1 mg L^{-1} IBA proved to be the best auxin source to induce adventitious roots from leaf explants of *M. citrifolia* [18]. Numerous reports ascribed the involvement of auxin in the initiation of adventitious roots and that division of root initials is dependent upon exogenous or endogenous auxins. IBA and NAA are proved to be more effective than the naturally occurring IAA [25]. On the contrary, *in vitro*-cultured explants may involve in organogenesis and develop shoots or roots depending on the morphogenetic potentiality of the cells. Three distinct stages during organogenesis, namely dedifferentiation, induction of organogenesis pathway and development of organs have been reported [26]. In case of *M. citrifolia*, it has been observed that the leaf explants developed conspicuous protuberances from the cut ends after 3 weeks under light compared to the cut ends under dark. These protuberances develop into adventitious roots directly without the callus phase after another 2 weeks [18].

The triggering process of differentiation and induction pathways depends on specific plant hormones and plant species. For example, 2, 4-dicholorophenoxy-acetic acid (2, 4-D) initiated callus formation, and IBA was responsible for adventitious

root development from the callus in *Panax notoginseng* [27]. In case of *Andrographis paniculata*, NAA proved to be the best auxin to induce adventitious roots from leaf explants directly without the callus phase [28]. The triggering of adventitious root induction from leaf explants of *M. citrifolia* was also observed with 1 mg L^{-1} IBA without the callus phase [18].

Light Quality on Induction Mechanism and Metabolites Content

The selected leaf explants collected from *in vitro*-grown plantlets were placed on a solid MS medium supplemented with 1 mg L^{-1} IBA, 30 g L^{-1} sucrose, and 2.3 g L^{-1} gelrite in a petri dish containing 25 mL medium. Cultures were maintained in a 16 h photoperiod under a 20 µmol m^{-2} s^{-1} PPF for fluorescent, red, blue, red + blue (1:1) and 4.5 µmol m^{-2} s^{-1} PPF for far-red LEDs at 23 ± 2 °C for 5 weeks. Light quality showed significant effects on adventitious root induction (Fig. 9.2). Under red light, numerous adventitious roots were induced (25.60 explants^{-1}), followed by a lesser number under blue light. Fluorescent light ranked third, followed by red + blue. The lowest roots were induced under far-red light (6.20 explants^{-1}). The combinations of red + blue light triggered the callus formation process that led to the compactness of adventitious roots. However, under far-red light, the lack of callus, as well as poor induction with browning of roots, was observed [18].

The organogenesis process with respect to root, callus, or somatic embryogenesis depends markedly on the light source or plant growth regulators. Callus production of *Cydonia oblonga* quince leaves increased by increasing 2, 4-D concentrations, while blue + far-red light reduced callusing response among different light sources [29]. This suggests the involvement of the blue absorbing photoreceptor system in the callus formation process and for root regeneration; phytochrome seemed to be the only photoreceptor involved. In case of wild carrot, callus production increased with the decreasing embryo production under lights other than red and green [30]. In *M. citrifolia*, NAA and dark environment have a marked effect on the callusing process. In contrast, induction of adventitious roots markedly decreased callus formation under fluorescent light, while red + blue or red light alone enhanced the callusing process [18].

Antioxidant enzyme (CAT, G-POD, SOD and APX) activities were varied with different light sources. CAT and G-POD activities were highest under red followed

Fig. 9.2 Effects of light quality on the induction of adventitious root from leaf explants (cultured on MS medium supplemented with 1 mg L^{-1} IBA and 30 g L^{-1} sucrose) of *M. citrifolia* after 5 weeks

by fluorescent light. The lowest activity occurred under the combination of red + blue light. APX activities were highest under fluorescent light, followed by blue light, while the lowest APX activities were observed under far-red light that was much closer to red + blue light. Higher but very close SOD activities were observed under red, red + blue and far-red light, but the lowest under fluorescent and blue light. However, SOD activities were not significantly affected by light sources [18]. The activities of those enzymes were lowest under red + blue and far-red light, which led to the accumulation of higher H_2O_2 in induced roots. However, the joint functions of CAT, G-POD, and APX under fluorescent and blue light mitigate the toxic effects of H_2O_2 by converting it to non-toxic H_2O [18].

Changes in antioxidant activities were greatly affected by light source and the stage of organ development. For example, In Toyonaka strawberry, SOD, CAT and G-POD activities were highest under the red films. These activities were decreased when almost all the calluses ceased to grow [31]. Shohael et al. [32] also reported the stimulated activities of G-POD, CAT, and reduced APX activity under red light in *Eleutherococcus senticosus* somatic embryos. They concluded that higher CAT activity than G-POD under red light was involved in the protection of embryos from stress conditions. In our study, red light stimulated CAT and G-POD activities, but higher APX activity under fluorescent light jointly functions with CAT and G-POD to eliminate the toxic effects of H_2O_2 and triggers the induction of healthy callus-free adventitious roots [18].

Light is an important factor affecting growth, organogenesis and the formation of plant products including both primary and secondary metabolites. Total anthraquinones (AQ), phenolics and flavonoid contents were significantly influenced by different light sources (Table 9.1). Far-red light, followed by red light stimulates higher AQ, phenolics and flavonoid contents in the induced roots. The formation of these secondary metabolites showed similar patterns with moderate content under fluorescent and blue light, while the lowest content was observed under red + blue light. The stimulatory effect of far-red light on secondary metabolite formation in the induced root is associated with oxidative stress due to higher accumulation of H_2O_2 and MDA content. On the other hand, lower secondary product formation under red + blue light might be due to fewer calluses with adventitious roots [18]. It is widely believed that

Table 9.1 Effects of light quality on secondary metabolite contents in induced adventitious roots of *M. citrifolia* after 5 weeks[a, b, c]

Light quality	AQ (mg g^{-1} DW)	Phenolics (mg g^{-1} DW)	Flavonoids (mg g^{-1} DW)
Fluorescent	15.14d	16.38b	10.23b
Red (R)	18.40b	17.75a	12.09a
Blue (B)	15.28c	16.41b	9.81b
R + B	11.04e	13.81c	7.30c
Far-red	23.05a	18.71a	13.24a

Experiments were performed three times

[a]Explants cultured on MS medium supplemented with 1 mg L^{-1} IBA with 30 g L^{-1} sucrose

[b]Each treatment consisted of 20 petridishes and each petridish contained four explants

[c]Mean separation within columns by Duncan's multiple range tests at 5 % level

the synthesis of secondary metabolites in plants is part of the defense response of plants to stress. Oxidation has been associated with the stress of plants [33].

Proliferation of Adventitious Roots

The induced adventitious roots were further proliferated in liquid MS media supplemented with 5 mg L^{-1} IBA, 30 g L^{-1} sucrose and 10 g L^{-1} inoculum. The cultures were agitated at 100 rpm on a gyratory shaker in darkness at 23 ± 2 °C for 4 weeks. The roots were subcultured at every 4 weeks [18, 19].

9.1.3 Establishment of Suspension Cultures in Shake Flask

Effect of Auxin on Root Growth and Metabolite Production

In order to determine the optimum concentrations of plant growth regulators, the adventitious roots were grown under different concentrations of IBA and NAA (1, 3, 5, 7 and 9 mg L^{-1}) in full strength MS medium containing 30 g L^{-1} sucrose and 10 g L^{-1} inoculum in 250 mL Erlenmeyer flasks containing 100 mL liquid medium for 4 weeks. Roots were also grown in a hormone-free medium that served as the control. The fresh weight (FW) and dry weight (DW) of the roots increased with increasing concentrations of both IBA (up to 5 mg L^{-1}) and NAA, and IBA was found to be more efficient in increasing the FW and DW compared to NAA. The maximum FW (54.02 g L^{-1}) and DW (4.48 g L^{-1}) were achieved at 5 mg L^{-1} IBA; the control FW was 6.44 g L^{-1} and control DW was 0.72 g L^{-1} [19].

Previous investigators have suggested that the response of adventitious root growth to growth regulators varies from species to species. In case of *Gymnema* cells, NAA was found to be more effective than IBA [34]. The highest dry mass production from adventitious roots *Echinacea angustifolia* was achieved at 2 mg L^{-1} IBA when compared to NAA [35]. In adventitious root cultures of *Panax ginseng*, IBA was found to be more effective than NAA in promoting the dry mass [36]. In *Karwinskia* root cultures, the highest root dry mass was produced under light on media supplemented with IBA when compared to NAA [37]. The authors have concluded that media enriched with NAA support the formation of callus-like masses with very short roots, resulting in a lower dry mass. On the other hand, IBA seems to be most effective substance for inducing and elongating roots in *Karwinskia* root cultures.

To continue our optimization, we also checked AQ, phenolics and flavonoid production under the same conditions. The maximum content of AQ (63.10 for IBA, 55.60 for NAA mg g^{-1} DW), phenolics (29.46 for IBA, 22.97 for NAA mg g^{-1} DW) and flavonoid (60.23 for IBA, 49.47 for NAA mg g^{-1} DW) were observed in roots cultured at 1 mg L^{-1} IBA and NAA, respectively [19]. High auxin levels are often deleterious to secondary metabolite accumulation [38]. Higher concentrations of

NAA (7 and 9 mg L^{-1}) decreased the secondary metabolite contents at higher rate than those of IBA in our study. This clearly shows that the effect of auxins on secondary product formation markedly depends on the types of auxin used and their concentrations. For example, in case of *M. citrifolia* cell culture, high concentrations of NAA reduced the production of AQ [14, 17]. On the other hand, the production of AQ was induced by NAA and inhibited by 2, 4-D in the same species [39]. They observed that in cultures treated with high concentrations of NAA, concomitant alkalization occurred in the cytoplasm, leading to cell death and subsequent drop in metabolite accumulation. However, 5 mg L^{-1} NAA enhanced the secondary metabolite accumulation when compared to the similar concentrations of IBA in our study. These results clearly suggest that secondary product formation can be separated from growth. Based on the growth of adventitious roots in terms of DW and production of metabolites, we selected 5 mg L^{-1} IBA as a suitable concentration for our further experiments.

Combined Effect of Auxin and Cytokinins on Root Growth and Metabolite Production

In order to elucidate the optimum concentrations of kinetin and TDZ for the production of secondary metabolites, adventitious roots were grown in full-strength MS medium containing 30 g L^{-1} sucrose and 10 g L^{-1} inoculum treated with different concentrations (0.1, 0.3 and 0.5 mg L^{-1}) of kinetin and TDZ after adding 5 mg L^{-1} IBA in shake flask. Roots that were grown in a medium containing only 5 mg L^{-1} IBA served as control. The FW and DW of adventitious roots were significantly suppressed by different concentrations of kinetin and TDZ when supplemented with 5 mg L^{-1} IBA. The maximum FW (55.16 g L^{-1}) and DW (4.88 g L^{-1}) of the roots were obtained at 5 mg L^{-1} IBA (control). The FW and DW of the roots decreased sharply, particularly with increasing TDZ levels [19]. Cytokinin can inhibit root growth, especially under dark conditions. In contrast, the inhibitory effect of kinetin on root growth may be due to a reduction in the auxin content in the root [40]. The fact that the auxin-cytokinin combinations applied decreased root DW in this study may be due to interactions among these growth regulators showing impact on root growth [19].

The combinations of auxin and cytokinins resulted in significant increase in secondary metabolite content, especially when the auxins were combined with different concentrations of TDZ. When increasing concentrations of cytokinins were combined with IBA, the contents of total AQ, phenolics and flavonoids also gradually increased (Table 9.2). This increasing trend was more conspicuous in the culture containing different levels of TDZ compared to those of kinetin. The maximum contents of total AQ (30.90 mg g^{-1} DW), phenolics (29.98 mg g^{-1} DW) and flavonoids (36.61 mg g^{-1} DW) were achieved when 0.5 mg L^{-1} TDZ were combined with the control [19].

Phenolics are considered to be the secondary metabolites that are synthesized in plants through the phenylpropanoid pathway and function as a defense mechanism that reacts to various biotic and abiotic stress conditions [41]. In addition to this,

Table 9.2 Combined effect of auxin and cytokinins on secondary metabolite contents in adventitious roots of *M. citrifolia* after 4 weeks of culture[a, b]

PGR[c]	Concentration (mg L^{-1})	AQ (mg g^{-1} DW)	Phenolics (mg g^{-1} DW)	Flavonoids (mg g^{-1} DW)
Control (IBA)	5.0	13.16 ± 0.01	17.89 ± 0.75	14.27 ± 0.49
Control + Kinetin	0.1	13.47 ± 0.01	20.14 ± 0.13	15.55 ± 0.24
	0.3	17.07 ± 0.01	21.83 ± 0.02	18.40 ± 0.99
	0.5	15.02 ± 0.02	20.35 ± 0.24	16.91 ± 0.76
Control + TDZ	0.1	20.57 ± 0.02	27.35 ± 0.61	23.54 ± 0.93
	0.3	27.78 ± 0.03	29.57 ± 0.76	33.05 ± 0.50
	0.5	30.90 ± 0.40	29.98 ± 0.20	36.61 ± 0.61

[a]Adventitious roots were cultured in full strength MS medium containing 30 g sucrose L^{-1} and 10 g inoculum L^{-1}

[b]Values are of three replicates along with standard error

[c]*PGR* plant growth regulator

oxidative stress also plays an important role in the production of secondary metabolites in plants. Ali et al. [42, 43] showed that H_2O_2 and O_2^- induced ginsenoside content in adventitious roots of *Panax ginseng*. TDZ, a substituted urea compound, is also known to be a synthetic growth regulator that acts by modulating endogenous plant growth regulators, either directly or as a result of stress induction such as callus induction, induction of defoliation and plantlet differentiation [44].

The accumulation of AQ was increased by about 134 % in TDZ-treated roots and by 29 % in kinetin-treated roots when compared to the control. At 0.5 mg L^{-1} TDZ, the H_2O_2 content increased about fourfold compared to the control, and at the same concentration of TDZ, the AQ, phenolics and flavonoids contents also increased [19]. A similar induction of AQ content was also noted with increasing H_2O_2 levels in *M. eliptica* cell cultures [15]. H_2O_2 is known to be the signal that induces antioxidant defense systems in plants in response to biotic and abiotic stresses [45]. This suggests that the TDZ-induced H_2O_2 accumulation observed in our study may play a significant role in the production of AQ [19].

Effect of Medium Salt Strength on Adventitious Root Growth and Metabolite Production

In an attempt to improve root growth and secondary metabolite production, 15 g L^{-1} (FW) adventitious roots of *M. citrifolia* were cultured in different strengths (0.25, 0.50, 0.75, 1.0, 1.5, and 2.0) of MS medium supplemented with 5 mg L^{-1} IBA and 30 g L^{-1} sucrose [18, 19]. Medium salt strength significantly influenced the growth and secondary metabolite content in adventitious root cultures of *M. citrifolia* [20]. A gradual decrease of FW and DW, as well as decrease of % dry weight and growth ratio was observed with the increasing medium salt strength (Table 9.3). The highest FW (53.25 g L^{-1}) and DW (5.18 g L^{-1}) weight of roots, maximum % of DW (9.71) and growth ratio (3.87) were achieved at 0.25 strength MS medium. High (1.5, 2.0 MS) salt

Table 9.3 Effects of MS salt strength on growth of adventitious roots of *M. citrifolia* after 4 weeks of culture[a]

MS salt strength	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	% dry weight	Growth ratio
0.25	53.25a	5.18a	9.71a	3.87a
0.5	50.88ab	4.05b	7.96b	3.03b
0.75	49.35ab	3.98bc	8.09b	2.97bc
1.0	48.45ab	3.83bc	7.93b	2.86bc
1.5	45.48b	3.55bc	7.84b	2.66bc
2.0	37.75c	3.33c	8.81ab	2.49c

Mean separation within columns by Duncan's multiple range test at 5 % level
[a]Adventitious roots were cultured in MS medium supplemented with 5 mg L^{-1} IBA, 15 g L^{-1} inoculum size and 30 g L^{-1} sucrose using 250 mL conical flask containing 100 mL medium

Table 9.4 Effect of MS salt strengths on secondary metabolite production from adventitious roots of *M. citrifolia* after 4 weeks of culture[a]

	AQ		Phenolics		Flavonoids	
MS salt strength	Contents (mg g^{-1} DW)	Yield[b] (mg L^{-1} DW)	Contents (mg g^{-1} DW)	Yield (mg L^{-1} DW)	Contents (mg g^{-1} DW)	Yield (mg L^{-1} DW)
0.25	20.41a	105.75a	17.15 cd	88.84a	14.28a	73.96a
0.50	15.98c	64.70c	17.22 cd	69.76c	11.55c	46.80b
0.75	14.92d	59.38e	18.02 cd	71.72c	10.38d	41.30c
1.0	16.04bc	61.45d	18.82c	72.10c	10.48d	40.01c
1.5	16.14b	57.30f	20.82b	73.92c	10.97 cd	38.93c
2.0	20.48a	68.18b	24.45a	81.41b	12.55b	41.80c

Mean separation within columns by Duncan's multiple range test at 5 % level
[a]Adventitious roots were cultured in MS medium supplemented with 5 mg L^{-1} IBA, 15 g L^{-1} inoculum size and 30 g L^{-1} sucrose using 250 mL conical flask containing 100 mL medium
[b]Yield = Dry weight (g L^{-1}) * content (mg g^{-1} dw)

strength inhibited root growth as evidenced from the decreased fresh and dry root biomass and growth ratio when compared to those recorded at low salt strength (0.25 MS). The best media for the production of total AQ, phenolics and flavonoids were 2.0 and 0.25 strength MS in terms of contents and yield, respectively (Table 9.4). Except 2.0 strength MS medium, the secondary metabolite content showed increasing trend with the increasing medium salt strength. However, the highest overall yields of total AQ, phenolics and flavonoids were recorded at 0.25 strength MS treated cultures as manifested in the higher accumulation of root dry mass and secondary metabolites [20]. These results imply that the culture of *M. citrifolia* adventitious root requires low levels of medium salt strength for the production of root dry mass and bioactive compounds. The results of our present study are inconsistent with the findings of Wu et al. [35] where 0.25 and 0.50 MS salt strength showed better performance to enhance dry biomass and secondary metabolite accumulation in adventitious root cultures of *Echinacea angustifolia*. The authors concluded that suitable interactions among the nutrients in low salt strength treated culture enhanced the availability of ions to the roots.

The appropriate concentration of medium constituents is crucial for the growth of isolated plant cells and organs. In contrast, the optimal concentration of macro and microelement also play a pivotal role to enhance the rate of organ development, morphology and secondary metabolite accumulation [10]. In *Panax ginseng* adventitious root cultures, both half and full strength media were suitable for root dry mass production whereas maximal secondary metabolite production was achieved with a full strength MS medium [46]. Sivakumar et al. [10] also observed that half strength MS medium ensured the highest ginsenoside content and yield in adventitious roots of *Panax ginseng* cultured in bioreactors, whereas 2.0 strength MS medium inhibited root growth instead led to high ginsenoside content but low yield. In *Bupleurum falcatum* adventitious root culture, a full-strength MS medium was found suitable for both root development and saikosaponin production [47].

Electrical conductivity (EC) value reflects the uptake of medium salts (ions) by the cells, so the conductivity measurements have been used as an indirect method of biomass estimation [48]. In our study, EC value progressively increased with the increasing medium salt strength. High EC value was observed in the residual media containing high salt strength due to lower uptake of ions by the roots. After 4 weeks of culture, high amounts of cations (Na^+, NH_4^+, K^+ and Ca^2) and anions (Cl^-, NO_3^-, PO_4^{3-} and SO_4^{2-}) were observed in the residual media treated with high salt strength. Meanwhile, adventitious roots cultured at 0.25 strength medium efficiently utilized almost all the cations and anions that led to decrease in EC value. However, the contents of cations and anions varied differently. For instance, in case of nitrogen sources like NH_4^+ and NO_3^-, the adventitious roots consumed preferably NH_4^+. PO_4^{3-} was totally exhausted from the media after 4 weeks in the cultures containing low salt strength (0.25–0.75 MS) while K^+ and NO_3^- were not crucial for the growth of *M. citrifolia* adventitious roots in this study [20]. These results indicate that *M. citrifolia* adventitious root cultures require low salt strength containing media as well as NH_4^+ and PO_4^{3-} regarded as the key elements for intensive growth and secondary metabolite production [20]. The requirement of nutrient elements varied with plant species according to their secondary metabolism. For example, in *Panax ginseng* adventitious root culture, NH_4^+ was proven as a key element while Na^+ and K^+ were not necessary for the root growth and ginsenoside production [10].

Water potential (WP), the chemical potential of water in the system was decreased with the increasing medium salt strength. Higher salt strength (1.5 and 2 MS) resulted in a more negative WP values caused by high concentration of ions in the residual media which indicated that ions were not properly utilized by the roots. In contrast, negative WP induced water deficit condition which negatively affected water uptake by the roots from their surrounding media. Therefore, root growth was strongly inhibited at high salt strength treated cultures [20]. It could be hypothesized that salt stress may damage plants due to a combination of causes, including mainly osmotic injury and specific ion toxicity [49] that affect a wide variety of physiological and metabolic processes in plants [50].

Proline is an important component of salt-stress induced responses in plants. The content of free proline was markedly increased with the increasing medium salt strength (Fig. 9.3a). More than twofold increase in free proline content was recorded

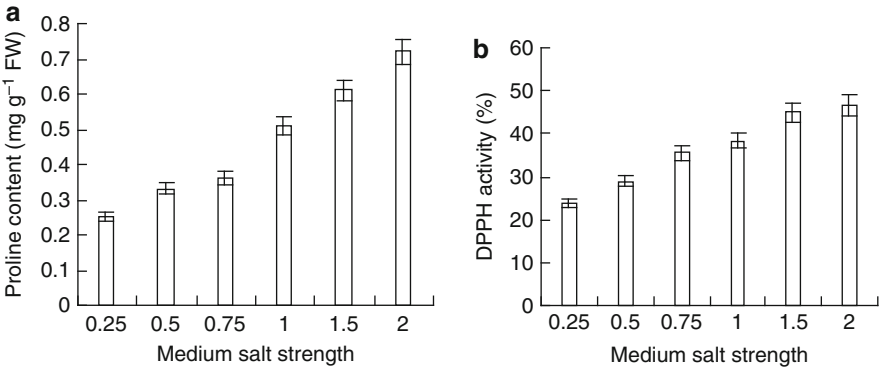

Fig. 9.3 Contents of free proline (**a**) and DPPH radical scavenging activity (**b**) in adventitious roots of *M. citrifolia* as affected by medium salt strength after 4 weeks. *Bars* represent means ± S.E. (n = 4)

in roots grown under higher salt strength compared to lower salt strength treated cultures [20]. Proline accumulation has been frequently observed in salt-stressed plants. Lutts et al. [51] observed an increase in proline accumulation in rice cultivars caused by salinity stress. In *Spathiphyllum* leaves, higher proline accumulation was also observed when plantlets were grown under higher EC levels [52]. Most of the attempts to account for the phenomenon have focused on the ability of proline to mediate osmotic adjustment, to stabilize sub-cellular structures and scavenge free radicals. On the other hand, proline accumulation may reduce stress-induced cellular acidification. The increased $NADP^+$/NADPH ratio mediated by proline biosynthesis is likely to enhance activity of the oxidative pentose phosphate pathway to support the demand for increased secondary metabolite production under stress [53]. Presumably, higher proline accumulation induced secondary metabolite content at high salt strength treated roots in this study.

1, 1-diphenyl-2-picrylhydrazyl (DPPH) activity is a proper indicator for investigating the free radical scavenging activities of phenolic compounds [54]. In our current study, DPPH radical scavenging activity was significantly elevated with the increasing medium salt strength (Fig. 9.3b). More than two-fold increase in DPPH radical scavenging activity was recorded in the culture treated with higher (2 MS) salt strength compared to lower (0.25 MS) salt strength. In contrast, a positive correlation was observed between DPPH radical scavenging activity and accumulation of phenolic compounds in roots relation to as increased medium salt strength. Higher salt strength resulted in a significant proline accumulation in roots which might be responsible for enhancing DPPH radical scavenging activity in this study [20].

Medium salt strength significantly influenced Phenylalanine ammonia lyase (PAL) enzyme activity (Fig. 9.4a). A gradual increase of PAL activity was observed as medium salt strength increased. A more than twofold PAL activity was induced in roots cultured at two strength MS medium when compared to the roots cultured at 0.25 strength MS medium [20]. Phenolic compounds are considered as secondary metabolites that are synthesized in plants and help in defense mechanism in response

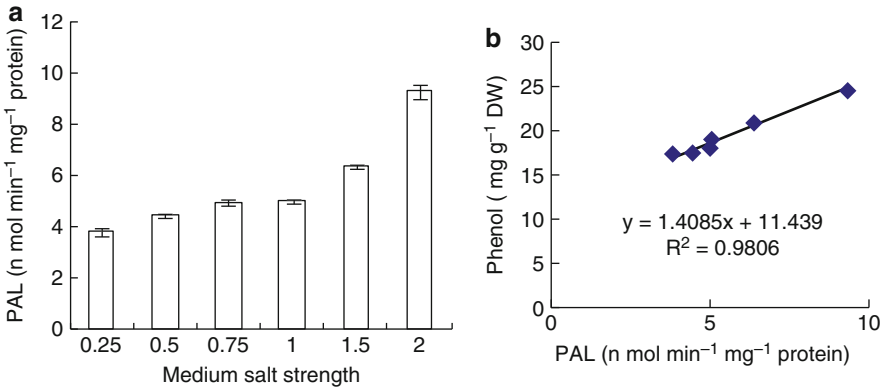

Fig. 9.4 Activities of phenylalanine ammonia lyase (PAL) (**a**) and relationship between PAL and phenol synthesis (**b**) in adventitious roots of *M. citrifolia* as affected by medium salt strength after 4 weeks. *Bars* represent means ± S.E. (n = 4)

to various stress conditions [55]. Most natural phenolic compounds in plants are derived from *trans*-cinnamic acid formed by deamination of L-phenylalanine by L-phenylalanine ammonia-lyase (PAL) [56]. We observed stimulatory effects of medium salt strength on PAL activity (Fig. 9.4a) in relation to accumulation of phenolic compound (Fig. 9.4b) that lead to an investigation of the role of a key regulatory enzyme in phenol synthesis [20]. PAL plays a pivotal role in phenol synthesis and many of reports emphasized the correlation between increase in the corresponding PAL gene expression/activity and increases in phenolic compounds in response to different stimuli [56]. A strong and positive correlation ($r^2 = 0.9806$) between PAL and phenol synthesis was observed at different salt strength treated roots in our study. In root suspension cultures of *Panax ginseng*, similar phenomenon was also reported by Ali et al. [43] in response to copper stress. It is worth to be mentioned here that an increase in phenol synthesis provoked by salt stress is not the aim of the current study. But the primary aim was to optimize culture conditions by revealing the suitable salt strength and thus to enhance biomass accumulation as well as AQ formation at the expense of phenol synthesis. We observed that phenol synthesis was positively correlated with PAL activity (Fig. 9.4b), and that reduction of PAL activity triggers AQ biosynthesis in relation to medium salt strength. Considering these phenomena, 0.25 strength MS medium was proven as the best salt strength to enhance biomass accumulation and AQ biosynthesis in root suspension cultures of *M. citrifolia* [20].

Effect of Sucrose on Adventitious Root Growth and Metabolite Production

To discern suitable sucrose concentration for enhancing root growth and metabolites, 15 g L^{-1} inoculum (FW) was inoculated in one-fourth strength MS medium supplemented with 5 mg L^{-1} IBA [19, 20] and different concentrations (0, 1, 3, 5, 7

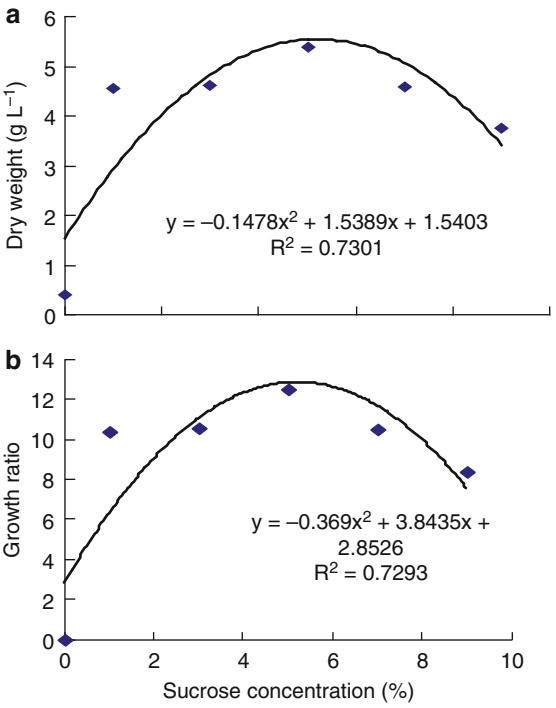

Fig. 9.5 Relationship between root dry weight and sucrose concentration (**a**), growth ratio and sucrose concentration (**b**) of *M. citrifolia* adventitious roots after 4 weeks

and 9 %) of sucrose [21]. Adventitious root cultures were agitated in 100 rpm on a gyratory shaker under darkness at 23 ± 2 °C for 4 weeks. A differential and significant effect of sucrose concentration on biomass accumulation and metabolite production was observed in this study with different concentrations of sucrose [21]. Figure 9.5a, b show the positive polynomial relationship among root dry weight (R^2 = 0.7301) and growth ratio (R^2 = 0.7293) with different sucrose concentrations, indicating root dry weight and growth ratio increased up to a certain concentration (5 %) then decreased with increasing sucrose concentration. The maximum root dry weight (5.40 g L^{-1}) and growth ratio (12.50) were observed in the culture treated with 5 % (w/v) sucrose, while higher concentrations of sucrose (7 and 9 % in w/v) decreased root dry weight and growth ratio. These results suggest that the biomass growth was repressed by relatively higher initial sucrose concentrations (7 and 9 %), which might be due to a relatively higher osmotic pressure in the cultured roots. Sucrose is considered as an important carbon and energy source in plant cell and tissue culture because; initial concentration can affect growth and the yield of secondary metabolites. On the contrary, higher amount of sucrose can retard the development of cultured cells [35] by causing a cessation of the cell cycle when nutrients are limited [57]. In cell cultures of *Coleus blumei*, a high initial sucrose concentration (6 %) led to a higher biomass accumulation without an obvious lag phase [58]. Similar phenomenon was also observed in our current study with up to 5 % sucrose without showing a lag phase. With the increasing initial sucrose

Table 9.5 Effects of sucrose concentration on secondary metabolite production in adventitious roots of *M. citrifolia* after 4 weeks of culture

Sucrose concentration (%)	Yield of AQ (mg L^{-1} DW)	Yield of phenolics (mg L^{-1} DW)	Yield of flavonoids (mg L^{-1} DW)
0	7.73f	10.52f	4.74e
1	251.89a	165.14a	163.56a
3	173.08d	115.90d	113.26c
5	213.55b	151.20b	139.94b
7	208.94c	135.39c	134.07b
9	114.64e	99.46e	74.67d

Mean separation within columns by Duncan's multiple range test at 5 % level
Adventitious roots were cultured in quarter-strength MS medium supplemented with 5 mg L^{-1} IBA and 15 g L^{-1} inoculum size using 250 mL conical flask containing 100 mL liquid medium
Yield = Dry weight (g L^{-1}) x content (mg g^{-1} DW)

concentration up to 5 % (w/v), growth ratio increased significantly. The increased biomass accumulation with 7 % sucrose was reported in root suspension cultures of *Echinacea angustifolia* [35] and with 5 % in *Panax ginseng* [46]. These results suggest that initial sucrose concentration in the culture media is important for the growth of plant roots and its effect is dependent on the specific plant species.

The trajectory of AQ, phenolics and flavonoids content in adventitious roots of *M. citrifolia* was significantly affected by the initial sucrose concentration. During 4 weeks of culture period, it was observed that AQ, phenol and flavonoid contents progressively increased after 3 weeks with 1 % sucrose followed by 7 %, 5 % and followed by 3 % sucrose. Higher sucrose concentration (9 %) or adventitious roots cultured in the absence of sucrose decreased the secondary metabolite contents [21]. Although, root dry weight increased at 5 % sucrose treated culture, the highest production of AQ (251.89 mg L^{-1} DW), phenolics (165.14 mg L^{-1} DW) and flavonoids (163.56 mg L^{-1} DW) were observed at 1 % sucrose treated culture (Table 9.5). The increased metabolite production at 1 % sucrose was accompanied with higher contents of AQ, phenolics and flavonoids in this treatment compared to others. Polysaccharide contents showed increasing trend with increasing sucrose concentration in the culture media up to 7 %. Polysaccharide content was the greatest at 7 % sucrose followed by 5 % sucrose, while higher sucrose concentration (9 %) decreased polysaccharide content and was comparable with 3 % sucrose treated culture [21]. Studies have shown that initial sucrose concentration can affect *in vitro* secondary metabolite production and it varied with species to species. Zhong et al. [59] have found that 4.5 % sucrose is the best for the production of anthocyanin in suspended cultures of *Perilla frutescens* cells. It has been reported that an increase in initial sucrose concentration above the normal level (2–3 %) increased saponin and polysaccharide content in cell cultures of *Panax ginseng* [60]. Higher sucrose concentration (5 %) enhanced the accumulation of phenolics, flavonoids and chlorogenic acid also reported in root suspension cultures of *Echinacea angustifolia* [35], and embryogenic cultures of *Eleutherococcus sessiliflorus* [32]. They concluded that increased metabolite production might be due to elevated levels of

osmotic stress. However, in our present study, lower sucrose concentration (1 %) activated AQ, phenolics and flavonoids in terms of contents and production. In contrast, polysaccharide contents enhanced with higher (7 %) sucrose treatment [21].

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) activity is a proper indicator for investigating the free radical scavenging activities of phenolic compounds [54]. The profile of DPPH radical scavenging activity in roots of *M. citrifolia* showed a significant variation with different concentrations of sucrose treatment. DPPH free radical scavenging activity observed relatively higher during the initial culture period (after 1 week) and gradually decreased with the increasing culture time in all the treatments. In contrast, adventitious roots cultured in the medium without sucrose or lower initial sucrose concentration (1–3 %) induced higher DPPH radical scavenging activity up to 3 weeks when compared to all the sucrose treated cultures. After 3 weeks of culture, it was observed that DPPH activity was not influenced significantly by the presence or absence of sucrose in the medium [21]. These results suggest that adventitious roots of *M. citrifolia* cultured at lower initial sucrose concentration or without sucrose induced DPPH radical scavenging activity during the initial culture period (1–2 week). Such elevated DPPH radical scavenging activities are considered beneficial for bioactive compound production during the later stage of culture period.

Sucrose, glucose and fructose are the most common sugars existing in all plants, and can be easily transported into plant cells, readily converted to each other and normally metabolized through glycolysis [61]. The hydrolysis of sucrose to glucose and fructose is a rapid process and occurred *via* external and internal invertases [62]. Sugar concentrations in the residual media were measured at 1 week intervals during 4 weeks of culture period to determine changes of the pattern of soluble sugar content (sucrose, glucose and fructose). After 1 week of culture, sucrose concentrations showed a sharp-drop and concentration was almost zero at the culture treated with 1 % sucrose (Fig. 9.6a). Similarly, almost all the sucrose was exhausted from the media after 2, 3 and 4 weeks in the cultures which were initially supplemented with 3, 5 and 7 % sucrose, respectively. Whereas less than 1 % sucrose was detected after 4 weeks in the culture media which were supplemented initially with 9 % sucrose. The opposite phenomenon was observed in case of glucose and fructose. The concentration of glucose in the culture media progressively increased up to 3 weeks of culture period thereafter decreased in all the treatments except in the cultures treated with 7 and 9 % sucrose (Fig. 9.6b). The concentration of fructose increased obviously up to 4 weeks in all the treatments except in the culture initially supplemented with 3, 5 % sucrose (Fig. 9.6c). Glucose and fructose, however, exhausted after 4 weeks from the culture media initially treated with 1 % sucrose. Although only sucrose was added in the culture medium, glucose and fructose were also detected [21]. These findings suggest that extracellular hydrolysis of sucrose occurred, which leads to the formation of glucose and fructose. This hydrolysis might be due to invertase which might have been secreted from adventitious root tissue into the medium or on the surface of epidermal cells of the tissue [63, 64]. Thus, the assay results of soluble sugar content reveal that supplementation of 1 % sucrose as an initial carbon source is optimal for enhancing secondary metabolite production from adventitious roots of *M. citrifolia*.

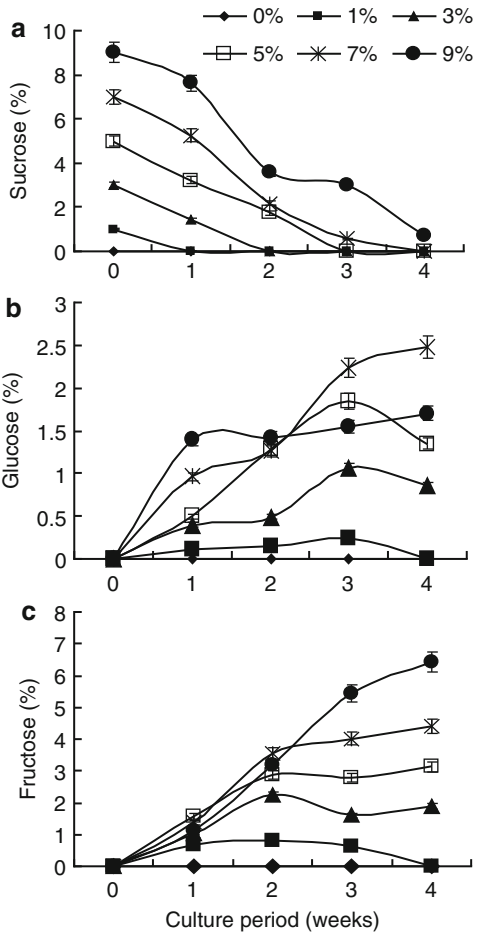

Fig. 9.6 Changes of soluble sugar contents; sucrose (**a**), glucose (**b**) and fructose (**c**) in the exhausted medium during 4 weeks of culture period in shake flask as affected by sucrose concentrations. *Bars* represent means ± S.E. (n = 4)

9.2 Establishment of Adventitious Root Suspension Cultures in Airlift Bioreactors

9.2.1 Optimization of Culture Conditions in Bioreactor

To determine the optimal aeration rate for biomass and bioactive compound production, adventitious roots were cultured in 3 L BTBBs (Fig. 9.7) containing 1.5 L of quarter-strength (0.25×) liquid MS medium supplemented with 5 mg L^{-1} IBA, 15 g L^{-1} inoculum size, and 10 g L^{-1} sucrose [19–21]. Cultures were agitated at 0.05, 0.1, 0.2, or 0.3 vvm (air volume / culture volume / min), or with gradual increase in the aeration rate at 1-week intervals (steps of 0.05, 0.1, 0.2, and 0.3 vvm). After the optimized aeration rate was determined, the effects of different inoculum densities (5, 10, 15, 20, and 30 g L^{-1} fresh roots) and strengths of MS

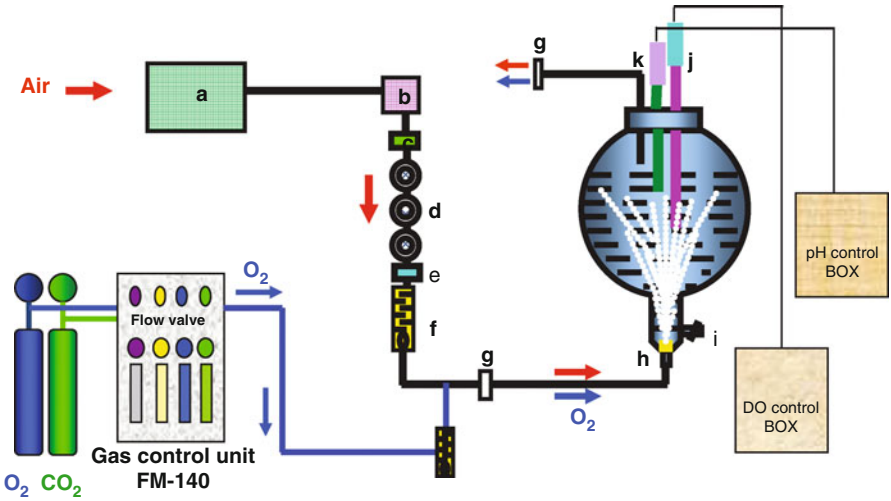

Fig. 9.7 Schematic diagram of an air-lift bioreactor. *a* air compressor, *b* air reservoir, *c* air after cooler, *d* air filter system, *e* air dryer, *f* air flow meter, *g* membrane filter, *h* glass sparger, *i* medium feeding port, *j* DO sensor, *k* pH sensor

medium (0.25×, 0.50×, 0.75×, 1.0×, and 1.5×) on biomass and bioactive compound production were tested. All cultures were maintained at 23 ± 2 °C in the dark for 4 weeks.

Effects of Air Supply on Biomass and Secondary Metabolite Production

Root FW and DW markedly decreased with increasing aeration rate (Table 9.6). The maximum root growth (4.59 g L^{-1} DW) was measured at an aeration rate of 0.05 vvm, followed by 4.12 g L^{-1} DW for the 0.05–0.3 vvm series. In contrast, the accumulation of secondary metabolites (AQ, phenols, and flavonoids) increased with increasing aeration rate. The highest concentrations of AQ (121.11 mg g^{-1} DW), phenolics (57.78 mg g^{-1} DW), and flavonoids (92.13 mg g^{-1} DW) were achieved at an aeration rate of 0.3 vvm (Table 9.6). In terms of productivity, the highest yields [content (mg g^{-1} DW) × dry weight (g L^{-1})] of AQ (386.59 mg L^{-1} DW) and phenolics (191.76 mg L^{-1} DW) were obtained at the 0.05 vvm aeration rate, while the highest yield of flavonoids (281.45 mg L^{-1} DW) was obtained at the 0.3 vvm aeration rate [65]. The accumulation of root or cell dry biomass and secondary metabolites varies with the plant species or explants used. For *Echinacea purpurea*, an aeration rate of 0.1 vvm was optimal for enhancing adventitious root growth compared to a high aeration rate [66]. Similarly, cell growth of *Gymnema sylvestre* was also enhanced by a 0.1 vvm aeration rate compared to a 0.3 vvm aeration rate [34]. In contrast, aeration of cell suspension cultures of *M. citrifolia* at 0.3 vvm resulted in the most cell growth [13]. In bioreactor culture, a gradual

Table 9.6 Effect of aeration rate on adventitious root growth and accumulation of secondary metabolites of *M. citrifolia* after 4 week of culture

Aeration volume(vvm)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio	AQ (mg g^{-1} DW)	Phenolics (mg g^{-1} DW)	Flavonoids (mg g^{-1} DW)
0.05	68.89 ± 1.56a[a]	4.59 ± 0.13a	10.07a	84.22 ± 0.05d	41.76 ± 0.72b	54.97 ± 0.93c
0.1	40.18 ± 0.27c	3.56 ± 0.01c	7.80c	88.16 ± 0.61c	43.72 ± 0.81b	56.68 ± 0.40c
0.2	32.91 ± 0.58d	3.10 ± 0.02d	6.81d	111.07 ± 0.29b	55.55 ± 1.47a	79.61 ± 0.29b
0.3	28.02 ± 1.06e	3.05 ± 0.04d	6.70d	121.11 ± 0.30a	57.78 ± 1.55a	92.13 ± 0.31a
0.05–0.3[b]	50.70 ± 1.73b	4.12 ± 0.14b	9.04b	81.54 ± 0.14e	45.67 ± 1.46b	54.95 ± 1.83c

[a]Means followed by the same *letter* within a set of values are not significantly different by Duncan's multiple range test at the 5 % level

[b]Stepwise increase from 0.05 to 0.3 vvm over the 4-week culture period

increase in aeration rate is often favourable for cell growth because of the high inflow of air agitating the cells, thereby elevating the oxygen concentration in the culture, resulting in accelerated cell growth [24, 34]. In general, a high aeration rate is beneficial for speeding up the transfer of oxygen into bioreactors, which improves both secondary metabolite accumulation and root growth [13, 24]. However, a high aeration rate is not always advantageous to the accumulation of metabolites in plant cultures. For example, high aeration rate (≥0.2 vvm) negatively affected the accumulation of AQ and alkaloid in cell suspension cultures of *M. citrifolia* and *Scopolia parviflora*, respectively [13, 67]. In the present study, high aeration rates (≥0.2 vvm) induced accumulation of AQ, phenolics, and flavonoids in adventitious roots of *M. citrifolia*, whereas a low aeration rate (0.05 vvm) enhanced root growth. The optimal aeration rate was 0.05 vvm for increasing production of both root biomass and bioactive compounds in this study.

PAL activity markedly increased with increasing aeration rate from 0.05 to 0.3 vvm. Adventitious root cultures agitated with a 0.3 vvm aeration rate had significantly higher PAL activity than other treatments, while much lower PAL activities were detected in adventitious roots cultured at low aeration rates (≤0.1 vvm). In addition, PAL activity showed a positive polynomial relationship with accumulation of phenolics ($R^2 = 0.8973$) and flavonoids ($R^2 = 0.9262$; data not shown). This suggests that over 89 % of the accumulation of phenolics and over 92 % of the accumulation of flavonoids was associated with PAL activity [65]. In concurrent with this study, increase in PAL activity in response to CO_2 and Cu stress and subsequent biosynthesis of phenolics and flavonoids has been reported by Ali et al. [42, 43]. Moreover, the H_2O_2 burst induced by shear stress plays an important role in inducing the biosynthesis of phenolic compounds through upregulation of PAL activity [68]. The upregulation of PAL activity in the present study may be due to H_2O_2 generation, which occurs as a primary reaction in response to shear stress, and it plays a major role in controlling the flux into phenolics and flavonoids. These results suggest that induction of PAL activity (the key enzyme of the phenylpropanoid pathway) by high aeration rate (≥0.2 vvm) not only triggers the accumulation of phenolic compounds but also induces a defense response in adventitious roots of *M. citrifolia* [65].

The activities of several antioxidant enzymes (SOD, CAT, G-POD, and APX) were significantly affected by aeration rate (Fig. 9.8a–d). CAT activity markedly increased at the 0.3 vvm aeration rate (Fig. 9.8a), while G-POD activity was highest at the 0.3 and 0.1 vvm aeration rates (Fig. 9.8b). In contrast, SOD activity was highest at the 0.3 vvm aeration rate followed by the 0.1 and 0.05–0.3 vvm aeration rates (Fig. 9.8c). APX activity was highest at the lowest aeration rate (0.05 vvm) (Fig. 9.8d) [65]. Antioxidant enzymes are critical components in preventing oxidative stress in plants. The activities of these enzymes are increased in plants exposed to stress conditions, and this elevated activity correlates with increased stress tolerance [43]. For instance, the stimulation of SOD activity is responsible for conversion of O_2^- to H_2O_2 and subsequently induction of CAT, G-POD, and APX activities that mitigate the toxic effects of H_2O_2. Although CAT and G-POD activities were elevated at the highest aeration rate (0.3 vvm) (Fig. 9.8a–d), the much lower APX

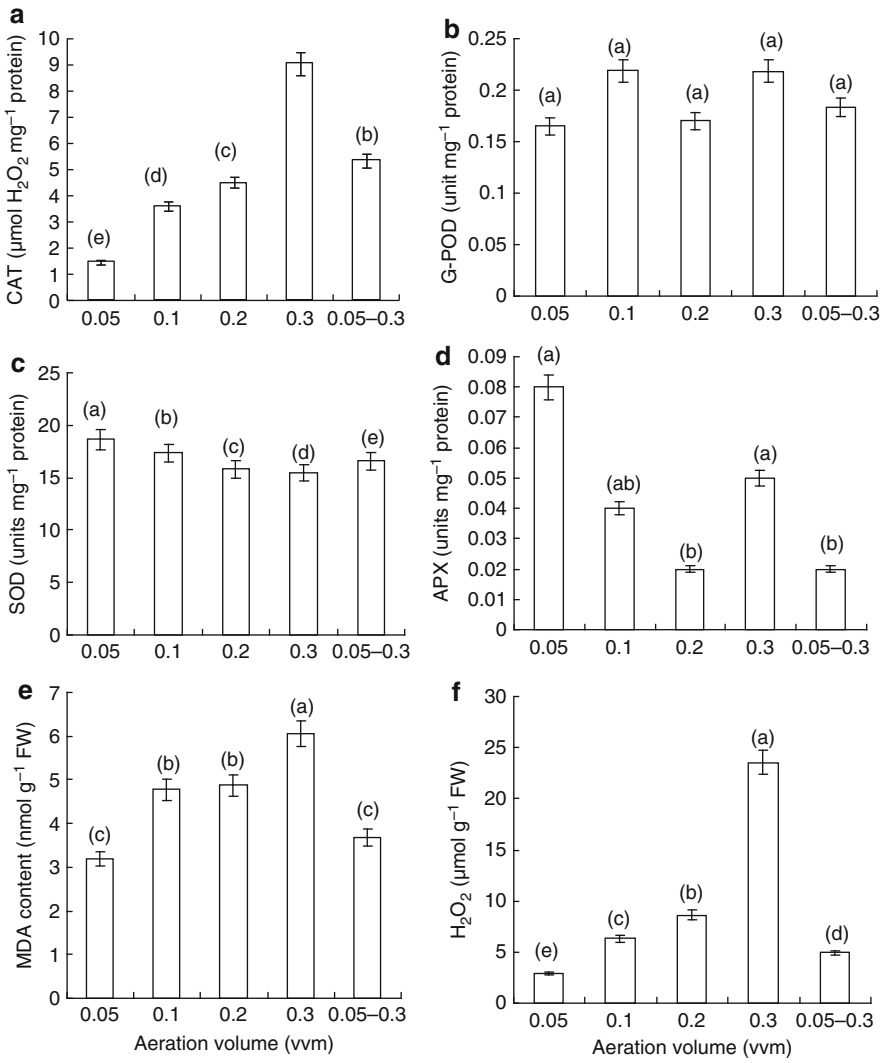

Fig. 9.8 Activities of antioxidant enzymes CAT (**a**), G-POD (**b**), SOD (**c**), and APX (**d**), and contents of MDA (**e**) and H_2O_2 (**f**), in adventitious roots of *M. citrifolia* as affected by air supply rate after 4 weeks of culture. Bars represent means ± S.E, n = 3

activity at this aeration rate indicates that the upregulated activities of CAT and G-POD were not sufficient to cope with toxic H_2O_2 accumulation. Therefore, higher levels of H_2O_2 and MDA were observed in adventitious roots cultured at 0.3 vvm than in the other treatments (Fig. 9.8e, f).

ROS are highly cytotoxic and can react with the vital biomolecules such as lipids, proteins, and nucleic acids, causing lipid peroxidation and DNA mutation [69]. MDA, a marker for lipid peroxidation or damage to plasmalemma and organelle

membranes increases with stress. Moreover, lipid peroxidation is linked to the activities of antioxidant enzymes, which are responsible for enhancing tolerance to oxidative stresses in plants by decreasing the accumulation of H_2O_2 and MDA [69]. The MDA content increased with increasing H_2O_2 accumulation and reached its highest level when culture was aerated at 0.3 vvm (Fig. 9.8e–f). The increase in MDA content at a high agitation rate, in parallel with the increase in H_2O_2 level, indicated that the CAT and G-POD activities were not sufficient to mitigate the harmful effects of H_2O_2 in adventitious roots cultured at a high aeration rate (0.3 vvm). Consequently, peroxidation of lipids occurred, which negatively affects root growth in highly aerated cultures [65].

Effects of Inoculum Density on Biomass and Secondary Metabolite Production

Inoculum density significantly affected the accumulation of root biomass of *M. citrifolia* during 4 weeks of culture. Root FW and DW increased with increasing inoculum density, while growth ratio decreased with the increasing inoculum density (Table 9.7). The maximum root FW (78.56 g L^{-1}) and DW (5.17 g L^{-1}) were measured at an inoculum density of 30 g L^{-1} (fresh root). The opposite phenomenon was observed in case of secondary metabolite accumulation. Higher (›10 g L^{-1}) inoculum density inhibited accumulation of AQ, phenolics, and flavonoids, while lower inoculum densities (5 and 10 g L^{-1}) stimulated accumulation of secondary metabolites (Table 9.7). In addition, vitamin E levels were highest at an inoculum density of 5 g L^{-1}, while at higher inoculum densities (›10 g L^{-1} FW) vitamin E levels in adventitious roots did not differ significantly among treatments [65]. The highest concentrations of AQ (123.14 mg g^{-1} DW) and flavonoids (57.09 mg g^{-1} DW) were obtained at an inoculum density of 10 g L^{-1}, whereas the maximum concentration of phenolics (56.84 mg g^{-1} DW) was detected at an inoculum density of 5 g L^{-1} (Table 9.7). Considering productivity, the 15 g L^{-1} inoculum density was proven optimal for enhancing the yields of AQ (375.36 mg L^{-1} DW) and flavonoids (159.44 mg L^{-1} DW; not significantly different from the highest value) in adventitious roots of *M. citrifolia* [65]. Inoculum density is an important factor affecting growth and bioactive compound production in a number of plant cell culture systems. For instance, high inoculum density stimulated root growth but inhibited biosynthesis of phenolics and flavonoids in adventitious root suspension cultures of *Echinacea angustifolia* [35], eleutheroside B and E in *Eleutherococcus koreanum* [24], and scopolamine in *Scopolia parviflora* [67]. In addition, the anthocyanin composition can be changed by cell inoculum size in strawberry suspension cultures [70]. In *Panax notoginseng* cell suspension cultures, cell growth and biosynthesis of saponin and polysaccharides were affected significantly by inoculum size [71]. We therefore conjecture that disparity in cell inoculum size could lead to large differences in cell density during cultivation and a number of culture parameters, such as concentration of dissolved oxygen and dissolved gaseous metabolites, and even the related enzymatic activities could be altered by accumulated cell mass.

Table 9.7 Effect of inoculum density on adventitious root growth and accumulation of secondary metabolites of *M. citrifolia* after 4 weeks of culture

Inoculum density (g L^{-1})	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio	AQ (mg g^{-1} DW)	Phenolics (mg g^{-1} DW)	Flavonoids (mg g^{-1} DW)
5	28.16 ± 1.59e[a]	2.57 ± 0.08c	5.47a	110.17 ± 0.23b	56.84 ± 0.36a	52.32 ± 0.15b
10	36.31 ± 0.72d	2.79b ± 0.07c	3.63b	123.14 ± 0.16a	42.73 ± 1.49b	57.09 ± 0.47a
15	45.79 ± 0.89c	3.81 ± 0.07abc	3.05bc	98.44 ± 0.20c	35.36 ± 0.03c	41.82 ± 0.46c
20	51.84 ± 0.97b	4.07 ± 0.34ab	2.35c	86.88 ± 0.14d	33.99 ± 0.86c	38.71 ± 0.66d
30	78.56 ± 1.49a	5.17 ± 0.76a	2.33c	62.46 ± 0.24e	28.08 ± 0.53d	28.41 ± 0.49e

[a]Mean values followed by the same *letter* within a set of values are not significantly different by Duncan's multiple range test at the 5 % level

Directly or indirectly, these changes could further affect cell metabolism [71]. However, it is still unclear exactly how inoculum size affects the biosynthesis of secondary metabolites.

Effects of Medium Salt Strength on Biomass and Secondary Metabolite Production

The root FW, DW and growth ratio and the levels of AQ, phenolics and flavonoids were significantly affected by MS salt strength. Root FW, DW and growth ratio increased up to 0.5× MS, thereafter decreasing with increasing salt strength of the culture medium (Table 9.8). Adventitious roots cultured at 0.5× MS showed maximum root FW (74.70 g L^{-1}), DW (4.38 g L^{-1}), and growth ratio (2.55). Root growth faced a strong challenge when cultured in 1.5× MS medium. Although 1.5× MS medium led to maximum concentrations of AQ (116.77 mg g^{-1} DW), phenolics (57.03 mg g^{-1} DW), and flavonoids (53.43 mg g^{-1} DW) in adventitious roots, the maximum yields of AQ (451.47 mg L^{-1} DW), phenolics (240.05 mg L^{-1} DW), and flavonoids (215.81 mg L^{-1} DW) were obtained with 0.5× MS medium [65]. This was mainly due to the increase in root DW combined with average metabolite levels in adventitious roots cultured in 0.5× MS medium.

The results of this study are concurrent with those of Sivakumar et al. [10], in which 0.5× MS medium provided the highest ginsenoside yield in bioreactor cultures of *Panax ginseng* adventitious roots, whereas 2.0× MS medium inhibited root growth, resulting in high ginsenoside content but low yield. The suitability of 0.5× MS medium for production of both root dry mass and secondary metabolites has also been reported for root suspension cultures of *Echinacea* [35] and *Hypericum perforatum* L. [72] due to suitable interactions among the nutrients that promote availability of ions to the roots. On the other hand, in case of *Echinacea angustifolia* the greatest root dry weight and accumulation of total phenolics, total flavonoids and total caffeic acid derivatives were obtained at quarter-strength MS medium [73]. These observations reveal that the requirement of medium salt strength for adventitious root suspension culture in bioreactor is species dependent.

Vitamin E is a fat-soluble antioxidant that stops the production of ROS when fat undergoes oxidation [74]. During this process, vitamin E protects cell membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reactions. This removes the free radical intermediates and prevents the oxidation reaction from continuing. In the present study, the concentration of vitamin E was significantly higher in roots grown on 0.5× MS medium than in other treatments. This increase in vitamin E level at 0.5× MS decreased the MDA and H_2O_2 contents in adventitious roots [65]. Although higher strengths of MS salt (1× and 1.5× MS) also produced relatively high vitamin E levels, the amounts may not have been sufficient to protect the chain propagation step in lipid auto-oxidation, resulting in higher concentrations of H_2O_2 and MDA in adventitious roots after 4 weeks of culture [65].

Table 9.8 Effect of medium salt strength on adventitious root growth and accumulation of secondary metabolites of *M. citrifolia* after 4 week of culture

MS salt strength	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio	AQ (mg g^{-1} DW)	Phenolics (mg g^{-1} DW)	Flavonoids (mg g^{-1} DW)
0.25	57.16 ± 1.0b[a]	4.08 ± 0.03ab	2.38a	88.29 ± 0.22e	48.72 ± 0.08e	39.64 ± 0.08e
0.5	74.70 ± 0.71a	4.38 ± 0.01a	2.55a	103.08 ± 0.15b	54.81 ± 0.19b	49.27 ± 0.18b
0.75	72.24 ± 1.16a	3.98 ± 0.06b	2.32a	94.92 ± 0.19d	49.67 ± 0.21d	44.17 ± 0.09d
1.0	52.12 ± 1.51b	3.49 ± 0.10c	2.03a	101.63 ± 0.36c	50.91 ± 0.03c	48.12 ± 0.05c
1.5	37.22 ± 3.48c	2.59 ± 0.20d	1.51a	116.77 ± 0.24a	57.03 ± 0.12a	53.43 ± 0.29a

[a]Means followed by the same *letter* within a set of values are not significantly different by Duncan's multiple range test at the 5 % level

To understand the extent of membrane injury of cultured roots, we analyzed the concentrations of secondary metabolites in the residual media. Adventitious roots grown with higher strengths of MS salts (>0.75× MS) had higher concentrations of AQ, phenols, and flavonoids in the residual media than those grown in 0.5× MS [65]. Therefore, it can be conjectured that accumulation of H_2O_2 in roots cultured at high salt strengths induced peroxidation of lipids, resulting in membrane injury and leakage of metabolites into the spent media [65]. These results clearly indicate that 0.5× MS medium has the optimum salt strength for root suspension cultures of *M. citrifolia* because it provides suitable growing conditions, through enhancement of vitamin E levels, which protects the chain propagation step in lipid auto-oxidation and acts as an effective free radical trap.

9.3 Scale-Up of Cultures

9.3.1 Growth Kinetics and Metabolites Production Pattern in Flask and Bioreactors

Bioreactor cultures are regarded as an efficient method to enhance biomass as well as metabolites production in plant cell or organ cultures. To determine the exact stage at which maximum biomass and bioactive compound production occurred and to discern the changes occurred in the culture during the cultivation period, 15 g L^{-1} (FW) roots were inoculated in shake flask (quarter-strength MS medium supplemented with 5 mg L^{-1} IBA and 10 g L^{-1} sucrose) [19–21] and in bioreactors (half-strength MS medium supplemented with 5 mg L^{-1} IBA and 10 g L^{-1} sucrose) [65] as a comparative study. The accumulation of root biomass (FW and DW) and growth ratio reflected that after 1 week of lag-phase, the adventitious roots grew exponentially from 1 to 4 weeks and then entered its stationary phase from 4 weeks onwards. After 5 weeks of cultivation, root biomass (FW and DW) and growth ratio reached its peak in both flask and bioreactors. A conspicuous declining phase of biomass accumulation was observed after 5 weeks in flask culture, whereas adventitious roots maintained its steady growth in bioreactors without showing conspicuous declining phase.

The accumulation of secondary metabolites (AQ, phenol, flavonoids and polysaccharide) showed differential pattern during 6 weeks of culture cycle both in flask and bioreactors. In case of bioreactor, AQ and phenolics content rapidly increased from 1 to 2 weeks, then slightly decreased on week 3 and again increased its maximum peak on week 5, thereafter decreased [75]. On the contrary, the accumulation pattern of AQ and phenolics in flask culture slightly altered. AQ and phenolics content slightly decreased from 1 to 2 weeks, then it increased up to 5 weeks and again decreased. Flavonoids content, however increased for 6 weeks both in bioreactor and flask culture except on week 4 for bioreactor cultures, showing a decreasing

trend from 3 to 4 weeks. Flask culture initially accelerated polysaccharide content (0~1 week) then slightly decreased up to 4 weeks followed by an increased up to 6 weeks, while bioreactor culture accelerated polysaccharide content and reached its maximum peak after 5 weeks [75]. However, bioreactor culture significantly enhanced biomass accumulation (FW and DW), as well as metabolite (AQ, phenol, flavonoids and polysaccharide) production compared to shake-flask culture, and 5 weeks of culture period regarded as an optimal time for the production of both root biomass and secondary metabolites.

In root suspension cultures of *Echinacea purpurea*, Jeong et al. [23] reported that the productivity of dry root mass was found to be higher in the bioreactor cultures than in the shake flask culture. A more than 1.5-fold increase in caftaric acid, cichoric acid, and more than fivefold increase in chlorogenic acid in the bioreactor cultures than in the shake-flask cultures has been reported in this species [76]. In addition, Ahmed et al. [13] reported 1.5-fold increase in cell dry mass, as well as threefold AQ and flavonoids and 1.5-fold increase in phenolics content of cell suspension cultures of *M. citrifolia* in bioreactor cultures than in the flask cultures. In this study, a 1.3-fold increase in root fresh mass, 1.33, 1.5, 1.3, and 1.14-fold increase in AQ, phenolics, flavonoids and polysaccharide contents respectively were achieved in the bioreactor cultures than in the flask cultures [75]. These results suggest that bioreactor culture is suitable for large-scale production of AQ, phenolics, flavonoids and polysaccharide of *M. citrifolia* adventitious roots for 5 weeks of culture period.

The soluble sugar (sucrose, glucose and fructose) content of spent medium was measured at weekly intervals during the culture period to discern the sugar uptake pattern of root suspension cultures of *M. citrifolia* in the flask and bioreactor cultures. As we added a very low concentration of sucrose (1 %) in the culture media for initial carbon source, after 1 week of culture sucrose was not detected in the spent medium instead glucose and fructose was observed. This result indicates that all the sucrose converted to glucose and fructose. The concentrations of fructose in the culture media increased during the initial 3 weeks and then decreased sharply in flask culture compared to bioreactor culture. The higher fructose content in the bioreactor implies that lesser amount of glucose and fructose was consumed by the roots [75]. In cell suspension cultures of *M. elliptica*, Abdullah et al. [5] observed a substantial amount of unutilized fructose in bioreactors compared to shake flasks. They finally concluded that this could be due to incomplete hydrolysis of sucrose and is attributed to insufficient invertase activities in the bioreactor. In addition, glucose concentration increased exponentially from 1 to 2 weeks followed by a rapid depletion from 2 to 3 weeks and slowly from 3 to 4 weeks. After a steady depletion from 4 to 5 weeks, again it rapidly depleted from the culture medium and was exhausted at the end of the culture period (6 weeks). Although, only sucrose was added in the culture medium extracellular hydrolysis of sucrose was occurred, that led to the formation of glucose and fructose. The hydrolysis of sucrose in the culture media may have been induced by acid invertase that was secreted from the adventitious root tissue into the medium [23].

Table 9.9 Effects of chitosan and pectin ratio on secondary metabolite content in adventitious roots of *M. citrifolia* after 4 weeks

Chitosan/Pectin (mg mL^{-1})	AQ (mg g^{-1} DW)	Phenolics (mg g^{-1} DW)	Flavonoids (mg g^{-1} DW)
Control (no elicitor)	49.30 ± 0.420 g	24.19 ± 0.023 g	41.44 ± 0.170f
0.2/0	115.16 ± 0.242a	48.57 ± 0.187c	75.32 ± 0.124a
0.2/0.1	98.90 ± 0.182b	50.45 ± 0.564b	65.89 ± 0.047b
0.2/0.2	93.33 ± 0.113c	52.45 ± 0.049a	61.22 ± 0.084c
0.4/0.2	64.293 ± 0.197e	42.01 ± 0.024e	45.44 ± 0.508e
0.4/0.4	67.40 ± 0.219d	42.69 ± 0.187d	48.04 ± 0.299d
0.8/0.8	50.94 ± 0.151f	33.88 ± 0.199f	35.89 ± 0.057 g

Mean separation within columns by Duncan's multiple range test at 5 % level. The various combinations of chitosan and pectin were added in the culture during inoculation. Control = without chitosan and pectin

9.3.2 Elicitation

As an enhancement strategy, various elicitors such as chitosan and pectin, MeJa, SA, lactalbumin hydrolysate (LH) were added in the culture to elucidate their optimal concentration and time of application. Adventitious root cultures treated with various combinations of chitosan and pectin or chitosan alone resulted in enhanced biosynthesis of secondary metabolites but inhibited root growth. The strong inhibition of root growth might be due to the lethal effect of elicitor as evidenced by 36–79 % cell death was measured [22]. The optimum concentration of elicitor for enhancing metabolite biosynthesis was found at the concentration of 0.2 mg mL^{-1} chitosan, in which highest accumulation of AQ, phenolics and flavonoids were achieved (Table 9.9).

Of the various elicitors tested (MeJa, SA, LH), the addition of 150 µM MeJa during inoculation was found to be the most effective elicitor on AQ biosynthesis (twofold over control) but it strongly repressed the root growth [3]. To overcome detrimental effect of MeJa on root growth, two-stage culture system was adopted: addition of 150 µM MeJa in the culture after 4 weeks and harvested after 1 week of elicitation was shown to be effective for enhancing biosynthesis of AQ, phenolics and enzymatic antioxidant vitamin C without decreasing root growth (Table 9.10). Later, we were able to increase AQ (up to 22–41 %), phenolics (24 %) and flavonoids (21–35 %) content in adventitious roots harvested from 500 L BTBB compared to 3–20 L BTBB by applying 150 µM MeJa (Table 9.11) as two-stage culture system [3].

In cell suspension cultures of *M. citrifolia*, Komaraiah et al. [16] observed a synergistic effect by simultaneously applying 150 µM MeJa and controlled feeding of 2 % sucrose, which increased the AQ production to 16.74 mg g^{-1} DW, which was more than fourfold over the cultures without MeJa treatment. We achieved 148.35 mg g^{-1} DW of AQ in adventitious roots (cultured in 500 L BTBB) by application of 150 µM MeJa after 5 weeks of culture (Table 9.11), which was 26-, 2.37-, 12- and 24-fold of AQ content in field grown madder roots, leaf of green house grown plant, leaf-originated cells and fruits of green house grown plants (Table 9.12),

Table 9.10 Growth, secondary metabolite content and antioxidant vitamin response of *M. citrifolia* adventitious roots as affected by MeJa in 5 weeks of bioreactor culture

Treatments	FW (g L^{-1})	DW (g L^{-1})	AQ (mg g^{-1} DW)	Phenolics (mg g^{-1} DW)	Flavonoids (mg g^{-1} DW)	Vitamin C (μg g^{-1} FW)	Vitamin E (μg g^{-1} FW)
Control	138.96a	8.96a	83.36b	42.84b	61.14a	171.44b	224.30a
150 μM MeJa	142.32a	9.02a	110.15a	54.31a	59.98b	282.26a	191.71b

Mean separation within columns by Duncan's multiple range tests at 5 % level
Adventitious root cultured in 5 L balloon type bubble bioreactor containing half-strength MS medium supplemented with 5 mg L^{-1} IBA with 10 g L^{-1} sucrose
15 g L^{-1} (FW) adventitious root used as initial inoculum size and 150 μM MeJa elicitor treated in the culture after 4 weeks and harvested after 1 week of elicitation. Control indicates no elicitor

respectively [3]. These results corroborate the feasibility for commercial exploitation of AQ from adventitious root cultures of *M. citrifolia* using large scale bioreactors.

9.3.3 Adventitious Roots in Large-Scale and Pilot-Scale Bioreactors

In order to scale-up, adventitious roots were cultured in half-strength MS medium supplemented with 5 mg L^{-1} IBA, 10 g L^{-1} sucrose and 15 g L^{-1} FW inoculum size (optimized culture condition in 3 L bioreactors). Adventitious roots cultured in large-scale bioreactors (3–20 L) showed enhancement in fresh and dry weights of roots compared to pilot-scale bioreactors (100–500 L). The maximum root fresh weight (127.92, 95.30 and 74 g^{-1}) and dry weight (9.60, 7.21 and 5.75 g L^{-1}) were achieved at 5, 10 and 20 L bioreactors (Fig. 9.9), respectively [75]. Whereas, the accumulation of secondary metabolites (AQ, phenolics and flavonoids) significantly increased in pilot-scale bioreactors compared to large-scale bioreactors (Table 9.11). The highest accumulation of AQ (205.75 mg g^{-1} DW) and phenolics (90.26 mg g^{-1} DW) was recorded in 500 L bioreactors, while the highest flavonoids (93.34 mg g^{-1} DW) and polysaccharide (153.23 mg g^{-1} DW) contents were observed at 100 L and 5 L bioreactors, respectively [3].

In general, growth and metabolite production are different phenomena. In case of pilot-scale bioreactors, higher accumulation of secondary metabolites compensates the reduction of root growth. It is worth noting that *M. citrifolia* adventitious roots are very much sensitive with changes in culture systems (shake-flask, large scale and pilot scale bioreactors), medium strategy (maintenance and production medium) and culture age. In case of production medium (optimized culture), higher accumulation of secondary metabolites induced root senescence and cell death that reduce root growth in the subsequent subculture. Therefore, it is very difficult to maintain root viability, as well as stable culture for long time [3]. On the other hand,

Table 9.11 Growth and secondary metabolite production of *M. citrifolia* adventitious roots using large scale and pilot scale bioreactors

Bioreactors volume (L)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Anthraquinone (mg g^{-1} DW)	Phenolics (mg g^{-1} DW)	Flavonoids (mg g^{-1} DW)	Polysaccharide (mg g^{-1} DW)
3[a]	79.22c	4.89d	87.54 g	47.90d	59.45 g	124.37e
5[a]	127.92a	9.60a	92.63f	42.78e	61.14f	153.23a
10[a]	95.30b	7.21b	109.99e	43.92de	69.50e	146.35b
20[a]	74.40d	5.75c	116.71d	47.30de	72.65d	143.82c
100[a]	38.80f	3.01e	154.74b	57.04c	93.34a	–
500[a]	31.33 g	2.80e	205.75a	90.26a	82.23c	128.97d
500[b]	64.99e	4.84d	148.35c	62.12b	92.08b	–

Mean separation within columns by Duncan's multiple range tests at 5 % level

Initial inoculum size was maintained 15 g L^{-1} (FW) and working volume 1.5, 3, 6, 12, 100 and 300 L, respectively for 3, 5, 10, 20, 100 and 500 L capacity bioreactor

[a]Adventitious roots cultured in half – strength MS medium supplemented with 5 mg L^{-1} IBA with 10 g L^{-1} sucrose

[b]Adventitious roots cultured in full strength MS medium supplemented with 5 mg L^{-1} IBA with 30 g L^{-1} sucrose (150 µmol MeJa treated after 4 weeks and harvested after 1 week of elicitation)

Table 9.12 Comparative analysis of bioactive compounds in the adventitious roots and mother plants of *M. citrifolia*

Sources	Anthraquinone (mg g^{-1} dw)	Phenolics (mg g^{-1} dw)	Flavonoids (mg g^{-1} dw)
Madder root (Field grown plant)	5.70e	6.93e	9.28c
Leaf (Green house grown plant)	62.39b	33.08b	16.68b
Fruit (Green house grown plant)	6.15e	20.70c	5.62d
AR_1	15.64c	16.38d	10.23c
AR_2	148.35a	62.12a	92.08a
Callus (C)	11.94d	30.27b	5.34d

Mean separation within columns by Duncan's multiple range tests at 5 % level

AR_1 adventitious root induced from leaf (*in vitro* grown plantlets), AR_2 150 µmol MeJa treated adventitious root after 5 weeks (MeJa treated after 4 weeks and harvested after 1 week of elicitation), *C* Callus after 2 weeks cultured in bioreactor

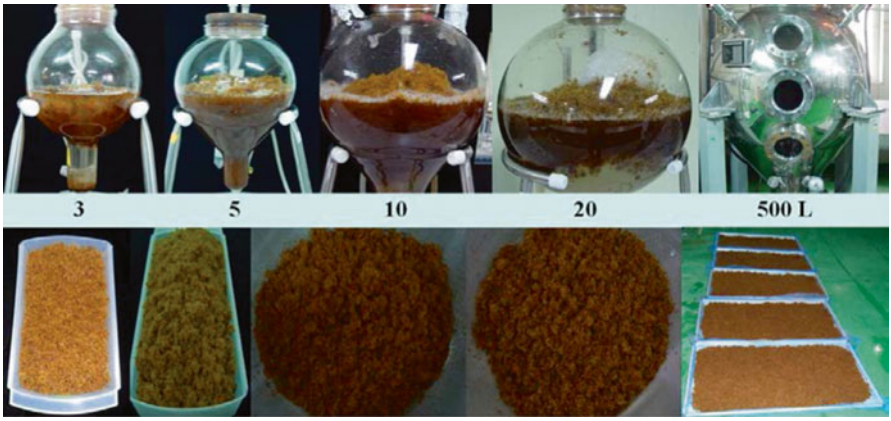

Fig. 9.9 Scale-up of *M. citrifolia* adventitious roots in large-scale and pilot-scale bioreactors

adventitious roots of *M. citrifolia* cultured in maintenance medium (full strength MS medium supplemented with 5 mg L^{-1} IBA and 30 g L^{-1} sucrose) can overcome the mentioned problem due to lower metabolite accumulation and higher cell viability. In addition, it has already been demonstrated that adventitious roots culture in shake-flask require low strength of MS salt (quarter-strength MS), while the nutrient requirement increased in large-scale bioreactor culture (half-strength MS). Taken together, this could be the reason to decrease root growth in pilot-scale bioreactors. To overcome the problem, adventitious roots were cultured in full-strength MS medium supplemented with 5 mg L^{-1} IBA, 30 g L^{-1} sucrose and an inoculum size of 15 g L^{-1} FW in pilot-scale bioreactors (500 L). Root growth (FW and DW) significantly increased in full-strength medium with 30 g L^{-1} sucrose compared to adventitious roots cultured with half-strength MS medium with 10 g L^{-1} sucrose in 500 L bioreactors after 5 weeks of culture period [3]. Although root DW in 500 L bioreactor still lower than 20 L bioreactor, the yield of AQ (718.01 mg L^{-1} DW), phenolics (300.66 mg L^{-1} DW) and flavonoids (445.67 mg L^{-1} DW) in 500 L bioreactor is higher than that of 20 L bioreactor (671.08, 271.98 and 417.74 mg L^{-1} DW, respectively), showing 6.99, 10.54 and 6.69 % (AQ, phenolics and flavonoids, respectively) yield over 20 L bioreactor [3].

9.3.4 Comparative Study Between Mother Plant and Adventitious Roots of M. citrifolia

The Accumulation of AQ, phenolics and flavonoids in different parts of mother plants, adventitious roots and cells of *M. citrifolia* were studied (Table 9.12). The highest accumulation of AQ (148.35 mg g^{-1} DW), phenolics (62.12 mg g^{-1}

Table 9.13 Quantification of rubiadin content in various plant parts of *M. citrifolia* by HPLC

Various plant parts	Rubiadin content (%)
Stem	Not detected
Leaf	Not detected
Fruit	Not detected
Ex-vitro roots	0.02
Adventitious roots	0.58

Adventitious roots cultured in 500 L balloon type bubble bioreactor (BTBB) containing full-strength of MS medium supplemented with 5 mg L^{-1} IBA, 30 g L^{-1} sucrose and 15 g L^{-1} of inoculum size with an aeration volume of 0.05 vvm for 5 weeks (150 µM MeJa elicited in the culture after 4 weeks and harvested after 1 week of elicitation)

DW) and flavonoids (92.08 mg g^{-1} DW) was observed in adventitious roots treated with 150 µmol MeJa followed by leaves of green house grown mother plant (62.39, 33.08 and 16.68 mg g^{-1} DW AQ, phenolics and flavonoids, respectively). The lowest accumulation of AQ, phenolics and flavonoids was observed in the cells (11.94, 30.27 and 5.34 mg g^{-1} DW) followed by fruits of green house grown plant (6.15, 20.70 and 5.62 mg g^{-1} DW) and followed by field-grown madder roots (5.7, 6.93 and 9.28 mg g^{-1} DW). Adventitious roots cultured in the optimized culture conditions and treated with 150 µmol MeJa resulted in 13-fold and 26-fold increase in AQ was observed compared to cells and madder roots (Table 9.12), respectively [3]. These results clearly indicate the feasibility of large-scale commercial application of *M. citrifolia* adventitious roots in the field of biotechnology for the production of AQ, phenolics and flavonoids.

Rubiadin, a major constituent of AQ is highly valued in pharmaceutical industry due to hepatoprotective [77], and antitumor activity [78], and also have been found to inhibit lipid peroxidation [79]. Rubiadin was identified and purified from MeJa treated adventitious roots of *M. citrifolia* harvested from pilot scale bioreactor. A reverse-phase HPLC assay method was also developed to quantify rubiadin content in adventitious roots. The HPLC assay of rubiadin was performed by C-18 column using a gradient solvent system of methanol and water with a UV detector at 280 nm [80]. It is worth noting here that rubiadin was not detected in the various parts (leaf, stem, fruit) of field grown plant of *M. citrifolia* or very few amounts (0.02 %) in madder roots; whereas, copious amount of rubiadin (≥0.58 %) was detected in adventitious roots compared to *ex-vitro* roots (Table 9.13). These results clearly indicate that adventitious root cultures of *M. citrifolia* using large scale bioreactors could be a useful tool for commercial production of AQ, rubiadin, phenolics and flavonoids [3]. Therefore, we have initiated further works, and the commercial application of adventitious root culture of this valuable medicinal plant is now under trail with 1,000 L BTBBs in our laboratory.

9.4 Conclusions

In this study, an efficient bioreactor technology is established through optimization of culturing conditions by employing an iterative series of experiments. The newly developed culture protocol is expected to be less prone to erratic metabolite production than undifferentiated cells and to display a lower sensitivity to shear stress. Such optimization of culture protocol will be beneficial for scaling up of adventitious root cultures in commercial scale bioreactor for the production of AQ and rubiadin.

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Chapter 10
Production of Biomass and Bioactive Compounds in Adventitious Root Cultures of *Eleutherococcus koreanum* Nakai

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Abstract *Eleutherococcus koreanum* Nakai is an endemic medicinal plant grown in Jeju Island, South Korea. Extracts from this plant have traditionally been used in Korea as a tonic and for treating rheumatism, diabetes and hepatitis. These extracts contain many useful bioactive substances, particularly eleutherosides, chlorogenic acid and other phenolic compounds. The quality and quantity of major bioactive compounds from naturally grown plants are greatly affected by harvest time and environmental conditions. Therefore, determination of suitable growing and harvesting conditions is necessary to achieve reliable supply of *E. koreanum*-based bioactive compounds for commercial use. To establish an efficient method for the year-round production of bioactive compounds, adventitious roots of *E. koreanum* were tested with various physical and chemical factors (inoculum density, aeration volume, salt strength, nitrogen source, and sucrose concentration) that affect root biomass and production of target bioactive compounds. Root biomass, concentrations of five target bioactive compounds (eleutherosides B and E, chlorogenic acid, total phenolics, and flavonoids), physiological responses of the adventitious roots and other environmental conditions in the culture vessels were determined. In addition, responses of roots subjected to chemical elicitors (methyl jasmonate and salicylic acid) were evaluated to determine a strategy for enhancing the final production of bioactive compounds. Finally, we compared the contents of bioactive compounds and typical DNA histograms for the adventitious roots and naturally grown plants to

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verify the competitive ability and genetic stability of cultured *E. koreanum* adventitious roots. The development of *in vitro* culture protocol, which controls the quality and quantity of elicited bioactive compounds, will be beneficial for the pilot-scale production of *E. koreanum*-based bioactive compounds for commercial use.

Keywords Adventitious roots • Bioactive compounds • Bioreactor culture • Eleutherosides • *Eleutherococcus koreanum*

Abbreviations

DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
FW	Fresh weight
H_2O_2	Hydrogen peroxide
HPLC	High-performance liquid chromatography
IBA	Indole-3-butyric acid
MJ	Methyl jasmonate
MS	Murashige and Skoog
PAL	Phenylalanine ammonia lyase
ROS	Reactive oxygen species
SA	Salicylic acid
TDZ	Thidiazuron; N-phenyl-N′-1,2,3,-thidiazol-5-ylurea
vvm	Air volume culture volume^{-1} min^{-1}

10.1 Introduction

Eleutherococcus koreanum Nakai (Araliaceae) is an endemic medicinal plant grown in Jeju Island, located off the southern coast of South Korea. Extracts from this plant have traditionally been used in Korea as a tonic and for treating rheumatism, diabetes and hepatitis [1]. The roots and stems of *E. koreanum* contain useful bioactive compounds such as eleutherosides, chlorogenic acid and other phenolic compounds. Among the various bioactive compounds in *E. koreanum*, eleutherosides B and E are considered to be the major saponins and have strong stimulant and anti-stress effects [2]. Chlorogenic acid has been shown to have a protective effect against gastric ulcers and to stimulate the activity of eleutheroside E [3]. Interest in *E. koreanum*-based bioactive substances for use in pharmaceuticals, therapeutics, cosmetics, food products and pigments is increasing, but the commercial supply of these compounds is insufficient [1, 4]. The propagation of *E. koreanum* by seed is difficult because germination of the zygotic embryos requires >18 months of stratification [5] and the quantity and quality of bioactive compounds obtained from field-grown plants is strongly affected by harvest time and environmental

conditions [6, 7]. Thus, a suitable method for the annual production of *E. koreanum*-based bioactive compounds is required for commercial purposes.

In recent years, plant biotechnology has enabled *in vitro* pilot-scale production of useful bioactive compounds from adventitious root, cell, embryo, and hairy root cultures, such as anthraquinones, echinacosides, ginsenosides, hypericin, rubiadin, and taxol [8, 9]. Among *in vitro* culture techniques, adventitious root culture is one of the most efficient methods for producing large amounts of biomass and bioactive compounds because it easily enables the establishment of a pilot-scale production system using bioreactors. Furthermore, the quality of bioactive compounds obtained from adventitious roots is very similar to that of the parent plants [10]. In addition, the accumulation of bioactive compounds in adventitious roots can be increased by elicitation.

To establish a successful bioreactor culture using adventitious roots, many physical parameters (e.g., aeration volume, gas composition, inoculum density, light intensity and quality, and temperature) and chemical factors (e.g., natural product, nitrogen source, salt strength, and sugar content) must be optimized according to the plant materials, explant types, and target bioactive compounds. We have been working to develop a bioreactor-based *in vitro* protocol for adventitious root culture that can control the quality and quantity of bioactive compounds, for the pilot-scale production of *E. koreanum*-based compounds for commercial use. This review focuses on the production of root biomass and bioactive compounds by optimizing *in vitro* culture conditions, and suggests strategies for enhancing the productivity of target bioactive compounds by chemical elicitation. Finally, we verify the competitive ability and genetic stability of *E. koreanum* adventitious roots.

10.2 Induction of Adventitious Roots and Adventitious Root Cultures in Airlift Bioreactors

Adventitious roots were directly induced from seed-derived plantlets [11]. Briefly, mature seeds maintained at <5 °C for 2 years to break dormancy were sterilized with 2 % (w/v) sodium hypochlorite solution for 15 min, and then washed three times with distilled water. Zygotic embryos were isolated from the sterilized seeds and placed on half-strength Murashige and Skoog (MS) medium [12] for germination. After 2 months, adventitious roots were directly induced from plantlets *in vitro*. Induced adventitious roots were maintained in half-strength MS medium (HN_4^+:NO_3^- = 5:25) supplemented with 5 mg L^{-1} indole-3-butyric acid (IBA), 0.01 mg L^{-1} thidiazuron (TDZ; N-phenyl-*N*′-1,2,3,-thidiazol-5-ylurea), 30 g L^{-1} sucrose, and 2.3 g L^{-1} gelrite at 22 ± 1 °C in dark conditions based on the experimental results (data not shown; [13]). Bioreactor cultures were initiated by inoculating with fresh adventitious roots (5.0 g L^{-1}) and the aeration volume in the bioreactors was automatically adjusted to 0.1 vvm (air volume culture volume^{-1} min^{-1}) using air flow meters. Adventitious roots were maintained in the bioreactors by subculturing to a fresh medium every 5 weeks (Fig. 10.1).

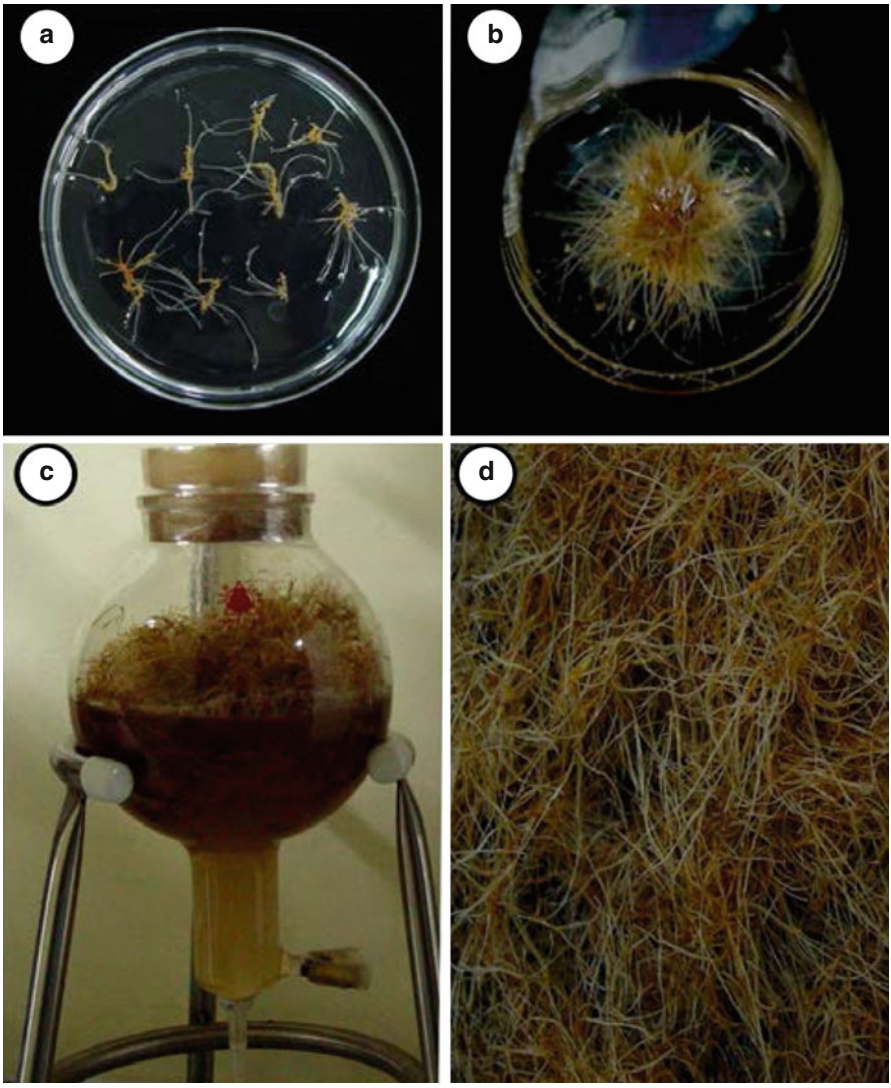

Fig. 10.1 (**a**–**d**) Culture procedure for producing *Eleutherococcus koreanum* adventitious roots. (**a**) Proliferation of adventitious roots in petri dishes. (**b**) Proliferation of adventitious roots in 250-ml erlenmeyer shake flasks. (**c**) Proliferation of adventitious roots in 3-L airlift bioreactors. (**d**) Harvested fresh roots

10.3 Optimization of Physical Factors in Airlift Bioreactor Culture

10.3.1 Effects of Inoculum Density on Biomass and Production of Bioactive Compounds from Adventitious Roots

To determine the parameters for enhancing the biomass productivity using 3-L airlift bioreactors over a 5-weeks period, fresh roots were inoculated at various initial densities ranging from 2.5 to 15.0 g L^{-1}. Fresh and dry weights increased with the increasing inoculum density, but the highest percentage dry weight was achieved using 5.0 g fresh roots L^{-1} (Table 10.1; [14]). In contrast, growth rate was negatively affected by increased inoculum density; the lowest inoculum density (2.5 g L^{-1}) resulted in the highest growth rate (0.35 day^{-1}). These results indicated that initial adventitious root densities >5.0 g L^{-1} resulted in low efficiency of biomass production in *E. koreanum* roots under the given experimental conditions. Wu et al. [15] and Min et al. [16] reported that a high initial inoculum density had a positive effect on biomass production and a negative effect on growth rate in adventitious root cultures of *Echinacea angustifolia* and *Scopolia parviflora*, respectively.

The accumulation of bioactive compounds in adventitious roots of *E. koreanum* was strongly affected by the initial inoculum density (Table 10.2). The highest total

Table 10.1 Effects of inoculum density on biomass accumulation and growth rate in adventitious roots of *Eleutherococcus koreanum* after 5 weeks of culture

Inoculum density (g roots L^{-1})	Fresh weight (g L^{-1})		Dry weight (g L^{-1})		Percentage dry weight	Growth rate (day^{-1})	
2.5	34.09	e[a]	4.64	d	13.61	0.35	a
5.0	46.65	d	6.73	c	14.43	0.25	b
7.5	50.90	c	7.02	bc	13.79	0.16	c
10.0	57.27	b	7.63	b	13.33	0.13	d
15.0	74.20	a	9.61	a	12.94	0.13	d

[a]Mean separation within columns by Duncan's multiple range test at $p = 0.05$

Table 10.2 Accumulation of bioactive compounds in adventitious roots of *Eleutherococcus koreanum* as affected by inoculum density after 5 weeks of culture

Inoculum density (g L^{-1})	Eleutheroside B (μg g^{-1} DW)		Eleutheroside E (μg g^{-1} DW)		Chlorogenic acid (mg g^{-1} DW)		Total phenolics (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)		Total target compounds (mg g^{-1} DW)	
2.5	46.21	a[a]	108.53	a	2.69	a	9.95	a	4.60	a	17.40	a
5.0	27.59	b	106.61	a	2.42	a	10.06	a	4.91	a	17.53	a
7.5	26.42	b	108.89	a	2.71	a	9.12	b	4.03	b	15.99	b
10.0	15.95	c	87.47	b	2.64	a	9.38	b	4.16	b	16.28	b
15.0	6.71	d	89.65	b	0.99	b	8.26	c	3.41	c	12.76	c

[a]Mean separation within columns by Duncan's multiple range test at $p = 0.05$

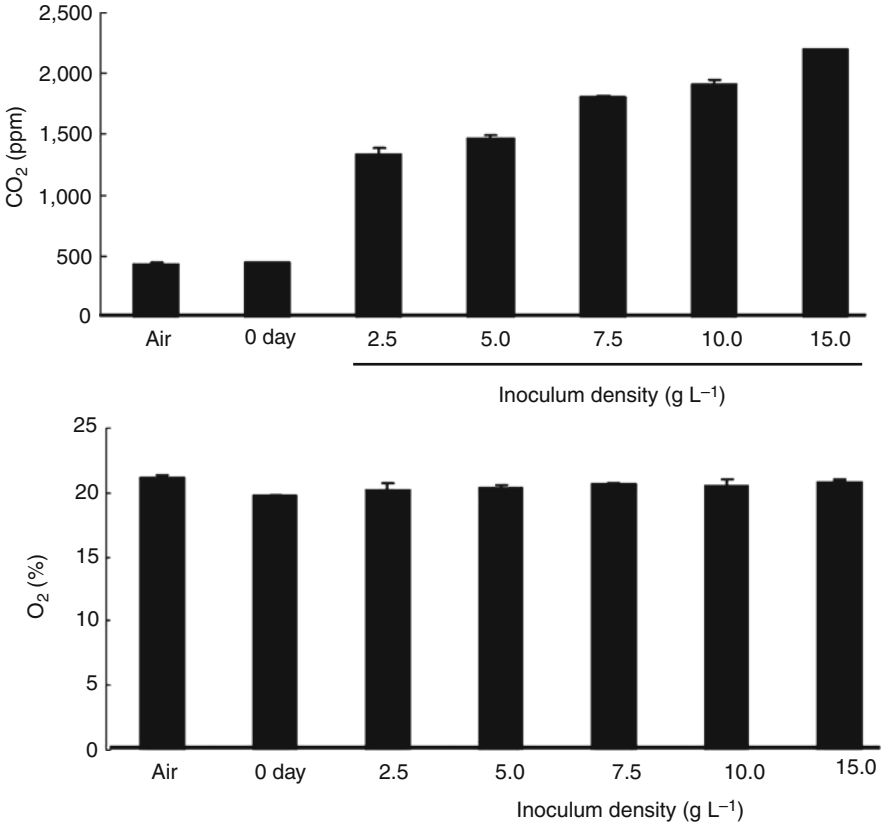

Fig. 10.2 Effect of inoculum density on CO_2 and O_2 concentrations inside the bioreactors after 5 weeks of culture. *Bars* represent means ± SE (n = 3)

content of the target compounds (17.53 mg g^{-1} DW) was obtained using 5.0 g roots L^{-1} and the contents of all bioactive compounds tended to decrease with increasing inoculum density. Wu et al. [15] reported that high initial inoculum densities resulted in low total phenol and flavonoid contents in adventitious roots of *E. angustifolia*, and Min et al. [16] also determined that high inoculum densities resulted in decreased production of bioactive compounds in roots.

Initial inoculum density directly affects the gaseous environment in the culture vessel, which affects primary and secondary metabolic processes in the roots. During the culture period, *in vitro* plantlets consume O_2 and release CO_2. Figure 10.2 shows the CO_2 and O_2 concentrations inside the bioreactor after 5 weeks of culture. CO_2 concentration increased significantly with the increasing inoculum density (a minimum threefold increase when compared with the initial bioreactor CO_2 concentration at all inoculum densities). The greatest accumulation of CO_2 (an approximately fivefold increase over the initial concentration) occurred at the highest inoculum density (15.0 g L^{-1}). High CO_2 concentrations probably caused

root death and inhibited the accumulation of bioactive compounds. However, no significant difference was observed in O_2 concentration initial inoculum density (2.5–15.0 g L^{-1}) because fresh air was supplied to the bioreactor throughout the whole culture period. Thus, CO_2 accumulation inside bioreactors is an important factor affecting primary and secondary metabolic processes in *E. koreanum* adventitious roots.

These results indicate that an inoculum density of 5.0 g L^{-1} is optimal for the production of biomass and bioactive compounds in adventitious roots of *E. koreanum* using 3-L airlift bioreactors for a 5-weeks culture period, as evidenced by high root biomass and production of the five target bioactive compounds (i.e., eleutherosides B and E, chlorogenic acid, total phenolics, and flavonoids) and the absence of physiological disorders caused by high CO_2 concentrations.

10.3.2 Effects of Aeration Volume on Biomass and Production of Bioactive Compounds from Adventitious Roots

Table 10.3 illustrates the effects of aeration volume (0.05–0.4 vvm) on biomass production of *E. koreanum* adventitious roots in 3-L airlift bioreactors over a 5-weeks period. Although percentage of dry weight increased slightly with the increasing aeration volume, there were no significant differences in biomass production among the different aeration volumes, except between the lowest and highest volumes. Root death showed a pattern similar to that of biomass accumulation; it was higher at the lowest and highest aeration volumes and lowest at 0.1 vvm (30.67 day^{-1}). Increase in aeration volume improved the oxygen transfer and mixing efficiency between explants and the culture medium. The lowest aeration volume (0.05 vvm) resulted in stunted root growth and stimulated root senescence, and the highest aeration volume (0.4 vvm) inhibited biomass accumulation and increased root death, probably because of physiological damage from excessive agitation and shear stress. Fischer and Alfermann [17] reported that supply of high aeration volumes at the initial *in vitro* culture stage exposed plantlets to excessive shear stress, which could directly induce metabolic damage. In contrast, poor aeration volumes

Table 10.3 Effects of aeration volume on biomass accumulation and root death in adventitious roots of *Eleutherococcus koreanum* after 5 weeks of culture

Aeration volume (vvm)	Fresh weight (g L^{-1})		Dry weight (g L^{-1})		Percentage dry weight		Root death (day^{-1})	
0.05	44.21	b[a]	5.51	c	12.46		35.33	ab
0.1	47.13	a	5.99	ab	12.70		30.67	b
0.2	47.52	a	6.22	a	13.09		33.33	ab
0.4	43.74	b	5.81	bc	13.27		39.33	a
0.05–0.4	48.89	a	6.37	a	13.02		35.00	ab

[a]Mean separation within columns by Duncan's multiple range test at $p = 0.05$

Table 10.4 Accumulation of bioactive compounds in adventitious roots of *Eleutherococcus koreanum* as affected by aeration volume after 5 weeks of culture

Aeration volume (vvm)	Eleutheroside B (μg g^{-1} DW)		Eleutheroside E (μg g^{-1} DW)		Chlorogenic acid (mg g^{-1} DW)		Total phenolics (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)		Total target compounds (mg g^{-1} DW)	
0.05	49.35	b[a]	106.88	a	3.02	a	9.09	a	5.34	a	17.60	ab
0.1	59.25	a	107.70	a	3.36	a	9.84	a	5.52	a	18.88	a
0.2	59.55	a	108.23	a	3.34	a	9.64	a	5.26	a	18.41	a
0.4	49.05	b	90.68	b	1.44	b	9.72	a	5.10	a	16.39	b
0.05–0.4	52.65	ab	98.78	a	2.96	a	9.58	a	5.20	a	17.89	a

[a]Mean separation within columns by Duncan's multiple range test at $p = 0.05$

resulted in stunted growth and stimulated senescence because of low rates of oxygen transfer and poor mixing ratios [18]. Each culture stage, culture method, explant type, and plant species requires a different aeration volume for primary and secondary metabolic processes and exhibits a different response to shear stress. In *E. koreanum* adventitious root cultures, the highest root biomass and lowest root death were achieved at 0.1 vvm. These results suggest that an aeration volume of 0.1 vvm is optimal for biomass production of *E. koreanum* adventitious roots and does not cause high shear stress that would induce physiological disorders (e.g., darkening of roots or inhibition of root elongation).

Accumulation of bioactive compounds in adventitious roots of *E. koreanum* as affected by aeration is shown in Table 10.4. No significant differences were found in total content of the target compounds, except for between the lowest and highest aeration volumes. High aeration volumes significantly inhibited the accumulation of eleutherosides B and E and chlorogenic acid in the roots. The highest content of the total target compounds was obtained at 0.1 vvm (18.88 mg g^{-1} DW). Min et al. [16] and Jeong et al. [19] also reported that optimization of aeration volume is an important factor for enhancing production of biomass and bioactive compounds in adventitious roots of *S. parviflora* and *Echinacea purpurea*, respectively.

The CO_2 and O_2 concentrations inside the bioreactor were strongly affected by the aeration volume after 5 weeks of culture (Fig. 10.3). The CO_2 concentration decreased significantly with increasing aeration volume. CO_2 concentrations in bioreactors with poor aeration (0.05 vvm) were 5.2 times higher than those in the inflow air, which probably inhibited respiratory function and affected root development. In contrast, high aeration (0.4 vvm) corresponded to the lowest CO_2 concentration inside the bioreactor after 5 weeks of culture. Excessive aeration may have induced stripping of essential gases such as CO_2 and ethylene, which are needed to stimulate the production of bioactive compounds [20, 21]. No significant changes were observed in O_2 concentration among the different aeration treatments.

The above aeration results indicate that an aeration volume of 0.1 vvm is optimal to prevent stripping of essential gaseous components and to supply adequate air for achieving maximum production of biomass and bioactive compounds in *E. koreanum*

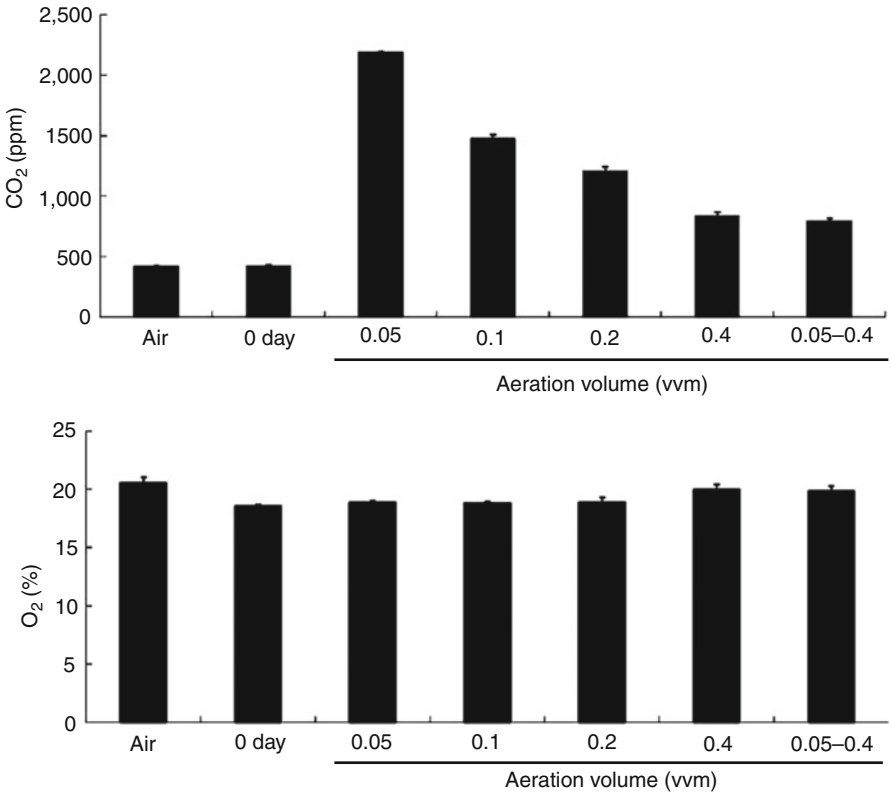

Fig. 10.3 Effect of aeration volume on CO_2 and O_2 concentrations inside the bioreactors after 5 weeks of culture. *Bars* represent means ± SE (n = 3)

adventitious roots. This was evidenced by the highest total production of the target bioactive compounds, the lowest rates of root death, and the absence of physiological disorders caused by excessive agitation and high shear stress.

10.4 Optimization of Chemical Factors in Airlift Bioreactor Culture

10.4.1 Effects of Salt Strength on Biomass and Production of Bioactive Compounds from Adventitious Roots

Plantlets grown *in vitro* must be provided with mineral nutrients from the culture medium for their survival, growth, and development, and mineral requirements differ according to culture stage and method, explant type, and plant species [22].

Table 10.5 Effects of strength of Murashige and Skoog (MS) medium on biomass accumulation in adventitious roots of *Eleutherococcus koreanum* after 5 weeks of culture

MS medium salt strength	Fresh weight (g L^{-1})		Dry weight (g L^{-1})		Percentage dry weight
1/4	39.51	ab[a]	4.95	ab	12.52
1/2	42.37	a	5.27	a	12.43
3/4	39.64	ab	4.99	ab	12.58
1	36.50	b	4.54	b	12.43
2	21.43	c	3.17	c	14.80

[a]Mean separation within columns by Duncan's multiple range test at $p = 0.05$

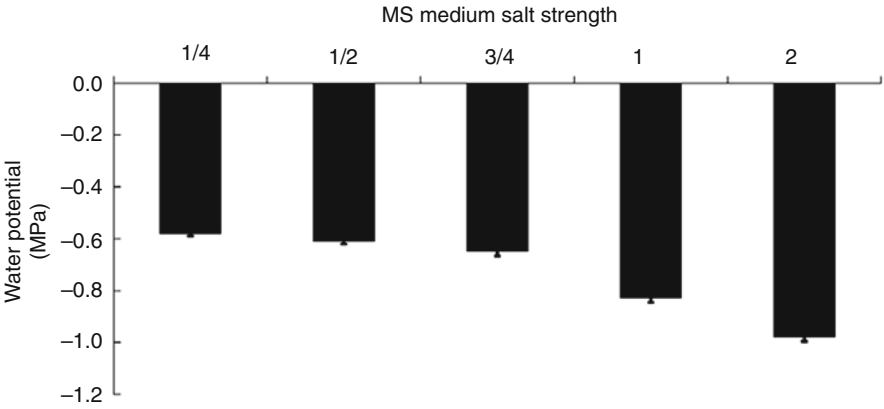

Fig. 10.4 Effect of strength of MS medium on water potential in the medium after 5 weeks of culture. *Bars* represent means ± SE (n = 3)

Table 10.5 [23] illustrates the effects of different strengths of MS medium on biomass production in adventitious roots of *E. koreanum*. Biomass production was greater at low salt strengths (1/4, 1/2, and 3/4 MS) than that at high salt strengths (1 and 2 MS), and the highest fresh and dry weights were achieved at 1/2 MS after 5 weeks of culture. In contrast, the highest percentage of dry weight was obtained at the highest salt strength; dry weight at 2 MS was >60 % lower than that at 1/2 MS. In addition, roots cultured at 2 MS were shorter, thicker, and less numerous compared to those cultured at other salt strengths, whereas roots cultured at 1/4 MS were long and numerous but were so thin that they could not be continually subcultured (data not shown). On the other hand, biomass accumulation in roots cultured at low salt strength (especially 1/4 MS) was inhibited because of insufficient essential minerals.

The total mineral content in the culture medium directly affects water potential, which influences the ability of plantlets to take up minerals, water, and other components from the culture medium [24]. Water potential decreased substantially with increasing strength of the MS medium; >1 MS retarded root growth by sharply decreasing water potential in the medium (Fig. 10.4). Low water potential caused dehydration that resulted in stunted primary and secondary metabolic processes

Table 10.6 Accumulation of bioactive compounds in adventitious roots of *Eleutherococcus koreanum* as affected by strength of Murashige and Skoog (MS) medium after 5 weeks of culture

MS medium salt strength	Eleutheroside B (μg g^{-1} DW)		Eleutheroside E (μg g^{-1} DW)		Chlorogenic acid (mg g^{-1} DW)		Total phenolics (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)		Total target compounds (mg g^{-1} DW)	
1/4	36.65	c[a]	92.94	d	4.99	a	9.80	a	5.25	a	20.16	a
1/2	56.35	a	143.82	b	3.61	bc	8.32	c	4.07	b	16.20	b
3/4	56.82	a	165.42	a	3.26	c	7.46	d	3.33	c	14.27	c
1	49.01	b	148.81	b	2.53	d	7.02	d	2.72	d	12.46	d
2	47.43	b	130.73	c	3.70	b	9.27	b	3.70	bc	16.85	b

[a]Mean separation within columns by Duncan's multiple range test at $p = 0.05$

such as cell elongation, biomass accumulation, and biosynthesis of secondary metabolites. Consistent with this observation, Baque et al. [25] reported that high-strength MS medium induced osmotic stress that inhibited biomass production in adventitious roots of *Morinda citrifolia* because of low water potential.

The effects of MS medium on accumulation of bioactive compounds in adventitious roots of *E. koreanum* are presented in Table 10.6. The highest contents of eleutherosides B and E were obtained at 1/2 and 3/4 MS, whereas the other bioactive compounds tended to decrease with the increasing MS strength, with the exception of 2 MS. Eleutherosides B and E (lignins), are synthesized by different metabolic pathways than are other bioactive compounds such as chlorogenic acid. Therefore, eleutherosides B and E may exhibit different patterns from that of other bioactive compounds, even in roots cultured in the same medium. Roots grown in 2 MS showed the highest percentage of dry weight (i.e., contained the least water) when compared to other high salt strength treatments, thereby, possessed greater quantities of bioactive compounds. The highest root biomass and the greatest accumulation of each bioactive compound were achieved at different MS strengths. To determine the optimum MS medium salt strength for production of biomass and the target bioactive compounds, total production of bioactive compounds in roots (L $medium^{-1}$) was calculated based on dry weight after 5 weeks of culture. Total production of target bioactive compounds decreased with increasing strength of the medium. However, the highest production of eleutherosides B and E was observed at 1/2 and 3/4 MS, respectively. Although the total production of the target bioactive compounds was higher at 1/4 MS (99.80 mg L^{-1}) than at 1/2 MS (85.36 mg L^{-1}), we propose 1/2 MS as the optimal strength for production of biomass and bioactive compounds in *E. koreanum* because eleutherosides B and E are the primary bioactive compounds in genus *Eleutherococcus*, and roots cultured at 1/4 MS were too thin to perform continual subculturing. In addition, the highest fresh and dry weights were achieved at 1/2 MS. Previous studies also proposed that optimal medium strength for production of biomass and bioactive compounds could differ. For instance, Yu et al. [26] reported the maximum biomass at 1 MS, while lower salt strengths (1/4 and 1/2 MS) were suitable for the production of phenolic compounds in adventitious root cultures of *Panax ginseng*. Min et al. [16] also determined that 1/2 MS was optimal for the production of both biomass and bioactive compounds in

E. angustifolia adventitious roots. These results suggest that the strength of the medium must be controlled by manufacturing the target bioactive compounds according to the required purposes.

These results indicate that 1/2 MS is the optimal salt strength for producing biomass and bioactive compounds and for preventing physiological disorders caused by high osmotic stress in *E. koreanum* adventitious roots, in 3-L airlift bioreactors for a 5-weeks culture period. Evidence for this conclusion included high root biomass, high total production of target bioactive compounds in roots, and appropriate root morphology.

10.4.2 Effects of Nitrogen Source on Biomass and Production of Bioactive Compounds from Adventitious Roots

MS medium, which is widely used for *in vitro* culture technology, contains higher amounts of nitrogen (60 mM) when compared with other *in vitro* culture media. The total nitrogen source and NH_4^+:NO_3^- ratio affects the biomass and production of bioactive compounds [27, 28]. In general, low NH_4^+:NO_3^- ratios are more optimal for *in vitro* plant culture. However, some species perform well when NH_4^+ is provided as the sole nitrogen source [15]. Therefore, to produce large amounts of biomass and bioactive compounds using *in vitro* culture technology, the ratio of NH_4^+ to NO_3^- must be optimized according to the plant species, explant type, culture method, and target bioactive compounds.

Biomass production in adventitious roots of *E. koreanum* was strongly affected by the NH_4^+:NO_3^- ratio at an initial total nitrogen content of 30 mM (data not shown; [4]). The greatest fresh and dry weights were obtained at NH_4^+:NO_3^- = 5:25 and 10:20 mM, respectively, and percentage dry weight decreased with the increasing NH_4^+:NO_3^- ratio. High NH_4^+ concentration had a negative effect on root growth; root growth was strongly inhibited at NH_4^+:NO_3^- > 15:15 mM, and dry weight at NH_4^+:NO_3^- = 30:0 mM was 25 % of that achieved with the optimal NH_4^+:NO_3^- ratio for high root biomass. Therefore, NO_3^- was more essential than NH_4^+ for the biomass production of adventitious roots in *E. koreanum* and the optimal NH_4^+:NO_3^- ratio was either 5:25 or 10:20 mM. The accumulation of bioactive compounds in *E. koreanum* adventitious roots was also affected by the NH_4^+:NO_3^- ratio at an initial total nitrogen content of 30 mM (data not shown). The contents of chlorogenic acid, total phenolics, and flavonoids in roots decreased sharply with the increasing NH_4^+:NO_3^- ratio, whereas high concentrations of eleutherosides B and E were obtained at NH_4^+:NO_3^- ratios from 10:20 to 20:10 mM after 5 weeks of culture.

The highest biomass and accumulation of each bioactive compound in roots were achieved at different NH_4^+:NO_3^- ratios. Thus, to determine the optimal NH_4^+:NO_3^- ratio for production of biomass and the target bioactive compounds, total production of bioactive compounds in roots (L medium^{-1}) was calculated based on dry weight after 5 weeks of culture (Table 10.7). The total production of the target bioactive compounds decreased significantly with the increasing NH_4^+:NO_3^- ratio, except for NH_4^+:NO_3^- = 0:30. In addition, when NH_4^+ levels were higher than NO_3^- levels

Table 10.7 Total production of bioactive compounds in adventitious roots of *Eleutherococcus koreanum* (L medium^{-1}) as affected by NH_4^+:NO_3^- ratio at an initial total nitrogen content of 30 mM, after 5 weeks of culture

NH_4^+:NO_3^- (mM)	Eleutheroside B (μg L^{-1})	Eleutheroside E (μg L^{-1})	Chlorogenic acid (mg L^{-1})	Total phenolics (mg L^{-1})	Total flavonoids (mg L^{-1})	Total target compounds (mg $^{-1}$)
0:30	153.99	488.19	35.86	49.02	29.94	115.46
5:25	241.30	830.88	44.31	71.48	37.44	154.30
10:20	291.03	969.11	20.89	58.83	31.01	111.00
15:15	245.85	897.45	10.72	39.13	15.27	66.26
20:10	213.05	729.44	6.65	31.39	9.39	48.25
25:5	143.65	368.29	1.21	17.75	4.58	24.05
30:0	55.41	147.44	0.44	10.15	1.11	11.90

Value of total production = mean of dry weight (g L^{-1}) × mean of each bioactive compound content (mg g^{-1} DW)

(NH_4^+:$NO_3^- \geq$ 15:15), total production of the target compounds was approximately one-third lower than that obtained at NH_4^+:NO_3^- = 5:25, the ratio that showed the highest production of bioactive compounds (154.30 mg L^{-1}). Therefore, the optimal NH_4^+:NO_3^- ratio for total production of the target bioactive compounds was 5:25, while production of eleutherosides B and E was slightly higher at NH_4^+:NO_3^- = 10:20 than 5:25. Consistent with this observation, the highest biomass of *P. ginseng* adventitious roots was obtained at NH_4^+:NO_3^- = 7.19:18.50 mM, while production of total ginsenosides was highest when NO_3^- was used as the sole nitrogen source [29]. Cui et al. [30] also reported that the optimal NH_4^+:NO_3^- ratios for biomass and bioactive compound production differed and that patterns of accumulation of target bioactive compounds differed according to NH_4^+:NO_3^- ratios in the growth medium. In *Eleutherococcus senticosus* cell culture, production of chlorogenic acid decreased with increasing NH_4^+:NO_3^- ratio, whereas the highest production of eleutheroside E was obtained at NH_4^+:NO_3^- = 1:1 [31]. Thus, the NH_4^+:NO_3^- ratio in the medium must be optimized to achieve maximum production of biomass and bioactive compounds according to the plant species, explant type, culture method, and target compounds.

These results indicate that an NH_4^+:NO_3^- ratio of 5:25 mM is optimal for production of biomass and bioactive compounds in *E. koreanum* adventitious roots. This was evidenced by the highest root biomass and total production of the target bioactive compounds in the roots per liter of medium obtained at this ratio.

10.4.3 Effects of Carbon Source on Biomass and Production of Bioactive Compounds from Adventitious Roots

In vitro plantlets require carbon sources from the culture medium for biological processes including survival, growth, development, and bioactive compound accumulation. Sucrose is an essential substrate for carbon and energy metabolism and

Table 10.8 Effects of sucrose concentration on biomass accumulation in adventitious roots of *Eleutherococcus koreanum* after 5 weeks of culture

Sucrose conc. (%)	Fresh weight (g L^{-1})		Dry weight (g L^{-1})		Percentage dry weight	
1	39.90	b[a]	3.88	c	9.71	
3	46.13	a	6.01	a	13.02	
5	25.64	c	4.15	b	16.17	
7	16.63	d	2.99	d	17.95	
9	11.89	e	2.18	e	18.35	

[a]Mean separation within columns by Duncan's multiple range test at $p = 0.05$

polymer biosynthesis in *in vitro* culture technology. Moreover, sucrose is the major transport carbohydrate in higher plants and sucrose levels in higher plants affect the impact of sucrose-specific signaling on development, gene transport, primary and secondary metabolism, and defense responses [32, 33].

Table 10.8 illustrates the effects of different sucrose concentrations (1–9 %) on biomass production in adventitious roots of *E. koreanum*. The greatest fresh and dry weights were obtained at 3 % sucrose and root growth was significantly inhibited at ≥5 % sucrose. In contrast, the highest percentage of dry weight was obtained at the highest sucrose concentration (>40 % higher at 9 % than that at 3 % sucrose; the latter was optimal for biomass production). Root morphology was also affected by initial sucrose concentration after 5 weeks of culture (data not shown). Roots cultured in >5 % sucrose were shorter and thicker and had fewer new roots than those cultured under optimal conditions. In particular, roots cultured in 9 % sucrose became enlarged but did not produce new roots. Adventitious roots consume carbon sources (e.g., sucrose, glucose, and fructose) from the culture medium, and the ability to utilize these carbon sources is reduced above a certain concentration because of osmotic stress [30, 34]. Plant cell size is also affected by source-induced osmotic stress. In particular, cell size under high osmotic stress is smaller than that under optimal culture conditions because of dehydration [35]. Therefore, percentage dry weight increased substantially with the increasing sucrose concentration because of a decline in cell water content.

High sucrose concentrations had a negative effect on water potential in the growth medium after 5 weeks of culture; water potential at >5 % sucrose decreased significantly with the increasing sucrose concentration (Fig. 10.5). These results indicate that roots cultured in >5 % sucrose were exposed to high osmotic stress, which could directly stimulate the defense responses. Plants have two antioxidant systems (enzymatic and non-enzymatic scavenging systems) that provide protection by controlling the contents of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), and hydroxyl radical (OH^-) [36].

The H_2O_2 content in roots increased significantly with the increasing sucrose concentration, indicating elevated stress levels (Fig. 10.6a). In particular, the H_2O_2 content in roots cultured in 5 % sucrose (the initial sucrose concentration that stunted root growth) was twofold higher than that in 3 % sucrose, which was the optimal sucrose concentration for biomass production. H_2O_2 is a well-known

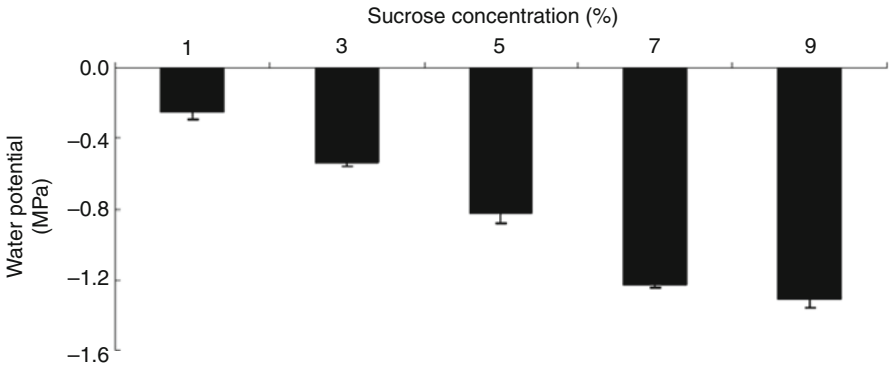

Fig. 10.5 Effect of sucrose concentration on water potential in the medium after 5 weeks of culture. *Bars* represent means ± SE (n = 3)

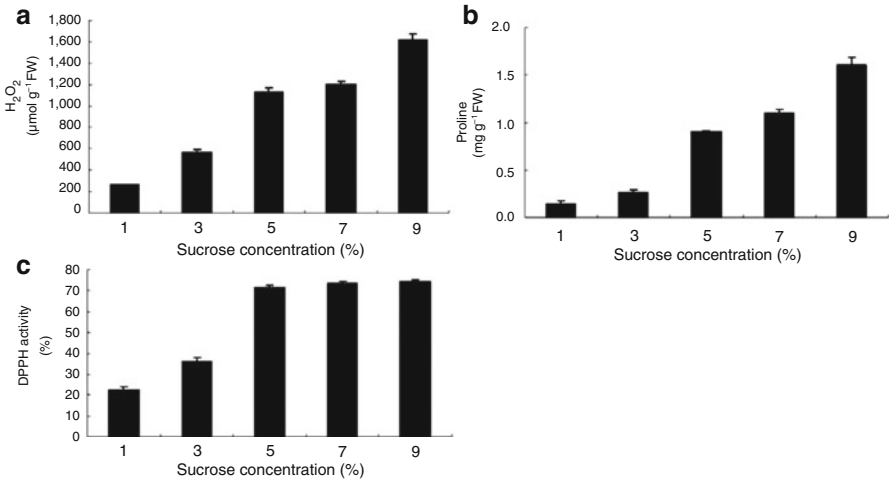

Fig. 10.6 (**a**–**c**) H_2O_2 content (**a**), proline content (**b**), and DPPH activity (**c**) in adventitious roots of *Eleutherococcus koreanum* affected by sucrose concentration after 5 weeks of culture. *Bars* represent means ± SE (n = 3)

substrate for inducing systemic acquired resistance [37], hypersensitivity resistance [38], senescence [39], and programmed cell death [40]. Therefore, the initial 5 % sucrose supply induced various defense mechanisms in *E. koreanum* adventitious roots as a result of sucrose-induced osmotic stress. After 5 weeks of culture, free proline content (Fig. 10.6b) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) activity (Fig. 10.6c) in roots showed a similar pattern as the H_2O_2 content, with the highest values achieved at the highest sucrose concentration. Accumulation of free proline is a plant adaptation, suggesting that the plants were exposed to salinity or water-deficit conditions [41], and DPPH activity is widely used to evaluate the antioxidant

activity of specific extracts [42]. Thus, the significant increase in free proline content and DPPH activity in roots with the increasing sucrose concentrations indicated elevated stress levels. Our results are similar to those reported by Cui et al. [43], who studied the responses of *Hypericum perforatum* adventitious roots using various sucrose treatments in suspension cultures. Similarly, free proline content in *M. citrifolia* adventitious roots was shown to increase significantly with the increasing osmotic stress [44].

The effect of sucrose concentration on the accumulation of bioactive compounds in *E. koreanum* adventitious roots is shown in Table 10.9. The highest contents of the target bioactive compounds was achieved at 5 % sucrose, which was the initial sucrose concentration that stunted root growth because of sucrose-induced osmotic stress, as evidenced by decreased root biomass and low water potential in the medium. The accumulation of each bioactive compound was stimulated under higher sucrose concentrations (5 and 7 %) compared to that under lower concentrations (1 and 3 %). In particular, the contents of chlorogenic acid, total phenolics, and flavonoids were lowest in the roots cultured in 1 % sucrose, in which no eleutheroside B was detected. These results indicated that the lack of a sugar source is a limiting factor for secondary metabolite synthesis in *E. koreanum* adventitious roots. The highest root biomass and accumulation of bioactive compounds were achieved at different sucrose concentrations. Thus, to determine the optimum sucrose concentration for production of biomass and the target bioactive compounds, total production of bioactive compounds in roots (L medium^{-1}) was calculated based on dry weight after 5 weeks of culture. The highest total production of the target compounds was observed with 3 % sucrose (158 mg L^{-1}), which was the optimal concentration for high root biomass. Consistent with this observation, the highest production of biomass, total phenolics, and flavonoids in adventitious roots of *H. perforatum* occurred in treatments with 3 and 5 % sucrose, respectively [43]. Shohael et al. [34] also reported that the contents of chlorogenic acid, total phenolics, and flavonoids in *Eleutherococcus sessiliflorus* embryos increased with increasing sucrose concentrations, but the productivity of eleutherosides B, E, and E_1 increased with 7 % sucrose. Thus, they insisted that the initial sucrose concentration must be optimized for the plant species, explant type, culture method, and target compounds [34].

Table 10.9 Accumulation of bioactive compounds in adventitious roots of *Eleutherococcus koreanum* as affected by sucrose concentration after 5 weeks of culture

Sucrose conc. (%)	Eleutheroside B (μg g^{-1} DW)		Eleutheroside E (μg g^{-1} DW)		Chlorogenic acid (mg g^{-1} DW)		Total phenolics (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)		Total target compounds (mg g^{-1} DW)	
1	0.00	d[a]	125.69	d	2.32	e	7.93	c	3.90	d	14.28	e
3	46.20	b	143.28	c	7.95	b	12.15	b	6.01	c	26.30	b
5	57.90	a	212.76	a	8.98	a	13.24	a	7.52	a	30.00	a
7	52.00	ab	214.84	a	7.13	c	13.06	a	7.11	b	27.57	b
9	27.11	c	168.65	b	5.61	d	11.67	b	6.13	c	23.61	d

[a]Mean separation within columns by Duncan's multiple range test at $p = 0.05$

These results indicate that 3 % sucrose is the optimal concentration of sugar for the production of biomass and bioactive compounds, and for the prevention of physiological disorders caused by high osmotic stress in *E. koreanum* adventitious roots. This was evidenced by the highest root biomass, high total production of the target bioactive compounds, and appropriate root morphology. Roots cultured in >5 % sucrose induced antioxidant system responses by controlling the contents of intracellular ROS, especially H_2O_2; these phenomena might increase free proline content and DPPH activity in roots.

10.5 Elicitation Strategies for Enhancing Productivity of Bioactive Compounds in Adventitious Roots

Synthesis of bioactive compounds in plants is a common response to biotic and abiotic stress, and accumulation of these compounds in plant cells and organs can be stimulated by exposure to stressors. Various elicitation strategies have been developed to stimulate synthesis of bioactive compounds in plant cells, tissues and organs. Exogenous elicitors usually interact with plant membrane receptors and activate specific genes, resulting in stimulation of synthesis of bioactive compounds [8, 45]. Among the various elicitors studied, methyl jasmonate (MJ) and salicylic acid (SA) are considered the most attractive in adventitious root culture for commercial purposes [46]. The addition of MJ and SA into liquid growth medium containing adventitious roots stimulates various signal-transduction pathways that catalyze the synthesis of several bioactive compounds in a range of plant species [47].

To enhance production of the target bioactive compounds in *E. koreanum* adventitious roots, various concentrations (0–400 μmol) of MJ and SA were applied to the 3-L airlift bioreactors for 1 week before harvest. After 6 weeks of culture, fresh and dry weights decreased with the increasing MJ and SA concentrations, but percentage dry weight was not influenced by MJ or SA elicitation (Table 10.10). The highest fresh and dry weights were obtained in the control treatment; roots cultured without MJ and SA elicitation for 6 weeks, and those to which 50 μmol MJ was added, did not exhibit negative effects on biomass production. Root growth at high MJ concentrations was better than that at high SA concentrations; dry weight at 400 μmol SA was >64 % lower than that in the control treatment. In general, the addition of MJ and SA as chemical elicitors to culture media induces various defense mechanisms in plants. Therefore, excess concentrations of elicitors have a negative effect on biomass production; up to 100 μmol MJ and 50 μmol SA inhibited root growth in *E. koreanum*, as evidenced by reduced fresh weight after 6 weeks of culture. Consistent with this observation, biomass production in *P. ginseng* adventitious roots [48], *S. parviflora* hairy roots [47], and *E. sessiliflorus* embryos [7] was significantly reduced with increasing MJ and SA concentrations. In particular, biomass production in adventitious roots of *P. ginseng* at high SA concentrations was lower than

Table 10.10 Effect of methyl jasmonate (MJ) and salicylic acid (SA) elicitation on biomass accumulation in adventitious roots of *Eleutherococcus koreanum* for 1 week[a]

Type	Conc. (μmol)	Fresh weight (g L^{-1})		Dry weight (g L^{-1})		Percentage dry weight	
Control		56.44	a[b]	7.36	a	13.03	
MJ	50	55.71	a	6.97	ab	12.52	
	100	51.56	b	6.53	bcd	12.66	
	200	50.48	b	6.32	cd	12.53	
	400	48.79	bc	6.03	d	12.36	
SA	50	51.89	b	6.83	abc	13.16	
	100	51.62	b	6.77	bc	13.12	
	200	46.56	c	6.01	d	12.91	
	400	40.64	d	4.70	e	11.58	

[a]Adventitious roots were cultured for 5 weeks before MJ and SA elicitation
[b]Mean separation within columns by Duncan's multiple range test at $p = 0.05$

Table 10.11 Accumulation of bioactive compounds in adventitious roots of *Eleutherococcus koreanum* subjected to methyl jasmonate (MJ) and salicylic acid (SA) elicitation for 1 week[a]

Type	Conc. (μmol)	Eleutheroside B (μg g^{-1} DW)		Eleutheroside E (μg g^{-1} DW)		Chlorogenic acid (mg g^{-1} DW)		Total phenolics (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)		Total target compounds (mg g^{-1} DW)	
Cont.		37.51	e[b]	124.48	b	9.10	b	13.30	e	7.42	e	29.97	e
MJ	50	39.57	e	171.26	a	11.22	a	22.22	ab	9.95	ab	43.61	a
	100	39.65	e	180.40	a	11.60	a	22.48	a	10.00	a	44.29	a
	200	39.50	e	177.92	a	9.81	b	21.66	b	9.70	b	41.39	b
	400	35.13	e	125.86	b	7.55	c	19.97	c	9.25	c	36.93	c
SA	50	65.64	d	134.56	b	9.66	b	14.68	d	7.98	d	32.52	d
	100	138.53	c	129.75	b	9.19	b	13.64	e	7.51	e	30.62	e
	200	293.12	b	101.46	c	6.35	d	10.85	f	5.30	f	22.89	f
	400	495.68	a	62.59	d	3.11	e	9.37	g	4.76	g	17.80	g

[a]Adventitious roots were cultured for 5 weeks before MJ and SA elicitation
[b]Mean separation within columns by Duncan's multiple range test at $p = 0.05$

that at similar MJ concentrations [48], and biomass production in *E. sessiliflorus* embryos decreased sharply to 200 μmol MJ [7]. In general, exogenous MJ and SA in plants regulate plant developmental responses and induce production of ROS, resulting in decreased biomass and increased productivity of bioactive compounds [49]. However, these two signal transducers act in different ways. The concentration of MJ in plants usually increases with insect and/or animal invasion and wounding responses, while SA concentration is usually involved in systemic acquired resistance to microbial pathogens [50]. Accordingly, the responses to root growth under SA elicitation were more inhibited than those under MJ elicitation in *E. koreanum*.

The effects of MJ and SA elicitation on accumulation of bioactive compounds are shown in Table 10.11. The highest total content of the target bioactive compounds

in roots was obtained at 100 μmol MJ (44.29 mg g^{-1} DW); contents of eleutheroside B, eleutheroside E, chlorogenic acid, total phenolics, total flavonoids, and total target compounds at 100 μmol MJ were 5.70, 44.92, 27.47, 69.02, 34.77, and 47.48 % higher, respectively, than that of the control treatments. MJ elicitation strongly stimulated accumulation of the target bioactive compounds but SA elicitation had a negative effect on their accumulation with the exception of eleutheroside B. Root eleutheroside B content increased sharply with the increasing SA, and was 12.21 % higher than that in the control treatments at 400 μmol SA. In general, the addition of MJ and SA to culture media stimulates the synthesis of bioactive compounds in adventitious roots. The different pathways of eleutheroside synthesis compared to those of other bioactive compounds [11] mean that eleutherosides may exhibit distinct patterns, including similar stress-signaling molecules. For example, the metabolic synthesis pathway of eleutheroside B was sharply stimulated by SA elicitation but was not influenced by MJ elicitation. Previous studies showed that the production of bioactive compounds in *P. ginseng* adventitious roots [48], *S. parviflora* hairy roots [47], and *E. sessiliflorus* embryos [7] was enhanced by MJ and SA elicitation, but the optimal elicitor type and concentration differed for each plant species, explant type, culture method, and target bioactive compound; the elicitor type and concentration must be optimized accordingly. The highest root biomass and accumulation of bioactive compounds were achieved at different MJ and SA concentrations. Thus, to determine the optimum elicitor type and concentration, total production of bioactive compounds in roots (L $medium^{-1}$) was calculated based on dry weight after 6 weeks of culture. Total production of the target bioactive compounds was increased by MJ elicitation compared to the control treatments. However, SA elicitation decreased the total production of the target compounds in roots with the exception of eleutheroside B, the contents of which were 744 % higher than that of the control at 400 μmol SA. The highest total production of the target compounds (304 mg L^{-1}) was observed at 50 μmol MJ, and was 38 % higher than that in the control treatment (221 mg L^{-1}). This elicitor type and concentration had no negative effects on biomass production after 6 weeks of culture.

Phenylalanine ammonia-lyase (PAL) activity in *E. koreanum* adventitious roots subjected to MJ and SA elicitation for 1 week is shown in Fig. 10.7. PAL activity in roots exhibited a similar pattern to that of bioactive compounds accumulated in the roots after 6 weeks of culture, and the highest PAL activity was obtained at 100 μmol MJ. PAL activity in roots at 100 μmol MJ was twofold higher than that in the control treatment and total accumulation of the target bioactive compounds in the roots at 100 μmol MJ was 1.5-fold higher than that in the control treatment. Addition of >200 μmol SA sharply decreased PAL activity and resulted in lower accumulation of biomass and target compounds in roots. High SA concentrations may inhibit primary and secondary metabolic processes in roots compared to that of control treatment. Elicitation strategies can modulate plant defense mechanisms, which may stimulate various metabolite synthesis pathways and lead to accumulation of large quantities of useful bioactive compounds [51]. PAL is a key enzyme initiating the transition from primary metabolism to phenylpropanoid metabolism and

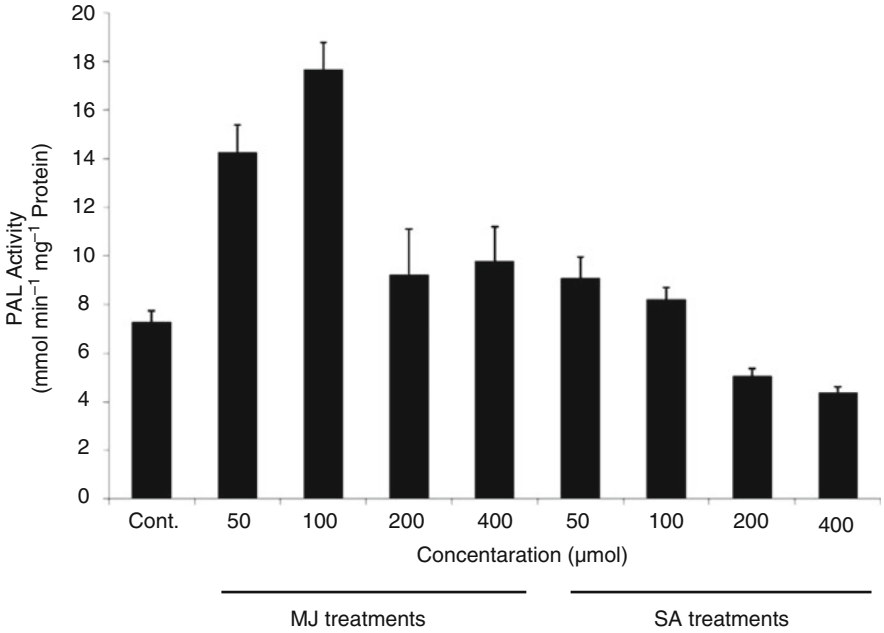

Fig. 10.7 Phenylalanine ammonia-lyase (*PAL*) activity in adventitious roots of *Eluetherococcus koreanum* subjected to methyl jasmonate (*MJ*) and salicylic acid (*SA*) elicitation for 1 week. *Bars* represent means ± SE (n = 3)

strongly stimulates the production of isoflavonoids and other phenolic compounds [32, 52]. Thus, enhanced PAL activity in plants can stimulate synthesis of bioactive compounds such as chlorogenic acid, total phenolics, and flavonoids in *E. koreanum* adventitious roots. There are several reports on the relationship between PAL activity and accumulation of bioactive compounds in *in vitro* explants. In *E. purpurea* hairy root cultures used for the production of caffeic acid derivatives and anthocyanin [53], *Daucus carota* hairy root cultures used for the production of total phenolics and flavonoids [54], and *Lupinus luteus* embryo axis cultures used for isoflavonoid production [32], PAL activity in the explants was high when they contained high concentrations of bioactive compounds. Wang et al. [52] confirmed that exogenous elicitors can increase accumulation of target bioactive compounds and PAL activity in cells.

These results indicate that the optimal elicitor type and concentration for enhancing total production of the target bioactive compounds was MJ elicitation at 50 μmol for 1 week, without harmful effects on biomass production in *E. koreanum* adventitious roots. In addition, increased PAL activity in the roots can enhance the production of phenolic compounds, especially chlorogenic acid, total phenolics, and flavonoids.

10.6 Comparison of Contents of Bioactive Compounds and Typical DNA Histograms Between Adventitious Roots and Field-Grown Plants

To verify the competitive ability of *E. koreanum* adventitious roots, contents of the target bioactive compounds were compared in three types of adventitious roots and four types of field-grown plants using high-performance liquid chromatography (HPLC). The following materials were analyzed: (1) adventitious roots without elicitation harvested after 6 weeks of culture; (2) adventitious roots subjected to 50 μmol MJ after 5 weeks of culture and harvested after 6 weeks of culture; (3) adventitious roots subjected to 50 μmol SA after 5 weeks of culture and harvested after 6 weeks of culture; (4) leaves of 3-years-old field-grown parental plants harvested in late September; (5) stems of 3-years-old field-grown parental plants harvested in late September; (6) thin roots (≤1 cm) of 3-years-old field-grown parental plants harvested in late September; and (7) thick roots (≥1 cm) of 3-years-old field-grown parental plants harvested in late September. The contents of bioactive compounds in adventitious roots and field-grown plants are presented in Table 10.12. The highest total content of the target bioactive compounds (DW basis) was achieved in adventitious roots subjected to 50 μmol MJ for 1 week (43.6 ± 0.56 mg g^{-1} DW), which was

Table 10.12 Bioactive compound contents in adventitious roots (*in vitro*) and field-grown (*ex vitro*) *Eleutherococcus koreanum*

Plant source	Eleutheroside B (μg g^{-1} DW)	Eleutheroside E (μg g^{-1} DW)	Chlorogenic acid (mg g^{-1} DW)	Total phenolics (mg g^{-1} DW)	Total flavonoids (mg g^{-1} DW)	Total target compounds (mg g^{-1} DW)
In vitro[a]						
AR-1	37.5 ± 3.66[b]	124.5 ± 6.54	9.10 ± 0.04	13.30 ± 0.25	7.4 ± 0.05	30.0 ± 0.34
AR-2	39.6 ± 1.34	171.3 ± 7.19	11.2 ± 0.80	22.2 ± 0.30	10.0 ± 0.08	43.6 ± 0.56
AR-3	65.6 ± 3.67	134.6 ± 4.50	9.7 ± 0.12	14.7 ± 0.30	8.0 ± 0.04	32.5 ± 0.31
Ex vitro[c]						
Leaves	135.4 ± 1.80	157.6 ± 14.87	6.2 ± 0.33	14.6 ± 0.74	9.4 ± 0.28	30.5 ± 0.62
Stems	88.1 ± 0.30	55.9 ± 1.43	0.6 ± 0.02	0.8 ± 0.12	0.6 ± 0.04	2.1 ± 0.11
Thin roots	86.6 ± 0.35	64.3 ± 6.41	1.7 ± 0.13	3.4 ± 0.32	2.0 ± 0.03	7.2 ± 0.38
Thick roots	86.0 ± 1.24	77.1 ± 8.86	1.6 ± 0.06	3.2 ± 0.02	1.9 ± 0.13	6.8 ± 0.14

AR-1 Adventitious root without elicitation, *AR-2* Adventitious roots with 50 μmol MJ elicitation for 1 week before harvesting, *AR-3* Adventitious roots with 50 μmol SA elicitation for 1 week before harvesting

[a]Data were taken after 6 weeks of bioreactor culture

[b]Values are means ± standard error (n = 5)

[c]Data were taken in late September in the field

the optimal elicitor type and concentration for biomass production. The contents of chlorogenic acid, total phenolics, and flavonoids in adventitious roots subjected to MJ and SA were strongly enhanced compared to those in field-grown plants, indicating that the culture conditions provided a competitive edge for commercial supply of *E. koreanum*-based bioactive compounds. Moreover, the content and quality of the bioactive compounds in the field-grown plants were strongly affected by environmental conditions and harvest time [6]. The highest concentration of bioactive compounds in field-grown *E. koreanum* was observed in late September; and these plants were unable to produce a controlled, year-round supply of bioactive compounds of adequate quality and quantity. However, using pilot-scale bioreactors, *in vitro E. koreanum* adventitious root cultures could produce a controlled supply of bioactive compounds appropriate for industrial use year round. Consistent with this observation, Ali et al. [55] reported that the accumulation of saponin in adventitious roots of *P. ginseng* and *P. quinquefolius* subjected to 200 μmol MJ was fourfold higher compared to that of untreated native ginseng roots. In addition, Wang et al. [10] identified ginsenoside composition in the native ginseng roots, adventitious roots, cells, and hairy roots using LC-MS and cluster analysis, and confirmed that the ginsenoside quality of the adventitious roots was similar to that in the native roots. In addition, total saponin content in adventitious roots was much higher than that in other *in vitro* explants.

The adventitious roots used in the present study were induced from *in vitro*-grown plants and were continuously maintained in half-strength MS medium (HN_4^+:NO_3^- = 5:25) supplemented with 5 mg L^{-1} IBA, 0.01 mg L^{-1} TDZ, and 30 g L^{-1} sucrose over a 3-years period. To verify the genetic safety of *E. koreanum* adventitious roots, ploidy levels of the adventitious roots and the 3-years-old field-grown plants were analyzed: (1) adventitious root tips (1 cm) after 3 weeks of culture, where the roots were undergoing an exponential growth phase and high rates of cell division; and (2) young leaves (0.5 × 0.5 cm) from a 3-years-old field-grown parental plant. The peak locations of stained nuclei from the adventitious roots and field-grown plants on typical DNA histograms were identical (Fig. 10.8). In general, adventitious root culture is an attractive method for producing large quantities of biomass and bioactive compounds because of rapid biomass accumulation, similar quality of bioactive compounds compared to their natural counterparts, stable productivity of bioactive compounds, and genetic stability compared to other plant tissue-culture methods [15, 46].

The present results indicate that adventitious root culture is an attractive method for year-round production of valuable *E. koreanum*-based bioactive compounds and that this method enables control of the quality and quantity of target compounds and verify genetic safety for industrial use. Adventitious roots subjected to 50 μmol MJ for 1 week demonstrated a competitive edge for commercial use as evidenced by their high concentrations of the target bioactive compounds and the identical peak location on typical DNA histograms compared to 3-years-old field-grown plants.

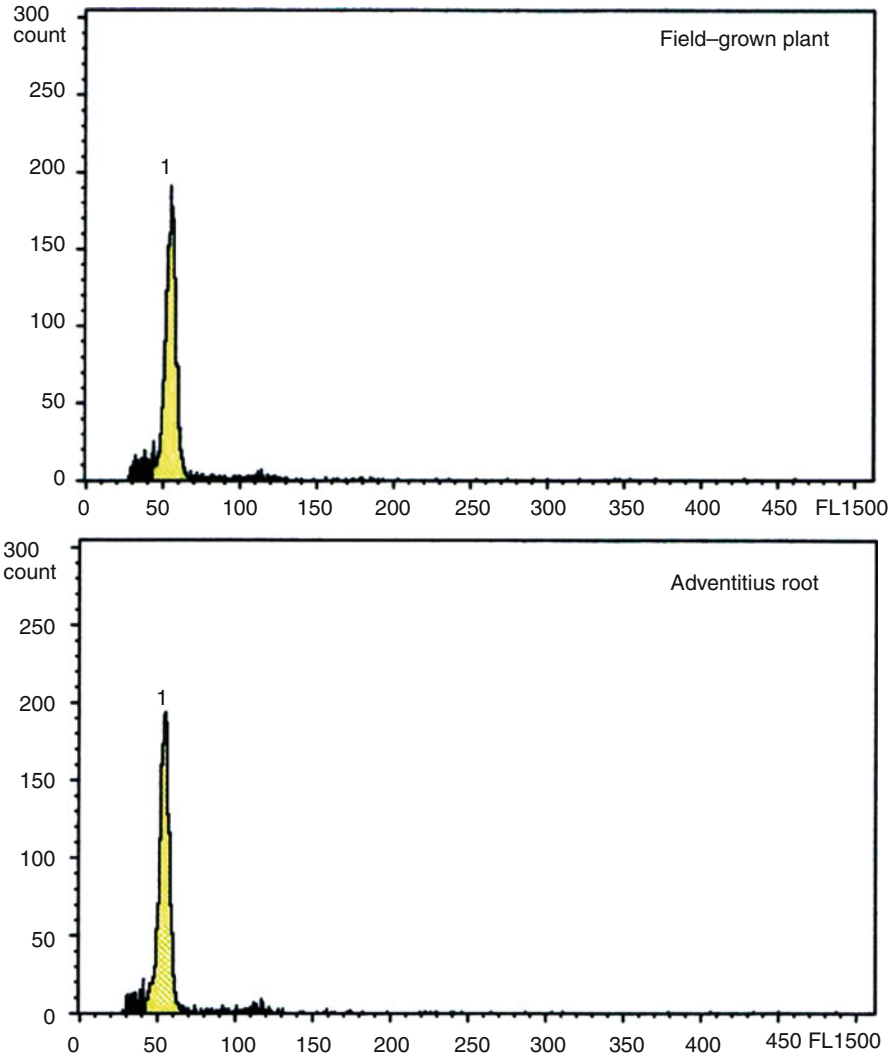

Fig. 10.8 Typical DNA histograms of stained nuclei of adventitious roots and field-grown *Eluetherococcus koreanum*

10.7 Conclusions

In vitro conditions strongly affect biomass and production of bioactive compounds in adventitious roots of *Eleutherococcus koreanum* Nakai cultured in 3-L airlift bioreactors. To establish an efficient method for year-round production of

E. koreanum-based bioactive compounds, the adventitious roots were tested with various physical and chemical factors (inoculum density, aeration volume, salt strength, nitrogen source, and sucrose concentration). An inoculum density of 5.0 g L^{-1} and aeration volume of 0.1 vvm were found to be optimal physical factors; half-strength MS medium, an NH_4^+:NO_3^- ratio of 5:25 mM, and 3 % sucrose were found to be optimal chemical factors for producing large quantities of biomass and bioactive compounds. Evidences for this conclusion included growth parameters (high root biomass and low root death), strong accumulation of the five target bioactive compounds (eleutherosides B and E, chlorogenic acid, total phenolics, and flavonoids), and the absence of physiological root disorders. Moreover, the addition of 50 μmol MJ for 1 week prior to harvest was an effective elicitation strategy for enhancing the final productivity of the target compounds; under these conditions, roots produced 304 mg L^{-1}, which was 38 % higher than that produced in the control treatment. Adventitious roots subjected to 50 μmol MJ for 1 week had a competitive edge and were genetically safe for commercial use as evidenced by the large concentrations of the target bioactive compounds and DNA histogram peaks identical to those of 3-years-old field-grown plants.

The development of such an *in vitro* culture protocol, which controls the quality and quantity of elicited bioactive compounds, will be beneficial for pilot-scale production of *E. koreanum*-based bioactive compounds for industrial use.

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Chapter 11
Production of Adventitious Root Biomass and Bioactive Compounds from *Hypericum perforatum* L. Through Large Scale Bioreactor Cultures

Xi-Hua Cui, Hosakatte Niranjana Murthy, and Kee-Yoeup Paek

Abstract *Hypericum perforatum* L. (St. John's wort) is a traditional medicinal plant with antidepressive and woundhealing properties. It contains a lot of constituents with documented biological activity including phenolics, a broad range of flavonoids, naphthodianthrones and phloroglucinols. In recent years plant cell, tissue and organ cultures have been developed as an important alternative sources for the production of high value secondary metabolites. The adventitious roots of *H. perforatum* are regarded as an effective means of biomass production due to their fast growth rates and stable metabolite productivity. To determine optimal culture conditions for the bioreactor culture of *H. perforatum* adventitious roots, experiments have been conducted on various chemical and physical parameters in flasks and bioreactors. Adoption of elicitation methods have shown enhancement in the accumulation of total phenolics and flavonoids. Based on these results, a large scale bioreactor system at the industrial level (100 and 500 L) was established for the production of biomass and secondary metabolites from the adventitious root cultures. To investigate the usefulness of the adventitious root cultures for the production of secondary metabolites, comparison of ploidy level and the contents of total phenolics and flavonoids in adventitious roots and field-grown plant was conducted. In addition, identification of the major constituents in adven-

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titious roots (hypericin, hyperin, quercetin and chlorogenic acid) were analyzed by Liquid Chromatography Coupled with Electrospray Ionization Tandem Mass Spectrometry (LC-MS/MS). Moreover, 12 isolated phenolic compounds from root cultures were evaluated for anti-inflammatory effects [Nuclear factor kappa B (NF-**κ**B) inhibition and Peroxisome proliferator-activated receptor (PPAR) activation effects] and five xanthones of them were also tested for antioxidant and anticancer activities.

Keywords Adventitious roots • Bioreactor cultures • *Hypericum perforatum* • Secondary metabolites

Abbreviations

ABTS	2, 2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)
B5	Gamborg
BA	N_6-benzyladenine
BTBB	Balloon type bubble bioreactor
DCFDA	2′,7′-dichlorfluorescein-diacetate
DPPH	1, 1-diphenyl-2-picrylhydrazyl
DW	Dry weight
EC	Electrical conductivity
ESI	Electrospray ionization
FW	Fresh weight
H_2O_2	Hydrogen peroxide
HepG2	Human hepatocarcinoma
IAA	Indole-3-acetic acid
IBA	Indole butyric acid
LC	Liquid chromatography
LH	Lactoalbumin hydrolysate
MDA	Malondialdehyde
MJ	Methyl jasmonate
MS	Murashige and Skoog
MS/MS	Tandem mass spectrometry
NAA	1-naphthalene acetic acid
NF-κB	Nuclear factor kappa B
PGR	Plant growth regulator
PPAR	Peroxisome proliferator-activated receptor
ROS	Reactive oxygen species
SA	Salicylic acid
TDZ	Thidiazuron
TNF-α	Tumor necrosis factor alpha
vvm	Air volume · culture volume^{-1} · min^{-1}

11.1 Introduction

Hypericum perforatum L. (St. John's wort) is an important traditional medicinal plant native to Europe that is grown worldwide for commercial purpose. It contains a large number of constituents with documented biological activity including phenolics, a broad range of flavonoids, naphthodianthrones, phloroglucinols and xanthones [1]. The herb of *H. perforatum* has been used in folk medicine as healing and anti-inflammatory agent since antiquity. Now-a-days, it has gained international popularity as it is used for the treatment of depression and as a dietary supplement, especially in Europe and the United States [2]. The antidepressant activity is attributed to hypericin, hyperforin, adhyperforin, which are the characteristic constituents of *H. perforatum* [3, 4]. Additionally, a broad range of flavonoids from this plant have also been shown to possess antidepressive activity but certainly act as co-effectors by improving pharmaceutical properties of other constituents such as hypericins [5, 6]. These bioactive compounds act by mutually expressing biological activities. Hence, the efficacy of medical preparations of *H. perforatum* is based on the whole mixture of metabolites, rather than the presence of a single constituent.

For commercial production of *H. perforatum*, field-grown plant material has generally been used. However, field production requires approximately 2 years and unfortunately, cultivars that are bred for increased secondary product accumulation are not necessarily disease resistant [7]. Moreover, the concentration of hypericin and pseudohypericin in *H. perforatum* extracts was not only very low (0.3 %), but also varied up to 50-fold in summer and winter grown plants [8]. Cell or organ cultures have emerged as a valuable route for biosynthesizing phytochemicals, and bioreactor-based systems have been developed for the production of ginsenosides [9], phenolics [10] and alkaloids [11]. There are reports on a large-scale *in vitro* growth system for the production of *H. perforatum* bioactive compounds using stem, but these techniques have limitations such as recalcitrance to liquid medium and the possibility of genetic variability in organ cultures [12]. In addition, cell suspension cultures of *H. perforatum* using flasks were established to study the production of bioactive compounds, but low biomass resulted in the drop of productivity of secondary metabolites [13, 14]. The bioreactor culture of adventitious roots is an effective means of biomass production due to fast growth rates, stable metabolite productivity and easy to scale up [15, 16]. In addition, compared to callus culture, adventitious roots are genetically stable and contain high secondary metabolites. In this review we present a protocol for *H. perforatum* adventitious root cultures for the production of bioactive compounds using bioreactor system.

11.1.1 Induction of Adventitious Roots and Their Maintenance

Adventitious roots of *H. perforatum* were induced by using leaf explants (0.5×0.5 cm) on full-strength Murashige and Skoog (MS) medium supplemented

with Gamborg (B5) [17] vitamins, 3 % (w/v) sucrose, 0.5 mg L^{-1} indole-3-acetic acid (IAA), and 2.3 g L^{-1} gelrite. Adventitious roots were maintained in MS liquid medium supplemented with B5 vitamins, 3 % (w/v) sucrose and 1.0 mg L^{-1} indole butyric acid (IBA) in 250 mL shake flasks (containing 70 mL of medium). Cultures were maintained in darkness at a temperature of 25 ± 1 °C on gyratory shakers at 100 rpm and were subcultured once in every 3 weeks.

11.1.2 Chemical and Physical Factors Affecting Adventitious Root Growth in Suspension Cultures

Effect of Plant Growth Regulators on the Adventitious Root Growth and Accumulation of Bioactive Compounds in Flask Culture

When adventitious root explants of *H. perforatum* were cultured in MS medium supplemented with various concentrations of auxins [IAA, IBA and 1-naphthaleneacetic acid (NAA); 0.5, 1.0, 2.0, 3.0 mg L^{-1}] over 2 weeks, lateral roots developed profusely in medium supplemented with IBA and IAA, whereas no lateral root development was observed in auxin-free medium (control) and in medium supplemented with NAA. Therefore, IBA and IAA were more effective for lateral root induction and root growth. In lower concentrations of IBA and IAA (0.5 mg L^{-1}) the roots were slender and elongated but in higher concentrations (3 mg L^{-1}), the lateral roots were shorter, thicker and numerous. As shown in Table 11.1, the greatest response in terms of biomass production (3.75 g $flask^{-1}$ FW and 0.27 g $flask^{-1}$ DW, respectively) and contents of phenolics and flavonoids (33.39 and 15.87 mg g^{-1} DW, respectively) was observed on medium containing 1 mg L^{-1} IBA. These responses were markedly suppressed when the medium was supplemented with >1 mg L^{-1} IBA. High auxin levels are often deleterious to secondary metabolite accumulation [18, 19]. Auxins are responsible for maintaining plant cell and tissue culture systems and are associated with the promotion of growth, callus proliferation, rooting and morphological diversity [20, 21]. Our experiments also demonstrated that the supplementation of the culture medium with NAA had a negative effect on the biomass and phenol and flavonoid contents. The positive effects of IBA on adventitious root growth in the present study are in concurrent with the reports by Wu et al. [16] and Kim et al. [22]. Biomass and phenolics contents in roots grown in medium containing IBA were higher than in medium containing IAA. Moreover, after 2 weeks, adventitious root growth and elongation (>1 cm) in the medium containing 1 mg L^{-1} IBA was better than the adventitious roots grown on the medium supplemented with other PGRs and the roots were adapted to continuous culture. Therefore, 1 mg L^{-1} IBA proved to be the best for adventitious root culture and phenolics accumulation among the growth regulators tested.

Table 11.1 Effect of different types and concentrations of auxins on adventitious root growth of *H. perforatum* and productivity of phenolics and flavonoids after 2 weeks of culture

Auxin (mg L^{-1})		FW (g $flask^{-1}$)		DW (g $flask^{-1}$)		Growth ratio	Total phenolics (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)	
Control	0	0.92	d[a]	0.12	d[a]	2.00	24.61	b[a]	12.35	bc[a]
IBA	0.5	3.49	ab	0.26	ab	5.50	31.36	a	17.19	a
	1.0	3.75	a	0.27	a	5.75	33.39	a	15.87	ab
	2.0	3.13	abc	0.22	c	4.50	16.67	c	8.49	c
	3.0	3.51	ab	0.23	bc	4.75	18.55	c	9.63	c
IAA	0.5	2.63	c	0.24	abc	5.00	27.15	b	14.41	ab
	1.0	3.10	bc	0.24	abc	5.00	25.34	b	15.38	ab
	2.0	3.30	ab	0.23	bc	4.75	24.05	b	14.17	ab
	3.0	3.34	ab	0.21	c	4.25	16.18	c	8.69	c
NAA	0.5	0.98	d	0.06	e	0.50	3.86	d	1.11	d
	1.0	1.03	d	0.06	e	0.50	2.74	d	0.50	d
	2.0	0.99	d	0.05	e	0.25	2.70	d	0.54	d
	3.0	0.94	d	0.06	e	0.50	2.79	d	0.69	d
Significance										
Auxin (A)		***		***			***		***	
Concentration (B)		NS		NS			***		**	
Interaction (A×B)		NS		NS			***		*	

NS, *, **, ***, Non-significant at $P \leq 0.1$, 0.01, 0.001, respectively
[a]Mean separation within columns by Duncan's multiple range test at 5 % level

Effect of Types and Concentrations of Cytokinins in Combination with 1 mg L^{-1} IBA on the Adventitious Root Growth and Accumulation of Bioactive Compounds in Flask Culture

When adventitious root explants of *H. perforatum* were cultured on MS medium supplemented with various concentrations of cytokinins combined with 1 mg L^{-1} IBA over 2 weeks (Table 11.2), the addition of 0.1 mg L^{-1} kinetin increased the root biomass and phenolic contents compared to the control (1 mg L^{-1} IBA). Especially, the addition of 0.1 mg L^{-1} kinetin increased fresh weight (4.00 g $flask^{-1}$) and dry weight (0.31 g $flask^{-1}$) by 9.5 and 19.2 %, respectively, when compared with the control. Similarly, phenolics (34.12 mg g^{-1} DW) and flavonoid contents (17.56 mg g^{-1} DW) increased by 15.7 and 20.7 % respectively. Also, in the present investigation, adventitious roots grown in liquid culture had higher content of secondary metabolites when compared to solid cultures. However, by the addition of N6-benzyladenine (BA), thidiazuron (TDZ) and >0.1 mg L^{-1} kinetin no adventitious roots were formed and only abnormal roots with thicker and elongated tips were observed. A combination of 0.1 mg L^{-1} kinetin and 1 mg L^{-1} IBA promoted root biomass, which may be due to the fact that adventitious root elongation nearly doubled when compared to

Table 11.2 Effect of different cytokinin treatments along-with 1 mg L^{-1} IBA on adventitious root growth of *H. perforatum* and productivity of phenolics and flavonoids after 2 weeks of culture

Cytokinin (mg L^{-1})		Fresh wt. (g $flask^{-1}$)		Dry wt. (g $flask^{-1}$)		Growth ratio	Total phenolics (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)	
Control (1 mg L^{-1} IBA)		3.65	f[a]	0.26	g[a]	5.50	29.48	b[a]	14.54	b[a]
Kinetin	0.1	4.00	ef	0.31	g	6.75	34.12	a	17.56	a
	0.5	4.52	de	0.43	f	9.75	24.42	c	11.86	bc
	1.0	4.77	cd	0.48	ef	11.00	22.08	cd	10.23	c
	2.0	5.12	bc	0.52	cde	12.00	22.05	cd	10.34	c
BA	0.1	4.99	bcd	0.5	de	11.50	21.41	cd	11.55	bc
	0.5	5.39	b	0.54	bcd	12.50	20.33	cd	10.20	c
	1.0	5.49	b	0.55	bcd	12.75	19.55	cd	8.86	c
	2.0	6.35	a	0.63	a	14.75	18.69	d	8.57	c
TDZ	0.01	6.25	a	0.59	ab	13.75	23.76	cd	11.22	bc
	0.05	6.06	a	0.56	bcd	13.00	20.04	cd	8.53	c
	0.1	6.12	a	0.56	bcd	13.00	22.41	cd	9.83	c
	0.2	6.39	a	0.57	abc	13.25	23.06	cd	8.58	c
Significance										
Cytokinin(A)		***		***			***		***	
Concentration(B)		***		***			**		***	
Interaction(A × B)		NS		**			*		NS	

NS, *, **, ***, Non-significant at $P \leq 0.1, 0.01, 0.001$, respectively
[a]Mean separation within columns by Duncan's multiple range test at 5 % level

the control. There are also reports by Lee [23] and Narayan et al. [24] where a combination of low cytokinin and high auxin levels increased biomass in cell cultures of *Eleutherococcus koreanum* and *Daucus carota* respectively.

Effect of Inoculum Density on the Adventitious Root Growth and Accumulation of Bioactive Compounds in Flask Culture

Inoculum density is an important parameter affecting the performance of suspended plant cells, tissues and organ cultures [25]. Final weights of root biomass of *H. perforatum* increased with elevated inoculum density but the growth ratio greatly decreased (Table 11.3). The maximum phenolic and flavonoid contents were obtained when 6 and 10 g L^{-1} FW of adventitious roots were used as inoculums. Moreover, when the inoculum density was lower than 6 g L^{-1} FW, only low numbers of lateral roots developed, whereas at inoculum densities of 8 and 10 g L^{-1} FW, high numbers of lateral roots developed resulting in growth ratio reductions of 31.8 and 44 %, respectively. Therefore, an inoculum density of 6 g L^{-1} FW is suitable to generate optimum adventitious root biomass and accumulation of phenolics and flavonoids during scale up cultivation.

Table 11.3 Effect of inoculum density on adventitious root growth and productivity of phenolics and flavonoids of *H. perforatum* after 2 weeks of culture

Inoculum density (g L^{-1} FW)	FW (g $flask^{-1}$)		DW (g flask)		Growth ratio	Total phenolics (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)	
2.0	3.03	d[a]	0.23	c	14.33	26.56	b	11.63	c
4.0	4.03	c	0.30	b	9.00	29.16	ab	13.45	b
6.0	4.15	bc	0.32	a	6.11	30.19	ab	13.98	ab
8.0	4.24	b	0.31	ab	4.17	27.86	b	13.00	b
10.0	4.38	a	0.33	a	3.40	33.44	a	15.98	a

[a]Mean separation within columns by Duncan's multiple range test at 5 % level

Effect of MS Salt Strength on the Adventitious Root Growth and Accumulation of Bioactive Compounds in *H. perforatum* During 5 Weeks of Flask Culture

Modified MS medium contains high mineral concentrations compared to other nutrient media [26]. In this study, adventitious roots were cultured in different strengths of MS medium (varied from 1/4, 1/2, 3/4, 1 to 2 MS) and harvested once in a week over a 5-week period. After 5 weeks, root growth (growth rate) at 1/2 MS, 3/4 MS and 1 MS were better than those at 1/4 MS and 2 MS (Fig. 11.1a–c). Higher salt strengths, especially 2 MS, inhibited root growth likely because of a low water potential which inhibited absorption of water and mineral nutrients from the medium. In contrast, at 1/4 MS, roots grew rapidly, but did not reach sufficiently high biomass levels, likely due to a lack of nutrients. At 1/2 and 3/4 MS, the peak growth rate (0.830 and 0.826, respectively) occurred at 4 weeks, but at 1 MS, the peak growth rate was after 5 weeks and reached 0.681. During 5th week, the growth pattern of adventitious roots typically showed a lag phase of 1 week, and an exponential phase from weeks 2 to 5 at 1 MS, but at 1/2 MS and 3/4 MS the exponential phase extended from week 2 to 4 and growth remained unchanged from week 4 onwards. Therefore, 1 MS was not optimal for biomass production due to a long exponential phase.

As shown in Fig. 11.1d–e, the accumulation of total phenolics and flavonoids decreased with the elevated MS salt strengths and total phenols and flavonoid contents at low nutrient levels (1/4 and 1/2 MS) were much higher than those at high nutrient levels. Therefore, 1/2 MS is preferable for achieving desirable root growth and phenolics accumulation. A similar observation was also made by Yu et al. [15] who reported that the maximum biomass was obtained in 1 MS medium, whereas lower salt strengths (1/2 and 2/3 MS medium) were suitable for both root growth and ginsenoside productivity. In Echinacea adventitious root cultures, 1/2 MS was suitable for biomass and metabolites [16].

Changes in the concentration of anions and cations in the medium were monitored continuously during the culture cycle (Fig. 11.2). Overall, mineral nutrient decreased over the time and the depletion of nutrients increased with the strength of MS medium. NH_4^+, K^+, NO_3^-, SO_4^{2-} and HPO_4^- were rapidly depleted from the medium and their uptake increased with MS salt strength.

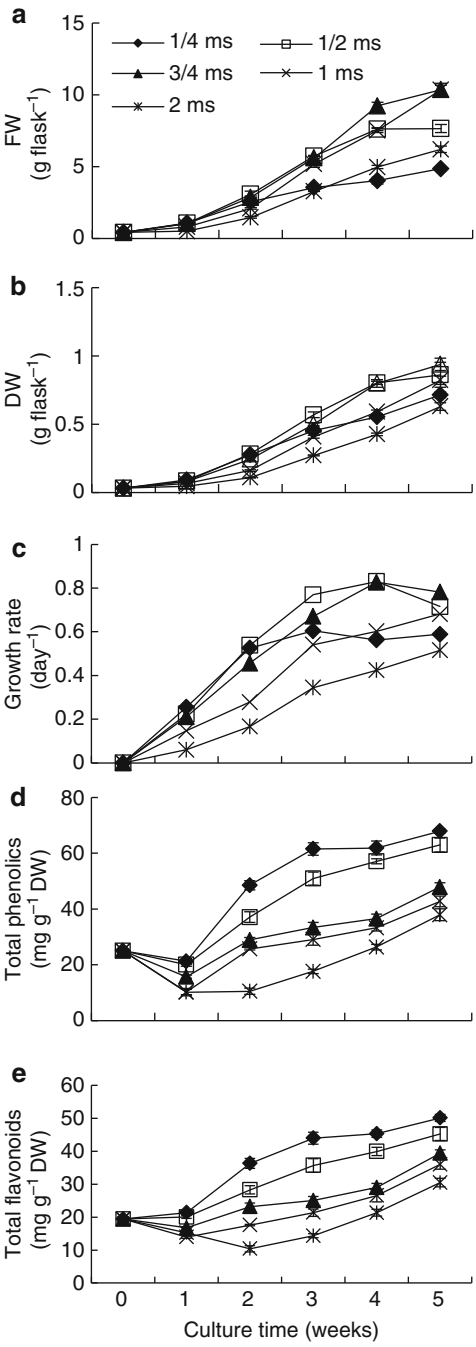

Fig. 11.1 Time profile for adventitious root growth (**a** FW, **b** DW, and **c** growth rate) and productivity of phenolics (**d**) and flavonoids (**e**) of *H. perforatum* as affected by MS medium salt strength cultured in 250 mL flasks. The *vertical bars* represent the standard error of three replicates

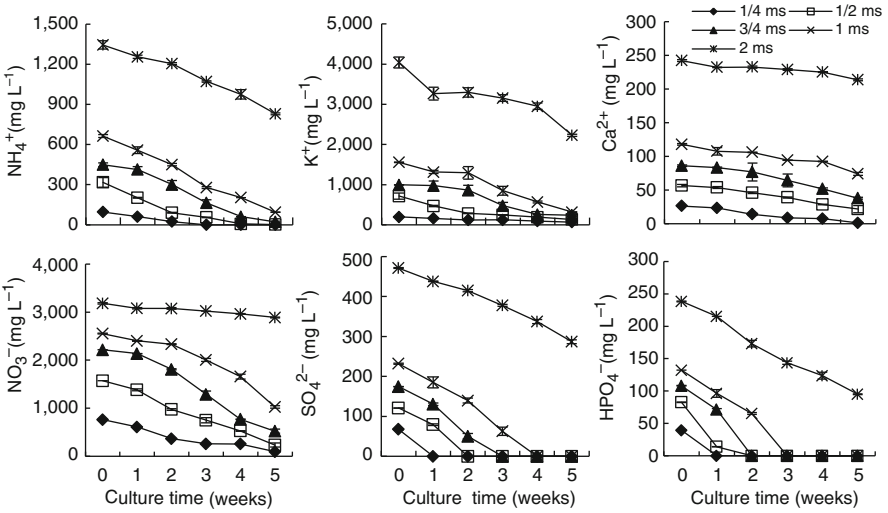

Fig. 11.2 Changes of cation and anion contents in the medium as affected by MS medium salt strength in adventitious root cultures of *H. perforatum* in 250 mL flasks. The *vertical bars* represent the standard error of three replicates

Preferential uptake of NH_4^+ at the beginning of cultivation was observed and the resulting acidification might have indirectly promoted NO_3^- uptake [27–29]. In 2 MS, NH_4^+ uptake did not promote NO_3^- uptake because the existing NH_4^+ supply made the uptake of NO_3^- unnecessary. The absorption of other nutrients showed a similar pattern in all treatments. At 1/2 MS, the absorption of mineral nutrients was rapid during the exponential phase from weeks 2 to 4. HPO_4^- was rapidly depleted from the medium and its concentration was near zero after 1 week, whereas NH_4^+ and SO_4^{2-} and were nearly depleted within 2 weeks. Preferential uptake of NH_4^+, HPO_4^- and SO_4^{2-} at the beginning of the culture has been observed for many species [30, 31].

Effect of Sucrose Concentration on the Adventitious Root Growth and Accumulation of Bioactive Compounds After 5 Weeks of Flask Culture

The results of accumulation of adventitious root biomass of *H. perforatum* cultured in the medium supplemented with different concentrations of sucrose [0, 1, 3, 5, 7 and 9 % (w/v)] over 5 weeks is presented in Table 11.4. The root biomass was optimum at 3 % (w/v) sucrose, resulting in maximum fresh weight and dry weight as well as growth ratio. However, higher sucrose concentrations [5, 7 and 9 % (w/v)] reduced dry weight by 31.51, 40.18 and 52.13 %, respectively, as compared with the response of 3 % (w/v) sucrose. In addition, the percentage dry weight increased with the increasing concentration of sucrose; however, the growth ratio was the

Table 11.4 Effect of sucrose concentration on biomass and metabolite accumulation of *H. perforatum* adventitious roots after 5 weeks of culture

Sucrose conc. % (w/v)	FW (g flask^{-1})		DW (g flask^{-1})		% of DW	Growth ratio	Chlorogenic acid (mg g^{-1} DW)		Total phenolics (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)	
0	0.26	f[a]	0.01	f	3.85	–	0	d	0.18	c	1.12	c
1	4.77	b	0.27	e	5.66	6.20	0.18	b	44.39	b	30.05	b
3	8.52	a	0.92	a	10.80	23.53	0.11	c	51.42	a	39.12	a
5	4.12	c	0.63	b	15.29	15.80	0.17	b	55.25	a	42.61	a
7	3.17	d	0.55	c	17.35	13.67	0.22	a	51.90	a	39.54	a
9	2.49	e	0.44	d	17.67	10.73	0.22	a	46.80	b	32.61	b

[a]Mean separation within columns by Duncan's multiple range test at 5 % level

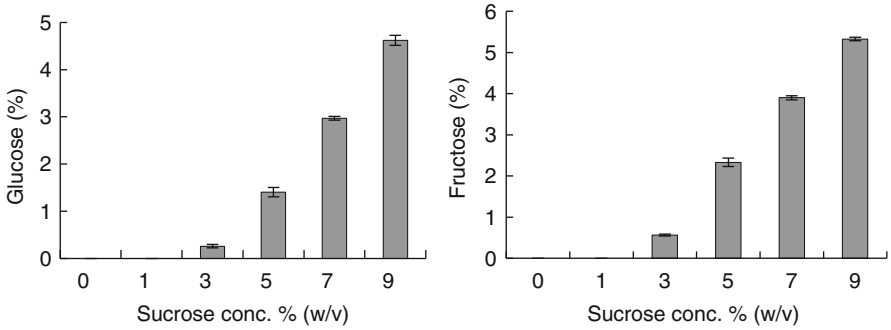

Fig. 11.3 Glucose and fructose contents in the medium in adventitious root cultures of *H. perforatum* as affected by the sucrose concentration after 5 weeks of culture. The *vertical bars* represent the standard error of three replicates

highest with the medium supplemented with 3 % (w/v) sucrose. These results indicate that higher sucrose concentration beyond 3 % (w/v) level inhibits root growth and this reduced root biomass accumulation might be due to the higher osmotic potential of cultured cells/organs. Similar observations of reduction in biomass with the increased osmotic stress are on record with cell suspension cultures of tobacco [32]. The accumulation of total phenols and flavonoids were optimum with cultures supplemented with 3, 5 and 7 % (w/v) sucrose (Table 11.4). Similarly, higher level of chlorogenic acid was accumulated with adventitious roots cultivated in the medium supplemented with higher concentrations of [3, 5 and 7 % (w/v)] sucrose. These findings demonstrate that higher initial sucrose concentration enhanced accumulation of total phenolics, flavonoids, and chlorogenic acid. This might be due to elevated levels of osmotic stress [16, 33].

The analysis of residual sugar levels (sucrose, glucose and fructose) of the spent medium from 5-week-old cultures revealed that all the exogenous sucrose had been utilized by the adventitious roots with the cultures supplemented with 1 % (w/v) sucrose, whereas, less than 1 % glucose and fructose was remained with the cultures which were initially supplemented with 3 % (w/v) sucrose (Fig. 11.3). However,

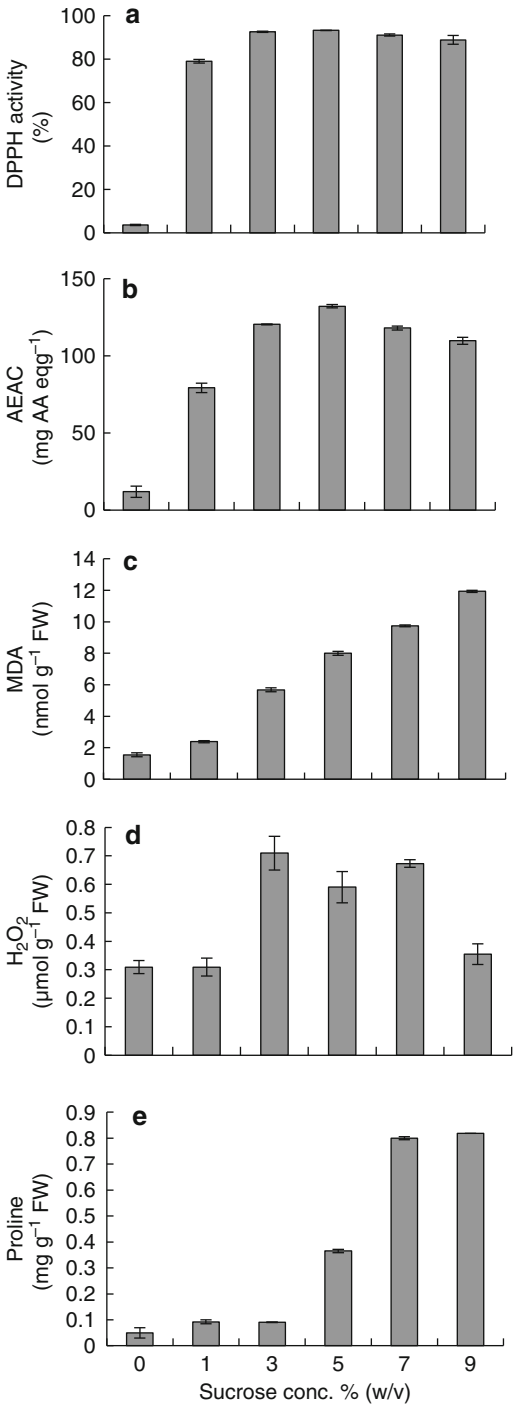

Fig. 11.4 Effect of sucrose concentration on DPPH activity (**a**), total antioxidant activity (AEAC **b**), MDA content (**d**), H_2O_2 content (**c**) and proline content (**e**) in adventitious roots of *H. perforatum* cultured for 5 weeks. The tested concentration was 1 mg mL^{-1}, respectively. The *vertical bars* represent the standard error of three replicates

higher levels of glucose and fructose were present in the cultures which were initially supplemented with higher concentration of sucrose [5, 7 and 9 % (w/v)]. Thus, supplementation of cultures with 3 % (w/v) sucrose was found suitable for the cultivation of adventitious roots of *H. perforatum*. Concentrations of 2–3 % (w/v) sucrose have frequently been reported as optimal for suspension cultures and higher concentrations of sucrose have been found to repress the growth [32].

Sucrose acts as an osmotic agent that may introduce osmotic stress above certain concentration [34]. In the present study, the osmotic stress effect of sucrose was assessed by using the free radical scavenging compounds like 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and 2, 2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The DPPH scavenging activities were 92.63, 93.32 and 91.17 % (1 mg mL^{-1}) with *H. perforatum* root extracts which have been cultivated in the 3, 5 and 7 % (w/v) of sucrose respectively (Fig. 11.4a). ABTS radical scavenging activities of the extracts were also showed similar effects (Fig. 11.4b). The roots which were grown in the medium supplemented with higher levels of sucrose accumulated higher amounts of phenolics (Table 11.4). Such elevated levels of phenolics in the roots grown in the medium with higher levels of sucrose are obvious and are helpful in free radical scavenging.

To verify the effect of osmotic stress on lipid peroxidation, the levels of hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and proline were determined in the adventitious roots. Formation of MDA was considered as a measure of lipid peroxidation that was induced by the roots exposed to osmotic stress. This was confirmed by the higher level of MDA in the adventitious roots treated with elevated sucrose (Fig. 11.4c). Osmotic stress in plant cells produces sub-cellular damage that impairs electron transport system and leads to the production of reactive oxygen species (ROS) [35]. ROS imbalances the cellular redox systems and in favor of oxidized forms, inactivate enzymes cause lipid peroxidation and ultimately leading to potential damage to DNA [36]. The production of H_2O_2 (Fig. 11.4d) was lower in the adventitious roots grown in the medium supplemented with lower levels of sucrose [0 and 1 % (w/v)], whereas the adventitious roots grown in higher concentrations of sucrose [3, 5 and 7 % (w/v)] showed higher levels of H_2O_2. Thus, it is speculated that a cascade of events, including lipid peroxidation and accumulation of H_2O_2 contents may be involved in the induction of secondary metabolite accumulation. The above observations reveal that osmotic stress is a pre-requisite for secondary metabolite synthesis.

Proline accumulation is one of the adaptations of plants to salinity and water deficiency [37]. In the present study, proline content in adventitious roots significantly increased with elevated sucrose concentration indicating the elevated stress levels (Fig. 11.4e). Such a phenomenon was also observed with *in vitro* culture of *Spathiphyllum cannifolium* shoots, where proline content increased with the elevated salt strengths in MS medium [38]. Therefore, when sucrose concentration was higher than 3 % (w/v), the adventitious roots of *H. perforatum* were much affected by water deficit stress. In this study, H_2O_2 content, MDA content and proline content increased with elevated sucrose concentration. Therefore, water deficit-stress might

Table 11.5 Effect of NH_4^+:NO_3^- ratio in half-strength MS medium on biomass and metabolite accumulation of *H. perforatum* adventitious roots after 5 weeks of culture

Nitrogen source (NH_4^+:NO_3^-)	FW (g flask^{-1})		DW (g flask^{-1})		Growth ratio	Chlorogenic acid (mg g^{-1} DW)		Total phenolics (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)	
0:30	4.18	c[a]	0.73	c	18.47	0.11	b[a]	39.95	a	32.02	b
5:25	9.96	a	1.08	a	27.80	0.10	bc	40.41	a	34.00	a
10:20	8.60	b	0.88	b	22.47	0.13	ab	41.96	a	34.87	a
15:15	3.77	c	0.54	d	13.40	0.14	a	40.86	a	34.20	a
20:10	4.20	c	0.44	e	10.73	0.14	a	39.87	a	31.97	b
25:5	0.99	d	0.16	f	3.27	0.06	c	22.72	b	15.42	c
30:0	0.32	e	0.05	g	0.33	0.02	d	21.55	b	12.56	d

[a]Mean separation within columns by Duncan's multiple range test at 5 % level

be considered to be alleviated or prevented by increased non-enzymatic scavenging system, such as amino acid proline accumulation.

Effect of Ammonium/Nitrate Ratio on the Adventitious Root Growth and Accumulation of Bioactive Compounds After 5 Weeks of Flask Culture

The effect of the NH_4^+:NO_3^- ratio on adventitious root biomass accumulation of *H. perforatum* was investigated after 5 weeks of culture by using a total initial nitrogen level of 30 mM (Table 11.5). In this study, nitrate, rather than ammonium, nitrogen was found to result in better root growth and higher accumulation of phenolics and flavonoids. The optimum biomass of 9.96 g/flask FW and 1.08 g/flask DW were obtained when the NH_4^+:NO_3^- ratio was 5:25. The highest phenolic and flavonoid contents (40.41 and 34 mg g^{-1} DW) were also obtained under a NH_4^+:NO_3^- ratio of 5:25 (Table 11.5). As shown in Table 11.5, chlorogenic acid was optimum when the NH_4^+:NO_3^- ratio was either 15:15 or 20:10. These results suggest that the NH_4^+:NO_3^- ratio of 5:25 is suitable for not only optimum biomass production of adventitious roots but also for the optimum accumulation of phenolics and flavonoids. It is a general observation that a lower NH_4^+ to NO_3^- ratio is more favourable for plant tissue and cell growth [39]. In *Panax notoginseng,* Zhang et al. [40] showed that the cell growth was negligible in a medium containing ammonium but no nitrate, and that of saponin production increased as the ratios of NH_4^+:NO_3^- decreased. The present results are also in accordance with this report, the nitrogen of nitrate rather than of the ammonium found to be more essential for root growth and the accumulation of phenolics and flavonoids.

The antioxidant potential of a methanolic extract, measured as the DPPH and ABTS radical scavenging activities of *H. perforatum* adventitious roots showed that antioxidant activity was higher in root extracts from the adventitious roots grown on higher concentrations of NO_3^- nitrogen (15, 20 and 25 mM) (Fig. 11.5a, b). Further, assessment of H_2O_2 and MDA content of the root extracts revealed that cultures

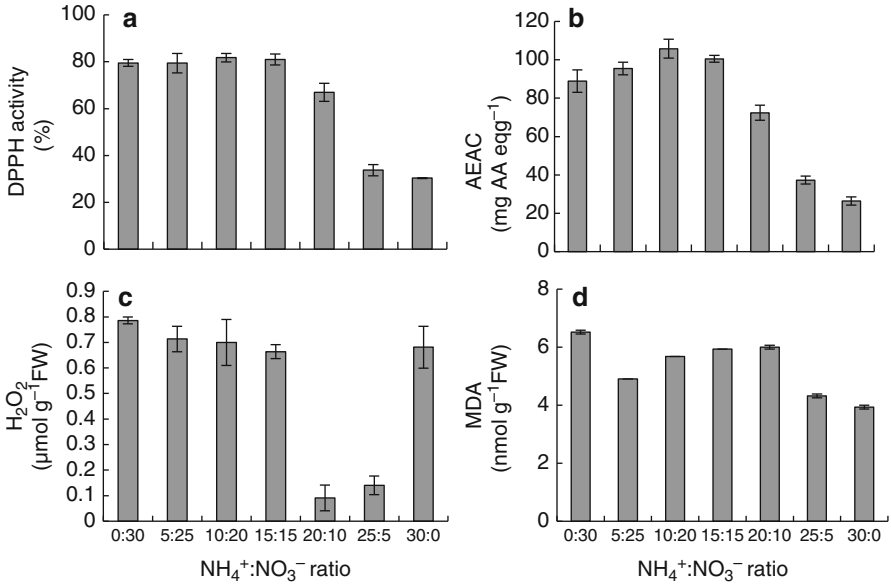

Fig. 11.5 Effect of NH_4^+:NO_3^- ratio on DPPH activity (**a**) and total antioxidant activity (AEAC **b**), H_2O_2 content (**c**) and MDA content (**d**) in adventitious roots of *H. perforatum* cultured for 5 weeks. The tested concentration was 1 mg mL^{-1}, respectively. The vertical bars represent the standard error of three replicates

supplemented with higher levels of NO_3^- nitrogen (15–30 mM) were under oxidative stress, which boosted the levels of secondary metabolites in the adventitious roots (Fig. 11.5c, d). These results indicate that adventitious roots experience oxidative stress when NO_3^- levels were higher than NH_4^+ levels or when NH_4^+ was provided as a sole nitrogen source. H_2O_2 content under NH_4^+ stress may be alleviated by the increased activities of scavenging enzymes. In concurrence to our observations, Nandwal et al. [41] reported that lipid peroxidation goes along with ethylene formation, which increases with elevated levels of NO_3^-. However, Wang et al. [42] and Nimptsch and Pflugmacher [43] found a reduced content of MDA under ammonia stress and speculated that the increased antioxidant response alleviated or prevented lipid peroxidation.

Effect of Inoculum Density on Biomass and Metabolites Accumulation in Bioreactor Culture

The relationship of inoculum density of the cultured cells and organs with biomass and metabolite accumulation has been studied repeatedly [44–47]. For example, Moreno et al. [46] investigated the production of ajmalicine from *Catharanthus roseus* suspension cultures at cell inoculum densities of 100 and 400 g FW L^{-1} and reported sixfold higher ajmalicine content and a 2.5-fold higher total ajmalicine

Table 11.6 Effect of inoculum density on biomass and metabolite accumulation of *H. perforatum* adventitious roots after 5 weeks of bioreactor culture

Inoculum density (g L^{-1} FW)	FW (g L^{-1})		DW (g L^{-1})		Growth ratio	Chlorogenic acid (mg g^{-1})		Total phenolics (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)		Total polysaccharides (mg g^{-1} DW)	
1.5	95.07	b[a]	13.83	b	85.42	0.65	b	55.14	b	38.04	b	65.92	c
3.0	110.45	ab	15.74	a	48.17	1.07	a	58.99	a	39.87	a	70.64	b
6.0	111.35	ab	15.66	a	23.46	1.08	a	60.47	a	43.41	a	72.49	ab
9.0	112.68	ab	15.59	a	15.15	0.95	ab	60.42	a	42.30	a	74.70	ab
12.0	124.11	a	16.11	a	11.53	1.07	a	59.12	a	40.13	a	75.83	a

[a]Mean separation within columns by Duncan's multiple range test at 5 % level

content in the low density cultures (inoculum density of 100 g FW L^{-1}). The effect of inoculum size on biomass and secondary metabolite accumulation determined in the present study are presented in Table 11.6. Accumulation of biomass was achieved with 3 g L^{-1} FW inoculum. Increased inoculum densities (6.0–12.0 g L^{-1} FW) were not beneficial as they were responsible for the decrease in biomass (Table 11.6). The accumulation of bioactive compounds was optimum with an inoculum density of 3 and 6 g L^{-1} FW (Table 11.6). Therefore, inoculum size of 3 g L^{-1} FW was suggested for bioreactor cultures as this inoculum size is responsible for both higher biomass and secondary metabolites. Figure 11.6a, b shows DPPH and ABTS radical scavenging activities of adventitious roots cultivated using different inoculum size. DPPH activity and ABTS activities were correlated with metabolites accumulation. H_2O_2 and MDA levels in the adventitious roots cultivated using different inoculum density are presented in Fig. 11.6c, d. MDA content decreased with increased inoculum density (Fig. 11.6c) and this might be due to increased antioxidant response of adventitious roots which might be due to accumulation of phenolics in the roots.

The Effect of Aeration Volume on Biomass and Metabolite Accumulation in Bioreactor Culture

The air supply into bioreactor cultures play two important roles: one is to supply dissolved oxygen for metabolic activities, and the other is improvement in agitation [48]. The factor that influences effective oxygen transfer in the plant cell cultures must be carefully analyzed for the bioreactors design [11, 49]. The adventitious root growth of *H. perforatum* under five models of aeration (0.05, 0.1, 0.2, or 0.3; or 0.05–0.3 vvm) over 5 weeks is presented in Table 11.7. The aeration of the bioreactor cultures at 0.05 and 0.1 vvm was optimal for the biomass accumulation and growth ratio. However, when the bioreactor was operated with 0.2 and 0.3 vvm, turbulent flow was produced and it was responsible for lower biomass accumulation and growth values (Table 11.7). The optimal production of chlorogenic acid, total phenolics and total flavonoids, as well as enhanced root growth was achieved

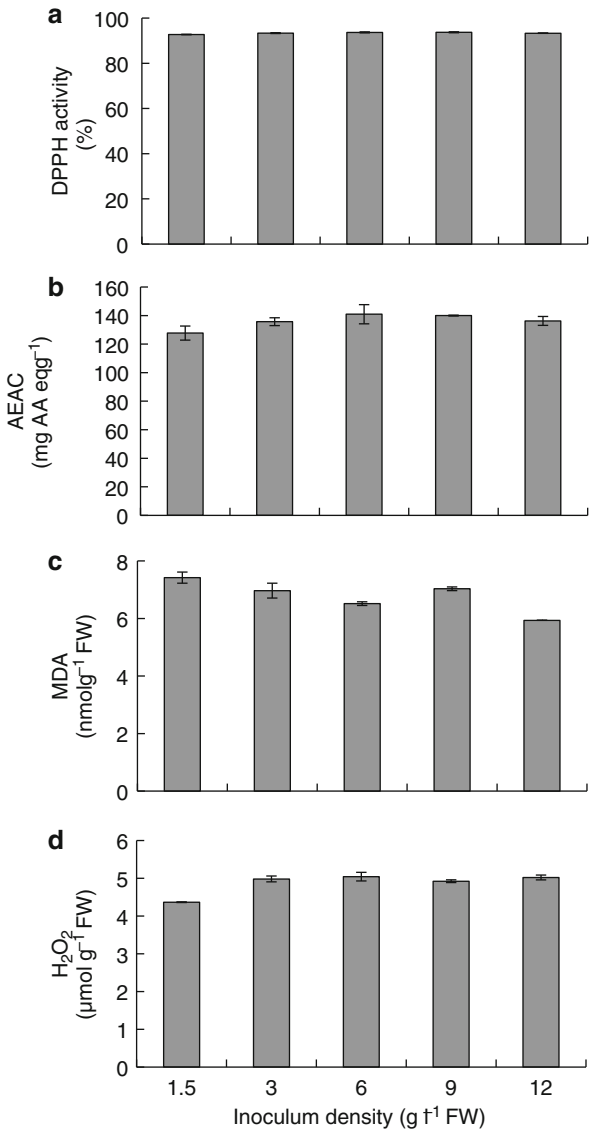

Fig. 11.6 Effect of inoculum density on DPPH activity (**a**), total antioxidant activity (AEAC) (**b**), MDA content (**c**) and H_2O_2 content (**d**), content of *H. perforatum* adventitious root extract after 5 weeks of culture. The tested concentration was 1 mg mL^{-1}, respectively. The *vertical bars* represent the standard error of three replicates

at 0.1 vvm (Table 11.7). It has been reported that maintaining a constant, high aeration volume throughout the culture period inhibited root growth due to sheer stress [21, 30]. However, Ahmed et al. [48] reported that cell growth of *Morinda citrifolia* was positively affected by a high aeration volume (0.3 vvm), since it resulted in the highest growth rate. The reason for this might be the faster cell growth at the beginning of the culture period (Days 2–3) [21] compared with that of the adventitious roots (Days 5–7) [30, 48]. The DPPH and ABTS radicals scavenging activities were also assessed and correlated with metabolites accumulation (Fig. 11.7a, b).

Table 11.7 Effect of aeration volume on biomass and metabolite accumulation of *H. perforatum* adventitious roots after 5 weeks of bioreactor culture

Aeration volume (vvm)	FW (g L^{-1})		DW (g L^{-1})		Growth ratio	Chlorogenic acid (mg g^{-1})		Total phenolics (mg g^{-1} DW)		Total flavonoids (mg g^{-1} DW)		Total polysaccharides (mg g^{-1} DW)	
0.05	104.21	a[a]	15.14	a	59.56	0.79	b	54.13	b	36.68	c	74.35	a
0.1	102.70	a	15.12	a	59.48	0.83	ab	56.22	a	39.07	abc	79.01	a
0.2	98.76	b	14.99	a	58.96	0.84	ab	56.23	a	39.46	ab	79.55	a
0.3	93.71	c	14.43	b	56.72	0.93	a	55.98	ab	40.96	a	74.29	a
0.05–0.3	104.17	a	15.28	a	60.12	0.79	b	56.39	a	38.01	bc	79.22	a

[a]Mean separation within columns by Duncan's multiple range test at 5 % level

Quantitation results of H_2O_2 and MDA levels in the adventitious roots cultivated with different aeration volume are presented in Fig. 11.7c, d. This could be due to either removal of key volatiles such as carbon dioxide and ethylene from the cultured medium or due to a direct oxygen toxicity resulting from a high level of dissolved oxygen [50, 51].

11.1.3 Growth Kinetics of Adventitious Roots

To determine the exact stage at which maximum biomass production occurs and to evaluate the accumulation of bioactive compounds, adventitious root biomass at a concentration of 3 g L^{-1} FW (0.25 g L^{-1} DW) was inoculated into the bioreactors. As shown in Fig. 11.8a, the adventitious root growth typically exhibited a lag phase from 0 to 1 week, an exponential phase from 1 to 5 weeks, stationary phase from 5 to 6 weeks, and a declining phase thereafter. After 6 weeks of cultivation, the biomass reached its peak (15.07 g L^{-1} DW), which was approximately 50 times higher than that of the dry weight (DW) of the initial inoculum. A similar pattern of growth behavior has been observed in *Echinacea purpurea* adventitious root cultures [31]. The specific growth rate (μ) of the *H. perforatum* adventitious roots was rapid during the initial days, with the peak (0.218) occurring at 14th day, which gave a doubling time (Td) of 3.18 days.

Figure 11.8b shows the electrical conductivity (EC) and hydrogen ion concentration (pH) of the medium during the course of the experiment. The conductivity of the culture medium decreased over time, which reflects the increase in biomass accumulation. It has been reported that the conductivity values reflect nutrient uptake by the cells; therefore, EC measurements have been used as an indirect method of biomass estimation [52]. As shown in Fig. 11.8c, linear correlations and a high calculated correlation coefficients (r^2 0.93) were observed between cell mass and medium conductivity. In biotechnological processes, cells serve as "factories" that convert substrates into products. Hence, monitoring cell growth during the

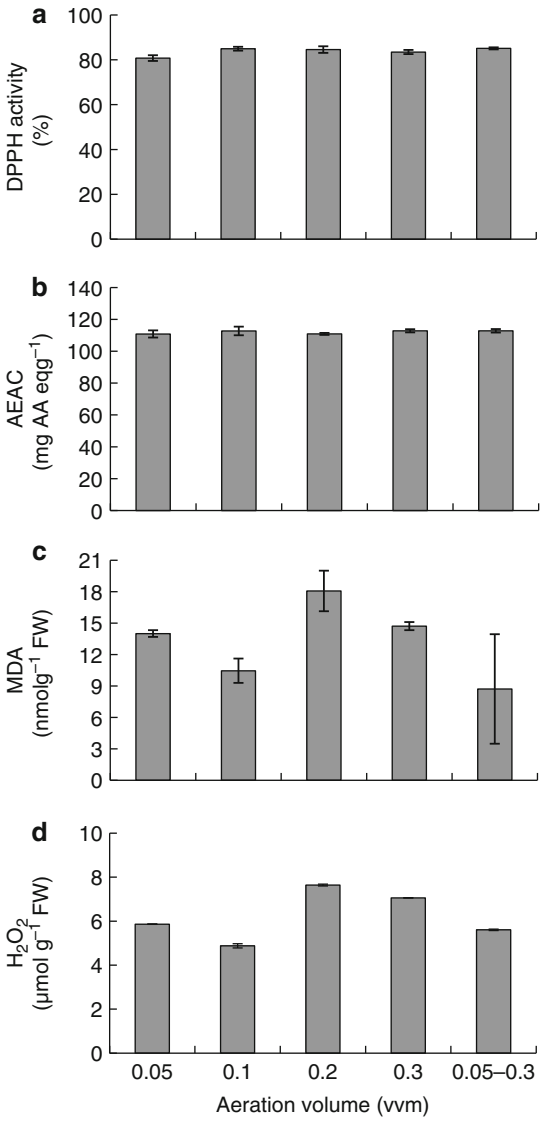

Fig. 11.7 Effect of aeration volume on DPPH activity (**a**), total antioxidant activity (AEAC) (**b**), MDA content (**c**) and H_2O_2 content (**d**) of *H. perforatum* adventitious root extract after 5 weeks of culture. The tested concentration was 1 mg mL^{-1}, respectively. The *vertical bars* represent the standard error of three replicates

cultivation is essential (especially during large-scale). Timely information on the physiological status of plant cells allows more effective control and management of the biosynthetic processes. Therefore, a prompt measure can be obtained by exploiting the known linear relationship between changes in medium conductivity and cell growth [53].

The pH of the medium increased gradually to 5.6 after the second week and then remained steady (Fig. 11.8b). In general, cell/organ cultures are effectively maintained and the nutrient uptake from the medium is accessible when the medium pH

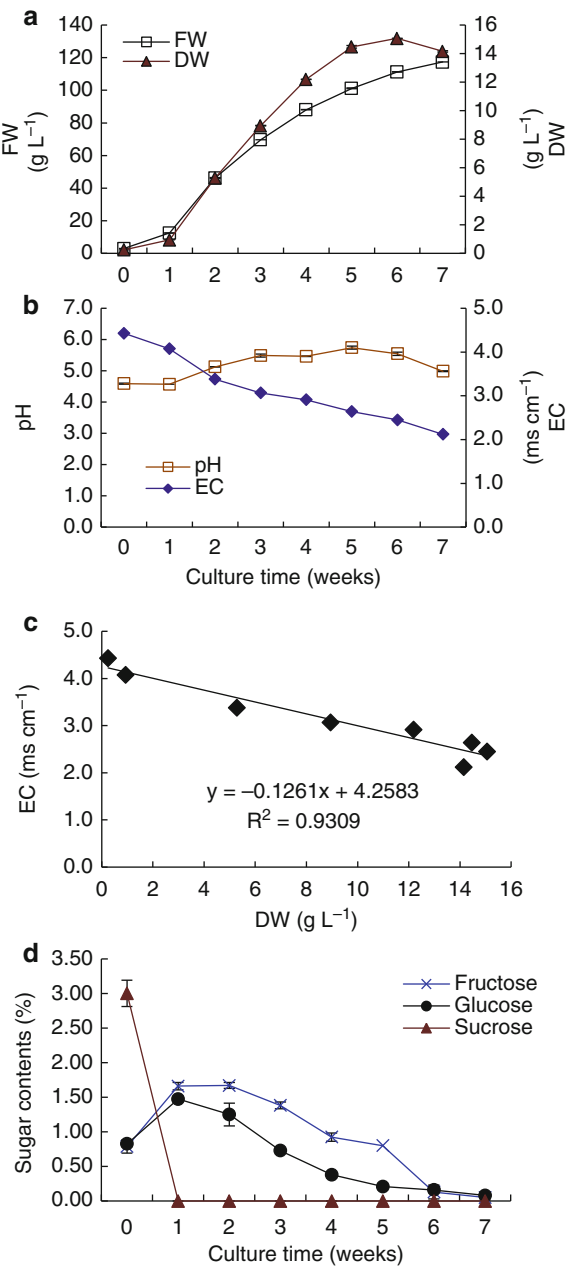

Fig. 11.8 Changes in fresh and dry weights of adventitious roots (**a**), changes in EC and pH (**b**) and sugar (**d**) contents of medium of *H. perforatum* during 7 weeks of culture. Relationship between changes in medium conductivity and biomass concentration (**c**) of *H. perforatum* during 7 weeks of culture. The *vertical bars* represent the standard error of three replicates

is between 5.0 and 6.0 [31, 45]. Therefore, in the present study, the change of medium pH might had no effect on the nutrient uptake from the medium.

The residual sugar levels in the media were measured at weekly intervals during the culture period to determine the sugar uptake patterns of the adventitious roots

(Fig. 11.8d). During the culture period, the entire carbon source for the culture medium was nearly exhausted. The sucrose concentration (3 %) rapidly decreased to zero after 1 week. In addition, even though only sucrose was added to the medium, monosaccharide utilization was noted over the cultivation period. Concentrations of glucose and fructose were increased significantly during the first week and then they gradually decreased. Sucrose apparently was being hydrolyzed to glucose and fructose likely by acid invertase that was secreted from the adventitious root tissue into the medium, as previously reported [54, 55].

Total phenolics and flavonoids production are shown in Fig. 11.9a. The accumulation of total phenolics and flavonoids increased linearly with time for the first 6 weeks, with the highest values being 61.1 and 45.7 mg g^{-1} DW, respectively. Chlorogenic acid, vitamin C, vitamin E and total polysaccharide accumulation in the *H. perforatum* adventitious root cultures are shown in Fig. 11.9b–e. The amount of chlorogenic acid (Fig. 11.9b) was initially low, but increased gradually and an optimum concentration (0.9 mg g^{-1} DW) was observed after 7 weeks. The amount of vitamin C (Fig. 11.9c) initially increased rapidly and reached its highest values of 0.7 mg g^{-1} FW at the end of the fourth week. The concentration of vitamin E (Fig. 11.9d) also initially increased quickly and a maximum amount of 4.3 mg g^{-1} FW was measured at the end of the fourth week. The amount of total polysaccharides (Fig. 11.9e) increased gradually, with the highest value of 71.9 mg g^{-1} DW at the end of the fourth week.

Figure 11.10a shows DPPH and ABTS radical scavenging activities of methanolic extracts of adventitious roots cultivated in bioreactors. The DPPH and AEAC activities were optimum with increase in accumulation of biomass and secondary metabolites when higher phenolics and flavonoids, chlorogenic acid, vitamin C and vitamin E were present (Fig. 11.9a–d). The amounts of H_2O_2 and MDA levels in the adventitious roots cultured in bioreactors are presented in Fig. 11.10b, c. The H_2O_2 levels increased for 4 weeks. However, the MDA levels were highest after 6 weeks. These results indicated that lipid peroxidation occurred in *H. perforatum* adventitious roots, probably as a consequence of the higher H_2O_2 levels. H_2O_2 in plants acts as a secondary messenger to signal subsequent defense reactions in plants [56]. Based on the present results, we speculate that accumulation of H_2O_2 content in adventitious roots may be involved in the induction of secondary metabolite accumulation.

11.1.4 Identification and Quantitation of Hypericin, Quercetin and Hyperoside by Liquid Chromatography Coupled with Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS)

To identify quercetin and hyperoside in the adventitious roots of *H. perforatum*, standards were analyzed by LC-MS/MS in electrospray ionization (ESI) negative mode. MS analyzed through ion scans revealed quercetin at m/z 300.92 and

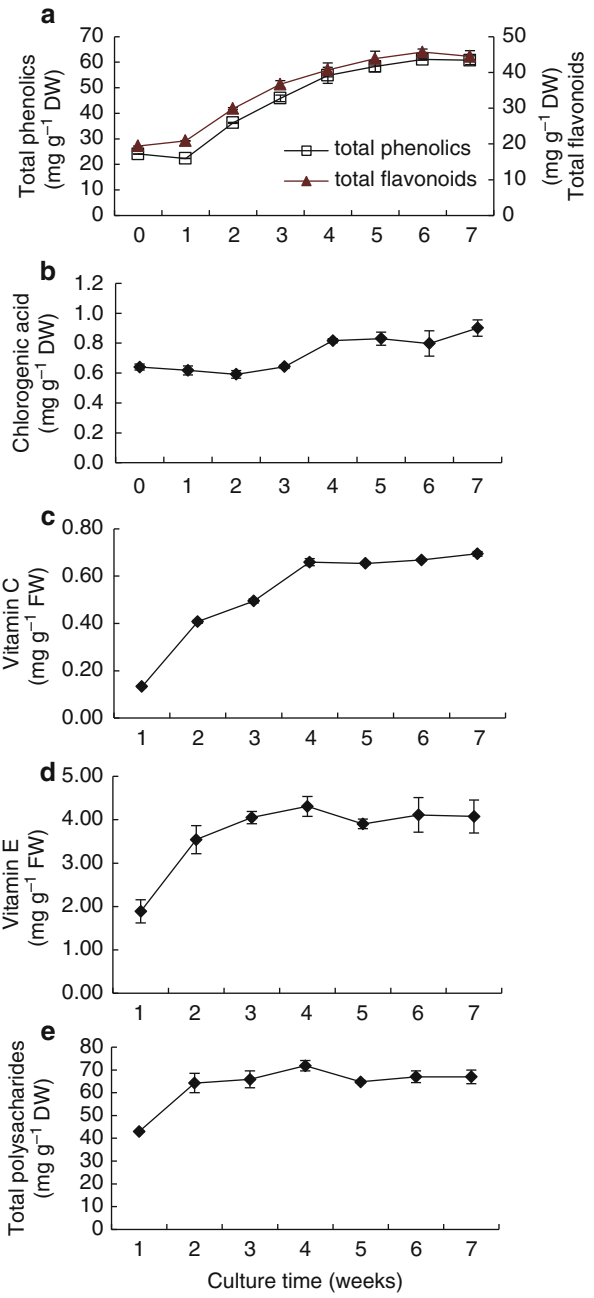

Fig. 11.9 Changes of total phenolics and flavonoids (**a**), chlorogenic acid (**b**), vitamin C (**c**), vitamin E (**d**) and total polysaccharide (**e**) contents in adventitious root of *H. perforatum* during 7 weeks of culture. The *vertical bars* represent the standard error of three replicates

hyperoside at m/z 463. Collision fragment ion (MS/MS) analysis of quercetin and hyperoside gave collision induced fragment ion spectra identical to those reported by Gadzovska et al. [57] and Tatsis et al. [58]. MS/MS data showed fragment ions

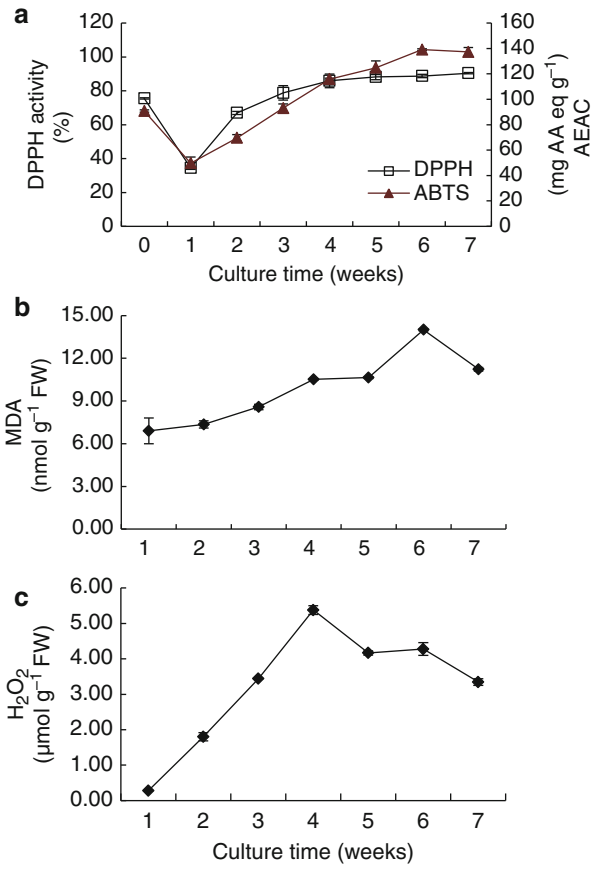

Fig. 11.10 Changes of DPPH activity, total antioxidant activity (AEAC) (**a**), MDA content (**b**) and H_2O_2 content (**c**) in *H. perforatum* adventitious root extract during 7 weeks of culture. The tested concentration was 1 mg mL^{-1} respectively. The *vertical bars* represent the standard error of three replicates

as follows: quercetin (m/z 178.92); hyperoside (m/z 301.00). Figure 11.11a, b shows the LC-MS/MS chromatogram of the standard and the extract from the adventitious roots of *H. perforatum* using ESI negative mode. In Fig. 11.11a, the standard of quercetin and hyperoside were detected at 19.53 and 17.27 min, respectively. In the adventitious roots, quercetin and hyperoside were also detected the same retention time (Fig. 11.11b).

The quercetin and hyperoside contents of adventitious roots analyzed by LC-MS/MS were 0.97–1.39 and 2.34–14.53 μg g^{-1} DW, respectively during 7 weeks of culture (Table 11.8). The quercetin content of adventitious roots remained steady during 7 weeks. However, the hyperoside content was low in the first 2 weeks and then increased quickly in subsequent weeks. In addition, MS analysed through ion scans approved that hypericin (*m/z* 503) were also present in adventitious roots (data not shown). These results indicated a clear identification of hypericin, quercetin and hyperoside in the adventitious roots of *H. perforatum* and the possibility of the production of quercetin, hyperoside and chlorogenic acid in bioreactor culture of adventitious roots.

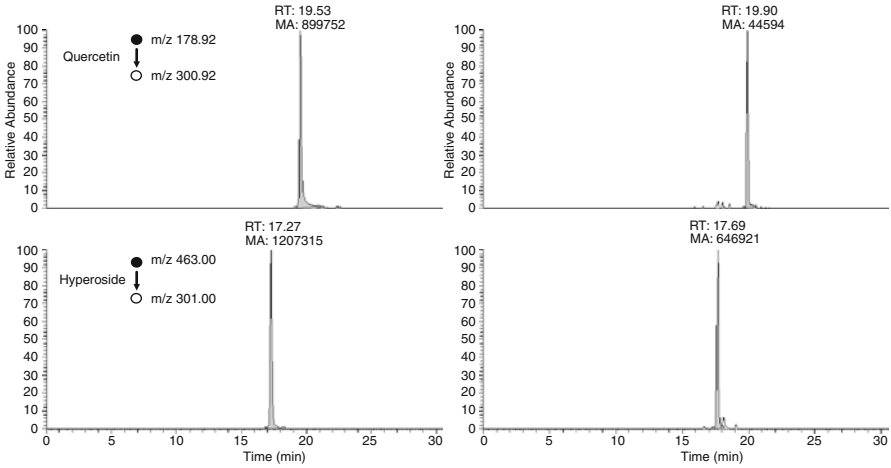

Fig. 11.11 LC–MS/MS chromatogram of quercetin and hyperoside standards (**a**) and adventitious root extract (**b**) from *H. perforatum* using ESI negative mode

Table 11.8 Quantification of quercetin and hyperoside by LC-ESI-MS/MS in the adventitious roots of *H. perforatum*

	Compounds (μg g^{-1} DW)	
Weeks	Quercetin	Hyperoside
1	1.006	2.337
2	0.974	3.684
3	1.251	9.125
4	1.390	12.665
5	1.309	14.534
6	1.328	14.005
7	1.342	13.655

The quercetin and hyperoside contents of adventitious roots are very small when compared to the 2–4 % flavonol glycosides concentrations of naturally grown plants [6]. It can be conjectured that the protected environment in which adventitious root are grown may be responsible for the low flavonoid accumulation due to the lack of the stress, normally found in nature. Therefore, enhancement of flavonoids using elicitors will be necessary.

11.1.5 Elicitation

Elicitation is generally the most effective strategy to enhance the production of secondary metabolites in plant cell and tissue cultures. Stress signaling molecules like methyl jasmonate (MJ) or salicylic acid (SA) are frequently used in elicitation experiments with adventitious roots. However, the growth of adventitious roots is

inhibited by application of elicitor in adventitious root cultures. Therefore, a two-stage culture method to maximize both biomass and secondary metabolite accumulation in *H. perforatum* can be adopted: the root biomass could be maximized on the medium determined to be optimal, cultured on the same medium until the highest biomass (without elicitor) is achieved and could be added with elicitors for the accumulation of secondary metabolites.

Addition of MJ (0, 50, 100, 150, 200 μmol) to the culture of *H. perforatum* before 1 week of harvest (5 weeks, 6 weeks) decreased the root dry mass but significantly increased phenolics compounds production (total phenolics, total flavonoids, and chlorogenic acid) compared to control. The maximum total production of phenolic compounds (per 1 L medium) was obtained at 100 μmol MJ treatment after 5 weeks of addition. Similar to the present study, Kim [25] reported that the total ginsenoside content linearly increased with increasing concentration of MJ up to 150 μmol but the growth of root was inhibited by MJ concentration.

SA (0, 50, 100, 150, 200 μmol) and lactalbumin hydrolysate (LH; 0, 25, 50, 100, 200 mg L^{-1}) addition before 1 week of harvest of *H. perforatum* (6 weeks) had no effect on enhancement of phenolics compounds. However, SA and LH significantly increased the accumulation of total polysaccharides without changing biomass. 50 μmol SA and 200 μmol LH resulted in the highest production of total polysaccharides but LH was more suitable for not causing reduction in phenolic compounds. Wang et al. [59, 60] reported that addition of 100 mg L^{-1} LH significantly enhanced ginsenoside and polysaccharide contents in *P. quinquefolium* cell cultures. Earlier studies reported that the treatment with SA increased H_2O_2 level [23, 61] which possibly played a key role in providing the system acquired resistance [62, 63] in plants. In this study, SA did not induce phenolic compound accumulation in adventitious root cultures of *H. perforatum* may be due to low content of H_2O_2 when compared with the control or may be due to insensitivity to SA.

11.2 Scale-Up Production of Secondary Metabolites Through Adventitious Root Culture

Based the above results of 3 L bioreactor cultures, we cultivated industrial scale cultures of *H. perforatum* adventitious roots in 500 L air lift bioreactors (Fig. 11.12). We could able to achieve 6.3 kg dry biomass of adventitious roots and these roots possessed higher amounts of total phenolics (66.9 mg g^{-1} dry mass) and total flavonoids (48.67 mg g^{-1} dry mass). The root growth pattern in the scale-up bioreactors was same as that of root growth pattern in small scale bioreactors. It can be regarded as one of the few successful plant cell/tissue culture scale-up examples for both biomass accumulation and secondary metabolite production.

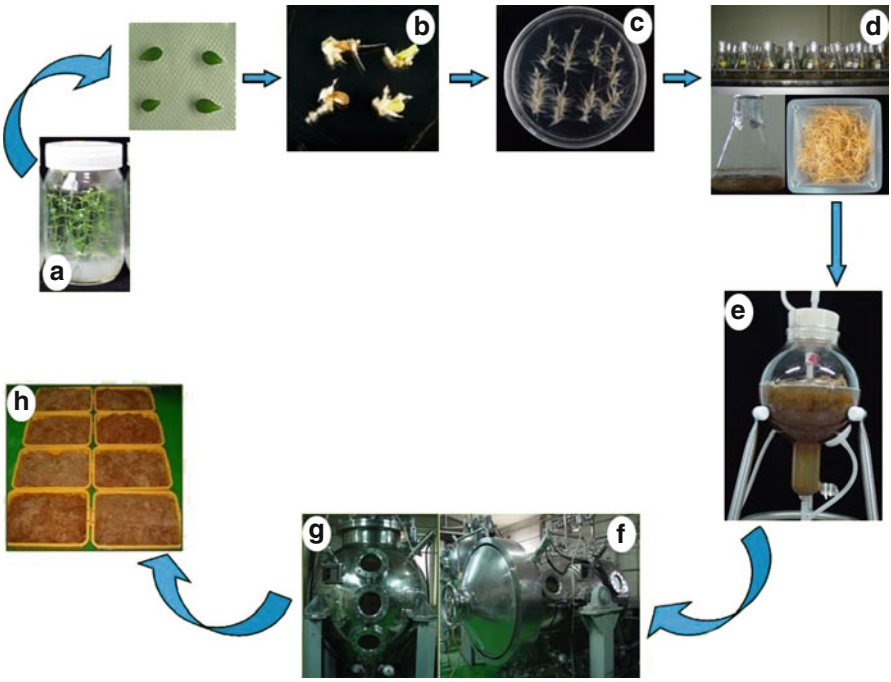

Fig. 11.12 Schematic diagram of *in vitro* adventitious roots production in *H. perforatum* by 500 L bioreactor. *A In vitro* cultured *H. perforatum* plantlets, *B* Induction of adventitious roots from leaves on full-strength MS medium supplemented with B5 vitamins, 3 % (w/v) sucrose, 0.5 mg L^{-1} IAA and 2.3 mg L^{-1} gelrite; *C* Induced adventitious roots were sub-cultured on the same medium for further proliferation and sub-cultured once in 4 weeks, *D* Adventitious roots were proliferated in 250-mL shake flasks (containing 70 mL of medium) containing MS liquid medium supplemented with B5 vitamins, 3 % (w/v) sucrose and 1.0 mg L^{-1} IBA, *E* Adventitious root were maintained and sub-cultured on the same medium in 3 L BTBB and sub-cultured once in 4 weeks, *F* and *G* Adventitious root production in 500 L (drum and balloon type bioreactors) capacity airlift bioreactors cultures containing 500 L of 1/2MS medium supplemented with B5 vitamins, 3 % (w/v) sucrose, 1.0 mg L^{-1} IBA, 0.1 mg L^{-1} kinetin and 3 g L^{-1} inoculum, *H* Harvested adventitious roots from 500 L bioreactor after 6 weeks

11.3 Comparison of Characteristics Between Adventitious Roots and Mother Plants

To investigate the usefulness of the adventitious root culture of *H. perforatum* for the production of secondary metabolites, comparison of the bioactive compound accumulation in adventitious roots (harvested after 6 weeks of culture) and mother plants (transferred to greenhouse for 1 year) was conducted. The content of total polysaccharide, chlorogenic acid, Vitamin C and E were higher in leaves of field-grown

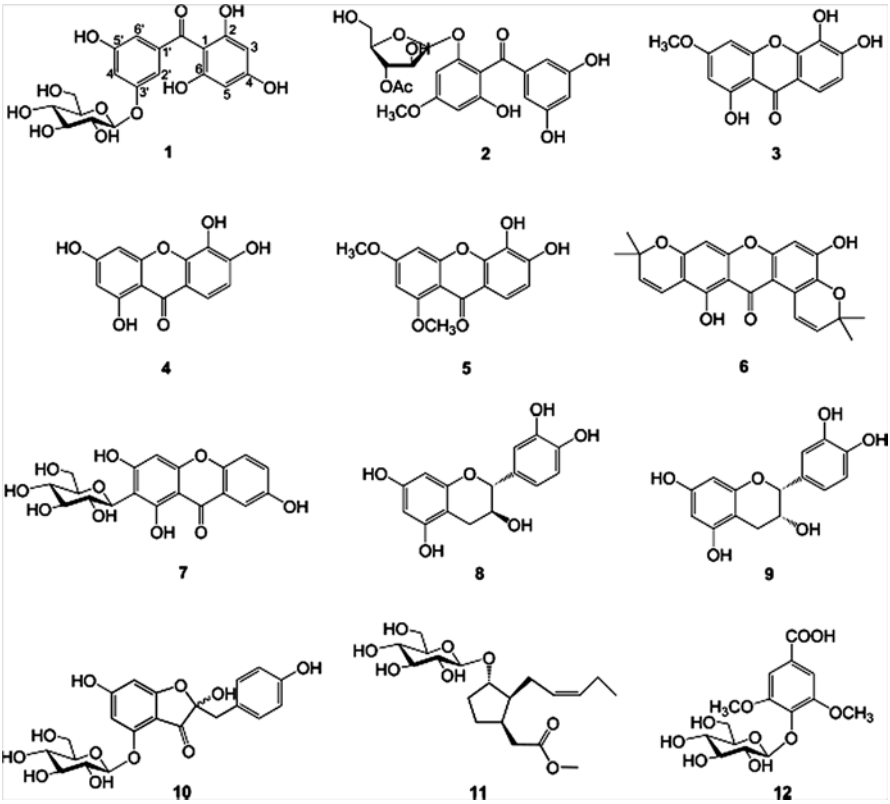

Fig. 11.13 Structure of compounds 1–12 from *H. perforatum*

plants compared with the adventitious roots. However, total phenolics and flavonoids were much higher in adventitious roots compared with field-grown plants. The ploidy levels of adventitious roots and the field-grown plants were tested, resulted in two typical DNA histograms at the same retention time. The result confirmed the genetic stability of the adventitious root in spite of long-term cultures *in vitro* (at least 3 years). In view of the above, adventitious roots can serve as a continuous source for obtaining secondary metabolites for stable metabolites and genetic stability.

11.4 Isolation of Phenolic Compounds from *H. perforatum* Adventitious Root

Li et al. [64] reported a new compound, perforaphenonoside A (1), along with 11 known compounds (2–12) isolated from a methanol extract of adventitious roots of *H. perforatum* (Fig. 11.13). Their chemical structures were elucidated using

chemical and physical methods as well as by comparing NMR and mass spectral data with previously reported data. Structures of compounds (1–12) were elucidated by comparing spectroscopic data to publish values and identified as perforaphenonoside A (1), acetylannulatophenonoside (2), 1,5,6-trihydroxy-3-methoxyxanthone (3), 1,3,5,6-tetrahydroxyxanthone (4), ferrxanthone (5), brasilixanthone B (6), neolancerin (7), (+)-catechin (8), (−)-epicatechin (9), hovetrichoside C (10), methyl 3-O-β-D- glucopyranosylcucurbate (11), and glucosyringic acid (12). Of these, compounds 2, 5–7, 10, and 11 were isolated from *H. perforatum* for the first time.

11.5 Evaluation of Anti-inflammatory Effects, Antioxidant and Cytotoxicity Activities of Isolated Compounds

11.5.1 NF-κB Inhibition and PPAR Activation by Isolated Phenolic Compounds (1–12) from Adventitious Roots

Their inhibition of NF-κB and activation of PPAR was measured in human hepatocarcinoma (HepG2) cells using a luciferase reporter system [64]. The results revealed that among the isolated compounds, 3, 6, 7 and 12 inhibited NF-κB activation stimulated by tumor necrosis factor alpha (TNFα) in a dose-dependent manner, with IC_{50} values ranging from 0.85 to 8.10 μM (Table 11.9). Moreover, compounds 1–3, 7, 11 and 12 activated the transcriptional activity of PPARs in a dose-dependent manner, with EC_{50} values ranging from 7.3 to 58.7 μM (Table 11.10). The transactivational effects of compounds 1–3, 7, 11 and 12 were evaluated on three individual PPAR subtypes (Table 11.11). Among them, compound 2 activated PPARα transcriptional activity, with 153.97 % stimulation at 10 μM, while compounds 1, 2 and 11 exhibited transcriptional activity of PPARγ with stimulation from 124.76 to 126.91 % at 10 μM.

Table 11.9 Inhibitory effects of compounds 1–12 from *H. perforatum* adventitious roots on the TNF**α**-induced NF-**κ**B transcriptional activity

Compound	IC_{50} (μM)
3	5.50 ± 2.62
6	8.10 ± 0.35
7	0.85 ± 0.07
12	0.93 ± 0.18
Sufasalazine[a]	0.9 ± 0.1

The values are mean ± SD (n = 3). Compounds 1, 2, 4, 5, and 8–11 were inactive at tested concentrations (IC_{50} > 10 μM)
[a]Positive control (10 μM)

Table 11.10 PPARs transactivational activities of compounds 1–12 from *H. perforatum* adventitious roots

Compound	EC_{50} (μM)
1	7.9 ± 0.8
2	58.7 ± 7.2
3	17.9 ± 1.8
4	>60[a]
5	>60
6	>60
7	57.8 ± 6.9
8	>60
9	>60
10	>60
11	7.3 ± 0.7
12	27.0 ± 1.9
Sufasalazine	1.05 ± 0.15

EC_{50}: the concentration of a tested compound that gave 50 % of the maximal reporter activity
The values are mean ± SD (n = 3)
[a]A compound was considered inactive with EC_{50} > 60 μM

Table 11.11 PPAR**α**, **γ**, and **β**(**δ**) transactivational activities of compounds 1–3, 7, 11 and 12 from *H. perforatum* adventitious roots

Compound	Concentration (μM)	Stimulation (%)		
		Gal4/ PPARα-LBD	Gal4/PPAR γ-LBD	Gal4/PPAR β(δ)-LBD
1	0.1	109.06 ± 1.25	106.31 ± 2.96	107.12 ± 4.25
	1	110.59 ± 1.38	106.89 ± 2.12	115.23 ± 1.68
	10	115.20 ± 3.84	126.91 ± 1.23	122.52 ± 3.30
2	0.1	116.44 ± 2.33	100.57 ± 1.11	103.94 ± 4.73
	1	142.13 ± 2.84	120.86 ± 2.32	100.71 ± 2.35
	10	153.97 ± 1.92	124.76 ± 3.79	110.43 ± 1.64
3	0.1	100.16 ± 1.25	100.62 ± 2.48	100.59 ± 1.50
	1	114.01 ± 1.43	101.31 ± 1.17	99.75 ± 1.38
	10	117.59 ± 2.35	114.22 ± 2.99	99.88 ± 1.02
7	0.1	106.68 ± 1.78	100.72 ± 1.39	100.47 ± 1.39
	1	118.13 ± 3.28	111.31 ± 3.79	101.86 ± 3.79
	10	105.38 ± 1.32	104.70 ± 1.35	99.74 ± 1.35
11	0.1	100.79 ± 2.02	119.78 ± 1.19	111.42 ± 2.58
	1	117.79 ± 1.47	124.83 ± 1.26	116.13 ± 1.09
	10	111.17 ± 1.39	126.66 ± 1.93	122.06 ± 1.25
12	0.1	102.20 ± 1.46	103.77 ± 2.53	102.28 ± 2.53
	1	100.42 ± 2.51	105.33 ± 0.89	112.53 ± 0.89
	10	102.77 ± 1.28	101.66 ± 1.27	101.05 ± 1.27
Ciprofibrate	1	214.57 ± 1.57		
Troglitazone	1		223.27 ± 2.33	
L-165041	1			266.04 ± 3.01

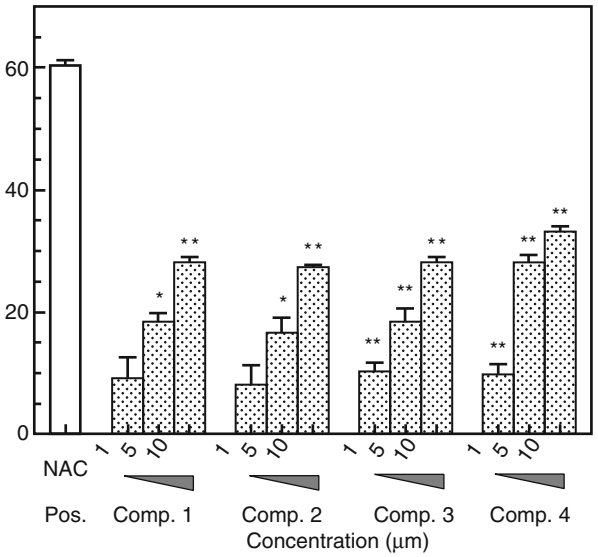

Fig. 11.14 Scavenging effect of four compounds from *H. perforatum* adventitious roots on intracellular ROS. Cells were treated with the samples at 1, 5, and 10 μM. After 30 min, 1 mM of H_2O_2 was added to the plate. After an additional 30 min, DCF-DA was added and the intracellular ROS generated were detected by spectrofluorometry. NAC was used as positive control (*Pos.*) at concentration of 2 mM. *Comp.* compound. *Comp. 1* 1,3,5,6-etrahydroxyxanthone, *Comp. 2* 1,5,6-trihydroxy-3-methoxyxanthone, *Comp. 3* Ferrxanthone, *Comp. 4* Brasilixanthone B. Statistical significance is indicated as *(P<0.05) and **(P<0.01) as determined by Dunnett's multiple comparison test

11.5.2 Antioxidant and Cytotoxic Activities by Xanthones from Adventitious Roots

Five xanthones, 1,3,5,6-tetrahydroxyxanthone, 1,5,6-trihydroxy-3-methoxyxanthone, ferrxanthone, brasilixanthone B and neolancerin were evaluated for antioxidant activities using the intracellular ROS radical scavenging 2′,7′-dichlorfluorescein-diacetate (DCFDA) assay and for cytotoxic activity against the HL-60 human promyelocytic leukemia cells [65]. Among them, 1,3,5,6-tetrahydroxyxanthone, 1,5,6-trihydroxy-3-methoxyxanthone, ferrxanthone and brasilixanthone B exhibited scavenging activity with inhibition values of 27.4–33.2 % at 10 μM (Fig. 11.14); 1,3,5,6-tetrahydroxyxanthone, 1,5,6-trihydroxy-3-methoxyxanthone and brasilixanthone B reduced the viability of HL-60 cells significantly, with IC_{50} values of 31.5, 28.9, and 27.7 μM respectively (Table 11.12).

11.6 Conclusions and Perspectives

A series of techniques for optimization of culture medium and physical conditions were conducted to establish efficient *H. perforatum* adventitious root growth and phenolic compounds production in liquid media and in a 3-L balloon type bubble

Table 11.12 IC_{50} values of five compounds from *H. perforatum* adventitious roots on the growth of HL-60 human leukemia cells

Compound	IC_{50} values (μmol)
1,3,5,6-tetrahydroxyxanthone	31.51 ± 1.69[a]
1,5,6-trihydroxy-3-methoxyxanthone	28.96 ± 1.11
Ferrxanthone	51.74 ± 5.89
Brasilixanthone B	27.73 ± 1.36
Neolancerin	86.63 ± 5.60
Mitoxantrone[b]	6.80 ± 0.90

[a]Results are the means ±SD of three independent experiments in triplicates
[b]Positive control

bioreactor (BTBB). Such cultures are expected to be less prone to erratic metabolite production than cultures of undifferentiated cells and to exhibit lower sensitivity to shear stress than cell suspension cultures. We also established effective elicitation methods to increase phenolic compounds content. For commercialization of *H. perforatum* adventitious roots, large-scale cultures have been achieved using airlift bioreactors at the industrial level. The results of our study contribute to some crucial information for optimization and development of bioreactor technology for adventitious root cultures of *H. perforatum* for the production of hypericin, quercetin, hyperoside and chlorogenic acid. In addition, isolated phenolic compounds (1–12) from root cultures of *H. perforatum* exhibited significant anti-inflammatory effects, antioxidant and anticancer activities. These results provide scientific support for the utilization of adventitious root cultures in functional food and nutraceuticals. In the light of current success, further research should be focused on establishment of effective elicitation methods to increase hypercin and flavonoid contents. Furthermore, biosafety assessment of *H. perforatum* adventitious roots needs to be researched, which is very important for its applications.

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Chapter 12
Production of Withanolides from Cell and Organ Cultures of *Withania somnifera* (L.) Dunal

Praveen Nagella and Hosakatte Niranjana Murthy

Abstract *Withania somnifera* (L.) Dunal. (Indian ginseng) is one of the most important medicinal plants used as a crude drug for its preventive and therapeutic purposes. Among the diverse constituents of *Withania*, withanolides are found to be the major components responsible for their biological and pharmacological actions. On the other hand, difficulty in supplying the pure withanolides in sufficient quantity prevents the development of *Withania* for clinical medicines. Field cultivation of *Withania* is time consuming and it needs extensive efforts for quality control as the growth is susceptible to many environmental factors including soil, climate, pathogens and pests. To overcome these problems, cell and organ cultures have been widely explored for more rapid and efficient production of *Withania* biomass and withanolides. Recently, cell and organ cultures of *W. somnifera* have been developed in laboratory scale with a view to establish large scale production using bioreactors. Various physical and chemical parameters affecting the biomass production and withanolide accumulation have been investigated.

Keywords Adventitious roots • Cell suspension culture • Hairy root culture • Secondary metabolites • *Withania somnifera* • Withanolides

Abbreviations

2,4-D	2,4- Dichlorophenoxy acetic acid
BAP	6-Benzylaminopurine
DW	Dry weight
FW	Fresh weight

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IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KN	Kinetin
MJ	Methyl jasmonate
MS medium	Murashige and Skoog medium
NAA	α- Naphthalene acetic acid
SA	Salicylic acid

12.1 Introduction

Withania somnifera (L.) Dunal, also known as ashwagandha, Indian ginseng and winter cherry is an important medicinal plant in Ayurvedic medicine of the traditional medicinal system of India [1, 2]. The roots and leaves of ashwagandha contain various alkaloids, viz., withanolides and withaferin. The withanolides are steroidal compounds, which resemble the active ginsenosides of Asian ginseng both in action and appearance. Studies show that the plant has been used as an antioxidant, adaptogen, aphrodisiac, liver tonic, anti-inflammatory agent, antitumor, astringent and more recently to treat ulcers, bacterial infection, venom toxins and senile dementia. Clinical trials and animal research support its use in treating anxiety, cognitive and neurological disorders, inflammation, hyperlipidemia and Parkinson's disease [1]. It has been used as a tonic and an antistress supplement. Pharmacological activities of ashwagandha include antiarthritic, antiaging, nerve tonic, cognitive function improvement in geriatric states, and recovery from neurodegenerative disorders [3, 4]. Various alkaloids, withanolides and sitoindosides have been isolated from this plant. Of the various withanolides reported, withaferin A and withanone are customary major withanolides of the plant, of which the amount of withanolide A is usually very low [5]. Recently, withanolide A has attracted interest due to its strong neuropharmacological properties of promoting outgrowth and synaptic reconstruction [6, 7]. Withanolide A is therefore important compound for the therapeutic treatment of neurodegenerative diseases, like Alzheimer's disease, Parkinson's disease, convulsions, cognitive function impairment [8].

For commercial withanolide production, field grown plant material has generally been used but the quality of these products may be highly affected by different environmental conditions, pollutants and pests and pathogens like insects, fungi, bacteria and viruses, which can result in a heavy loss in yield and alter the medicinal content of plant. Plant cell and organ cultures are promising technologies to obtain plant-specific valuable metabolites [9]. Cell and organ cultures have a higher rate of metabolism than field grown plants because the initiation of cell and organ growth in culture leads to fast proliferation of cells/organs and to a condensed biosynthetic cycle. Further, plant cell/organ cultures are not limited by environmental, ecological and climatic conditions and cells/organs can thus proliferate at higher growth rates than whole plant in cultivation [10].

12.2 Induction of Callus, Adventitious Roots and Hairy Roots

12.2.1 Induction of Callus

Callus was induced from leaf explants of *W. somnifera* cv. Jawahar on full strength MS [11] gelled (0.8 % agar, w/v) medium supplemented with 30 g L^{-1} sucrose (w/v) and 2.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg L^{-1} kinetin (KN) [12]. Sivanandhan et al. [13] induced the callus from roots of 4-week old *in vitro* grown seedlings and MS medium supplemented with 2 mg L^{-1} picloram.

12.2.2 Induction of Adventitious Roots

Rani et al. [14] induced adventitious roots from leaf explants of *Withania* by dip method. The leaf explants were dipped in different concentrations of IBA for different intervals of time and cultured the explants on the MS medium of different strengths. Adventitious roots were induced directly when leaf explants were cultured on half strength MS medium supplemented with a combination of IBA and IAA at 9.85 and 2.85 μM respectively. Other auxin combinations resulted in the formation of callus along with adventitious root induction. The adventitious roots were cultured in a 2.5 L bubble column reactor with 1,000 mL of half strength MS medium with the same hormonal concentration for 6 weeks and the roots were harvested and analyzed for the withanolide production. A maximum yield of 10 mg g^{-1} dry weight was obtained in the bubble column reactor [15]. Adventitious roots were also induced directly from the leaf explants by Praveen and Murthy [16]. They cultured the leaf explants on full and half strength MS medium with various concentrations of auxins (2, 4-D, NAA, IBA and IAA; at 0.1, 0.5, 1.0, 2.0, and 5.0 mg L^{-1}) for the induction of adventitious roots. The explants cultured on full strength MS medium produced only callus in all the concentrations tested, which hindered the induction of roots. Explants cultured on half MS medium supplemented with IBA and IAA individually were able to induce the adventitious roots. On medium supplemented with 0.5 mg L^{-1} IBA, explants developed maximum number of roots. Explants cultured on 2, 4-D and NAA medium were not potent in induction of adventitious roots (Table 12.1). The adventitious roots were maintained on half strength MS medium supplemented with 0.5 mg L^{-1} IBA concentration. Callus mediated induction of adventitious roots were induced from leaf, internode and cotyledonary explants by Sivanandhan et al. [17] that were cultured on MS medium supplemented with 2.0 mg L^{-1} 2, 4-D and 0.2 mg L^{-1} KN. Four week old calli obtained from different explant sources were used as explants for the induction of adventitious roots. The calli were cultured separately on half strength MS medium supplemented with IBA, IAA, and NAA individually or in combinations of IBA/IAA or IBA/NAA. Highest number of adventitious roots was obtained from leaf

Table 12.1 Effect of different auxins on adventitious root induction from leaf explants of *Withania somnifera* cultured for 4 weeks on half strength MS medium[a]

Auxins	Concentration (mg L^{-1})	Explants cultured	% of response	Nature of response	Mean no. of roots ± SE
2,4-D	0.1	12	72.22	Friable callus	–
	0.5	12	83.33	Friable callus	–
	1.0	12	83.33	Friable callus	–
	2.0	12	91.66	Friable callus	–
	5.0	12	86.11	Friable callus	–
NAA	0.1	12	69.44	Friable callus	–
	0.5	12	69.44	Friable callus	–
	1.0	12	81.66	Friable callus	–
	2.0	12	83.33	Friable callus	–
	5.0	12	80.55	Friable callus	–
IBA	0.1	12	91.66	Roots	6.41 ± 0.87de
	0.5	12	100	Roots	17.50 ± 0.37a
	1.0	12	91.66	Roots	13.75 ± 1.30b
	2.0	12	91.66	Roots	9.83 ± 1.02c
	5.0	12	83.33	Roots	6.50 ± 0.71de
IAA	0.1	12	100	Roots	4.91 ± 0.28ef
	0.5	12	100	Roots	6.25 ± 0.30de
	1.0	12	91.66	Roots	8.08 ± 0.82 cd
	2.0	12	83.33	Roots	4.91 ± 0.71ef
	5.0	12	83.33	Roots	3.16 ± 0.50f

[a]Data represents means ± SE of 12 replicates; each experiment was repeated twice. Mean separation within column by Duncan's multiple range test at $P < 0.05$

derived callus explants when cultured on combination of IBA and IAA at 0.5 and 0.1 mg L^{-1} concentrations. Further, the adventitious roots thus produced were maintained on the half strength MS medium supplemented with 0.5 mg L^{-1} IBA.

12.2.3 Induction of Hairy Roots

Transformed roots (=hairy roots) were induced by infecting the shoots of *Withania* with *A. rhizogenes* strain LBA 9402 for the production of withanolide D [18]. Pawar and Maheshwari [19] used 15 days old *in vitro* plants for stem segments, hypocotyls and leaves from 2 months old *in vitro* plants as explants for the induction of hairy roots by infecting them with *A. rhizogenes* strain MTCC 2364 and MTCC 532. Only leaf explants responded to the infection with the *A. rhizogenes* and induced hairy roots while stem segments and hypocotyl explants became necrotic after infection with *A. rhizogenes*. Kumar et al. [20] also induced hairy roots by infecting leaf explants with *A. rhizogenes* and the transformants were confirmed by PCR using rol A gene specific primers. Further, these transgenics were confirmed by

southern blot analysis. Bandyopadhyay et al. [21] used leaf explants from the *in vitro* grown plantlets obtained through shoot tip meristem culture for the induction of hairy roots by infecting with two virulent wild-type agropine strains of *A. rhizogenes*, LBA 9402 (pRi 1855) and A4 (pRi A4). The transgenic nature of the hairy roots was confirmed by PCR using specific primers. Hairy roots were also induced by Murthy et al. [22] by infecting roots, stems, hypocotyls, cotyledons, cotyledonary nodes and leaf segments from *in vitro* raised seedlings with *A. rhizogenes* strain R1601. Only cotyledons and leaf explants responded to the infection and induced hairy roots with 3.33 and 40.3 % efficiency respectively. The transgenic nature of hairy roots was confirmed by PCR using *npt*II and *rol*B gene specific primers and transgenicity was also confirmed by southern blot analysis. Sivanandhan et al. [23] induced hairy roots by infecting the leaf explants from 45 day old *in vitro* seedlings with agropine type strain of *A. rhizogenes* R1000. Hairy roots induced by using *A. rhizogenes* stains R1601 and R1000 are found stable and could be used for optimization of cultural parameters for biomass and metabolite production.

12.3 Establishment of Cell and Organ Suspension Cultures

12.3.1 Effect of Growth Regulators on Biomass Accumulation and Withanolide Production

Proliferation of cells in the suspension cultures depends on the growth regulators supplemented to the culture medium. The type and concentration of auxin or the auxin/cytokinins ratio alters dramatically both the growth and the production of secondary compounds in cultured plant cells [24, 25]. For example, Nagella and Murthy [12] reported the variation in biomass accumulation and withanolide content with varied concentrations of auxins. They observed the maximum accumulation of biomass and the highest production of withanolide in the medium supplemented with 2 mg L^{-1} 2, 4-D. However, the highest accumulation of biomass and withanolide production was observed with the cultures supplemented with 2 mg L^{-1} 2, 4-D + 0.5 mg L^{-1} KN (Table 12.2). The combination of 2,4-D (1 mg L^{-1}) and KN (0.2 mg L^{-1}) has also been reported for the culturing of cell suspension and the production of withaferin A [26]. Sabir et al. [27] noticed that 2,4-D at 3 mg L^{-1} and KN at 0.5 mg L^{-1} showing withanolide production. However, Sivanandhan et al. [13] observed that when root explants were cultured with 1 mg L^{-1} picloram, highest accumulation of biomass and withanolides were observed. Further, inclusion of 0.5 mg L^{-1} KN along with 1 mg L^{-1} picloram proved to be the best suitable combination when compared with all other cytokinin concentrations for the maximum accumulation of biomass and withanolide production. The differences in withnolide synthesis might be due to the explant type, initiation of cell line, physical and chemical conditions of the medium.

Table 12.2 *Withania somnifera* cell suspension culture: effect of 2.0 mg L^{-1} 2, 4-dichlorophenoxy acetic acid (2, 4-D) in combination with different concentrations of cytokinins on biomass accumulation and withanolide A production[a, b]

Cytokinins	Concentration (mg L^{-1})	Dry weight (g L^{-1})	Withanolide A content (mg g^{-1} DW)
Benzylaminopurine (BAP)	0.1	5.48 ± 0.06d	1.36 ± 0.02d
	0.5	5.76 ± 0.18d	1.42 ± 0.06d
	1.0	6.51 ± 0.09c	1.82 ± 0.03b
	2.0	6.69 ± 0.06c	1.63 ± 0.02c
Kinetin (KN)	0.1	7.17 ± 0.12b	1.78 ± 0.02b
	0.5	10.79 ± 0.05a	2.26 ± 0.01a
	1.0	6.74 ± 0.16c	1.81 ± 0.06b
	2.0	6.66 ± 0.10c	1.78 ± 0.04b

[a]0.5 g of cells were cultured in 50 mL of MS medium for 4 weeks
[b]Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

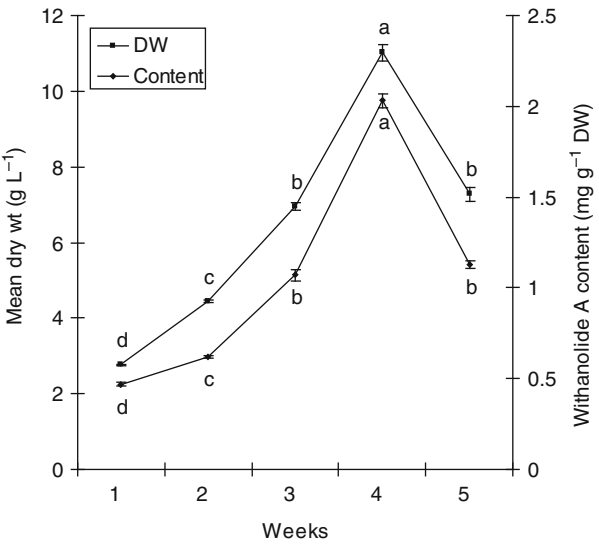

Fig. 12.1 Time-profile of cell growth and withanolide A production in shake flask cultures of *Withania somnifera*. Five hundred milligram of cells were cultured in 50 mL of MS medium supplemented with 2.0 mg L^{-1} 2, 4-D + 0.5 mg L^{-1} KN for 4 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean values with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT)

12.3.2 Growth Kinetics of Biomass Accumulation and Withanolide Production

Growth kinetics of *W. somnifera* cell and adventitious root suspension and production of withanolide is presented in the Figs. 12.1 and 12.2. The biomass accumulation and withanolide production of the cultured cells followed typical growth culture and reached their optimum within 4 weeks of time [12, 13]. Growth kinetic pattern of hairy roots also followed similar pattern [22]. However, Sivanandhan et al. [23, 37] reported that cultured cells and hairy roots took 36 days to reach their

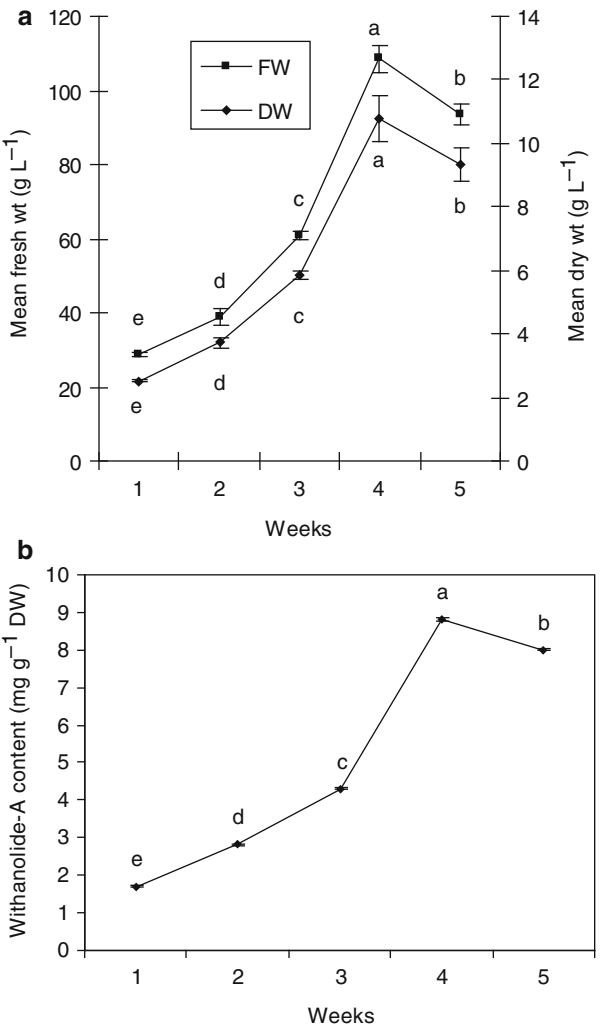

Fig. 12.2 Time profile of adventitious root growth (**a**) and kinetics of production of withanolide A (**b**) in flask scale cultures of adventitious root suspension cultures of *Withania somnifera.* The roots were cultured in 50 mL of MS medium supplemented with 0.5 mg L^{-1} IBA. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P < 0.05$ according to Duncan's multiple range test (DMRT)

optimal growth, whereas withanolide accumulation was highest after 40 days of culture. Such variation in growth kinetics and accumulation of secondary compound in the cultured cells might be due to genotype specificity.

12.3.3 Effect of Inoculum Density on Biomass Accumulation and Withanolide Production

Plant suspensions are initiated using relatively high cell density as there is a minimum inoculation density below which growth does not occur or is preceded by a lag

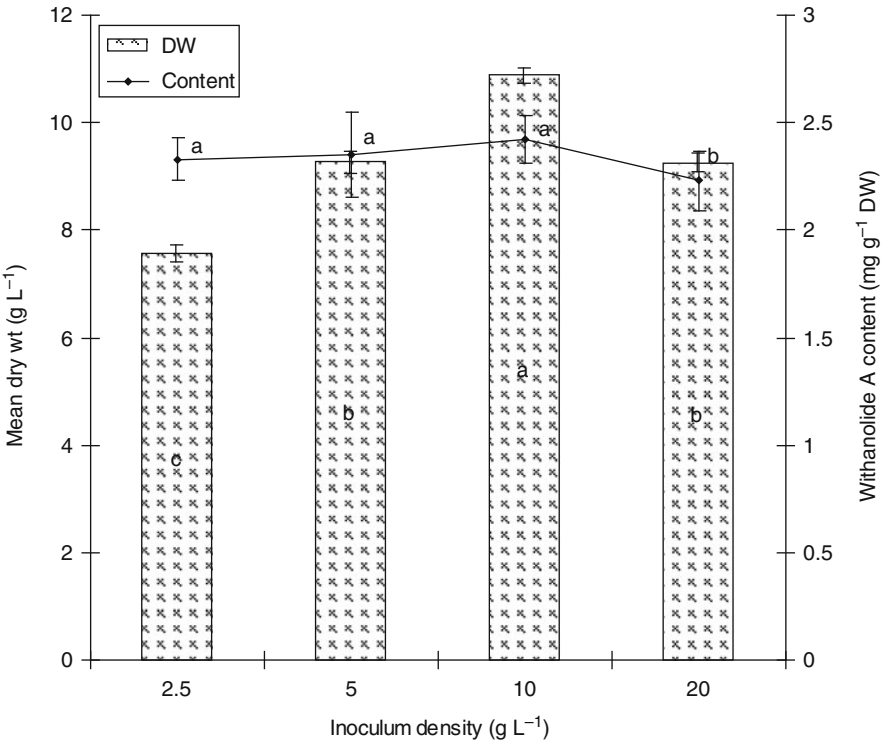

Fig. 12.3 *Withania somnifera* cell suspension culture: effects of inoculum densities on biomass accumulation and withanolide A production. Five hundred milligram cells were cultured in 50 mL of MS medium supplemented with 2.0 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} KN for 4 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean values with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT)

phase. Medium conditioning can be used to reduce the minimum inoculum density, however, the chemical basis of the conditioning effect has not been fully defined and it is primarily empirical [28]. Inoculum density ranging 2.5–20.0 g L^{-1} were tested by Nagella and Murthy [12, 16] for biomass and withanolide accumulation in cell and adventitious root cultures of *W. somnifera* and they reported that the inoculum density of 10.0 g L^{-1} was suitable for biomass growth and withanlide accumulation (Figs. 12.3 and 12.4). Increased inoculum density 20.0 g L^{-1} was not suitable for biomass growth and secondary metabolite accumulation. In the hairy root cultures, 5 g L^{-1} inoculum favoured the maximum accumulation of biomass and withanolides contents [22]. These results reveal that inoculum density is a critical factor for both biomass growth and metabolite production, it might be species and cultivar specific and it should be worked out at the beginning of establishment of cell and organ suspension cultures.

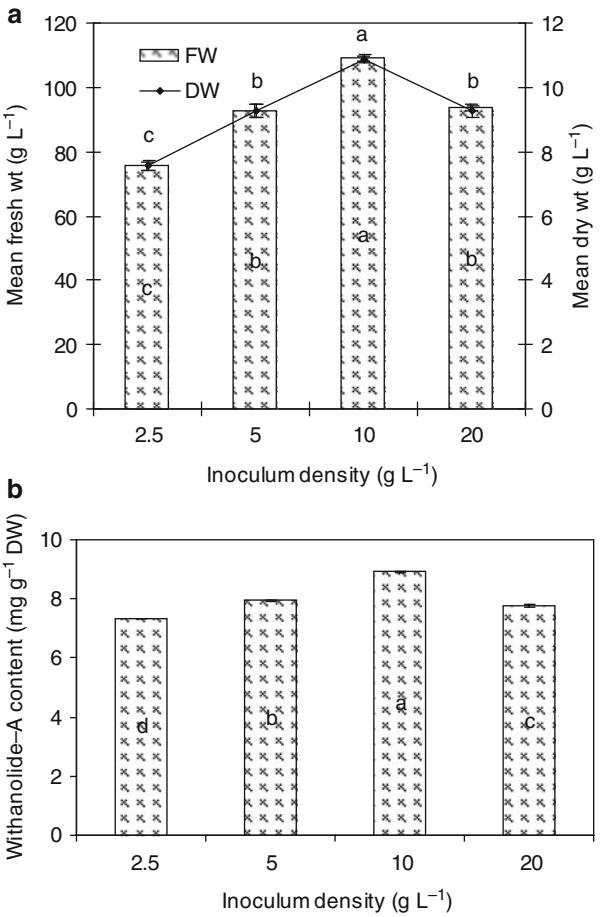

Fig. 12.4 Effect of different inoculum density on the biomass accumulation (**a**) and withanolide-A production (**b**) by adventitious root suspension cultures of *Withania somnifera* after 4 weeks of culture in 50 mL of MS medium supplemented with 0.5 mg L^{-1} IBA. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P < 0.05$ according to Duncan's multiple range test (DMRT)

12.3.4 Effect of Different Media on Biomass Accumulation and Withanolide Production

Various media such as MS, B5, NN and N6 media were tested for culturing of *W. somnifera* cells and adventitious roots by Nagella and Murthy [12, 16] and they have reported that MS medium was superior to other media i.e. Gamborg's (B5; [29]), Nitsch and Nitsch (NN; [30]), Chu's (N6; [31]) media for both biomass accumulation and withanolide production (Figs. 12.5 and 12.6). Murthy et al. [22] also reported that MS medium was optimal compared to other media like Chu's (N6; [31]), Schenk and Hildebarndt (SH; [32]), Linsmaier and Skoog (LS; [33]) for the accumulation of biomass in the hairy root cultures of *W. somnifera*. These results confirm the view of Ramachandra Rao and Ravishankar [10] that constituents of culture media are important determinants of biomass growth and metabolite accumulation.

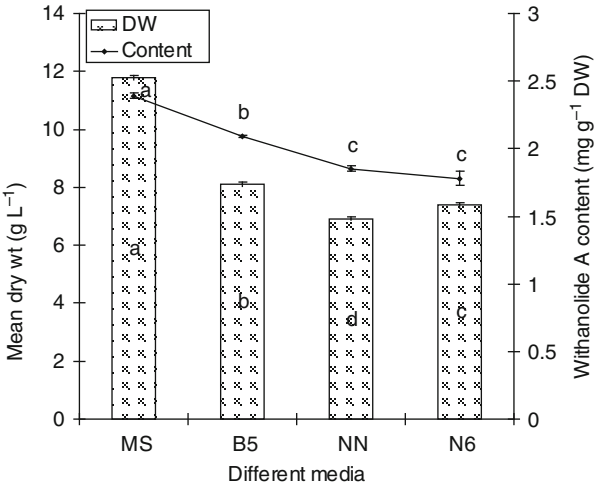

Fig. 12.5 *Withania somnifera* cell suspension culture: effects of different media on biomass accumulation and withanolide A production. Five hundred milligram of cells were cultured in 50 mL of medium supplemented with 2.0 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} KN for 4 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean values with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT)

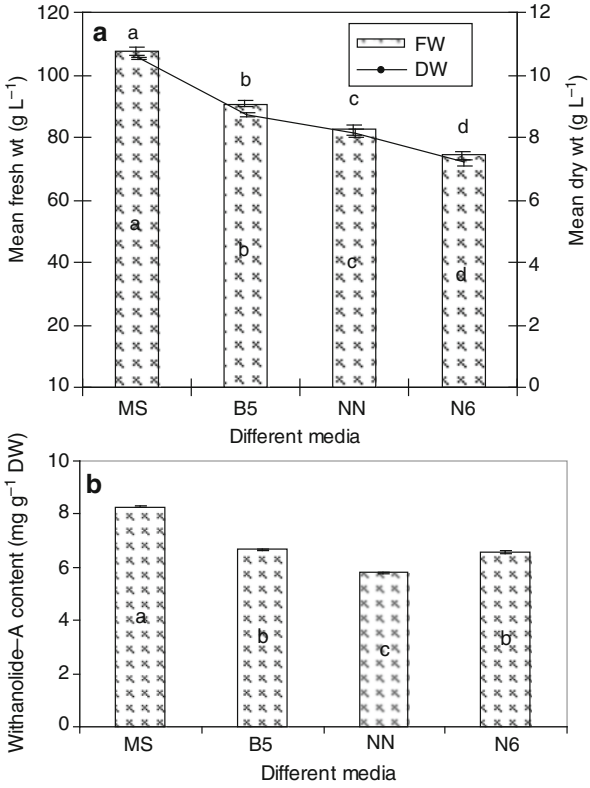

Fig. 12.6 Effect of different media on the biomass accumulation (**a**) and withanolide-A production (**b**) by adventitious root suspension cultures of *Withania somnifera* after 4 weeks of culture in 50 mL of medium supplemented with 0.5 mg L^{-1} IBA. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P < 0.05$ according to Duncan's multiple range test (DMRT)

12.3.5 *Effect of Medium Salt Strength on Biomass Accumulation and Withanolide Production*

The optimum nutrient concentration is a critical determinant in controlling the growth of cells/organs and the accumulation of secondary metabolites [10]. Different salt strengths (0.25, 0.5, 0.75, 1.0, 1.5 and 2.0×) of the MS medium were employed to determine the optimum growth of the biomass and metabolite production. In cell suspension cultures, full salt strength (1.0×) MS medium favored the highest biomass accumulation and withanolide production (Fig. 12.7; [12]). In adventitious root cultures, half strength (0.5×) MS medium favored the maximum accumulation of biomass and withanolide production. Higher salt strengths (1.5 and 2.0×) MS media were not suitable as they were responsible for the decrease in biomass and metabolite accumulation (Table 12.3; [16]). Such variations of utilization of media might be due to the selection of different strains of cells and adventitious roots. Therefore, selections of cell/organ clone and selection of suitable medium and salt strengths are important for the establishment of cell and organ cultures.

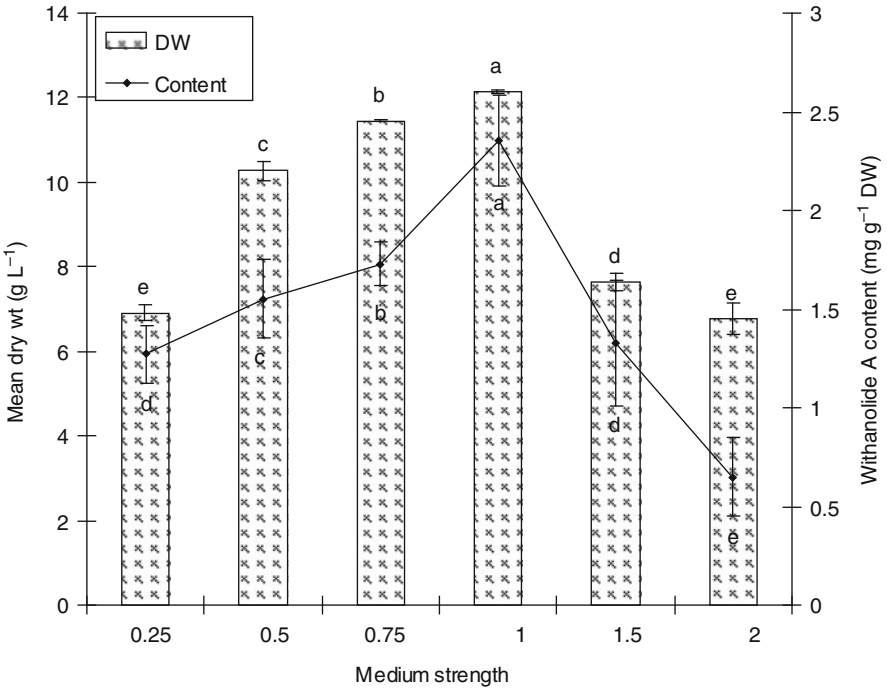

Fig. 12.7 *Withania somnifera* cell suspension culture: effects of medium strength on biomass accumulation and withanolide A production. Five hundred milligram of cells were cultured in 50 mL of MS medium supplemented with 2.0 mg L^{-1} 2,4-D and 0.5 mg L^{-1} KN for 4 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean values with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT)

Table 12.3 Biomass growth and withanolide-A production of *Withania somnifera* adventitious roots as affected by MS medium salt strength. Cultures were maintained in 250 mL Erlenmeyer flasks for 4 weeks[a]

Medium strength (g)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio	Withanolide A content (mg g^{-1} DW)
0.25	72.27 ± 1.09 cd	6.94 ± 0.07d	5.33	7.12 ± 0.01c
0.50	107.48 ± 1.13a	10.53 ± 0.04a	8.10	8.65 ± 0.02a
0.75	81.66 ± 1.13b	7.76 ± 0.08b	5.97	8.25 ± 0.04b
1.00	79.46 ± 0.92b	7.64 ± 0.06b	5.88	8.24 ± 0.02b
1.50	74.80 ± 0.40c	7.36 ± 0.08c	5.66	6.48 ± 0.03d
2.00	69.82 ± 0.37d	6.89 ± 0.07d	5.37	4.89 ± 0.02e

[a]Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean separation within column by Duncan's multiple range test at $P < 0.05$

Table 12.4 *Withania somnifera* cell suspension culture: effect of different carbohydrate sources on biomass accumulation and withanolide-A production[a, b, c]

Carbohydrate sources (3 %)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio	Withanolide-A content (mg g^{-1} DW)
Sucrose	115.63 ± 2.10a	10.47 ± 0.12a	9.52	2.95 ± 0.10a
Glucose	98.68 ± 0.56b	8.67 ± 0.06c	7.88	2.25 ± 0.02b
Fructose	84.48 ± 0.68 cd	7.36 ± 0.04ef	6.69	1.83 ± 0.01c
Maltose	82.53 ± 0.57d	7.06 ± 0.07f	6.42	1.70 ± 0.02d
Glucose + fructose (1:1)	90.48 ± 1.89c	7.85 ± 0.11d	7.14	1.85 ± 0.03c
Fructose + sucrose (1:1)	82.04 ± 5.27d	7.73 ± 0.20de	7.03	1.90 ± 0.08c
Sucrose + glucose (1:1)	103.01 ± 1.44b	9.32 ± 0.20b	8.47	2.18 ± 0.15b

[a]0.5 g of cells were cultured in 50 mL of MS medium supplemented with 2.0 mg L^{-1} 2,4-D and 0.5 mg L^{-1} kinetin for 4 weeks

[b]Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean separation within column by Duncan's multiple range test at $P < 0.05$

[c]Growth ratio is the quotient of the dry weight of harvested biomass (cells) and the dry weight of the inoculum

12.3.6 Effect of Different Carbon Sources on Biomass Accumulation and Withanolide Production

Sugars have been recognized as molecules that act as energy sources and can also act as signaling molecules that affect growth, development and metabolism of cultured cells [34]. Sucrose is a major carbon as well as energy source for plant cultures and the utilization of carbon is correlated with the accumulation of biomass as well as metabolic status of cells and organs. To improve the biomass growth and metabolite production, various sugars such as sucrose, glucose, maltose, glucose + fructose (1:1), fructose + sucrose (1:1) and sucrose + glucose (1:1) of carbon sources were tested by Nagella and Murthy [12] and they have reported 3 % (w/v) sucrose was superior for both biomass and withanolide A accumulation in *W. somnifera* cell suspension cultures (Table 12.4). Shivanandhan et al. [13] tested supplementation of sucrose, glucose, maltose and fructose in range 1–6 % to MS medium and reported that 2 % sucrose best for biomass accumulation and 5 % sucrose

Table 12.5 Biomass growth and withanolide A production of *Withania somnifera* adventitious roots as affected by different carbon sources in the MS medium. Cultures were grown in 250 mL conical flasks containing 50 mL medium for 4 weeks

Carbon sources (3 %)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio	Withanolide A content (mg g^{-1} DW)
Sucrose	105.63 ± 2.10a	10.47 ± 0.12a	8.05	8.73 ± 0.04a
Glucose	88.68 ± 0.56c	8.67 ± 0.06c	6.67	7.94 ± 0.02c
Fructose	74.48 ± 0.68e	7.36 ± 0.04e	5.66	7.72 ± 0.02c
Maltose	72.53 ± 0.57e	7.06 ± 0.07e	5.43	7.53 ± 0.01d
Glucose + fructose (1:1)	80.48 ± 1.89d	7.85 ± 0.11d	6.04	7.24 ± 0.03e
Fructose + sucrose (1:1)	78.71 ± 1.71d	7.73 ± 0.20d	5.95	7.49 ± 0.02d
Sucrose + glucose (1:1)	100.68 ± 0.89b	9.99 ± 0.12b	7.68	8.38 ± 0.03b

Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

Table 12.6 Biomass growth and withanolide A production of *Withania somnifera* hairy roots as affected by different carbon sources in the MS medium. Hairy roots (500 mg) were cultured in 250 mL Erlenmeyer's flasks containing 50 mL of MS medium for 4 weeks

Carbohydrate sources (30 g L^{-1})	Dry weight (g L^{-1})	Growth ratio	Withanolide-A content (mg g^{-1} DW)
Sucrose	11.92 ± 0.12a	9.46	11.96 ± 0.34a
Fructose	8.20 ± 0.10d	6.51	9.43 ± 0.17d
Glucose	9.20 ± 0.17c	7.30	10.91 ± 0.23b
Maltose	7.77 ± 0.15e	6.17	9.90 ± 0.28c
Glucose + fructose (1:1)	8.37 ± 0.12d	6.64	8.55 ± 0.23e
Sucrose + glucose (1:1)	10.79 ± 0.05b	8.56	11.55 ± 0.40a
Fructose + sucrose (1:1)	9.17 ± 0.12c	7.28	9.30 ± 0.40d

Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

optimal secondary metabolite accumulation. Sucrose (3 %, w/v) was also ideal carbon source for the cultivation of adventitious/hairy roots of *W. somnifera,* which facilitated biomass and withanolide accumulation (Tables 12.5 and 12.6; [35–37]). Doma et al. [38] found that 4 % sucrose and 5 % glucose in the MS medium favored the hairy root growth and withanolide production when supplemented individually. Thus, sucrose is generally most preferred carbon source for culturing the cells and organs of *W. somnifera*.

12.3.7 Effect of Sucrose Concentrations on Biomass Accumulation and Withanolide Production

It was reported that initial carbon source and its concentration is essential for cell and organ growth and secondary metabolite accumulation [10] and to improve cell growth and withanolide production, different types and concentrations of sugar were tested and Nagella and Murthy [12] found that the optimal sucrose concentration

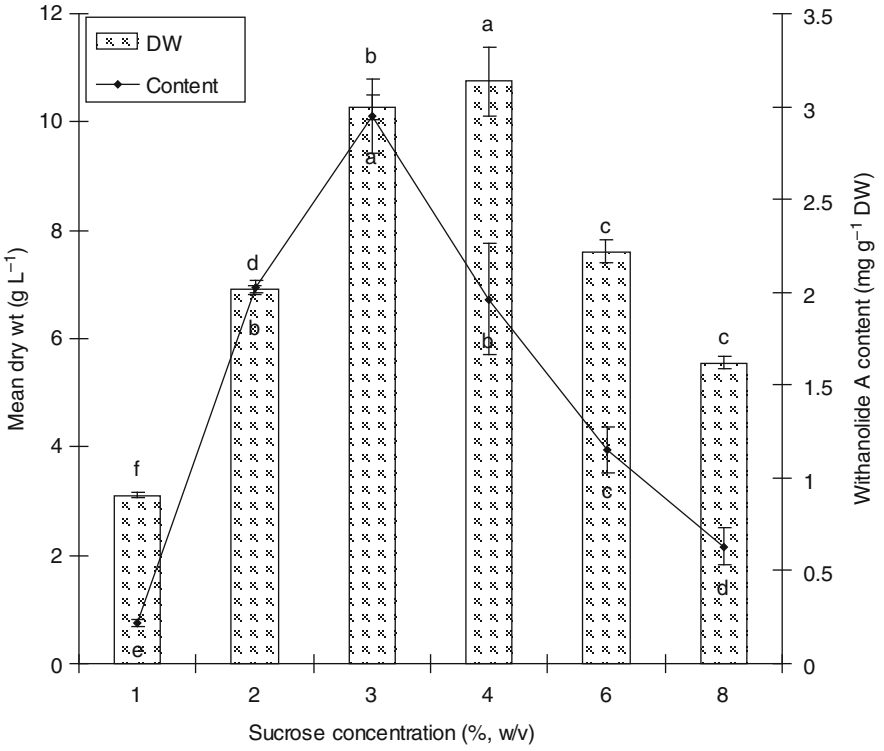

Fig. 12.8 *Withania somnifera* cell suspension culture: effects of different sucrose concentrations on biomass accumulation and withanolide A production. Five hundred milligram of cells were cultured in 50 mL of MS medium supplemented with 2.0 mg L^{-1} 2, 4-D + 0.5 mg L^{-1} KN for 4 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT)

for *Withania* cell growth was 4 %, above which cell growth was inhibited. However, accumulation of withanolide was optimum with cultures supplemented with 3 % sucrose (Fig. 12.8). Whereas, Sivanandhan et al. [13] reported that in cell suspension cultures of *Withania*, 2 % sucrose concentration promoted the biomass accumulation and with the increase in sucrose concentration there was decrease in the biomass and with 5 % sucrose concentration highest content of withanolide production. In the adventitious root cultures of *Withania*, 2 % sucrose concentration favored both the biomass accumulation and withanolide production (Table 12.7; [35]). Murthy et al. [22] reported that 4 % sucrose concentration was optimal for biomass accumulation in the hairy root cultures of *Withania*. While, Praveen and Murthy [36] reported that 3 % sucrose concentration was optimal for the hairy root growth and 4 % sucrose favored the maximum accumulation of withanolide content (Fig. 12.9). Doma et al. [38] observed that at 4 % sucrose concentration the accumulation of hairy root biomass was maximum and withaferin A content was highest. At 3 % sucrose concentration the production of withanolide A and withaferin A was almost at the same levels. Whereas at 5 % glucose concentration the production of

Table 12.7 Biomass growth and withanolide A production of *Withania somnifera* adventitious roots as affected by different concentrations of sucrose in the MS medium. Cultures were grown in 250 mL conical flasks containing 50 mL medium for 4 weeks

Concentration of sucrose (%)	Fresh weight ($g\ L^{-1}$)	Dry weight ($g\ L^{-1}$)	Growth ratio	Withanolide-A content ($mg\ g^{-1}$ DW)
1	50.98 ± 1.25e	4.88 ± 0.07e	3.75	2.48 ± 0.03e
2	113.58 ± 2.97a	11.33 ± 0.19a	8.70	8.93 ± 0.01a
3	107.35 ± 1.12b	10.19 ± 0.16b	8.24	8.73 ± 0.02a
4	90.12 ± 0.75c	8.27 ± 0.07c	6.36	8.16 ± 0.03b
6	59.02 ± 2.16d	5.44 ± 0.19d	4.18	6.50 ± 0.02c
8	43.96 ± 2.41f	4.55 ± 0.25f	3.50	3.61 ± 0.03d

Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

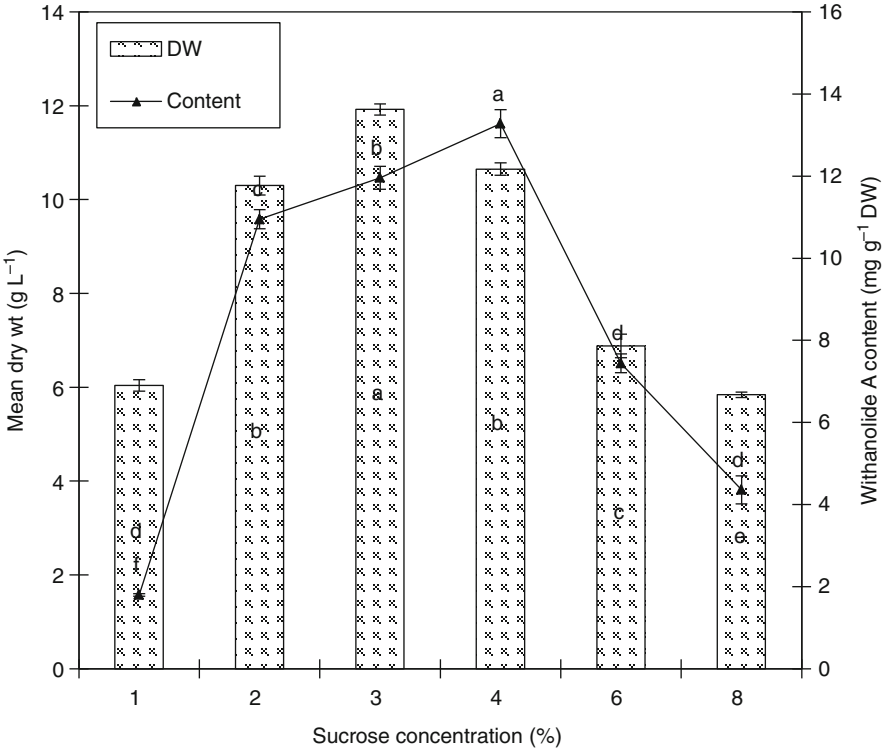

Fig. 12.9 *Withania somnifera* hairy root culture: effects of different sucrose concentrations on biomass accumulation and withanolide A production. Hairy roots (500 mg) were cultured in 250 mL Erlenmeyer's flasks containing 50 mL of MS medium for 4 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT)

withaferin A content was optimal. All these experimental evidences clearly demonstrate that the effect of carbon and energy source is dependent on specific cell or organ lines and it should be worked out thoroughly and specific carbon source and its concentration should be established for attaining optimal results.

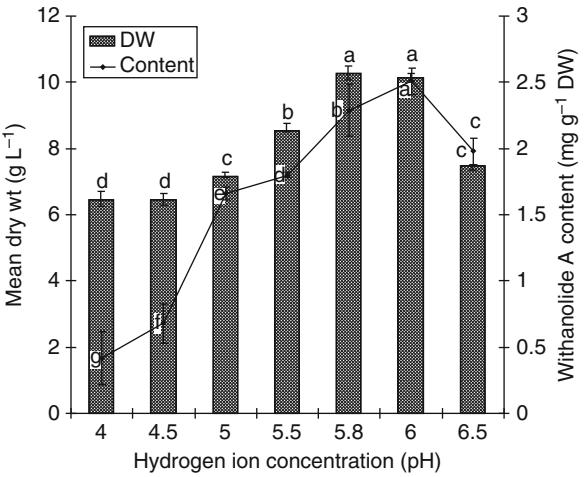

Fig. 12.10 *Withania somnifera* cell suspension culture: effect of hydrogen ion concentration (pH) on biomass accumulation and withanolide A production. Five hundred milligram of cells were cultured in 50 mL of MS medium supplemented with 2.0 mg L^{-1} 2,4-D and 0.5 mg L^{-1} KN for 4 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT)

12.3.8 Effect of Agitation Speed on Biomass Accumulation and Withanolide Production

Agitation speed is one of the important parameters for a successful establishment of plant cell suspension cultures. A proper agitation speed promotes better growth and secondary metabolite synthesis by enhancing the transfer of nutrients from liquid and gaseous phases to cells and dispersion of air bubbles for effective oxygenation [39]. In the cell suspension cultures of *Withania*, Sivanandhan et al. [13] found that the agitation speed of 120 rpm was found suitable for the maximum accumulation of biomass and withanolides production. At higher rpm of 140–160, biomass accumulation and withanolides production were highly affected, whereas at lower rpm of 80–100, the cells aggregated into hard clumps at 80 rpm and resulted in cell death; at 100 rpm, the cells were loosely attached in the clump.

12.3.9 Effect of Initial Medium pH on Biomass Accumulation and Withanolide Production

The hydrogen ion concentration (pH) of the culture medium one more factor which affects the biomass accumulation and metabolite production during *in vitro* culturing of plant cells and the concentration of hydrogen ions in the medium changes during the culture period [25, 40]. Different ranges of pH (4.0, 4.5, 5.0, 5.5, 5.8, 6.0, and 6.5) were employed to improve the biomass accumulation and metabolite production. The highest accumulation of biomass was observed when the medium pH was set at 5.8 and the maximum withanolide production was recorded when the initial medium pH was 6.0 in the cell suspension cultures of *W. somnifera* (Fig. 12.10;

Table 12.8 Biomass growth and withanolide A production of *Withania somnifera* adventitious roots as affected by initial medium pH in the MS medium. Cultures were grown in 250 mL conical flasks containing 50 mL medium for 4 weeks

Medium pH	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio	Withanolide-A content (mg g^{-1} DW)
4.0	55.83 ± 1.17 g	5.52 ± 0.07f	4.25	5.87 ± 0.02f
4.5	60.39 ± 0.52f	5.96 ± 0.03f	4.58	7.22 ± 0.01d
5.0	74.56 ± 0.80e	7.43 ± 0.16e	5.71	7.99 ± 0.02c
5.5	96.32 ± 3.01c	9.50 ± 0.26c	7.31	9.09 ± 0.02a
5.8	113.26 ± 0.66a	11.33 ± 0.09a	8.71	8.92 ± 0.02a
6.0	109.08 ± 1.05b	10.88 ± 0.07b	8.37	8.49 ± 0.02b
6.5	85.80 ± 1.05d	8.51 ± 0.07d	6.55	6.97 ± 0.04e

Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

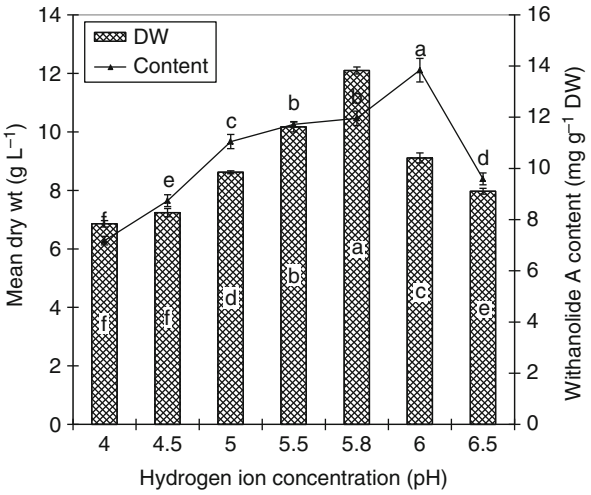

Fig. 12.11 *Withania somnifera* hairy root culture: effect of hydrogen ion concentration (pH) on biomass accumulation and withanolide A production. Hairy roots (500 mg) were cultured in 250 mL Erlenmeyer's flasks containing 50 mL of MS medium supplemented with 3 % sucrose for 4 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT)

[12]). In the adventitious root cultures of *W. somnifera*, the highest accumulation of biomass was observed with the initial medium pH of 5.8, whereas maximum production of withanolide was noticed with the medium pH of 5.5 followed by 5.8 (Table 12.8; [35]). Whereas, in the hairy root cultures of *W. somnifera*, the highest accumulation of biomass was observed when the medium pH was set at 5.8 and the maximum withanolide production was recorded when the initial medium pH was 6.0 (Fig. 12.11; [36]).

12.3.10 Effect of Macroelements on Biomass Accumulation and Withanolide Production

Various nutrient factors especially macroelements and microelements play an important role in biomass accumulation, and secondary metabolite production in cell and organ suspension culture systems [41] and they should be worked out systematically for obtaining optimized results. The role of macroelements has been studied in cell and adventitious/hairy root cultures of *W. somnifera* and following are the results of such efforts.

12.3.11 Effect of NH_4NO_3 on Biomass Accumulation and Withanolide Production

The cells of *W. somnifera* cultured in the medium containing the lower concentration of NH_4NO_3 (0.5× strength) accumulated the highest biomass (Table 12.9), while the highest production of withanolide content was recorded in the NH_4NO_3 – free medium (Fig. 12.12; [42]). The medium supplemented with 0.5× strength of NH_4NO_3 resulted in the maximum growth of adventitious roots and hairy root cultures (Tables 12.10 and 12.11) and withanolide production of *W. somnifera* (Figs. 12.13 and 12.14; [43, 44]).

Table 12.9 Biomass growth of *Withania somnifera* cell suspension culture as affected by concentrations of macro elements in the MS medium. Cultures were grown in 250 mL conical flasks containing 50 mL medium for 4 weeks[a]

Macro elements	Concentration (×times)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio
NH_4NO_3	0.0	128.00 ± 3.39b	12.47 ± 0.50b	11.34
	0.5	147.81 ± 2.39a	14.02 ± 0.04a	12.74
	1.0	111.06 ± 0.63d	10.24 ± 0.03cd	9.31
	1.5	94.34 ± 1.66ef	8.70 ± 0.33fghi	7.91
	2.0	87.10 ± 1.16fgh	8.13 ± 0.08hi	7.39
KNO_3	0.0	75.02 ± 0.56jk	7.09 ± 0.03jk	6.44
	0.5	90.04 ± 0.69efg	8.68 ± 0.05fghi	7.89
	1.0	107.81 ± 1.36d	9.88 ± 0.08de	8.98
	1.5	118.04 ± 2.46c	11.05 ± 0.38c	10.04
	2.0	143.75 ± 1.91a	14.33 ± 0.27a	13.03
$CaCl_2$	0.0	74.20 ± 1.50k	5.87 ± 0.11m	5.34
	0.5	90.74 ± 2.26efg	6.80 ± 0.17k	6.18
	1.0	121.50 ± 5.27c	10.18 ± 0.35cd	9.25
	1.5	96.62 ± 1.35e	8.01 ± 0.10hi	7.28
	2.0	84.50 ± 1.57gh	7.00 ± 0.12jk	6.36

Table 12.9 (continued)

Macro elements	Concentration (×times)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio
$MgSO_4$	0.0	75.75 ± 2.37ijk	6.54 ± 0.85kl	5.94
	0.5	82.31 ± 1.84hi	8.28 ± 0.14ghi	7.53
	1.0	91.04 ± 1.77efg	8.64 ± 0.09fghi	7.85
	1.5	93.12 ± 0.78ef	8.94 ± 0.11fgh	8.13
	2.0	82.40 ± 0.76hi	8.11 ± 0.02hi	7.37
KH_2PO_4	0.0	73.24 ± 0.42k	6.97 ± 0.02jk	6.34
	0.5	81.82 ± 0.26hij	7.79 ± 0.13ij	7.08
	1.0	93.16 ± 1.12ef	7.88 ± 0.68ij	7.16
	1.5	97.45 ± 0.67e	9.41 ± 0.26def	8.55
	2.0	109.42 ± 2.46d	9.11 ± 0.23efg	8.28

[a]Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

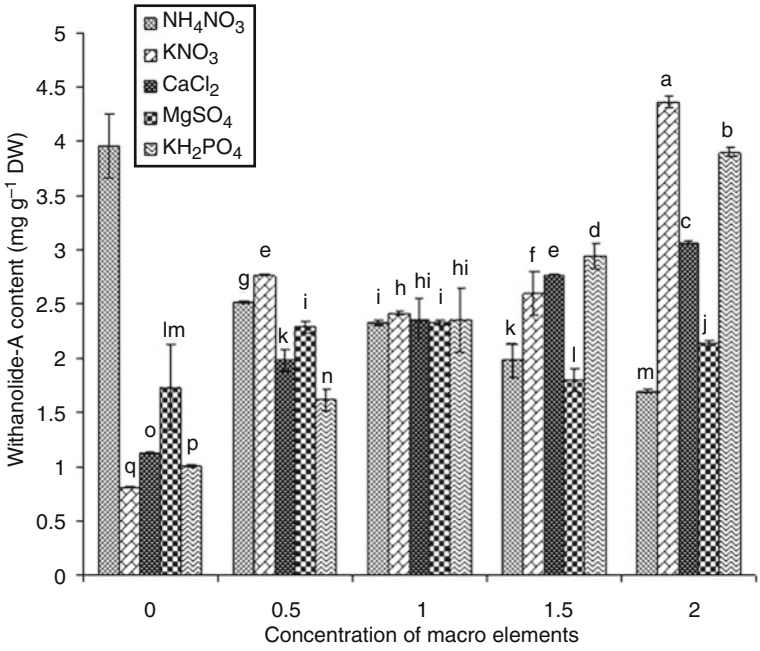

Fig. 12.12 Withanolide A content in *Withania* cell suspension after 4 weeks of culture as affected by different concentrations of macro elements. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT)

Table 12.10 Biomass growth of *Withania somnifera* adventitious roots as affected by concentrations of macro elements in the MS medium. Cultures were grown in 250 mL conical flasks containing 50 mL medium for 4 weeks

Macro elements	Concentration (×times)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio
NH_4NO_3	0.0	117.62 ± 1.29b	11.66 ± 0.12b	8.97
	0.5	127.52 ± 0.69a	12.45 ± 0.14a	9.58
	1.0	104.87 ± 1.84de	10.29 ± 0.10e	7.91
	1.5	78.04 ± 1.52h	7.68 ± 0.05i	5.91
	2.0	63.62 ± 1.52j	6.31 ± 0.14jk	4.85
KNO_3	0.0	59.98 ± 0.87j	5.82 ± 0.08l	4.48
	0.5	79.87 ± 0.51h	7.89 ± 0.02i	6.07
	1.0	111.20 ± 1.90c	11.00 ± 0.09c	8.46
	1.5	118.08 ± 0.84b	11.72 ± 0.05b	9.01
	2.0	126.40 ± 1.43a	12.51 ± 0.13a	9.62
$CaCl_2$	0.0	61.13 ± 1.44j	6.04 ± 0.08kl	4.65
	0.5	85.80 ± 0.81g	8.35 ± 0.05h	6.42
	1.0	103.53 ± 2.14e	10.21 ± 0.20e	7.85
	1.5	94.73 ± 0.26f	9.31 ± 0.04f	7.16
	2.0	116.92 ± 1.15b	11.53 ± 0.13b	8.87
$MgSO_4$	0.0	67.91 ± 1.33i	6.55 ± 0.14j	5.04
	0.5	85.85 ± 0.48g	8.36 ± 0.03h	6.43
	1.0	107.46 ± 0.48d	10.59 ± 0.01d	8.15
	1.5	126.92 ± 1.00a	12.48 ± 0.07a	9.60
	2.0	87.63 ± 0.85g	8.84 ± 0.06g	6.80
KH_2PO_4	0.0	62.13 ± 1.29j	6.02 ± 0.13kl	4.63
	0.5	87.18 ± 0.86g	8.50 ± 0.06h	6.54
	1.0	104.83 ± 1.18de	10.27 ± 0.15e	7.90
	1.5	118.18 ± 0.38b	11.69 ± 0.04b	8.99
	2.0	124.50 ± 0.76a	12.31 ± 0.07a	9.47

Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

Table 12.11 Biomass growth of *Withania somnifera* hairy root cultures as affected by concentrations of macro elements in the MS medium. Cultures were grown in 250 mL Erlenmeyer's flasks containing 50 mL medium for 4 weeks[a]

Macro elements	Concentration (×times)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth rate
NH_4NO_3	0.0	96.10 ± 1.30h	9.34 ± 0.07ij	7.41
	0.5	127.65 ± 0.75d	12.74 ± 0.08d	10.11
	1.0	118.23 ± 1.71f	11.70 ± 0.19g	9.29
	1.5	88.30 ± 1.94jk	8.74 ± 0.16k	6.94
	2.0	79.50 ± 1.20l	7.83 ± 0.06l	6.21

Table 12.11 (continued)

Macro elements	Concentration (×times)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth rate
KNO_3	0.0	77.18 ± 0.67l	7.69 ± 0.10l	6.10
	0.5	86.84 ± 1.13k	8.83 ± 0.02k	7.01
	1.0	115.68 ± 0.62fg	11.54 ± 0.11g	9.16
	1.5	124.07 ± 0.35e	12.34 ± 0.02ef	9.79
	2.0	137.87 ± 0.75ab	13.69 ± 0.06a	10.86
$CaCl_2$	0.0	78.51 ± 1.76l	7.76 ± 0.18l	6.16
	0.5	95.66 ± 0.68h	9.51 ± 0.09i	7.55
	1.0	124.08 ± 1.63e	12.23 ± 0.13f	9.71
	1.5	127.39 ± 1.27de	12.62 ± 0.12de	10.02
	2.0	133.18 ± 1.14c	13.28 ± 0.11bc	10.54
$MgSO_4$	0.0	76.15 ± 0.47lm	7.48 ± 0.06l	5.94
	0.5	91.25 ± 0.74ij	9.08 ± 0.03jk	7.21
	1.0	118.04 ± 0.59f	11.70 ± 0.08g	9.29
	1.5	134.85 ± 0.82bc	13.50 ± 0.18ab	10.71
	2.0	118.35 ± 0.91f	11.71 ± 0.06g	9.29
KH_2PO_4	0.0	73.24 ± 0.42m	6.97 ± 0.02m	5.53
	0.5	91.82 ± 0.26i	8.93 ± 0.01k	7.09
	1.0	113.16 ± 1.12g	10.75 ± 0.11h	8.53
	1.5	117.45 ± 0.67f	11.41 ± 0.26g	9.06
	2.0	139.42 ± 2.46a	13.11 ± 0.23c	10.40

[a]Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

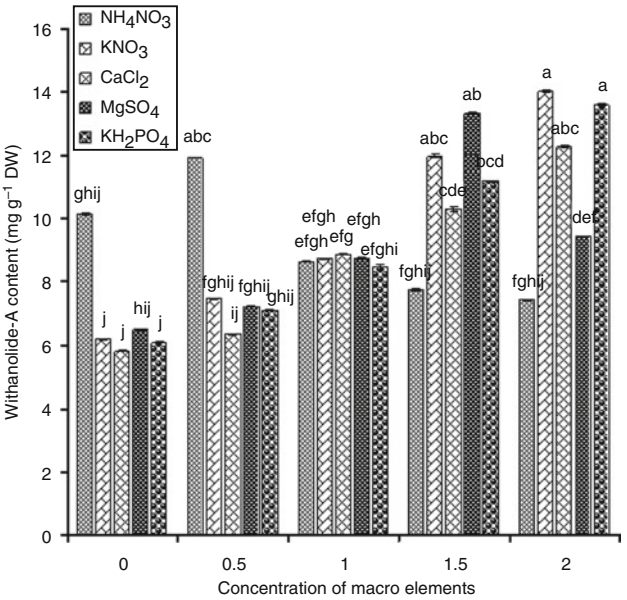

Fig. 12.13 Withanolide A content in *Withania* adventitious root culture after 4 weeks of culture as affected by different concentrations of macro elements. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P < 0.05$ according to Duncan's multiple range test (DMRT)

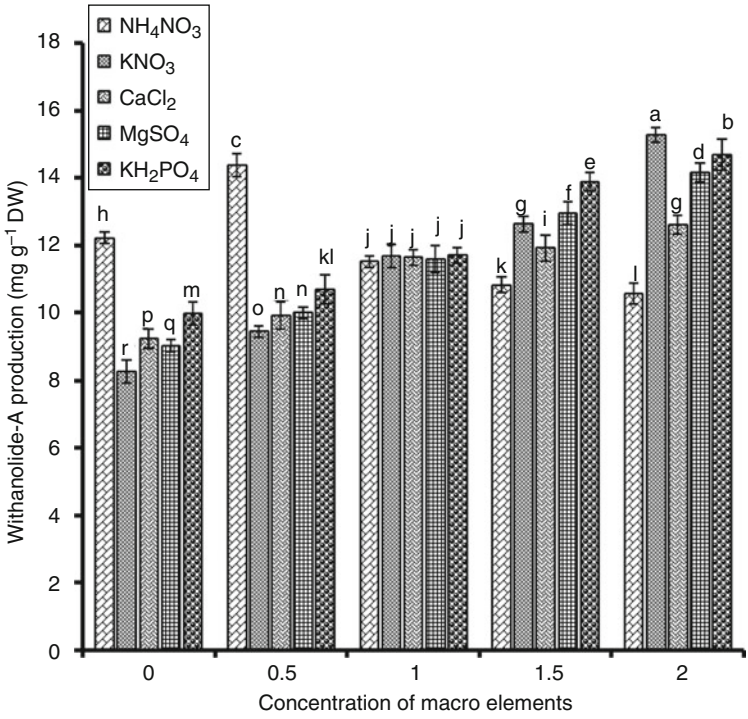

Fig. 12.14 Withanolide A content in *Withania* hairy root cultures as affected by different concentrations of macroelements. Hairy roots (500 mg) were cultured in 250 mL Erlenmeyer flasks containing 50 mL of MS medium supplemented with 3 % sucrose for 4 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT)

12.3.12 Effect of KNO_3 on Biomass Accumulation and Withanolide Production

In cell suspension, adventitious roots and hairy root cultures of *W. somnifera*, highest accumulation of biomass (Tables 12.9, 12.10, and 12.11) and withanolide content was observed in the MS medium supplemented with 2.0× KNO_3 (Figs. 12.12, 12.13, and 12.14; [42–44]).

12.3.13 Effect of $CaCl_2$ on Biomass Accumulation and Withanolide Production

Nagella and Murthy [42] reported that in cell suspension cultures, 1.0× strength of $CaCl_2$ promoted the highest accumulation of biomass (Table 12.9) and 2.0× strength of $CaCl_2$ favored the maximum production of withanolide content (Fig. 12.12). In the

adventitious roots and hairy root cultures, among the different concentrations of $CaCl_2$ tested, 2.0× strength of $CaCl_2$ favored both the root biomass accumulation (Tables 12.10 and 12.11) and withanolide production (Figs. 12.13 and 12.14; [43, 44]).

12.3.14 *Effect of $MgSO_4$ on Biomass Accumulation and Withanolide Production*

In the cell suspension cultures of *W. somnifera*, of the $MgSO_4$ concentrations tested, the highest accumulation of biomass was recorded in the medium with 1.5× strength of $MgSO_4$ (Table 12.9) and the highest production of withanolide content was recorded in the medium supplemented with 1.0× strength of $MgSO_4$ (Fig. 12.12; [42]). Murthy and Praveen [43] reported that 1.5× strength of $MgSO_4$ was favorable for the biomass accumulation (Table 12.10) and withanolide production (Fig. 12.13) in the adventitious root cultures. In the hairy root cultures, 1.5× strength of $MgSO_4$ favored the biomass accumulation (Table 12.11) whereas higher concentration of 2.0× strength favored the withanolide production (Fig. 12.14; [44]).

12.3.15 *Effect of KH_2PO_4 on Biomass Accumulation and Withanolide Production*

Of the different concentration of KH_2PO_4 tested in the cell and organ suspension cultures of *Withania*, the highest accumulation of biomass and withanolide content was observed in the medium supplemented with higher concentration of 2.0× strength of KH_2PO_4 (Tables 12.9, 12.10, and 12.11; Figs. 12.12, 12.13, and 12.14; [42–44]).

12.3.16 *Effect of Ammonium/Nitrate Ratios on Biomass Accumulation and Withanolide Production*

Nitrogen concentration affects the level of proteinaceous or amino acid products in cell suspension cultures. The ratio of the ammonia/nitrate and overall levels of total nitrogen markedly affect the production of secondary plant products. Table 12.12 and Fig. 12.15 illustrate the effects of ammonium/nitrate ratios in MS medium on the growth of cell suspension culture and accumulation of withanolides. Nitrate rather than ammonium was necessary for both cell growth and withanolide production. A low concentration of ammonium (7.19 mM) with the moderate concentration of nitrate (18.80 mM) was favorable for the highest accumulation of biomass. Maximum withanolide yield was achieved at a ratio of 14.38 (mM) ammonium to 37.60 (mM) nitrate followed by the ammonium free medium [42].

Table 12.12 Biomass growth of *Withania somnifera* cell suspension cultures as affected by NH_4^+/NO_3^- ratios in the MS medium. Cultures were grown in 250 mL conical flasks containing 50 mL medium for 4 weeks[a, b]

NH_4^+/NO_3^- ratios in MS medium (mM)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio
0.00/18.80	100.12 ± 1.04b	8.47 ± 0.10b	7.70
7.19/18.80	110.45 ± 1.92a	9.29 ± 0.16a	8.44
14.38/18.80	107.42 ± 0.67a	9.13 ± 0.05a	8.28
21.57/18.80	86.89 ± 0.98c	6.54 ± 0.07cd	5.94
28.75/18.80	73.70 ± 1.29e	5.15 ± 0.08e	4.68
14.38/0.00	38.66 ± 1.24f	3.03 ± 0.11f	2.75
14.38/9.40	84.06 ± 1.07c	6.32 ± 0.06d	5.74
14.38/18.80	107.31 ± 1.10a	9.05 ± 0.02a	8.23
14.38/28.20	97.46 ± 0.74b	8.27 ± 0.08b	7.52
14.38/37.60	78.84 ± 0.70d	6.70 ± 0.22c	6.09

[a]NH_4^+/NO_3^-=NH_4Cl/KNO_3 (mM/mM)

[b]Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

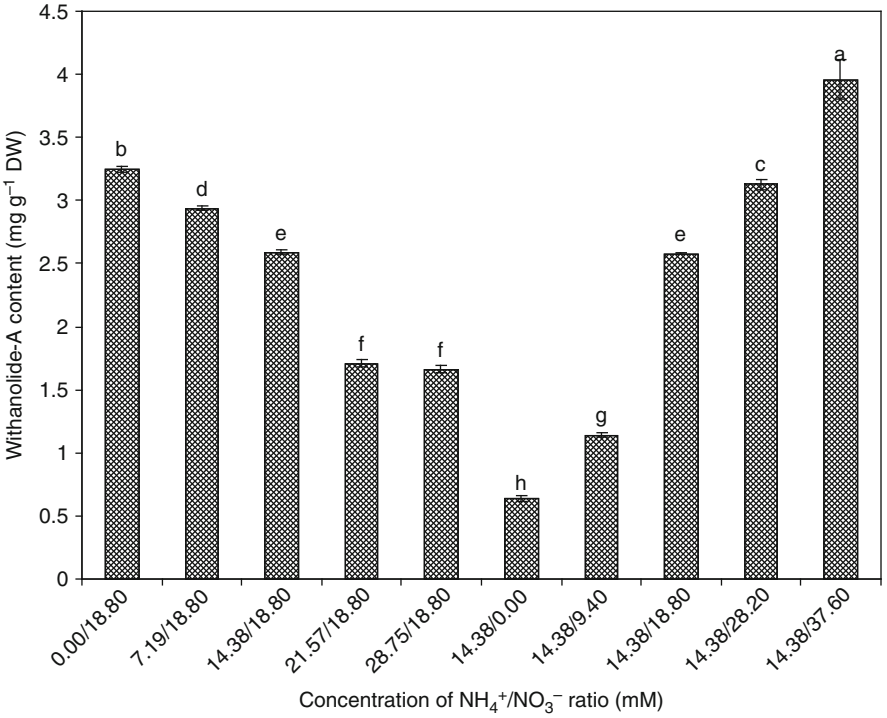

Fig. 12.15 Withanolide A content in cell suspension cultures after 4 weeks of culture as affected by different ratio of NH_4^+/NO_3^- in the MS medium. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT)

Table 12.13 Biomass growth of *Withania somnifera* adventitious roots as affected by NH_4^+/NO_3^- ratios in the MS medium. Cultures were grown in 250 mL conical flasks containing 50 mL medium for 4 weeks[a, b]

NH_4^+/NO_3^- ratios in MS medium (μM)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio
0.00/18.80	100.12 ± 1.04e	9.47 ± 0.10d	7.28
7.19/18.80	130.45 ± 1.92b	12.29 ± 0.16b	9.45
14.38/18.80	117.42 ± 0.67d	11.23 ± 0.05c	8.53
21.57/18.80	82.56 ± 0.90f	8.24 ± 0.14e	6.34
28.75/18.80	74.09 ± 0.73g	7.15 ± 0.08f	5.50
14.38/0.00	36.32 ± 1.29h	3.56 ± 0.20g	2.74
14.38/9.40	75.73 ± 0.80g	7.48 ± 0.11f	5.75
14.38/18.80	117.31 ± 1.10d	11.05 ± 0.02c	8.50
14.38/28.20	125.46 ± 1.61c	12.27 ± 0.08b	9.44
14.38/37.60	145.84 ± 1.01a	14.49 ± 0.09a	11.15

[a]NH_4^+/NO_3^-=NH_4Cl/KNO_3 (mM/mM)

[b]Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

Table 12.14 Biomass growth of *Withania somnifera* hairy root cultures as affected by NH_4^+/NO_3^- ratios in the MS medium. Cultures were grown in 250 mL Erlenmeyer's flasks containing 50 mL medium for 4 weeks[a, b]

NH_4^+/NO_3^- ratios in MS medium (μM)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth
0.00/18.80	110.12 ± 1.04e	10.47 ± 0.10d	8.31
7.19/18.80	140.45 ± 1.92b	13.29 ± 0.16b	10.55
14.38/18.80	127.31 ± 1.10d	12.05 ± 0.20c	9.56
21.57/18.80	85.22 ± 0.65f	8.54 ± 0.07e	6.78
28.75/18.80	75.09 ± 0.58g	7.15 ± 0.08f	5.68
14.38/0.00	36.66 ± 1.21h	3.70 ± 0.22g	2.94
14.38/9.40	74.06 ± 1.07g	7.32 ± 0.06f	5.81
14.38/18.80	127.31 ± 1.10d	12.05 ± 0.20c	9.56
14.38/28.20	135.46 ± 1.61c	13.27 ± 0.08b	10.53
14.38/37.60	148.17 ± 0.19a	14.79 ± 0.06a	11.74

[a]NH_4^+/NO_3^-=NH_4Cl/KNO_3 (mM/mM)

[b]Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

Generally, lower concentrations of ammonium to nitrate ratios are beneficial to plant cell cultures [45]. Similar observations were also reported with adventitious and hairy root cultures where maximum root growth was achieved at an ammonium/nitrate ratio of 14.38/37.60 mM (Tables 12.13 and 12.14) and the highest withanolide production was found with an ammonium/nitrate ratio of 0.00/18.80 mM (Figs. 12.16 and 12.17). Root growth and withanolide productivity reached only one-third of the maximum values when ammonium was used as the sole nitrogen source [43, 44]. Based on these results, it can be suggested that the nitrate and ammonium ions have differential effects on secondary metabolism in plant cell and organ cultures.

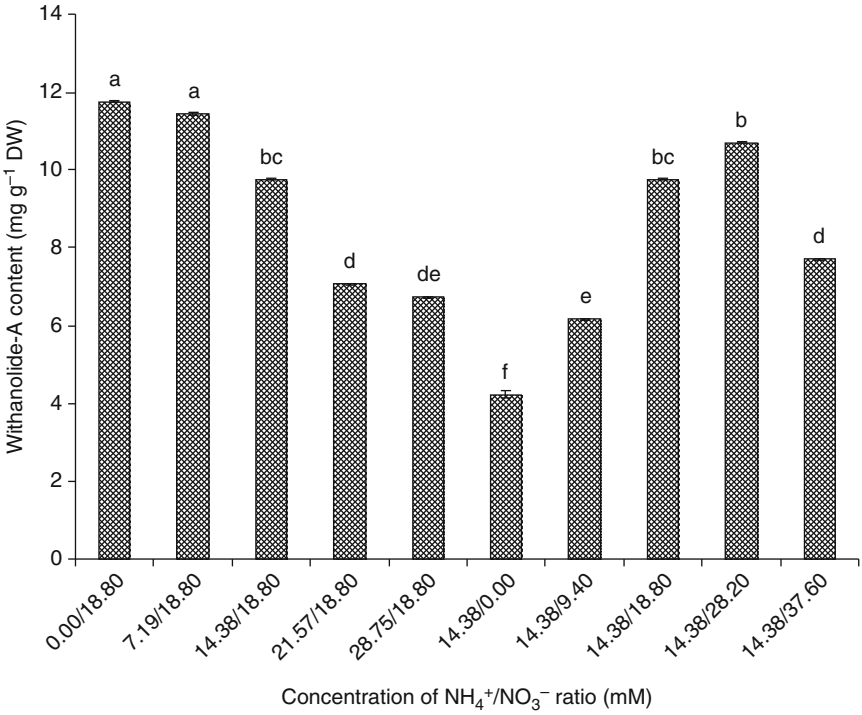

Fig. 12.16 Withanolide-A content in adventitious root cultures after 4 weeks of culture as affected by different ratio of NH_4^+/NO_3^- in the MS medium. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT)

12.4 Effect of Elicitors on Biomass Accumulation and Withanolide Production

Various attempts have been made to increase the biomass accumulation and withanolide production *in vitro* cultures of *Withania*. Baldi et al. [46] studied the effect of various abiotic (arachidonic acid, methyl jasmonate, calcium chloride and copper sulphate) and biotic elicitors (*Alternia alternate*, *Fusarium solani* and *Verticilium dahaliae*) on the production of withaferin A from transformed cell cultures and found 5.4 and 9.7 times higher production respectively with copper sulphate (100 μM) and the cell extract of *V. dahaliae* (5 % v/v). The dual elicitation strategy by the combined addition of these two elicitors resulted in 13.8 fold enhancement of withaferin A content in comparison to control cultures. The effect of various organic additives (L-glutamine, casein hydrolysate, adenine sulphate, coconut water and malt extract) and seaweed extracts (*Sargassum wightii* and *Gracilaria edulis*) were studies by Sivanandhan et al. [13] on the biomass accumulation and withanolide production. Among the different organic additives supplemented in the cell

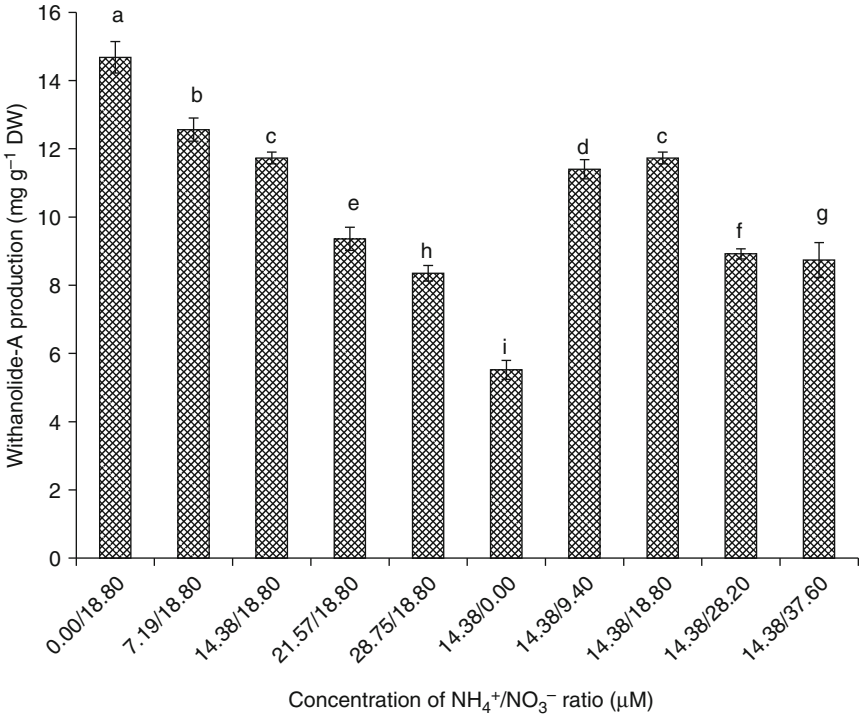

Fig. 12.17 Withanolide A content in hairy root cultures as affected by different ratio of NH_4^+/NO_3^- in the MS medium. Hairy roots (500 mg) were cultured in 250 mL Erlenmeyer flasks containing 50 mL of MS medium supplemented with 3 % sucrose for 4 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test (DMRT)

suspension cultures, L-glutamine at 200 mg L^{-1} resulted in the highest accumulation of biomass and withanolides production followed by 150 mg L^{-1} casein hydrolysate. The supplementation of 40 and 50 % extract of *G. edulis* resulted in the highest accumulation of biomass and withanolide production compared to *S. wightii*.

In the adventitious root cultures of *Withania*, salicylic acid and methyl jasmonate at different concentrations and different exposure time were studied for the biomass accumulation and withanolide production and the results suggested that addition of 30-day-old adventitious root cultures with 150 μM salicylic acid for 4 h elicitor exposure period and harvesting the adventitious roots at 40 days interval resulted in the highest production of withanolides compared to the methyl jasmonate treatments [47]. In another study, Sivanandhan et al. [17] reported that chitosan at 100 mg L^{-1} with 4 h exposure time stimulated the higher withanolides production at the end of 6 weeks of culture period when compared with different concentrations and exposure time of chitosan and aluminium chloride.

Various concentrations of methyl jasmonate and salicylic acid and specific exposure times were studied for the production of withanolides from hairy root cultures.

Enhanced production of biomass and withanolides were achieved from 40 day old harvested hairy roots elicited with 150 μM SA for 4 h exposure time when compared with other concentration of SA (0, 50, 100, 150, and 200 μM) or MJ (0, 5, 10, 15 and 20 μM) with several exposure times (0, 2, 4, 6, and 8) [27]. Doma et al. [38] studied the effect of different elicitors viz., chitosan, jasmonic acid, acetyl salicylic acid, sodium nitroprusside and triadimefon at different concentrations on hairy root biomass accumulation and withanolides production and found that triadimefon at 10 mg L^{-1} was optimal for the accumulation of hairy root biomass whereas 100 mg L^{-1} triadimefon was found suitable for the withaferin A production and withanolide A was not detected in most of the treatments. Hairy roots elicited with 1 mg L^{-1} acetyl salicylic acid improved both withaferin A and withanolide A production.

12.5 Conclusions and Perspectives

Extensive research work has been carried out on *Withania* cell and organ cultures, such as optimization of culture medium, physical conditions and strategies to improve flask scale cultures. The work also included maximizing biomass yield and withanolides contents. *Withania* cell and organ cultures have been evolved as an alternative to field grown plants and optimized biomass and metabolite production is possible by adopting optimized culture conditions including elicitation.

In addition to the production of *Withania* biomass and withanolides, other opportunities still exist for the utilization of cell and organ cultures for the production of valuable ingredients such as saponins, biophenols, alkaloids, acylsteryl glucosides and various ubiquitous compounds such as fatty acids, amino acids and vitamins. In the light of current success, future research should be focused on development of large scale and bioreactor culture processes such as continuous culture, process monitoring, modeling and control which may be useful for reducing the production cost and meeting the supply of the active constituents.

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Chapter 13
Production of Bioactive Compounds from Somatic Embryo Suspension Cultures of Siberian Ginseng in Bioreactors

Abdullah Mohammad Shohael, Sayeda Mahfuja Khatun, Hosakatte Niranjana Murthy, and Kee-Yoeup Paek

Abstract *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim (*Acanthopanax senticosus*), popularly known as 'Siberian ginseng', is a woody medicinal plant which is used in traditional medicine as an adaptogen. It is marketed throughout the world as a health supplement. The major active ingredients are lignan glycosides called eleutherosides. Efforts have been made recently to produce bioactive compounds from suspension cultures of somatic embryos. Bioreactor cultures have been established for the production of embryogenic biomass and bioactive compounds. In this review, we have presented bioreactor scale production of bioactive compounds and explained various bioprocess strategies for the production of eleutheroside B, E, E1 and chlorogenic acid.

Keywords Bioreactor cultures • Chlorogenic acid • Embryos • Eleutherosides • Siberian ginseng

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Abbreviations

2 4-D	2, 4-Dichlorophenoxy acetic acid
C_2H_4	Ethylene
CO_2	Carbon dioxide
DW	Dry weight
FW	Fresh weight
GA_3	Gibberellic acid
MJ	Methyl jasmonate
MS medium	Murashige and Skoog medium
O_2	Oxygen
vvm	Air volume per medium volume per minute

13.1 Introduction

Eleutherococcus senticosus (Rupr. & Maxim.) Maxim (*Acanthopanax senticosus*) is a woody medicinal plant popularly known as 'Siberian ginseng.' It is distributed in southeast Russia, Northeastern China, Korea and Japan [1, 2]. This plant is used in traditional Chinese medicine as an adaptogen and to strengthen spleen and kidney [3]. It is popular in other parts of the world as an health supplement and recorded as a pharmacopeial species in monographs of European Pharmacopoeia and American Herbal Pharmacopoeia. The natural products isolated from *Eleutherococcus senticosus* have been shown to have various activities including antibacterial, anticancer, anti-inflammatory, antigout, antihepatitis, antihyperglycemic, antileishmanicidic, antioxidant, haemostatic, immuno-stimulatory, and hypocholesterolemic effects [2]. The main chemical substances of *E. senticosus* are eleutherosides and these are lignan glycosides (Fig. 13.1). Among various eleutherosides, eleutheroside B (synarin; a phenyl propanoid glycoside), eleutheroside E ((−) syringaresinol-di-O-β-D-glucoside) and eleutheroside E1 are known to be the main active principles of *E. senticosus* [4].

Plant cell and organ cultures have emerged as useful techniques for the production of bioactive compounds, and bioreactor based systems have been developed for the production of ginsenosides [5], phenolics [6] and alkaloids [7]. Somatic embryos have been induced in various species of *Eleutherococcus* and the bioreactor cultures of embryos were established [8–10]. In this review, summerize bioreactor culture of somatic embryos of *E. senticosus* for the production of bioactive compounds.

13.1.1 Induction of Embryos and Establishment of Suspension Cultures in Shake Flasks

Embryogenic callus of *E. senticosus* was induced by using root explants on Murashige and Skoog (MS) [11] medium supplemented with 3 % (w/v) sucrose, 1.0 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) and 2.3 g L^{-1} gelrite [8].

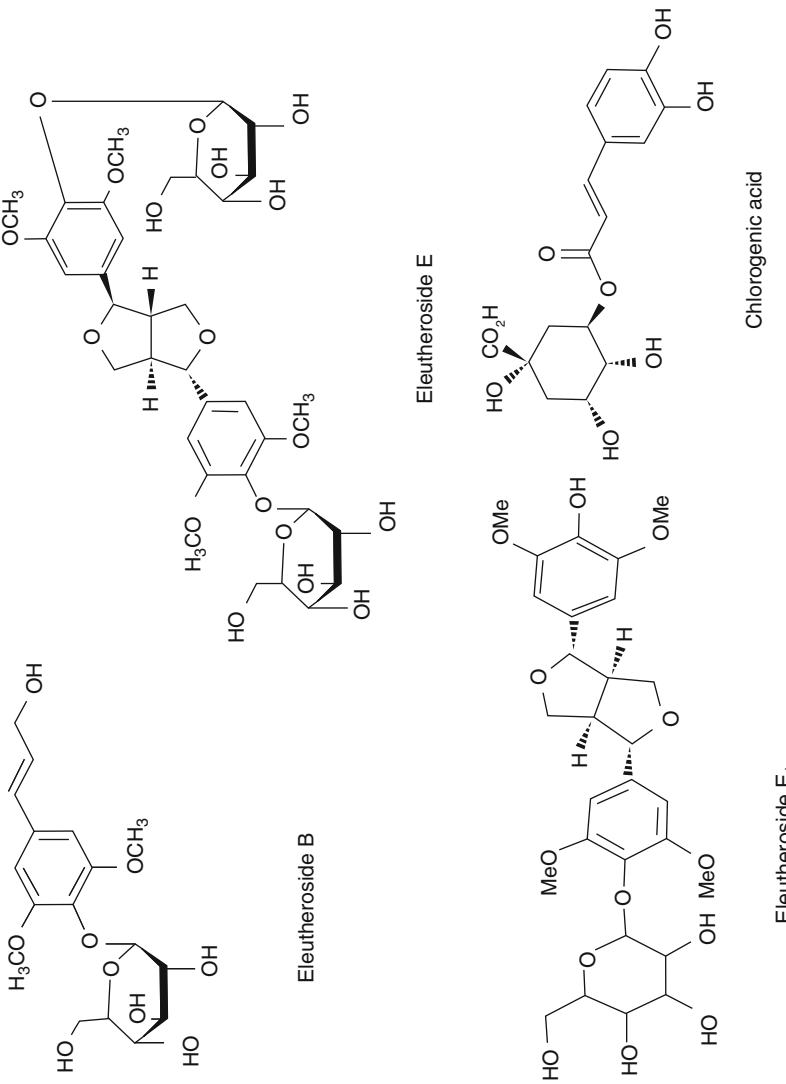

Fig. 13.1 Eleutherosides and chlorogenic acid present in *E. senticosus*

Embryogenic cells were maintained in MS liquid medium with 3 % (w/v) sucrose, 1.0 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) by sub-culturing in fresh medium in 250 mL shake flasks (containing 100 mL of medium). Cultures were maintained in darkness at a temperature of 25 °C on gyratory shaker at 100 rpm and were subcultured once in every 3 weeks.

About 500 mg of embryogenic cell clumps were cultured in 250 mL shake flasks containing 100 mL of MS medium with 30 g L^{-1} sucrose, without growth regulators for the embryo development and to study the growth kinetics of somatic embryos (Fig. 13.3a). The cultures were maintained in darkness at a temperature of 25 °C on gyratory shaker at 100 rpm for 63 days. A typical growth curve was observed during somatic embryo development and biomass accumulation with a lag-phase until 7 days, an exponential phase of growth from day 7 to day 35 and a stationary phase from day 35 till day 49 (Fig. 13.2a). The biomass doubling time (*td*) was 4.85 h (7 days) and the specific growth rate (μ) during the exponential phase was 0.022 day^{-1} (Table 13.1). The changes in the hydrogen ion concentration (pH) and electrical conductivity (EC) during culture is presented in Fig. 13.2b. The pH of the culture increased from 5.68 to 5.94 on day 7, after that the pH reached to 6.13. The changes in the pH reveal the absorption of specific mineral elements by the developing embryos in the medium [12]. EC levels decreased gradually and this also reflects the increase in biomass accumulation that occurred over the time lapse. This finding is similar to the results of studies conducted by Ryu et al. [13] and Taya et al. [14], who have correlated the conductivity parameters with the cell biomass.

13.2 Establishment of Bioreactors Cultures

Bioreactor based systems have been developed for the production of bioactive compounds in many plants [8, 9, 15, 16] and various parameters such as inoculum size, medium components, efficient oxygen transfer and mixing and other physico-chemical parameters have been investigated. We established bioreactor cultures with the objective of production of eleutherosides and chlorogenic acid. Ten grams of embryogenic cells were cultured in 3 L balloon type airlift bioreactors containing 2 L MS medium with 30 g L^{-1} sucrose and investigated growth regulators (Fig. 13.3b). The volume of input air was adjusted to 0.1 vvm. All the bioreactors were maintained at 25 °C in dark. Complete embryo development was achieved after 30 days in this medium. We investigated various factors such as the effect of bioreactor type, aeration volume, temperature, light, gaseous nutrients such as oxygen, carbon dioxide and ethylene on biomass and secondary metabolite accumulation.

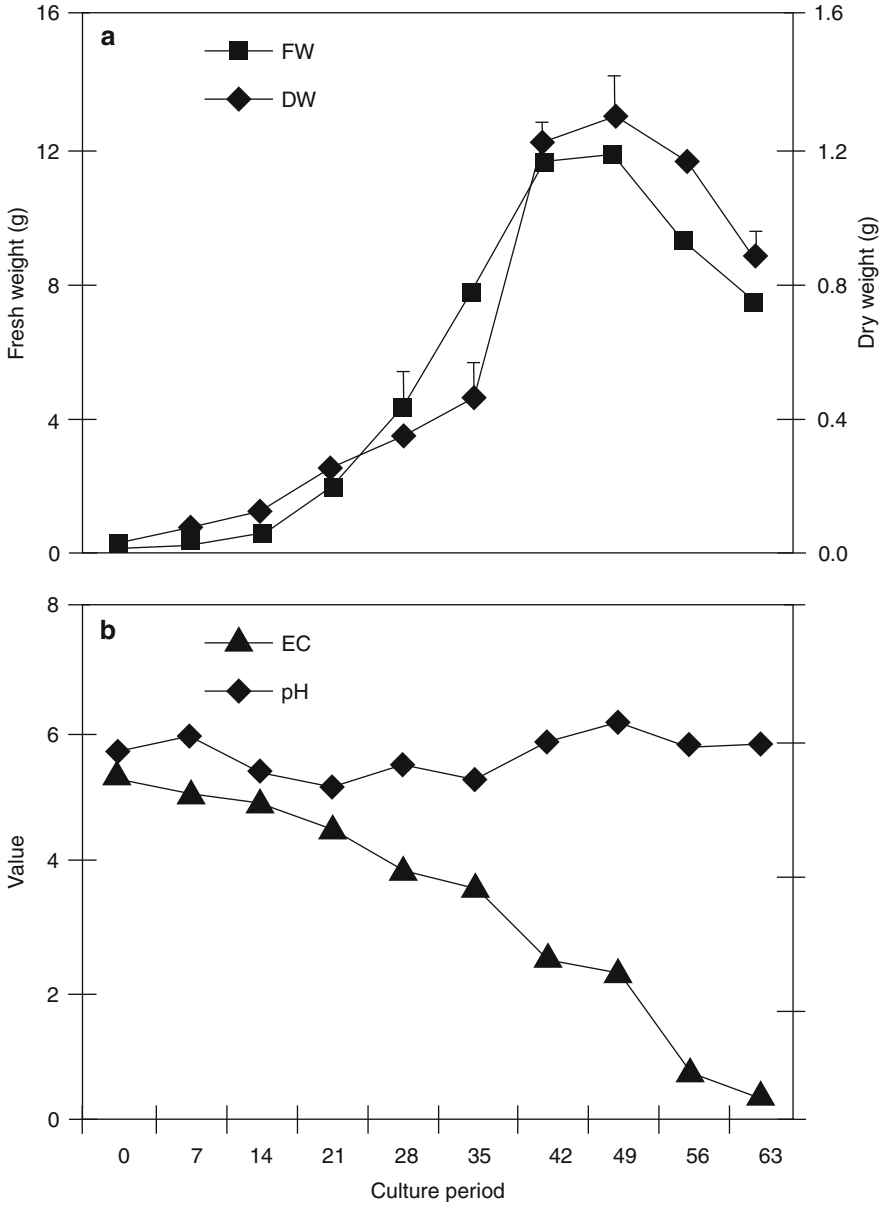

Fig. 13.2 Changes in (**a**) fresh weight, dry weight, (**b**) pH, electrical conductivity (*EC*) of *E. senticosus* embryos during suspension cultures

Table 13.1 Specific growth rate (μ) and doubling time (T_d) of *E. senticosus* somatic embryos

Day	7	14	21	28	35	42	49	56	63
μ	0.142	0.071	0.048	0.036	0.029	0.023	0.020	0.018	0.016
$T_d = 1n\ 2/\mu_{max}$(day)				4.85					

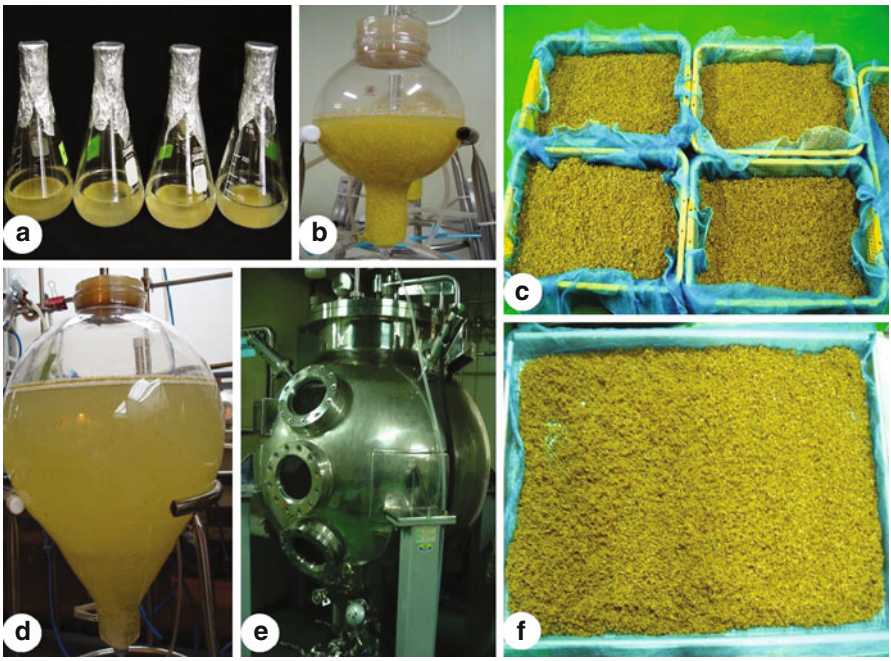

Fig. 13.3 Suspension cultures of somatic embryos of *Eleutherococcus senticosus*: (**a**) Embryogenic cells in MS liquid medium supplemented with 30 g sucrose L^{-1} and 1.0 mg 2,4-dichlorophenoxy acetic acid L^{-1}. (**b**) Embryogenic suspension in 3 L capacity balloon-type airlift bioreactor containing 2 L MS medium with 30 g sucrose L^{-1}. (**c**) Biomass harvested from 500 L balloon bioreactor after 30 days of culture. (**d**) Embryogenic suspension in 20 L balloon type airlift bioreactor. (**e**) Embryogenic suspension in 500 L balloon-type airlift bioreactor. (**f**) Biomass harvested from 20 L balloon bioreactor after 30 days of culture

13.2.1 The Effect of Bioreactor Type on Production of Biomass and Bioactive Compounds

To facilitate accumulation of biomass and bioactive compounds, selection of suitable bioreactors is essential [17, 18]. We have used balloon, bulb, cone and cylinder type bioreactors for culturing of *E. senticosus* somatic embryos and got highest biomass with balloon type bioreactors (Table 13.2). 102.3 g L^{-1} fresh biomass and 11.3 g L^{-1} dry biomass was obtained in balloon type airlift bioreactors and the growth ratio was also optimum (17.0). Optimal amount of eleutheroside

Table 13.2 Effect of bioreactor type on growth of *E. senticosus* somatic embryos and secondary metabolites production

Bioreactor type	Initial k_La (h^{-1})	Biomass g L^{-1}			Growth ratio[a]	Eleutherosides (μg g^{-1} DW)				Chlorogenic acid (mg g^{-1} DW)
		FW	DW	% DW		B	E	E_1	Total	
Balloon	6.98	102.3 a[b]	11.3 a	11.1	17.0	20.0 b	47.2 a	34.4 a	101.6 a	1.1 a
Bulb	6.95	98.6 a	11.3 a	11.4	16.9	17.2 c	48.7 a	26.5 b	92.4 b	1.0 b
Cone	5.69	85.0 b	9.8 b	11.5	14.5	22.8 b	42.7 b	20.2 c	85.7 c	0.8 c
Cylinder	5.25	73.8 c	8.2 c	11.1	12.0	29.4 a	35.5 c	16.6 d	81.5 c	0.8 c

[a]Growth ratio is the quotient of the dry weight after culture and the dry weight of the inoculum size
[b]Mean separation within column by Duncan's multiple range test at $p \leq 0.05$

B (20.0 μg g^{-1} DW), eleutheroside E (47.2 μg g^{-1} DW), eleutheroside E1 (34.4 μg g^{-1} DW) and chlorogenic acid (1.1 mg g^{-1} DW) were accumulated in embryos cultivated in balloon type bioreactors. The volumetric oxygen transfer coefficient (k_La) is an important factor for biomass growth during bioreactor cultures and the optimal k_La values (6.98) of balloon type bioreactors might be responsible for enhanced accumulation of biomass and secondary metabolites. Since we got optimal results with balloon type airlift bioreactors further experiments were carried out by using these bioreactors.

13.2.2 The Effect of Aeration Volume on Production of Biomass and Bioactive Compounds

In bioreactor cultures, the volumetric airflow rate is an important parameter that affects oxygen transfer, degree of turbulence and medium circulation [19]. Constant air supply of 0.05, 0.1, 0.2, 0.3 vvm and variable air supply of 0.05/0.1/0.2/0.3 vvm (air supply were changed once in every 7 days over the culture period) were tested on biomass and metabolite accumulation and the results are presented in Table 13.3. Maximum fresh and dry biomass accumulation was observed with the bioreactors supplemented with variable supply of air volumes over the culture period (Table 13.3). 25.7 μg g^{-1} DW of eleutheroside B, 52.5 μg g^{-1} DW of eleutheroside E, 30.2 μg g^{-1} DW of eleutheroside E1 and 1.0 mg g^{-1} DW of chlorogenic acid were recorded with cultures supplemented with variable air volume over the culture period and such variable supply of aeration volume facilitates proper agitation and also it prevents setting of the embryogenic biomass at the bottom of bioreactors.

13.2.3 The Effect of Inoculum Density on Production of Biomass and Bioactive Compounds

Inoculum density/size is one of the factors that determine the accumulation of biomass and the productivity of bioactive compounds from *in vitro* cultures [19, 20]. In this study, the inoculum density was found to have profound influence on growth, accumulation of biomass and the production of eleutherosides and chlorogenic acid (Table 13.4). The maximum biomass was obtained (103.7 g L^{-1} FW and 11.5 g L^{-1} DW) when 5 g L^{-1} of embryogenic cells were fed into the bioreactors. Optimal productivity of eleutherosides (21.2 μg g^{-1} DW of eleutheroside B, 49.9 μg g^{-1} DW of eleutheroside E, 28.9 μg g^{-1} DW of eleutheroside E1) and chlorogenic acid (1.2 mg g^{-1} DW) were also obtained when the inoculum density was 5 g L^{-1}.

Table 13.3 Effect of air volume on growth and secondary metabolite production of *E. senticosus* somatic embryos in bioreactor

Air volume (vvm)	Initial k_La (h^{-1})	Biomass g L^{-1}			Growth ratio[a]	Eleutherosides (μg g^{-1} DW)				Chlorogenic acid (mg g^{-1} DW)
		FW	DW	% DW		B	E	E_1	Total	
0.05	4.95	97.7 a[b]	11.1 a	11.3	16.6	18.5 c	41.1 b	17.5 d	77.1 d	0.9 b
0.1	7.84	96.1 a	10.1 a	10.5	15.0	25.5 a	43.8 b	26.5 b	95.8 b	1.0 a
0.2	11.42	90.7 b	10.0 a	11.0	14.9	21.0 b	42.8 b	21.4 c	85.2 c	0.7 c
0.3	16.81	78.0 c	8.3 b	10.6	12.2	23.0 b	37.8 c	22.1 c	82.9 c	0.8 c
0.05/0.1/0.2/0.3[c]	5.0–16.58	99.2 a	11.3 a	10.9	16.5	25.7 a	52.5 a	30.2 a	108.5 a	1.0 a

[a]Growth ratio is the quotient of the dry weight after culture and the dry weight of the inoculum size
[b]Mean separation within column by Duncan's multiple range test at $p \leq 0.05$
[c]Air volume increased at 10 day intervals

Table 13.4 The effect of inoculum density on biomass accumulation and secondary metabolite production of *E. senticosus* somatic embryos in bioreactor

Inoculum density (g L^{-1})	Biomass g L^{-1}			Growth ratio[a]	Eleutherosides (μg g^{-1} DW)				Chlorogenic acid (mg g^{-1} DW)
	FW	DW	% DW		B	E	E_1	Total	
1	97.7 a[b]	10.8 a	11.0	19.4	12.9 c	50.5 a	26.6 c	90.0 b	1.0 b
3	98.1 a	11.0 a	11.2	19.8	20.5 b	47.9 b	25.2 c	93.6 a	1.1 a
5	103.7 a	11.5 a	11.1	20.8	21.2 a	49.9 a	28.9 b	100.0 a	1.2 a
7	98.0 a	10.3 b	10.5	18.5	22.3 a	42.2 c	32.8 a	97.3 a	0.8 c
9	94.2 b	9.3 c	9.9	16.6	18.2 b	26.1 d	29.5 b	73.8 c	0.6 d

[a]Growth ratio is the quotient of the dry weight after culture and the dry weight of the inoculum size
[b]Mean separation within column by Duncan's multiple range test at $p \leq 0.05$

Table 13.5 The effect of temperature on biomass accumulation and secondary metabolite production of *E. senticosus* somatic embryos in bioreactor

Temp. (°C)	Biomass g L^{-1}			Growth ratio[a]	Eleutherosides (μg g^{-1} DW)				Chlorogenic acid (mg g^{-1} DW)
	FW	DW	% DW		B	E	E_1	Total	
12	68.3 b[b]	5.1 c	7.4 d	8.6 c	ND[c]	43.1 a	12.7 b	55.8 b	0.5 b
18	78.0 b	8.4 b	10.7 a	12.3 b	15.9 b	26.9 b	11.7 b	54.5 b	1.0 a
24	102.1 a	11.10 a	10.8 b	16.6 a	21.2 a	42.0 a	39.6 a	102.8 a	1.0 a
30	48.3 c	4.6 c	9.6 c	7.8 c	ND	ND	ND	ND	ND

[a]Growth ratio is the quotient of the dry weight after culture and the dry weight of the inoculum size
[b]Mean separation within column by Duncan's multiple range test at $p \leq 0.05$
[c]Not detected

13.2.4 The Effect of Incubation Temperature on the Production of Biomass and Bioactive Compounds

It has been shown that the optimal temperature treatment of suspension cultures is necessary for the accumulation of biomass and secondary metabolites [21, 22]. In the present study, bioreactor cultures were maintained at four different temperature regimes i.e., 12, 18, 24 and 30 °C to verify their effect on accumulation of biomass and bioactive compounds. The accumulation of biomass was optimum with cultures incubated at 24 °C, 102.1 g L^{-1} fresh biomass and 11.10 g L^{-1} dry biomass was evident (Table 13.5). Production of eleutherosides B (21.2 μg g^{-1} DW), eleutheroside E1 (39.6 μg g^{-1} DW) and chlorogenic acid (1.0 mg g^{-1} DW) was also highest with cultures incubated at 24 °C. However, highest accumulation of eleutheroside E (43.1 μg g^{-1} DW) was recorded with the cultures incubated at temperature 12 °C.

13.2.5 The Effect of Oxygen, Carbon Dioxide and Ethylene on Production of Biomass and Bioactive Compounds

Oxygen is an important gaseous nutrient, which is available in the bioreactor cultures as dissolved oxygen or in the form of bubbles with airlift bioreactors with the incoming air. Plant cells have lower metabolic rates than microbial cells and therefore require comparatively low oxygen supply. Further, oxygen requirement of the cultured cells and organ vary from species to species and it affects metabolic activity and energy supply. To facilitate enhanced oxygen supply the incoming air was supplemented with 30, 40 and 50 % oxygen and the results revealed that extra supplementation of oxygen was not beneficial for both biomass and metabolite accumulation in embryogenic suspensions of *E. senticosus* (Table 13.6). However, the improvement of secondary metabolite accumulation in oxygen enrichment cultures was reported by Gao and Lee [23], Han and Zhong [24] and Thanh et al. [25].

Table 13.6 Effect of oxygen and carbon dioxide on growth and total eleutherosides production in *E senticosus* somatic embryos in bioreactor

Name of the gases	Concentration used	Biomass (g L^{-1})			Growth ratio[a]	Total eleutherosides (μg g^{-1} DW)
		FW	DW	% DW		
O_2 (%)	Control	105.3 a[b]	10.2 a	9.7	15.2	97.8
	30	101.1 ab	10.0 a	9.9	14.9	90.0
	40	94.3 ab	9.7 a	10.3	14.5	74.8
	50	89.2 b	9.9 a	11.1	14.7	67.9
CO_2 (%)	Control	105.6 a	10.6 b	10.0	19.0	89.6
	1.0	84.7 b	10.5 b	12.4	18.8	69.1
	2.5	84.6 b	11.3 a	13.4	20.4	49.6
	5.0	79.3 d	10.7 ab	13.5	19.2	46.4
	10.0	83.4 c	10.6 b	12.7	19.0	33.8

[a]Growth ratio is the quotient of the dry weight after culture and the dry weight of the inoculum size
[b]Mean separation within column by Duncan's multiple range test at $p \leq 0.05$

Maurel and Preilleux [26] observed the increased growth of *Catharanthus roseus* cultures in carbon dioxide (CO_2) environment. Similarly, CO_2 enrichment was found to cause an enhancement of secondary metabolites in *Digitalis purpurea* [27] and *Panax ginseng* [28]. Experiments were conducted with embryogenic suspension cultures of *E. senticosus* with the supplementation of incoming air with 1, 2.5, 5.0, and 10.0 % CO_2 and results showed the supplementation of CO_2 was not beneficial both for biomass and metabolite accumulation (Table 13.6).

Effect of ethylene was also studied by many researchers [29, 30] and reported the inhibition of growth and stimulation of secondary metabolite production. For example, Kim et al. [29] found stimulating effect of ethylene on production of alkaloids in cell suspensions of *Thalictrum rugosum. E. senticosus* embryogenic cultures were supplemented with 1.0, 2.5, 5.0 and 10.0 ppm of ethylene and results showed that inhibitory effect on biomass accumulation (Table 13.7). However, there was increment in accumulation of eleutheroside B (32.9 μg g^{-1} DW), eleutheroside E (89.2 μg g^{-1} DW) and eleutheroside E1 (174.7 μg g^{-1} DW) with the supplementation of 2.5 ppm of ethylene. Increment in chlorogenic acid (1.9 mg g^{-1} DW) was documented with supplementation of 5.0 ppm of ethylene (Table 13.7).

13.2.6 The Effect of Light Quality on the Production of Biomass and Bioactive Compounds

Light is an important factor for the accumulation of cell biomass and the formation of secondary metabolites. The stimulatory effects of light on the productivity of secondary metabolites have shown in *Petroselinum hortense* [31], *Beta vlugaris*

Table 13.7 Effect of ethylene on growth and secondary metabolite production in *E. senticosus* somatic embryos in bioreactor

Name of the gases	Conc. used	Biomass g L^{-1}			Growth ratio[a]	Eleutherosides (μg g^{-1} DW)				Chlorogenic acid (mg g^{-1} DW)
		FW	DW	% DW		B	E	E_1	Total	
C_2H_4 (ppm)	Cont.	105.6 a[b]	10.6 a	10.0	19.0	22.5 d	29.6 e	33.8 e	85.9 e	1.1 d
	1.0	104.3 a	9.8 b	9.4	17.5	25.6 c	59.5 d	40.7 d	125.8 d	1.4 c
	2.5	92.5 b	8.8 c	9.6	15.7	32.9 b	89.2 a	53.5 a	175.6 a	1.5 c
	5.0	90.1 b	8.5 c	9.5	15.1	36.0 a	70.0 b	44.5 c	150.5 b	1.9 a
	10.0	83.8 c	7.9 d	9.5	14.0	24.0 c	65.1 c	48.4 b	137.5 c	1.6 b

[a]Growth ratio is the quotient of the dry weight after culture and the dry weight of the inoculum size

[b]Mean separation within column by Duncan's multiple range test at $p \leq 0.05$

[32], *Perilla frutescens* [33], *Panax ginseng* [22]. On the other hand, light has an inhibitory effect on accumulation of secondary metabolites such as nicotine and shikonin [34]. In the current studies, cultures were incubated under fluorescent, blue, red and blue plus far red lights and compared their impact on biomass growth and metabolite accumulation. The cultures which were incubated in dark were used as control and the results are presented in Table 13.8. The experimental results showed the stimulatory effect of light on secondary metabolite accumulation, but the effect of light was not significant on biomass accumulation. Cultures treated with red light were responsible for stimulating the accumulation of eleutheroside E (54.5 μg g^{-1} DW), eleutheroside E1 (50.4 μg g^{-1} DW), whereas cultures treated with blue light stimulated the accumulation of eleutheroside B (27.9 μg g^{-1} DW). Therefore, to enhance the accumulation of eleutherosides, it is suggested to illuminate the cultures with red and blue lights at least on the final week of culture period.

13.2.7 The Effect of Gibberellic Acid on the Production of Biomass and Bioactive Compounds

It was reported that amount of eleutherosides are in highest quantities in germinated embryos/young plantlets compared to that of matured somatic embryos of *Eleutherococcus koeanum* [9]. It is also known that gibberellic acid is the key phytohormone, which controls the germination process of somatic embryos. Therefore, we treated the embryogenic cultures of *E. senticosus* with gibberellic acid (GA_3) in the last week of culture process. The effect of gibberellic acid on biomass accumulation and production of secondary metabolites is presented in Table 13.9. Among the various concentrations of GA_3 tested (0, 1, 2, 3, 4, 8 mg L^{-1}), embryos which were treated with 4.0 mg L^{-1} accumulated highest biomass (121.9 g L^{-1} and 12.6 fresh and dry biomass) and growth ratio (22.7). This treatment was also superior for accumulation of secondary metabolites and the cultures accumulated 41.0 μg g^{-1} DW of eleutheroside B, 72.9 μg g^{-1} DW of eleutheroside E, 77.1 μg g^{-1} DW of eleutheroside E1 and 2.1 mg g^{-1} DW of chlorogenic acid, thus 2.2, 2.4, 1.8 and 2.2 fold increment was evident.

13.2.8 Elicitation

The accumulation of secondary metabolites in plants is part of the immune response against the pathogenic attack. The accumulation of secondary compounds is triggered and activated by the elicitors and signal compounds of plants' defense system [35]. Therefore, the treatment of plant cells with biotic and/or abiotic elicitors has been useful strategy to enhance secondary metabolite production in cell or organ cultures. Methyl jasmonate (MJ) is the most frequently used elicitor and improved taxol production in *Taxus chinensis* [36], ginsenoside production in *Panax ginseng*

Table 13.8 Effect of light quality on biomass accumulation and secondary metabolite production of *E. senticosus* somatic embryos in bioreactor

Light quality	Biomass g L^{-1}			Growth ratio[a]	Eleutherosides (μg g^{-1} DW)				Chlorogenic acid (mg g^{-1} DW)
	FW	DW	% DW		B	E	E_1	Total	
Dark	105.6 a[b]	10.6 a	10.0 a	19.0 a	21.3 b	26.7 c	39.7 b	87.7 b	21.3 b
Fluorescent	109.0 a	11.1 a	10.1 a	19.8 a	23.1 a	42.9 b	48.6 a	114.6 a	23.1 a
Blue	104.4 a	9.9 a	9.5 a	17.7 a	27.9 a	25.0 c	24.6 c	77.5 c	27.9 a
Red	93.7 b	8.6 b	9.1 b	15.2 b	14.9 c	54.5 a	50.4 a	119.8 a	14.9 c
Blue + Far red (1:1)	108.0 a	10.7 a	9.7 a	19.1 a	22.6 b	37.2 b	35.8 b	95.6 b	22.6 b

[a]Growth ratio is the quotient of the dry weight after culture and the dry weight of the inoculum size

[b]Mean separation within column by Duncan's multiple range test at $p \leq 0.05$

Table 13.9 Effect of gibberellic acid on biomass accumulation and secondary metabolite production of *E. senticosus* somatic embryos in bioreactor[b]

GA_3 (mg L^{-1})	Biomass g L^{-1}			Growth ratio[a]	Eleutherosides (μg g^{-1} DW)				Chlorogenic acid (mg g^{-1} DW)
	FW	DW	% DW		B	E	E_1	Total	
0.0	105.6 c	9.9 c	9.4	17.7	20.1 d	28.9 c	40.5 c	89.6 d	1.1 c
1.0	107.5 c	10.6 c	9.9	19.1	31.2 b	35.4 b	55.4 b	122.0 c	1.8 c
2.0	110.7 c	10.7 c	9.6	19.2	43.1 a	71.6 a	73.7 a	188.4 b	2.3 a
3.0	117.2 b	11.5 b	9.8	20.8	45.2 a	75.4 a	74.9 a	195.5 a	2.4 a
4.0	121.9 a	12.6 a	10.3	22.7	41.0 a	72.9 a	77.1 a	191.0 a	2.1 b
8.0	108.0 c	8.3 b	7.7	14.8	25.5 c	39.9 b	17.9 d	83.3 e	2.1 b

[a]Growth ratio is the quotient of the dry weight after culture and the dry weight of the inoculum size
[b]Mean separation within column by Duncan's multiple range test at $p \leq 0.05$

Table 13.10 Effect of methyl jasmonate (MJ) on biomass accumulation and secondary metabolite production of *E. senticosus* somatic embryos in bioreactor[b]

MJ (Conc.) μM	Biomass g L^{-1}			Growth ratio[a]	Eleutherosides (μg g^{-1} DW)				Chlorogenic acid (mg g^{-1} DW)
	FW	DW	% DW		B	E	E_1	Total	
0	102.6 a[b]	11.3 a	11.0	20.3	25.5 c	28.6 e	34.5 f	88.7 f	1.1 e
50	103.1 a	10.6 b	10.2	19.0	26.7 c	89.0 c	119.9 e	235.7 e	2.0 d
100	104.6 a	10.1 b	9.6	18.0	27.9 c	93.9 b	150.0 d	271.9 d	2.3 c
150	102.5 a	9.5 c	9.2	16.9	32.7 b	89.0 c	315.5 b	437.2 b	4.0 b
200	99.2 b	9.2 c	9.3	16.5	33.0 b	99.4 a	517.5 a	649.9 a	4.4 a
300	88.8 c	7.5 d	8.5	13.3	37.4 a	90.9 c	238.1 c	366.4 c	2.4 c
400	18.3 d	2.9 e	15.9	4.4	31.5 b	85.2 d	159.5 d	276.3 d	1.3 e

[a]Growth ratio is the quotient of the dry weight after culture and the dry weight of the inoculum size
[b]Mean separation within column by Duncan's multiple range test at $p \leq 0.05$

[15, 37, 38] cell and organ cultures. The growth and secondary metabolite accumulation by the embryos of *E. senticosus*, cultivated in bioreactor cultures are presented in Table 13.10. The embryos in the untreated cultures reached 102.6 and 11.3 g L^{-1} fresh and dry weight. The fresh and dry weight and growth ratio were decreased with the increasing concentration of MJ (Table 13.10). On the other hand, eleutheroside content was significantly enhanced by the addition of MJ. Amount of total eleutherosides and chlorogenic acid increased with the increasing MJ concentration and reached a maximum at 200 μM MJ representing 7.3 fold (649.9 μg g^{-1} DW) and 3.9 fold (4.4 μg g^{-1} DW) increase over control respectively. There were 1.4, 3.4 and 14.9 fold increments in eleutheroside B, E and E1 respectively when compared to control [39]. Eleutheroside E1 content was highest among the different eleutherosides produced by the suspended embryos. Similar to the present observations, differential accumulation of secondary metabolites have been reported during cell/organ cultures of *Panax ginseng* [15, 38].

13.3 Cultivation of Somatic Embryos in Large Scale Cultures

Somatic embryos of *E. senticosus* were cultured in 20 L balloon type bioreactor (Fig. 13.3d), 500 L drum type and balloon type bioreactors (Fig. 13.3e) based on the results of 5 L cultures. The embryos were cultured in MS basal medium with 3 % (w/v) sucrose and without growth regulators. The cultures were established by using 3 g L^{-1} inoculum and aerated with 0.1 vvm. Optimal of 1.9 kg (Fig. 13.3f) and 216 g of fresh and dry biomass and 161.5 mg kg^{-1} DW total eleutherosides were obtained from 20 L balloon type airlift bioreactors. Large scale bioreactors were also efficient in accumulation of biomass and secondary metabolites. Biomass of 56.6 kg, 63.0 kg fresh weight (Fig. 13.3c) and 5.3 and 5.7 kg dry weight of embryos could be achieved in 500 L balloon and drum bioreactors respectively (Table 13.11). 163.6 and 158.8 mg kg^{-1} DW of total eleutherosides were accumulated in the biomass obtained from 500 L balloon and drum bioreactors respectively. These results are promising and show the possibilities of culturing embryogenic biomass of *E. senticosus* for obtaining bioactive compounds.

13.4 Conclusion and Perspectives

Eleutherococcus senticosus Rupr. & Maxim (Siberian ginseng) is important medicinal plant which yields bioactive compounds known as eleutherosides. These active components showed positive effects on cellular defense and physical strength in man [40] and it is one of the most popular ingredients of nutracetuicals/functional food. We have induced somatic embryos in *E. senticosus* and cultured them in airlift bioreactors and investigated chemical and physical parameters which influence the biomass and secondary metabolite accumulation. We have also developed elicitation technology for enhanced accumulation of eleutherosides using methyl jasmonate and established large scale bioreactor systems for cultivation of somatic embryos.

In addition to the production of Siberian ginseng embryogenic biomass and eleutherosides, there is still scope for exploring the possibilities of accumulation of other bioactive compounds. Improvement of bioprocess parameters such as medium

Table 13.11 Biomass and eleutheroside production from somatic embryos of *E. senticosus* in 20 L and 500 L bioreactors

Bioreactor types and volume	Biomass production			Total eleutherosides (mg kg^{-1} DW)
	Fresh weight (kg)	Dry weight	% dry weight	
Balloon (20 L)	1.9 kg	216 g	11.1	161.5
Balloon (500 L)	56.6 kg	5.2 kg	9.1	163.6
Drum (500 L)	63.0 kg	5.7 kg	9.0	158.8

feeding, high density cultures, continuous cultures are still to be investigated. Establishing drying technology of embryogenic biomass and extraction procedures should be worked out. Evaluation of biosafety and efficacy tests of bioactive ingredients of embryogenic cultures is also the present need.

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Chapter 14
Bioreactor Culture of Shoots and Somatic Embryos of Medicinal Plants for Production of Bioactive Compounds

So-Young Park and Kee-Yoeup Paek

Abstract Plant cell and tissue culture technology has been considered as a powerful tool for the clonal production of medicinal plants. Plant tissue culture is an excellent alternative to traditional methods of plantation, as it offers a controlled supply of biochemicals independent of plant availability. In the past decade, tremendous progress has been made in this area, and its importance has rapidly increased because of increased need for medicinal plant substances as sources of medicine and health food ingredients. Bioreactor culture system was applied for biomass and secondary metabolite production in medicinal plants. The bioreactor system has been also refined to enhance the efficiency in terms of productivity and for cost reduction. For an efficient large-scale bioreactor culture, a perpetual explant source that is stable and fast growing is important, and till now, five types of culture materials have been commonly used: (1) hairy roots, (2) adventitious roots, (3) suspension cells, (4) somatic embryos and (5) multiple shoots. Majority of studies have been conducted on the cell and root cultures for biomass and secondary metabolite production for commercial purposes. In comparison, only limited studies have been conducted on somatic embryo and multiple shoot cultures as sources of medicinal compounds, even though it has been found that intact plants contain more pharmaceutical chemicals than that of the cells or roots. This review provides an updated and comprehensive overview of somatic embryo and multiple shoot induction in various medicinal plants for the production of biomass and secondary compounds. Future perspectives of biomass and bioactive compound production *via* somatic embryogenesis and shoot culture have been also discussed.

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Keywords Somatic embryogenesis • Multiple shoots • Biomass • Antioxidant • Balloon-type bubble bioreactor

Abbreviations

2, 4-D	2, 4-Dichlorophenoxy acetic acid
ABA	Abscisic acid
APX	Ascorbate peroxidase
BTBB	Balloon-type bubble bioreactor
CAT	Catalase
CI	Continuous immersion
CIN	Continuous immersion with net
E&FC	Ebb and flood culture
EC	Embryogenic callus
GC-MS	Gas chromatography and mass spectroscopy
GPx	Glutathione peroxidase
GR	Glutathione reductase
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IMA	Immersion culture with air supply
MDA	Malondialdehyde
MJ	Methyl jasmonate
MRC	Modified raft culture
MS medium	Murashige and Skoog medium
NAA	α-Naphthalene acetic acid
RC	Raft culture
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TDZ	Thiadiazuron (*N*-phenyl-*N*′-(1 2, 3-thiadiazol-yl))
TI	Temporary immersion
TIN	Temporary immersion with net
vvm	Air volume per medium volume per minute

14.1 Introduction

Plants have been a major source of pharmacologically active substances for thousands of years and the earliest drugs were plant extracts. It is estimated that approximately one quarter of all prescribed drugs contain plant extracts or active ingredients obtained from modeled on plant substances [1]. Recently, increased emphasis is on the research of bioactive products from plants with potential pharmacological

activity. The most popular analgesic, aspirin was originally derived from the species of *Salix* and *Spiraea.* It is well known that some of the most valuable anti-cancer agents such as paclitaxel and vinblastine are derived solely from plant sources, and some species of the genus *Swertia* are used for the industrial production of preparations used to treat acute viral hepatitis [2]. In nature, the consistent, year-round production of these plants is limited. Thus, there is a renewed interest in plant cell and tissue culture technology in the past decade. The large-scale culture of plant cells and tissues has been considered as a powerful tool for the mass production of overexploited and important medicinal plants, and at the same time, also as an alternative resource for biochemicals. Routien and Nickel [3] obtained the first plant tissue culture patent; its potential for the production of secondary metabolites has been presented by many researchers [2].

Since the main factor in the production cost of *in vitro* propagated plants is the high input of manual labour, a reduction of labour by automation is one of the prime aims of commercial tissue culture. Generally, liquid culture systems are more amenable to automation, and thus result in faster growth and propagation, but these systems are often of limited use due to ensuing physiological abnormalities of the plants, especially hyperhydricity [4]. For an efficient large-scale culture with reduced physiological abnormalities in the plants, a bioreactor system has been used for the production of horticultural and medicinal plants. This automated micropropagation system has been promoted as a possible method to reduce the costs and labour-intensive nature of clonal propagation [5]. For this, it is necessary to have a stable, fast-growing, perpetual explant source to secure the culture materials. For the production of biomass that contains active biomolecules, five kinds of culture materials are generally used: (1) hairy roots, (2) adventitious roots, (3) cells, (4) somatic embryos, and (5) multiple shoots. Suspension cell cultures are widely used in large-scale production of biomass and secondary metabolite, as it is easy to subculture and convenient for scale-up culture. However, it is found that in many plants, the biochemical contents are comparatively low and sometimes fluctuated, thus causing the productivity of suspension cell cultures to be unstable, even though the doubling time of suspension cells is shorter than that of other tissue and organ cultures. Compared to this, organs such as roots (hairy and adventitious roots) and shoots have been regarded as good materials for the productivity and reproducibility [6, 7] of biomass and secondary metabolites [8, 9].

Along with organ cultures, somatic embryos are also regarded as a suitable source material for *in vitro* natural compound production. The embryos offer an excellent experimental system to study the physiological and biochemical aspects of embryo development. Somatic embryogenesis has numerous benefits, including rapid propagation and a high reproduction coefficient without the restriction of natural conditions. In terms of biomass production, embryogenic callus production is small during the maintenance and proliferation stages. However, after being transferred onto a hormone-free medium, biomass increases rapidly and usually pharmaceutical compounds also increase as the plants develop further [10]. Researchers have not focused solely on development, but they have also studied the regulation of metabolic pathways. Since 2000, high frequency propagation of somatic embryos

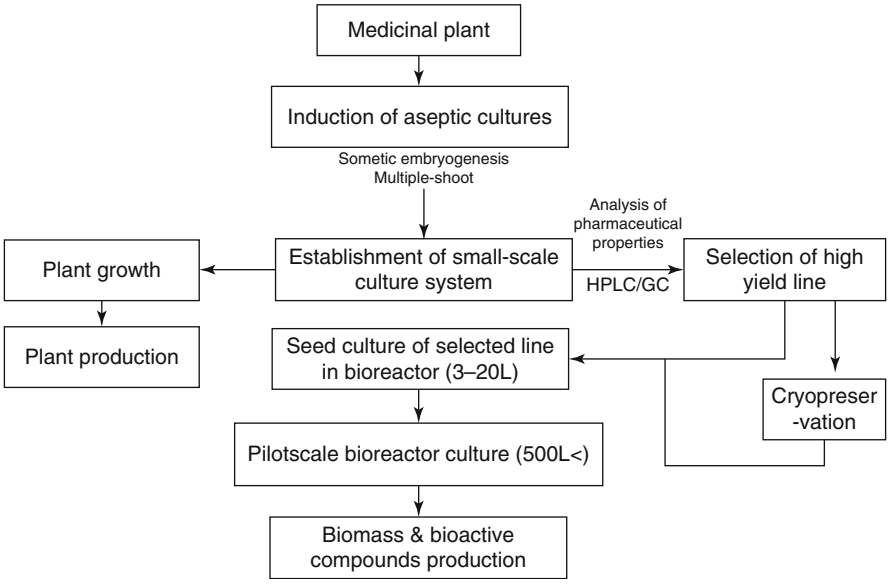

Fig. 14.1 Scheme of biomass and bioactive compound production from medicinal plants *via* shoot and somatic embryogenesis using bioreactor system

through bioreactor culture in various medicinal plants including *Panax ginseng* and *Eleutherococcus senticosus* [11, 14, 15] has been reported [5, 11–13]. This chapter focuses on the general methods of biomass and secondary metabolite production *via* shoot and somatic embryo culture in bioreactors (Fig. 14.1). Some successful examples are described in detail.

14.2 Establishment of Culture System

14.2.1 Induction of Multiple-Shoots and Embryogenic Cultures

Multiple-Shoot Induction

Since the first *Begonia* bioreactor culture reported by Takayama and Misawa [16], the culture method has been proven applicable to many species and various explant types, including shoots, bulbs, microtubers, roots, and somatic embryos [5]. Among the explants, the shoots are suitable explants in terms of secondary metabolite accumulation and biomass. In *Eleutherococcus koreanum*, the shoots were found to contain more eleutheroside E than that of adventitious roots and somatic embryos [10]. As expected, shoot cultures produce similar secondary metabolites like that of plants. For large-scale cultures such as bioreactors, the initial shoot culture is usually initiated in solid medium gelled with agar or gelrite. Three main factors are needed for successful

multiple-shoot formation: (1) selection of responsive explants, (2) suitable basal medium and (3) optimum concentrations and combinations of auxins and cytokinins.

Numerous factors are reported to influence the success of the *in vitro* propagation of shoot cultures in various medicinal plants [17–19]. The effects of auxins and cytokinins on the shoot multiplication of various medicinal plants have been reported. Benjamin et al. [20] showed that 6-benzylaminopurine (BAP), at high concentrations (1.0–5.0 mg L^{-1}) stimulates the development of the axillary shoots of *Atropa belladonna*. Lal et al. [21] observed a rapid proliferation rate in *Picrorhiza kurroa* using kinetin at 1.0–5.0 mg L^{-1}. In *Nothapodytes*, the highest shoot multiplication was achieved on 2.2 μM thidiazuron-containing medium. Regeneration capacity and specific response for the exogenous hormones in plant tissue cultures is related to the physiological status of the plant and its endogenous hormone levels, and is known to be a species-specific characteristic [22]. Earlier, it was reported that the regeneration capacity varies even among the genotypes of a single species and the endogenous cytokinin/abscisic acid (ABA) ratio may have influence over the regeneration of *Kalopanax septemlobus* individuals [23].

Induction of Embryogenic Culture

Somatic embryogenesis is the process by which groups of somatic cells will produce the somatic embryos, which resemble the zygotic embryos of intact seeds and can grow into seedlings on suitable media. Plant regeneration *via* somatic embryogenesis from single cells that can be induced to produce an embryo, and eventually a complete plant has been demonstrated in many medicinal plant species. Supplementation of growth regulators was found to be essential for embryo induction and development in many species [24–26]. However, in some cases, chemical and physical stresses, for example, high sucrose concentration [27], high temperature [28] and heavy metal ions [29] have been reported as more important stimuli for somatic embryogenesis than exogenous growth regulators.

Growth regulators and nutrient components of the media have profoundly influenced the embryogenesis process in many plant species. So, a suitable media composition should be identified in order to improve embryo induction, development, maturation and conversion. Medium composition is a key factor in successful embryo induction and subsequent plant regeneration. Sugar, a carbon source, also acts as an osmotic regulator in the medium during embryogenesis [27, 30]. Sucrose level played a major role in triggering embryogenesis in many plant species, including *Cucumis sativus* [31].

14.2.2 Multiple-Shoot Cultures in Bioreactors

Various researchers use bioreactors for large scale cultivation of medicinal plants *via* shoot multiplication and to increase multiplication efficiency, micropropagation should be scaled-up. Bioreactor systems have been used for mass propagation of

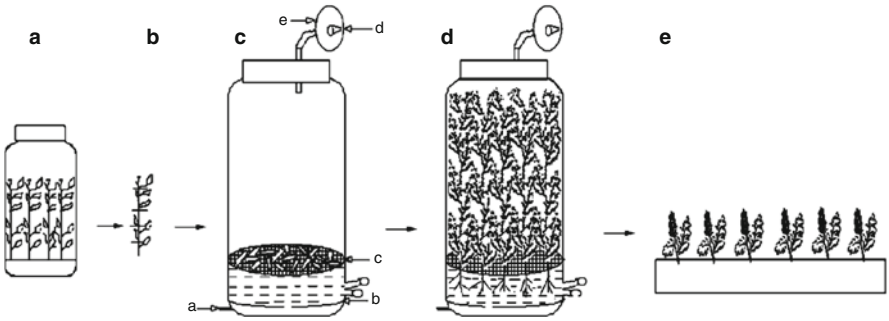

Fig. 14.2 Schematic diagram of bioreactor system for Chrysanthemum shoot production. (**a**) Shoot induction; (**b**) Single node stems; (**c**) Bioreactor culture; (**d**) Shoot multiplication in a bioreactor; (**e**) *Ex vitro* rooting of cuttings (Adapted from Hahn and Paek [36])

horticultural plants such as the *Phalaenopsis* [32], oriental lily [33], cacao [34], coffee [35], and chrysanthemum [36] and they have proved their potential for large-scale micropropagation (Fig. 14.2).

14.2.3 Embryogenic Cultures in Bioreactors

Somatic embryogenesis in liquid media is a powerful alternative to other biomass production techniques. Three important advancements have led to the scaling-up of somatic embryogenesis in medicinal plants: (1) liquid cultures for the multiplication of embryogenic cells and for the production of embryos, (2) temporary immersion systems for the culture and further development of embryos (beyond cotyledonary embryos), and (3) increase in the range of utilization of somatic embryos. Based on these improvements, many researchers have recently reported successful results in Siberian ginseng, coffee, and cacao [13, 34, 35, 37].

In coffee, currently about 2.5–3.0 million cotyledonary embryos can be produced each year using bioreactor systems [35]. In addition to the production of plantlets, somatic embryos are useful for biomass and pharmaceutical compound production in medicinal plants. Our previous study on *Eleutherococcus koreanum* revealed that somatic embryos contained greater amounts of bioactive compounds than adventitious roots or cells [10].

14.2.4 Explant and Bioactive Compound Biosynthesis

Plant secondary metabolites (Fig. 14.3) have various functions throughout the life cycle of a plant. It is clear that those metabolites may play important roles in defense mechanisms and signaling in plants [38]. In the past decade, their importance has

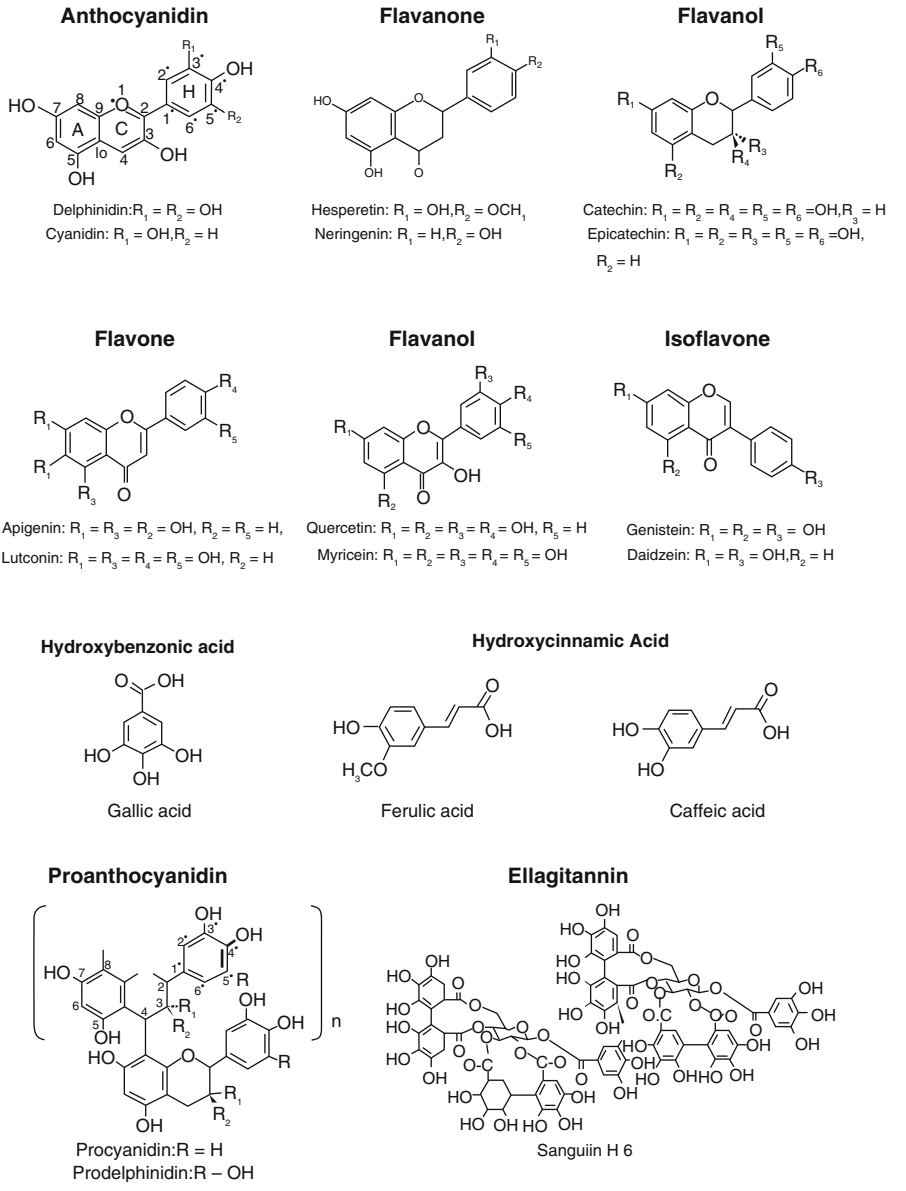

Fig. 14.3 Structures of flavonoids, phenolic acids and tannins (Adapted from Dai and Mumper [38])

increased rapidly as these molecules also determine important aspects of human food quality and have pharmaceutical effects. Thus, it is important to increase the production of these useful bioactive compounds and to exploit plant materials through the large-scale culture of medicinal plants. During the culture period of a medicinal plant, the contents of bioactive compounds change with the physiological

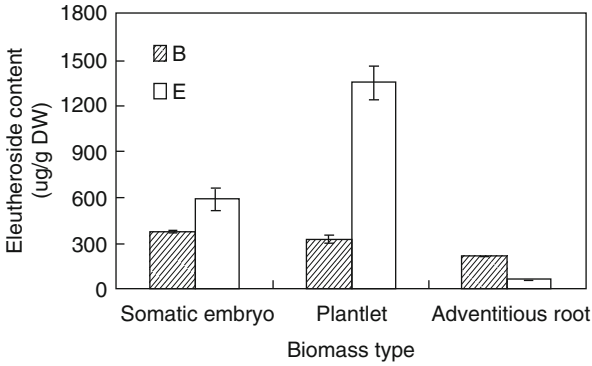

Fig. 14.4 Eleutheroside B and eleutheroside E contents of somatic embryos, plantlets and adventitious roots harvested from bioreactor (Adapted from Park et al. [10])

changes in the propagules such as their proliferation and development. As a result, it is important to know the relationship between bioactive compounds and plant development or tissue type in a bioreactor culture in order to determine the proper tissue (or explant) type and harvesting time. During the culture period of *Glycyrrhiza uralensis* cells in a bioreactor, the contents of triterpenoid saponins and flavonoids showed a correlation with cell growth pattern, but Wang et al. [39] presumed that it might be caused due to the reduction of EC in the medium. Secondary metabolites often accumulate in special types of cells or organs, as their biosynthesis are often coupled with certain morphological differentiations [40].

In *Eleutherococcus koreanum*, HPLC analyses of the extracts of somatic embryos, embryo-derived plantlets, and adventitious roots harvested from the bioreactor culture confirmed the presence of eleutherosides (Fig. 14.4). The HPLC data demonstrate that these secondary metabolites are produced in the embryos developed *in vitro*, plantlets and adventitious roots. The eleutheroside B and E content were 377 μg g^{-1} DW and 588 μg g^{-1} DW in somatic embryos (torpedo stage); 409 μg g^{-1} DW and 1,796 μg g^{-1} DW in *in vitro* plantlets; and 220 μg g^{-1} DW and 68 μg g^{-1} DW in adventitious roots, respectively (Fig. 14.4). Greater eleutheroside B and E contents were detected in plantlets grown *in vitro* than in adventitious roots and embryos. This shows that *in vitro*-grown plantlet biomass can be conveniently used for the extraction of eleutherosides, and that may be useful not only for mass propagation of the endangered *E. koreanum* but also for securing a source of eleutherosides.

14.2.5 Bioreactor System for Organ Cultures

Bioreactor technology provides the potentiality for the economical and efficient production of a large number of plants [5]. The most commonly used bioreactor system is immersion culture. However, this method is limited by oxygen supply, and aeration is a major concern in bioreactor design and scale-up for plant organ cultures [41]. Temporary Immersion Systems (TIS), i.e. flooding of plant tissue at

regular time intervals open possibility of automating some *in vitro* cultures [42] and increase the biological efficiency and productivity of propagated material [43]. The system supplies enough oxygen to the propagules throughout their cultivation [44]. This is one of benefits of using this system for shoot and somatic embryo cultures in bioreactors. Oxygen requirement of plant cells is relatively low for cell growth. Although sufficient oxygen is essential for organ cultures, it may significantly increase during metabolite synthesis [41]. Niemenak et al. [34] made a simple TIS (1-L scale) based on the "twin flasks" type and this system was used by Escalona et al. [45] and Hempfling and Preil [46], for culturing of cacao somatic embryos.

Among bioreactor designs, the balloon-type bubble bioreactor has been used for cell and root cultures of many species including Siberian ginseng [47, 48] and *Panax ginseng* [5]. However, its round shape was considered unsuitable for plantlet growth. So, a modified bioreactor for plant culture was designed by Paek et al. [5]. At first, it was slightly modified (Fig. 14.5) from its original balloon like shape (Fig. 14.5a) ant the redesigned bioreactor increased the volume for plant growth and this system was used culture [36].

14.3 Biomass Production of Medicinal Plants in Bioreactors: Successful Examples

14.3.1 Eleutherococcus koreanum

Eleutherococcus koreanum Nakai (syn. *Acanthopanax koreanum* Nakai, Araliaceae) is a medicinal woody plant that grows in Jeju Island, located 50 miles off the southernmost tip of the Korean Peninsula [49]. The species is currently considered to be an endangered species because of its excessive random harvest from their natural stands. Propagation of this species through seeds is difficult because it requires over 18 months of stratification for the germination of zygotic embryos [50]. The rooting of stem cuttings and division of roots are the main methods of propagation, but their efficiency is low [51].

Eleutherococcus species contains eleutheroside as a major compound, and their roots and stems have traditionally been used to treat rheumatism, diabetes, and hepatitis [13, 52–55]. The *Eleutherococcus* family contains both saponins and lignans with a large number (over 200) of different molecules being detected as either aglycones or glycosides. The main bioactive compounds appear to be eleutheroside B (syringin) and eleutheroside E (a syringaresinol diglucoside) (Fig. 14.6).

Among those, eleutheroside E (liriodendrin) and B (syringin) were known to possess the most pronounced stimulant and anti-stress effects [55]. Eleutheroside E is reported to have a counteracting effect on stressed animals, androgenic effects on immature male mice, and to increase the RNA content of the seminal vesicles and the prostate [1]. Along with eleutherosides, acanthoic acid has an important pharmacological activity. It was isolated from *E. koreanum* root, and it was revealed that

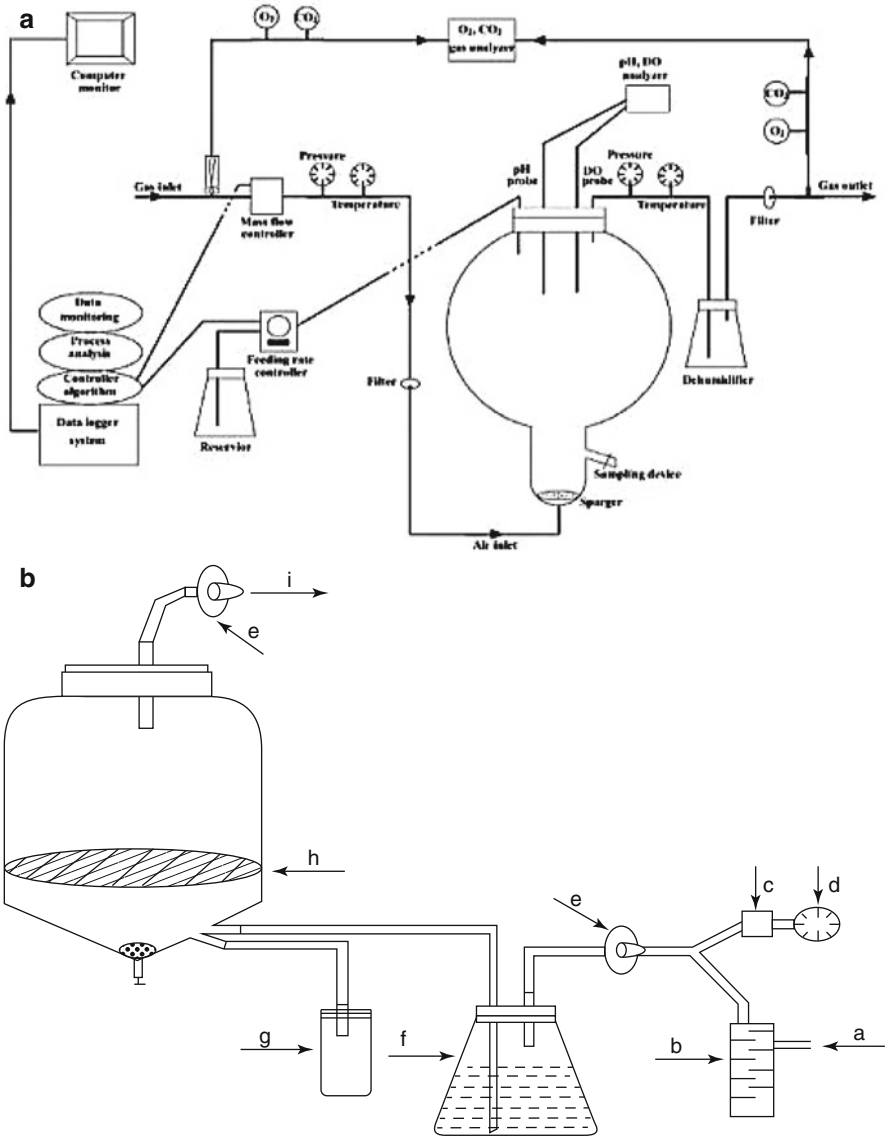

Fig. 14.5 Layout of balloon-type bubble bioreactor (**a**) and redesigned bioreactor for organ culture (**b**). (*a*) Air inlet; (*b*) Air flow meter; (*c*) Timer; (*d*) Solenoid valve; (*e*) Membrane filter; (*f*) Medium reservoir; (*g*) Sampling port; (*h*) Supporter (net); (*i*) Air outlet (Adapted by Paek et al. [5])

the compound had analgesic and anti-inflammatory activities, without acting on the central nervous system, and showed inhibition of lipid peroxidation [53].

We have previously developed a methodology for mass cultivation of the adventitious roots of *E. koreanum* for the production of eleutherosides [47]. During the

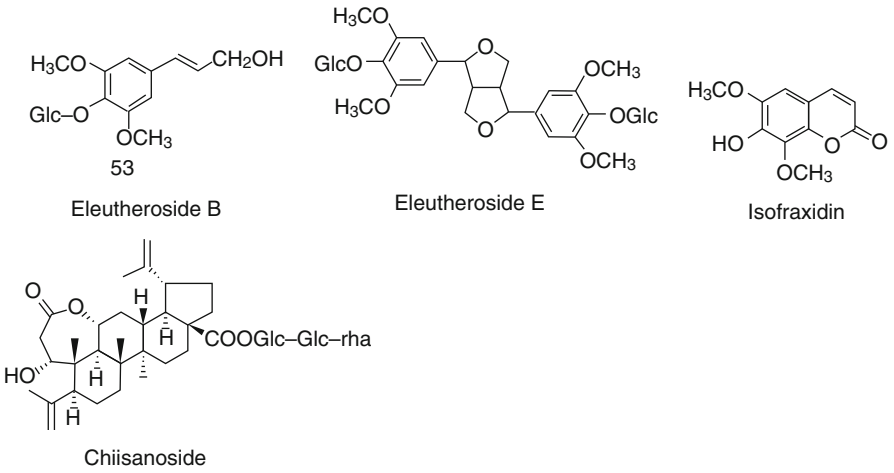

Fig. 14.6 Structure of bioactive compounds in *Elutherococcus* family

mass cultivation of the adventitious roots in liquid medium, we observed the development of somatic embryos on the adventitious roots. Here, we have reported plantlet production *via* somatic embryogenesis from root cultures of *E. koreanum*. Embryos and plantlets produced during the culture were also examined for their ability to accumulate eleutherosides. The eleutherosides were extracted and analyzed by using the protocol by Ahn et al. [56]. The eleutheroside fraction was analyzed using the Thermo Separation Products HPLC system equipped with a UV detector on a Spherisorb ODS column (GroB-Umstadt, Germany), with water and acetonitrile as the mobile phase. The ratio of water and acetonitrile for the initial 10, 30, 40, 45, 46 and 50 min, were 95:5, 90:10, 60:40, 50:50, 45:55 and 95:5, respectively. Flow rate of the mobile phase was 0.6 mL·min^{-1} and the eleutherosides were detected at 220 nm. Authentic eleutheroside B and E were purchased from Nakarai Inc. (Japan) and Sigma (USA) to compare with the accumulation pattern from the cultured plantlets. Total eleutheroside content was calculated as the sum of the eleutheroside fractions.

Plant Material

Seeds of *E. koreanum* were collected from the experimental forest of the Korean National Forest Research Institute. They were surface-sterilized and dissected to isolate the zygotic embryos. The embryos were placed on one half MS [57] medium for germination. After 2 months, roots were excised from the seedlings and cultured in 200 mL of MS medium containing 3.0 mg L^{-1} indolebutyric acid (IBA), 0.01 mg L^{-1} thiadiazuron (TDZ: *N*-phenyl-*N*′-(1,2,3-thiadiazol-yl)urea; Sigma Chemical Co., St. Louis, MO), and 30 g L^{-1} sucrose in 500 mL Erlenmeyer flasks

and kept on orbital shaker at 110 rpm. The adventitious roots were maintained in the same medium by subculturing at 4-week intervals.

Culture Establishment

Induction of Somatic Embryos from Root

In the absence of growth regulators, root segments cultured in one third, one half, full, and double strength MS media developed globular embryos directly on the root surface without callus mediation after 4 weeks of culture. Subsequently, these globular embryos developed into torpedo and cotyledonary embryos when left in the same medium for 2 more weeks. Root segments were proliferated along with the embryos. Histological examination showed the direct development of embryos on root surfaces without callus mediation. Embryos were matured and converted into plantlets on the same medium after 8 weeks of culture. The increase in the fresh mass of roots, fresh mass of embryos, and number of matured embryos per explant were compared with respect to the medium strength. Among the four media tested (one third, one half, full, and double strength), one third strength medium was found most suitable for embryogenesis since it produced the highest number of somatic embryos per explant. However, one half strength MS medium was found to be optimal for the production of embryo biomass. This observation is concurrence with the previous report that a one half strength MS medium that had a lesser concentration of mineral ions, especially nitrates and ammonium was beneficial for embryo formation from the root segments of spinach [51]. The results of the present studies support this view, and one third strength MS medium was found suitable for embryo formation in *E. koreanum*.

In a separate experiment, root segments were cultured in one half-strength MS medium supplemented with 0.5–2.0 mg L^{-1} 2, 4-dichlorophenoxyacetic acid (2,4-D) and 0.01–1.0 mg L^{-1} TDZ to enhance the embryogenesis process. The results revealed that supplementation with 2, 4-D and TDZ was not beneficial. The medium supplemented with 2, 4-D suppressed embryo formation. Embryos developed on the root explants in the medium supplemented with TDZ, but the frequency of embryo formation was less when compared to the hormone-free medium. In the present study, root segment cultures of *E. koreanum* developed embryos in hormone-free medium, and the embryogenic response of these explants did not favour the hormone-supplemented medium. Similarly, successful direct embryogenesis was induced from the root segment cultures of *Lotus corniculatus* [58] in hormone-free medium.

To verify the effect of sucrose concentration on embryogenesis, root segments were cultured in medium supplemented with 15–90 g L^{-1} sucrose. Higher sucrose concentrations were found to be beneficial for promoting embryogenesis and 11.8 mature embryos were recorded in the medium supplemented with 60 g L^{-1} sucrose. This medium also favoured an increase in the biomass of the roots, but the biomass of the embryos was lower than that of the medium supplemented with 30 g L^{-1}

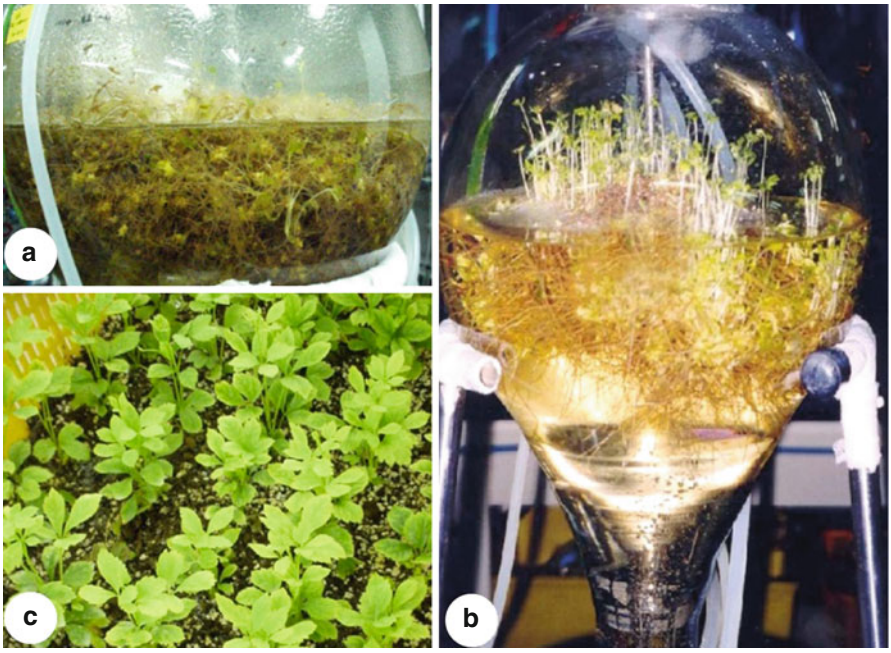

Fig. 14.7 Plantlets production *via* somatic embryogenesis in a 20 L air lift bioreactor, (**a**) Embryo formation after 4 weeks of culture in one third MS medium containing 60 g L^{-1} sucrose, (**b**) Plantlets in bioreactor after 12 weeks of culture, (**c**) Plantlets grown on the peat moss and perlite mixture after 8 weeks of acclimatization (Adapted from Park et al. [10])

sucrose. The present studies of *E. koreanum* also confirmed the importance of sucrose level in the medium on the induction and development of embryos.

In the present study, embryogenesis was induced from the root segments of *E. koreanum* in one third-strength MS, hormone-free medium supplemented with 60 g L^{-1} sucrose. Since this medium has been found suitable for embryo induction, development, maturation, and germination, this makes the protocol simple and efficient. Based on the above experiment, we attempted large-scale production of embryos and plantlets in the bioreactor system. Bioreactor systems were established containing 18 L of one third-strength MS, hormone-free medium supplemented with 60 g L^{-1} sucrose. The results revealed that the globular embryos were produced rapidly from the root segments and simultaneously they were developed into cotyledonary embryos. The embryos that developed from the roots passed through all stages of development and matured embryos were converted into plantlets in the same medium after 12 weeks of culture. The plantlets that were harvested from the bioreactor culture acclimatized very well after transplantation (Fig. 14.7). The total fresh biomass of adventitious roots, plantlets, and somatic embryos are presented in Table 14.1 and the bioreactor culture was found to be suitable for the highest biomass accumulation. Similarly, bioreactor cultures were efficiently utilized for mass propagation [30, 59] and biomass production [60] in many systems. The most

Table 14.1 Plantlet production *via* somatic embryogenesis of *E. koreanum* cultured in 18 L of one third MS medium supplemented with 60 g L^{-1} sucrose in 20 L balloon-type air-lift bioreactors after 12 weeks

Total biomass (g)	Biomass of adventitious roots (g)	Plantlets		Somatic embryos	
		Number	Biomass (g)	Number	Biomass (g)
840.5	123.0	1,446	55.7	3,774	163.2

Adapted from Park et al. [10]

important finding of the current study was the development of a simple somatic embryo regeneration system for *E. koreanum* in hormone-free liquid medium. The current study demonstrated the potential of root explants of *E. koreanum* for both plantlet and phytochemical production.

14.3.2 Kalopanax septemlobus

Kalopanax septemlobus Nakai (Syn. *Kalopanax pictus*; common name, castor aralia) is a medicinal woody species of the family Araliaceae that is mainly distributed in northeast Asia, and has been used in furniture making and for important medicinal materials. Stem cortical tissues of *K. septemlobus* contain large amounts of chemical constituents such as the phenolic glycosides liriodendrin, syringin and hederagenin, glycosides of *Kalopanax* saponin as triterpene, and these stem cortical tissues are being used in traditional medicine [63, 64]. The main pharmacological effects of *K. septemlobus* are anti-rheumatic [11], anti-diabetic [64], and anti-inflammatory [53]. It has also been shown to have neuritogenic activity, which is effective against Alzheimer's and Parkinson's diseases [54]. For medicinal purpose, the edible shoots are picked by farmers during early spring along with that of *Aralia elata*; the bark and stem of the trees are used for making soup during summer. Propagation of this tree has been achieved through seeds or stem cuttings, but with very low efficiency [61]. Under natural conditions, this plant requires 2-year germination period and stem cutting is impractical for mass propagation of the species due to low efficiency [61]. As an alternative way to propagate this species, somatic embryogenesis was achieved from the immature seeds of a 100-year-old plant [62].

Plant Material

Young, expanding leaves of 3–5 cm in length were excised from each graft and used as explants. For surface disinfection, five to ten leaves were placed into a 500 mL flask and washed by vigorous shaking in tap water containing a few drops of Tween 20® (Sigma, St. Louis, USA). Thereafter, the leaves were disinfected in 70 % ethanol for 1 min, in 2 % (w/v) NaClO for 5 min, and rinsed five times with sterile distilled water in aseptic conditions. Explants were finally immersed in sterilized distilled water for about 30 min. Leaf segments measuring approximately 5 mm^2 were prepared with a

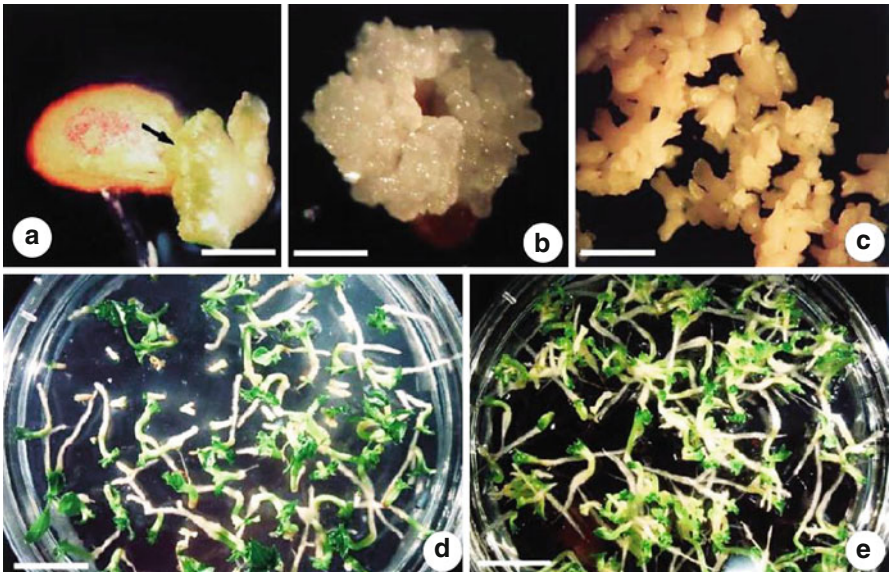

Fig. 14.8 Somatic embryogenesis and plantlet production of *K. septemlobus*. (**a**) Immature zygotic embryos producing a somatic embryo, (**b**) Embryogenic callus formed from zygotic embryos, (**c**) Somatic embryo development, (**d**, **e**) Conversion of embryos on one half-strength MS medium (Adapted from Moon et al. [62])

surgical blade, with each explant segment possessing some leaf vein tissue. The explants were inoculated onto MS basal medium supplemented with 4.5 μM 2, 4-D and 3 % (w/v) sucrose (Sigma). The cultures were maintained at 25 ± 1 °C with a 16-h photoperiod provided by cool-white fluorescent lamps (40 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Induction and Maintenance of Embryogenic Callus

White and pale yellow, friable embryogenic calli were induced after 8 weeks of culture from leaf segments. To stimulate proliferation, these calli were carefully selected under a microscope and subcultured onto MS medium supplemented with 4.5 μM 2,4-D, 1.0 g L^{-1} L-glutamine, 5 % sucrose, and 0.5 % gellan gum. Further, they were subcultured onto fresh medium of the same type at 3-week intervals. For proliferation of calli, around 0.2 g embryogenic callus was transferred on new callus induction medium in petridish and five plates were maintained for replication in each cell line.

Somatic Embryo Formation and Maturation

For the formation of somatic embryos, 10 mg of embryogenic callus (Fig. 14.8) was cultured on one half-strength MS medium containing 5 % sucrose, 5 % (w/v)

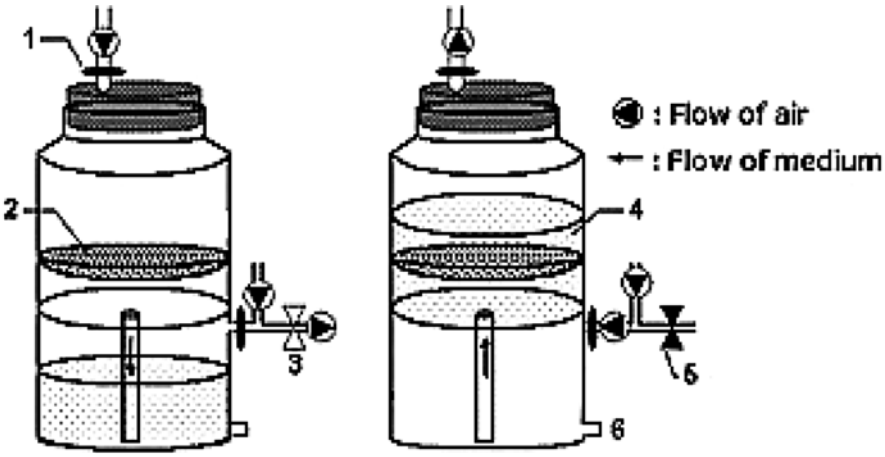

Fig. 14.9 Bioreactor design for somatic embryo culture of *K. septemlobus*. (**a**) Withdrawal phase (**b**) Immersion phase

polyethylene glycol (PEG), 0.38 μM ABA, 0.2 % (w/v) activated charcoal (AC), and 5 g L^{-1} gelrite (Duchefa, The Netherland). Cultures were incubated in tissue culture room and maintained at 24 ± 1 °C under a 16-h photoperiod under cool white fluorescent lamps at 35–40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux (PPF).

Culture Establishment

A suitable bioreactor system (Fig. 14.9) for the large-scale embryo-to-plantlet conversion of *K. septemlobus* was established. In the temporary immersion with net (TIN) bioreactor, 85 % of the embryos successfully produced plantlets, whereas in the continuous immersion with net (CIN) bioreactor, a conversion rate of only 29.3 % was obtained. Embryos cultured in the TIN bioreactor produced plantlets more vigorously in terms of fresh weight, height, root length, and quantity of roots and leaves. In the CIN bioreactor, *Kalopanax* plantlets showed high malondialdehyde (MDA) content and increased activities of reactive oxygen species (ROS)-processing enzymes such as ascorbate peroxidase (APX) and glutathione reductase (GR), indicating the occurrence of oxidative stress. However, superoxide dismutase (SOD) and catalase (CAT) showed similar activities in plantlets growing in different bioreactors. *Kalopanax* plantlets grown in both TIN and CIN bioreactors were harvested and transferred to greenhouse for their acclimatization. Plantlets grown in the CIN bioreactor exhibited low survival rate (75.8 %) when compared to those grown in the TIN bioreactor (100 % survival; Figs. 14.10 and 14.11). MDA content decreased with the progression of acclimatization, indicating a decrease in oxidative stress. However, MDA level in the CIN-derived plantlets was higher than in the TIN-derived plantlets. In the TIN-derived plantlets, an increase in SOD and GR activities was observed after 1 week, though it decreased thereafter. CAT activity

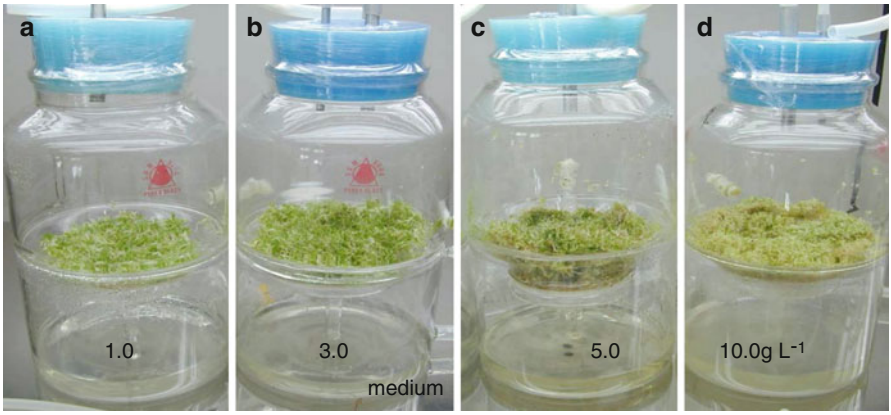

Fig. 14.10 Effect of inoculation density and culture methods of somatic embryos (*SEs*) on biomass production. (**a**) SEs 1.0 g L^{-1} medium, (**b**) SEs 3.0 g L^{-1} medium, (**c**) SEs 5.0 g L^{-1} medium, (**d**) SEs 10.0 g L^{-1} medium

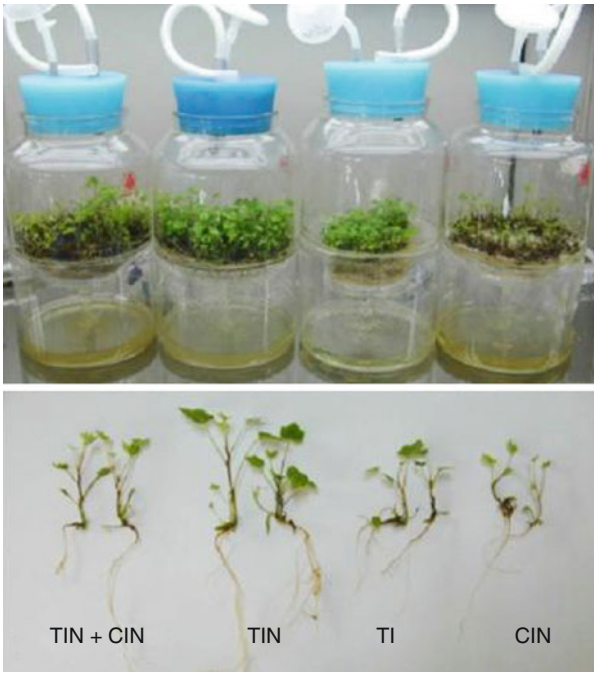

Fig. 14.11 Effect of bioreactor culture system on somatic embryo conversion in *K. septemlobus*. (*TI* temporary immersion, *TIN* temporary immersion with net, *CIN* continuous immersion with net, TIN + CIN) (Adapted from Kim et al. [44])

decreased while APX activity started to increase after 1 week of acclimatization. These results indicated that *Kalopanax* plantlets were able to overcome oxidative stress mainly through SOD activity. However, levels of antioxidant enzyme activities were higher in CIN-derived plantlets than TIN-derived plantlets. *Kalopanax* plantlets obtained from the TIN bioreactor performed better during the

acclimatization phase and showed a higher survival rate than the material obtained from the CIN bioreactor or conventional culture systems.

14.3.3 Allium victorialis var. *platyphyllum Makino*

Allium victorialis var. *platyphyllum* Makino is a perennial herb belonging to Liliaceae family, which is distributed around Korea, Japan, and China. It is commonly considered as a broad-leaved species of wild onion (victory onion, Alpine leek, or caucas). The subspecies *platyphyllum* occurs widely in meadows and woodlands across the Amur River system in China and Russia, extending its range up to Japan, there it is an important cultural food source for the Ainu people. Unlike other *Allium* spp. such as garlic and onions, which have been studied extensively, research on *A. victorialis* var. *platyphyllum* is rare [65], and only reports on chemical compounds [64, 66] and taxonomic classification [67] exist.

Gitogenin 3-*O*-lycotetroside, an active constituent extracted from *Allium victorialis,* exerted certain cytotoxic activities against cancer cell lines. It seems that the disulfides produced secondarily were the principle anti-tumor molecules [53]. Chung et al. [68] reported on the major essential oil composition of *A. victorialis* L. var. *platyphyllum*, and its immunotoxicity effects. These analyses were carried out using gas chromatography and mass spectroscopy (GC-MS), which revealed the occurrence of essential oils in *A. victorialis* L. var. *platyphyllum* stems. The yield of essential oils from the stem of *A. victorialis* L. var. *platyphyllum* was 1.45 %, and GC-MS analysis revealed that its major constituents were allyl methyl disulfide (24.36 %), dimethyl trisulfide (11.78 %), allyl *cis*-1-propenyl disulfide (9.17 %), dipropyl trisulfide (7.22 %), and allyl methyl trisulfide (4.13 %). The essential oils had a significant toxic effect on early fourth-stage larvae of *Aedes aegypti* L. [68]. The methanol extraction from *A. victorialis* displayed both pro-oxidant and antioxidant properties, but no anti-human immunodeficiency virus (HIV) or anti-*Helicobacter pylori* activity [69]. The possibility of *in vitro* mass propagation, *via* shoot and somatic embryogenesis of this valuable plant was reported by Lim et al. [66]. They obtained shoots formed directly from bulb explants of *A. victorialis*. However, their work was performed only in a flask-scale level, and no further mass production system or cultures was investigated.

Plant Material

The bulbs of *Allium victorialis* var. *platyphyllum* were provided by the Korean National Arboretum in 2003. The bulbs were surface sterilized with 2 % sodium hypochlorite for 20 min and used to induce *in vitro* plants. The bulbs were cut to 0.5 × 0.5-cm size and cultured on MS medium supplemented with BA 3.0 mg L^{-1} and 1-naphthalene acetic acid (NAA) 0.1 mg L^{-1}. The cultures were maintained at 25 ± 1 °C with a 16-h photoperiod provided by cool-white fluorescent lamps

60–80 μmol·m^{-2}·s^{-1}. After 4 weeks of culture, shoots were formed directly on the surface of bulb explants. Proliferated shoot clumps were excised to 10 × 10 mm in size and sub-cultured on the fresh medium of same composition at 4-week intervals. Enough materials (shoot clumps) were obtained for bioreactor cultures after approximately 6 months of shoot clump culturing.

Culture Establishment

Multiple Shoot Formation and Bulblet Enlargement

Shoot clumps weighing approximately 0.1 g were cultured in MS medium containing BA (0–5.0 mg L^{-1}) and thiadiazuron (0.1–2.0 mg L^{-1}). A relatively high concentration of BA (3.0–5.0 mg L^{-1}) induced more vigorous multiple-shoots than the somatic embryos, while thiadiazuron treatment produced more somatic embryos than shoots at 1.0 mg L^{-1} (Figs. 14.12 and 14.13).

The effects of ABA and MJ on bulblet formation from the culture of *A. victorialis* var. *platyphyllum* were studied. Shoot clumps were cultured on MS medium containing ABA (0, 0.01–2.0 mg L^{-1}) and MJ (0.01–5.0 mg L^{-1}). Low concentrations of ABA (0.01 mg L^{-1}) induced shoot proliferation without bulblet formation. However, bulblet formation did start on the medium containing MJ after approximately 4–6 weeks of culture. Furthermore, 1.0 mg L^{-1} MJ resulted in higher frequency bulblet formation (100 %) than the control (46.1 %). Cortical cells of the bulblets enlarged on the medium with MJ and had a dense, protein-like substance in expanded and round cells when examined under the microscope. The data described here show that the formation and enlargement of *Allium victorialis* bulblets can be improved by the addition of an appropriate concentration of MJ.

Establishment of the Bioreactor Culture

A bioreactor was used for the mass production of biomass in *A. victorialis* var. *platyphyllum*. An appropriate bioreactor culture system for shoot proliferation and bulb formation was investigated (Figs. 14.14, 14.15, and 14.16; Tables 14.2 and 14.3) and the uptake of soluble carbohydrates in different culture systems was also analyzed throughout the entire culture period. Optimal results for multiple shoot formation were observed in raft culture (RC) and modified raft culture (MRC; 13–15 per explant; Table 14.2), in which the explants were placed on a sieve in contact with the liquid medium. MRC was a better method to avoid hyperhydricity by controlling the medium supply using a medium reservoir. For bulb formation, 93.4 % of shoot clumps were formed into bulbs at the basal part, which were uniform in size when cultured with the ebb and flood culture (E&FC) system. Bulbs harvested from RC and MRC showed vigorous rooting, however, bulb growth was not uniform. With respect to biomass production, the immersion culture with air supply (IMA) was the best culture system, but bulbs were malformed without

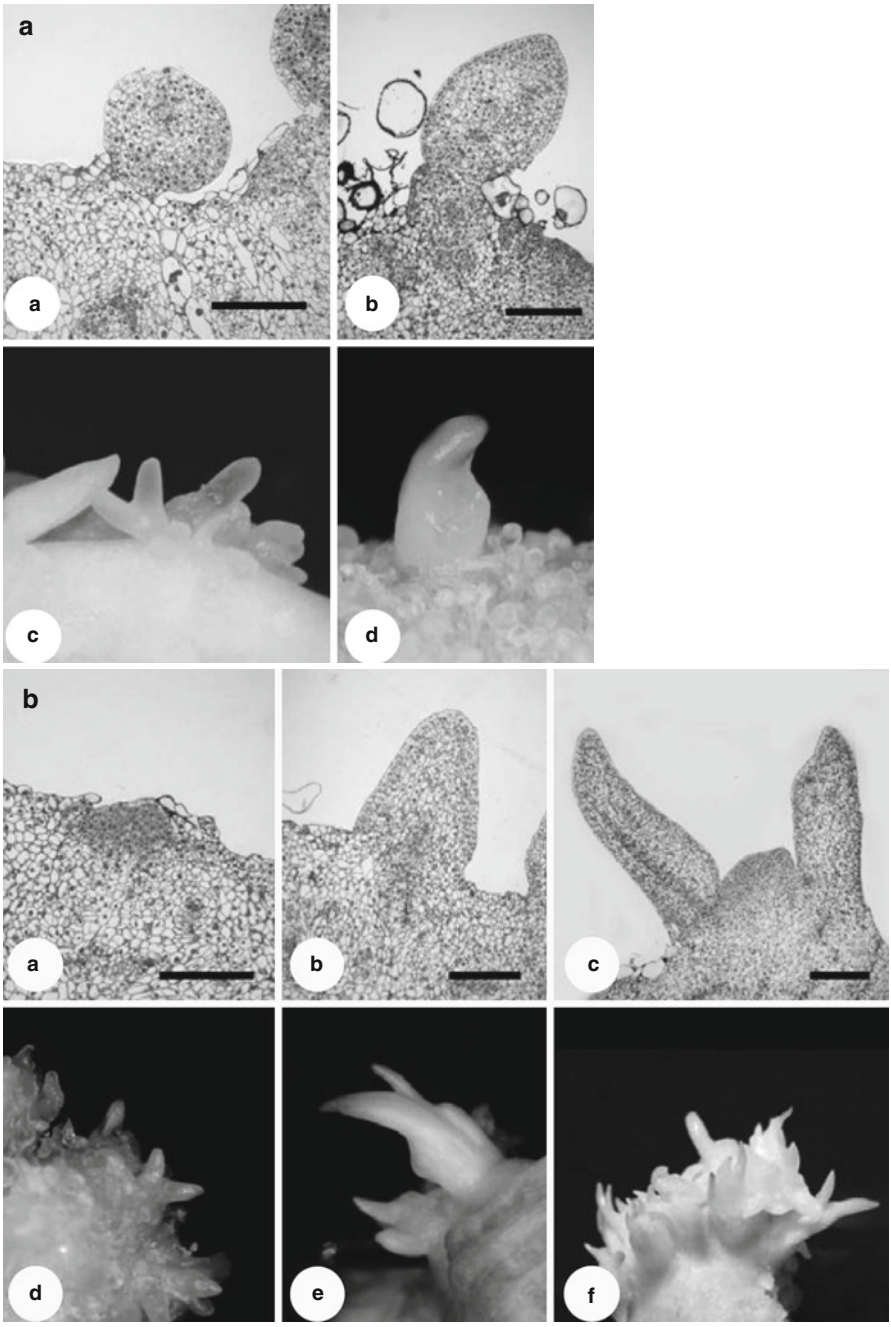

Fig. 14.12 Direct regeneration of Somatic embryo (Upper '**a**') and shoots (lower '**b**') from bulb segment of *A. victorialis* var. *platyphyllum.* (**a**) Globular embryo formation and development into bulblet, (**b**) Direct shoot formation from basal part of bulblet

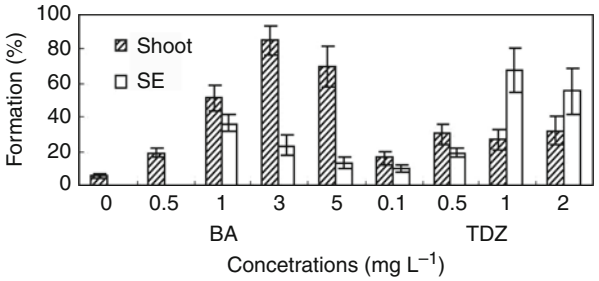

Fig. 14.13 Effect of BA and thidiazuron (*TDZ*) on shoot formation from bulblet segment in *A. victorialis* var. *platyphyllum*

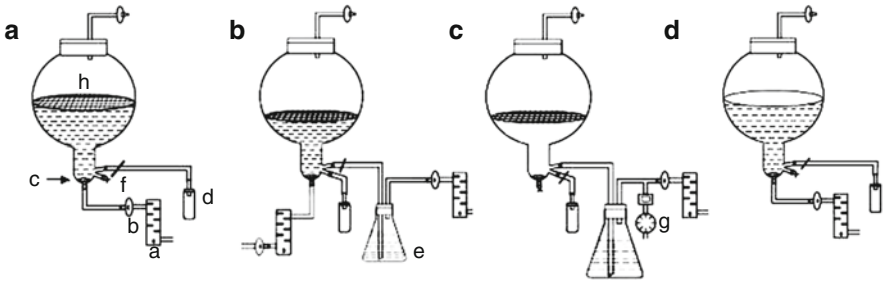

Fig. 14.14 Scheme of 5 L balloon type air-lift bioreactors using for this study. (**a**) Raft culture (*RC*); (**b**) Modified raft culture with medium reservoir (*MRC*); (**c**) Ebb and flood culture (*E&FC*); (**d**) Immersion culture with air supply (*IMA*) ((**a**) Air flow meter, (**b**) Membrane filter, (**c**) Glass sparger, (**d**) Sampling port, (**e**) Medium reservoir, (**f**) Connector for medium exchange and sampling, (**g**) Timer, (**h**) Net) (Adapted from Park et al. [43])

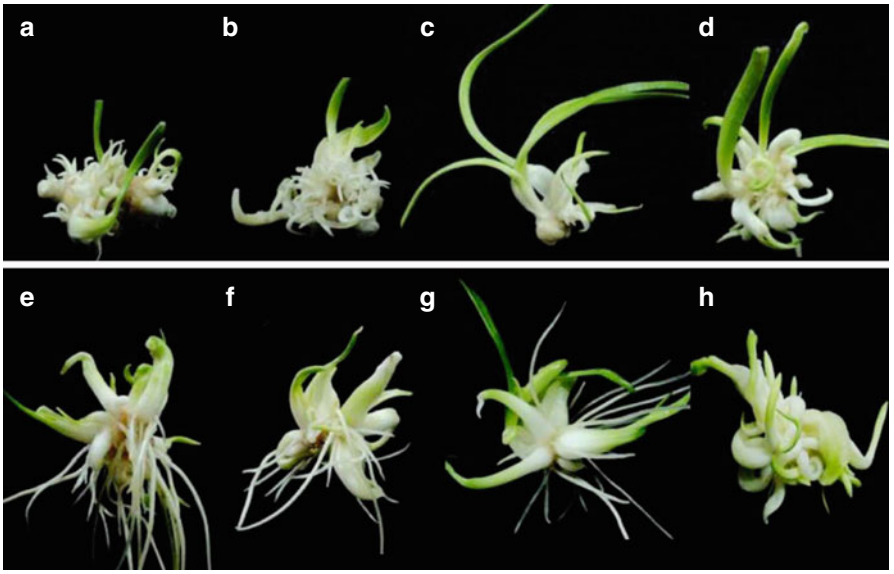

Fig. 14.15 Shoot (**a**–**d**) and bulblet clumps (**e**–**h**) of *Allium victorialis* harvested from 5 L balloon type air-lift bioreactors after 8 weeks of culture. (**a**, **e**) Shoot and bulblet clump cultured on RC, (**b**, **f**) Shoot and bulblet clump cultured on MRC, (**c**, **g**) Shoot and bulblet clump cultured on E&FC, (**d**, **h**) Shoot and bulblet clump cultured on IMA

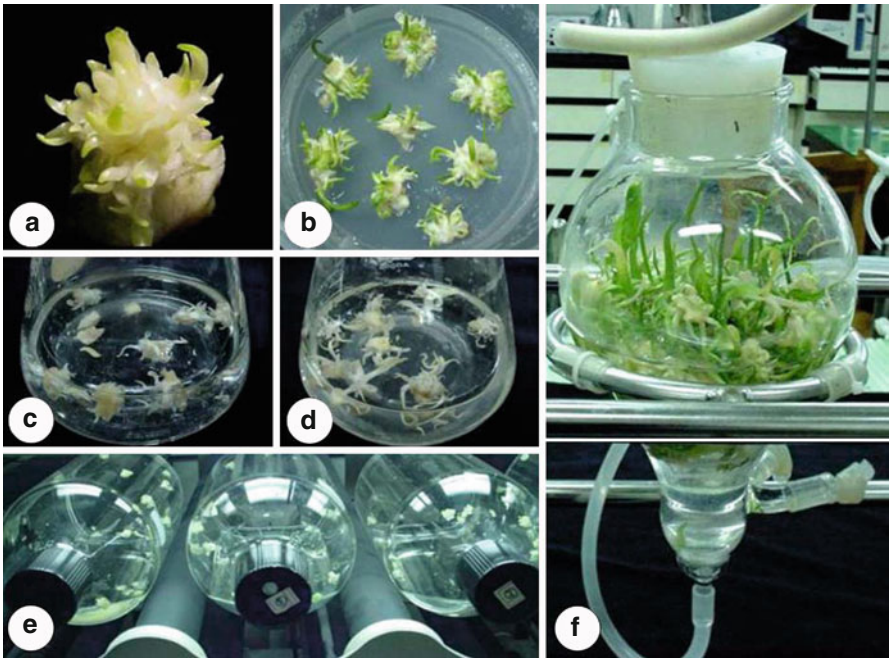

Fig. 14.16 Biomass production process of *A. victorialis* var. *platyphyllum via* multiple shoot culture. (**a**, **b**) Shoot induction (**a**) and proliferation (**b**), (**c**–**e**) Shoot multiplication in liquid medium, (**f**) Mass production in balloon-type bubble bioreactor

Table 14.2 Effect of bioreactor culture system on shoot proliferation in *Allium victorialis* after 8 weeks of culture

Bioreactor system	Total fresh wt (g)	Plantlet wt (g)	No of shoots per explant	Leaf length (cm)
Raft culture (RC)	90.8	1.5a[a]	15.0a	8.7b
Modified raft culture (MRC)	68.8	0.8b	13.1a	8.1b
Ebb and flood system (E&FS)	110.6	1.6a	9.4b	6.6b
Immersion culture with air (IMA)	122.7	1.6a	8.3b	13.0a

[a]Mean separation within columns by Duncan's multiple range test ($P \leq 0.05$)

showing roots. Shoot clumps did not grow properly without an air supply. Air supply was an important factor for growth when cultured with continuous immersion in the liquid medium. Depending upon different bioreactor culture systems, changes in concentration of sugar in the medium were measured during the culture period and in bulbs at the harvesting stage. The soluble carbohydrate content of the bulb cultured with the E&FC system was the lowest, but the starch content was higher than that of the others, with the exception of the bulbs grown in IM. Sucrose, glucose,

Table 14.3 Effect of bioreactor culture system on bulblet formation and enlargement in *Allium victorialis* after 8 weeks of culture

Bioreactor system	Total fresh wt (g)	Bulblet formation (%)	Fresh wt of bulblets (g)	Dry matter of bulblet (%)
Raft culture (RC)	69.3	59.6bc[a]	0.61c	32.7ab
Modified raft culture (MRC)	82.2	77.0b	0.99b	35.7a
Ebb and flood culture (E&FC)	72.1	93.4a	0.74c	27.2b
Immersion culture with air (IMA)	137.5	46.7c	1.34a	33.1ab

[a]Mean separation within columns by Duncan's multiple range test ($P \leq 0.05$)

and fructose concentrations in the medium of the E&FC system decreased concurrently with bulb formation and growth. It is indicated that external sucrose is taken into the cell before it is hydrolyzed. Sucrose was maintained at a minimum of 6 % in the medium until harvest time under all culture systems; hence, it can be deduced that only 3 % of the sucrose was taken for plant growth, and the remaining sucrose was used to control the osmotic pressure of the medium.

14.3.4 Rosa rugosa

Rugosa rose (*Rosa rugosa* Thunb.; syn. rugosa rose, Japanese rose, or Ramanas rose) is a deciduous shrub belonging to the Rosaceae family. This species grows on the sea coast and in sand dunes in Eastern Asia, including Northeastern China, Korea, Japan and Southeastern Siberia. *Rosa rugosa* is one of the most important genetic resources for the breeding of roses (*R. hybrida*) because of its useful horticultural traits such as disease resistance, cold hardiness and flower fragrance. In Asia, the rose has been used as a medicinal plant and food in tea [70]. It is also well known as a folk remedy for treating mastitis and diabetes mellitus. Several workers have systematically investigated the chemical components of the flowers, fruits, leaves, roots, and galls of this plant. Specifically, the flower is known to be an astringent, stomachic, and is traditionally used as an agent for activating blood circulation to relieve blood stasis, aiding in menstrual regulation, and counteracting toxins. It has been shown that this native rose is a fairly good source of aromatics, phenolics, terpenoids, fatty acid derivatives, sugars and other polar compounds [71]. The positive effects of *R. rugosa* on lipid peroxidation, alanine transaminase (ALT), aspartate transaminase (AST), glutathione, and protein oxidation levels was reported in carbon tetrachloride (CCl_4)-treated male Wistar rats [77]. Ng et al. [78] reported that *R. rugosa* flower extract increases the activities of antioxidant enzymes and their gene expression and reduces lipid peroxidation. In that report, the activities of catalase (CAT) and glutathione peroxidase (GPX) in 9-month-old senescence-accelerated mice (SAM mice) were lower than those in 6-month-old SAM mice [78].

In roses, embryogenic callus formation has been shown to occur from various explants, including the leaves, stems, filaments, petioles, roots, zygotic embryos, and protoplasts [72–76] for stock material propagation *in vitro* and genetic manipulation of the plant.

Plant Material

Mature seeds of the *Rosa rugosa* were collected from the natural habitat in Cheonnam (Fig. 14.20a). They were soaked in 70 % ethanol for 1 min, rinsed with sterile deionized water, and placed into 2 % (v/v) sodium hypochlorite solution supplemented with three to four drops of Tween-20 for 20 min. The seeds were rinsed three times in sterile deionized water before removal of the zygotic embryos with a scalpel and forceps. Zygotic embryos were placed on full-length MS medium containing 3.0 mg L^{-1} 2,4-D at 25 °C in the dark. After 8 weeks of culture, clumps of embryogenic calli and somatic embryos were isolated and subcultured on MS medium containing 10.0 mg L^{-1} 2,4-D. The embryogenic callus clumps were proliferated on the same fresh medium for embryogenic cultures. For shoot cultures, somatic embryos were transferred onto half strength MS medium without plant growth regulator, and developed plants from somatic embryos were used as culture material for multiple shoot formation.

Culture Establishment

Shoot Culture

Somatic embryo-derived plantlets were maintained on MS medium supplemented with 0.1 mg L^{-1} BA, 3 % (w/v) sucrose, and gelled with 2.5 g L^{-1} gelrite for 6 months. First, 2-cm long shoot-tips were dissected from the plantlets, and placed on MS medium supplemented with a wide range of BA (0–3.0 mg L^{-1}) or thiadiazuron (TDZ; 1.0–3.0 mg L^{-1}), or in combination with IBA 0.5 mg L^{-1} containing 3 % (w/v) sucrose to investigate the effect of cytokinins on multiple shoot formation. Later, multiplied shoots were transferred to a 3 L balloon-type bubble bioreactor (BTBB) to establish a scale-up culture for biomass production. Five types of bioreactor cultures were tested; (1) control (solid and liquid culture in flask), (2) continuous immersion BTBB (CI), (3) continuous immersion with net (CIN), (4) temporary immersion (TI), and (5) temporary immersion with net (TIN).

Multiple Shoot Cultures

The highest number of shoots was achieved in 1.0 mg L^{-1} BA treatment. However, the best biomass, in both fresh and dry weight, was in thiadiazuron in combination

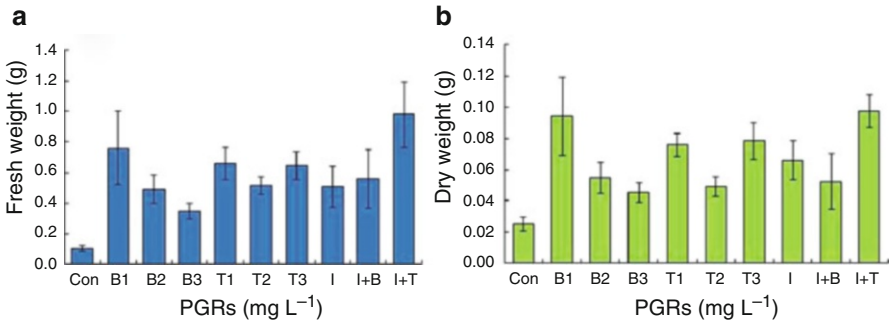

Fig. 14.17 Effect of different concentrations of plant growth regulators (*PGRs*) on multiple shoot formation from nodal culture in *R. rugosa* after 8 weeks of culture. (**a**) Fresh weight of explant, (**b**) Dry weight of explant (*B* BA, *T* TDZ, *I* IBA, *I* + *B* IBA 0.5 mg L^{-1} + BA 1.0 mg L^{-1}, *I* + *T* IBA 0.5 mg L^{-1} + TDZ 1.0 mg L^{-1})

Table 14.4 Effect of culture methods of balloon-type bubble bioreactor system on biomass and bioactive compounds production in *R. rugosa* after 8 weeks of culture

Culture methods	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Dry matter	Total phenolics (mg g^{-1} DW)	Flavonoids (mg g^{-1} DW)
Solid culture (flask)	43.00c[a]	2.99c	6.95	56.55a	13.82a
Liquid culture (flask)	67.00b	7.76b	11.58	37.94b	8.52b
Continuous immersion (CI)	91.90a	12.10a	13.01	39.21b	13.28a
Continuous immersion with net (CIN)	61.00b	7.10b	11.63	39.04b	13.12a
Temporary immersion (TI)	44.10c	7.45b	16.89	23.61c	7.92bc
Temporary immersion with net (TIN)	14.50d	1.00c	6.89	25.68c	11.00ab

[a]Mean separation within columns by Duncan's multiple range test ($P \leq 0.05$)

with IBA (Fig. 14.17). When BA concentration was increased, the number of adventitious shoots also increased (data not shown), but the biomass did not decrease.

The results obtained from biomass production using different culture systems are presented in Table 14.4. Among the different culture systems used, the maximum biomass was recorded in continuous immersion (CI) in a BTBB bioreactor, followed by continuous immersion with net (CIN), and liquid culture in a 300 mL conical flask (Fig. 14.18). Variable results were recorded with respect to the dry matter (%) and culture system. However, significantly higher dry matter was recorded in temporary immersion (TI) bioreactor (16 %), followed by CI bioreactor culture (13 %). The highest dry weight of biomass was achieved in the CI bioreactor, and it is evident that the cultures grew well directly in the liquid medium. The

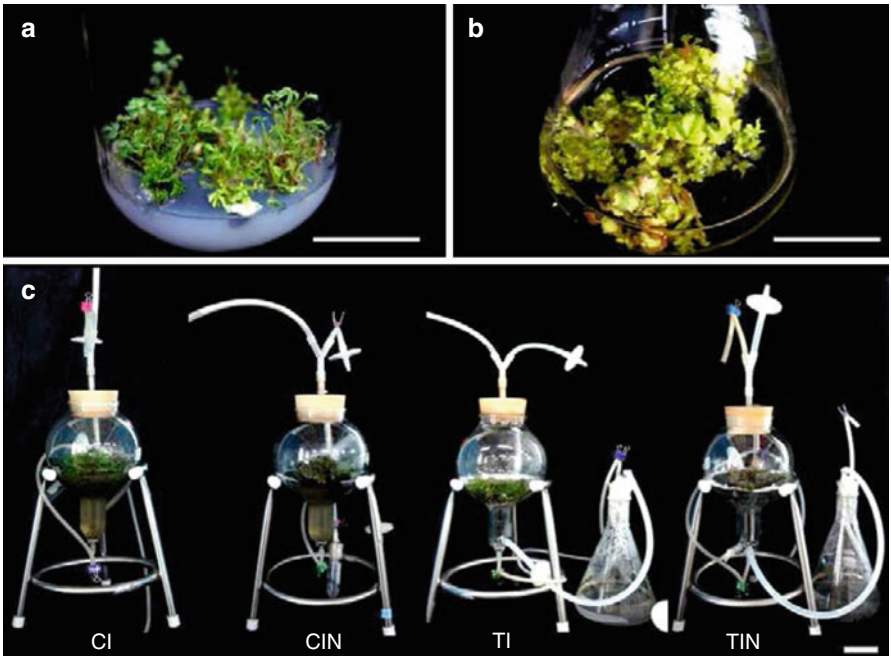

Fig. 14.18 Biomass production *via* balloon-type bubble bioreactor (*BTBB*) culture in *R. rugosa*. (**a**) Solid culture, (**b**) Liquid culture, (**c**) Four-types of BTBB system (*CI* continuous immersion, *CIN* continuous immersion with net, *TI* temporary immersion, *TIN* temporary immersion with net, *Scale bar* 5 cm

only factors that made differences in biomass between the two culture methods were agitation method and air supplement. The CI bioreactor was provided with 0.1 vvm aeration because of the large volume of the liquid medium.

Aeration rate is one of the factors that may affect the growth of propagules/explants in liquid cultures [59]. Plant tissue culture vessels with their caps or closures create boundaries between the internal microenvironment and the external environment of outside air [19]. They reported that the conical glass flask had the smallest air exchange rate while the bioreactor culture supplied enough aeration [19]. The type of vessel and culture system affects the gaseous composition inside the vessel as well as the dissolved O_2 content in the medium, and it affected cell and tissue growth. In the present study, the CI bioreactor with 0.1 vvm aeration showed maximum fresh and dry weight biomass production. In terms of bioactive compounds, total phenolics were highest in plantlets grown in solid culture, while flavonoid accumulation was high in the CI and CIN bioreactors (Table 14.4).

Somatic Embryo Culture

For embryogenic callus induction, immature seeds, leaves, and petals were placed on Murashige–Skoog (MS) medium supplemented with 3 % (w/v) sucrose, 0.3 % (w/v) gelrite, and plant growth regulators (PGRs): 2,4-dichlorophenoxyacetic acid

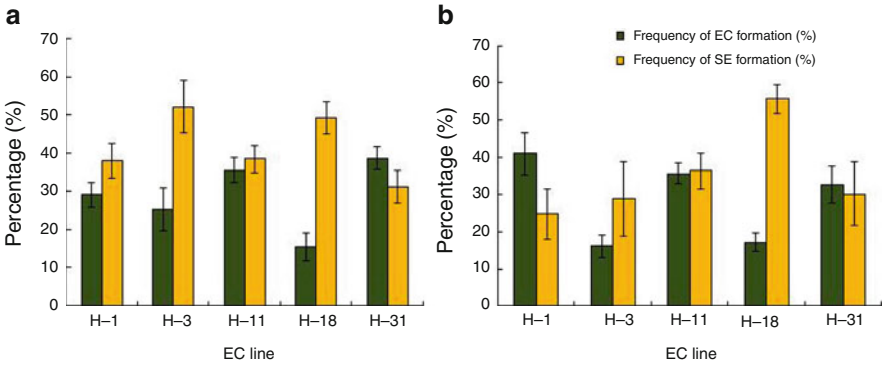

Fig. 14.19 Effect of 2,4-D concentrations on embryogenic callus proliferation from different cell lines (genotype) in *R. rugosa* after 4 weeks of culture. (**a**) MS medium containing 2,4-D 5.0 mg L^{-1}, (**b**) MS medium containing 2,4-D 10.0 mg L^{-1} (Unpublished data)

(2,4-D; 0–10.0 mg L^{-1}) alone or in combination (0.02–1.0 mg L^{-1}) with thidiazuron (TDZ), kinetin, benzyladenine (BA), zeatin, and 2-isopentyladenine (2iP). For selection and proliferation of embryogenic calli, five lines of embryogenic calli (H1, H-3, H-11, H-18, and H-31) were induced from immature seeds and used to investigate their embryogenic capacity by proliferation. The calli were proliferated on MS medium containing 3 % sucrose, 0.3 % gelrite, and 2,4-D (5.0 and 10.0 mg L^{-1}).

After 4 weeks of culture, each explant showed different responses to the various concentrations of PGRs. The highest rate of embryogenic callus (28.5 %) was obtained when immature zygotic embryos were cultured on the medium containing 1.0 mg L^{-1} 2,4-D + 0.5 mg L^{-1} TDZ, while no embryogenic callus was produced when immature zygotic embryos were cultured on the medium containing 2.0 mg L^{-1} 2,4-D or 1.0 mg L^{-1} 2,4-D + 0.5 mg L^{-1} kinetin. These results indicated that TDZ plays an important role in somatic embryogenesis in *R. rugosa* by regulating endogenous hormone levels and cell division. Unlike immature embryos, leaves produced embryogenic calli after treatment with a high concentration of 2,4-D (5.0 mg L^{-1}, 1.3 %), whereas low concentrations of 2,4-D failed to induce embryogenic calli from leaf segments. Similarly, the highest induction rate of embryogenic calli was obtained with higher concentrations of 2,4-D. After treatment with 10.0 mg L^{-1} 2,4-D, a 14.06 % embryogenic callus formation was achieved. We postulate that the reason for the differences in the responses to 2,4-D concentration was because immature embryos contain pre-embryogenic determined cells, but leaves and petals induced-embryogenic determined cells.

Five selected embryogenic callus lines showed different characteristics in embryogenic callus and somatic embryo formation (Fig. 14.19). After 4 weeks of culture, all embryogenic callus lines showed embryogenic callus and heart, torpedo, and cotyledon stages of somatic embryos (Fig. 14.20). H-1 produced a higher rate of embryogenic callus formation after treatment with 10.0 mg L^{-1} 2,4-D, while H-3 and H-18 produced a higher rate of somatic embryo formation regardless of 2,4-D concentration. This indicated that the genotype influenced somatic embryogenesis because of the differences in endogenous hormonal level resulted in lower embryogenic capacity.

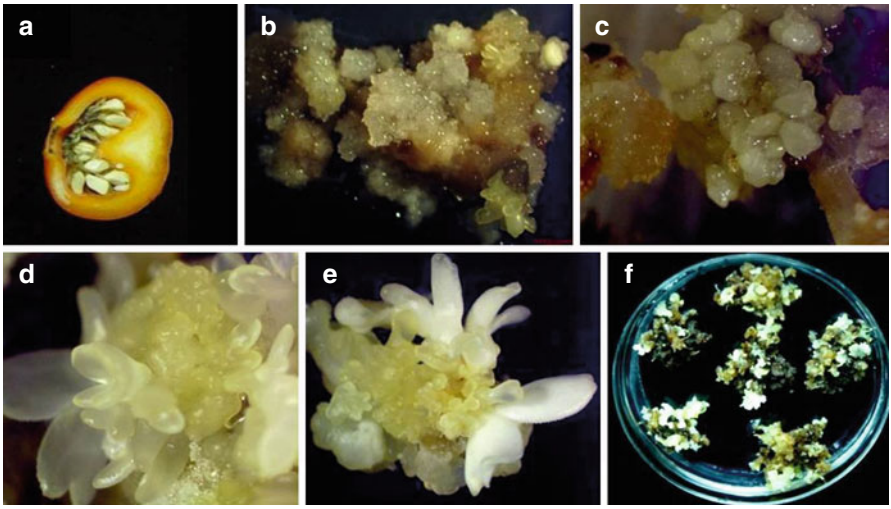

Fig. 14.20 Somatic embryogenesis from mature zygotic embryo-derived embryogenic calli in *R. rugosa*. (**a**) Mature fruit and seeds, (**b**) Embryogenic callus induction, (**c**) Globular-stage somatic embryos, (**d–f**) Well-developed somatic embryos on hormone-free medium after 4 weeks of culture

14.4 Conclusions

Multiple-shoot and somatic embryo cultures are among the most valuable and underexploited sources for biomass and useful secondary metabolite production. They are easier to manipulate, and as shown in the above cases, long-term and large-scale culture of multiple-shoots and somatic embryos is possible in bioreactors. Bioreactor technology provides the potentiality for producing economical and efficient amount of biomass and bioactive compounds from medicinal plants. Furthermore, cultures may be more conducive to scale-up in a bioreactor, making secondary accumulation an acceptable biotechnological process for further applications such as enhancement in the production of pharmaceutical molecules, introduction of foreign genes into plant genomes to produce recombinant proteins, or over-expression of useful proteins.

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Chapter 15
Hairy Roots: Production of Metabolites to Environmental Restoration

N.S. Desai, P. Jha, and V.A. Bapat

Abstract Hairy roots (HRs) have been proven as a potential source of secondary metabolites and also, for the biotransformation of desirable metabolites. Recently, HRs have emerged as an efficient *in vitro* model systems for screening the capabilities of different plant species to tolerate, accumulate, and/or to remove environmental pollutants. HRs offer benefits of greater genotypic and phenotypic stability than the dedifferentiated cultures, thus providing a more reliable and a reproducible experimental system, and even for flexibility of insertion of gene of interest to the HR gene construct for efficient applications. Additionally, absence of soil matrix and microbes is the key advantage in HRs for precise removal of toxic products as well as for elucidating metabolic pathways for conversion of hazardous chemicals to non hazardous products. The feasibility of scale up of HRs in bioreactors offers an attractive avenue for industrial processes both for metabolite synthesis as well as for phytoremediation. The present review highlights current knowledge, recent progress, areas which need to be explored and future perspectives related to the application and improvement of the efficiency of HRs for phytoremediation research.

Keywords Hairy roots (HRs) • Inorganic pollutant • Organic pollutant • Phytoremediation

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Abbreviations

DDT	Dichloro diphenyl trichloroethane
DU	Depleted uranium
FTIR	Fourier transform infrared spectroscopy
GCMS	Gas chromatography–mass spectrometry
HMX	Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
HPLC	High pressure liquid chromatography
HR	Hairy roots
PCBs	Polychlorinated biphenyls
PCR	Polymerase chain reaction
RDX	Hexahydro-1,3-5-trinitro-1,3-5-triazine

15.1 Introduction

Hairy root (HR) cultures are preferred over plant cell/callus and suspension cultures due to their genetic/biochemical stability, hormone-autotrophy, multi-enzyme biosynthetic potential mimicking that of the parent plants and relatively low-cost cultural requirements.

Infection of wounded plant parts with *Agrobacterium rhizogenes* and subsequent transfer of a DNA segment of root inducing (Ri) plasmid into the plant genome, result into hormonal imbalance leading to the formation of HRs. These HRs are also susceptible to genetic transformation, which help in gene transfer and can be characterized in a system that may lead to minimum health damages or environmental concerns.

HR cultures have entered into an array of junctures of functional research from generation of high value secondary metabolites [1] and pharmaceutical lead compounds by the process of chemical transformations aided through their inherent enzyme resources [2] to their use as an *in vitro* systems for phytoremediation research [3].

HRs often produce valuable secondary metabolites for a long period of time, and many examples have been reported in the literature (Table 15.1). In some cases, changes in growth conditions (like medium composition, carbon source, pH, light/dark, aeration, etc.) as well as the use of elicitors have been successfully employed to increase the HR growth from different plant species and also for the production or secretion of secondary metabolites [4, 5]. Cellular and molecular events induced by elicitors in HRs and the correlation with enhanced secondary metabolite synthesis has been discussed in a recent review [6]. However, new approaches are directed on kinetic models for HR growth improvement [5]. In this aspect, Goel and coworkers [6] proposed the combination of elicitation with *in silico* approaches to understand and identify the rate-limiting steps of biosynthetic pathways existing in HRs, in order to improve the productivity of different compounds, by using metabolic engineering pathways.

Table 15.1 Application of HRs in the production of secondary metabolites from different expression systems

Secondary metabolites	Expression systems	References
Withanolide A	*Withania somnifera*	[7]
Camptothecin	*Ophiorrhiza alata* Craib	[8]
Glycyrrhizin	*Glycyrrhiza inflate*	[9]
Dopa and dopamine	*Beta vulgaris*	[10]
Serpentine	*Catharanthus roseus*	[11]
Sesquiterpenes	*Hyoscyamus albus*	[12]
Purpurin	*Rubia cordifolia*	[13]

Table 15.2 Applications of HRs for the production of recombinant proteins from different expression systems

Recombinant proteins	Expression systems	References
Murine IgG1	*Nicotiana tabacum*	[16]
Human IgG1 and IgG4	*N. tabacum*	[17]
Hepatitis B surface antigens (HBsAg)	*Solanum tuberosum* (var. Kufri bahar)	[18]
Murine interlukin-12	*N. tabacum* cv Xanthi	[19]
Human acetylcholinesterase	*N. benthamiana*	[20]
β-Glucuronidase (GUS)	*N. tabacum*	[21]
Ricin-B	*N. tabacum* cv Xanthi	[22]

It is well known that transgenic plants have been used widely for the expression of therapeutic proteins, such as vaccines, antibodies, and mammalian enzymes [14]. Similarly heterologous proteins for pharmaceutical and industrial use have been successfully expressed in HR based bioreactor systems. HRs have an enormous potential because this system combines the advantages of plant-based or "green" technologies such as intrinsic biosafety, scalability, low production and downstream costs, and the existence of eukaryotic folding and assembling machinery available in *in vitro* technology. Moreover, growth under controlled and optimized conditions in confined bioreactors, continuous production, utilization of simple nutritional requirements, exclusion of transgene dissemination, reproducible product yield and easy regulatory compliance are the other advantages. A pioneering work of Wongsamuth and Doran [15] stated the first application of HRs for the synthesis of a full length murine IgG monoclonal antibody. So far 15 recombinant proteins including several antibodies, antigens, immunomodulators, reporter proteins, enzymes etc. have been successfully produced in HRs cultures (Table 15.2).

Recent progress in plant biotechnology has enabled the employment of HRs in phytoremediation research for clear understanding of metabolic events involved in conversion of toxic chemicals to non toxic compounds. Among the plant *in vitro* systems, HRs have been astutely and judiciously exploited by the researchers for the development of an easy and cost effective alternative for the removal of toxic metabolites (Table 15.3).

Table 15.3 Phytoremediation of various environmental pollutants by HR cultures

Plant species model	Pollutant	References
Black nightshade (*Solanum nigrum*)	PCBs	[25]
Periwinkle (*Catharanthus roseus*)	RDX and HMX	[29]
Rapeseed (*Brassica napus*); Sunflower (*Helianthus annuus*)	Phenol	[31, 33]
Sunflower (*H. annuus*)	Tetracycline and oxytetracycline	[35]
Chicory (*Cichorium intybus*) and Indian mustard (*Brassica juncea*); *Chenopodium amaranticolor*	DDT; Chlorpyrifos	[37, 38]
Marigold (*Tagetes patula*); Indian mustard (*B. juncea*); Gooseberry (*Physalis minima*)	Reactive red 198; Methyl orange: Reactive black 8	[39–41]
Wild mustard (*Alyssum bertolonii*) and Alpine pennygrass (*Thlapsi caerulescens*)	Nickel; cadmium	[44, 46]

PCBs polychlorinated biphenyls, *RDX* hexahydro-1,3-5-trinitro-1,3-5-triazine, *HMX* oxtahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine, *DDT* dichloro-diphenyl-trichloroethane

One more advantage of using HRs for studying phytoremediation, is their capability to produce large amount of exudates which contain enzymes and few metal chelating compounds that may decontaminate organic and inorganic pollutants [23, 24]. It is well known that HRs are able to metabolize per se hazardous compounds by common metabolic pathways. As roots are the first organs to have contact with the soil pollutants, they have evolved specific mechanisms to deal with the stress created due to the pollutants. However, to study precisely the metabolic events occurred due to the soil pollutants, natural roots may not be an appropriate option. Hence, by using HRs metabolic pathways and enzymatic catalyzed reactions involved in pollutants detoxification can be elucidated accurately. In addition, the mechanisms of uptake, transformation, conjugation, and compartmentation of pollutants in vacuoles and/or cell walls, which are important detoxification sites in plants can be elucidated by HRs.

15.2 Application of HRs in Phytoremediation

15.2.1 Removal of Organic Pollutants

PCBs

Polychlorinated biphenyls (PCBs) chemicals have certain hazardous properties mainly dielectric fluids, hydraulic fluids, and other applications requiring stable, fire-retardant materials. In this context, PCBs bioaccumulation can lead to reduced ability to control infection, increased rate of autoimmunity, cognitive and behavioral problems and hypothyroidism [25]. The HRs of black nightshade (*Solanum nigrum*) have been demonstrated to metabolizes and remove PCBs from solutions spiked with PCB congeners [26, 27].

Explosives

Hexahydro-1,3-5-trinitro-1,3-5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) are the two most common pollutants found in military sites where explosives are commonly tested [28]. Bhadra et al. confirmed that HRs of *Catharanthus roseus* have inherent capabilities to remove RDX and HMX from the medium [29].

Phenols

Phenols are commonly used in various agricultural applications. They are released from coal and petroleum refining activities, and they pose a threat to human health. HRs have been used to check plants for their ability to tolerate high levels of phenols. In HRs of carrot (*Daucus carota*) and other plant species, the role of peroxidase enzymes might be the key factor in the removal of phenol and chlorophenols from the culture medium [30]. Also, the inherent activity of peroxidases in HRs of rapeseed (*Brassica napus*), was associated with the effective removal of 2, 4-dichlorophenol and phenol from the medium for several cycles and the removal process was aggravated by the addition of hydrogen peroxide [31]. The HRs of carrot, kangaroo apple (*Solanum aviculare*) and sweet potato (*Ipomoea batatas*) are able to incorporate and conjugate the phenolic compounds with polar cellular materials (possibly sugars and proteins) as well as with insoluble materials. Peroxidase isozymes involved within a species may show variation in substrate preference and catalytic efficiency of phenol metabolism [32]. Another recent study pointed out phenol (100 mg L^{-1}) removal in 144 h by hairy roots of *Helianthus annuus* L. [33].

Antibiotics

Environmental analytical studies show that trace concentrations of antibiotics occur in hospital and municipal wastewaters and in the nearby aquatic environments [34]. HRs of sunflower (*Helianthus annuus*) were effective in extracting and metabolizing antibiotics including tetracycline and oxytetracycline through a process that was thought to involve reactive oxygen intermediates [35].

Insecticides

There is a controversy regarding the continuous use of the insecticide DDT to combat mosquitoes that spread malaria in developing countries [36]. Some studies suggested that DDT might have detrimental effects on human health. HRs of chicory (*Cichorium intybus*) and Indian mustard (*Brassica juncea*) have been used to study their potential in removing DDT from contaminated sites [37]. HRs of *Chenopodium amaranticolor* have been used to degrade Chlorpyrifos, a commonly used pesticide [38].

Dyes

HRs of *Tagetes patula* were able to remove a dye concentration up to 110 mg L^{-1} and could be successively used at least for five consecutive decolourization cycles [39]. The HR cultures of *Brassica juncea* L. have shown 92 % decolourization of Methyl orange within 4 days. The enzyme laccase was purified and characterized from the decolourized samples which contributed to a better understanding of the enzymatic process involved in phytoremediation of textile dyes [40]. In a recent study, role of antioxidant enzymes of HRs of *Physalis minima* during degradation of Reactive Black 8 have been investigated [41].

15.2.2 Removal of Inorganic Pollutants

Heavy Metals

Depleted uranium (DU) is an emerging environmental pollutant that is introduced into the environment primarily by military activity. Adult animals that were exposed to depleted uranium during development display persistent alterations in behaviour, and a variety of changes in brain chemistry and pose a radiologic hazard [42]. Eapen et al. have demonstrated that HRs of *Brassica juncea* and *Chenopodium amaranticolor* could uptake 20–23 % and 13 % uranium, respectively from solutions of 5,000 μM within 10 days [43]. HRs have exhibited that they can be utilized as a source of selecting a range of plant species for their ability to extract and absorb metals [44]. A comparison between nickel tolerance of HRs and whole plants revealed that the translocation of nickel to shoots may not be required for nickel tolerance and hyperaccumulation in certain species of *Alyssum* [45, 47]. Boominathan and Doran have demonstrated that HRs of alpine pennygrass (*Thlaspi caerulescens*) extracted cadmium and accumulated it in complexes with organic acids inside the cell walls [46].

15.3 Transgenic Plants

Several examples of transgenic plants for phytoremediation have been reported. Bernejee et al. used an approach to express a mammalian cytochrome P450 enzyme in deadly nightshade (*Atropa belladonna*) and the transgenic plants were able to metabolize the environmental pollutant TCE [48]. Doty et al. were successful in transforming poplar (*Populus tremula* x *Populus alba*) with this mammalian enzyme to generate plants with a superior capacity to remove various organic pollutants from hydroponic solutions and air [49]. Over-expression of a tomato (*Lycopersicon esculentum*) *tpx*1 gene encoding peroxidase in HR generated roots with enhanced capacity

of removing phenols from the medium [50]. These studies demonstrated that transgenic approaches may be adopted to produce plants with novel and improved phytoremediation capacity [51]. Therefore, in the near future the use of transgenic HR systems may become more common in testing the efficacy of transgenes and the enzymes they encode for the removal of hazardous environmental pollutants. Though the generation of transgenic plants with enhanced phytoremediation capacity might seem as a feasible solution, public skepticism and reluctance to transgenic organisms might make this option less favourable for its application. The alternative is the selection of local plant species with enhanced phytoremediation capacity through HR screens which may be a more practical solution to remove contaminants from the environment.

15.4 Case Study (Phytoremediation of Textile Azo Dye, Direct Blue 71 by HRs of Tagetes patula)

15.4.1 Induction of HRs in Explants of Tagetes

Different explants including cotyledonary leaf, hypocotyl and epicotyl of *in vitro* grown seedlings of *Tagetes* were used for HR induction using *A. rhizogenes* MTCC532. After 3 weeks, the cotyledonary leaf explants showed the maximum response (86.67 %) as well as the maximum root numbers (3.7 roots per explant). Similar variable response was also observed in *Taraxacum platycarpum* explants when infected with *Agrobacterium rhizogenes* (ATCC15834) [52]. The molecular confirmation of transgenic nature of the roots of the infected explants was confirmed by PCR amplification of *rol*C gene from genomic DNA of HRs, using gene specific primers. The best medium for maximum growth of HRs was formulated on the basis of growth kinetics (Fig. 15.1).

15.4.2 Screening of Different Textile Azo Dyes Tagetes

Among the 25 textile azo dyes tested for their decolourization by HRs of *Tagetes*, more than 50 % decolourization was observed in 19 dyes after 6 days (Fig. 15.2). The maximum and minimum decolourization was obtained for Direct Blue 71 (99 %) and Reactive Orange 4 (21.1 %), respectively. Direct Blue 71 (DB71) was used as model dye for rest of the study. The variation observed in decolourization efficiency and the time required for decolourization, might be due to the molecular complexity of the dyes and the enzymes produced during decolourization [53].

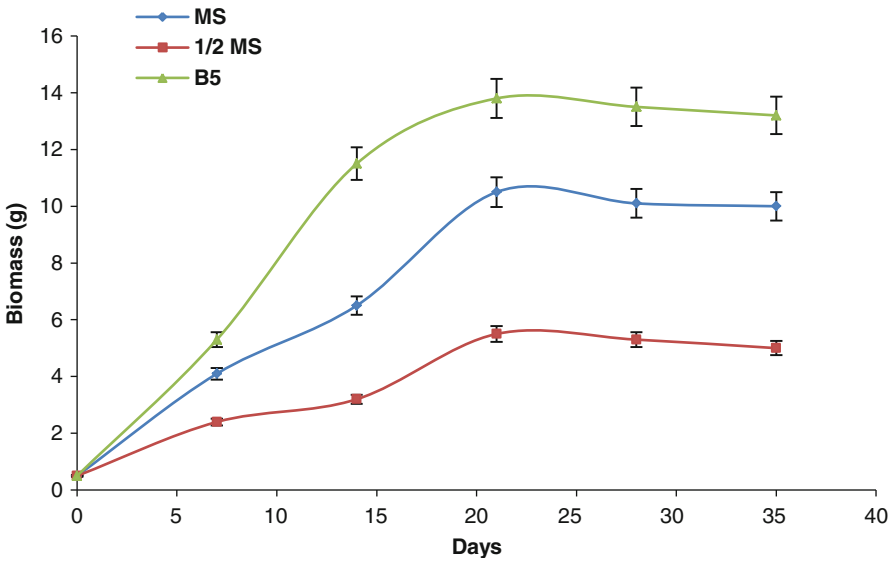

Fig. 15.1 Growth kinetics of HRs

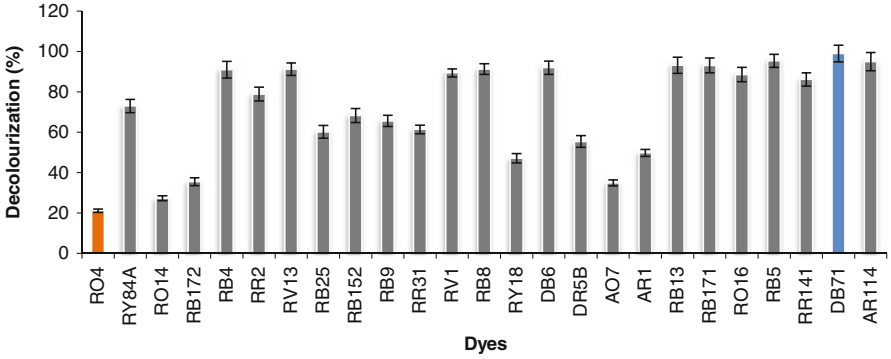

Fig. 15.2 Screening of textile azo dyes using HRs

15.4.3 Effect of Different Parameters on Decolourization of DB71

When various pH conditions (1.8–7.8) were assessed, pH 4.8 and 5.8 was observed to be optimum pH for the maximum decolourization of DB71 (Fig. 15.3a). There was no appreciable effect of temperature changes on decolourization of DB71 (Fig. 15.3b). It was also found that 30 g L^{-1} of biomass dosage was optimum (Fig. 15.3c). HRs of *Tagetes* were able to decolourize DB71 ranging from 97 % (60 mg L^{-1}) to 32 % (150 mg L^{-1}) (Fig. 15.3d). Considering these data, all further DB71 decolourization experiments were carried out at pH 5.8, temperature 25 ± 2 °C, biomass dosage 30 g L^{-1} and initial dye concentration of 60 mg L^{-1}.

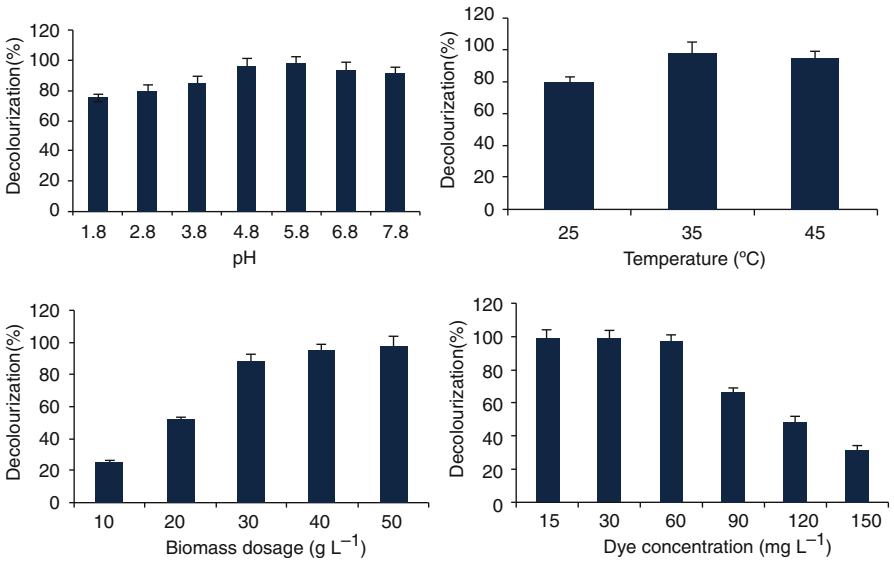

Fig. 15.3 Effect of pH, temperature, biomass dosage and initial dye concentration on decolourization of DB71

15.4.4 Biosorption of DB71 by HRs

Percentage Adsorption of DB71 onto HRs

See Fig. 15.4a.

Adsorption Isotherms

See Fig. 15.4b, c.

The Langmuir constants (Q_{max} = 76.92 mg g^{-1}, b = 0.041, R^2 = 0.979) and Freundlich constants (k = 3.236, 1/n = 0.803, R^2 = 0.967) were calculated.

The results obtained fitted well in the linear forms of Langmuir and Freundlich adsorption isotherms (Fig. 15.4) which indicated that both monolayer adsorption and heterogeneous surfaces conditions exist under the experimental conditions used [54].

15.4.5 Phytodegradation Analysis

Different analytical techniques like Ultraviolet-visible (UV-Vis) spectroscopy, HPLC, FTIR spectroscopy and GCMS were used to confirm the degradation of DB71 by HRs of *Tagetes*.

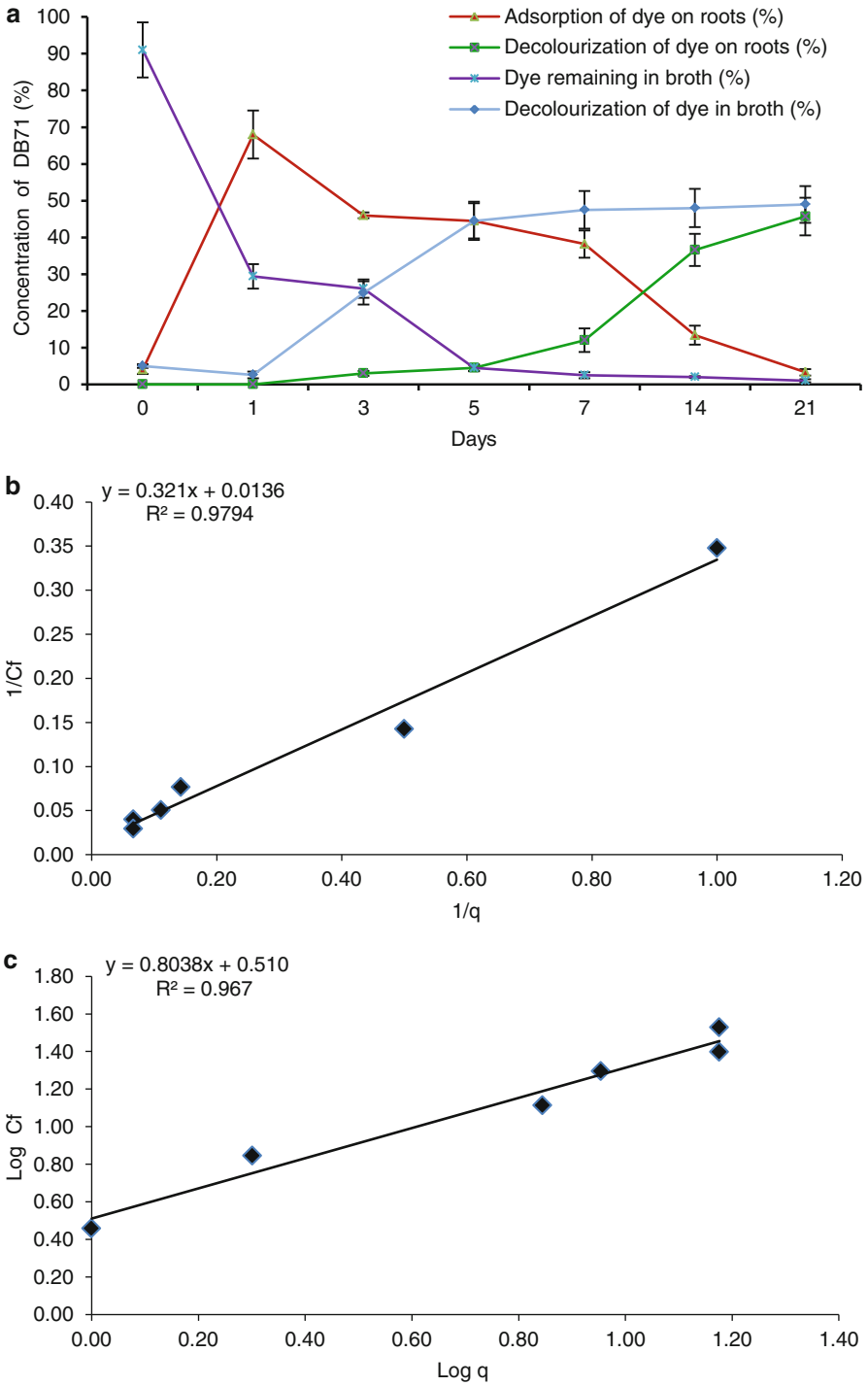

Fig. 15.4 (**a**) Adsorption studies of DB71 onto HRs. (**b**) Langmuir isotherm. (**c**) Freundlich isotherm

UV-Vis Spectra During Decolourization of DB71

Absorption spectra of different days in 200–800 nm during decolourization DB71 by HRs (Samples) were compared to the spectrum of untreated DB71 (Control) (Fig. 15.5).

Determination of Differential Expression of Enzymes and Aromatic Amines Under DB71 Stress

Lignin peroxidase and azoreductase showed a gradual increase in their activities during the decolourization of DB71 by HRs. After 1 day of DB71 treatment, a significant increase of sevenfold in the extracellular activity of lignin peroxidase was recorded when compared to control.

This was followed by a gradual increase in intracellular activities of lignin peroxidase and azoreductase. The maximum increase in lignin peroxidase and azoreductase was observed at tenfold and eightfold respectively when compared to the initial values after 14 days of treatment. In a recent study on decolourization of DB71 by *Brevibacterium* sp. UVS, the induction of tyrosinase, DCIP reductase, riboflavin reductase and azoreductase has been reported [55]. The concentration of aromatic amines were determined in both intracellular and extracellular samples for 21 days at regular intervals (Fig. 15.6). The maximum concentration of aromatic amine was observed on same days as for the maximum induction of enzymes.

HPLC Analysis of Degraded Products of DB71

The eluent profile of untreated DB71 showed the presence of a peak at 1.767 min (Fig. 15.7a). The eluent profile of DB71 treated with HRs of *Tagetes*, showed three major peaks at 2.810, 3.631 and 3.763 min and four minor peaks (Fig. 15.7b).

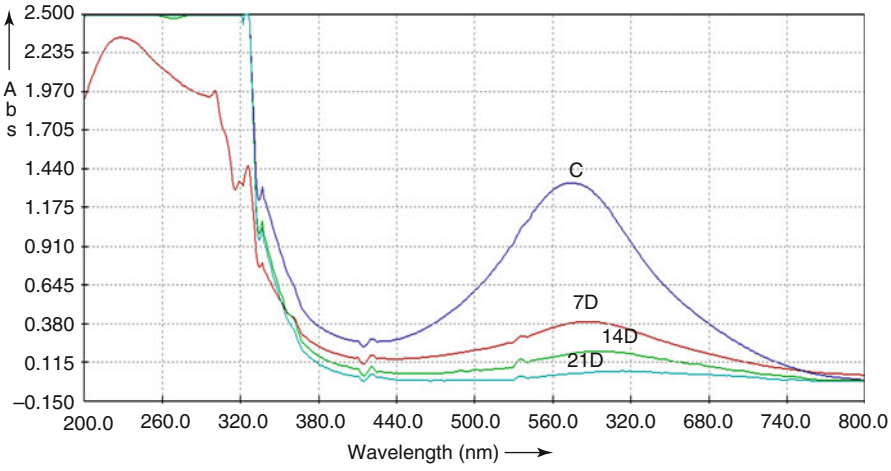

Fig. 15.5 UV–vis spectra of DB71 (C) and during its decolourization by HRs of *Tagetes* after 7, 14 and 21 days

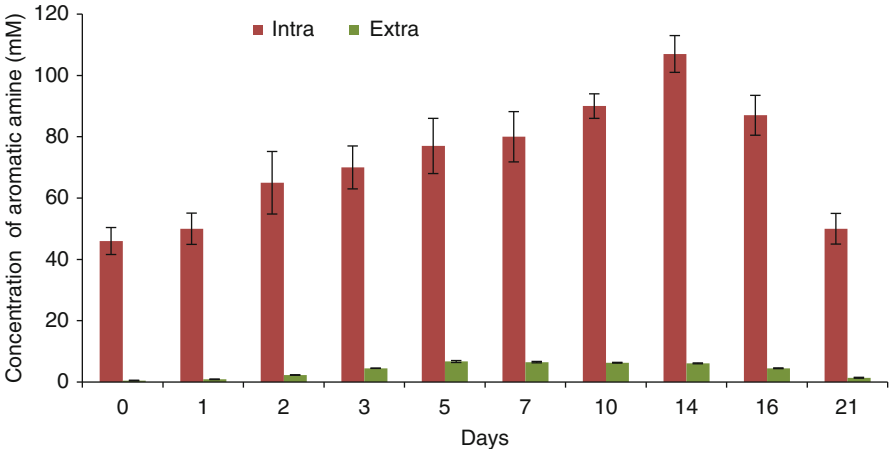

Fig. 15.6 Aromatic amine concentrations in intracellular (*intra*) and extracellular (*extra*) samples during decolourization of DB71

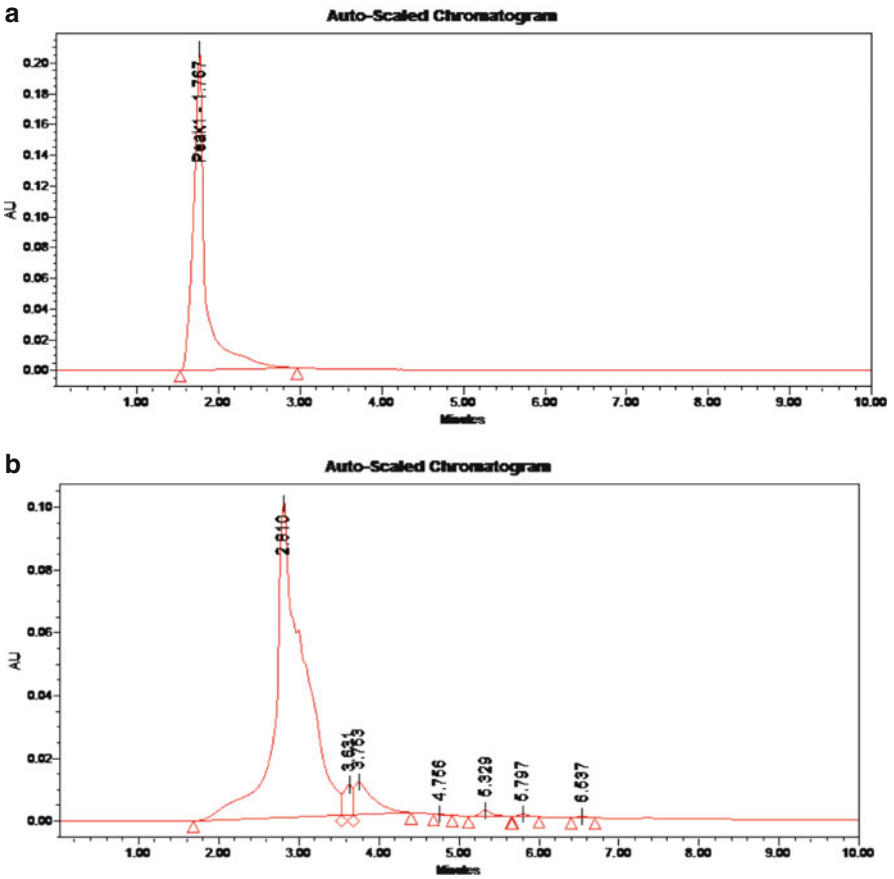

Fig. 15.7 HPLC eluent profile of DB71 and its degraded products. (**a**) Eluent profile of DB71. (**b**) Eluent profile of degraded products of DB71

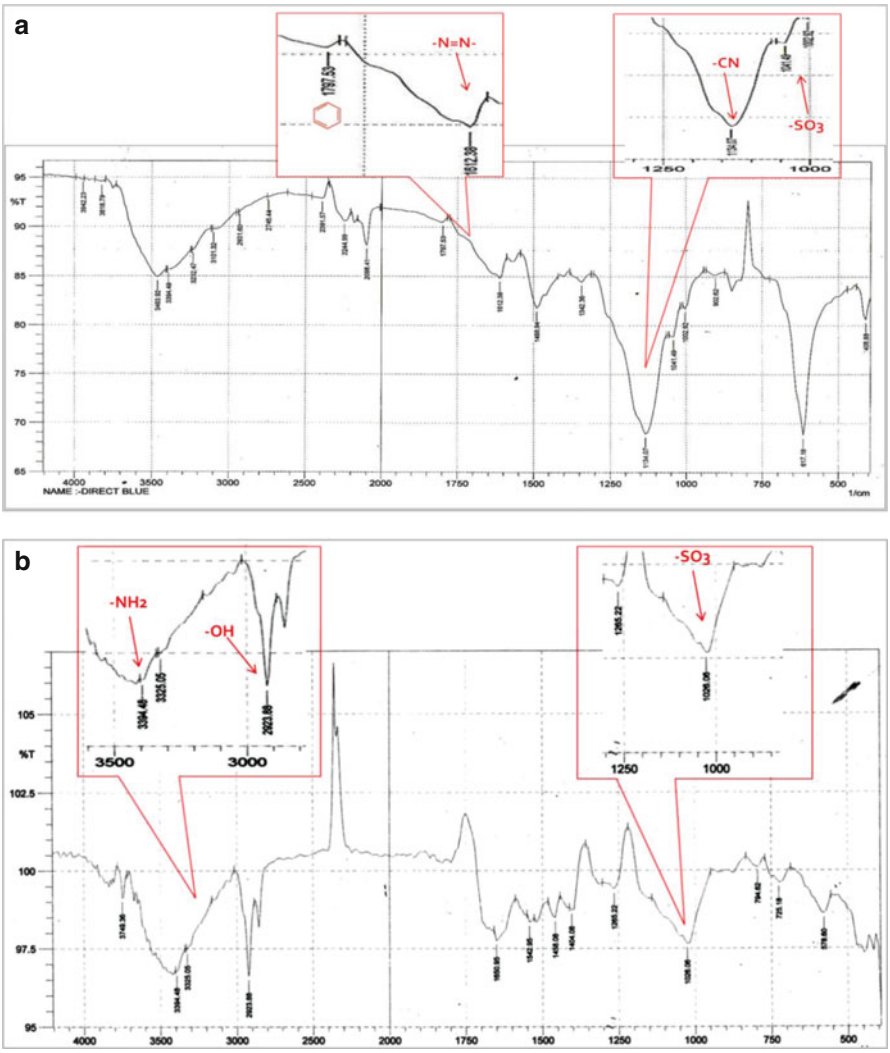

Fig. 15.8 FTIR spectra of DB71 and its degraded products. (**a**) FTIR spectrum of DB71. (**b**) FTIR spectrum of degraded products of DB71

FTIR Spectrum of Degraded Products of DB71

The FTIR spectrum of DB71 (Fig. 15.8a) showed the presence of a peak at 1612.38 cm^{-1} which suggested the presence of azo bond. The occurrence of peak at 1797.53 cm^{-1} indicated the presence of the substituted benzene rings. The presence of peaks at 1041.49 and 1134.07 cm^{-1} implied the presence of $–SO_3$/C-N stretching. The FTIR spectrum of DB71 degraded by HRs (Fig. 15.8b) showed a different spectral pattern. The presence of peak at 1026.06 cm^{-1} indicated the presence of $–SO_3H$. Occurrence of peaks at 3394.48 and 2923.88 cm^{-1} represented the presence of primary amine and O–H stretching, respectively. In particular, there was absence of

the peak indicative of azo (-N=N) group which meant that there was removal of azo bond after decolourization of DB71. The absence of groups, $–SO_3$ (1041.49 cm^{-1}) and C-N stretching (1134.07 cm^{-1}) in sample confirmed the phytodegradation of DB71.

GCMS Analysis

The metabolites formed during the phytodegradation of DB71 were predicted by GC mass spectral data as sodium 3, 7-diamino-4-hydroxynaphthalene-2-sulfonate (mw 276, m/z 276) and naphthalene-2-sulfonic acid (mw 208, m/z 207). On the basis of enzymes induced, HPLC chromatograms, FTIR spectra and GC mass spectral data, the probable phytodegradation pathway of DB71 was predicted (Fig. 15.9).

15.4.6 Phytotoxicity Studies of DB71 and Its Degraded Products

The drastic morphological changes like stunted plumule and radicle were quite evident on the growth of seedlings obtained from seeds treated with DB71 (1,000 ppm). Seeds treated with degraded dye (DD) (1,000 ppm), the results were almost similar to the seeds grown in distilled water (D/W) (Table 15.4). Thus, phytotoxicity studies confirmed the non toxicity of degraded DB71 as well as toxicity of DB71 with respect to *Phaseolus mungo* and *Triticum aestivum*.

15.4.7 Reuse Efficiency of HRs in DB71 Decolourization

HRs of *Tagetes* were able to efficiently decolourize DB71 for eight repetitive continuous cycles (Fig. 15.10). In an earlier study, repeated decolourization (>70 %) of DB71 for six cycles by *Comamonas* sp. UVS has been reported [56].

Hence, the approach of phytoremediation of textile dyes by hairy roots used in case study have proved the efficacy of these hairy roots in remediation of dyes. Further, to implement this on commercial scale, a pilot study has to be carried out.

15.5 Conclusions and Future Prospects

The flexibility to induce HRs from many plant species by infecting them with *Agrobacterium rhizogenes* has facilitated a wide range of plants for phytoremediation studies. Depending upon the nature of the pollutant, a suitable plant can be selected for hairy root induction for detoxification of a specific hazardous pollutant. However, it is a prerequisite to establish a reproducible protocol for growth and

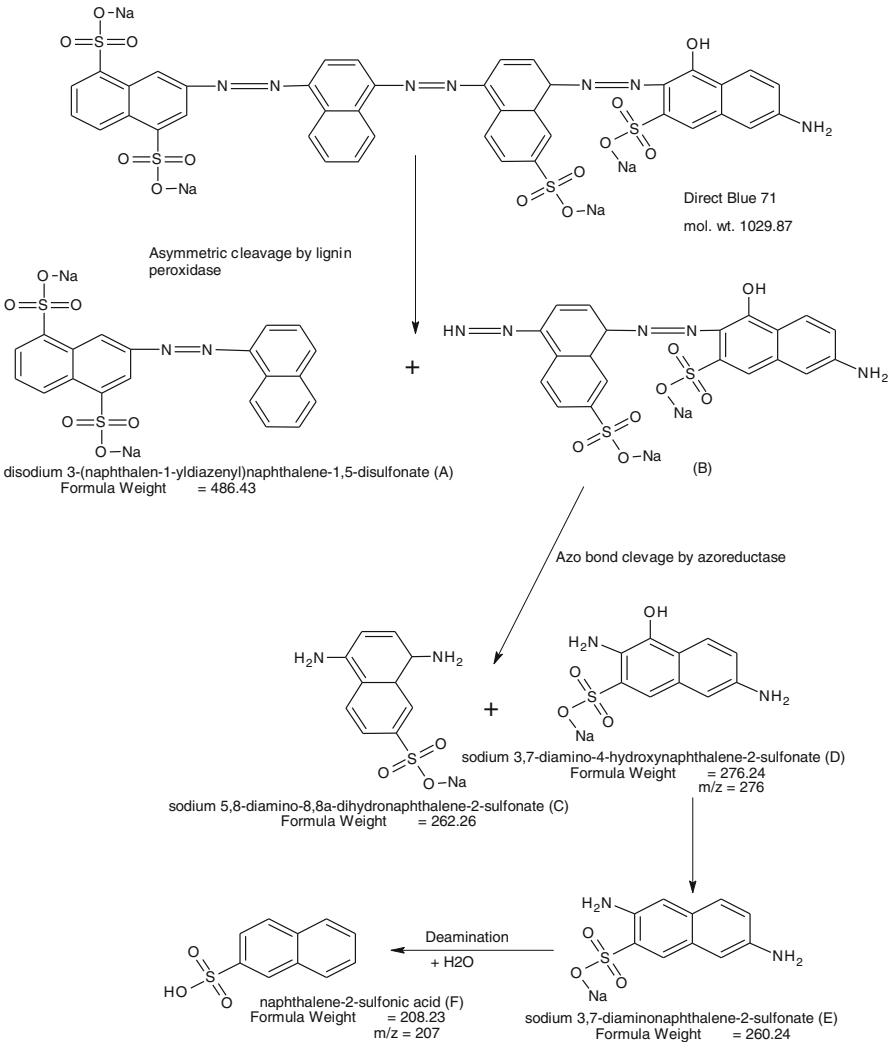

Fig. 15.9 Proposed phytodegradation pathway of DB71

development of hairy roots along with optimization of all the parameters necessary for scale up in a bioreactor. For these conceptual twin objectives, hairy roots stand out superior among other biological sources. Plants have an inherent potential to extract and metabolize contaminants and their cooperation with rhizospheric microorganisms enhance the removal of contaminants from the environment. As HRs are amenable to genetic transformation, transgenic approaches may be used to study candidate genes that affect the removal of contaminants. Genes from numerous microbial systems which have been demonstrated to detoxify the toxic molecules effectively can be isolated and cloned and such genes can be integrated into hairy

Table 15.4 Phytotoxicity study of DB71 and its degraded products

	Phaseolus mungo			*Triticum aestivum*		
Parameters	D/W	DB71	DD	D/W	DB71	DD
Germination (%)	90	70	80	90	60	75
Plumule (cm)	11.97 ± 0.23	8.97 ± 0.75***	11.78 ± 0.62***	11.13 ± 0.87	6.20 ± 0.3***	10.57 ± 0.76***
Radicle (cm)	9.07 ± 0.16	3.2 ± 0.2***	8.57 ± 0.66**	3.57 ± 0.31	2.17 ± 0.25***	3.27 ± 0.21***

Data were analyzed by one-way ANOVA followed by Tukey–Kramer multiple comparison test and mentioned values are the mean of n = 10 germinated seeds ± SE. Symbol in the table indicates statistical significance $^{**}p < 0.01$, $^{***}p < 0.001$

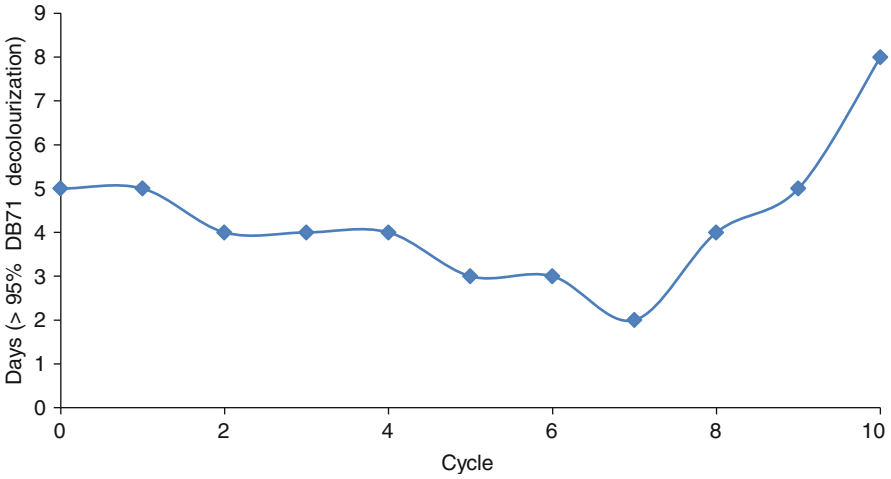

Fig. 15.10 Reuse efficiency of HRs in decolourization of DB71

root gene construct for hyper remediation of pollutants. Remarkable advances in genomics and proteomics coupled with metabolic engineering will be of a great assistance to formulate a well designed strategy for the development of hairy roots for phytoremediation relevancies.

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Chapter 16
Mass Production of *Lilium* Bulblets in Bioreactors

Mei-Lan Lian, Xuan-Chun Piao, and So-Young Park

Abstract *Lilium* is an important floricultural crop for bulb and cut flower production. Culturing *Lilium* bulblets from bulbscales is one of the main methods for vegetative propagation; however, the traditional tissue culture requires numerous vessels and considerable labour. This study evaluates the efficiency of using air-lift bioreactor systems to produce *Lilium* bulblets from bulbscale segments. The factors that affect bulblet formation and enlargement *in vitro* and specific factors that affect bulblet production in air-lift bioreactor systems were investigated. A simple method to mass produce bulblets by using a one-step bioreactor culture is indicated, and a pilot-scale bioreactor culture is introduced. The effects of storage temperature and duration on the carbohydrates and related enzymes of *in vitro*–produced bulblets are determined during breaking of dormancy. A suitable bulblet size for culturing is determined after observing the emergence of cauline leaves from different sized bulblets that are produced in solid and bioreactor cultures. Finally, an efficient method to mass produce high-quality *Lilium* bulblets is selected based on the production cost.

Keywords Bioreactor • Bulblet enlargement • Bulblet formation • Bulblet size • Bulbscale segment • Immersion culture • Ebb and flood culture • Stem leaf

Abbreviations

BA	6-Benzyladenine
BTBB	Balloon-type bubble bioreactor
DIF	Difference

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LEDs	Light emitting diodes
MS	Murashige-Skoog
NAA	α-Naphthalene acetic acid
SPS	Sucrose phosphate synthase
SS	Sucrose synthase

16.1 Introduction

Lilium is an important floricultural crop for bulb and cut flower production. At present, the vegetative propagation of lilies is realized by regenerating bulblets from excised *Lilium* bulbscales [1–8]. Bulblet culture is advantageous for the mass propagation of *Lilium* propagules because bulblets can easily be handled, transported, stored and can be pathogen free, if the culture is started with a pathogen-free material [9]. However, the commercial use of *Lilium* micropropagation is limited by high production costs that result from the high coagulant and labour costs in solid cultures; hence, liquid culture systems have been developed [7, 10].

The use of large-scale liquid culture and automation can eliminate the manual handling of the various stages of *in vitro* cultures and can decrease the production costs. Recent studies have used bioreactors to examine the mass propagation of plant tissues or organs (like plantlets, microtubers, protocorm like-bodies and rhizomes) in various plant species [11–16]. For *Lilium*, Son et al. [17] and Seon et al. [18] have studied the feasibility of using bioreactor systems for culturing *Lilium* bulblets in liquid media. Lian et al. [9, 19, 20] have systematically investigated the factors that affect bulblet formation and enlargement in bioreactors and have established a bioreactor culture system. Ahn et al. [21] used pilot bioreactor systems to produce *Lilium* bulblets. They introduced large-scale culture methods and provided solutions to existent problems.

The size of *in vitro*–produced bulblets affects the emergence of cauline leaves. Generally, the size of *in vitro*–produced bulblets must be suitable to allow the emergence of cauline leaves. Studies that involved the direct field planting of bulblets produced in solid media revealed a higher leaf emergence rate (90 %) in the large bulblets (3 g) during transplantation when compared to the smaller bulblets [22]. However, cauline leaves could exhibit a 90 % emergence rate from bioreactor-produced bulblets weighing >1.1 g [20]. Therefore, producing the bulblets of an appropriate size is important for solid and bioreactor cultures.

This chapter describes the medium factors [plant regulators, nitrogen concentration, Murashige and Skoog (MS) [23] medium strength and sucrose] and microenvironmental factors (temperature and light) which affect the formation and growth of *Lilium* bulblets *in vitro*. Then, we indicate the factors that affect bulblet production in air-lift bioreactor systems. A simple method to mass produce bulblets by using a one-step bioreactor culture is indicated, and a pilot-scale bioreactor culture is introduced. The effects of storage temperature and duration during breaking of dormancy on the carbohydrates and related enzymes of *in*

vitro–produced bulblets are determined. A suitable bulblet size for culturing is determined after observing the emergence of cauline leaves from different sized bulblets that are produced in solid and bioreactor cultures. Finally, an efficient method to mass produce high-quality *Lilium* bulblets is selected by calculating the production cost.

16.2 Culture Medium

The optimal growth and morphogenesis of plant tissues may vary among different plants according to their nutritional requirements. A plant tissue culture medium generally contains macronutrients, micronutrients, vitamins, amino acids or nitrogen supplements, a carbon source, undefined organic supplements, growth regulators and solidifying agents. The optimum concentration of each nutrient for achieving maximum growth rate varies among different plant species. For bulblet formation and enlargement, the suitable culture medium is slightly different among *Lilium* species, cultivars and hybrids. Therefore, culture medium components should be modulated to optimize *in vitro Lilium* culture systems. For large-scale production, the culture medium is generally screened in smaller vessels by using solid or liquid medium. Hence, factors (nitrogen, medium strength, plant growth regulators and sugar) that affect *Lilium* bulblet formation and enlargement should be verified in advance. Moreover, the culture medium should be optimized for the subsequent large-scale bioreactor culture.

16.2.1 MS Medium

Minerals are important components of the culture medium. MS medium is commonly used because most plant cultures favourably react to it. MS is a high salt containing medium with high levels of nitrogen, potassium and some micronutrients [24]. However, this nutrient medium is not always optimal for the growth and development of explants *in vitro* because of its high salt content.

The strength of MS medium affects plant organogenesis during micropropagation [25, 26]. The fitted MS strength differs among *Lilium* species, cultivars and hybrids. In *Lilium* bulblet production, bulblet formation is favoured when the strength of MS medium is increased to two fold in the bulbscale segment culture of *Lilium auratum* Lindl. [27]. However, the strength of MS medium does not affect bulblet formation in the Oriental hybrid ‘Casa Blanca’. By comparison, 1/2-strength MS medium supports the highest bulblet formation in the Asiatic hybrid, ‘Mona’ and the *longiflorum* hybrid, ‘Hinomoto’ [28]. For *Lilium* bulblet growth, many cultivars or hybrids are promoted by 2-strength MS medium [27, 28].

In addition, MS medium contains high amounts of nitrogen, with a high ratio of NO_3^-/NH_4^+. However, the plant growth is affected not only by nitrogen concentration

but also by forms of nitrogen. We observed that the MS medium with a low nitrogen amount (30 mM) can effectively support *Lilium* bulblet formation in 'Casa Blanca', 'Mona', and 'Hinotomo'. The optimum bulblet growth was obtained at 120 mM nitrogen in 'Casa Blanca' and 'Hinomoto', whereas nitrogen concentration elicited no significant effect on bulblet growth in 'Mona' [28].

16.2.2 Plant Growth Regulators

Plant growth regulators are chemicals applied to plant tissue cultures to regulate culture growth. Different plant growth regulators affect different plant processes. Auxins and cytokinins are by far the most important for regulating growth and morphogenesis in plant tissue and organ cultures; synthetic regulators have biological activities that equal or exceed those of their natural counterparts. *Lilium* bulblets easily form from the bulbscales of field-cultivated bulbs or *in vitro*–cultured bulblets. This finding can be attributed to the good organogenesis ability or essential substances in bulbscales. 6-benzyl adenine (BA) generally promotes bulblet primordium differentiation but inhibits bulblet development during *Lilium* bulbscale segment culture. Low auxin amounts are sufficient for bulblet formation, whereas cytokinins do not elicit the same effect [29, 30]. A culture medium supplemented with a low amount of auxins or without any plant growth regulator promotes the enlargement of *Lilium* bulblets *in vitro* [31]. A culture medium supplemented with a low amount of auxins or without any plant growth regulator promotes the enlargement of *Lilium* bulblets *in vitro* [29]. In *Lilium* hybrids 'Casa Blanca', 'Mona', and 'Hinomoto', a medium supplemented with a mixture of 0.3 mg L^{-1} α-naphthalene acetic acid (NAA) and 1.0 mg L^{-1} BA effectively and rapidly induces bulblet formation when compared with a medium supplemented with either BA or NAA and without any plant growth regulators. However, the bulblet formation rate does not vary among the cultivars after 8 weeks of culture in a medium with or without BA or NAA [32].

16.2.3 Sugar

Most plant tissues require an exogenous source of carbohydrates because they do not efficiently photosynthesize. The optimum sugar concentration may vary with the stages of differentiation in the culture. Sucrose is the principal carbon source in plant tissue culture; it is involved not only in metabolism-related organogenesis and respiration but also in osmoregulation [33]. During *Lilium* bulblet enlargement, sucrose is more crucial in regulating osmotic pressure than in being a carbon source in metabolism [34]. Low sucrose concentrations benefit bulblet formation, whereas high sucrose concentrations promote bulblet enlargement. Takayama and Misawa [27] indicated that large *Lilium* bulblets can be obtained by increasing the sucrose concentration in the medium; they obtained the maximum bulblet biomass after

supplying the culture medium with 90 to 120 g L^{-1} of sucrose. However, they also found that high sucrose concentrations delay bulblet formation. Nevertheless, a high sucrose concentration (90 g L^{-1}) should be added to the culture medium during long-term bulbscale segment culture (>4 weeks) to promote bulblet formation and enlargement because a low initial sucrose concentration (30 g L^{-1}) will be depleted after 2–3 weeks of culture [18].

16.3 Culture Environment

The maximum growth and development of cultures *in vitro* are determined by their genes. However, their actual rates are limited by microenvironmental factors, including temperature, light, humidity and carbon dioxide. Controlling these environmental factors can promote the growth and development of cultures. Therefore, an appropriate microenvironment must be established to allow cultures to exhibit their hereditary characteristics in a highly efficient and stable manner.

16.3.1 Temperature

Air temperature is the most important environmental factor that affects *Lilium* bulblet formation and enlargement. Previous studies suggested that 25 °C is the optimum temperature for the bulblet formation and growth of many *Lilium* species, cultivars and hybrids such as *Lilium rubellum* [10], 'Casa Blanca' and 'Mona' [35], and *Lilium longiflorum* 'Ace' and 'Nellie White' [36, 37]. Meanwhile, a high temperature of 30 °C suppresses bulblet formation and growth by increasing the respiration rather than the assimilation of sugar by bulblets.

The air temperature mentioned above remains constant throughout the day. However, the culture growth and morphology require the air temperature in culture vessels during the photoperiod at normal light intensities to be slightly higher than that during the dark period in many cases. *Lilium* bulblet formation and growth are also affected by day and night temperature differences (DIF). Lian et al. [35] determined that zero DIF and negative DIF (−7) promote the bulblet formation of 'Casa Blanca' and 'Hinomoto' but do not affect that of 'Mona'. During the bulbing stage, bulblet growth is favourable at +7 DIF for 'Casa Blanca', at zero DIF for 'Mona', and at −7 DIF for 'Hinomoto'.

16.3.2 Light

Light (spectral quality, photon flux and photoperiod) is another environmental factor that affects the overall growth and development of cultures *in vitro* [38, 39]. The results of light experiments on *Lilium* bulblet formation vary among the various

research reports. Maesato et al. [40] stated that continuous illumination during culture stimulates the bulblet production of *Lilium japonicum.* By contrast, Stimart and Ascher [30] found that continuous darkness increases the size and number for *Lilium longiflorum* bulblets. In our study, we investigated the effect of light intensity on *Lilium* bulblet formation and growth *in vitro* and found that light promoted bulblet formation in 'Casa Blanca' and 'Hinomoto' but did not affect that in 'Mona'. During the bulbing stage, light induced better bulblet growth of 'Casa Blanca' and 'Mona' when compared with the darkness. Bulblet growth in 'Hinomoto' increased at high light intensity. We also examined the effects of lighting conditions on *Lilium* 'Casa Blanca' bulblet formation in an ebb and flood bioreactor culture system and found that the bulblet formation rate increased under 16 h illumination (30 μmol m^{-2} s^{-1}) than under 24 h darkness. However, the number of bulblets formed per explant was not affected by lighting conditions. A similar response was observed by Varshney et al. [8] during the *in vitro* mass propagation of a *Lilium* Asiatic hybrid. Such variations in potential regeneration under different lighting conditions can be attributed to the physiological status of the material (cultivar, age, culture time, etc.).

Fluorescent tubes or lamps with spectral emission wavelengths ranging from 350 to 750 nm are the principal light sources for maintaining tissue cultures [41]. Recently, light emitting diodes (LEDs) have arisen as an alternative light source for plant culture systems [42–45]. LED lighting systems have several unique advantages including small size, durability, long operating lifetime, wavelength specificity, relatively cool emitting surfaces, photon output, and the capacity to control spectral composition that is linear with the electrical input current. In addition, blue and red LEDs have been used to study photosynthesis [46], chlorophyll synthesis [47] and morphogenesis [48]. The formation of *Lilium* bulblets was better under LED treatments than under dark conditions. The number of bulblets increased under blue + red LEDs and fluorescent lamps. Bulblet fresh weight was promoted by fluorescent lamp treatment in 'Casa Blanca' and 'Hinomoto'. In 'Mona', LEDs and darkness did not affect bulblet formation, and bulblet fresh weight peaked after fluorescent lamp treatment.

16.4 Bulblet Formation in Bioreactors

Studies have reported that bioreactors can be used to enlarge *in vitro*–induced *Lilium* bulblets [9, 17–20]. However, information on *Lilium* bulblet formation using bioreactor systems is limited [9]. During the formation of *Lilium* bulblets from bulbscale segments in solid cultures, explant incisions gradually dry up with the time, after which bulblets start to regenerate. However, the most explants in liquid immersion culture systems are brown and swollen, and it is difficult to induce bulblets from the explants. Therefore, a special method to induce bulblet formation is needed for liquid bioreactor cultures. Accordingly, the present study introduces a new bioreactor culture method for bulblet formation and recognizes the factors affecting this system.

16.4.1 Culture Method

The morphology of microcultures in liquid media or in closed vessels is generally restricted because of excess ethylene production from the cultures at a high relative humidity. Hence, a viable and efficient method of *Lilium* bulblet formation in bioreactors should be explored. *Lilium* bulblet formation varies in solid and liquid cultures. Thus, we have designed four experimental groups, namely, solid culture, agitated flask liquid culture, bioreactor immersion, and ebb and flood liquid culture. 'Casa Blanca' bulbscale segments (1 mm × 1 mm) were inoculated into four culture systems. The culture medium used was MS medium supplemented with 0.3 mg L^{-1} of NAA, 1 mg L^{-1} of BA, and 30 g L^{-1} of sucrose. For the bioreactor culture, a 5 L balloon-type bubble bioreactor (BTBB) with 1 L of culture medium was used, and the air volume was adjusted to 0.1 vvm (air volume/culture volume, min). In the immersion-type system, the bulblets were submerged in liquid during the entire period. In the ebb and flood liquid culture, the bulblets were immersed into the medium for 0.3 h and then dried for 5.7 h using a timer and a solenoid valve. All cultures were maintained at 25 °C for a 16 h photoperiod (light intensity of 40 μmol m^{-2} s^{-1}). After 4 weeks of culture, the bulblet formation was sufficient in the solid culture, whereas no bulblet formation was observed in the agitated flask liquid culture and immersion-type bioreactor cultures. This result indicates that immersing the entire bulbscale segments into the liquid medium inhibits the bulblet formation. The ebb and flood bioreactor system, which works on the principle of temporary immersion, facilitated a constant supply of nutrients and aeration to the explants and promoted the formation of bulblets (Fig. 16.1). Although the percentage of bulblet formation was lower in the ebb and flood system (51.7 %) than in solid culture system, we harvested over 1,000 bulblets from the bioreactor system.

16.4.2 Temporary Immersion Cycles

The ebb and flood bioreactor culture method involves temporarily wetting the entire culture or plant tissue with nutrient medium, followed by draining the excess nutrient medium under gravity. This method has been used to micropropagate many plant species [49–54]. Similar to other bioreactor culture types, the ebb and flood culture method of plant micropropagation is affected by many factors such as culture medium, aeration, inoculation density, temperature, and light intensity. The ebb and flood bioreactor culture is particularly influenced by the temporary immersion cycle.

Modulating the temporary immersion cycle can improve *Lilium* bulblet formation. We examined the effects of the number (4, 6 and 8 times per day for 30 min each) and duration (4 times per day for 15, 30, 60 or 120 min) of medium supply on bulblet formation during ebb and flood bioreactor culture. The highest percentage of bulblet formation (75.8 %) was observed when the medium was supplied 4 times per day for 15 min, with the efficiency of bulblet formation nearly similar to that of

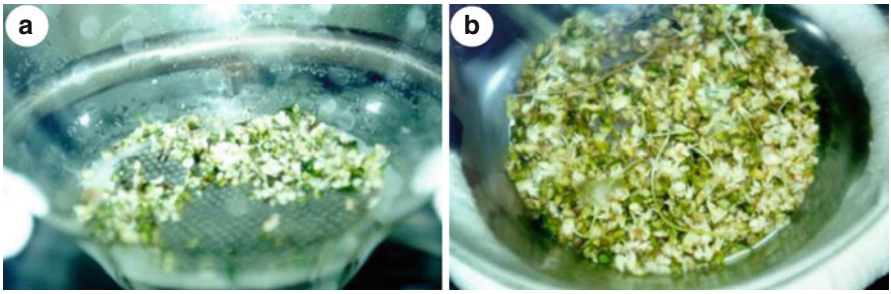

Fig. 16.1 Bulblet formation of *Lilium* 'Casa Blanca' in ebb and flood bioreactor system (**a**), initial inoculated bulbscale segments (**b**), right, bulblets formed from bulbscale segments after 4 weeks of culture

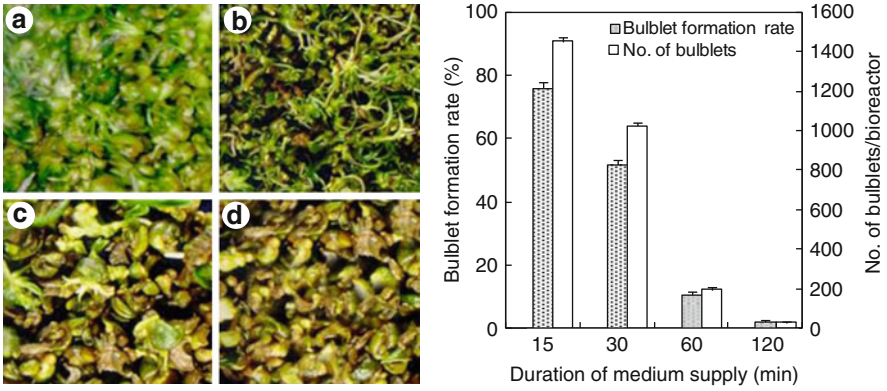

Fig. 16.2 Effects of duration of medium supply on bulblet formation in scale segment culture of *Lilium* 'Casa Blanca' after 4 weeks of culture in ebb and flood type bioreactor (**a**) 15 min, (**b**) 30 min, (**c**) 60 min, (**d**) 120 min

the solid culture. The percentage of bulblet formation and number of bulblets per bioreactor decreased when the medium was supplemented for more than 15 min. Immersion of the bulbscale segments in the liquid medium for a longer time inhibited the bulblet formation (Fig. 16.2). This result may be related to the fact that by increasing the surface contact of the bulbscales with air stimulates the formation of bulblets during bioreactor culture.

16.4.3 Cytokinins and Auxins in Culture Medium

Cytokinin or auxin concentration can control the quantity and morphology of *Lilium* bulblets being formed. Tufty and formless bulblets are often observed in culture media supplemented with high BA amounts; this finding proves again that BA promotes bulblet primordium differentiation but inhibits bulblet development [55]. The bulblets

formed in culture media with low NAA amounts display visible morphology and proper development. We examined the effects of BA and NAA concentrations on bulblet formation and further development in the ebb and flood bioreactor system. 'Casa Blanca' bulblets were immersed into the medium 4 times per day for 15 min each. The culture medium was supplied with a mixture of 0.3 mg L^{-1} NAA and 1.0 mg L^{-1} BA or without any plant growth regulators. More bulblets were formed in the medium supplemented with 0.3 mg L^{-1} NAA and 1.0 mg L^{-1} BA than in the medium without plant growth regulators. However, numerous abnormal bulblets were observed when the bulblets that were formed in the medium supplemented with 0.3 mg L^{-1} NAA and 1.0 mg L^{-1} BA were cultured in a medium with 90 g L^{-1} sucrose but without growth regulators for 8 weeks. Conversely, the bulblets that were initiated in the medium without growth regulators showed no signs of abnormality even after 16 weeks of bulbing. The addition of growth regulators may cause morphological abnormalities and hyperhydricity [56]. On the basis of the results showing the lower survival rates of the abnormal bulblets during transplantation, we recommend the use of medium without growth regulators for the formation of *Lilium* bulblets in bioreactors.

16.5 Bulblet Enlargement in Bioreactors

Recently, the mass propagation of storage organs in bioreactors has been examined in several plant species, including lilies. Factors such as medium renewal, sucrose concentration and bioreactor type influence the growth of *Lilium* bulblets in bioreactor cultures. We explored the optimum culture conditions during bioreactor culture to obtain the mass and high-quality *Lilium* bulblets.

16.5.1 Bioreactor Culture Type

Bulblet development is affected by different bioreactor culture methods. We used two types of bioreactors (immersion and temporary immersion liquid culture using ebb and flood; Fig. 16.3) to culture 'Casa Blanca' bulblets. A total of 200 *in vitro*–cultured bulblets each weighing 0.1 g were transferred to a 5 L BTBB containing 4 L of MS liquid medium supplemented with 90 g L^{-1} of sucrose. The pH of the medium was adjusted to 5.8 before autoclaving at 121 °C and 1.2 kg cm^{-2} pressure for 40 min. The air volume was adjusted to 0.1 vvm. The bulblets in the immersion-type bioreactors were submerged into the liquid medium during the entire culture period. Meanwhile, the bulblets in the ebb and flood system were immersed into the medium for 60 min and then dried for 30 min using a timer and a solenoid valve. The bioreactors were maintained at 25 °C in the dark for 16 weeks before harvesting. After 16 weeks of culture, the bulblets cultured under the ebb and flood system showed lower growth rates when compared with those cultured under the immersion system (Fig. 16.4). The total number of large bulblets also increased in the

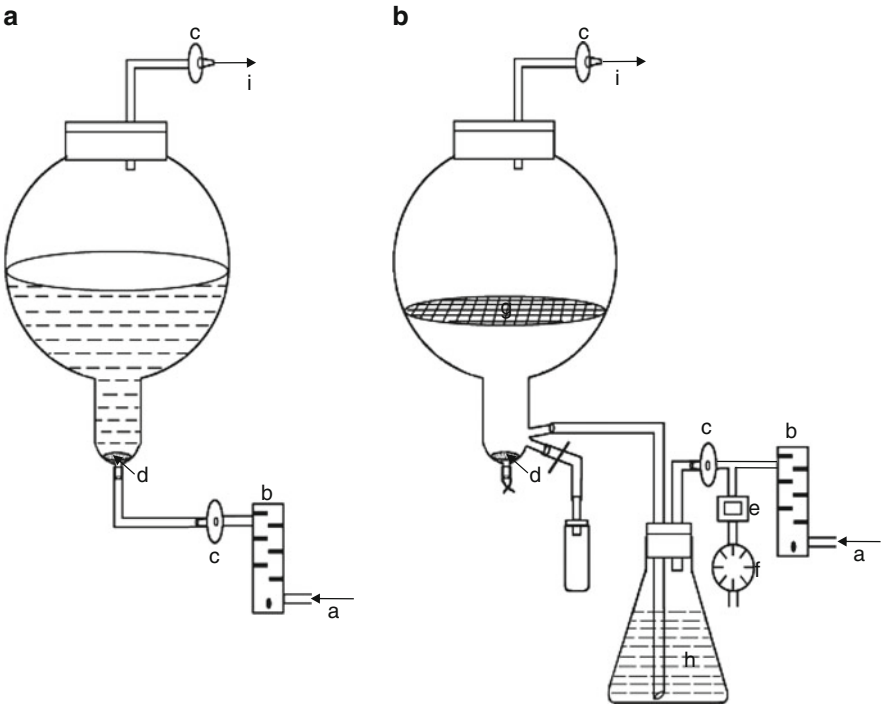

Fig. 16.3 Schematic diagram of immersion (**a**) and ebb and flood (**b**) bioreactor system. (*a*) air inlet, (*b*) air flow meter, (*c*) membrane filter, (*d*) glass sparger, (*e*) solenoid valve, (*f*) timer, (*g*) supporter (net), (*h*) medium reservoir, (*i*) air outlet

immersion-type bioreactor. This result indicates that the immersion system permits an efficient nutrient uptake, whereas the ebb and flood system allows aeration but not the complete surface contact with the nutrient medium. Hyperhydricity is often presented in an immersion system, but it has never been reported in lily liquid culture for the bulblet enlargement. In addition, it is not present in our bioreactor culture system. Immersion culture systems can be easily assembled and they are simpler when compared with the ebb and flood system. Therefore, the former is suitable for *Lilium* bulblet enlargement.

16.5.2 Methods of Medium Supply in Immersion Bioreactor Culture

A long-term culture characterizes the *in vitro* bulblet enlargement of *Lilium*. Approximately 0.1 g of bulblet inoculum must be subcultured at least 3 times for nearly half a year using the traditional solid medium to obtain approximately 3 g of bulblets. However, in bioreactors the entire culture period can be completed by

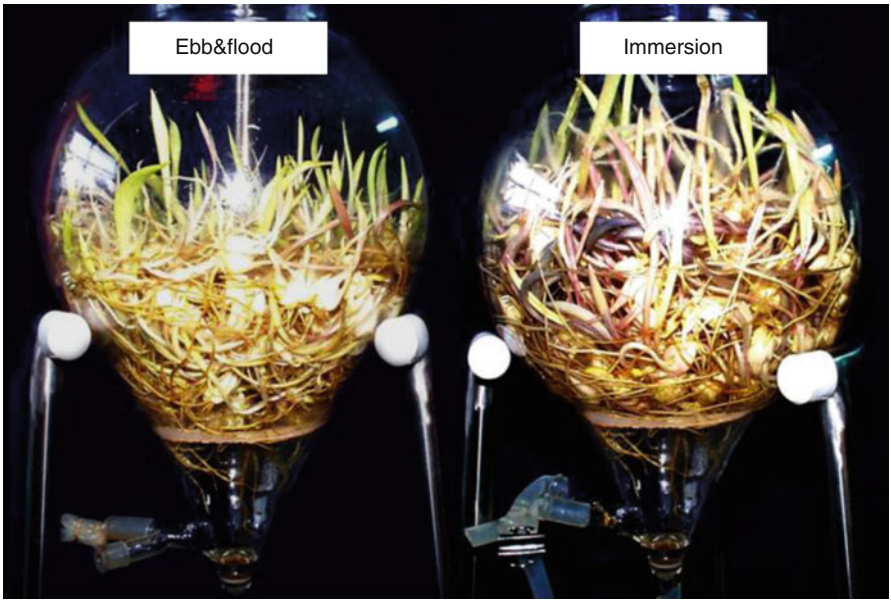

Fig. 16.4 Bulblet growth of *Lilium* Oriental hybrid 'Casa Blanca' in ebb and flood and immersion bioreactor system after 16 weeks of culture

Table 16.1 Effect of culture method on bulblet growth and distribution of bulblet size in bulblet culture of *Lilium* Oriental hybrid 'Casa Blanca' after 16 weeks in bioreactor

Methods of medium supply	No. of different size bulblets/bioreactor			Bulblet weight (g/bioreactor)	
	≤1.0 g	1.1–2.0 g	≥2.1 g	Fresh	Dry
Addition	61.2 a[a]	94.6 a	45.4 b	291.5 b	92.0 b
Exchange	40.0 b	65.1 b	95.7 a	425.0 a	135.5 a

[a]Mean separation by Duncan's multiple range test at $P \leq 0.05$

medium renewal, which avoids the need for subculture for *Lilium* bulblet production. We examined the two methods of medium renewal, namely, medium exchange and medium addition. For medium exchange, 200 bulblets (0.1 g) were inoculated using the immersion bioreactor system filled with 1 L of MS medium supplemented with 30 g L^{-1} of sucrose at initial culture. After 2, 6 and 12 weeks of bioreactor culture, the old medium was replaced with 2, 3 and 4 L of fresh medium (MS + 90 g L^{-1} sucrose). For medium addition, 1 L of MS medium supplemented with 30 g L^{-1} of sucrose was used when the bulblets were inoculated. An aliquot of 1 L fresh medium (MS + 90 g L^{-1} sucrose) was added after 2, 6 and 12 weeks of culture. Bulblet biomass significantly increased when the medium was replaced with fresh medium after 16 weeks of bioreactor culture (Table 16.1). The enhanced bulblet growth by the medium replacement method was due to an increase in number of large bulblets; 95 bulblets heavier than 2.1 g were produced (i.e. nearly 50 % of inoculated explants produced bulblets ≥2.1 g). This result is nearly a 2.0-fold improvement compared

with the medium addition method, which yielded 45 bulblets ≥2.1 g. On the basis of this experiment, we conclude that an appropriate medium supply is essential for enhancing the bulblet growth, which is a key criterion to achieve large sized bulblets.

16.5.3 Frequency of Medium Exchange

Well-grown bulblets can be obtained by the medium replacement method, but the frequency of medium replacement is also an important parameter to optimize the culture system. To determine the optimum number of medium replacement, we designed four medium replacement levels using a 5 L BTBB with MS liquid medium supplemented with 90 g L^{-1} of sucrose: 0 (4 L of medium was used without medium replacement during the entire period); 1 (2 L of medium was used upon inoculation of the bulblets and was replaced with 4 L of fresh medium after 8 weeks of culture); 2 (1 L of medium during inoculation replaced with 2 and 4 L of medium after 4 and 12 weeks of culture respectively); and 3 (1 L of medium during inoculation, replaced with 2, 3 and 4 L of medium after 4, 8 and 12 weeks of culture, respectively). After 16 weeks of culture, minimal bulblet growth was observed in the no- replacement and one-time medium replacement conditions. Meanwhile, the rate of bulblet growth increased in the 2 and 3 medium replacement levels. The distribution of bulblet size also indicates a strong influence of medium replacement on individual bulblet growth. The highest number of large bulblets (≥2.1 g) was achieved after 16 weeks of culture in an immersion system with 2 or 3 times medium renewal (Table 16.2). This result indicates that 2 times medium replacement in the immersion culture system of BTBB is an appropriate method for obtaining abundant large sized *Lilium* bulblets.

16.5.4 Sugar in Culture Medium

In most bulbous plants, sucrose is converted into starch and is stored in bulbscales, which is the tissue responsible for storage. The available carbohydrates, particularly sucrose, in the medium are responsible for increasing the weight of bulblets. Apart from sucrose being a suitable carbon source for easy assimilation and conversion into starch to develop the bulblets, it also provides a favourable osmolarity for bulblet development [57]. We already mentioned the effects of sugar on *Lilium* bulblet culture and indicated that sugar critically affects the bulblet formation and enlargement *in vitro*. To confirm the influence of sugar on bulblet enlargement in bioreactors, we cultured 'Casa Blanca' bulblets in a 5 L BTBB immersion culture system that contains 4 L of MS culture medium supplemented with different concentrations of sucrose alone (30, 60, and 90 g L^{-1}) or in combination with mannitol (30 g L^{-1} sucrose + 32 g L^{-1} mannitol, 60 g L^{-1} sucrose + 16 g L^{-1} mannitol). Most bulblets in the medium supplemented with 30 g L^{-1} of sucrose grew to a small to medium size

Table 16.2 Effect of medium exchange numbers on bulblet growth and distribution of bulblet size in bulblet culture of *Lilium* Oriental hybrid 'Casa Blanca' after 16 weeks in bioreactor

No. of medium exchanges[a]	No. of different size bulblets/bioreactor			Bulblet weight (g/bioreactor)	
	≤1.0 g	1.1–2.0 g	≥2.1 g	Fresh	Dry
0	62.5 a[b]	126.1 a	4.6 c	224.0 b	68.0 b
1	45.5 b	113.9 a	40.5 b	276.5 b	86.5 b
2	14.7 c	55.5 b	129.5 a	529.0 a	170.0 a
3	15.1 c	50.5 b	136.5 a	578.5 a	186.5 a

[a]0: No medium exchange, 1: exchanged medium after 8 weeks of culture, 2: exchanged medium after 4 weeks and 12 weeks of culture, 3: exchanged medium after 4, 8 weeks and 12 weeks of culture
[b]Mean separation by Duncan's multiple range test at $P \leq 0.05$

(≤2.1 g) after 16 weeks of culture; meanwhile, large bulblets were found in the medium supplemented with 90 g L^{-1} of sucrose. The medium supplemented with a mixture of sucrose and mannitol did not influence bulblet growth during bioreactor culture. This result agrees with the aforementioned finding in solid culture that high sucrose concentrations promote *Lilium* bulblet growth. Based on the findings that the presence of large bulblets indicates a high survival rate during transplantation, we recommend using 90 g L^{-1} of sucrose for *Lilium* bulblet production during bioreactor culture.

16.5.5 Inoculation Density

Inoculation density is a relevant physical parameter that influences the culture growth during micropropagation [58]. The ratio of explant number to media volume affects the proliferation rate [59]. Many studies have proven through micropropagation systems that the appropriate initial explant number depends on the plant species, organs and culture methods [60–62]. Plant micropropagation is used to obtain the maximum number of healthy cultures from a small inoculum quantity [63]. We inoculated 100, 200, 300 and 400 bulblets (0.1 g) into a 5 L BTBB with 4 L of MS medium supplemented with 90 g L^{-1} of sucrose and then cultured them for 16 weeks. At densities of 100 and 200, more number of larger bulblets (≥2.1 g) were produced than the smaller bulblets in the bioreactor. However, these two densities were inadvisable because of the limited total bulblet number in the bioreactor. The total number of bulblets larger than 2.1 g peaked at the inoculation density of 300 (Fig. 16.5). In practice, producing large *Lilium* bulblets *in vitro* is not advisable because it requires high labour and production costs. Therefore, cultivating *in vitro*–produced bulblets in the field to promote bulblet enlargement is an efficient approach for the commercial production of high-quality *Lilium* bulblets. *Lilium* bulblets heavier than 3 g produced in solid medium [22] or 1.1 g produced in liquid medium [20] can form cauline leaves. Accordingly, bulblets of ≥1.1 g are considered as an appropriate size for bioreactor liquid cultures. Most bulblets were heavier than 1.1 g at the inoculation density of

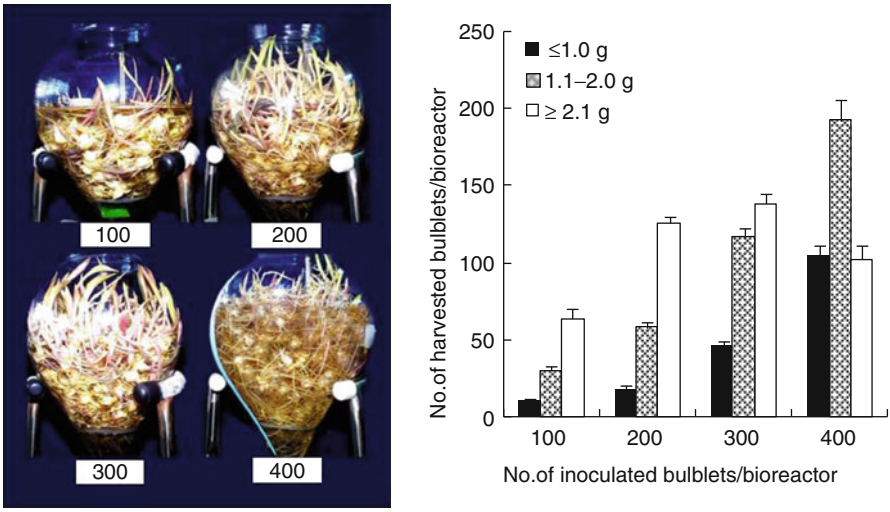

Fig. 16.5 Effect of inoculation density on bulblet growth and distribution of bulblet size in bulblet culture of *Lilium* Oriental hybrid 'Casa Blanca' after 16 weeks of bioreactor culture

300. Thus, we suggest that the inoculation density of 300 is suitable for *Lilium* bulblet production using a 5 L BTBB with a 4 L working volume.

16.5.6 Kinetics of Nutrient Uptake in Medium

The medium components dynamically changed in the bioreactor culture of *Lilium* bulblets. We inoculated 200 'Casa Blanca' bulblets (0.1 g) into a 5 L BTBB with 1 L of MS liquid medium supplemented with 30 g L^{-1} of sucrose at initial culture. After 2, 6 and 12 weeks of bioreactor culture, the old medium was replaced with 2, 3 and 4 L of fresh medium (MS + 90 g L^{-1} sucrose) respectively. The bioreactor was aerated at 0.1 vvm. The medium samples were collected after every 2 weeks of bioreactor culture, and the sugar, ion contents as well as the pH level were measured.

Glucose and fructose were determined in the culture medium, although only sucrose was added into the original medium. The presence of these sugars may be due to the invertase secretion from *Lilium* bulblets into the culture medium or onto the epidermal cell surface of the tissue, resulting in the hydrolysis of extracellular sucrose into glucose and fructose [64, 65]. The sucrose was rapidly hydrolyzed into glucose and fructose, and almost equal amounts of glucose and fructose were measured in the medium (Fig. 16.6). This result indicates equal utilization of these reducing sugars. We observed that a high sucrose concentration was maintained in the medium. This result proves that sucrose is not only a suitable carbon source for easy assimilation and conversion into starch but also it maintains a favourable osmolarity in bulblet development [57].

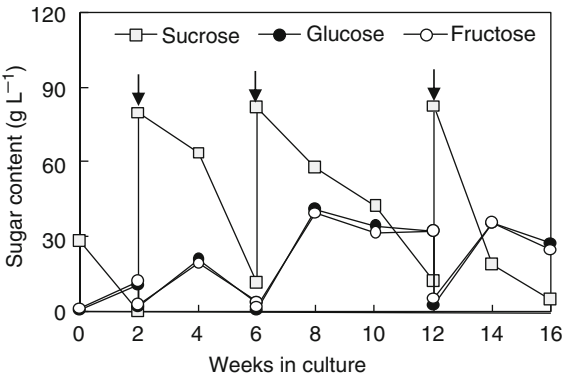

Fig. 16.6 Changes in sucrose, glucose, and fructose content in the culture medium during the bioreactor culture of *Lilium* 'Casa Blanca' bulblets. *Arrow* indicates the time of medium exchange

The kinetics of change in ion concentration showed three approaches in mineral absorption (Fig. 16.7): a rapid depletion of NH_4^+ and $H_2PO_4^-$, a steady absorption rate for NO_3^- and SO_4^{2-}; and a slow consumption of K^+, Mg^{2+}, Ca^{2+}, and Cl^- in the medium during bulblet growth. The depletion of a particular nutrient from the medium cannot be used as an indicator of imminent nutrient deficiency. However, the availability of $H_2PO_4^-$ and NH_4^+ is correlated with the bulblet growth.

A rapid decrease in pH was also observed after the exchange with fresh medium during bulblet growth (Fig. 16.8). The acidic pH of the culture medium may be attributed to the rapid uptake of NH_4^+, which led to the efflux of protons during NH_4^+ absorption. The apparent relationship between nitrogen absorption and pH change demonstrates depletion of ammonium in the medium. Based on these results, we speculate that the uniform availability of NH_4^+ and $H_2PO_4^-$ ions as well as the high concentration of sucrose primarily functions in achieving optimum bulblet growth.

16.6 One-Step Bioreactor Culture

A separate method for bulblet formation and enlargement was described using the bioreactors that are mentioned in previous sections. The production cost can be reduced and the culture procedure can be simplified by applying the one-step bioreactor culture technique in *Lilium* bulblet production. One-step culture requires the completion of the two stages of bulblet formation and enlargement in a single bioreactor; this technique will become the most cost-efficient method in the industrial production of *Lilium* bulblets although it has not been optimized at present. The one-step culture system (Fig. 16.9) and its procedures are given as follows:

(1) Addition of medium: An aliquot of 6 L of the medium for bulblet enlargement (MS + 90 g L^{-1} sucrose) is added into a 5 L BTBB bioreactor through the bioreactor cap. Valve g3 is opened, the medium flows down to reservoir bottle d1 (the medium will be used for bulblet enlargement), and valve d3 is reclosed. Then, 1 L of the medium for bulblet formation (MS + 30 g L^{-1} sucrose) is added into the bioreactor

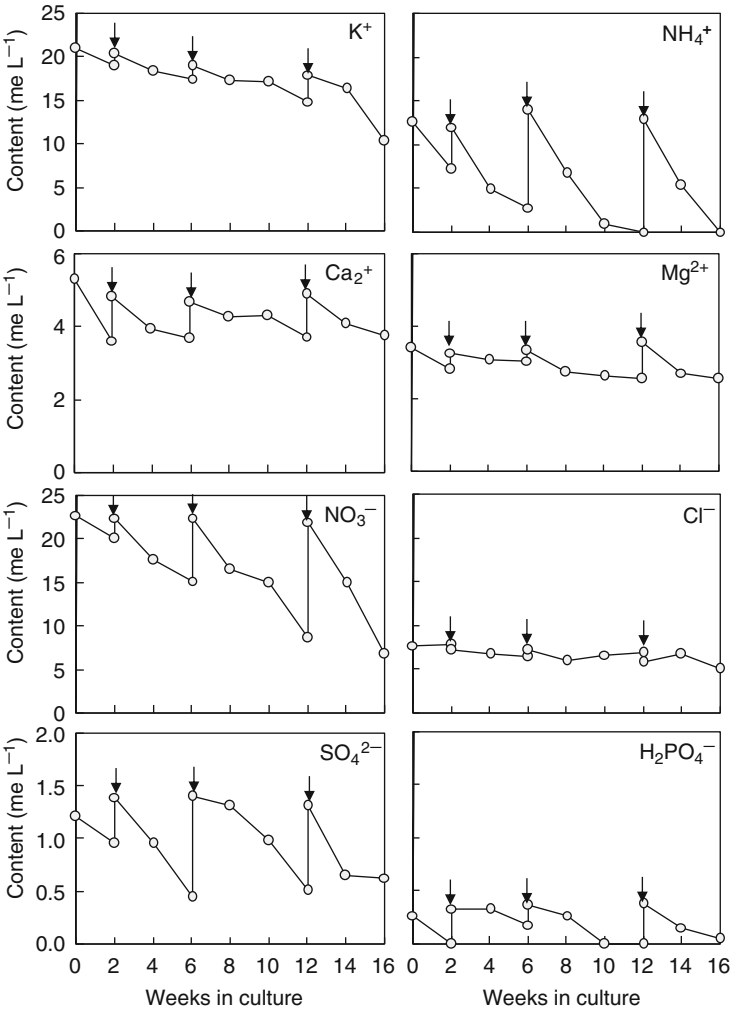

Fig. 16.7 Changes of cation and anion content in the culture medium during the bioreactor culture of *Lilium* 'Casa Blanca' bulblets. *Arrow* indicates the time of medium exchange

through the bioreactor cap, and valve g2 is opened. (2) Ebb and flood culture for bulblet formation. 'Casa Blanca' *in vitro*–cultured bulblets (approximately 2 g) are randomly cut into 1 mm × 1 mm segments. Approximately 17 g of the total bulblets is inoculated into the bioreactor. The ebb and flood system is programmed to immerse the bulbscale segments into the medium 4 times per day for 15 min each. The bioreactor is maintained at 25 °C in a 16 h photoperiod (light intensity of 30 μmol m^{-2} s^{-1}). After 30 days of culture, each explant segment can form one to two bulblets with good morphology (Fig. 16.10a). At this time, the ebb and flood culture can be stopped; meanwhile, valve g2 is reclosed after the medium in the bioreactor has

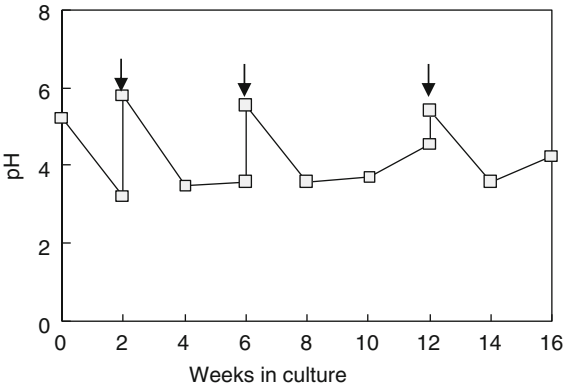

Fig. 16.8 Changes of pH in the medium during the bioreactor culture of *Lilium* 'Casa Blanca' bulblets. *Arrow* indicates the time of medium exchange

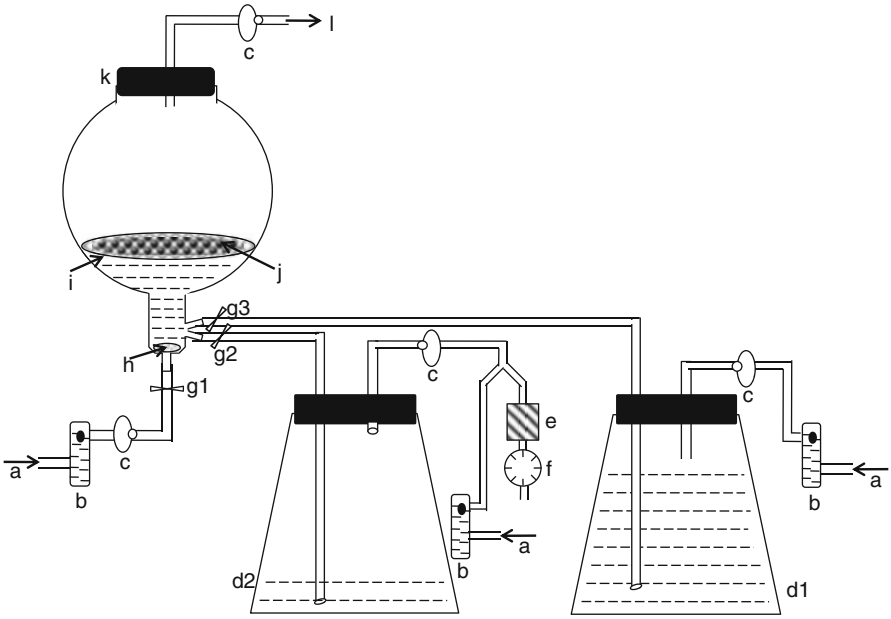

Fig. 16.9 One-step bioreactor culture system for bulblet formation. (*a*) air inlet, (*b*) air flow meter, (*c*) membrane filter, (*d*) medium reservoir, (*e*) solenoid valve, (*f*) timer, (*g*) valve, (*h*) glass sparger, (*i*) supporter (net), (*j*) induced bulblets, (*k*) cap, (*l*) air outlet

completely flowed down to bottle d2. (3) Immersion culture for bulblet enlargement. Valve g3 is opened; then, the air that is forced to bottle d1 leads the medium (MS + 90 g L^{-1} sucrose) in bottle d1 to enter the bioreactor. Aeration is immediately stopped when the medium in the bioreactor measures 1 L (a scale mark is drawn in advance on the bioreactor), and valve g3 is simultaneously reclosed. Valve g1 is opened and the bioreactor is aerated at 0.1 vvm to begin the immersion culture for bulblet enlargement under dark conditions. After 4 weeks of bulblet enlargement culture, the old medium in the bioreactor is discarded to bottle d2, and 2 L of fresh

Fig. 16.10 One-step culture of *Lilium* 'Casa Blanca' bulblet in bioreactor (**a**), the formed bulblets after 4 weeks of ebb and flood culture in MS medium supplemented with 30 g L^{-1} sucrose (**b**), bulblet enlargement after 4 weeks of immersion culture in MS medium supplemented with 90 g L^{-1} sucrose (**c**), further bulblet enlargement after 16 weeks of immersion culture in MS medium supplemented with 90 g L^{-1} sucrose (**d**), the bulblets were taken from (**c**)

medium in bottle d1 is added to the bioreactor to complete the medium replacement. Meanwhile, the bulblets grow to approximately 0.3 g in the bioreactor (Fig. 16.10b). The old medium is again replaced with 4 L of fresh medium after 8 weeks of bulblet enlargement culture. The culture will be maintained till 16th week for bulblet enlargement. Finally, bulblets (Figs. 16.10c, d) weighing an average of 2 g are harvested.

16.7 Scale Up Bulblet Production in Bioreactors

Applying the optimized culture conditions in small bioreactors to scale up culture is the ultimate goal for the industrial production of plant propagules. Ahn et al. [21] conducted a 500 L horizontal pilot-scale bioreactor culture for the formation and enlargement of 'Casa Blanca' bulblets. They set up the bioreactor with the ebb and flood system in which the inocula were immersed into the medium 8 times per day for 30 min each. In this culture system, 47.2 % of bulbscale segments formed bulblets, and the regenerated bulblets grew to an average of 1.5 g (12.9 mm diameter) after 20 weeks of culture for bulblet formation. This type of bioreactor system was also used for bulblet enlargement culture [21]. The inoculated bulblets (0.1 g) grew to an average of 2 g after 12 weeks of culture. However, several disadvantages were observed in the 500 L horizontal pilot-scale bioreactor with the ebb and flood

system. The inoculated explants (bulbscale segments or bulblets) were not placed uniformly on the supporter in the ebb and flood system. So, the inocula get accumulated in some areas of the supporter, and some areas were clear during the culture. This phenomenon may result in low bulblet formation rate and poor growth. Therefore, the design of an air sparger in the horizontal bioreactor is suggested to uniformly spray air into the bioreactor to avoid inoculum accumulation on the supporter. Ahn et al. [21] used a 100 L balloon-type air-lift bioreactor with an immersion system to produce bulblets. The inoculated *Lilium* bulblets (0.1 g) grew to an average of 3.3 g after 16 weeks of culture. These bulblets showed a favourable growth and a culture period that was evidently shorter than that in the 500 L horizontal bioreactor with the ebb and flood system. However, harvesting bulblets with this culture system is difficult because the bioreactor-grown bulblets are tightly accumulated at the bottom of the bioreactor at the later culture period. Accordingly, a high pressure pump should be set up at the bottom part of the balloon-type bioreactor for the vertical movement of the bulblets during immersion culture.

16.8 Carbohydrates and Related Enzymes in Bulblets During the Breaking of Dormancy

Plant growth, development and physical activity are temporarily stopped during dormancy [66]. The dormancy of *Lilium* starts from around the formation of storage organs to the formation of vegetative organs (leaf or stem), although the beginning of dormancy varies between cultivars or hybrids of *Lilium*. In general, *in vitro*–produced *Lilium* bulblets exhibit dormancy. The degree of dormancy is influenced by sugar content in the culture medium; deep dormancy is exhibited by bulblets cultured in media supplemented with high sugar concentrations. Culture period and temperature also affect bulblet dormancy. *In vitro*–produced bulblets should be stored at low temperatures [37] or treated with gibberellin [67] before transplantation to break the dormancy. Furthermore, the enlargement of dormancy-breaking bulblets in the field is closely associated with the size of *in vitro*-cultured bulblets and the formation of bulblets by the cauline leaves. During transplantation, physiological and biochemical changes in bulblets are noticeable and are affected by storage temperature and duration. We stored the *in vitro*–produced bulblets at 4, 10 and 25 °C for 9 weeks and then determined the changes in carbohydrates and associated enzymes in the bulblets. Storage at 4 °C stimulated starch breakdown when compared with that of other temperatures. Starch content did not change at 25 °C during the storage period (Fig. 16.11). The activities of α- and β-amylase exhibited a tendency to change starch content, and high enzyme activities were observed at 4 °C. The activity of β-amylase was considerably higher than that of α-amylase (Fig. 16.12). The activities of sucrose phosphate synthase (SPS) and sucrose synthase (SS) increased with decreasing storage temperature. The activity of SS was higher than that of SPS. With the decrease in starch content and increment of sucrose content, the activity of SS was obtained after 5 weeks of storage. Glucose content

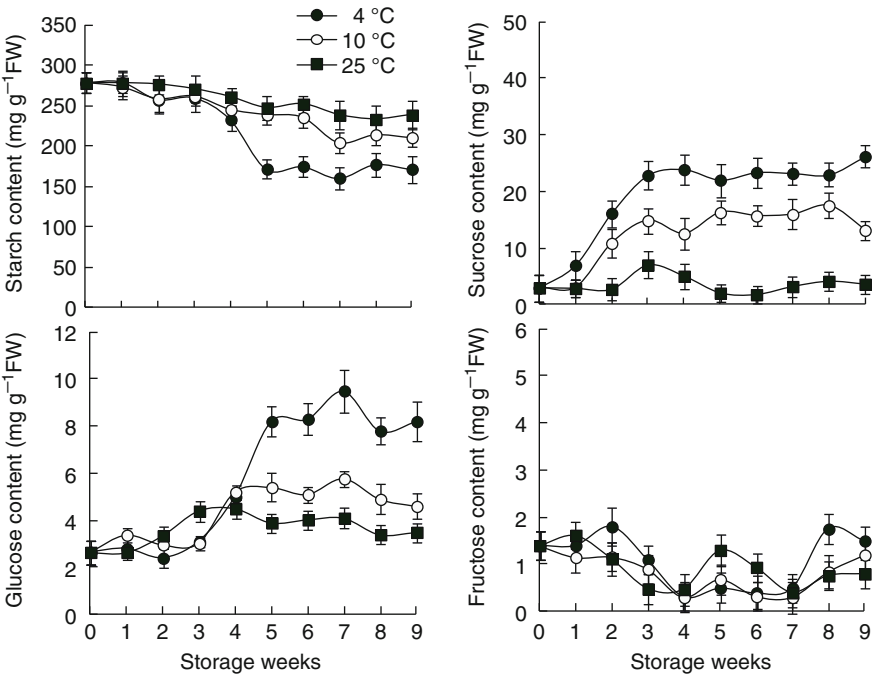

Fig. 16.11 Changes in content of starch, sucrose, glucose, and fructose of *in vitro*-produced of *Lilium* 'Casa Blanca' bulblets as affected by storage temperature and duration

and α- and β-amylase activities increased after 7 weeks of storage at 4 °C. The contents of starch and sucrose decreased and increased respectively with the decreasing storage temperature, which promoted bulblet emergence (Fig. 16.13).

16.9 Transplantation of Bulblets

In either traditional solid or liquid bioreactor cultures, the appropriate *Lilium* bulblet size is considered when bulblets can generate cauline leaves. Culturing of large bulblets *in vitro* requires prolonged culture period, which increases the production cost. Bulbing in the field is more cost effective than bulbing *in vitro*. As described previously, *Lilium* bulblets cultured in media supplemented with high sucrose concentrations are dormant; such media inhibit the emergence of leaves from these bulblets. After breaking the dormancy, bulblet emergence is influenced by factors such as bulblet size, physical culture conditions (solid or liquid culture), and environmental conditions. To break the dormancy, the bulblets harvested from solid medium or bioreactors should be rinsed several times and maintained in a storage room at 4 °C under dark conditions for at least 8 weeks before soil transplantation. We classified the harvested 'Casa Blanca' bulblets from solid and bioreactor cultures into three groups (≤1 g,

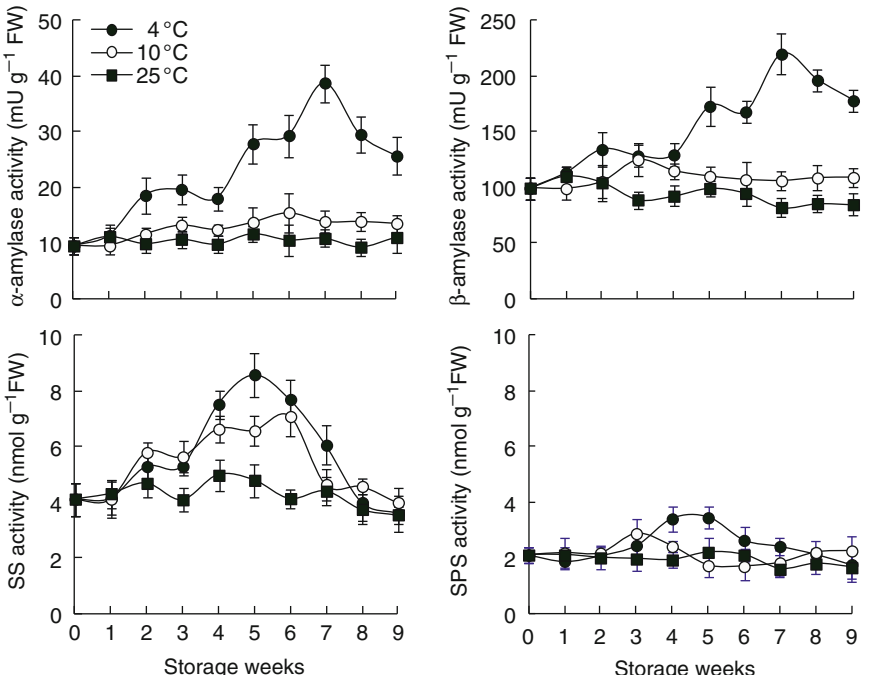

Fig. 16.12 Changes of **α**-amylase, **β**-amylase, SS, and SPS activity of *in vitro*-produced of *Lilium* 'Casa Blanca' bulblets as affected by storage temperature and duration

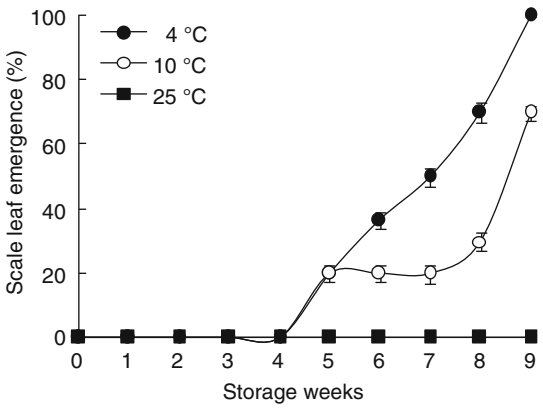

Fig. 16.13 Scale leaf emergence as affected by storage temperature and duration *in vitro* produced bulblets of *Lilium* 'Casa Blanca' into soil after 6 weeks

1.1 g to 2 g, and ≥2.1 g) according to their sizes and then separately transplanted them into boxes that contain equal amounts of perlite and peat moss. These boxes were maintained in a growth room at 24 °C and a 16 h photoperiod under fluorescent lights (30 μmol m^{-2} s^{-1}). After bulblet transplantation, scale (Fig. 16.14a) and cauline leaves (Fig. 16.14b) formed from the bulblets at 1 week, the leaf emergence rate increased with the number of transplanting weeks, and all bulblets formed scale leaves at the 6th

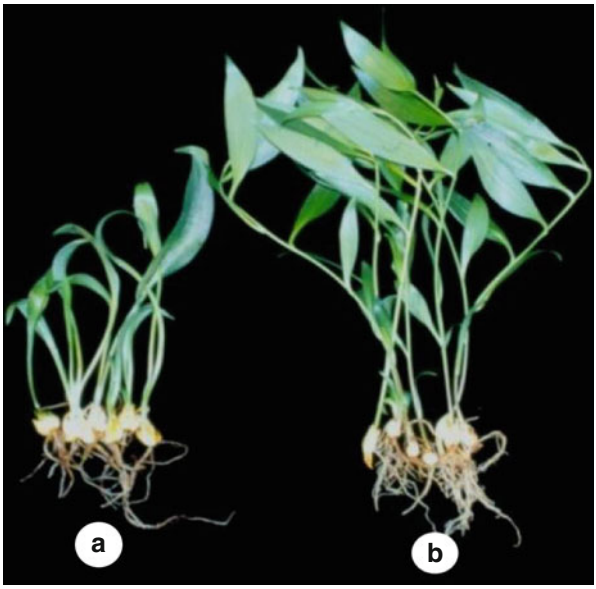

Fig. 16.14 Scale leaves (**a**) and stem leaves (**b**) formed from bioreactor-grown bulblets

week (Fig. 16.13). Although the scale leaves are easily formed, they often die soon after their emergence, which hinders further bulblet enlargement in soil. The emergence of cauline leaves occurred after the emergence of scale leaves. Emergence was observed in only 64 % of the large bulblets (≥2.1 g) produced from solid cultures but in almost all bulblets (1.1 g) produced in the bioreactors at the 6th week after transplantation (Fig. 16.15). As mentioned above, other reports stated that the emergence rate of cauline leaves can reach more than 90 % in large bulblets (3 g) produced in solid medium but can only reach 30 % in small bulblets (1.4 g) [22]. This result indicates that the formation of cauline leaves is affected not only by bulblet size but also by culture methods. Scale leaf emergence is important for further bulblet enlargement in the field because the formed cauline leaves can provide photosynthates to the underground parts and thus promote bulblet development.

16.10 Production Costs of Bulblets

The production costs of *Lilium* bulblets produced in solid and bioreactor culture systems are significantly different. Solid culture has a high production cost. Bioreactor culture, which includes bulblet formation in solid medium and bulbing in bioreactors, has 50 % lower production cost when compared with the solid culture. The production cost in one-step culture (both stages of bulblet formation and bulbing in one bioreactor) was half of that in bioreactor culture (Table 16.3). We considered the one-step bioreactor culture to be the best approach for *Lilium* bulblet production in terms of production cost. However, this technique needs to be further developed because many abnormal bulblets were observed at the bulbing stage

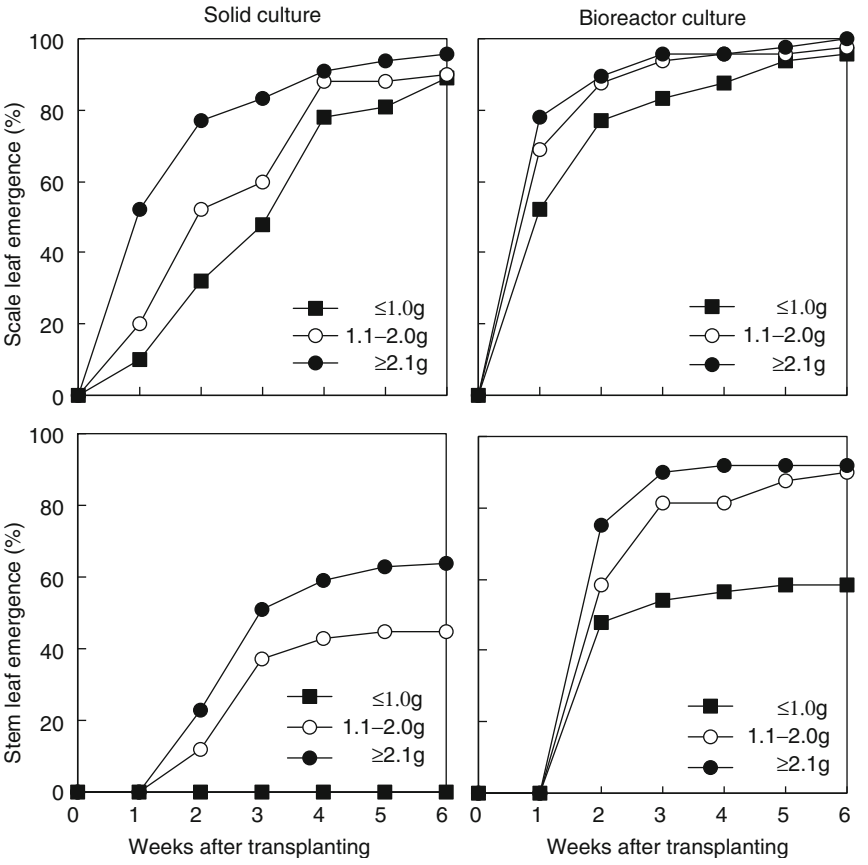

Fig. 16.15 Leaf emergence of *Lilium* 'Casa Blanca' bulblet produced from solid and bioreactor culture as affected by bulblet size after transplanting

when this approach was used. Consequently, bioreactor culture involving bulblet formation in solid media and bulbing in bioreactors is strongly suggested because it is well developed and cost effective.

16.11 Conclusion

Bulblet production using bioreactors is an efficient approach to realize the industrial production of *Lilium* propagules. The bulblet formation and enlargement in bioreactors are affected by numerous factors, including culture medium and microenvironment. For bulblet enlargement in bioreactors, the immersion culture system is more favourable than the ebb and flood system. During long-term culture of bulblet growth in immersion bioreactors under dark conditions, the old medium should be replaced twice with fresh medium within the culture period to promote bulblet enlargement.

Table 16.3 Effect of medium exchange numbers on bulblet growth and distribution of bulblet size in bulblet culture of *Lilium* Oriental hybrid 'Casa Blanca' after 16 weeks in bioreactor[a]

Method	Stage	Medium	Production cost[b]					
			Medium (US$)				Labor (US$)	Total (US$)
			MS	Agar	Sucrose	Total		
Solid culture	Bulblet formation	Solid	0.02	0.15	0.07	0.25	1.60	14.35
	Bulbing	Solid	0.23	1.90	0.86	2.99	9.52	
Bioreactor culture	Bulblet formation	Solid	0.02	0.15	0.07	0.25	1.60	8.36
	Bulbing	Liquid	0.18	–	0.68	0.86	5.65	
One-step bioreactor culture	Bulblet formation	Liquid	0.02	–	0.03	0.05	0.94	3.74
	Bulbing	Liquid	0.18	–	0.68	0.86	1.88	

[a]For producing 400 bulblets
[b]For the price in Korea (2002)

Sucrose, which is the carbon source in the culture medium is involved in increasing the weight of bulblets. Specifically, bulblet development needs a high sucrose concentration; a sucrose concentration of 90 g L^{-1} is considered to be appropriate. With regard to bulblet formation from bulbscale segments, the ebb and flood system must be applied because the explants cannot form bulblets in the immersion culture system. In the ebb and flood system, the temporary immersion cycle is a specific influencing factor. For *Lilium* bulblet formation, the bulbscale segments must be immersed in the culture medium 4 times per day for 15 min each under 30 μmol m^{-2} s^{-1} light intensity for a 16 h photoperiod. Supplementing the culture medium with BA and NAA results in the rapid production of *Lilium* bulblets (1 mg L^{-1} BA and 0.3 mg L^{-1} NAA) with well-defined morphology and further enlargement. Moreover, the culture medium does not require supplementation with any plant growth regulator or minimal amount of NAA. The one-step bioreactor culture method, wherein two stages of bulblet formation and enlargement are completed in one bioreactor, can be applied for *Lilium* bulblet production to reduce the cost and simplify the culture procedure. An average of 2 g of bulblets from bulbscale segments can be harvested from the one-step bioreactor culture system after 15 weeks of culture. Bulblet production using bioreactors is an efficient approach to realize the industrial production of *Lilium* propagules. However, problems should first be addressed beforehand. These problems include the non-uniform explants on the supporter in the ebb and flood culture system of horizontal pilot bioreactors and the tightly accumulated bulblet growth at the bottom of balloon-type pilot bioreactors in the later culture period. Bioreactor-cultured bulblets heavier than 1.1 g can form cauline leaves. Thus, bulblets should only be harvested when most of them weigh >1.1 g in the bioreactors. Bioreactor culture, which includes bulblet formation in solid medium and bulbing in bioreactors, is more well-developed and cost effective when compared with the solid cultures. These results suggest that bioreactor culture is an efficient method for the mass production of high-quality *Lilium* bulblets.

Acknowledgments This work was supported by Korea Science and Engineering Foundation (KOSEF) through Research Center for the Development of Advanced Horticultural Technology at Chungbuk National University, Cheongju, 361–763, Korea and National Science Foundation of China (30860176).

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Chapter 17
In Vitro Production of *Digitalis purpurea* Biomass Using Temporary Immersion Cultures

Anika Schumann, Diana Claus, and André Gerth

Abstract The annual turnover of the herbal active substances exceeds yearly 10 billion Euros. The biotechnological production of these secondary metabolites leads year round supply of these compounds for the pharmaceutical use and it also helps to some extent to a conservation of natural resources in their countries of origin to avoid wild collections. Furthermore, the pharmaceutical production supports the product safety, constancy of price and quality of the secondary metabolites. During the past years, numerous different bioreactor types have been set up allowing a biotechnological production of secondary compounds in plants and the temporary immersion system is one among them which is considered as the simplest method. In this article, we have discussed the production of *Digitalis purpurea* shoot biomass using temporary immersion system.

Keywords Bioreactor • Biomass production • *Digitalis* • Secondary metabolites • Temporary immersion system

Abbreviations

LEDs	Light emitting diodes
PET	Polyethylene terephthalate
TIS	Temporary immersion system

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17.1 Introduction

Production of bioactive compounds from *in vitro* cultivation of cell, organ or plantlets using bioreactor technology is an active field of research during recent years. *In vitro* production of biomass and bioactive compounds to some extent help in the protection of valuable natural resources. In the pharmaceutical production of herbs in bioreactors imply for the product security, price stability and a constant quality of active substances. Commercial production of bioactive substances through traditional agriculture methods or by collection of plants from the wild has several limitations [1]. For example, the contents of the cardiotonic glycosides in field grown plants (e.g. *Digitalis* sp.) are greatly affected by climatic and soil conditions [2], especially with respect to the biotic factors [3]. Large scale production of plant cells and tissues has been considered as an alternative technique for the production of phytochemicals when compared to the traditional methods of field cultivation [3]. A well defined *in vitro* production system offers an advantage to result in higher yields and more consistent quality of the secondary products [4]. But, despite of the efforts made to produce plant active compounds from cell cultures, only a few industrial applications have been successfully established. Wilken et al. [5] made an effort to use *in vitro* organ cultures for the production of medicinally important compounds, as the production of secondary metabolites is generally higher in different tissue cultures. More than 200 different species of herbs and rare medicinal plants with traditional medicinal use were taken for *in vitro* culture. Root, shoot and cells were cultivated under different growth conditions in fully automated bioreactors with essential nutrients and gas exchange. Combining the bioreactor technology with a high throughput screening methods high yielding plants can be selected according to their biomass production and the contents of their active compounds. Advantages of *in vitro* production of medicinal plant raw material over field cultivation or collection from natural stand is presented in Table 17.1.

Strategies based on *in vitro* culture methods (Fig. 17.1) have been extensively used to improve the production of plant specific secondary metabolites by culturing the cells or plant organs in liquid nutrient medium systems such as plant bioreactors [4, 6–8]. Bioreactors are usually designed for an intensive production of biomass and secondary compounds in culture and to afford a maximal opportunity for monitoring and controlling over the microenvironmental conditions [4]. For this purpose, several configurations of bioreactors for plant biomass production are used; one of such configurations is a temporary immersion system (TIS). This type of bioreactor technology is economical for the automation of the process for the production of *in vitro* plant tissues. It is also a representative and an attractive alternative for the production of secondary metabolites in plants due to the facilities found in TIS for the large scale culturing of plant organs [5, 9–11]. Numerous different types of bioreactors have been set up during the last few years to allow the production of plant material (e.g. plants organs such as shoot or roots) as demonstrated in Fig. 17.1 with different graduations.

Table 17.1 Advantages and limitations of *in vitro* methods for the production of medicinal plant raw material in comparison with the conventional methods

Sources of raw material	Advantage	Disadvantage
Wild collection/natural population	Favorable price of the drug	Possible risk of extinction of rare plants Challenging acquisition of material depending on the plant species High diversity of content of active substances
Agricultural production/ greenhouse production	Favorable price of the drug Predictable availability	Erratic quality and quantity of the active compounds Partially insufficient quality of the active substances Production is limited by the season Danger of crop failure due to the natural hazards
Biotechnological production (*in vitro* cell culture techniques, bioreactor production)	Controlled production Independent of season, climate or weather Increased compound content by modification of culture conditions Cheaper costs for quality control	Higher costs of production

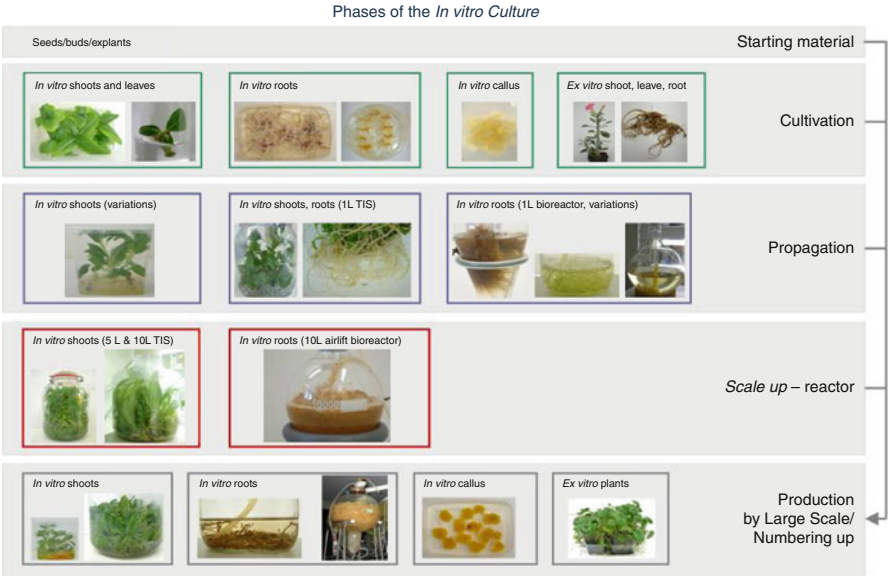

Fig. 17.1 Different phases of *in vitro* culture for the production medicinal plant raw material

17.1.1 Bioreactor Cultures

Bioreactors are vessels for large-scale cell, tissue or organ culture in liquid media. Plant culture bioreactors may be of two types: those in which cultures are immersed temporarily in the medium, and those in which the cultures are submerged continuously. Bioreactors provide more precise control over illumination, gaseous exchange, medium agitation, temperature and pH [12]. Bioreactors are used in the biotechnological production of substances such as pharmaceuticals and health foods [13].

The plant cells and organs in bioreactors can be cultured under aerobic conditions. Commonly, these bioreactors are cylindrical, ranging in size from liters to cubic meters (1,000 L), and are often made of stainless steel. The parameters such as aeration and agitation are usually controlled in the bioreactors. Depending upon the type of cultures, probes such as pH, temperature and dissolved oxygen can be maintained or regulated in the bioreactor cultures.

17.1.2 Parameters Influencing the Cell or Organ Growth in Bioreactors

Under optimal culture conditions, the growing cells or organs are able to perform their desired function with a 100 % success rate. Thereby, the environmental conditions of the bioreactor are gas (e.g., composition of the air atmosphere, content of oxygen and carbon dioxide), flow rate, temperature, pH and dissolved oxygen levels, and agitation speed or circulation rate. These parameters need to be monitored and controlled realizing an optimal growth of the cells in the bioreactor.

Figure 17.2 shows different parameters influencing on the growth of cells or organs in the bioreactors. Of prime importance is the aseptic environment of the bioreactor. Contamination by bacteria or fungi reduces the efficiency of the

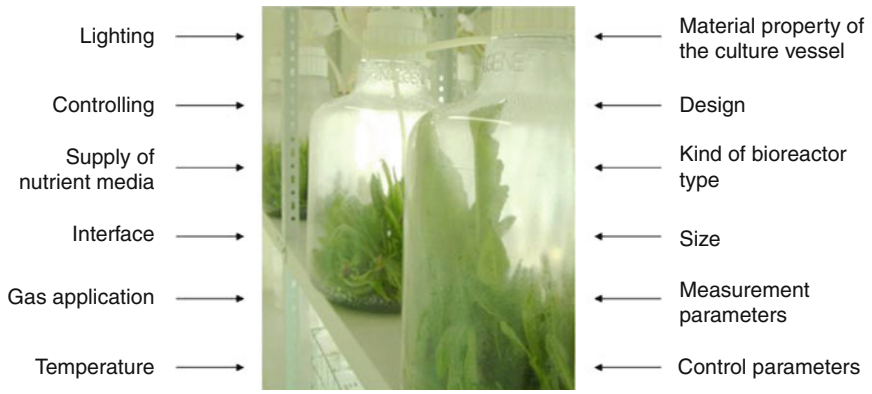

Fig. 17.2 Parameters which influence on the growth of bioreactor culture

bioreactors. Furthermore, an optimal oxygen transfer in aerobic processes is perhaps the most difficult task for achieving the growth under controlled conditions as oxygen is poorly soluble in water, and also is relatively scarce in the air composition (20.95 %), but oxygen is made available to the cultured cells through the agitation of medium which facilitates the availability of dissolved oxygen in the medium or additionally it can be supplemented through forced aeration. Agitation helps in the mixing of nutritional components of the medium. Excessive agitation may cause shear stress to the cultured cells; therefore, the method of agitation should be thoroughly investigated and standardized for individual cell types. Furthermore, bioreactor volumes range from small scale to large scale and most of the industrial manufacturers of bioreactors use vessels, sensors and a controlling system network altogether.

17.1.3 Temporary Immersion System (TIS) for Biomass Production

The biggest challenge for pharmaceutical application is the fluctuation in quality and quantity of plant raw material and their metabolic profile. As well, the potential risk of extinction of the natural populations in the wild is one of the major problems. For solving the aforementioned problem, the production of plants in closed systems is established. Therefore, the commercial laboratories need to produce a large number of plants with high quality at a low and cost efficient manner. A large scale *in vitro* production of medicinal plants is often criticized due to the cost intensive laboratory requirements. Thus, scaling up and the automation of production processes are the two factors necessary to reduce the production costs [14]. Therefore, using liquid nutrient medium either with temporary and/or by permanent immersion during the routine micropropagation of well-chosen plants is considered to be ideally suited for automation as well as for the cost efficiency [15–20]. Tisserat and Vandercook [21] first implemented the idea of temporary immersion systems (TIS) in plant tissue culture by designing a system consisting of a large elevated culture chamber that was drained and then refilled with fresh medium at certain intervals. Alvard et al. [15] applied this method to grow banana meristems by using a standard autoclavable filtration unit with two compartments. A similar apparatus was commercialized, namely Recipient for Automated Temporary Immersion (RITA®, Vitropic, France). In all these cases, compressed air is made to overflow the plant material with a liquid nutrient medium for a short time (10–30 min) for two to four times a day. After stopping the air flow, the liquid medium returns to the bottom of the vessel by gravity. Whereas Teission and Alvard [22], Escalona et al. [23], Etienne and Berthouly [24], and Jimenez [25] used compressed air in TIS with two flasks (Twin Flasks System: one flask for the plant material and the second flask for the liquid nutrient medium; both flasks were connected by tubes with one another and also a tube was connected for the movement of compressed air; compressed air regulated the flow of the liquid medium inside the plant culture vessel and the return flow into the second flask). Other authors demonstrated that compressed air was

more effective in providing an appropriate environment (e.g., gas exchange rate) combined with nutrient supply than immersion systems using only gravity without a gaseous exchange [20].

In summary, bioreactors with temporary immersion systems represent an advanced technology for commercial mass propagation of plants. Furthermore, several plant species are commercially propagated using this type of culture techniques with different regeneration pathways such as shoot, bulblet/tuber and embryo multiplication. Various modifications have been also carried out depending upon the methods or modes of plant propagation.

17.1.4 Modified Temporary Immersion Cultures for Large-Scale Biomass Production

Figure 17.3 shows different vessels used in the *in vitro* culture bioreactor technique. Ranging from small scale (volume: 250 mL) up to larger scale (volume: 10 L, possibly 40 L), they can be used for the production medicinal plant biomass. Besides the volume of bioreactor, the shape of culture vessels can be different according to the cultivated plant organ. In case of root culture, balloon or bulb shaped airlift bioreactors are the most suitable type for culturing.

For the cultivation of plant organs such as shoots, bulblets and corms in bioreactors a two bottle separated system has been used; one vessel meant for the plant organ

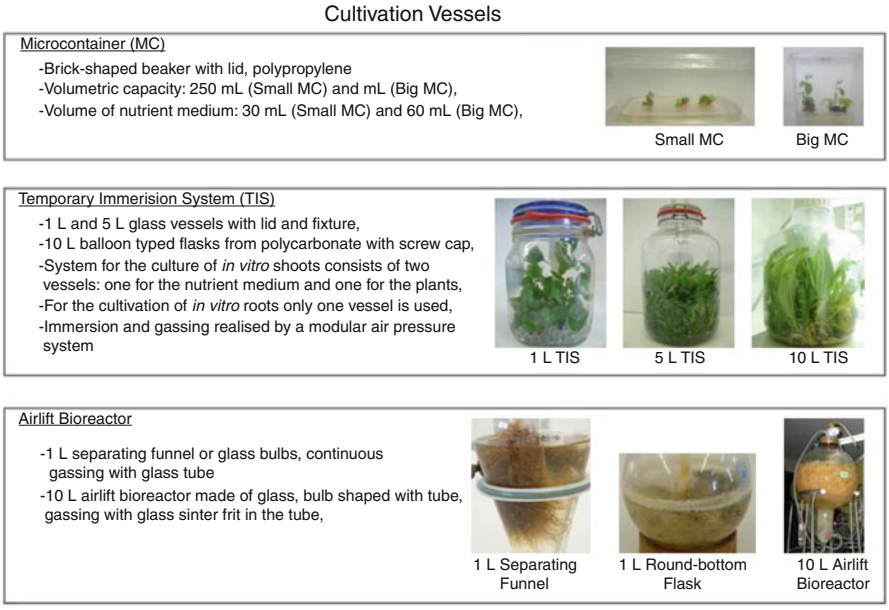

Fig. 17.3 Presentation of different culture vessels used in the *in vitro* culture of tissues

Fig. 17.4 Automation of an industrial applicable culture system for bioreactors

and another vessel for the liquid nutrient medium (medium reservoir). The medium is transferred between the two culture vessels connected through tubes by using the compressed air. Another possibility for the transfer of the nutrient medium can be performed by gravity, for example, the advantage of such a chosen temporary immersion system is the production of plant organs or plantlets without hyperhydricity. Moreover, it is possible to scale up the process as shown in Fig. 17.4.

17.2 Production of Shoot Biomass of *Digitalis purpurea* Using TIS

17.2.1 Culture Procedure

Digitalis purpurea cv. Berggold shoot cultures were initiated from nodal explants initially on Murashige and Skoog (MS) [26] semi-solid medium supplemented with 1.0 mg L^{-1} thiamine-HCl, 1.0 mg L^{-1} 6-benzylaminopurine (BAP), 0.1 mg L^{-1} indole acetic acid (IAA), 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose and 8.0 g L^{-1} agar. All cultures were incubated under a 16 h photoperiod from cool white fluorescent lamps at a photosynthetic photon flux density of 40 µmol m^2 s^{-1} at 24 °C ± 2 °C. Cultures were maintained by subculturing the shoots on fresh culture medium for every 28 days.

A temporary immersion system was used for the large-scale shoot multiplication of *D. purpurea*. TIS consisting of two glass vessels, 5 L capacity each, one culture vessel is used for culturing the shoots and the other as culture medium reservoir. Each TIS contained 3 L shoot multiplication liquid medium as described above and 60 individual shoots were cultured per TIS [weighing about 5.0 g fresh weight (FW) per TIS]. The nutrient medium supply was controlled from medium reservoir vessel to culture vessel using a solenoid valve and a timer. Medium supply was programmed in such a way that the medium should enter the culture vessel from the reservoir 6 times in a day (once in every 4 h) and should stay there for 5 min each time and goes back to reservoir. Four TIS were inoculated per treatment and the experiment was repeated twice.

Dynamics of biomass production were monitored by weighing the cultures every week by measurement of total FW per TIS. Biomass accumulation was expressed and determined as fresh and dry weight (g) produced per TIS after a culture period of 28 days. Also, the length and the number of shoots produced per TIS were determined. Cardiac glycosides were isolated and quantified by HPLC by using the methods described by Wichtl et al. [27].

For investigation of the biomass productions as well as the synthesis of secondary metabolites different experiments were performed. To check the influence of light, two various sources of light were used: (a) fluorescent light with a light intensity of 40 μmol m^2 s^{-1} and (b) white (blue plus red light emitting diodes) LEDs with a light intensity of 40 μmol m^2 s^{-1}. Furthermore, the influence of the application of the nutrient medium transfer was investigated: (a) transfer by compressed air and (b) transfer by gravity. As well, the influence of the material of culture vessel was tested: (a) culture vessel with glass material and (b) the plastic disposable culture vessel.

17.2.2 The Parameters Which Influence on the Biomass Production

Effect of Light Quality

A range of wavelengths of light responsible for photosynthesis is in the visible region and is also called white light and that ranges from 400 to 700 nm. The chlorophyll a and b are photosynthetically active pigments especially absorbing the light in blue and red region. For investigating the effects of light quality, in *in vitro* growth conditions fluorescent lamps with a white emission part were used.

The experimental results on effect of light quality showed that fluorescent light was superior for shoot multiplication of *Digitalis purpurea* when compared to red-blue LEDs. As shown in Fig. 17.5, a significant decrease in the biomass production was observed using red-blue LEDs. Furthermore, a higher multiplication rate of shoots was identified by applying white fluorescent light (7.9-fold in contrast to 5.0-fold). As well, the maximum length of shoots was achieved under these conditions.

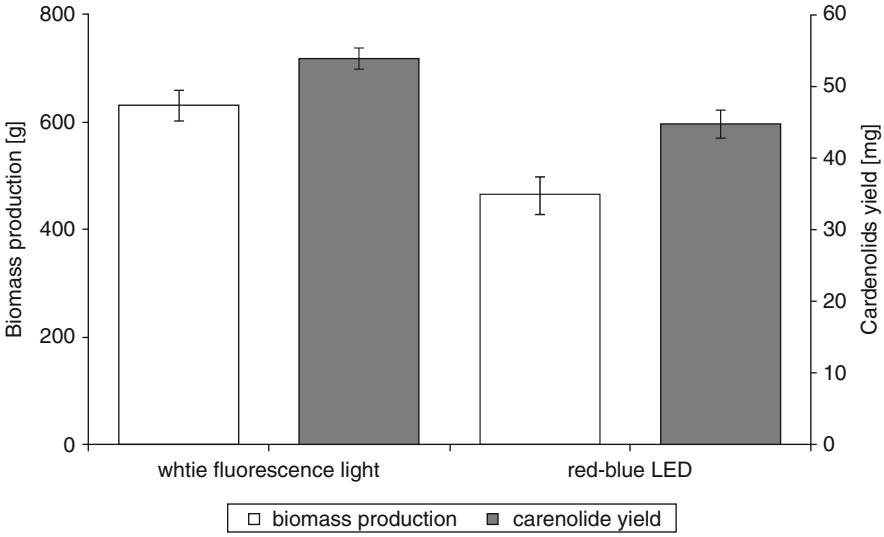

Fig. 17.5 Influence of lighting source (white fluorescent light and LEDs) on the biomass production and the net production of the cardenolides of *Digitalis purpurea in vitro* shoots cultured in TIS for a culture period of 4 weeks

Regarding the net production of cardiotonic glycosides, their production was higher when white fluorescent light was used.

Effect of Light Intensity

Besides the quality of the light, the quantity of light is also a parameter that influences the growth of *in vitro* plant organs in bioreactors. As well, the angle of incident light on the surface of the bioreactor vessel is decisive. The reduction of the area of lighting by 50 % results in 13.3 % decrease in the biomass production. The multiplication rate, the maximum length of the *in vitro* shoots, the fresh weight and the dry weight of the *in vitro* shoots was significantly reduced with reduced light intensity. Furthermore, the content of cardiotonic glycosides was reduced under the same conditions.

Effect of Type of Culture Vessel

The transmission of light through the bioreactor material is influenced by the type of material. Especially, the transmission of the ultraviolet region is important for the production of several secondary metabolites in plants. Thus, the material for the culture vessel with a wide range of transmission is desirable. The transmission area of glass as well as of PET (plastic disposable) opens up to the wavelength from 300 nm.

The rate of shoot multiplication, accumulation of biomass (Fig. 17.6) and cardiac glycoside were comparatively higher with cultures grown in glass vessel compared

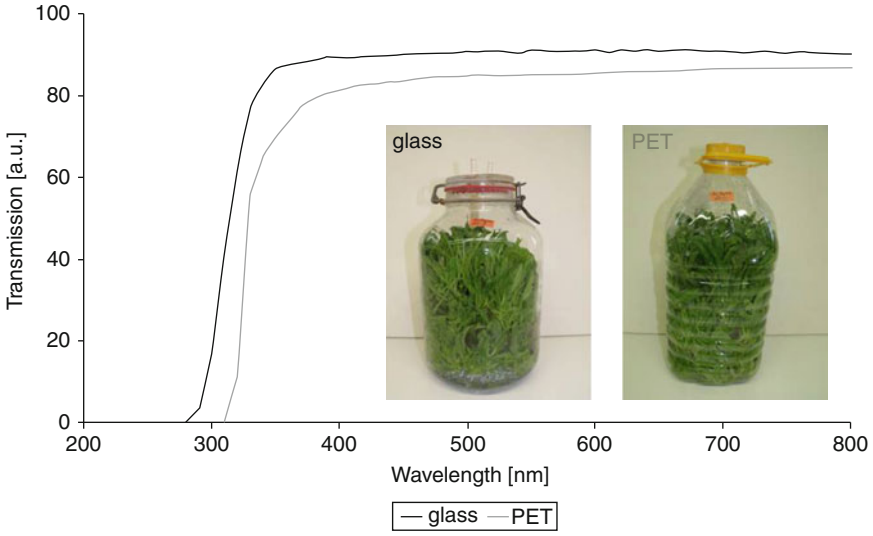

Fig. 17.6 Influence of materials of the culture vessel (*black line*: glass, *grey line*: plastic disposables (*PET*)) used for the bioreactor culture on the biomass production and the net production of the cardenolides of *Digitalis purpurea in vitro* shoots cultured in TIS for a culture period of 4 weeks

to PET vessel. However, it was easy to handle PET vessels and since they are disposable, cultivation of *Digitalis purpurea* shoots in PET vessels is recommended.

Effect of Air Supply

Many investigations were performed to check the influence of the type for the transfer of the nutrient medium from reservoir vessel (storage for the nutrient supply) to culture vessel (culture vessel for the growth of plant material). In first method, compressed air was supplemented along with incoming medium to the culture vessel from the reservoir vessel, whereas in the second method medium was supplied to the culture vessel from the reservoir vessel without supplementation of the air. Experimental results showed that supplementation of compressed air showed higher multiplication rate as well as secondary metabolite production (32 % higher than control; Fig. 17.7).

17.3 Conclusion

Bioreactors are usually designed for an intensive production of biomass and secondary compounds in culture. The advantages of temporary immersion system (TIS) for *in vitro* cultivation of plants using liquid medium have been highlighted in this review. It has a number of advantages including several fold increase in multiplication rates, and the reduction in space, energy and labour. Biomass production of

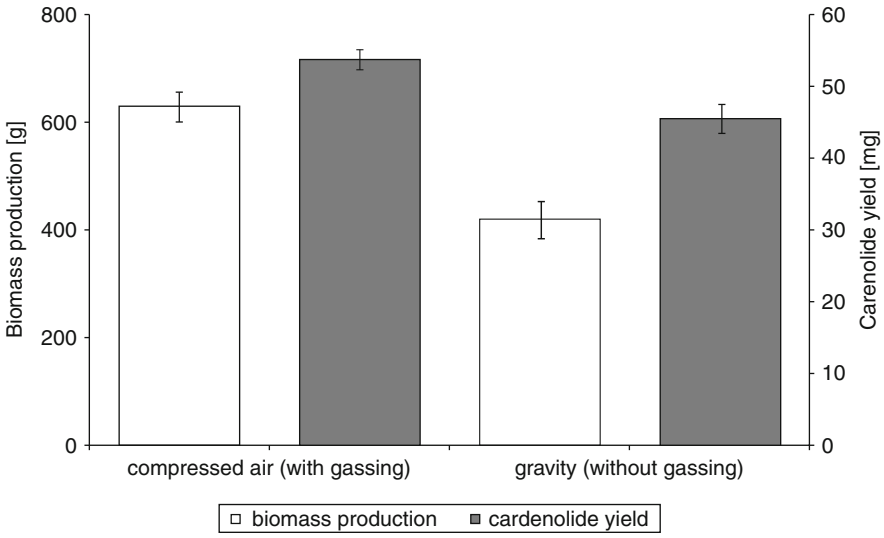

Fig. 17.7 Influence of the type of transfer for the supply of nutrient medium in the culture vessel (transfer by compressed air with gassing and transfer by gravity without gassing) on the biomass production and the net production of the cardenolides of *Digitalis purpurea in vitro* shoots cultured in TIS for a culture period of 4 weeks

Digitalis purpurea has been illustrated as case study. However, there are many disadvantages with liquid cultures including contamination, hyperhydricity and these problems can be minimized with tactful modifications of culture conditions.

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Chapter 18
Role of Nitric Oxide in Adventitious Root Development

Rajesh Kumar Tewari and Kee-Yoeup Paek

Abstract Nitric oxide (NO) is a gaseous free radical and a diffusible signalling molecule. NO influences plant growth and development. NO also affects the plant responses to various stresses. Treatments of NO producers (SNP, sodium nitroprusside; SNAP, S-Nitroso-N-acetylpenicillamine) and NO scavenger (PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide) revealed that NO is involved in the induction of new rootlets. Severe decline in the number of new rootlets by PTIO treatment indicates that NO acts downstream of auxin action in the process of root development. NO producers (SNP and SNAP) activated NADPH oxidase-like activity, resulting in a greater superoxide anion generation and a higher number of new rootlets from the adventitious root explants. A severe inhibition of NADPH oxidase-like activity and decline in root biomass of SNP and SNAP treated root explants in the presence of the NADPH oxidase inhibitor (diphenyl iodonium, DPI), further supports the involvement of NADPH oxidase-like activity in adventitious root development. The number of rootlets induced per explant and NADPH oxidase-like activity were related to NO content present in adventitious root of *Panax ginseng*. NO and superoxide anion generation at the site of root emergence strongly suggest the key roles of NO and ROS in root development.

Keywords Adventitious roots • Antioxidants • NADPH oxidase • Nitric oxide • *Panax ginseng* • Root growth • Reactive oxygen species

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Abbreviations

DAF-2DA	4,5-diaminofluorescein diacetate
DPI	Diphenyl iodonium
NO	Nitric oxide
PTIO	2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide
SNAP	S-Nitroso-N-acetylpenicillamine
SNP	Sodium nitroprusside
ROS	Reactive oxygen species

18.1 Introduction

Nitric oxide (NO), a biologically active diatomic, diffusive, water and lipid soluble gaseous free radical has been implicated as an effective regulator in cellular signalling even at a nanomolar concentration (1.0 nmol L^{-1}). The discovery of NO as an essential regulator in biology came in the mid-1980s from three main findings: the identification of NO as an endothelial relaxing factor in the vascular system, as a key cytotoxic agent of the immune system and as a signalling molecule in the nervous system [1, 2].

18.1.1 NO Is an Ubiquitous Signalling Molecule

NO is an interesting signalling molecule having homeostatic properties for the coordination and synchronization of cellular metabolism [3]. NO contributes in the broad spectrum of pathophysiological and developmental processes of living organisms [3]. NO simultaneously acts on several biochemical aspects through the modulation of the cellular redox status and cytosolic calcium ion (Ca^{2+}) concentrations. It has been demonstrated that the NO and reactive oxygen species (ROS) can considerably impact on the plant growth and development [4, 5]. NO and ROS can interact with each other and these interactions can be cytotoxic (result in cellular death) or cytoprotective depending on its concentrations and the cellular milieu [6]. The cytotoxic effects of elevated NO are caused by its reactivity as a radical with molecular oxygen, ROS and various transitional metal ions. The interaction of NO with iron-containing proteins can lead to inhibition of enzymes such as the cytochrome c oxidase of mitochondrion [7]. Now it has been accepted widely that NO protects plant cells against oxidative stress by scavenging Fenton active iron and thus avoids the generation of one of the most deleterious ROS, the hydroxyl radical ($OH^{\cdot}$) [6]. High concentration of NO can either be beneficial (for example, it activates defence responses or together with ROS, it directly kills the pathogen) or detrimental to the plant cell if its generation exceeds beyond the required limit [8]. NO protects plant cells from photooxidative damage [9] and retards cell death induced

by phytohormones, abscisic acid [10], gibberellins [11] and cytokinin, 6-benzyl aminopurine [12]. Therefore, NO has been proposed to have both antioxidant and prooxidant effects [8, 13].

18.1.2 Spatial Sites of NO Generation in Plant Tissues and Cells

There are reports of NO generation in various plant species and their organs – root, stem, and leaf of pea [14, 15]; *Arabidopsis* leaves and roots [16, 17]; maize leaves and roots [18–20]; *Panax ginseng* adventitious roots under Cu-toxicity [21] and salicylic acid treatment [22]; sunflower hypocotyls [23]; and senescing Phalaenopsis flower tepals [24]. Moreover, NO generation in the vascular tissues of pea leaves, xylem and phloem [14] and in differentiating xylem of *Zinnia elegans* [25] had also been observed. It seems that NO plays a key role in cell differentiation and organogenesis in plants. NO also accumulated in cryptogenin-treated leaf epidermis and chloroplasts [26] and chitosan-treated stomatal guard cells [27]. In plant cells, NO is generated both in the apoplast (the space outside of the plasma membrane including cell wall) [28] and symplast (space inside the plasma membrane including cellular organelles) [29]. NO generation in various subcellular compartments such as rapeseed leaves cytoplasm [29], pea leaves peroxisomes [14], barley root mitochondria in the presence of nitrite and NADH [30, 31], soybean leaf chloroplasts in the presence of arginine, $NaNO_2$ and GSNO [32], rapeseed leaves mesophyll chloroplasts [33] and soybean cotyledon chloroplasts [34] have also been reported.

18.1.3 NO Regulates Various Physiological and Developmental Processes

NO is a biological mediator that plays a central role in key physiological processes such as neurotransmission, immunological and inflammatory responses and relaxation of vascular smooth muscle [35] in animal system. In plants, NO is involved in seed germination [36] and induction of lateral roots in tomato [37] and adventitious roots in *Panax ginseng* [38]. It delays senescence [39, 40], regulates stomatal movement [41] modulates the influx of extracellular Ca^{2+} and actin filament organization during cell wall construction in *Pinus bungeana* pollen tubes [42], and directs targeted (oriented) pollen tube growth towards ovules in *Lilium logiflorum* [43] and *Arabidopsis thaliana* [44]. It has been reported that NO mediates cytokinin functions in cell proliferation and meristem maintenance in *Arabidopsis thaliana* [45]. NO also participates in cell wall lignification by modulating activities of basic peroxidases [46]. Nonetheless, exogenous application of NO also down-regulates xanthine oxidase-mediated generation of superoxide anion in Phalaenopsis flowers [24].

18.1.4 *Nitric Oxide Induces Biosynthesis of Phenolics and Ginsenosides*

It has been reported that NO plays a crucial role in the accumulation/synthesis of secondary metabolites after elicitation with a chemical, methyl jasmonate [47]; physical, ultrasound [48]; or microbial [49] elicitors in cell suspension culture. Exogenous application of NO producer (SNP), induced synthesis of phenolics, flavonoids, and caffeic acid derivatives in *Echinacea purpurea* adventitious roots [50]. NO is also involved in the biosynthesis of ginsenosides in *Panax ginseng* adventitious roots [49, 51]. Moreover, Salicylic acid applied to *Panax ginseng* adventitious roots also induced an accumulation of ginsenosides in a NO-dependent manner that was mediated by the associated increase in superoxide anion radical [22].

18.2 Adventitious Root Development in *Panax Ginseng*

Ginseng (*Panax ginseng* C. A. Meyer; family Araliaceae), a classical herb widely used in East Asia, provides resistance against stress and exhaustion. Ginseng has been practiced as a healing medicine and health tonic since ancient time [52]. In *Panax ginseng*, the active ingredients are believed to be the glycosylated triterpene saponins, which are also known as ginsenosides [52]. The leading source of ginsenosides is roots of *Panax ginseng* where it accumulates in significantly large quantities when compared to other plant parts. Field cultivation of *Panax ginseng* is a time-consuming and a labour-intensive process [53, 52] and therefore, in recent years a new approach of cultivation of cell and adventitious roots in bioreactors, have been developed [52]. Adventitious root cultures demonstrated the ability of higher accumulation of biomass and desired compounds at levels comparable to naturally grown roots [53, 54]. In brief, adventitious root cultures were generated from 4-year-old *Panax ginseng* through callus culture as described by Yu et al. [55]. Working under sterile conditions, the selected roots were proliferated further in 5 L airlift balloon type bioreactors containing 4 L of 3/4 strength Murashige and Skoog (MS) liquid, or maintained in Petri dishes containing 3/4 MS solid medium [56] supplemented with 5.0 mg L^{-1} IBA, 0.1 mg L^{-1} kinetin and 5 % sucrose for 4 weeks. These cultured roots were used as explants for various studies about the role of NO in adventitious root development described in this manuscript.

18.2.1 *Exogenous Application of NO Releasing Compounds Enhances Endogenous NO Levels in Adventitious Roots*

In situ localization of NO using 4,5-diaminofluorescein diacetate (DAF-2DA), a NO specific dye, revealed that exogenous application of NO releasing compounds such as sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP)

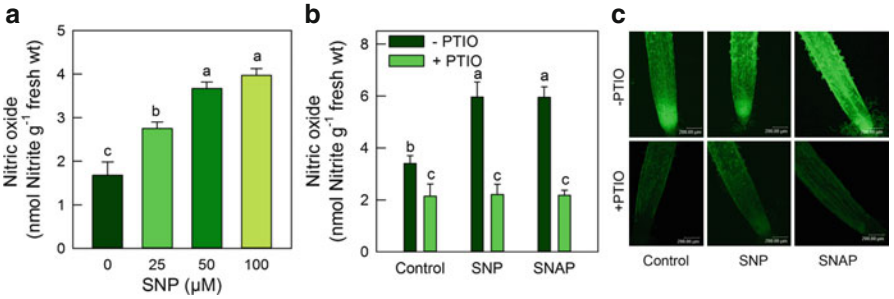

Fig. 18.1 Effects of graded supply of NO producers (*SNP*) on NO (nitrite) levels in the adventitious roots (**a**), comparative effect of various NO producers (100 μM SNP and 100 μM SNAP) in the absence and presence of NO scavengers (100 μM PTIO) on NO (nitrite) levels (**b**), and *in situ* localization of NO by DAF-2DA assay in the adventitious roots of *Panax ginseng* (**c**), scale bar = 200 μm. Adventitious root explants were grown in 3/4 MS medium containing 5 mg L^{-1} IBA, 5 % sucrose for 4 weeks. Data are mean ± SE (n = 6). Bar with different letters are statistically significant by Fisher LSD methods ($P \leq 0.05$)

enhanced endogenous NO level which was reflected as an elevated nitrite concentration (Fig. 18.1a, b) and intense DAF-2 T fluorescence (Fig. 18.1c) in the adventitious roots of *Panax ginseng*. Both intensity of NO specific DAF-2 T fluorescence (Fig. 18.1c) and nitrite levels (Fig. 18.1a, b) were decreased on incorporation of NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), in the growth medium. These observations suggested that the application of NO releasing compound indeed enhanced internal NO level in adventitious roots of *Panax ginseng*.

18.2.2 NO Is Involved in the Proliferation of Adventitious Roots

Panax ginseng root explants cultured on MS medium supplied with graded concentrations of a NO releasing compound, SNP, produced an increased number of rootlets (Fig. 18.2a). Moreover, adventitious root explants cultured on MS medium in the presence of another NO releasing compound, SNAP, were also proliferated well and generated an increased number of rootlets (Fig. 18.2b, c). Proliferation of adventitious rootlets was inhibited when a NO scavenger, PTIO was incorporated in the growth medium with the SNP and SNAP (Fig. 18.2b, c). An individual treatment of PTIO also inhibited root proliferation (Fig. 18.2b, c). PTIO-treated root explants, however, elongated well but they developed a very few adventitious rootlets (Fig. 18.2b, c). Mechanism of PTIO-induced explant elongation is currently unknown. Induction of increased numbers of adventitious rootlets along with enhanced biomass in the presence of NO releasing compounds, SNP and SNAP and an inhibition in number of rootlets in the presence of NO scavenger, PTIO suggests a key role of NO in adventitious root development [38, 51]. The involvement of NO

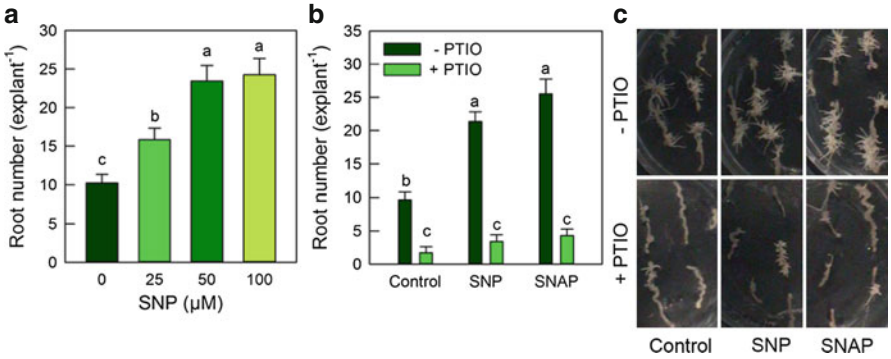

Fig. 18.2 Effects of graded (0, 25, 50 100 μM) supply of NO producers (*SNP*) on root proliferation (**a**), comparative effect of various NO producers (100 μM SNP and 100 μM SNAP) in the absence and presence of NO scavengers (100 μM PTIO) on adventitious root proliferation (**b**, **c**). Adventitious root explants were grown in 3/4 MS medium containing 5 mg L^{-1} IBA, 5 % sucrose and 2.2 g L^{-1} gelrite for 4 weeks. Data are mean ± SE (n = 20 counted explant for root number). Bar with different letters are statistically significant by Fisher LSD methods ($P \leq 0.05$)

in promoting the root elongation has previously been observed in maize plants by Gouvea et al. [57]. Moreover, carbon monoxide, alkamide (N-isobutyl decanamide) and N-acyl-homoserine-lactone (N-3-oxo-decanoyl-L-homoserine-lactone) also induced lateral roots in a NO dependent manner [58–60]. NO is also involved in phosphorus deficiency- and iron deficiency- induced [61, 62] cluster-root development in lupin. It appears from our studies with *Panax ginseng* [51, 38, 63] and those reported for other plant species that NO plays a fundamental role in the adventitious root growth and development in *Panax ginseng* or the lateral root growth and development in tomato [37, 64, 65].

18.2.3 NO Acts Downstream to Auxin Induced Root Proliferation

Auxin has been known for a long time to be the main plant hormone involved in lateral root development. We demonstrated that despite the presence of auxin in the growth medium, a number of rootlets were declined tremendously to a level lower than control in the presence of PTIO (Fig. 18.2b). This observation suggests that NO elicitation modulates cell division and it act downstream of auxin action in the process of root induction. Pagnussat et al. [66] also reported that NO acts downstream of auxin action leading to the accumulation of cGMP, which modulates expression of cell cycle regulatory genes [65]. NO mediates the induction of the *CYCD3*;*1* gene and the repression of the cyclin-dependent kinase (CDK) inhibitor, *KRP2* gene, at the beginning of lateral root primordium formation [65]. Lombardo et al. [67] demonstrated that NO is involved in the auxin-signalling cascade leading to root hair formation in lettuce and *Arabidopsis thaliana*. NO synthase inhibitor,

100 μM Nω-nitro-L-arginine methyl ester (L-NAME), did not show any effect on the development of rootlets. It seems that NOA1/NOS1 [68] is not involved in the synthesis of NO in *Panax ginseng* [38]. However, many studies indicate existence L-NAME inhibitory NO synthase-like activity in plants but the concentrations of NO synthase inhibitors (L-NAME or L-NNA) used in such studies [69, 61] are quite high (1–10 mM) and therefore, it might have induced secondary inhibitory effect. NO has probably been synthesized either by coordinating activity of the root-specific plasma membrane-bound enzymes, nitrate reductase and nitrite: NO reductase [70] or by non-enzymatic sources in the adventitious roots of *Panax ginseng*.

18.2.4 Nitric Oxide-Induced Superoxide Anion Generation is Related to Adventitious Root Development

Superoxide anion has previously been implicated in the root elongation of maize plants [71], and root hair [72] and pollen tube growth [73]. Increased superoxide anion generation (Fig. 18.3a–d) along with an increase in the NADPH oxidase-like activity (Fig. 18.4a–d) in the roots treated with NO producers (SNP and SNAP) and

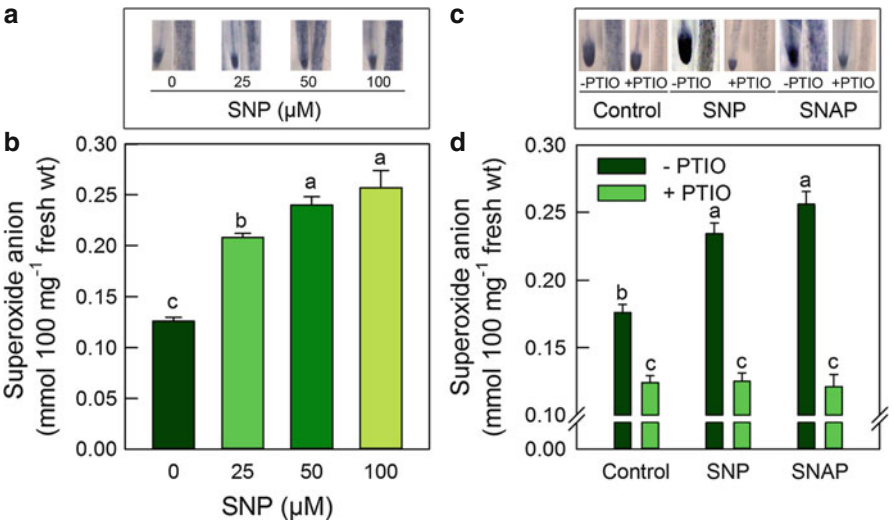

Fig. 18.3 Effects of graded (0, 25, 50 100 μM) supply of NO producers (*SNP*) on *in situ* superoxide anion localization (**a**), superoxide levels (**b**), and comparative effect of various NO producers (100 μM SNP and 100 μM SNAP) in the absence and presence of NO scavengers (100 μM PTIO) on *in situ* superoxide anion localization (**c**) and superoxide anion levels (**d**) in adventitious roots of *Panax ginseng*. Adventitious root explants were grown in 3/4 MS medium containing 5 mg L^{-1} IBA and 5 % sucrose for 4 weeks. Data are mean ± SE (n = 6). Bar with different letters are statistically significant by Fisher LSD methods ($P \leq 0.05$)

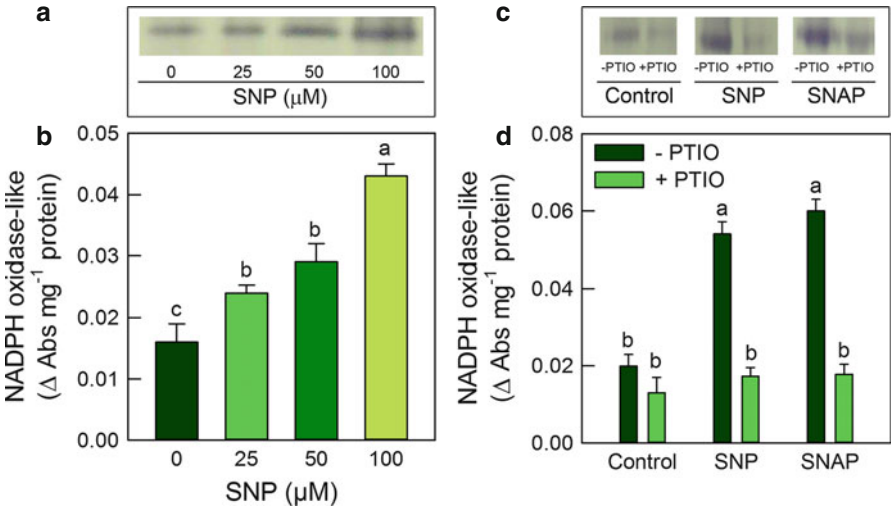

Fig. 18.4 Effects of graded (0, 25, 50 100 μM) supply of NO producers (*SNP*) on *in gel* NADPH oxidase-like activity (**a**) and spectrometric assay of NADPH oxidase-like activity (**b**), and comparative effect of various NO producers (100 μM SNP and 100 μM SNAP) in the absence and presence of NO scavengers (100 μM PTIO) on *in gel* (**c**) and spectrophotometric assay of NADPH oxidase-like activity (**d**) in adventitious roots of *Panax ginseng*. Adventitious root explants were grown in 3/4 MS medium containing 5 mg L^{-1} IBA and 5 % sucrose for 4 weeks. Data are mean ± SE (n = 6). Bar with different letters are statistically significant by Fisher LSD methods ($P \leq 0.05$)

its reversion on incorporation of NO scavenger, PTIO, suggests that superoxide anion is indeed involved in NO-induced adventitious root development in *Panax ginseng*. Involvement NADPH oxidase-like activity and superoxide anion in the adventitious root growth was investigated further by treating adventitious root culture with an NADPH oxidase inhibitor, 50 μM diphenyl iodonium (DPI), in combination with 100 μM SNP and 100 μM SNAP. A severe slowdown in the growth (Fig. 18.5a) along with decreased NADPH oxidase-like activity (Fig. 18.5b) and concomitant suppressed level of superoxide anion generation (Fig. 18.5c) were observed in the adventitious roots of *Panax ginseng* treated with DPI. *Arabidopsis* mutant defective in root hair development, *rhd2*, which had previously been defined as *Arabidopsis thaliana* respiratory burst oxidase homolog C (*Atrboh C*), could not produce sufficient superoxide anion and uptake Ca^{2+} ion required for root hair development [74], also support involvement superoxide anion in root organogenesis. Thus enhanced NADPH oxidase-like activity-mediated superoxide anion generation appears to be responsible for an enhanced cell division and enhanced root proliferation by exogenous application of NO producers, SNP and SNAP. Abolishment of root growth and superoxide anion generation by DPI, an NADPH oxidase inhibitor, further suggests a vital role of superoxide anion in adventitious root development in *Panax ginseng*.

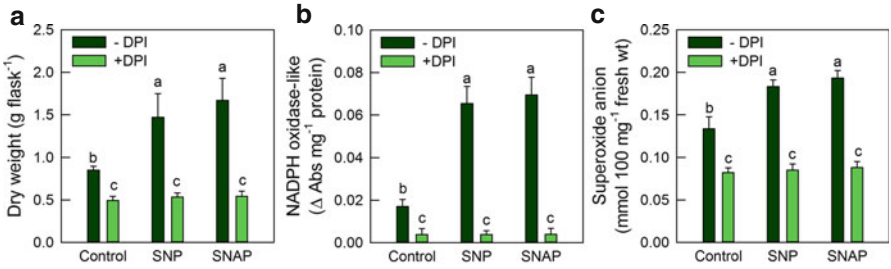

Fig. 18.5 Adventitious root growth (**a**) NADPH oxidase-like activity (**b**) and superoxide anion (**c**) in the adventitious roots of *Panax ginseng* grown in liquid suspension of 3/4 MS medium containing 5 mg L^{-1} IBA, 5 % sucrose for 3 weeks and subjected to the treatment of SNP and SNAP in the absence and presence DPI (NADPH oxidase inhibitor) or DPI alone for 1 week. Concentration applied for SNP and SNAP was 100 μM. Concentration of DPI was 50 μM. The data presented are mean ± SE (n = 6). Bar with different letters are statistically significant by Fisher LSD methods ($P \leq 0.05$)

18.2.5 Relationship of Root Growth with Superoxide Anion, NADPH Oxidase-Like Activity and NO Content

To have an insight into up-regulation in root proliferation in the NO-treated adventitious roots, we have drawn relationships among the some important parameters. We observed a correlation between superoxide anion content (r = 0.96), NADPH oxidase activity (r = 0.87), NO (nitrite) content (r = 0.80) with root number induced per explants (Fig. 18.6a–c). Moreover, a correlation exists between NADPH oxidase–like activity and NO (nitrite) content (r = 0.95) (Fig. 18.6d). These strong correlations in NO (nitrite) levels, NADPH oxidase-like activity, superoxide anion and numbers of adventitious root induced in *Panax ginseng* root explants strongly suggests an involvement of NO-induced superoxide anion in root proliferation. Notable inhibition of NADPH oxidase-like activity along with decreased root biomass by DPI treatment further support our hypothesis that the NO-induced NADPH oxidase-like activity-dependent superoxide anion generation is beneficial for the adventitious root growth.

18.3 Conclusions and Future Research Directions

In conclusion, our studies with *Panax ginseng* root explants on exogenous application of NO producers (SNP and SNAP) and NADPH oxidase inhibitor (DPI) revealed that NO is involved in adventitious root proliferation. Exogenous application of NO producers (SNP and SNAP) activated superoxide anion generation by inducing NADPH oxidase-like activity in adventitious root which concomitantly result adventitious root proliferation in *Panax ginseng* (Fig. 18.7). There are ten

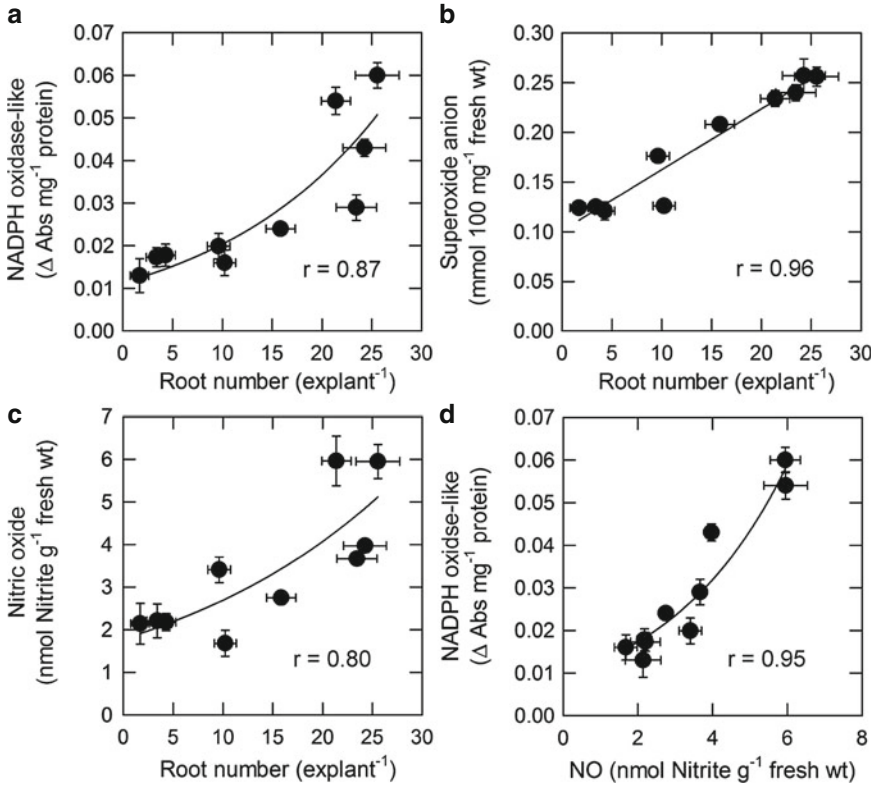

Fig. 18.6 Relationships between NADPH oxidase-like activity and root number (**a**), superoxide anion and root number (**b**), nitric oxide (nitrite) concentration and root number (**c**) and NADPH oxidase-like activity and nitric oxide (nitrite) concentration (**d**) in the adventitious roots of *Panax ginseng*

genes of respiratory burst oxidase homologs (RBOH A-J) which encode different NADPH oxidases in *Arabidopsis thaliana* [75]. These genes have been reported to express in different tissues in response to different environmental stimuli [75]. The specific NO-induced RBOH gene involved in superoxide anion generation in *Panax ginseng* adventitious root is still unknown. Moreover, the underlying downstream regulatory events of NADPH oxidase-dependent superoxide anion in root development in *Panax ginseng* are also unknown. Therefore, further genetic and molecular investigations are required to unravel underlying signalling events involved in the process of adventitious root development.

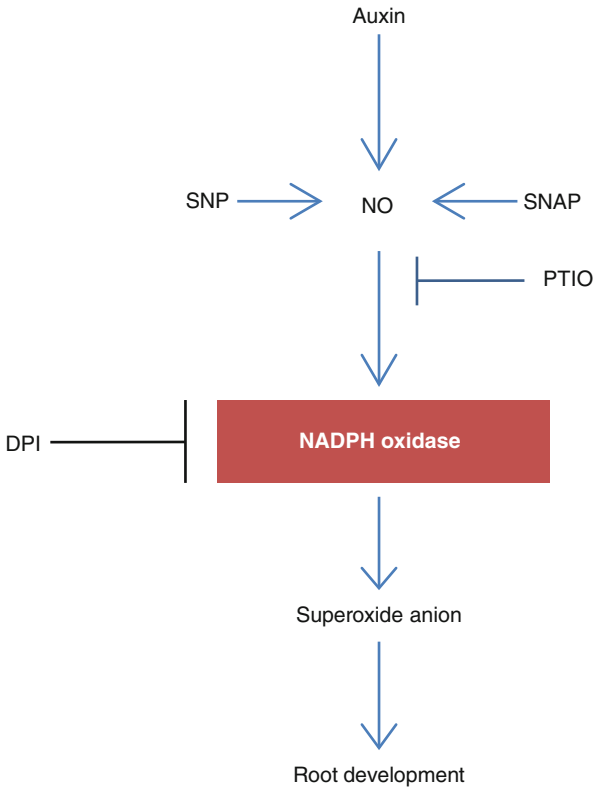

Fig. 18.7 Schematic representation of the possible *in vivo* mechanism by which nitric oxide induces adventitious root proliferation in *Panax ginseng*. NO activate NADPH oxidase-like activity resulting in the generation of superoxide anion radical and which consequently induces root proliferation

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Chapter 19
Melatonin Rich Plants: Production, Significance in Agriculture and Human Health

Vikramjit Bajwa, Susan J. Murch, and Praveen K. Saxena

Abstract Melatonin (*N*-acetyl-5-methoxytryptamine) has many therapeutic benefits for humans such as regulation of sleep cycle, aging, depression, and cancer. Melatonin was first discovered in fruits and vegetables in the mid-1990s. Since then, Melatonin has been recognized in large number of species including medicinal plants such as St. John's wort, feverfew and *Echinacea*. The melatonin content varies from plant to plant due to genetic and environmental factors; therefore there is a need to have integrated system (such as bioreactors) for large-scale propagation of the high melatonin containing elite plant germplasm under controlled environmental conditions. Recently, major advances have been made to understand the melatonin biosynthetic pathway in plants. Melatonin performs important roles in plants working as a growth regulator as well as environmental stress protector. The enhancement of endogenous melatonin levels in plants is beneficial in both agriculture as well as in human health.

Keywords Bioactive molecules • Medicinal plants • Melatonin

19.1 Introduction

Melatonin, often referred to as 'hormone of darkness', is secreted by the pineal gland at night in humans and in most diurnal mammals. In animals, the circadian rhythm of melatonin production is regulated by the light-dark cycle. Therefore, the nocturnal circulating melatonin levels are higher in animals when compared to the diurnal melatonin levels [1]. Melatonin regulates sleep-wake cycle and other cyclical body activities and is therefore, considered to be the body's chronological pacemaker or

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'Zeitgeber' [2]. The secretion of melatonin in animals is regulated by Suprachiasmatic nucleus (SCN) in response to the light/dark cycle [3]. Melatonin was first isolated from the bovine pineal gland [4] and in 1959 it was identified in humans [5]. Later, melatonin was also identified in other parts of the central nervous system, retina, the gastrointestinal tract, skin, bone marrow and in lymphocytes [2, 6–8]. Detectable quantities of melatonin are not exclusively present in blood but also in other body fluids such as cerebrospinal fluid, bile, fluid of the anterior chamber of eye, and ovarian follicle fluid [9, 10]. The presence of melatonin in various organs and body fluids of mammals heralds the diverse actions of the melatonin in the body [11]. Melatonin, discovered in plants in mid-1990s, is a relatively new compound for plant biologists. Melatonin is an indole amine and is structurally related to another well-known plant hormone indole-3-acetic acid (IAA). High amounts of melatonin have been identified in medicinal plants and common beverages [12–14] (Tables 19.1 and 19.2), indicating the importance of phytomelatonin for therapeutic purposes [14].

In plants, melatonin is derived from L-tryptophan [25]. The biosynthesis of melatonin involves a multi-step biosynthetic pathway in which L-tryptophan is first converted into serotonin *via* two enzymatic steps; in the first step, L-tryptophan is converted into tryptamine and in the second step, tryptamine is converted into

Table 19.1 High melatonin containing medicinal plants

Common name (plant part)	Scientific name	Detection method	Melatonin content (ng g^{-1})	References
Feverfew (fresh green leaf)	*Tanacetum) parthenium*	HPLC-ECD	2,450	[12]
Feverfew (fresh golden leaf)	*Tanacetum parthenium*	HPLC-ECD	1,920	
Feverfew (freeze-dried green leaf)	*Tanacetum parthenium*	HPLC-ECD	2,190	
Feverfew (freeze-dried golden leaf)	*Tanacetum parthenium*	HPLC-ECD	1,610	
Feverfew (oven-dried green leaf)	*Tanacetum parthenium*	HPLC-ECD	1,690	
Feverfew (oven-dried golden leaf)	*Tanacetum parthenium*	HPLC-ECD	1,370	
St John's wort (flowers)	*Hypericum perforatum*	HPLC-ECD	4,390	
St John's wort (leaves)	*Hypericum perforatum*	HPLC-ECD	1,750	
Huang-qin (herbal powder derived from flowers, seeds, leaves, roots, and stems)	*Scutellaria biacalensis*	HPLC-ECD	7,110	

Table 19.1 (continued)

Common name (plant part)	Scientific name	Detection method	Melatonin content (ng g^{-1})	References
Chantui (herbal powder derived from flowers, seeds, leaves, roots, and stems)	*Periostracum cicadae*	HPLC-FD on-line with MS	3,771	[14]
Diding (herbal powder derived from flowers, seeds, leaves, roots, and stems)	*Viola philipica*	HPLC-FD on-line with MS	2,368	
Gouteng (herbal powder derived from flowers, seeds, leaves, roots, and stems)	*Uncaria rhynchophylla*	HPLC-FD on-line with MS	2,460	
Shiya tea-leaf	*Babreum coscluea*	HPLC-FD on-line with MS	2,120	
Sangye (herbal powder derived from flowers, seeds, leaves, roots, and stems)	*Morus alba*	HPLC-FD on-line with MS	1,510	
Huangbo (herbal powder derived from flowers, seeds, leaves, roots, and stems)	*Phellodendron amurense*	HPLC-FD on-line with MS	1,235	
Sangbaipi (herbal powder derived from flowers, seeds, leaves, roots, and stems)	*Mori Albae*	HPLC-FD on-line with MS	1,110	
Yinyanghuo (herbal powder derived from flowers, seeds, leaves, roots, and stems)	*Epimedium brevicornum* Maxim	HPLC-FD on-line with MS	1,105	
Huanglian (herbal powder derived from flowers, seeds, leaves, roots, and stems)	*Coptis chinensis* Franch	HPLC-FD on-line with MS	1,008	
Dahuang (herbal powder derived from flowers, seeds, leaves, roots, and stems)	*Rheum palmatum* L.	HPLC-FD on-line with MS	1,078	

serotonin [26, 27]. Serotonin is then converted into melatonin through the last two biosynthetic reactions in which serotonin is first acetylated to N-acetyl serotonin followed by conversion of N-acetyl serotonin into melatonin in the final reaction [28, 29]. In recent years, four genes namely tryptophan decarboxylase (*TDC*), tryptamine 5-hydroxylase (*T5H*), serotonin N-acetyl transferase (*SNAT*) and N-acetyl serotonin methyl transferase (*ASMT*) encoding consecutive enzymes required for melatonin biosynthesis have been identified, cloned and characterized in rice [26, 28–30] (Fig. 19.1).

Table 19.2 High melatonin content in common edible plants and beverages

Edible plant or beverage	Scientific name	Melatonin content (ng g^{-1}) (FW or DW of tissue)	Detection method	References
Cabbage	*Brassica oleracea capitata*	0.1 FW	RIA, HPLC-FD and LC-MS	[15]
Chinese cabbage	*Brassica rapa*	0.1 FW		
White radish	*Raphanus sativus*	0.7 FW		
Rice seed	*Oryza sativa*	1.0 FW		
Barley seed	*Hordeum vulgare*	0.4 FW		
Sweet corn	*Zea mays*	1.4 FW		
Oat seeds	*Avena sativa*	1.8 FW		
Ginger	*Zingiber officinale Ros.*	0.6 FW		
White mustard	*Brassica hirta*	189 DW	RIA, HPLC-ECD	[16]
Black mustard	*Brassica nigra*	129 DW		
Wolf berry	*Lycium barbarum*	103 DW		
Fenugreek	*Trigonella foenum-graecum*	43 DW		
Almond	*Prunus amygdalus*	39 DW		
Sunflower	*Helianthus annuus*	29 DW		
Fennel	*Foeniculum vulgare*	28 DW		
Alfalfa	*Medicago sativum*	16 DW		
		15 DW		
Green cardamom	*Elettaria cardamomum*	12 DW		
Flax	*Linum usitatissimum*	7 DW		
Walnut	*Juglans regia*	3.5 DW	HPLC	[17]
Wine grapes	*Vitis vinifera*	>100 μg g^{-1}	UPLC-MS/MS	[18]
Grapevine	*Vitis vinifera*	3–18 FW	UPLC-MS/MS	[19]
Tomato fruit	*Solanum lycopersicum*	4.1–114.5 FW	HPLC-MS/MS	[20]
Strawberry	*Fragaria × ananassa* Duch.	1.4–11.2 FW		
Pomegranate	*Punica granatum*	0.5–5.5 FW	HPLC-MS/MS	[21]
Pineapple	*Ananas comosus*	0.3 FW		[22]
Coffee beans (robusta)	*Coffee canephora*	5,800–8,000 DW	HPLC/ESI-MS	[23]
Coffee beans (arabica)	*Coffee arabica*	6,800–9,600 DW		
Sweet cherries	*Prunus avium*	8–120 FW	HPLC	[24]

More and more new studies are presenting the important physiological roles of melatonin in plants either as a growth regulator or as an environmental stress alleviator. This review discusses the importance of phytomelatonin as a therapeutic and as a physiologically important compound in plants, and methods for selection

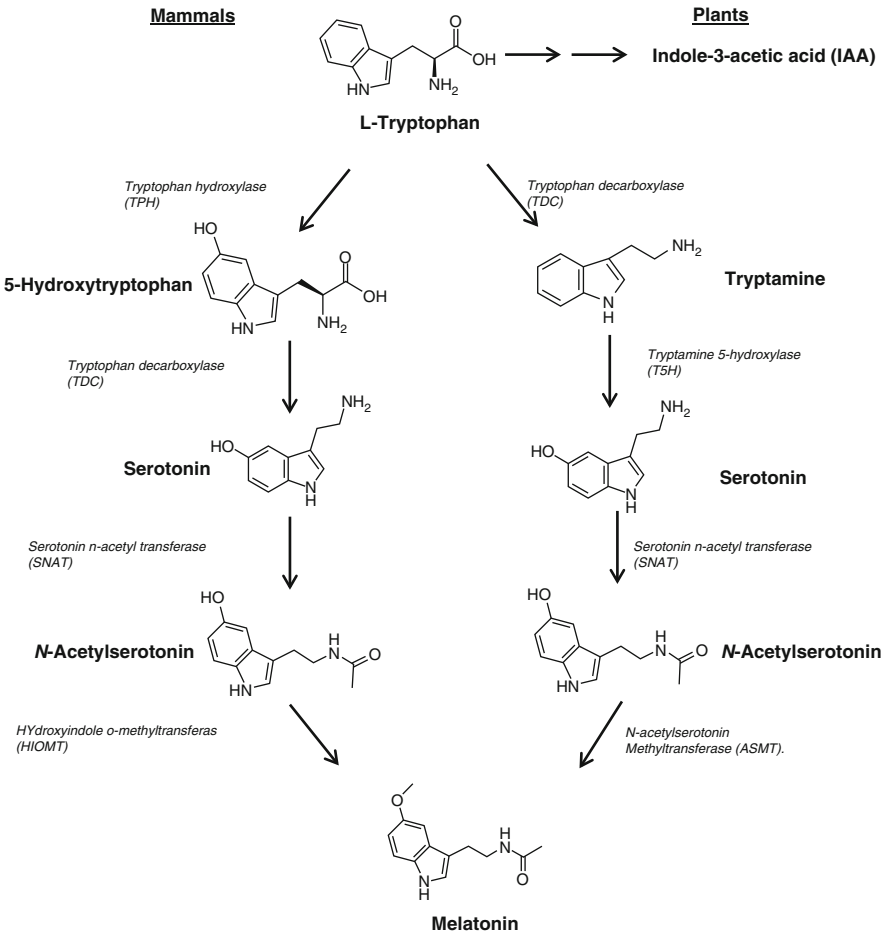

Fig. 19.1 The biosynthesis of melatonin

and propagation of melatonin rich medicinal plants. The potentiality to improve crop yield and stress tolerance through genetic manipulation of melatonin biosynthesis are also discussed.

19.2 Therapeutic Benefits of Melatonin

Melatonin regulates the normal circadian rhythm in animals; therefore, it is regularly used to treat jet lag, sleep and certain chronic neurological disorders. Melatonin has been helpful in treatment of sleep disorders and chronic insomnias such as Angelman Syndrome and Age-related insomnia [31–33]. Melatonin treatment is helpful in treating sleep disorders by advancing sleep onset time, reducing sleep

latency time, increasing total sleep time and reducing the number of night wakes [32]. Two to ten milligrams of melatonin was helpful in the treatment of sleep disorder of blind and neurologically disabled children without any side-effects [34]. Melatonin reduced sleep latency in children with developmental disabilities [35].

Therapeutic melatonin treatment is helpful in several circadian rhythm associated neurological disabilities, psychiatric and cognitive impairments such as seasonal affective disorder, bipolar disorder, unipolar depression, bulimia, anorexia, schizophrenia, panic disorder, obsessive compulsive disorder and cognitive disorders, dementia, epilepsy, attention deficit hyperactive disorder (ADHD) and other learning disabilities [2, 6, 36–40].

Melatonin not only controls circadian rhythm but also serves other important functions such as a powerful free radical scavenger making it an endogenous antioxidant that can scavenge a number of reactive oxygen (ROS) and nitrogen species [41]. The free radical scavenging capacity of melatonin and its metabolites, N1-acetyl-5-methoxykynuramine (AMK) and N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) are well established [42–45]. Melatonin has both lipophilic and hydrophilic antioxidant property, making it a broad-spectrum antioxidant [46]. Melatonin plays a significant role in regulating the activity and expression of enzymes such as glutathione peroxidase, superoxide dismutase and catalase, that act as the first line of defense against free radicals in organisms [47]. Recently melatonin has been identified as a scavenger of ROS produced in the ovarian follicle, and therefore contributes to oocyte maturation, embryo development and luteinization of granulosa cells, making a case for melatonin as a new cure for the treatment of infertile women [48]. A large number of publications on the anticancer effects of melatonin suggest its use for the treatment and prevention of oncological diseases [49]. Melatonin also has immunomodulatory/immune-enhancing properties, and therefore, is helpful in treating patients with compromised immune system [50–55]. Melatonin levels decrease with age and certain medical conditions such as coronary artery diseases and neurodegenerative disorders like Alzheimer's disease [56–59]. Thus, the therapeutic benefits of melatonin are pleiotropic including the treatment of insomnia, depression, cancers, and heart disease, as well as neuroprotectant, anti-ageing and immunostimulatory [31, 49, 50, 60–62].

19.3 Melatonin, a Bioactive Molecule in Medicinal Plants

Approximately four decades after the first report of melatonin isolation from the bovine pineal gland in 1958, melatonin was first detected in vegetables in 1995 using radioimmunoassay (RIA), high pressure liquid chromatography-fluorescence detection (HPLC-FD) and HPLC-mass spectrometry (MS) [15, 63, 64]. High levels of melatonin, several orders of magnitude higher than those detected in animal blood, have been found in medicinal plants, feverfew (*Tanacetum parthenium*), St John's Wort (*Hypericum perforatum*), and Huang-qin (*Scutellaria biacalensis*) (Table 19.1) [12]. Higher levels of melatonin in plants can be explained based on

the hypothesis that in eukaryotes, mitochondrion and chloroplast are the original sites of melatonin biosynthesis, and since plants contain both mitochondrion and chloroplast they have more number of sites to produce melatonin compared to mammals [65]. The higher levels of melatonin in plants may serve an important protective role by reducing oxidative damage to macromolecules such as lipids, proteins and DNA [16].

Chen et al. [14] identified melatonin in more than 100 commonly used Chinese medicinal herbs using HPLC-MS [14]. Melatonin levels in a number of these herbs was well above 1,000 ng g^{-1} compared to the normal average physiological plasma levels of melatonin of 10–60 pg mL^{-1} in humans (Table 19.1). The presence of high levels of melatonin virtually in all these herbs traditionally used to treat diseases provides important information regarding melatonin as one of the crucial bioactive constituents in medicinal plants [14]. Very high levels of melatonin have been reported in two popular beverages, coffee and tea, with seeds of freshly harvested *Coffea canephora* containing 115 ± 6 μg melatonin g^{-1} fresh weight as analyzed by HPLC and LC-MS-ESI and Chinese Longjing tea (green) containing melatonin 2.12 μg g^{-1} fresh weight (Table 19.1) [14, 23]. Serum melatonin levels and total antioxidant capacity of serum increases significantly after feeding subjects with melatonin-rich food stuff, indicating phytomelatonin may be an important protecting agent against oxidative damage in animals [15, 17, 66, 67]. In one of such studies done on rats, the authors concluded that walnuts contain melatonin and eating walnuts can increase the melatonin levels of the blood to levels where it could protect the animals against cardiovascular damage and cancer initiation and growth [17]. In two separate studies, consumption of melatonin containing vegetables raised urinary 6-sulfatoxymelatonin (aMT6-s) levels giving an indication that phytomelatonin may be a good source, providing health benefits associated with melatonin consumption [68, 69]. In a recent study done on healthy volunteers, dietary intake of melatonin from tropical fruit enhanced the urinary aMT6-s, with most significant increase in the aMT6-s concentrations observed with the consumption of pineapple (266 %) and banana (180 %) [70]. In animal studies, dietary intake of 5 μg day^{-1} of dietary melatonin for 3 weeks elevated amplitude and duration of nocturnal melatonin levels in tumor-bearing rats. The dietary melatonin uptake resulted in growth prevention in rat hepatome 7288 CTC in these rats [71]. The increased levels of melatonin in animals fed with plant foods containing melatonin as well as the ability of melatonin to readily cross both the placenta and blood-brain barrier and get into cells indicates its imminent significance in affecting biological functions in animals [15, 17, 67].

Leaf material, flowers, products, preparations and beverages of medicinal plants have sufficient melatonin and the consumption of these plants may be a good alternative to synthetic melatonin supplements such as tablets, pills, capsules, liquids etc. (Table 19.1). Medicinal plants such as St. John's wort and other plant species with high melatonin have been used for the treatment of neurological disorders and depression for a long time, giving a possibility that consumption of plants with high melatonin may have beneficial psychoactive effects in humans [12, 72–74]. Dietary melatonin from medicinal plants could be a potential medicinal component

important in protecting animals against oxidative damage [67]. Although melatonin is not the sole active ingredient in many medicinal plants it is found in considerably high levels in many medicinal plants and foods [12, 14, 75]. The efficacy of medicinal plant products is usually due to the combined effect of several different medicinally active phytochemicals or secondary metabolites [76]. The use of medicinal plants to treat human ailments have been tested for over thousands of years with minimum side-effects, providing best example for the use of medicinal plants as a rich source of phytomelatonin over the synthetic melatonin supplements.

19.4 Integrated Systems for Selection and Propagation of Melatonin Rich Medicinal Plants

The efficacy of plant-based medicinal products typically cannot be attributed to a single phytochemical, and there is a complex influence of the genetic make-up of the plant population used as well as the environmental conditions in which the plants are grown. It is therefore very difficult to produce plant products with uniformity and consistency in each of the active constituents of the medicinal plants [76]. The variability in the end product could be attributed to : (a) lack of genetic uniformity among the same species of medicinal plants, (b) fluctuating environmental conditions (including abiotic and biotic stresses) during the growth of the plants, (c) complex interaction among these factors (genetic × environment) influencing composition of medicinal plants, (d) contamination with biological and environmental pollutants, (e) adulteration with misidentified species, and (f) degradation of medicinal metabolites during harvesting, processing and handling [25, 76–78]. The problems associated with the variable genetic makeup and fluctuating growing conditions of the medicinal plants could be overcome by integrating *in vitro* plant propagation with the controlled environmental conditions. The superiority of this integrated production system employing large-scale clonal propagation through *in vitro* techniques combined with controlled environmental conditions in green house have been demonstrated in St. John's wort [72].

In vitro techniques provide a practical method for producing a large number of uniform plant materials. Some of the examples where *in vitro* plant multiplication is used to multiply medicinal plants rich in melatonin include – Huang-qin (*Scutellaria baicalensis*) [79], St. John's wort cv. 'New Stem' [80] and St. John's wort cv. 'Anthos' [73]. The propagation of various medicinal plant species in sterile controlled environments indicates a clear possibility of improving plant based medicinal products using *in vitro* clonal propagation techniques [25]. The genetic stability of melatonin rich medicinal plant such as Huang-qin has been evaluated using flow cytometry analysis with morphological and chemical profiling, indicating high level of genetic stability in *in vitro* cultures for a period of over 6 years [81].

Medicinal plants either grown in controlled environmental conditions or propagated *in vitro* using bioreactors with objective of production of medicinal plant biomass containing higher amounts of melatonin content. A range of bioreactors such

as modified air-lift, bubble column bioreactors, together with temporary immersion systems are available for the propagation of whole plants or isolated organs like shoots, bud-clusters and somatic embryos [82]. The use of bioreactor system has been described for the large scale propagation of *Anoectochilus*, apple, Chrysanthemum, garlic, ginseng, grape, *Lilium*, *Phalaenopsis*, potato, St. John's Wort and *Echinacea purpurea* [80, 83–86]. The use of bioreactors can also be helpful to test the effect of melatonin on the production of secondary metabolites, biomass accumulation and accumulation of enzymatic and non-enzymatic antioxidants in the medicinal and non-medicinal plants. Factors influencing the levels of secondary metabolites under controlled environment conditions include – temperature, humidity, light intensity, the supply of water, minerals, and CO_2 influence on the growth of the plant and secondary metabolite production [87]. The influence of physiological stage of plant on variation in melatonin levels in floral tissues of St. John's wort plantlets using hydroponic systems in the greenhouse has also been reported [88].

The effectiveness of this integrated system of combining propagation *in vitro* and in greenhouse could be further improved by selecting and developing elite varieties with higher amounts of medicinally active phytochemicals with the stability in their performance. Some attempts have been made to improve upon the chemical composition of the medicinal plants with melatonin. A melatonin-rich line of St John's wort was successfully developed using *in vitro* mutagenesis [72]. Several lines of St John's wort with different melatonin concentration ranging from 1 to 30 μg g^{-1} were isolated following mutagenesis and a stable melatonin-rich germplasm line containing >12-fold (1,200 %) melatonin content when compared with the wild-type plant was selected for multiplication of this elite germplasm [72]. This example indicates that naturally existing elite plant germplasm with high melatonin content or high melatonin germplasm generated through mutagenesis can be selected by quantifying the melatonin content of various lines through the analyses of the source material by liquid chromatography-mass spectrometry (LC/MS). The selected elite germplasm lines with high melatonin content can be clonally propagated *in vitro*, in greenhouses, and under different controlled environmental conditions including bioreactors to see the effect of environment on melatonin production and overall growth of plants.

19.5 Biosynthesis of Melatonin in Plants

The first investigation to identify melatonin biosynthetic pathway in plants was executed in St John's wort plants using radiolabel ^{14}C-tryptophan as an isotope tracer. A number of metabolites of melatonin biosynthetic pathway including ^{14}C-tryptamine, ^{14}C-5-hydroxytryptophan, ^{14}C-serotonin and ^{14}C-melatonin as well as most highly characterized metabolite of tryptophan, the plant hormone indole acetic acid (IAA) were recovered. This study revealed that similar to diurnal animals, plant melatonin and serotonin are biosynthesized from L-tryptophan [25].

In plants, L-tryptophan is a precursor of numerous primary and secondary metabolites such as IAA, indole glucosinolates and melatonin [28, 89].

Recent studies have shown that plants utilize a slightly different melatonin biosynthetic pathway when compared to animals. In diurnal animals, melatonin is biosynthesized from L-tryptophan *via* multistep metabolic pathway in which tryptophan is first converted to serotonin (5-hydroxytryptamine, 5-HT) by two enzymes, the first enzyme, tryptophan hydroxylase converts Trp to 5-hydroxytryptophan (5-HTP) and second enzyme, an aromatic amino acid decarboxylase gives 5-HT. Serotonin is further acetylated by aryl alkyl amine N-acetyl transferase (AANAT) to N-acetyl serotonin (NAS) followed by methylation catalyzed by hydroxyl indole-O-methyl transferase (HIOMT) to produce melatonin [90, 91] (Fig. 19.1). Serotonin is generally present in high levels and is not a rate limiting factor for melatonin biosynthesis [92]. The daily rhythm in melatonin production from serotonin is generally coupled to the activity of AANAT in animals [93]. In plants, the order of reactions for serotonin production from tryptophan seems to be reverse when compared to animals with hydroxylation reaction occurring before carboxylation in plants [94] (Fig. 19.1). However, Park et al. [30] showed that similar to animals, a hydroxylation reaction can occur before decarboxylation in rice as well, albeit at a low reaction rate. The plant genome also seems to lack the homolog for *AANAT,* however, another enzyme *SNAT* has been identified in rice and is encoded by a member of GCN5-related N-acetyl tryptamine (*GNAT*) super family. The mRNA expression studies show that *SNAT* is constitutively expressed whereas *ASMT* expression is inducible, suggesting that ASMT is the rate-limiting enzyme controlling the melatonin production in plants rather than AANAT as in animals [28–30, 95].

The pathways of melatonin biosynthesis seems to be complex in plants, therefore, more *in vivo* studies are necessary to identify melatonin biosynthetic pathway mutants in plants and to characterize orthologs for the biosynthetic pathway genes in higher plants. The *sekiguchi* mutant rice lacking functional tryptamine 5-hydroxylase (T5H) activity has been utilized to understand the serotonin and melatonin synthesis pathway in plants [27, 30]. However, identification of more mutants lacking melatonin in plants such as rice and *Arabidopsis* could be a significant advancement in our understanding of the biosynthetic pathway and role of melatonin in plants.

19.6 Role of Melatonin in Higher Plants

Melatonin has been hypothesized to play multiple roles in plants including a regulator of the circadian rhythm, regulator of reproductive development, protector against abiotic and biotic stresses like cold, heat, drought, salinity and diseases, and modulator of various growth and developmental activities in plants such as root and shoot development and increase in biomass (Table 19.3). However, only a limited number of studies have been conducted to determine the physiological function of melatonin in plants. Some of the studied physiological roles of melatonin are discussed below.

Table 19.3 Important biological roles of melatonin in higher plants

Reported biological role	References
Circadian rhythm oscillations and reproductive development	[74, 96–98]
Root biomass enhancement	[99–104]
Chlorophyll protection and photosynthesis induction	[77, 104–107]
Salinity stress tolerance	[108–111]
Cold and cryopreservation stress tolerance	[78, 112–115]
Water stress and drought tolerance	[106]
Light and high temperature stress tolerance	[116]
UV-B radiation stress tolerance	[117]
Herbicide-induced oxidative stress	[107]
Chemical agent stress tolerance	[77]
Delaying in dark-induced senescence	[77, 106, 118]
Biotic stress tolerance (apple blotch)	[119]

19.6.1 Circadian Rhythm Oscillations and Reproductive Development

Few experimental studies have been performed to test if melatonin acts as a chemical signal of darkness in plants, similar to its role in animals. The photoperiodic modulation in mammals and birds is a function of the duration and timing of the melatonin signal [120]. An oscillating behavior of melatonin was observed in the short-day plant *Chenopodium rubrum* L. with increase in the levels of melatonin observed 4–6 h after darkness in 12 h light: 12 h dark cycle and after 2 h in 16 h light: 8 h dark cycle, indicating that plants have a diurnal trend of melatonin production similar to animals. However, these results indicated that timing rather than the duration of photoperiod determined the levels of melatonin [96]. In another study conducted on *Pharbitis nil* and tomatoes (*Solanum lycopersicum*) no significant difference in the melatonin levels was observed with respect to light/dark cycle. This study gave in conclusive results regarding the circadian rhythm of melatonin production as observed previously in *Chenopodium rubrum*, indicating that photoperiodic change in melatonin levels might be species dependent in plants [121]. Although the role of melatonin has been implicated, evidence is still lacking to describe any definitive role of melatonin in regulating the circadian rhythms, plant light/dark responses [105].

Melatonin and its agonists (2-I-melatonin, 6-Cl-melatonin, CGP 52608) and 5-hydroxytryptamine also affected flowering in *Chenopodium rubrum*, as their application decreased flowering of plants in 12 h light: 12 h dark cycle. This effect of melatonin was observed only if melatonin was applied before the dark period or during the first half of the dark period, indicating that melatonin affects some early steps of the floral development [122]. In a study on *Datura metel*, the highest levels of melatonin were detected in immature buds and developing ovules with the levels

decreasing as the flower buds and the fruit matured, indicating that melatonin might play an important role in protecting the reproductive tissues during flower and seed formation [74]. The presence of high levels of melatonin in seeds may be required to prevent oxidative damage, particularly to highly vulnerable germ tissue [16]. Recently, Park et al. [123] observed that melatonin synthesis was induced in panicles of rice during the reproductive stages, whereas no induction was observed in flag leaves [123]. Together, these results indicate an important role of melatonin in regulating circadian rhythm and reproductive development in plants. However, studies have to be carried out to understand the mechanism of regulation of these important processes in plants.

19.6.2 Enhancement of Root Biomass, Chlorophyll Protection and Photosynthetic Induction

There are a number of studies that reported the enhancement of plant biomass, especially root biomass by melatonin. The increase in root biomass and altered architecture are mainly related to the growth regulating property of melatonin in plants similar to IAA. Murch et al. [124] showed that melatonin and serotonin, a tryptophan derivative and a precursor of melatonin, play an important role in plant morphogenesis [25]. In St. John's wort plants, higher endogenous levels of melatonin were associated with *de novo* root formation, whereas higher serotonin levels corresponded to increase in the shoot formation on the explants, indicating that changes in endogenous concentrations of serotonin and melatonin accompany plant morphogenesis *in vitro* [124]. A potential role of melatonin as a growth regulator in plants is hypothesized [99]. Melatonin (0.1 μM) application stimulated root growth in young (2-days old) and etiolated *Brassica juncea* seedlings, however, the higher concentration of melatonin (100 μM) inhibited root growth and the older seedlings (4-days old) were less responsive to the stimulatory and inhibitory effects of melatonin on root growth. The application of lower levels of melatonin (0.1 μM) was also shown to increase the levels of free indole 3-acetic acid (IAA) in the roots of *Brassica juncea* seedlings [100]. Similar to IAA, melatonin promoted vegetative growth in etiolated lupin (*Lupinus albus*) hypocotyls [125]. Both melatonin and IAA were also shown to promote lateral and adventitious roots in the etiolated hypocotyls of lupin [101]. In a study on *Arabidopsis thaliana* plants, the application of melatonin modulated root architecture by increasing the production of lateral and adventitious roots without affecting primary root morphology. These changes in root architecture elicited by melatonin were believed to be independent of auxin signaling, as melatonin application had no effect on activity of auxin-responsive marker constructs, *DR5:uidA, BA3:uidA* and *HS::AXR3NT-GUS* [102]. In another study, transgenic rice plants overexpressing sheep serotonin N-acetyltransferase with enhanced melatonin levels showed increased seminal root growth (higher seminal root length and root weight) compared with the wild-type plants. Treatment of wild-type rice with 0.5 and 1 μM melatonin also promoted seminal root growth

under continuous light [103]. The RNA-sequence analysis of the cucumber seedlings primed with melatonin and grown in saline conditions, showed higher expression level of peroxidase-related genes and some transcription factors in the melatonin treated tissue when compared to the control. The authors suggested that increase in the lateral root formation in the melatonin treated plants was probably due to the ROS scavenging activity of melatonin that is independent of auxin-related activity. Serotonin also stimulated lateral root development in *Arabidopsis thaliana* at concentrations ranging from 10 to 160 μM putatively by acting as a natural auxin inhibitor in the plants [126]. Sarropoulou et al. [104] discovered that cherry rootstock plants PHL-C (*Prunus avium* L. × *Prunus cerasus* L.) from shoot tip explants cultured in MS medium containing low levels of melatonin showed enhanced root regeneration, photosynthetic pigments, biomass, total carbohydrate and proline content [104]. Application of 1 μM melatonin to the explants increased the root length of the plants by 2.5 times and the fresh weight of the roots was four times higher than the control plants. However, the higher concentrations of melatonin had a negative effect on root biomass, reducing the total number of roots, the fresh weight and rooting percentage [104]. The lower concentrations of melatonin were also effective in increasing the photosynthetic pigments, carbohydrate levels and proline content in the plants. Based on this study, the optimum melatonin level to increase biomass production of cherry plants is between 0.05 and 1 μM [104]. Due to its high antioxidant activity, melatonin has a potential to prevent chlorophyll degradation in plants. There have been a few studies to support the role of melatonin as a protector of chlorophyll and other pigments in plants and thereby enhancing the photosynthetic capacity.

As an antioxidant, melatonin certainly has the capability of protecting against the degradation of chlorophyll, resulting from the oxidative damage in the plant cell. The ROS species generated by oxidative stresses such as high light, cold and hot temperatures can lead to chlorophyll degradation and reduction in the photosynthetic activity [127]. The protection of chlorophyll degradation is crucial for the survival, growth and development of the plant. First evidence of the chlorophyll protective role of melatonin came from Arnao and Hernandez-Ruiz [77], where the authors showed that barley leaves incubated in 1 mM melatonin for 48 h contained twice as much chlorophyll content when compared to the untreated control plants [77]. Melatonin has been shown to be important in protecting photosystem II against the oxidative damage in *Chara australis* and *Malus hupehensis*, and thereby helped to maintain better function of PSII, suggesting that melatonin protects chlorophyll and possibly photosynthetic proteins as well [105, 106].

These studies provide evidence for melatonin being an important compound in plants for regulating plant morphogenesis, especially root architecture. Due to its important role in *de novo* root regeneration, it should be tested in *in vitro* studies in some difficult to root plant species. Exogenously applied melatonin has been shown to increase the root biomass in several species as described above; therefore, its use as a bioactive compound to enhance root biomass in various bioreactor systems should be tested. Several studies on medicinal and recalcitrant plants can play a significant role in establishing the role of melatonin as an important regulator of

plant morphogenesis and as an enhancer of biomass and secondary metabolite production in different plant species. Also, studies at molecular level are required to understand the mechanism through which melatonin induces root regeneration and biomass accumulation in higher plants.

19.6.3 Abiotic and Biotic Stress Tolerance

The role of melatonin as a protector of various environmental stresses has been proposed in several studies. Melatonin has been hypothesized to have a role in reducing abiotic and biotic stresses including salinity, light, high temperature stress, cold and cryopreservation stress, water stress and drought, UV-B radiation, stress from chemical agents such as sodium chloride, zinc sulfate and hydrogen peroxidase [77, 106, 108, 113–116, 119, 128, 129]. Severe environmental stress imposed on plants lead to oxidative stress, which occur due to the generation of ROS in the stressed plants and tissues. If the ROS are not scavenged properly it can lead to irreversible damage to the plant tissue resulting in physiological incompetence and eventually cell death [130]. Melatonin can scavenge ROS generated in the plant tissue exposed to the environmental stress, thereby reducing damage to the tissues by acting as a cyto-protective agent. A large number of studies conducted over the past decade have shown that the primary role of melatonin in the plant is to work as an antioxidant and protect it from different types of environmental stresses. The role of melatonin in attenuating environmental stresses that affect plant growth and development and biomass production may be very useful for the production of bioactive compounds in bioreactors.

Tan et al. [131] made an observation that the plants subjected to cold stress contained significantly higher concentrations of melatonin, suggesting that melatonin might be involved in environmental stress tolerance in plants [131]. Lei et al. [78] showed that exogenous application of melatonin to carrot suspension cells attenuated cold-induced apoptosis [78]. They also observed an increase in the melatonin levels in St John's wort and *Aloe vera* plants that were transferred from room temperature to 4 °C. The authors suggested that the protective effect of melatonin against cold stress might be related to induction of polyamines [78]. Melatonin treatment also improved the survival and recovery of cryopreserved *Rhodiola* callus and cryopreserved American elm (*Ulmus americana*) shoot tips [113, 114]. More recently, exogenous melatonin treatment was shown to alleviate cold stress damage to *Arabidopsis* plants [115]. Many important cold acclimation genes including the C-repeat-binding factors (CBFs)/Drought Response Element Binding factors (DREBs), *COR15a*, a cold-responsive gene, *CAMTA1*, a transcription factor involved in freezing and drought-stress tolerance and transcription activators of reactive oxygen species (ROS)-related antioxidant genes, *ZAT10* and *ZAT12* were up-regulated by melatonin following cold treatment [115].

Melatonin has also been hypothesized to play a role in salinity tolerance in plants. The salinity stress causes hyper-osmolarity which leads to the change in ion

homeostasis and as a result secondary stresses such as oxidative damage occur to the tissue [132]. In *Malus hupehensis* melatonin treatment reduced salt stress damage by preventing chlorophyll damage and photosynthetic activity decline, generally caused by salt stress. Melatonin reduced the oxidative stress from salinity by directly scavenging H_2O_2, activation of the antioxidant enzymes, ascorbate peroxidase, catalase, and peroxidase and possibly *via* up-regulation of the expression of ion-channel genes, *MdNHX1* and *MdAKT1* [108]. Melatonin pre-treatment increased lateral root primodia in cucumber (*Cucumis sativus* L) plants compared to the control plants grown in saline growth conditions [109]. Endogenous melatonin levels increased in the barley roots exposed to different chemical stresses including salinity (NaCl) stress [77]. In another study, to determine the change in the endogenous levels of melatonin in lupin plant tissues exposed to different stresses, it was found that salinity stress resulted in the most pronounced change in endogenous melatonin levels [111].

In recent years many studies have shown the importance of melatonin in the alleviation of various plant stresses. Melatonin has been linked to physiological delay in dark-induced senescence in apple and barley observed through physiological and molecular analysis [106, 110, 118]. In cucumber (*Cucumis sativus*), melatonin application promoted water-stress tolerance, lateral root formation, and seed germination rate [128]. Melatonin played a significant role in the germination process of negatively photoblastic and the thermosensitive *Phacelia tanacetifolia* seeds by reversing the inhibitory effect of light and high temperature [116]. Melatonin treatment was also helpful in providing resistance against the biotic stress caused by Marssonina apple blotch disease to apple trees [119]. The apple plants pre-treated with melatonin showed less severe symptoms of the apple blotch compared to the non-treated plant, indicating the importance of melatonin as a promising cultivation strategy to protect plants against this pathogen [119].

The antioxidant capacity of melatonin is the most probable reason for its role in the mitigation of the environmental stress damage to plants. In animals, the antioxidant and cyto-protective role of melatonin is well established [2, 41, 47, 133–138]. Similar antioxidant and cell protective role of melatonin is emerging in plants also; however, more evidence is necessary especially at the biochemical and molecular levels to better understand the protective mechanisms of melatonin in plants.

19.7 Melatonin as a Biotechnological Target to Increase Crop Yield and Environmental Stress Tolerance

Melatonin is a naturally occurring plant compound that is medicinally important and has been shown to attenuate several abiotic and biotic environmental stresses and enhance plant growth and development. Therefore, the genetic manipulation to enhance melatonin biosynthesis offers a unique opportunity to increase crop yields by enhancing biomass and protecting plants from environmental stresses. Genetic transformation can also be utilized to increase melatonin levels of medicinal and

non-medicinal food plants, thereby, adding value to crop plants. Modulating endogenous levels of melatonin by direct manipulation of genes involved in melatonin biosynthesis could increase crop yield and plant performance in a uniform and predictable manner, thereby providing economic advantages to growers around the world.

Genetic transformation to increase medicinal and nutritional components in plants is widely used for plant improvement. Genetic manipulation has been applied to obtain biofortified crops such as provitamin A rich rice (golden rice), tomato, potato and maize; folate rich tomato and rice; and iron rich rice [139–145]. Genetic engineering methods have also been used to improve the secondary metabolite yields in culture systems such as *Catharanthus roseus* [146]. Therefore, these methods can likely be used to increase melatonin content in plant culture systems for medicinal and physiological benefits.

In order to increase the melatonin content in plants, Okazaki et al. transformed Micro-Tom tomato plants with a cDNA-coded *AANAT*, a rate limiting enzyme in melatonin biosynthesis, from *Chlamydomonas reinhardtii*. The resulting transformed plants were shown to contain higher levels of melatonin. This study demonstrated that the gene transfer can be used to increase melatonin levels in food plants, thereby increasing their medicinal value [147]. These melatonin-rich plants will be useful to elucidate the physiological role of melatonin in plants. In a very recent study, Wang et al. introduced sheep *AANAT* and *HIOMT* genes *via* genetic transformation into Micro-Tom tomato. These transgenic plants contained higher melatonin levels but reduced IAA levels, resulting in loss of 'apical dominance' in the transgenic plants. The authors also reported increased drought tolerance in *HIOMT* lines as a putative effect of higher melatonin levels in the plants [148]. In another study by Zhang et al., transgenic tobacco (*Nicotiana sylvestris*) plants expressing two melatonin synthetase genes, human *AANAT* gene and human *HIOMT* were obtained [117]. Higher levels of melatonin were present in transgenic plants when compared to the non-transgenic control plants. The authors also observed that less DNA damage caused by ultra-violet (UV)-B radiation was present in protoplasts isolated from transgenic plants producing more melatonin when compared to the non-transgenic plants, indicating that the enhanced melatonin production in crop plants such as tobacco can be useful in preventing them against the damage caused by environmental stresses such as UV-B damage.

The impact of melatonin-rich transgenic (MRT) rice plants expressing sheep serotonin *N*-acetyl transferase on providing resistance to herbicide-induced oxidative stress was observed by Park et al. [107]. The MRT rice plants showed a resistant phenotype, high chlorophyll levels, and low malondialdehyde and hydrogen peroxide contents when exposed to the singlet-oxygen-generating herbicide butafenacil (0.1 μM) whereas the control plants necrotized under the same conditions. This study is the first report showing that the melatonin gain-of-function rice mutants have a practical advantage in scavenging ROS, and therefore, has potential to be used as a strategy for coping with environmental stresses in plants [107]. In a separate study, transgenic rice seedlings producing higher melatonin by ectopic overexpression of human *SNAT* showed better cold resistance, indicating that melatonin

plays an important role in cold resistance in plants [112]. These studies provide sufficient evidence that biotechnological approaches to enhance melatonin production have a practical advantage for increasing resistance of plants against environmental stresses. Therefore, genetic manipulation to increase melatonin production can be a practical strategy toward generating high-yielding transgenic plants. All the above studies have used melatonin biosynthesis genes from animals; therefore, the acceptance of such plants for agricultural use may be very low at least in the near future. In the further research, non-transgenic approaches for higher melatonin production should also be explored. The plant genes from melatonin rich medicinal plants should be used for genetically modifying crops for higher melatonin production. The use of plant genes might also be beneficial in enhancing the concentration of melatonin in plants, as plants generally have higher endogenous melatonin levels compared to mammals. For example, *SNAT* and *ASMT* from higher melatonin producing plant such as St. John's wort might be much more beneficial in increasing the melatonin levels in lower melatonin producing plant such as tobacco. Therefore, it is essential to clone and characterize melatonin biosynthesis genes from high melatonin producing medicinal plants such as feverfew, St John's wort, and Huangqin and other herbs and food crops including tea and coffee.

19.8 Conclusions and Future Directions

Melatonin was first identified as a key hormone that regulates circadian rhythm in animals. Later, a number of therapeutic uses other than regulating sleep/wake cycle were recognized in animals and humans. Further research established melatonin as a powerful antioxidant that has beneficial effects in the treatment/prevention of neurological and sleep disorders, cancers, immunological disorders etc. In plants, melatonin is detected in medicinal plants, beverages, fruits and vegetables. Animal studies with dietary intake of phytomelatonin increased melatonin levels in body fluids indicating that phytomelatonin is bioavailable and can be a good source for human consumption. In this context, the selection of melatonin rich plants and their large scale multiplication in integrated controlled environment systems has tremendous potential for novel natural health products. The production of melatonin rich cells, tissues and organ in large-scale bioreactors as a source of phytomelatonin for developing formulations to treat specific health conditions will be of a special interest.

In addition to a huge potential of phytomelatonin as a therapeutic, melatonin and related compounds can be important tools for increasing the yields of crops through their action as a growth regulator and environmental stress protector. Several studies in the past decade have shown the relevance of melatonin as a growth regulator and as an environmental stress protector alleviating biotic and abiotic stress in plants and increasing overall agricultural productivity. However, a great deal of work remains to be established to understand the molecular mechanisms of melatonin biosynthesis and its multiple modes of action to fully explore the potential of this

unique metabolite. New systems to investigate the effects of endogenous and exogenously applied melatonin as well as mutants with altered biosynthesis and/or function will be very useful for research on melatonin mediated responses in plants and their use in agriculture.

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Part IV
Strategies for Production of Bioactive Compounds, Large-Scale Cultures and Metabolic Engineering of Selected Metabolites

Chapter 20
Strategies for Enhanced Production of Plant Secondary Metabolites from Cell and Organ Cultures

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Abstract Plant cell and organ cultures have emerged as a potential source of secondary metabolites which are used as pharmaceuticals, agrochemicals, flavours, fragrances, colouring agents, pesticides and food additives. Various strategies have been developed over past decades for biomass accumulation and synthesis of valuable compounds. Biosynthesis of secondary metabolites are generally not directly associated with cell growth. For the enhanced production of secondary metabolites, selection of high-yielding cell or organ clones, optimization of medium and physical factors which regulate the growth and accumulation of biomass are usually done at first, then in the secondary metabolite production stage, various strategies such as elicitation, precursor feeding, replenishment of nutrients are conducted. Permeabilization and immobilization are also proved to be important for the biosynthesis of secondary metabolites in some cases. By these strategies, it is possible to produce enormous biomass with improved accumulation of secondary metabolites.

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Abbreviations

2, 4-D	2, 4-dichlorophenoxy acetic acid
2-iP	2-isopentenyladenine
ABA	Abscisic acid
B5	Gamborg's medium
BA	Benzyladenine
DMSO	Dimethylsulfoxide
DW	Dry weight
FW	Fresh weight
GA	Gibberellic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
LS	Linsmaier and Skoog medium
MS	Murashige and Skoog medium
PUFAs	Polyunsaturated fatty acids
SH	Schenk and Hildebrandt medium

20.1 Introduction

Secondary metabolites are a diverse group of organic compounds that are generally produced by plants to interact with biotic environment and for the establishment of defense mechanism [1]. Most secondary metabolites such as terpenes, phenolics and alkaloids which are classified based on their biosynthetic origin and chemical structures, show different biological activities and many of them have been used as pharmaceuticals, agrochemicals, flavours, fragrances, colours and food additives [2]. Secondary metabolite production is traditionally produced through field cultivation of plants, but this conventional approach suffers various disadvantages such as low yields, instability of their contents due to geographical, seasonal and environmental variations, occupation of large amount of lands, and heavy use of labors. Therefore, plant cell and organ cultures have emerged as an attractive alternative to whole plant cultivation for production of secondary metabolites [2]. However, there are still drawbacks in the production of metabolites through cell and organ cultures due to the instability of cell lines, low yields, slow growth and scale-up problems [3]. An important requirement in secondary metabolite synthesis is to understand the metabolic pathways and the enzymology of product formation as proposed by Dornenburg and Knorr [4], but unfortunately the knowledge of plant metabolic pathways is still

Table 20.1 Strategies to enhance the production of secondary metabolites in plant cell and organ cultures

Stage 1 – Biomass accumulation
1. Selection of efficient cell lines or clones
2. Medium optimization
(a) Selection of suitable medium and salt strength
(b) Carbohydrate source and concentration
(c) Nitrate levels
(d) Phosphate levels
(e) Growth regulator levels
3. Inoculum size
4. Optimization of the cultural environment
(a) Temperature
(b) Illumination
(c) Quality of light or combination of lights
(d) Medium pH
(e) Aeration and agitation
State 2 – Accumulation of bioactive compounds
5. Elicitation
6. Nutrient feeding
7. Precursor feeding
8. Permeabilization
9. Immobilization
10. Two phase system
11. Biotransformation
12. Organ cultures
13. Large-scale cultures

very limited. In spite of all these odds, cell and organ cultures have enormous potentialities for the production of industrially useful secondary compounds. Various strategies like strain improvement, optimization of medium and culture environments, elicitation for the accumulation of secondary compounds, nutrient and precursor feeding, permeabilization, immobilization and biotransformation methods have been developed over the years for high biomass accumulation and efficient synthesis of secondary compounds (Table 20.1). Biomass accumulation and biosynthesis of metabolites through cell and organ cultures may be looked at as a two step process in many cases: (1) involvement of cultured cells and organs in growth, multiplication and accumulation of biomass at the initial stage, and (2) biosynthesis of metabolites from the accumulated cells in the later stage. In the earlier reports, accumulation of biomass and production of secondary metabolite events were dealt simultaneously, however, it is possible to achieve both higher biomass accumulation and enhanced metabolite production by following a two step process, i.e. focusing on the accumulation of biomass strategy in the first step, and applying strategies for metabolite biosynthesis stimulation in the second step of the cultivation. The experimental strategies for the production of secondary metabolites by plant cell and organ cultures with suitable examples are summarized in this article.

20.2 Selection of Cell Lines and Clones

Initiation of cell and organ cultures begins with the choice of a parent plant with high content of the desired product for callus or organ induction to obtain high-yielding cell/organ lines. Secondary metabolite accumulation in plants is specific to its genotype. For example, the concentration of bacoside A (a triterpenoid saponin) varies among different genotypes ranging from 3.53 to 18.36 mg g^{-1} DW (Table 20.2) [5]. Similarly, the amount of camptothecin (a quinoline alkaloid) varies among the different species (*Camptotheca* spp., *Ervatamia* spp., *Ophiorrhiza* spp., *Nothapodytes* spp.) and even in different organs of the plant (0.03–0.4 % DW) [6]. Bacoside A is a nootropic and camptothecin is an anticancerous drug. A choice for suitable genotype and a suitable organ is essential for callus or organ induction to raise the cell or organ cultures for the production of these compounds.

Isolation and selection of cell and organ lines for growth (i.e. for higher biomass accumulation) as well as for accumulation of metabolites are most important. Earlier, selection of cell lines was carried out by visual screening if the product of interest would be a pigment. In *Euphorbia milli* and *Daucus carota*, enhanced anthocyanin production by clonal selection and visual screening has been reported [7, 8].

Table 20.2 Bacoside A concentration in *Bacopa monnieri* from different locations of Karnataka, India

Sl. No.	Accession no.	Location	Concentration of bacoside (mg g^{-1} DW)
1.	Bm1	Bangalore	6.55 ± 0.52
2.	Bm2	Belgaum	18.36 ± 1.65
3.	Bm3	Belgaum	5.19 ± 0.34
4.	Bm4	Belgaum	3.59 ± 0.41
5.	Bm5	Belgaum	7.81 ± 0.55
6.	Bm6	Belgaum	5.46 ± 0.42
7.	Bm7	Dharwad	3.53 ± 0.49
8.	Bm8	Dharwad	6.43 ± 0.36
9.	Bm9	Dharwad	5.70 ± 0.24
10.	Bm10	Gadag	6.83 ± 0.42
11.	Bm11	Haveri	10.56 ± 0.82
12.	Bm12	Haveri	6.90 ± 0.43
13.	Bm13	Haveri	6.67 ± 0.38
14.	Bm14	Kolar	8.52 ± 0.62
15.	Bm15	Mysore	7.24 ± 0.47
16.	Bm16	Shimoga	5.53 ± 0.26
17.	Bm17	Shimoga	7.93 ± 0.35
18.	Bm18	Shimoga	9.42 ± 0.77
19.	Bm19	Uttara Kannada	6.63 ± 0.34
20.	Bm20	Uttara Kannada	10.82 ± 0.86
21.	Bm21	Uttara Kannada	5.04 ± 0.31
22.	Bm22	Uttara Kannada	4.84 ± 0.18

However, selection made by analysis of growth of cell lines or root clones (adventitious or hairy roots) in suspension cultures followed by quantification of the desired product is considered to be superior to visual selection techniques. The growth kinetic analysis method is also followed in some cases. In *Orthosiphon stamineus* two cell lines were selected and identified which produced higher amount rosmarinic acid through cell suspension culture [9]. Quantification of metabolites by high pressure liquid chromatography and radioimmuno-assay are also followed for screening high yielding cell lines [10, 11].

20.3 Medium Optimization

A number of chemical and physical factors influence biomass accumulation and synthesis of secondary metabolites in plant cell and organ cultures. Medium composition is a basic and critical factor affecting the cell physiology and metabolism. Some of the key factors are choice of culture medium, suitable salt strength of the medium, sugar levels, nitrate levels, phosphate levels and growth regulator levels in the medium [2, 4, 12, 13].

20.3.1 Influence of Nutrient Medium and Salt Strength

Various types of media formulations were tested and utilized earlier for the establishment of cell and organ suspension cultures for the production of secondary metabolites. Murashige and Skoog (MS) [14], Gamborg's (B5) [15], Schenk and Hildebrandt (SH) [16], Linsmaier and Skoog (LS) [17] media are widely used. The B5 medium of Gamborg et al. [15] was initially used for callus and suspension cultures. This medium differs from MS medium in having much lower amounts of nitrates in the form ammonia hence, suitable for certain cell cultures. The appropriate concentration of medium constituents (salt strength) is crucial for the growth of isolated cells and organs. In ginseng adventitious root cultures, maximum biomass and growth rate were obtained in 0.75 strength MS medium and ginsenoside content and yield were higher in 0.5 salt strength MS medium [18]. The full strength MS medium was suitable for both biomass and gymnemic acid accumulation (Fig. 20.1) in cell suspension cultures of *Gymnema sylvestre* [19]. Among the 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 strength MS medium tested, full strength (1.0) medium was found better for biomass accumulation and withanolide A production in *Withania somnifera* cell suspension cultures [20]. Interestingly, some medium salts like calcium chloride and sodium chloride could be working as signal inducers to stimulate secondary metabolism. The inducing effects of calcium ion and sodium ion on the intracellular calcium signaling pathway were well demonstrated in higher plant and mushroom cell cultures for production of bioactive secondary metabolites like ginseng saponin and ganoderic acid [21, 22].

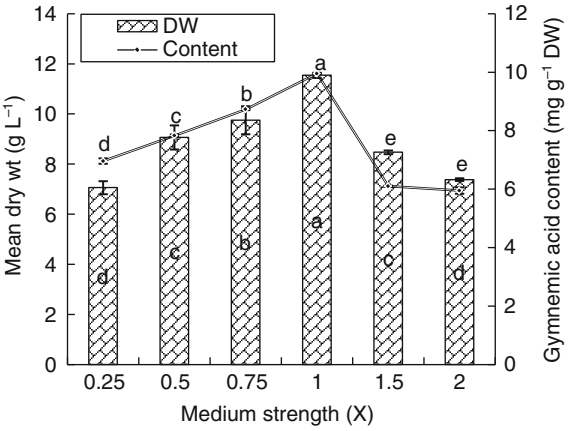

Fig. 20.1 Effect of medium strengths on biomass accumulation and gymnemic acid production in cell suspension cultures of *Gymnema sylvestre*. Five hundred mg of cells were cultured in 50 mL of MS medium supplemented with 2.0 mg L^{-1} 2,4-D + 0.1 mg L^{-1} KN and 30 g L^{-1} sucrose for 4 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test

20.3.2 Influence of Carbon Source and Concentration

Plant cell cultures are usually grown heterotrophically using simple sugars such as glucose, fructose, maltose, sucrose and their combinations as an energy and carbon source. Among the various sugars tested, sucrose was found to be an ideal carbohydrate source for the biomass accumulation (11.56 g L^{-1} DW) and the highest production of gymnemic acid content (9.95 mg g^{-1} DW) (Table 20.3) [19]. Wang and Weathers [23] tested the effect of sugars on production of artemisinin in hairy root cultures of *Artemisia annua* and found a maximum production of artemisinin when hairy roots were grown in the medium supplemented with glucose, whereas the level of artemisinin produced in the medium supplemented with fructose was twice that in the medium supplemented with sucrose. Similarly, concentration of carbohydrate supplemented to the medium greatly affects the biomass and metabolite production. For example, of the various levels of sucrose (1–8 % w/v) tested in *Gymnema sylvestre* cell cultures, 3 % sucrose in the medium favoured the accumulation of biomass (Fig. 20.2), whereas the highest amount of gymnemic acid (10.1 mg g^{-1} DW) was accumulated in the medium supplemented with 4 % sucrose. In *Ginkgo biloba* cell cultures, 3 % sucrose was good for biomass accumulation whereas higher concentration of 5 and 7 % sucrose favoured the production of ginkgolides and bilobalides [24]. In *Bacopa monnieri* shoot cultures, 2 % sucrose was found optimal in the tested range (0–6 %, w/v) for biomass accumulation and sucrose-free medium accumulated maximum amount of bacoside-A [25]. The initial sucrose concentration (i.e. 20, 30, 40 and 60 g L^{-1}) had a significant effect on the production of ginseng saponin in suspension

Table 20.3 Effect of different carbohydrate sources on biomass accumulation and gymnemic acid production in *Gymnema sylvestre* cell suspension culture

Carbohydrate source (3 %)	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	Growth ratio	Gymnemic acid content (mg g^{-1} dry weight)
Sucrose	125.67 a	11.56 a	10.16	9.95 a
Glucose	118.00 a	10.66 a	9.69	8.56 d
Fructose	100.91 b	9.23 b	8.39	6.58 f
Maltose	86.50 c	7.68 c	6.98	6.99 e
Glucose + fructose (1:1)	100.90 b	9.07 b	8.24	8.72 c
Fructose + sucrose (1:1)	94.25 c	8.36 c	7.60	9.26 b
Sucrose + glucose (1:1)	117.22 a	10.66 a	9.69	9.24 b

Cultures were grown in 250 mL conical flaks containing 50 mL of MS medium supplemented with 2.0 mg L^{-1} 2, 4-D + 0.1 mg L^{-1} KN for 4 weeks. Mean values with common letter within each column are not significantly different at $P \leq 0.05$ according Duncan's multiple range test

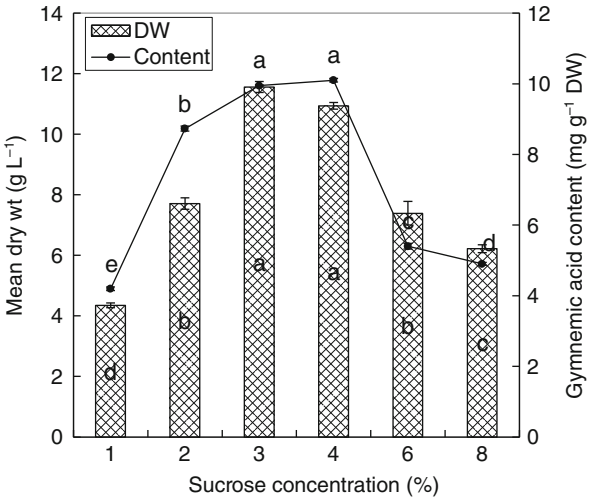

Fig. 20.2 Effect of different sucrose concentrations on the biomass accumulation and gymnemic acid production in cell suspension cultures of *G. sylvestre.* Five hundred mg of cells were cultured in 50 mL of MS medium supplemented with 2.0 mg L^{-1} 2,4-D + 0.1 mg L^{-1} KN for 4 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test

cultures of *Panax notoginseng* [26]. A high sugar level was favorable to the synthesis of ginseng saponin, may be due to the high osmotic pressure which was caused by high sugar concentration [27]. But, the cell growth was reduced at an initial sucrose concentration of 60 g L^{-1}, the maximum production of crude ginseng saponins (0.86 g L^{-1}) was achieved at an initial sucrose concentration of 40 g L^{-1} [26]. The osmotic stress created by sucrose alone or with other osmotic agents was found to regulate anthocyanin production in *Vitis vinifera* cell

suspension cultures [28]. A dual role of sucrose as a carbon source and an osmotic agent was observed in *Solanum melongena* [29]. Recently, sugars have been recognized as signaling molecules that affect growth, development and metabolism of cultured cells [23]. Therefore, the selection of a suitable carbohydrate source at appropriate concentration is a key criterion for secondary metabolite production in cell and organ cultures.

20.3.3 Influence of Nitrogen Source

The growth and metabolite accumulation in cell and organ suspension cultures was found to be influenced by a suitable nitrogen source and its concentration. The plant tissue culture media such as MS, LS, SH, and B5 contain both nitrate and ammonium as source of nitrogen. However, nitrogen present in the ammonium/nitrate and overall levels of total nitrogen have markedly affected both biomass accumulation and production of secondary plant products. In the shoot cultures of *Bacopa monnieri*, the effect of macro elements was tested by varying the levels of NH_4NO_3, KNO_3, $CaCl_2$, $MgSO_4$ and KH_2PO_4 in the MS medium each at 0.05, 1.0, 1.5 and 2.0 strengths and optimum number of shoots (99.33 shoots explant^{-1}), biomass (0.150 g DW) and the highest production of bacoside A (17.9 mg g^{-1} DW) were obtained with 2× strength NH_4NO_3 [30]. The effect of nitrogen supplements like $NH4^+$/$NO3^-$: 0.00/18.80, 7.19/18.80, 14.38/18.80, 21.57/18.80, 28.75/18.80, 14.38/0.00, 14.38/9.40, 14.38/18.80, 14.38/28.20 and 14.38/37.60 (mM mM^{-1}) when tested they found that shoot biomass and bacoside A content were optimum when the $NO3^-$ concentration was higher than that of $NH4^+$ (ratio of 14.38/37.60 mM; Fig. 20.3). In another report, reduced level of $NH4^+$ and increased levels of $NO3^-$ promoted the production of gymnemic acid and withanolide A [31, 32, 33]. Reduced levels of total nitrogen improved the production of capsaicin in *Capcicum frutescens*, and anthraquinones in *Morinda citrifolia* [34, 35]. However, complete elimination of nitrate in cultures of *Chrysanthemum cinerariaefolium* induced a twofold increase in pyrethrin accumulation in the second phase of culture [36]. The effects of the nitrate to ammonium ratio on the cell growth, the production of ginseng saponin and polysaccharide as well as consumption of major nutrients by suspension cultures of *Panax notoginseng* cells were investigated at total nitrogen of 60 mM in a 250-mL Erlenmeyer flask [37]. The biosynthesis of saponin was more susceptible to the ratio of NO_3^-/NH_4^+ than that of polysaccharides. Ammonium was unfavorable for saponin formation. The relationship between initial nitrate concentration (including both intracellular and medium nitrate) and specific cell growth rate based on active biomass could be described by Monod equation. The maximum production of crude saponin and polysaccharide was 0.85 and 1.59 g L^{-1}, respectively, with initial nitrate concentration of 60 mM.

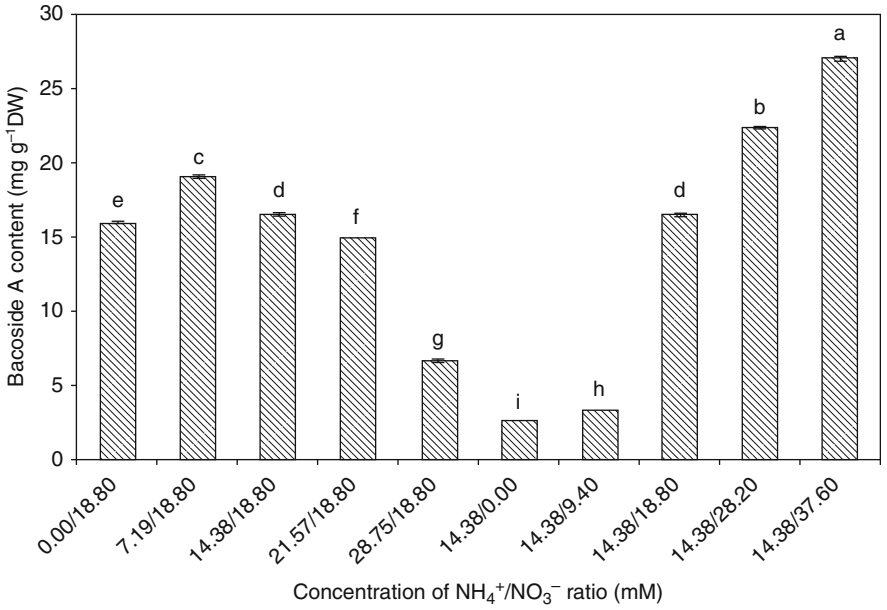

Fig. 20.3 Bacoside A content in *Bacopa monnieri* adventitious shoot culture after 8 weeks of cultivation as affected by different ratio of NH_4^+/NO_3^- in the MS medium. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test

20.3.4 Influence of Phosphate Levels

The phosphate concentration in the medium shows an excitatory effect on the production of secondary metabolites in plant cell and organ cultures. An increased phosphate level has been shown to stimulate synthesis of digitoxin in *Digitalis purpurea* [38]. Liu and Zhong [39] have reported that the highest saponin production at an initial phosphate concentration of 1.04 mM in *Panax ginseng* and 1.25 mM *Panax quinquefolium* respectively. Twice the phosphate levels of standard MS medium (1.25 mM) has proved better for the production of rosamarinic acid from *Lavandula vera* suspension cultures [31], gymnemic acid production from *Gymnema sylvestre* cell cultures [40] and solamargine production by *Solanum paludosum* multiple shoot cultures [41]. On the other hand, there are a number of reports showing that phosphate limitation could improve the production of metabolites, caffeine content in cell suspension cultures of *Coffea arabica* increased under phosphate limitations [42]. In grape cell suspensions, Dedaldechamp et al. [43] have reported enhancement of anthocyanin synthesis in response to phosphate deprivation.

20.3.5 Influence of Growth Regulator Levels

Growth regulators affect the growth and metabolite accumulation significantly because cell, adventitious root or shoot cultures generally need exogenous supply of growth regulators for growth, proliferation of biomass and metabolite accumulation. Whereas, hairy root cultures are genetically transformed roots which are produced by using *Agrobacterium rhizogenes* and they have the ability to grow without the addition of plant growth regulators [44]. However, a few recent reports showed that exogenous application of growth regulators also influence the growth and metabolite accumulation in hairy root cultures [45, 46]. In general, type of plant growth regulator and its concentration are crucial factors in cell and organ growth, proliferation and metabolite accumulation [47]. The type and concentration of auxin or cytokinin or the auxin/cytokinin ratio alter dramatically both the growth and the product formation in cultured cells [48]. Among auxins, indole acetic acid (IAA) and naphthalene acetic acid (NAA) have shown triggering effect on the production of anthocyanins in suspension cultures of populus and carrot, nicotine in tobacco, and anthraquinones in noni [34, 49, 50]. 2, 4-Dichlorophenoxyacetic acid (2, 4-D) has also shown a stimulatory effect on the accumulation of carotenoids in carrot [51] and anthocyanin in oxalis [52]. In suspension cultures of *Panax quinquefolium* strain Q91625, the highest content of crude ginsenoside saponins, i.e. 10.9 % by dry weight, was reached under a combination of the growth regulators of 2.5 mg L^{-1} indole-3-butyric acid (IBA) and 0.1 mg L^{-1} kinetin without addition of 2,4-D [53]. Among cytokinins, benzyladenine (BA) addition has improved the production of saponins in ginseng and addition of kinetin stimulated the production of anthocyanin in slender golden weed, but inhibited the production of anthocyanins in populus [49, 51]. 2-Isopentenyladenine (2-iP) inhibited root growth, however, stimulated artemisinin production in *Artemisia annua* [46].

The effect of gibberellins (GA) was specific to species and culture time. For example, Vanhala et al. [45] observed that addition of GA_3 decreased the accumulation of hyoscyamine in henbane. In contrast, GA_3 stimulated production of artemisinin in *Artemisia annua* and coumarin content in *Cichorium intybus* [54, 55]. Ethylene stimulated artemisinin production in plantlet cultures of *A. annua* [56] and it enhanced the growth of hairy roots of *Hyoscyamus muticus* [57]. A little is known about the effects of exogenous abscisic acid (ABA) on cell and organ cultures. Usually ABA inhibits growth and accumulation of secondary metabolites. ABA inhibited hyoscyamine accumulation in hairy root culture of *H. muticus* [45] with no adverse effect on biomass. In *Lotus corniculatus*, ABA application stimulated growth, but inhibited the accumulation of tannin [58].

20.4 The Influence of Inoculum Size/Density

Inoculum size/density is an important factor for plant cell and organ suspension cultures, which can influence the growth, biomass accumulation and metabolite formation [59, 60]. There is a critical minimum inoculum size below which cell

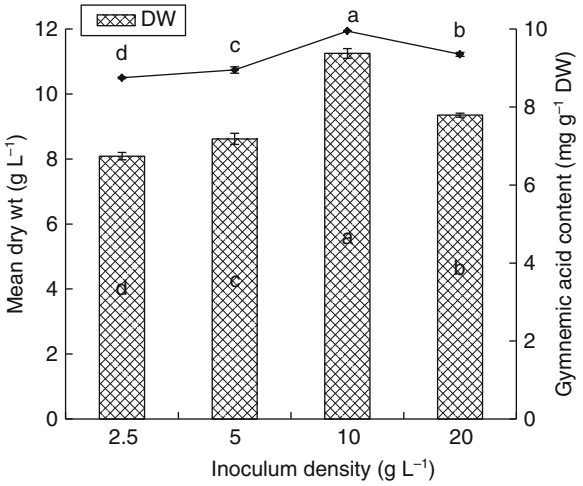

Fig. 20.4 Effect of inoculum density on the biomass and gymnemic acid accumulation in cell suspension cultures of *G. sylvestre*. Five hundred mg of cells were cultured in 50 mL of MS medium supplemented with 2.0 mg L^{-1} 2,4-D + 0.1 mg L^{-1} KN for 4 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test

growth will normally fail, while a suitable level of inoculum density could interestingly promote not only the cell growth but also the secondary metabolite biosynthesis. There are many reports on the influence of inoculum size/density of the cultured cells on biomass and metabolite accumulation [61–64]. In suspension cultures of *Perilla frutescens*, maximum cell density of 38.3 g DW L^{-1} was obtained at an elevated inoculum size of 50 g wet cells L^{-1} and anthocyanin production was enhanced 23-fold [65]. In cell suspension cultures of *Gymnema sylvestre*, the various quantities of inoculums (2.5, 5.0, 10.0 and 20.0 g L^{-1}) were tested, optimum density of biomass (11.25 g L^{-1}) as well as gymnemic acid (9.95 mg g^{-1} DW) was achieved with 10.0 g L^{-1} inoculum (Fig. 20.4). A higher (20.0 g L^{-1}) and lower (2.5 g L^{-1}) inoculum was not suitable for biomass and gymnemic acid accumulation. Another effect of inoculum size/density was at the induction of enzymes involved in the general phenylpropanoid metabolism when cells were transferred to a fresh medium. This is called 'transfer effect' or 'dilution effect'. Hahlbrock and Wellmann [66] have reported that the phenylalanine ammonia-lyase induced by transfer to fresh medium, decreased with increased inoculum size signifying that inoculum density may affect secondary metabolism. Morphology of the roots is another factor which influences biomass growth and synthesis of secondary compounds [67, 68] in the root suspension cultures. The adventitious root inoculum which was chopped (1–3 or 4–6 mm) or un-chopped, were responsible for lower yield of dry weight as well as ginsenosides. The root inoculum chopped to 7–10 mm was responsible for higher yield of 10 g L^{-1} DW and they also possessed highest content of ginsenosides of 5.5 mg g^{-1} DW [68].

20.5 Optimization of Culture Environment

Conditions of culture environment such as light, temperature, medium pH, and gases have been examined for their effects on biomass and secondary metabolite accumulation in cell and organ cultures.

20.5.1 Influence of Temperature

Since the early development of plant biotechnology, temperature effect has been investigated in cell and organ cultures and a temperature range of 17–25 °C is normally used for the maintenance of cultured cells and organs. However, each plant species may show better growth and metabolism under different temperature regimes. Morris [69] studied *Catharanthus roseus* cell line C87 and found maximum growth rate at 35 °C, maximum dry weight yield (0.47 g g^{-1}) was observed at 25 °C. Scragg et al. [70] investigated *Catharanthus roseus* cell line ID1 at 20, 25 and 30 °C but maximum biomass yield of 0.65 g g^{-1} at 25 °C. Courtois and Guern [71] found an optimum temperature of 16 °C for production of ajmalicine. Morris [69] reported that an optimum temperature of 25 °C for serpentine production and 20 °C for ajmalicine production. Toivonen et al. [72] estimated an optimum temperature of 25 °C for production of alkaloid from cell suspension cultures of *Catharanthus roseus*. Shohael et al. [73] studied the effect of low temperature (12 and 16 °C) and higher (30 °C) temperature, and reported that low and high temperatures cause significant decrease in biomass and reduction of phenolics and flavonoids, while low temperatures boost the accumulation of eleutheroside E in somatic embryos of *Eleutherococcus senticosus* and they correlated the increased accumulation of eleutheroside E for the oxidative stress. Yu et al. [74] studied the growth of hairy roots of ginseng under differential temperatures such as 13/20, 20/13, 25/25, and 30 °C/25 °C for 16/8 day and night cycles; got highest hairy root biomass with the cultures incubated at 20 °C/13 °C (Table 20.4). However, total ginsenosides was optimum (10.5 mg g^{-1} DW; Table 20.4) with the cultures incubated at 25 °C/25 °C and ginsenoside production was also highest (133.4 mg L^{-1}) at this temperature.

Table 20.4 Effect of incubation temperature (with 16 h/8 h/night cycles) on growth and ginsenoside production of ginseng hairy roots cultivated in bioreactors for 4 weeks

Growth	Biomass		Growth ratio	Ginsenoside (mg g^{-1} DW)	Ginsenoside (mg L^{-1})
	FW (g)	DW (g)			
13/20	431 ± 1.0	28 ± 1.0	19.7	4.5 ± 0.1	31.5 ± 1.5
20/13	892 ± 0.9	65 ± 0.8	45.8	8.2 ± 0.1	133.9 ± 0.9
25/25	889 ± 0.6	51 ± 0.7	35.9	10.5 ± 0.1	133.4 ± 1.2
30/25	764 ± 0.8	64 ± 0.9	45.1	6.4 ± 0.1	71.6 ± 0.5

Values within each column represent the mean of three replicates ± S.E.

20.5.2 *Influence of Light Intensity and Quality*

Light may be used as an energy source or just as an elicitor which affects the growth and accumulation of secondary metabolites in cultured cells and organs. Zhong et al. [75] demonstrated the effects of light quality, intensity, and irradiation period on the cell growth and anthocyanin pigment production by suspended culture of *Perilla frutescens*, and finally they optimized and successfully scaled-up the cell culture process from shake flasks to bioreactors based on the key factor of light irradiation. Chan et al. [76] also investigated the effects of different light intensity and irradiance (continuous radiance and continuous darkness) on cell biomass yield and anthocyanin production in cultures of *Melastoma malabathricum*. Moderate light intensity (300–600 l×) induced higher accumulation of anthocyanins, the cultures exposed to continuous darkness for 10-days showed the lowest pigment content, while the cultures exposed to continuous irradiance for 10-days showed the highest pigment content. The stimulatory effect of light on the formation of secondary compounds has been reported including flavonoids in *Petroselinum hortense* [77], anthocyanins in *Centaurea cyanus* [78], betalains in red beet [79], artemisinin in *Artemisia annua* [80]. On the contrary, light has an inhibitory effect on the accumulation of secondary metabolites such as nicotine and shikonin in *Lithospermum erythrorhizon* [81], monoterpenes in *Citrus limon* [82]. In some species, such as *Fragaria ananassa* [83]; and sweet potato [84], cell cultures have been reported to produce anthocyanin in the dark. Yu et al. [74] have studied the effect of fluorescent light, metal halide light, blue light, red light and blue plus red light on biomass growth and synthesis of ginsenosides in ginseng hairy root cultures and reported that hairy root growth was stimulated by red light than dark (Table 20.5). Fluorescent irradiation enhanced the accumulation of ginsenosides (5.3 mg g^{-1} DW). They also noticed differential accumulation of Rb and Rg group of ginsenosides in dark grown and light grown cultures, Rb group ginsenosides were highest in the cultures grown in dark (4.5 mg g^{-1} DW; Table 20.5) and Rg group of ginsenosides were optimal in the cultures grown in light (5.3 mg g^{-1} DW). These results suggest that manipulation of secondary metabolite accumulation is possible by manipulating light and dark regimes.

Table 20.5 Effect light quality on growth and ginsenoside production in ginseng hairy roots cultivated in bioreactors for 4 weeks

Light sources	Biomass		Growth ratio	Ginsenoside (mg g^{-1} DW)		Ginsenoside production (mg L^{-1})
	FW (g)	DW (g)		Rg	Rb	
Dark	270 ± 1.0	24 ± 0.6	11.4	2.8 ± 0.1	4.5 ± 0.2	27.8 ± 1.0
FL	226 ± 0.8	21 ± 0.6	10.1	5.3 ± 0.1	3.7 ± 0.1	30.2 ± 0.9
MH	193 ± 1.1	19 ± 0.3	8.9	3.5 ± 0.4	3.4 ± 0.3	23.3 ± 0.2
B	236 ± 0.2	24 ± 0.9	11.3	3.8 ± 0.4	3.9 ± 0.5	26.6 ± 0.4
R	284 ± 0.9	25 ± 1.0	11.6	3.1 ± 0.8	4.1 ± 0.7	20.9 ± 0.4
B + R	183 ± 0.9	21 ± 0.9	10.1	3.4 ± 0.1	2.9 ± 0.2	24.2 ± 0.7

Values within each column represent the mean of three replicates ± S.E.
FL fluorescent light, *MH* metal halide light, *B* blue light, *R* red light, *B + R* blue plus red light

20.5.3 *Influence of Hydrogen Ion Concentration*

The medium pH is usually adjusted between 5 and 6 before autoclaving and extremes of pH are avoided. The concentration of hydrogen ions in the medium changes during the course of culture due to nutrient uptake or due to the accumulation of metabolites in cultures. For example, decrease of medium pH due to ammonium assimilation and increase due to nitrate uptake was reported by McDonold and Jackman [85]. In *Withania somnifera* hairy root cultures, initial pH of the medium which was set at 5.8 was favourable for the accumulation of biomass (12. 1 g L^{-1} DW) and medium pH of 6.0 favoured the accumulation of withanolide A in the roots (13.84 mg g^{-1} DW; Fig. 20.5) [86]. In hairy root cultures of *Tagetes patula,* medium pH of 5.7 was suitable for growth and accumulation of thiophene [87]. In hairy root cultures of *Panax ginseng*, the medium pH set at 6.0 and 6.5 favoured both biomass accumulation and ginsenoside production [88]. The strategy of alteration of medium pH which results in the release of secondary products into the culture medium by changing the membrane permeability of the cells was reported in many culture systems [89, 90]. For example, betalains normally accumulate in roots of *Beta vulgaris*, but are released into the medium at pH 5.5 [90]. Up to 50 % of the total pigment was released at the time of exposure and roots continued to grow and accumulate betalains at later stage. When the roots were exposed to pH 2 for 20–30 min, they failed to grow, suggesting that low pH causes lysis of mature-pigment cells. A short exposure (10 min) to pH 2 followed by return to standard growth medium (pH 5.5) was beneficial for continuous release of pigments in the medium.

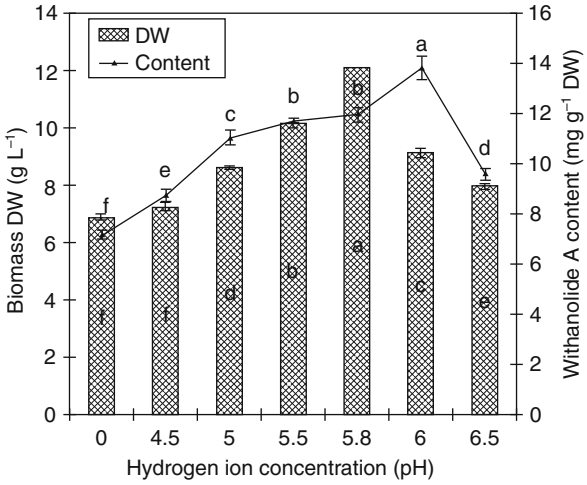

Fig. 20.5 *Withania somnifera* hairy root cultures: effect of pH on biomass accumulation and withanolide A production. Hairy roots (500 mg) were cultured in 250 mL Erlenmeyer flasks containing 50 ml of MS medium supplemented with 3 % sucrose for 4 weeks. Data represents mean values of three replicates. Means values with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test

20.5.4 *Influence of Agitation and Aeration*

Agitation is one of the important criteria which should be controlled in flask-scale to large-scale bioreactor cultures. The mixing of cultures promotes better growth by enhancing the mass transfer and uptake of nutrients from liquid and gaseous phases by cells/organs and the dispersion of air bubbles for effective oxygenation. Although plant cells have higher tensile strength in comparison to microbial cells, their shear sensitivity to hydrodynamic stresses restricts the use of high agitation speed for efficient mixing. The high shear rate and shear time that accompanies good mixing reduce the mean aggregate size, but also have an adverse effect on cell viability. Plant cells are therefore, often grown in stirred tank bioreactors at very low agitation speeds. Shifting from cell cultures to organ cultures such as adventitious or hairy root, shoot and embryo cultures for the production of secondary metabolites may be advantageous to overcome rheological problems [91, 92]. Many bioprocess techniques have been worked out by chemical engineers to overcome shear sensitivity, oxygen supply and mixing problems for the cultivation of plant cells in bioreactors [93–97].

Aeration is another important factor which should be controlled in bioreactor cultures for culture process optimization [93, 96, 97]. Aeration of plant cell cultures fulfills three main functions: maintenance of aerobic conditions, desorption of volatile products and removal of metabolic heat by mixing and air flow [97]. Oxygen requirement of plant cells is comparatively lower than that of microbial cells due to their low respiratory rates. However, oxygen supply has been shown significantly affecting the secondary metabolite production in cell cultures [93, 98, 99]. The effects of oxygen supply within the range of 20.8 %, 30, 40 and 50 % was studied by Thanh et al. [100] with ginseng cell cultures and a 40 % oxygen supply was found to be beneficial for the production of both cell biomass and spawning yield respectively (Fig. 20.6). In some cases, high oxygen concentration was even

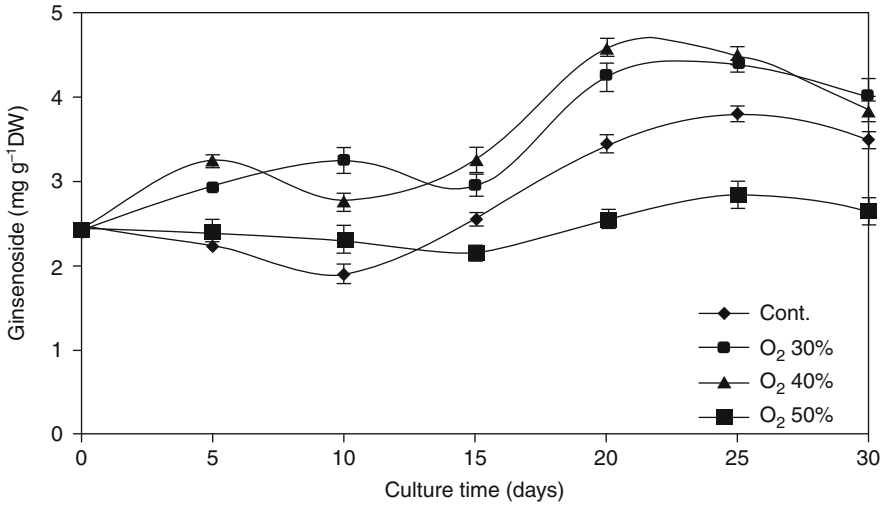

Fig. 20.6 Kinetics of ginseng saponin production by bioreactor-cultivated *Panax ginseng* cells

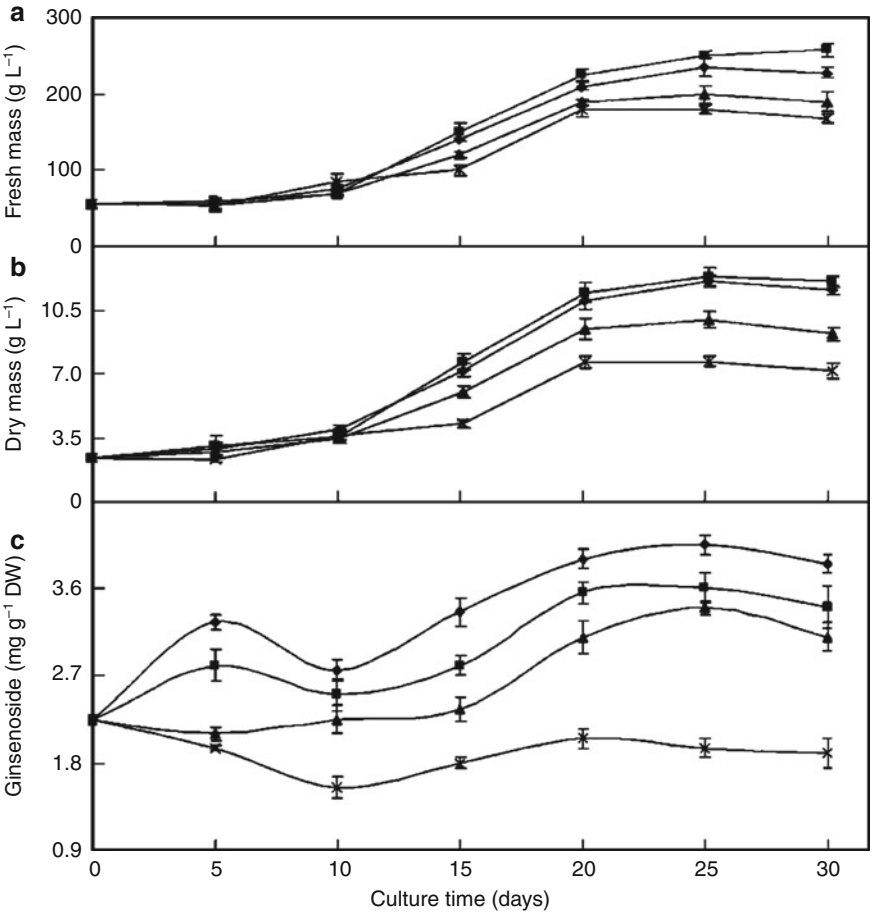

Fig. 20.7 Effect of CO_2 concentration on accumulation of cell fresh mass (**a**), dry mass (**b**) and production of saponins (**c**) of *Panax ginseng* cells cultivated in balloon type bubble bioreactors (*rhomboidal dots* – control, CO_2, *square* – 1 % CO_2, *triangle* – 2.5 % CO_2, *cross* – 5 % CO_2)

toxic to the metabolic activities of cells and may strip nutrients such as carbon dioxide from the culture broth [97]. Carbon dioxide is often considered as an essential nutrient in plant cell culture and has a positive effect on growth. The effects of carbon dioxide supply at 0.03, 1.0, 2.5 and 5.0 % in ginseng cell cultures was analyzed by Thanh et al. [101] and on improvement in biomass accumulation with 1 % carbon dioxide supply was observed. However, supplementation of carbon dioxide was not beneficial for saponin accumulation (Fig. 20.7). The beneficial effect of carbon dioxide on secondary metabolite production has been demonstrated in cell cultures of *Thalictrum minus* [102], *T. rugosum* [103], and *Stizoloibum hassjoo* [104].

20.6 Elicitation

Secondary metabolites are synthesized and accumulated in plant cells in response to a varied kind of stresses like biotic stresses such as a pathogen or insect attack and abiotic stresses like temperature, salinity, water stress, radiation stress, heavy metal and mineral stresses [105]. These varied stress conditions are generally designated as 'elicitors' [4] and elicitation has been widely used to enhance the production of secondary metabolites in plant cell and organ cultures [4, 105]. Elicitors of fungal, bacterial or yeast origin, viz. polysaccharides, glycoproteins, inactivated enzymes, purified crudlan, xanthan and chitosan salts and heavy metals are reported for the enhanced production of various secondary metabolites. Signaling molecules like methyl jasmonate and salicylic acid are also widely used for increased accumulation of secondary metabolites in cell and organ cultures [106–110]. Elicitor concentration, duration of exposure and age or stage of culture for elicitor treatment is also important for the successful production of secondary metabolites. Yu et al. [106] have studied the effect of jasmonic acid (0, 1.0, 2.0, 5.0 and 10.0 mg L^{-1}) on ginseng adventitious root cultures and increase in concentration of jasmonic acid resulted in a decrease in both fresh and dry biomass (Table 20.6). Whereas, ginsenoside content increased with higher concentrations up to 5.2-fold (Table 20.7). Decrease in biomass was tackled

Table 20.6 Effect of jasmonic acid on growth of ginseng adventitious roots after 5 weeks of flask culture

	Biomass		
Jasmonic acid (mg L^{-1})	Fresh weight (g)	Dry weight (g)	Growth ratio
0.0	16.15 ± 0.03	1.47 ± 0.07	4.08
1.0	12.48 ± 1.03	1.02 ± 0.07	2.83
2.0	8.52 ± 0.15	0.89 ± 0.01	2.47
5.0	6.33 ± 0.77	0.59 ± 0.07	1.64
10.0	4.67 ± 0.09	0.41 ± 0.01	1.13

Values within the columns represent the mean of three replicates ± S.E

Table 20.7 Effect of jasmonic acid on the biosynthesis of ginsenosides after 5 weeks of culture

Jasmonic acid (mg L^{-1})	Ginsenoside content (mg g^{-1} DW)			Rb/Rg	Ginsenoside production (mg L^{-1})
	Rb	Rg	Total		
0.0	7.49 ± 0.89 c	3.92 ± 0.34 c	11.42 ± 0.55 c	1.95 ± 0.39 c	167.58 ± 8.04 c
1.0	13.29 ± 0.49 d	2.83 ± 0.02 d	16.09 ± 0.46 d	4.68 ± 0.21 d	164.12 ± 4.69 c
2.0	24.29 ± 0.94 c	4.46 ± 0.25 b	28.69 ± 1.16 c	5.45 ± 0.16 c	255.39 ± 9.32 a
5.0	34.69 ± 0.89 b	4.15 ± 0.26 bc	38.82 ± 1.34 b	8.43 ± 0.71 b	229.04 ± 7.91 b
10.0	54.29 ± 1.04 a	5.53 ± 0.14 a	59.87 ± 0.90 a	9.83 ± 0.43 a	245.47 ± 3.69 a

Mean values of three replicates are represented with standard error

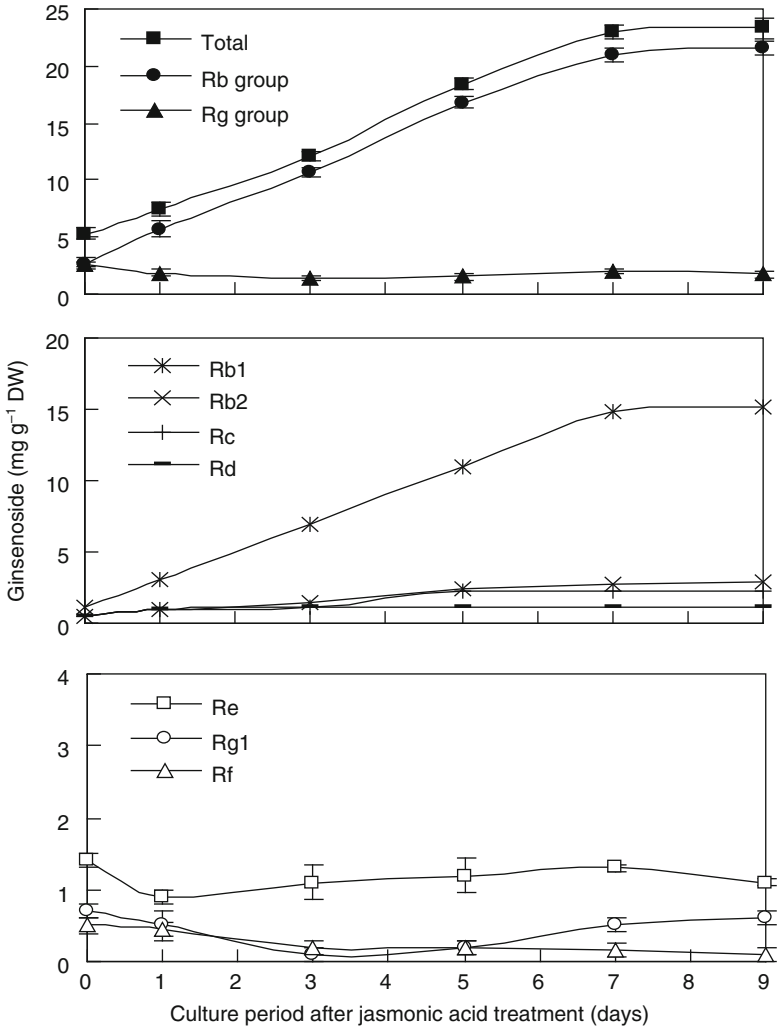

Fig. 20.8 Accumulation of total, Rb and Rg group ginsenosides during 9 days cultivation after jasmonic acid treatment (2 mg L^{-1}). The adventitious roots were grown for 25 days before jasmonic acid treatment

by following two step strategies i.e. by growing the adventitious roots in cultures for 25 days without elicitor and then by adding jasmonic acid (2 mg L^{-1}) and later there was increase in total ginsenosides and Rb group of ginsenosides by 5 and 5.6-fold respectively (Fig. 20.8). Hence, by following two step strategies it was possible to achieve both biomass growth and ginsenoside accumulation. Similarly, jasmonates have been used to elicit accumulation of paclitaxol in cell cultures of various *Taxus* species [111, 112], saikosaponins in root cultures of *Bupleurum falcatum* [113], eleutherosides in embryo cultures of *Eleutherococcus senticosus* [114].

Zhong and his co-workers have successfully developed a couple of new elicitors by chemical structure modification of traditional methyl jasmonate [115–117]. The novel chemically synthesized hydroxyl-containing jasmonates are more powerful

Table 20.8 The effect of linoleic and α-linolenic fatty acids on biomass production of *Panax ginseng* adventitious roots

Fatty acid concentration (μ mol L^{-1})	Biomass			Growth ratio
	Fresh weight (g L^{-1})	Dry weight (g L^{-1})	% dry weight	
Control				
0.0	134.3 ± 0.3 c	9.9 ± 0.3 cd	7.4 ± 0.2 abc	18.2 ± 0.6 cd
Linoleic acid (18:2) ±				
1.0	128.5 ± 1.2 d	9.6 ± 0.1 de	7.4 ± 0.1 abc	18.1 ± 0.1 cde
2.5	125.5 ± 1.2 d	8.8 ± 0.3 de	7.0 ± 0.2 dc	16.6 ± 0.7 de
5.0	120.7 ± 0.3 ef	8.8 ± 0.1 de	7.3 ± 0.1 bc	16.6 ± 0.7 de
10.0	120.8 ± 0.6 ef	8.7 ± 0.1 de	7.2 ± 0.1 bc	16.6 ± 0.1 de
20.0	120.1 ± 2.0 f	8.6 ± 0.1 e	7.1 ± 0.1 c	16.2 ± 0.1 e
α-Linolenic fatty acid (C18:3)				
1.0	136.9 ± 0.3 c	11.2 ± 0.3 ab	8.2 ± 0.2 a	21.4 ± 0.6 ab
2.5	150.3 ± 1.6 a	11.9 ± 0.6 a	8.0 ± 0.5 ab	22.9 ± 1.1 a
5.0	145.2 ± 3.2 b	11.1 ± 0.9 ab	7.7 ± 0.5 abc	21.6 ± 1.9 ab
10.0	145.7 ± 0.3 b	11.6 ± 0.1 ab	7.9 ± 0.1 abc	22.1 ± 0.7 ab
20.0	138.9 ± 1.0 c	10.4 ± 0.1 c	7.5 ± 0.1 abc	19.9 ± 0.1 bc
Significance (ANOVA)				
Elicitor (E)	***	***	***	***
Elicitor concen. (EC)	**	**	**	**
E × EC	**	*	–	–

Adventitious roots were cultured in 5 l balloon-type bioreactors containing 4 L MS liquid medium supplemented with 5 mg L^{-1} IBA and 5 % sucrose. Filter sterilized elicitors were added to culture medium on day 40. Roots were harvested and assayed at day 47. Mean values of three replicates are with ANOVA, at $P \leq 0.05$

than methyl jasmonate in inducing taxoid biosynthesis [115], and a new strategy of repeated elicitation plus sucrose feeding greatly enhanced the taxoid production titer to nearly 1 g L^{-1} in cell cultivation of *Taxus chinensis* [116]. The dynamic responses of defense signals and biosynthetic gene transcription to the new elicitor addition were also elucidated in the plant cell cultures [117]. Recently, polyunsaturated fatty acids (PUFAs) are known to possess biological activities in tissue cultures. For instance, exogenous PUFAs increased accumulation of secondary metabolites in suspension cultures of *Lycopersicon esculentum*, *Tinospora cordifolia*, *Erythrina cristagalli* and *Eschscholzia californiaca* [118]. In addition, elicitation with α-linolenic acid enhanced the activity of lipoxynase, the key enzyme of oxilipin biosynthesis [119]. When linoleic and α-linolenic acid were used as elicitors at a concentration ranging from 0 to 20 μM L^{-1} in adventitious root cultures of *Panax ginseng*, it was found that the effect of linoleic and α-linolenic acid was concentration dependent. In the cultures, linoleic acid significantly reduced root biomass growth and α-linolenic acid promoted biomass growth (Table 20.8) [120]. The content of protopanaxadiol and protopanaxatriol ginsenosides was elevated with the addition of α-linolenic acid (Fig. 20.9). Similarly, in the cell cultures of *Agrostis tenuis*, *Rauvolfia serpentina* and *Nicotiana tabacum*, addition of α-linolenic acid induced accumulation of jasmonic acid and was accountable for biosynthesis of pentacyclic oxylipins [118].

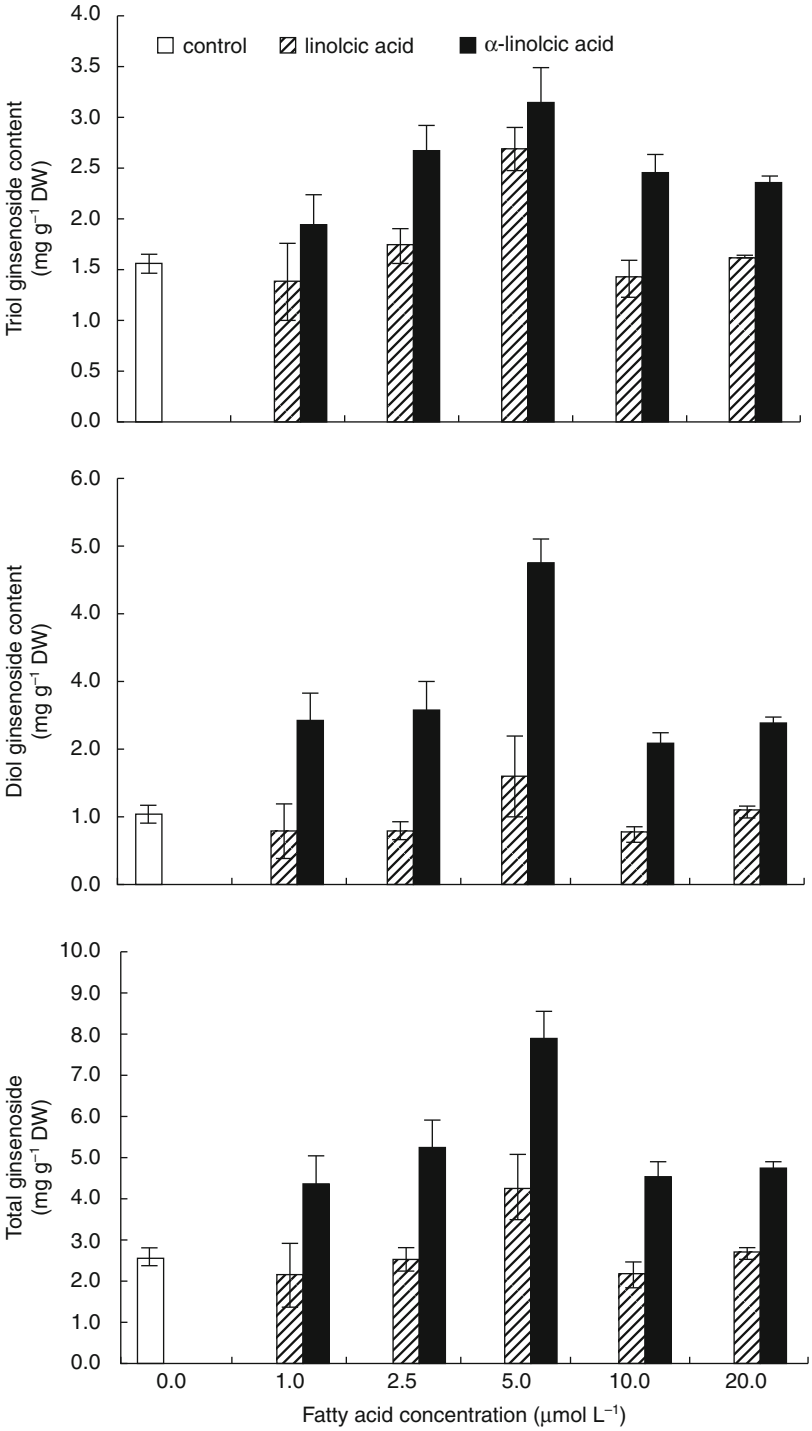

Fig. 20.9 Ginsenoside content of *Panax ginseng* adventitious roots as affected by elicitation with linoleic acid and α-linolenic acid fatty acids. All values are the means from three replicates with their standard errors

20.7 Nutrient Feeding

Medium or nutrient feeding strategy is one among the various approaches used to enhance the production of secondary metabolites after optimizing the basic chemical and physical parameters for the cultivation of cells/organs in large-scale [93, 121]. For instance, various nutrients of culture medium were exhausted by the end of 40 days of culture during ginseng adventitious root cultures (Fig. 20.10); with the objective of meeting the nutrient requirements of ginseng adventitious root cultures, and enhancing the biomass as well as ginsenosides production, Jeong et al. [121] replenished the cultures with 0.75 and 1.0 strength media after 10 and 20 days of cultivation. The cultures replenished with fresh medium (1.0-strength MS medium after 20 days of culture) showed a 27.45 % increase in dry biomass (28.66 g L^{-1} with replenishment treatment) and 8.25 % increase in ginsenoside content (4.93 gm g^{-1} DW; Table 20.9). The similar positive effect of media exchange strategy has been

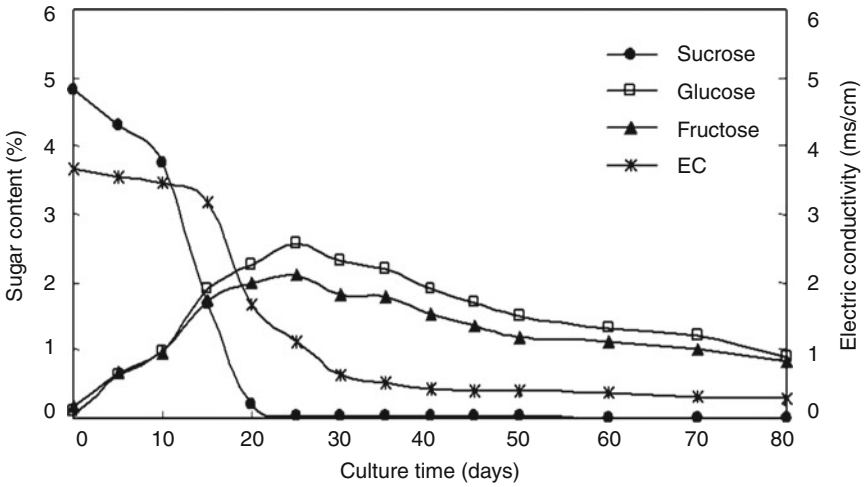

Fig. 20.10 *Panax ginseng* adventitious root cultures: concentrations of residual sugar (*circles*, sugars; *rhombuses*, glucose; *triangles*, fructose; *inverted triangles*, electrical conductivity)

Table 20.9 Effect of medium replenishment on ginsenoside production following 50 days of culturing on ginseng adventitious roots in 5 L balloon type bubble bioreactor containing 4 L of 1.5 strength MS medium

Medium strength and replenishment schedule	Dry weight (g L^{-1})	Ginsenoside content (mg g^{-1} DW)
Control	16.32 ± 0.54	4.17 ± 0.14
Replenishment after 10 days		
0.75 MS	23.49 ± 0.79	4.01 ± 0.16
1.0 MS	24.72 ± 0.51	4.27 ± 0.21
Replenishment after 20 days		
0.75 MS	24.03 ± 0.50	4.94 ± 0.17
1.0 MS	28.66 ± 0.70	4.92 ± 0.15

Each value within the columns represents mean of three replicates ± S.E.

reported in adventitious root cultures of *Echinacea purpurea* [122], cell cultures of *Lithospermum erythrorhizon* [123] and cell suspension cultures of *Taxus chinensis* [124]. Based on the investigation on initial sucrose effect on the cell growth and ginseng saponin biosynthesis, a sugar feeding strategy was formulated to enhance the saponin accumulation by *P. notoginseng* cells [26]. The highest production of crude saponins of 1.77 g L^{-1} (on day 26) was obtained by intermittent sugar feeding during cultivation; the production and productivity of ginseng saponin increased 2.3- and 2.1-fold compared with those of control, respectively.

A major disadvantage of batch processes is that significant amount of time is taken up for system and media sterilization, filling and emptying and cleaning the system. Thus, to improve the cost effectiveness of culturing plant cells, various operational modes including fed-batch, repeated fed-batch, semi-continuous and continuous cultivation have been developed by biochemical engineers and information on these aspects are well documented [93, 97]. The fed-batch operation involves the addition of one or more nutrients continuously or intermittently to the initial medium after the initiation of cultivation or at the stage of the batch process. Continuous cultivation includes variants without feedback control (e.g. in chemostats, where the substrate is fed at a constant rate) and with feedback control (e.g. in trubidostats, where the turbidity of the culture is kept constant by adjusting the rate at which substrate is fed, and auxostats, where the pH or dissolved oxygen of the medium is maintained at the set value). Perfusion cultivation is carried out by continuously feeding fresh medium to the bioreactor and constantly removing the cell-free medium while retaining the biomass in the reactor.

20.8 Precursor Feeding

Many plant cell cultures are also used to convert precursors into products by utilizing enzyme systems present in them. The addition of loganin, tryptophan and tryptamine enhanced the production of secologanin [125] and indole alkaloids [126] by *Catharanthus roseus* suspension cultures. Similarly, phenylalanine feeding improved accumulation of paclitaxel in *Taxus cuspidata* [127], and cholesterol feeding influenced the production of conessine in *Holarrhena antidysenterica* [128] cell cultures. For effective precursor feeding, factors such as the concentration of the precursor and the time of addition should be considered when applying it to the cell culture medium.

20.9 Permeabilization

Plant secondary metabolites formed by plant cell cultures are usually stored in the vacuoles and it is, therefore, desirable to extract the products into the culture medium such that the purification procedure may become easier and continuous recovery

and production of the product may be conducted. Removal of secondary metabolites from the vacuoles of the cells would also reduce the possible product inhibition thus increasing the productivity. Many attempts have been made to permeabilize the plant cell membranes in a reversible manner with organic solvents. Organic solvents such as isopropanol, dimethylsulfoxide (DMSO) and polysaccharides like chitoson have been used as permeabilizing agents [129–131]. Hexadecane, decanol and dibutylphthanlate are used for paclitaxol permeabilizaiton in *Taxus chinensis* [124]. However, when various chemicals are used as pemeabilizing agents they affect the cell viability. Therefore, selection of chemical agent with due consideration to its effect on cell growth may lead to substantial release of secondary metabolites. Other permeabilization methods such as electric field stress [132] and ultrasound methods [133] have also been used for recovery of secondary metabolites.

20.10 Immobilization

Immobilization of plant cells with a suitable matrix has been followed to overcome the problems of low shear resistance and the tendency for cell aggregation [4]. The advantages of immobilization include: (1) the extended viability of cells in stationary stage, enabling maintenance of biomass over a prolonged time period; (2) simplified downstream processing (if products are secretory); (3) high cell density within relatively small bioreactors showing reduced cost and risk of contamination (4) reduced shear stress (5) increased product accumulation (6) flow-through reactors to enable greater flow rates and (7) minimization of fluid viscosity which in cell suspensions causes mixing and aeration problems [134]. There are two major methods for cell immobilization: (a) gel entrapment and (b) surface immobilization. The widely used technique for immobilization involves the entrapment of cells in a specific gel or combination of gels, which polymerize around the cells. Calcium alginate is more widely used matrix, other than this, agar, agarose, gelatin, carrageenan and polyacrylamide have also been used [2, 4, 135]. The matrix used for cell entrapment should be non toxic to cells, should show good polymerization activity and it should be cheaper. Immobilization in *Morinda citrifolia*, *Digitalis purpurea* and *Catharanthus roseus* cultures was first reported by Brodelius et al. [136]. Surface immobilization is another method which takes advantage of the propensity of cultured plant cells to adhere to inert surfaces immersed in the liquid. DiCosmo et al. [137] have reviewed the work on plant cell adsorption to surfaces and immobilization on glass fibers. The surface immobilization of cultured cells in *Catharathus roseus*, *Nicotiana tabacum* and *Glycine max* has been reported for the production of metabolites [138, 139].

Some of the reports which showed dramatic effects of immobilization of cells for secondary metabolite production in plant cell cultures are: 100-fold increment in capsaicin production from immobilized cells with foam and gel [33, 140], 13 and 3.4-fold increment in methylxanthin and ajmalicine accumulation from gel immobilized cells of *Coffea arabica* and *Catharanthus roseus* respectively [138, 141].

Search for new biological and synthetic polymers is an extended research now and some immobilization strategies have been identified to increase bioproduction of secondary metabolites in plant cells [142].

20.11 Selective Adsorption of Plant Metabolites/ Two-Phase Systems

A low accumulation level of secondary metabolites in cell cultures in a number of instances may not be due to lack of key biosynthetic enzymes but rather due to feedback inhibition, enzymatic or non-enzymatic degradation of the product in the medium or volatility of compounds produced. In such cases, it is necessary to develop a separation technique which can concentrate the product. For *in situ* product separation of plant cell cultures, liquid-solid culture systems ('two-phase systems') for plant cells consisting of an aqueous nutrient phase and solid polar adsorbents have been preferred because many plant cells are expected to be of a polar character and bind weakly in the lipophilic phase of liquid-liquid systems. The removal and sequestering of the product in a non-biological compartment may increase total production of secondary compounds [143]. Polycarboxylic ester resin, neutral polymeric resin – XAD-7 could absorb berberine, a secondary metabolite from immobilized (alginate trapped) *Thalictrum rugosum* cells [144]. The advantages of adsorbents are that they can be used in bioreactor operation and allow easy separation of adsorbents from cells for the repeated use of cells and adsorbents [144, 145].

Activated charcoal, RP-8 (lipophilic carrier), Zeolith, XAD-2, XAD-4, XAD-7 (XAD is a neutral resin and ion exchanger), polyethelene glycol, β-cyclodextrin, polydimethylsiloxan, wofatite have been tested and used successfully for separation of secondary metabolites in cell suspension cultures of several systems [4]. Among all these, Ambrilte XAD-7 was efficiently used for adsorption and overproduction of paclitaxol from suspension cultures of *Taxus* [146], anthraquinones from suspension cultures of *Rubia akane* [147], and triptolide from adventitious root cultures of *Tripterygium wilfordi* [148].

20.12 Biotransformation

Biotransformation is a process of regio-selective and stereospecific chemical transformation that is catalyzed by the biological systems or entrapped enzymes or permeabilized cells [149, 150]. Biotransformation is another strategy followed for the production of high value metabolites using plant cell and organ cultures. Reactions carried out by such cultures include hydroxylation, glycosylation, glucosylation, oxidoreduction, hydrogenation, hydrolysis, methylations, acetylations, isomerization and esterification of various substrates [149].

Even though, plant cell cultures have high biochemical potential for the production of specific secondary metabolites, sometimes their desired products are not accumulated due to certain metabolic reasons. However, such cultures may retain an ability to transform exogenous substrates into products of interest. The chemical compounds, which can undergo biotransformation mediated by plant enzymes, are varied in nature which includes aromatic, steroid, alkaloid, coumarin, terpenoid, lignin and other molecular species. It is not always necessary for the compounds to be natural intermediates of plant metabolism but even substrate may be of synthetic origin. Plant cell cultures and enzymes have the potential to transform cheap and plentiful substances, such as industrial byproducts, intro rate and expensive products. For example, podophyllotoxin, a precursor of a semisynthetic anticancer drug is generally extracted from its source plant *Podophyllum* species. Kutnye [151] demonstrated that a cell line of *P. paltaum*, active in the biosynthesis of podophyllotoxin, was able to maintain repeated biotransformation of butanolide to the podophyllotoxin analogue. Ramachandra Rao and Ravishankar [152] used freely suspended and immobilized cells of *Capsicum frutescens* for conversion of protocatechuic aldehyde and caffeic acids to vanillin and capcinin. Li et al. [153] used ginseng cultured cells and roots for bioconversion of paeonol into its glycosides that have the radical scavenging effects.

20.13 Organ Cultures as a Source of Secondary Metabolites

Production of secondary metabolites by cell suspension culture is not always satisfactory, and organ cultures such as root, embryo and shoot culture methods have been developed in various plant species as an alternative for the production of secondary metabolites [44, 91, 92, 154]. Shoot cultures have been established in many medicinal plants which can accumulate secondary metabolites higher than that of natural plants. For example, shoot cultures were established in *Bacopa monnieri* for the production of bacoside A and regenerated shoots possessed threefold higher bacoside A than field grown plants [155]. Similarly, the shoots of *Nothapodytes nimmoniana* which were regenerated in the semisolid and liquid medium had several fold higher camptothecin compared to the mother plants (Fig. 20.11) [156]. Hairy roots can be obtained by transformation with *Agrobacterium rhizogenes*, which can grow with or without the supplementation of growth hormones, and have a growth rate, which is similar to cell suspension cultures [44]. Also these hairy root cultures are good producers of secondary metabolites, for example the terpenoid compound withanolide A was produced in optimum quantity in hairy root cultures [157]. The hairy roots were having high multiplication capability and contained withanolide A 2.7-fold higher than non-transformed roots (Table 20.10). Natural adventitious roots are induced in many medicinal plants and are cultivated in flask scale to bioreactors for the production various bioactive compounds [91, 92]. Adventitious root cultures of *Morinda citrifolia* grown in bioreactors showed several fold increment in anthraquinone content compared to field grown or plants grown in greenhouse [92].

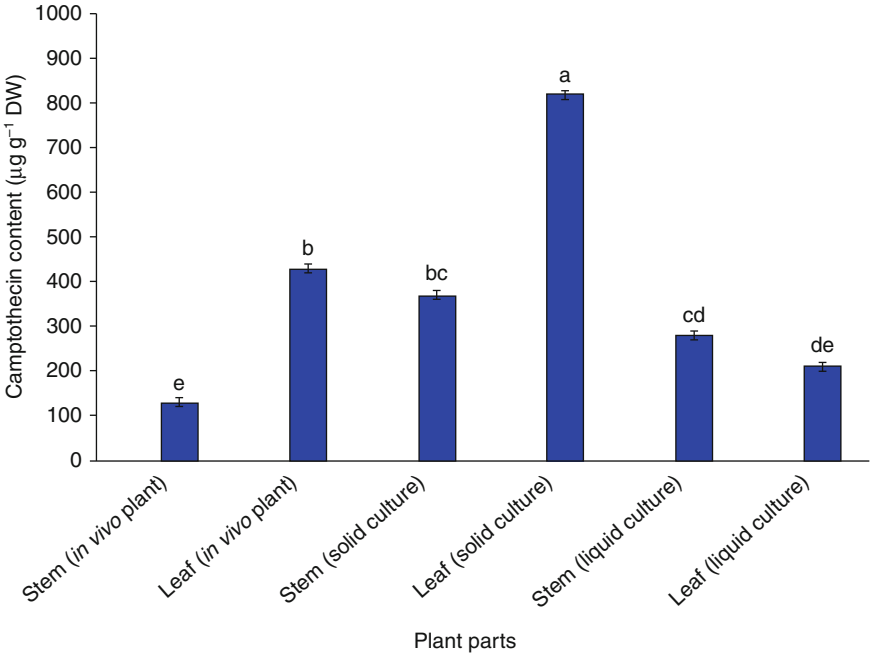

Fig. 20.11 Camptothecin content in plants regenerated on MS semisolid and liquid medium supplemented with or without cytokinins and control plants. *Bars* represent mean ± S.E. Mean values marked with *different letters* are significantly different ($P \leq 0.05$) according to Duncan's multiple range test

Table 20.10 Growth and withanolide content of transformed root lines in MS-based liquid medium after 28 days of culture

Hairy root lines	Doubling time (d)	Dry weight (mg)	Withanolide A (µg g^{-1} DW)
Control (non transformed roots)	60.0	120 ± 1.4 c	57.9 ± 1.9 c
Line 1	8.9	580 ± 2.1 a	148.0 ± 2.6 b
Line 2	13.9	520 ± 2.6 ab	155.6 ± 1.8 a
Line 3	9.8	600 ± 3.0 a	157.4 ± 2.0 a
Line 4	14.6	480 ± 3.5 b	146.5 ± 1.5 b

Values represent mean ± S.E.; $n = 5$ throughout. Values followed by different letters are significantly different ($P \leq 0.05$ Duncan's multiple range test)

20.14 Scale-Up of Plant Cell and Organ Cultures

Plant cells show unique characteristics such as less stability in productivity, higher shear sensitivity, slow growth rate and low oxygen requirements. A wide variety of bioreactor designs have been tested and used for plant cell cultures. Stirred tank reactors, airlift reactors and bubble column reactors for cultivation of plant cells are simply extensions of microbial culture systems with some modifications [154],

and the world's largest plant cell culture facility which was established in Germany (up to 75,000 L) is based on stirred tank models [97]. Centrifugal impeller bioreactor based on the principle of a centrifugal pump has been developed by Wang and Zhong [94, 95] especially for shear sensitive systems, such as culturing plant cells with high shear sensitivity. Successful scale-up of *Azadirachta indica* suspension cultures was developed in stirred tank reactors equipped with centrifugal impeller for the production of azadirachtin [158]. Scale-up of high-density cultivation of *Panax notoginseng* cells in a novel centrifugal impeller bioreactor (CIB) was demonstrated, in which initial k_La was identified to be a key factor affecting cell growth and production of ginseng saponin and polysaccharide [159]. Based on initial k_La level, the CIB high-cell-density cultivation process was successfully scaled up from 3 to 30 L in laboratory. A maximum dry cell weight (DW) and production titer of ginseng saponin and polysaccharide in a 30-L CIB reached 25.5, 1.7, and 2.9 g L^{-1} (on day 15) at an initial k_La value of 28.7 h^{-1}, respectively. Furthermore, by adopting a fed-batch cultivation strategy, a maximum DW and concentrations of total saponin and polysaccharide in the 30-L CIB were enhanced to 30.3, 2.1, and 3.5 g L^{-1}, respectively. The work suggests that the CIB may have great potential in large-scale high-density plant cell cultures for efficient production of useful secondary metabolites [159].

Mechanically driven 'wave reactors' have been recently developed for high shear-stress sensitive plant cells by Ebil and Ebil [160] and absence of air bubbles and wall growth as well as reduced foaming seems to make these reactors suitable for cultivating plant cell and organs [161]. Another reactor called 'slug bubble reactor' consists of vertical, flexible plastic cylinder in which aeration is achieved *via* the generation of large cylindrical bubbles, which move from the bottom to the top of reactors again for useful for cultivation of plant cells which are high-stress sensitive [162].

Dornenburg and Knorr [4] summarized the advantage and disadvantages of few standard bioreactor systems and airlift bioreactors seemed to be ideal for some plant cell cultures which are not highly shear sensitive. Further, airlift bioreactors which spread the air from the base of the reactor through sparger are suitable for cultivation of hairy roots and adventitious roots of various medicinal plants. They are also suitable for scale-up and pilot scale cultivation. Inoculation of 500 g fresh weight adventitious roots of ginseng into 500 L balloon type bubble bioreactors can produce 74.8 kg of root biomass after 8 weeks of culture. The saponin content obtained in small-scale (20 L) to pilot scale (500 L) bioreactors was 1 % based on dry weight [163]. These experimental results have led to the establishment of pilot and plant scale bioreactors (up to 10,000 L; Fig. 20.12) for obtaining ginseng adventitious root biomass and production of ginsenosides for commercial exploitation.

During the scale-up of plant cell and organ cultures, oxygen supply is generally very important as mentioned above. Partial pressure of oxygen may also be critical for secondary metabolite production as shown in high density cell cultures of *Panax notoginseng* [164]. Furthermore, minor gas composition like ethylene and carbon dioxide was identified as a key factor for scaling-up the suspension culture of *Taxus chinensis* for production of taxane diterpene [165]. Other important scale-up factors include shear force and mixing time, as demonstrated in suspension cultures of *Perilla frutescens* [166] and *Taxus chinensis* [167], respectively.

Fig. 20.12 Large-scale (10,000 L) bioreactors developed for cultivating adventitious roots of ginseng

20.15 Conclusions and Perspectives

Plant cell and organ cultures are promising techniques for the production of valuable secondary metabolites which have pharmaceutical, nutriceuticals and industrial importance. This technology is even more attractive with advanced biotechnology approaches such as signal transduction engineering for highly induced biosynthesis of specific targeted products among various heterogeneous metabolites with similar chemical structures but very different bioactivities [168–170]. The recent developments in plant tissue culture techniques and bio-processing have shown promising results to improve biomass growth and the productivity by several folds. Optimization of medium ingredients and culture environmental factors are the basic approaches which should be dealt with individual plant species at flask scale level in the first stage. Various other parameters such as inoculum density, agitation/aeration, elicitation, nutrient feeding, precursor feeding, permealization, and immobilization should be worked out in small scale bioreactor cultures. Care should be taken for the selection of bioreactor types and application of bioprocess parameters at this stage. Adoption of organ culture techniques and scale-up process can lead to significant enhancement in productivity of secondary metabolites. Proper understanding and rigorous analysis of these strategies (Fig. 20.13) would pave the way towards successful commercialization of plant cell bioprocesses.

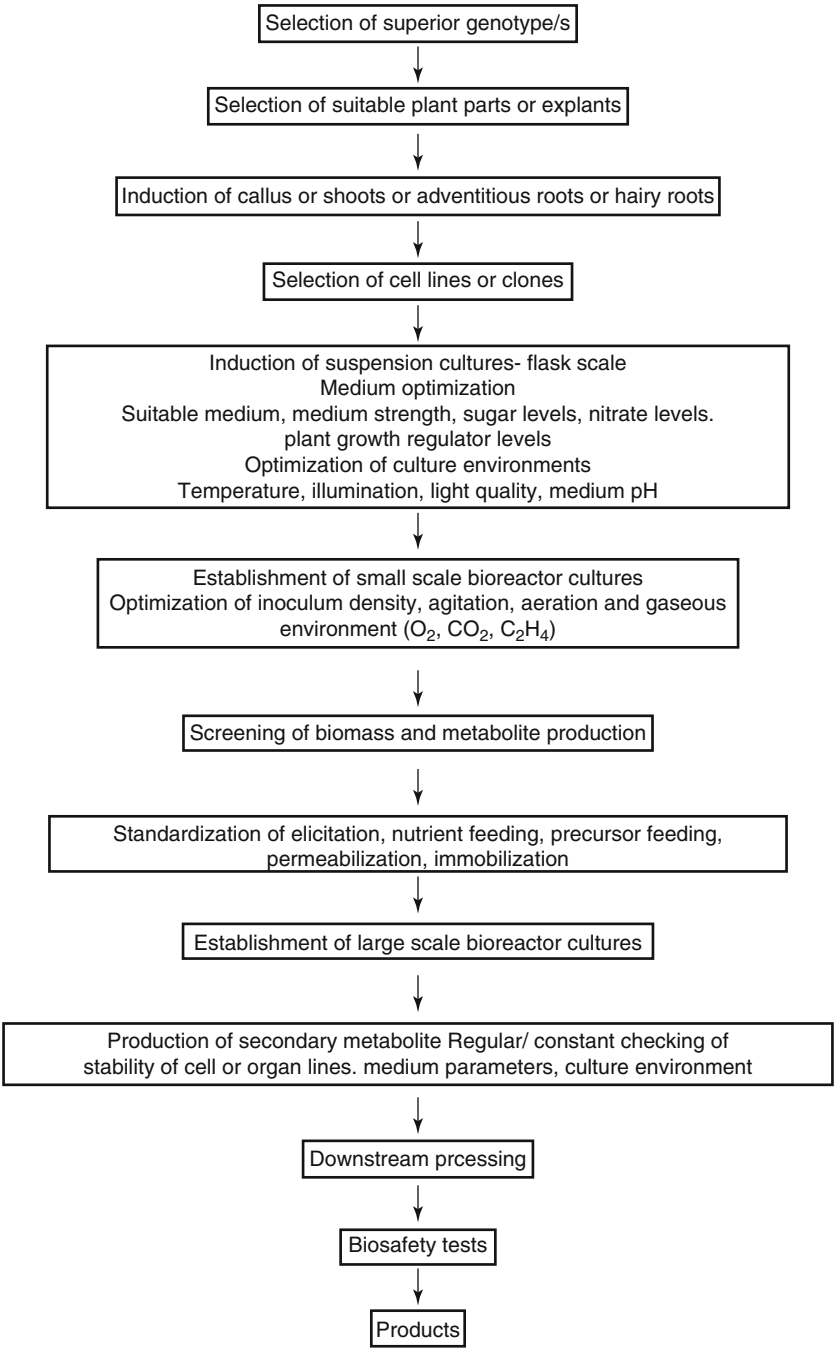

Fig. 20.13 Flow chart of general strategies followed for production of secondary metabolites from plant cell and organ cultures

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Chapter 21
Metabolic Engineering of Selected Secondary Metabolites

Jutta Ludwig-Müller

Abstract The demand for the production of valuable secondary metabolites is increasing rapidly. While many metabolites can be directly extracted from intact plants, others are routinely produced using cell or organ cultures. The latter, also called Hairy roots when generated through the transformation with the bacterium *Agrobacterium rhizogenes*, are also amenable to molecular modifications. Similar to intact plants metabolic pathways can be altered by introducing homologous or foreign genes. The better the knowledge of a given pathway, the more efficient will be the genetic alteration. Some of the general requirements for metabolic engineering of secondary metabolites will be discussed together with methodological considerations, especially the analysis of secondary metabolites and also the transformation methods. In addition, some examples for successful establishment of transgenic plants for metabolite production will be described. Finally, some alternative plant production systems will be discussed.

Keywords Genetic engineering • Hairy roots • Metabolite analysis • Metabolite production • Transformation

Abbreviations

AGO	Argonaute
BL-SOM	Batch-learning self-organizing map analysis
DCL	Dicer-like
DSB	Double stranded DNA breaks
ER	Endoplasmic reticulum
GC	Gas chromatography
GMO	Genetically modified organism
GSL	Glucosinolate

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HCA	Hierarchical cluster analysis
HPLC	High performance liquid chromatography
IEE	Intercistronic expression element
IR	Infrared
JA	Jasmonic acid
JA-Ile	JA-Isoleucine
LC	Liquid chromatography
MEP	Methylerythritol-phosphate
miRNA	microRNA
MS	Mass spectrometry
MVA	Mevalonate
NMR	Nuclear magnetic resonance
PA	Pyrrolizidine alkaloid
PCA	Principal component analysis
PLS-DA	Partial least squares discriminant analysis
QTL	Quantitative Trait Loci
RISC	RNA-induced silencing complex
SCF	*S*KP, *C*UL, *F*-box
TALEN	Transcription activator-like effector nuclease
Ti	Tumor-inducing
ZNF	Zinc finger nuclease

21.1 Introduction

Bioactive compounds are used by mankind for many different purposes and the requirement for compounds with better or even novel properties is increasing continuously. A need for biotechnological production of secondary metabolites with interesting properties is therefore obvious [1]. Thus, the genetic manipulation of medicinal plants has received a lot of attention over the last decade (reviewed in [2]). The exploitation of natural compounds of plants can be traced back generally to their medicinal use (i.e. pilocarpin, scopolamine), but also secondary metabolites are used in food as flavours or spice components as well as in cosmetics industry as aroma compounds and antioxidants. Examples are the spice capsaicin from peppers, which has also antimicrobial activity, vanillin from vanilla, but also the isothiocyanates released from the glucosinolates upon tissue disruption. Finally, there are coloured substances (i.e. betanidine, indigo, shikonin) that receive much attention in food industry and as natural dyes in other applications. In addition, their use in agriculture as biopesticides has received attention [3]. Among the pharmaceuticals, which have been released in the last 30 years, over 25 % were derived from plant metabolites and roughly 50 % of the "top chemicals" were generated from plant secondary metabolism [3]. The FDA gave approvement for clinical application of seven novel plant compounds, which include taxol/

paclitaxel (*Taxus brevifolia*), vinblastine and vincristine (*Catharanthus roseus*), topotecan and irinotecan (*Camptotheca acuminata*) and etoposide and teniposide (*Podophyllum peltatum*) [2].

Plants are considered as the organisms of choice for the production of metabolites relying in their synthesis on complex pathways [4]. The realisation of such complex biosynthetic pathways in microbes is very difficult, because of missing intermediates and potential problems with protein folding and modifications. Yeasts can be seen as alternative, but there reactions which need different plant compartments cannot be imitated. In addition, antimicrobial compounds pose a problem for the production also in eukaryotic microbes.

Also, many plants with beneficial properties are either difficult to cultivate and/or they are in the list of endangered species [2]. Therefore, their harvest is difficult or sometimes even prohibited and regulations have been implemented for harvesting these plants. This implies that novel methods for cultivation of such species are needed to ensure the production of beneficial compounds from medicinal plants. Laboratory cultures have also the advantage that gene alterations by molecular biological methods are possible to change pathways in favour of a desired metabolite [5, 6]. Therefore, plant cell or organ cultures have been established in the last decade for the production of bioactive metabolites (see Chaps. 6, 7, and 8; this book). These contribute to a high quality production of compounds, because of the controlled environment of the cultures. In addition, large-scale bioreactors for plant cultures are also available nowadays (see Chaps. 1, 2, and 3; this book). Under these controlled conditions the induction of secondary metabolites by elicitors is a feasible and easy to perform method. Elicitors could be stress signaling compounds or they may be changes in culture conditions like oxygen depletion [7].

21.2 General Aspects of Metabolic Engineering

In previous sections of this book the use of cell (Part II) and organ cultures (for example shoot, embryo and adventitious root cultures; Part III) is described in detail. Firstly, the most suitable plant organ for the generation of the cell or organ culture has to be selected for non-genetically modified organ or cell cultures. To this end the metabolite pattern of the respective organs has to be analyzed, but also intact plants as sources have to be considered (Fig. 21.1).

To identify or quantify a compound of interest, the metabolite has to be extracted from the plant tissues. The chemical properties of the substances are of high importance to devise a purification scheme [8]. Important issues to be taken into account are: (1) It has to be defined whether a compound that is already known, should be extracted and quantified (targeted approach), or whether a broad range of unknown (bioactive) compounds should be identified (untargeted approach). The latter experimental approach is often coined to metabolomics. (2) For individual compounds it has to be determined which properties are already known and whether standard compounds are available or need to be synthesized. (3) The purity of the compound

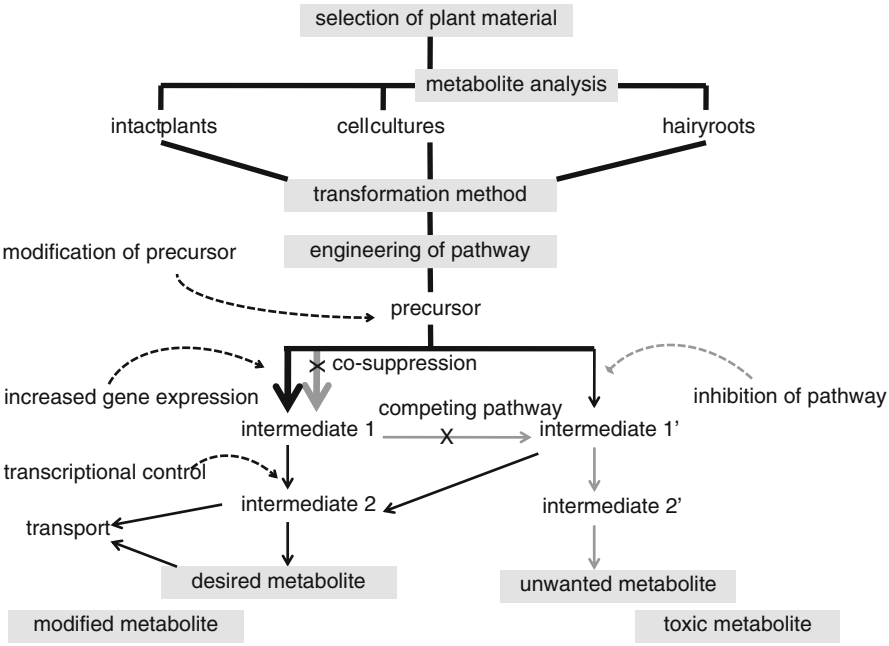

Fig. 21.1 Summary of planning a metabolic engineering experiment, starting with plant material, metabolite analysis (see also Fig. 21.2) and transformation (see also Figs. 21.3 and 21.4). Various possibilities to influence the biosynthetic fluxes for a given metabolite using metabolic engineering. On the *left* side (*black*) the increases of individual steps in a pathway are shown, on the *right* side (*grey*) the possibilities to decrease a metabolite (toxic metabolite) or pathway (competing pathway, *bold cross*). The heterologous expression of genes encoding enzymes leading to modifications of precursors or intermediates of a pathway are also indicated. The *bold arrow* shows that increases in mRNA might lead to a reduction (*bold cross*) by co-suppression. Increased expression of a heterologous gene with the same function (*second arrow*) might be the solution here

is important for identification, but also for bioactivity assays, so, in this case the metabolite needs to be further purified by chromatographic methods. (4) Is identification and quantification the only purpose, so the sample does not need to be recycled, but if should be subjected to further assays for bioactivity, the sample needs to be recovered. (5) The amount of sample required after purification might be critical, because if tests for bioactivity have to be carried out a larger amount of sample is needed (see Sect. 21.2.1).

After the identification of suitable plant materials, transformation methods have to be employed (see Sect. 21.2.2). For transformations, either *Agrobacterium-mediated* or biolistic (for plastids transformation) methods are used. Once a transformation protocol has been selected, the genetic engineering steps are to be carried out (see Sect. 21.2.3). First, suitable gene(s) for a pathway need to be identified (see "Metabolic Genes" in Sect. 21.2.3). These may be metabolic genes or the genes encoding for either transcription factors (see "Transcriptional Control" in Sect. 21.2.3) or transporters (see "Transport" in Sect. 21.2.3). Here, many precautions

Table 21.1 Applications and outcomes for secondary metabolite analysis (see also Fig. 21.1)

Application	Respective outcome	
Quality control of medicinal plants	Quality control results	Global metabolite correlations
Activity-related compounds in medicinal plants	Identification of novel (bioactive) compounds	
Chemotaxonomy		
Interaction with other organisms	Reprogramming plants against stressors	
Abiotic stress response		
QTL analysis	Identification of QTLs for breeding	
Evaluation of GMO	Biosafety	
Identification of metabolic genes	Identification of genes involved in metabolism	

GMO genetically modified organisms, *QTL* quantitative trait loci

have to be taken into account, for example that the strong overexpression of homologous genes does not result in a co-suppression phenomenon (Fig. 21.1; [9]). In addition, especially when genes should be expressed in a heterologous organism, it can be advantageous to adopt the codon usage of the foreign gene to the organism, in which it will be transformed since codon usage differs significantly between organisms (e.g. [10]).

21.2.1 Analysis of Secondary Metabolites

After it has been established for which application the extraction protocol will be used (Table 21.1), the first decisions to be made concern the extraction solvent (Fig. 21.2; [8]). It is important to know whether the extracted compound is polar or nonpolar. Further, different pH values will result in differentiated extracts according to molecular properties of the compounds. After evaporation of organic solvents the remaining aqueous phases can be extracted using organic solvents with different polarities. The extract may contain many molecules other than the desired one, which will either be analyzed using a nontargeted approach or have to be further enriched by chromatographic methods (Fig. 21.2). Different chromatographic techniques are available encompassing size fractionation and separation according to their charges/polarity or affinity chromatography [8]. For affinity chromatography more information has to be gathered about the compound. In this case, ligands which can bind to the molecule of interest are attached to a suitable resin and will retain ideally only one compound.

The analytical methods employed for the identification and quantification of secondary metabolites are thin layer chromatography, high performance liquid chromatography, gas chromatography, mass spectrometry, NMR-spectroscopy, IR-spectroscopy and spectrophotometry (Fig. 21.2). Some methods can be combined

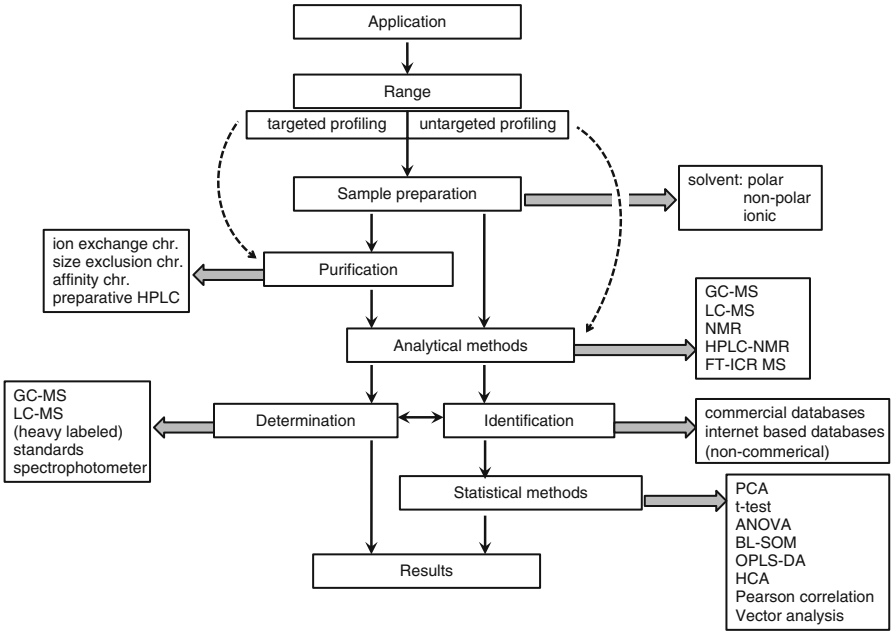

Fig. 21.2 Some aspects for the use of different analytical procedures in biotechnology. For possible applications and resulting outcomes of such a protocol see also Table 21.1. In the middle the main steps are shown and the possible branches by *thin arrows*. For some features, i.e. purification, analysis, statistics etc. in the boxes examples are shown, which are connected to the respective feature by *grey arrows*. The *dashed arrows* indicate that preferably for targeted profiling an additional step is needed, whereas for untargeted approaches direct analysis of the sample is possible. *Chr* chromatography

i.e. gas or liquid chromatography with mass spectrometry as the detector. Some techniques also enable the quantification of metabolites in addition to the identification of a compound, but in that case a reference substance should be available. If a mass spectrometer is used as a detector for either liquid or gas chromatography, then identification *via* the mass spectrum is possible. The quantification can also be achieved if reference compounds available are labelled with heavy isotopes [11]. They are assumed to behave like the natural compounds and if added prior to extraction, they can be used to determine the efficiency of the extraction procedure i.e. recovery of a specific compound. The accurate identification of a compound is sometimes not possible by mass spectrometry but can often be achieved using NMR- or IR-spectroscopy [12].

Metabolomics, a term used for the mostly unbiased parallel analysis of as many compounds as possible, has gained importance in the identification of novel metabolites and also in biotechnology [13]. This approach can give an insight into the changing metabolic pattern under various conditions during the development, but also changing environmental conditions under abiotic and biotic stress situations, and thus help to identify novel compounds. Using modern biotechnological

approaches, plants with different biological properties can be compared to find additional bioactive substances in medicinal plants. Other applications are quality control of medicinal plants, identification of Quantitative Trait Loci (QTLs), chemotaxonomy, evaluation of GMOs and identification of metabolic genes (Table 21.1; [13]).

The choice whether targeted or untargeted approaches are chosen determines the suitable solvent(s) used for extraction (Fig. 21.2). However, it should be noted that the perfect universal solvent for all applications does not exist. The following step determines the intensity, reliability and potentiality for identification of novel compounds, the analytical techniques to be used for the analysis of the compounds. While the pioneering metabolomics work was carried out using gas chromatography-mass spectrometry (GC-MS), this method is complemented or replaced by other techniques such as liquid chromatography-mass spectrometry (LC-MS), nuclear magnetic resonance spectroscopy (NMR), or combinations of the aforementioned [12].

The identification of individual compounds within gas or liquid chromatograms relies on deconvolution software, which is needed to separate peaks from each other and databases, either open source internet based ones or commercial ones (reviewed in [13]). Finally, the large datasets need to be evaluated. For this purpose various statistical methods are in use, of which some are described in the following (Fig. 21.2). A commonly used method in metabolomics is the principal component analysis (PCA), a multivariate analysis method. The PCA method basically provides an overview of the datasets by clustering each sample and highlighting the differences in a given sample set and between the samples [13]. Other statistical analytical methods have been used to analyze such metabolomic datasets, for example hierarchical cluster analysis (HCA), partial least squares discriminant analysis (PLS-DA) and batch-learning self-organizing map analysis (BL-SOM) [13]. The outcome of such an experiment could then be identification of QTLs, reprogramming metabolism against stress, global metabolite correlations, identification of genes involved in metabolism, identification of novel (bioactive) compounds, and quality control of results (Table 21.1).

21.2.2 Transformation Methods

The transformation of plants with either foreign genes or homologous genes under the control of a strong promoter relies always on the selection of a suitable method. In case of hairy root generation, the transformation is achieved by using a soil borne bacterium, *Agrobacterium rhizogenes*. This bacterium naturally transforms its hosts by stably integrating the *rol* genes, which lead to the hairy root phenotype. This happens also in nature, but the phenomenon is explored for the production of secondary metabolites. Therefore, the addition of genes encoding for the desired proteins from a given pathway together with a suitable promoter has been used for metabolic engineering of these organ cultures. This has been used mainly for the enhancement or modification of metabolites as described above (e.g. [5, 6]).

The first part of the characterization of new cell cultures or hairy root lines is the verification of transformation at the genetic level. This includes selection of transgene-containing lines, detection of the inserted construct *via* PCR and, in case of hairy roots, to prove the insertion of the transfer DNA [14]. A common selection marker uses hygromycin resistance. Suitable concentrations of the antibiotics may vary in different plant species, which makes it reasonable to investigate an effective lethal dose for untransformed cell lines or hairy roots generated with a wild type of *Agrobacterium* before the selection experiments. Alternatively, individual lines can be investigated for the integration of the transgene by PCR methods. This can be used together with a protocol needed for the verification of integration events of the *rol* genes as well as confirmation that bacteria are not present any more. Grabkowska et al. [15] established primer sequences for the detection of *rol*B and *rol*C. These primer pairs can be used in a multiplex PCR, in some plant backgrounds even together with the *vir*G primers [16]. If cell lines or hairy roots contain additional transgenes, the integration has to be shown. Most important for this aim are specific insert primers that bind to the transgene. Additionally, other parts of the construct, for example the selection marker, can be analyzed.

A biolistic transformation method by particle gun predominantly used for plastid engineering (see below) relies on an entirely physical DNA delivery process, and therefore is not limited with respect to the size of the transforming DNA (reviewed in [17]). However, the coating of the gold particles used as microprojectiles with the transforming DNA involves especially vigorous vortexing, which poses a relatively harsh mechanical treatment to the DNA, but it is necessary to keep the particles in suspension and prevent them from aggregation. Thus, the transformation of the plastid genome with very large DNA molecules has to be expected to occur with considerably less efficiency, because of the increased risk of mechanical shearing during particle coating with large DNA molecules [17].

Why is the transformation of intact plants probably less suitable for the production of secondary metabolites? The variation in secondary materials extracted from naturally grown plants is very high due to unfavorable environmental conditions, such as biotic and abiotic stresses, seasonal changes, but also the limitation of the plant material itself, if it is a protected species or does not contain reasonable amounts of the metabolite. Intact plants are routinely transformed with a close relative of *A. rhizogenes*, namely *Agrobacterium tumefaciens*. In nature, the phytopathogenic bacteria cause tumors on their hosts by stable transformation with genes encoding enzymes for plant hormone (auxin and cytokinin) biosynthesis. The oncogenic hormone biosynthetic genes in the T-DNA of *Agrobacterium* have been removed to enable technologically useful plant transformation. They were replaced by multiple cloning sites where genes of interest as well as dominant selectable markers can be integrated. These "disarmed" *A. tumefaciens* are used to introduce transgenes into plants that can be transformed by using these bacteria. However, it is possible to use the same strategy for hairy root generation by using a desired transgene in addition to the tumor-inducing genes. This should result in a stably transformed tumor harboring desired transgenes. These tumors should, in theory be grown like other cell cultures, may be even without or at least with reduced levels of growth hormones in the medium. However, the problem may arise due to the use of

huge Ti-plasmids. Even for the use of disarmed plasmids, a so-called binary vector system has been generated to circumvent handling these large plasmids [18], especially when the gene to be inserted into the plasmid for transformation is also big.

Similarly, the plasmids from *A. rhizogenes* used for hairy root induction (see above) are very large, posing sometimes equally the problem that large plasmids are difficult to transform. A different strategy for hairy root induction was introduced by Zang et al. [19], who used the binary vector system of *A. tumefaciens* in which they introduced the complete set of *rol* genes from *A. rhizogenes*. The transformation with *A. tumefaciens* yielded hairy roots in Chinese cabbage, so this might be an alternative to substitute *A. rhizogenes* with the (smaller) *rol* carrying binary vector systems.

Nevertheless, alternative transformation methods using plastid transformation might be successful so that the disadvantages of the metabolite production in intact plants can be overcome. Plastids (chloroplasts) harbor a small gene-dense genome that is amenable to genetic manipulation by transformation (reviewed in [17]). Due to the large prokaryotic genome structure and gene expression machinery, the high transgene expression levels attainable in transgenic chloroplasts, and the very low production costs in plant systems, the chloroplast transformation has been increasingly used to produce metabolites. Clearly, the advantages lie mainly in the high copy number of transgenes that can be achieved. Also, plastids possess a highly efficient homologous recombination machinery facilitating the targeted integration of DNA [17]. Further advantages are that different selection markers can be used than for nuclear transformation, the absence of epigenetic gene silencing (co-suppression; see above) and the predominantly maternal inheritance of plastid DNA [20, 21]. The latter greatly reduces the probability of transgene escape *via* pollen flow. Finally, the plastids, due to their evolutionary origin, are capable to express (even large) polycistronic messages (Fig. 21.3).

This method has been used to generate intact plants with high alpha-tocopherol levels [22]. First, the authors used plastid transformation in the model plant tobacco to test single-gene expression constructs for their effects on the flux through tocochromanol biosynthesis. The individual overexpression of the three key plastid-localized enzymes specific to tocopherol bio synthesis [23] yielded only stronger tocopherol accumulation in case of one enzyme [22]. Further overexpression of these genes encoding the three key, and probably rate-limiting enzymes of alpha-tocopherol biosynthetic pathway from *Synechocystis* in an operon-like manner in plastids of tobacco (*Nicotiana tabacum*) unexpectedly resulted only in moderate increases in alpha-tocopherol. This problem could be overcome by inserting short fragments of RNA, so-called intercistronic expression elements (IEE) in the construct within the individual transcripts. Indeed, such constructs showed a much higher accumulation of a-tocopherol [22]. Additionally, the transplastomic plants with high tocopherol levels were much less affected by oxidative stress.

However, there are also limitations for the application of this method in a more general way. Even though in recent years a toolbox for plastid transformation has been established, the method is more or less confined to one higher plant species, *Nicotiana tabacum* [24–26]. In some cases, the production of secondary metabolites in a species producing toxic compounds, i.e. the alkaloid nicotine, might not be

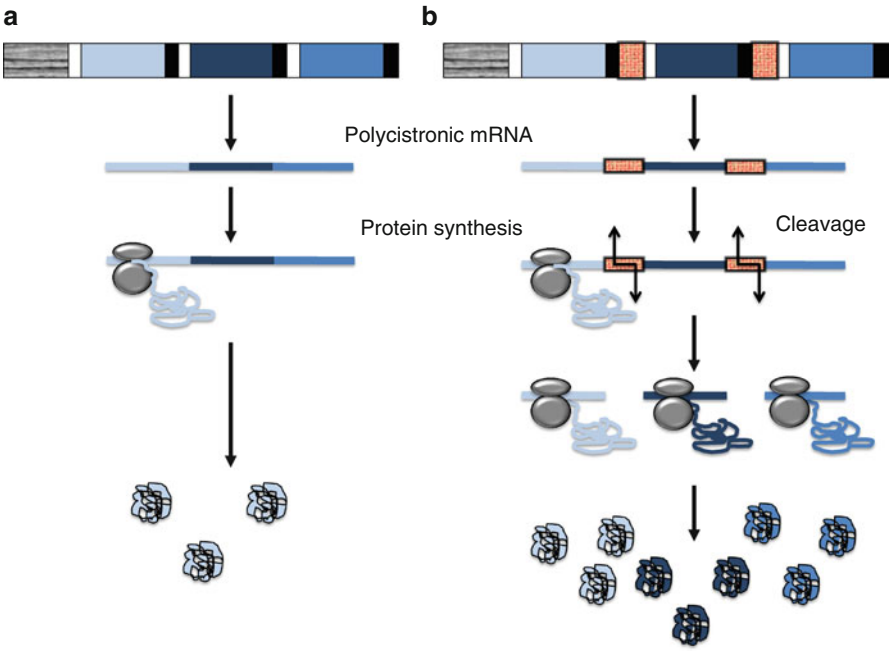

Fig. 21.3 Model representing the modular design of synthetic plastid operons for large-scale metabolic engineering in chloroplasts [22, 24]. (**a**) The case where translation of the polycistronic message is hampered because of missing intermediary parts (intercistronic expression element; IEE) is shown here. A construct with (from left) promoter, and three genes each with 5′ and 3′ end is shown. The result is only one protein type, which is encoded by the first part of the operon. (**b**) Here the three genes are separated by the IEE elements (shown in different patterns), which are later cleaved (*arrows*), resulting in three separate messages, which are then all translated, resulting in three different proteins (Adapted from [17])

feasible. Also, the routine method used for DNA delivery into plastids is by particle gun, which is not always accessible to every laboratory. Further advantages and disadvantages of plastid transformation are discussed in detail in a review [17].

Other aspects of transformation protocols, such as markers for further selection of transgenes will not be discussed here.

21.2.3 Examples for Metabolic Engineering

In the following step, the decision for the establishment of cell or organ cultures has to be made. Alternatively, depending on the method used for transformation (see below), intact plant material can be used. Cell cultures are dependent on the continuous addition of plant hormones, while the so-called hairy roots are hormone autonomous and therefore cheaper to cultivate (see Chap. 2; this book). Besides hairy roots, other organ cultures are also used to produce efficiently selected

secondary metabolites (see Part III, this book). The hairy roots can be induced by using the soil bacterium *Agrobacterium rhizogenes* from a variety of different plant tissues, for example from leaves, flowers, and also from roots. This phytopathogenic bacterium transforms plant roots with its tumor-inducing DNA (T-DNA), located on the Ti-plasmid, in nature to yield the hairy root symptoms [27]. The genes transformed into the host genome are termed *rol* genes. In addition to these *rol* genes, the bacterium can be transformed by other genes taken from plants or other organisms to change the metabolic pathways or profiles in a desired manner. The resulting organ cultures are also termed hairy roots, similar to the naturally occurring root disease. Such cultures are used now-a-days often for secondary metabolite production, either transformed with wild type of bacteria or with genetically modified ones. Already genetically optimized cell or organ cultures have to be adapted to cultivation conditions in bioreactors and this leads sometimes to stress situations due to possible depletion of nutrients or shearing forces in the reactor (see Part I; this book). The cultivation can also use to introduce deliberately stress factors, which can increase the synthesis of the product and also use elicitors, plant signalling molecules for biotic stresses [7].

Metabolic Genes

Since the plants do not leave the laboratory during cultivation in a bioreactor, there is no (legal) conflict with these genetic modifications. Detailed knowledge on the biosynthetic pathways, transport between compartments and their (transcriptional) regulation are necessary for targeted alterations. Many metabolites are now-a-days produced by cell and organ cultures.

Which factors can influence the outcome of the production of secondary metabolites by a given biosynthetic pathway? The best results can be obtained by using simple changes in pathways where only one enzyme or transcription factor is activated. For example, the synthesis of the alkaloid scopolamine *via* hyoscyamine and an intermediate is catalyzed in a two-step reaction by the same enzyme [28]. To modify complex biosynthetic pathways many things have to be taken into account to ensure that the desired metabolite can be produced. Among these are: (1) identification of the step determining the rate of production, (2) availability of precursors, (3) limitation of cofactors, (4) competing pathways using the same precursor or intermediates, (5) inhibition by final product, (6) transport over cellular compartments and (7) transcription factors [6, 29].

An attempt should be made to determine the critical step for the overall momentum of the pathway (compiled in [29]). This step is an ideal candidate for metabolic engineering. Despite a number of success reports the concept that single gene manipulations alter efficiently whole pathways and thus product formation, has to be revisited [30]. Some data have shown that the manipulation of such a single "bottleneck" or "rate limiting step" gene was not as effective as anticipated by experimental data on gene expression or flux data using labeled compounds. This observation suggests that in many pathways such individual key enzymes do not

exist. Therefore, over-expression of the gene for one enzyme in the pathway might render subsequent reactions more limiting. Nevertheless, to keep the approaches for metabolic engineering simple, transformation with single genes encoding metabolic enzymes is still feasible.

If genes for the pathway are not known, then an alternative could be, to use genes from other organisms to alter the metabolite pattern. If at least parts of the pathway need to be identified, methods of transcript and metabolite analysis (see above) may be applied. Even if a pathway is known at the genetic level, it needs confirmation by metabolic studies. Here, labeled precursors can be used to follow the fate of the precursor or selected intermediates of the pathway. This method is also useful to determine the flux rates for competing or to determine alternative pathways (e.g. [31]).

Changes can be generally achieved by overexpressing gene(s) encoding proteins responsible for limiting steps (Fig. 21.1). By increasing the flux from a precursor for a desired metabolite, the second undesired metabolite is not synthesized and therefore production of the desired product should be enhanced. However, when homologous genes are used, it has to be guaranteed that there is no co-suppression effect [9] reducing the expression of the transgene and the original gene itself. In the example shown here one intermediate would not be made any more, leading to the accumulation of another intermediate or the precursor. This in turn could lead to the undesired accumulation of an undesired compound. In principle, any of the metabolic steps leading to the synthesis of an intermediate in the pathway could be targeted with modifications, but to achieve optimum results the bottleneck of the pathway should be altered (Fig. 21.1).

There are experimental data available on modifications and/or overexpression of a pathway to generate a specific metabolite leading to the – unexpected – identification of novel compounds also. This was described for the metabolic engineering of carotenoids in rice callus, where a novel carotenoid, 4-keto-α-carotene was identified [32]. The engineered carotenoid pathway in rice callus was extended further by including a bacterial ketolase gene, which is able to form astaxanthin. This carotenoid is of a high value, but not a typical plant carotenoid. The novel carotenoid did not fit into the pathway leading to astaxanthin, so the authors postulated that this carotenoid may be formed from α-carotene *via* combined reactions of the heterologous gene products and endogenous rice enzymes [32]. Therefore, the evaluation of not only the expected end-product could be suggested for novel experimental systems.

So-called metabolons are emerging as structures where proteins from a specific biosynthetic pathway are organized as multi-enzyme assemblies that are in very close proximity to each other and in which the proteins catalyze sequential reactions, as shown for flavonoid biosynthesis [33]. The term "metabolic channeling" describes the idea that the intermediates between enzymatic reactions are not freely available, but are channeled through these large complexes [34]. For metabolic engineering these large complexes could be an advantage, because all proteins occur in the same subcellular compartment, making transport a lesser issue (see "Transport" in Sect. 21.2.3). However, the stoichiometry within such complexes might be a problem when only one gene is overexpressed and the reaction might be

imbalanced. Therefore, the absence of metabolons in many dedifferentiated tissues possibly resulted in the failure to detect the accumulation of desired metabolites in cell cultures [34].

The availability of precursors is also dependent on the number of biosynthetic pathways competing for a substrate. For example tryptophan is a precursor for a wealth of different secondary metabolites, but also for protein synthesis and the synthesis of the plant hormone indole-3-acetic acid [35]. Therefore, overexpression of tryptophan decarboxylase could lead to a reduction of other indole metabolites like indole glucosinolates. For laboratory cultures this is of no importance, but in the field, such plants could be less tolerant to various biotic stress factors [36, 37]. Also, the inhibition of an enzymatic reaction by the product or an intermediate could also constitute a problem (Fig. 21.1). In this case, even when the transgene is highly expressed, no product would be accumulating. This is also the case, when an essential cofactor is not present, but such a compound could be added to the culture, if not too expensive, or also engineered together with the gene for the biosynthetic enzyme.

In addition, bacterial genes can be used to alter the metabolite spectra of hairy roots (Fig. 21.1). *Catharanthus roseus*, the producer of the anticancer drugs, vinblastine and vincristine has been successfully transformed using bacterial tryptophan halogenase genes, resulting in the accumulation of halogenated terpene indole alkaloids. In general, the introduction of a halogen (halogenation) is important to biological activity [38] and bioavailability [39, 40] of such compounds. Chlorinated natural products, which are predominantly produced by terrestrial organisms, constitute the majority of halogenated products [39]. Transfer of two of these halogenases, PyrH [41] and RebH [42], which chlorinate the indole ring of tryptophan at positions five and seven respectively, yielded a different spectrum of halogenated indole alkaloids. This could be achieved because the chlorinated tryptophan variants were accepted by the enzyme tryptophan decarboxylase catalyzing the conversion to tryptamine and in this case to chlorinated tryptamine analogs [43]. Such approaches can also be exploited to discover novel bioactive compounds and/or to improve the biological value of already known natural compounds.

In addition to changes in pathway fluxes, the molecular properties of plant enzymes can be altered to modulate pathways. The genes are mutagenized in a way that is likely to modulate the activity of the encoded protein by, for example, altering the substrate-binding domain [44]. This can be done only with proteins, whose structure is known in detail. The enzymatic properties can be first tested by gene expression in microorganisms and if the desired mutant enzyme has been produced, then it is transformed into the plant. An alternative is to switch complete domains with important properties between enzymes. These proteins with novel structural elements could have completely new enzymatic properties. For example, the main biosynthetic pathway is preferentially catalyzed compared to side pathways. This has been shown to work for three genes encoding all dioxygenases in opium poppy (*Papaver somniferum*) morphine biosynthesis, where after combinations of various parts of the resulting protein novel mutant proteins were created, of which one had the desired property [45].

An alternative to the up regulation of the major pathway is the suppression of side pathways (Fig. 21.1) using techniques like antisense or RNA interference techniques.

This would result in the availability of more precursors for the main pathway. *C. roseus* produces vinblastine and vincristine *via* dimerization of the monoterpenoid indole alkaloids, vindoline and catharanthine (reviewed in [46]). Tryptophan decarboxylase was determined to be a key enzyme for the pathway. Next to several approaches to increase the metabolic flux through this pathway (reviewed in [47]), Runguphan et al. [48] have silenced tryptamine biosynthesis in hairy roots of *C. roseus* to produce an unnatural alkaloid metabolite spectrum. For this, tryptamine analogs, which can be used by the respective enzymes as substrates, were added during the cultivation process to the organ cultures and indeed novel alkaloids could be extracted.

In addition, such a strategy can be used to reduce toxic metabolites from otherwise beneficial plant materials (Fig. 21.1). In some cases not only the increase in active compound production is the most important aspect in generation of cells or tissues for metabolite production, but also a decrease in toxic by-products is necessary. Examples for such metabolites are pyrrolizidine alkaloids (PA), which occur in many medicinal plant species within the families of Asteraceae, Boraginaceae and Fabaceae [49, 50]. After ingestion, these alkaloids are metabolized and form toxic derivatives, which can harm human liver, capillaries and lung (reviewed in [50]). In addition, mutagenic and probably carcinogenic effects have been shown for these PA metabolites [51]. It is clear that the use of such plants in pharmacology or medicine requires complex cleaning processes and is often limited to a given maximum dose per year or to external application. Since the extract of a whole plant is more effective than the isolated main bioactive compound, the toxicity problem cannot be avoided by chemical synthesis. The choice of the knock down strategy depends on the target enzyme and possible off targets. Use of short hairpin RNAs, for example, is a well tested and a common method [52]. More recent and potentially more specific is the development of artificial microRNAs [53]. Both are post-transcriptional silencing methods, which do not eliminate the target gene but inhibit enzyme synthesis by mRNA degradation or translation inhibition. The principle of miRNA generation is shown in Fig. 21.4a. To reach silencing near to 100 %, careful planning of the construct as well as a strong promoter is necessary. Targeted knock out methods based on sequence-specific nucleases (zinc-finger nucleases and *t*ranscription *a*ctivator-*l*ike *e*ffector *n*ucleases; TALEN) for plants evolved during the last few years [54] and should be considered in future experiments. These nucleases enable targeted DNA sequence modifications by creating double-strand breaks in the genomic loci to be altered. The nucleases typically bind to a small number of nucleotides and the recognition sites can be engineered to recognize specific target sequences (Fig. 21.4b). With TAL effectors virtually any DNA sequence can be targeted [55]. The repair of the breaks, through either homologous recombination or, more often in higher plants, non homologous end joining, can be controlled to achieve the desired sequence modification (reviewed in [54]).

If the desired metabolites should be produced in several cell or organ systems, the choice of the production system can also be an option to reduce or exclude unwanted metabolites. Some complex substances are produced only in certain cell types and by that way excluded from systems that do not contain these cells. Huizing et al. [56] found that callus of *Symphytum officinale*, which was cultivated for 2

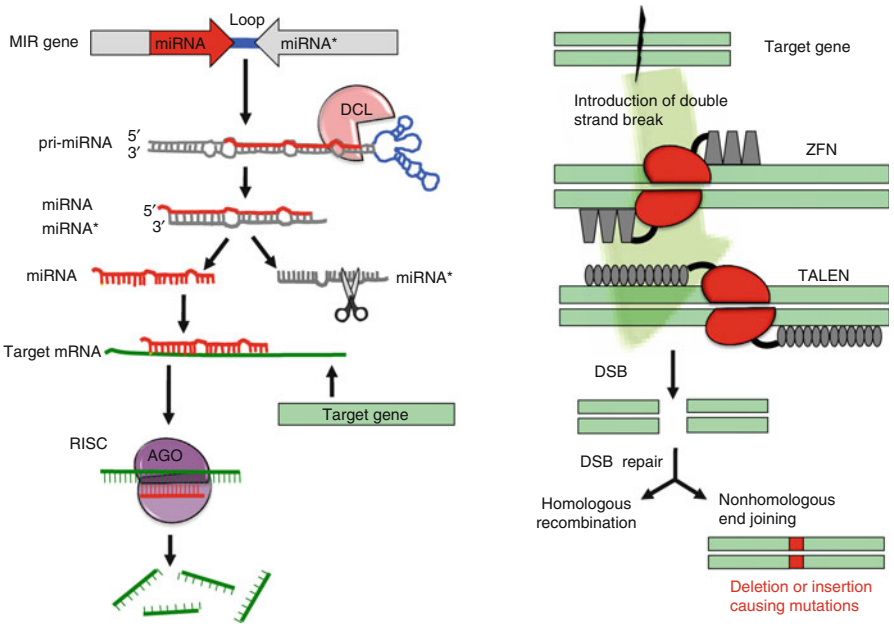

Fig. 21.4 Two possible mechanisms to reduce unwanted transcription products. (**a**) The generation of microRNAs is taking place in the plant to regulate mRNA levels post-transcriptionally. The design of artificial miRNAs allows the same process to reduce unwanted side products or toxic compounds in metabolic engineering. The miRNA is generated from a precursor which is encoded in the genome. After transcription, the miRNA folds in a way that a loop structure is cleaved by the enzyme DCL (dicer-like). Only the miRNA, which is complementary to the target mRNA is retained; the so-called miRNA* is degraded. Finally, the double-stranded RNA molecule is cleaved in the RISC (RNA-induced silencing complex), where the protein AGO (argonaute) plays an important role. (**b**) The use of nucleases to induce double stranded DNA breaks (*DSB*). Two nucleases are shown here, one is the zinc finger nuclease (*ZFN*), the other the transcription activator-like effector nucleases (*TALEN*). Both bind to DNA with specific recognition motifs, which can be genetically engineered. The nuclease generates breaks, which are then repaired by either homologous recombination (mechanism not shown here), or in plants more often by non homologous end joining. In both methods often mistakes are found, which result in mutations. The DNA is not transcribed correctly any more (Adapted from [54])

months did not produce PA any more. Regenerated plants again produced the typical alkaloid patterns.

Transcriptional Control

Transcriptional regulation is another option to determine the fate of a given metabolic pathway. Several families of transcription factors have been shown to participate in controlling the biosynthesis and accumulation of secondary metabolites (reviewed in [57]). Some genes are regulated only by one, others by two or even

three different transcription factors, depending on the signal(s) to which they respond. In other cases, only a few transcription factors control a complete pathway, and in such a case maybe overexpression of one transcription factor may lead to the production of a desired metabolite. The overexpression of a gene encoding a maize transcription factor in other plants is a good example for this kind of manipulation [58]. The transcription factor controls many steps in anthocyanin biosynthesis, so, various tissues of the resulting transgenic plants are colored.

Many transcription factors, which are involved in the control of secondary metabolite synthesis, are also controlled by signalling molecules such as jasmonic acid (JA). JA and salicylic acid (SA) are important signaling molecules in plant defense reactions against microbes and herbivores. Jasmonates can regulate many transcription factors, for example the MYB family, AP/ERF family and WRKY family [59, 60]. This implies that JA can regulate the synthesis of a wide spectrum of secondary metabolites. Among them are quite different structures such as glucosinolates and camalexin in *Arabidopsis*, flavonoids and anthocyanidins in many plant species, terpene indole alkaloids in *C. roseus*, nicotine in tobacco and artemisinin in *Artemisia*. Therefore, JA is often used to elicit the synthesis of secondary metabolites in biotechnology. The role of jasmonate has been confirmed in the regulation of terpene indole alkaloids, where it was shown to increase the levels of the transcription factor family ORCA 2 and 3 (octadecanoid-derivate responsive *Catharanthus* AP2-domain protein). Thereby the transcription of several genes involved in the terpene indole alkaloid pathway is enhanced [61, 62].

In case of the JA response, proteinaceous inhibitors of jasmonic acid-induced transcription have to be degraded before gene activation is possible. In this case not jasmonic acid itself, but especially its conjugate with the amino acid leucine is triggering this process [63]. Under non-inducing conditions a transcriptional repressor (JAZ) binds to the promoter of jasmonate-inducible genes [64]. This prevents transcriptional activation by the MYC transcription factors. To degrade the repressor, the amino acid conjugate of jasmonic acid with isoleucine is needed as a signal. Binding of the jasmonate conjugate together with the repressor protein to a receptor (COI1), which is an F-box protein, targets the repressor to proteolytic degradation in the 26S proteasome (reviewed in [65]). For this, the target protein has to be ubiquitinated, because only proteins with a polyubiquitin tail are recognized by the proteasome. Ubiquitination is achieved in the SCF-complex (SCF stands for SKP, CUL, F-box), composed of the JA-receptor itself (the F-box protein), the ubiquitin ligase and other adaptor proteins (Fig. 21.5). Once the repressor is degraded, the MYC-type transcription factor can activate the transcription of the jasmonate-inducible gene(s). Since these processes have been elucidated in the recent years, one might find interesting targets for genetic engineering also the signal transduction components.

JA plays a major role in the transcriptional control of taxane synthesis in *Taxus* species. It was shown that JA can induce transcripts of eleven genes encoding biosynthetic enzymes in the pathway [66]. Furthermore, this study provided an important finding namely that upon elicitation with the methyl ester of JA one of the two alternative branches of the taxane pathway was favored at the expense of the other and this in turn provided some insights into why unelicited cell lines failed to produce taxol.

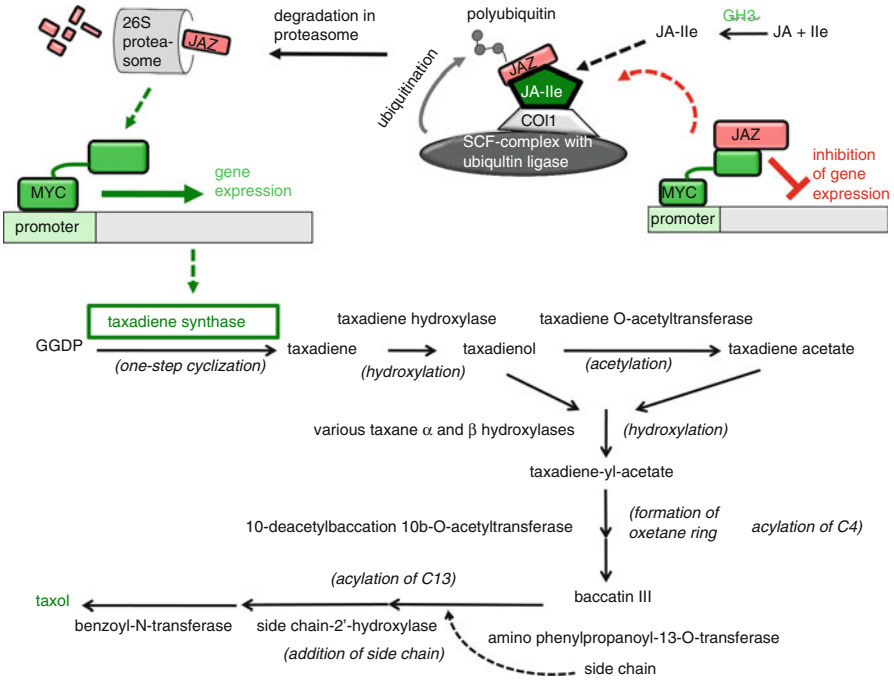

Fig. 21.5 Synthesis and possible transcriptional control of taxanes. One rate-limiting enzyme taxane synthase, which was used in experiments to increase synthesis, is indicated. Only an overview of the general pathway is shown here. Based on information from [71]. As an example for gene induction by jasmonate (*JA*) the current knowledge on JA-induced signal transduction is shown, which could also most likely induce transcripts encoding enzymes involved in taxane synthesis. Jasmonate (*JA*)-induced proteolytic degradation of transcriptional repressors (*JAZ*) has to happen to make gene activation possible. The F-box protein COI1 is part of the SCF-complex and the receptor for the isoleucine conjugate of JA (JA-Ile). The active JA-Ile is synthesized by members of the GH3 protein family. Another part of the SCF-complex (SCF: Skip, Cullin, F-box) has ubiquitin ligase activity, thereby polyubiquitinating the JAZ repressor proteins. These are targeted for proteolysis. Once the repressor is degraded in the 26S proteasome of the cell, the transcriptional activation of a transcriptional cascade *via* MYC transcription factors can take place

Transport

A major problem is the transport of intermediates between organelles, because the transporters would also need to be increased. Therefore, metabolic pathways occurring in only one compartment are easier to manipulate. A modification of compartmented pathways might be possible by adding signal sequences to the heterologously expressed genes. Alternatively, the synthesis of metabolites in the culture medium could be attempted by adding secretion sequences to the protein(s). The secretion of the protein or reaction product ensures easy purification of the product from the culture supernatant. Since the molecular transport mechanisms of secondary metabolites [67] are less well understood than other aspects, i.e. biosynthesis or transcriptional control, it is difficult to engineer this part of a compartmented pathway.

However, recently the engineering of glucosinolate transport has been reported [68], so that these techniques may become available also for intermediates. In some cases also long distance transport of molecules plays a role in the availability of precursors.

Examples for Successful Pathway Alterations

Pioneering work on pathway alterations using hairy roots and transformation approaches, which have been described here, has already been carried out for more than 30 years. For example Hamill et al. [69] were able to overexpress an ornithine decarboxylase from yeast in *Nicotiana rustica*, which resulted in enhanced production of nicotine. Many successful examples using this technique were to follow.

In the case of *Taxus baccata*, which is used for the production of taxanes, the reasonable concentration within the natural plant material of the bioactive compound is too low for commercial use [70], so, for the current demand alternative methods are needed. Till now, a complete chemical synthesis is not an alternative for taxol production, due to the complex structure of taxol that needs a lengthy and expensive synthetic routes. The genes and enzymes for the synthesis of this powerful anticancer drugs have been identified and characterized [71]. There are many steps involved in the universal diterpenoid progenitor geranylgeranyl diphosphate derived from the plastidial methyl erythritol phosphate pathway for isoprenoid precursor supply [72], selected steps are shown in the biosynthesis scheme in Fig. 21.5. The complexity of the pathway to taxanes makes one to realize that the genetic engineering is a challenge. Despite the challenges arising from the biosynthetic pathway, recent efforts have allowed to increase paclitaxel production in cell cultures by selecting for high biosynthesis lines and improving culture conditions (reviewed in [73]). Furthermore, now-a-days molecular approaches are used to improve the plant material producing taxanes (reviewed in [74]). It was possible to determine some genes that control the main flux limiting steps in taxane biosynthesis. It was also demonstrated using transcriptomic profiling, that some elicitors (i.e. JA; Fig. 21.5) induce a dramatic reprogramming of gene expression in *Taxus* cell cultures [75], which likely accounts for the enhanced production of taxol and related taxanes. Among the upregulated genes were those for taxadiene synthase (marked in Fig. 21.5), several intermediate hydroxylation steps, one acetylation step and several genes involved in the final biosynthetic steps, for example baccatin III-3-amino-13-phenylpropanoyl transferase and 3′*N*-benzoyl transferase. Taxadiene synthase is viewed as the first committing enzyme in the biosynthesis pathway [76]. Based on this information it is possible in the future to change the production levels by using metabolic engineering.

C. roseus produces the bisindole alkaloids vinblastine and vincristine, which are used as anticancer drugs (Fig. 21.6). Therefore, various modifications of these interesting metabolites have been genetically engineered in this species (reviewed in [47]). The biosynthesis of terpenes can proceed *via* two different pathways, one is termed mevalonate (MVA) pathway, the other methylerythritol-phosphate (MEP)

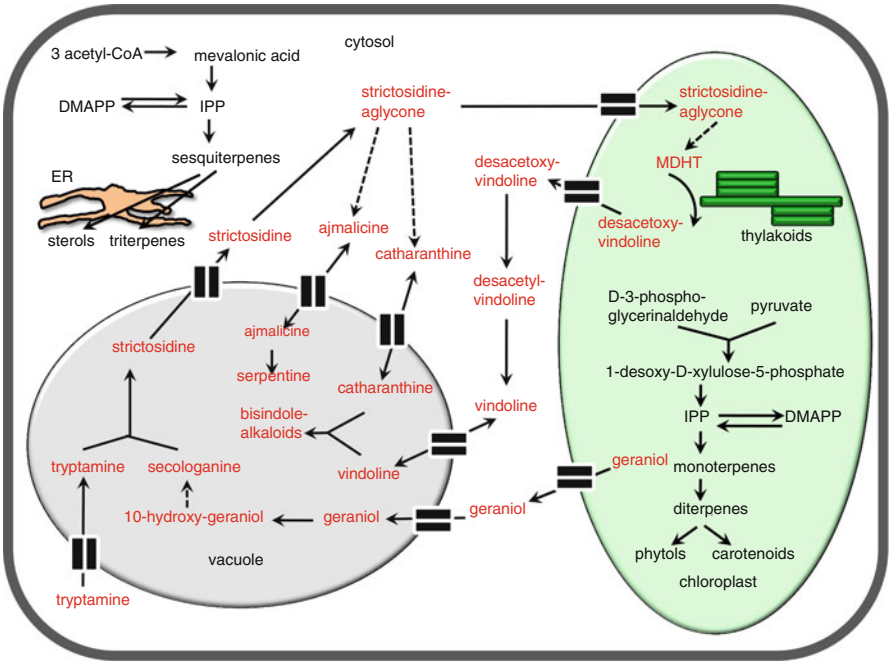

Fig. 21.6 General scheme for the two pathways of terpene synthesis with the general subcellular compartments where synthesis steps are taking place and compartmentation of terpene indole alkaloid biosynthesis in *Catharanthus roseus*. The compartments plastids, cytosol, vacuole and endoplasmic reticulum (*ER*) participate in the biosynthetic steps. ▬: Putative transporters needed; *solid arrows*: single enzymatic reaction step; *dashed arrows*: several reaction steps; *MDHT* 11-methoxy-2,16-dihydro-16-hydroxy-tabersonine, *G10H* geraniol-10-hydroxylase, *NMT* S-adenosyl-methionine hydroxytabersonine-*N*-methyltransferase, *DAT* acetyl-coenzyme A deacetylvindoline 17-*O*-acetyltransferase, *OHT* 2-oxoglutarate-dependent dioxygenase, *POX* peroxidase, *SSβG* strictosidine-ß-glycosidase, *SSS* strictosidine synthase (Adapted from [116])

pathway [77]. Names are derived from the major metabolites produced in the pathways. The MVA pathway is located in the cytosol, the MEP pathway in the plastids. Both can occur in the plant at the same time, but some organisms use only one pathway for the synthesis of terpenes. In the cytosol the sesqui- and triterpenes are synthesised, whereas the plastid is responsible for the synthesis of the mono-, di- and tetraterpene structures. Coupled to the endoplasmic reticulum oxidation steps are catalyzed by cytochrome P450-dependent monooxygenases. Terpenes can be made also in non-green plastids. It is assumed that these two pathways interact with each other. The terpene moiety of terpene indole alkaloids is synthesised predominantly *via* the MEP pathway in the plastids. The biosynthesis of these metabolites begins with the generation of indole and terpene precursors by the shikimate and MEP pathways. It is therefore clear that the pathway for terpene indole alkaloids is especially highly compartmented. Therefore, numerous transporters are also involved (see "Transport" in Sect. 21.2.3).

A key enzyme for the pathway is tryptophan decarboxylase (see above), which has been used many times as a target for genetic engineering, but several upstream enzymes can also be used. *C. roseus* hairy root cultures expressing genes encoding anthranilate synthase and/or tryptophan decarboxylase have been generated with enhanced fluxes through the tryptophan branch of the terpene indole alkaloid pathway and enhanced levels of tryptamine and serpentine as a result. Additional treatment of anthranilate overexpressing cultures with terpenoid precursors resulted in an elevation of various compounds including catharanthine, ajmalicine, lochnericine, and tabersonine. Tabersonine is a direct precursor for the pharmacologically important molecules vinblastine and vincristine, and therefore its increase could result in better production rates. On the contrary, increased synthesis of side products such as lochnericine could result in lower concentration of the precursors for the desired compounds. Increasing the expression of the gene encoding strictosidine synthase, an enzyme that catalyzes another rate-limiting step for terpene indole alkaloid biosynthesis in the second branch – the terpene moiety – of the pathway, also enhances the synthesis of downstream metabolites, and their production is further enhanced if one or both precursors loganin and tryptamine are added. Overexpression of other genes from the pathway also alters metabolite composition (reviewed in [47]).

A third example is the engineering of glucosinolases. Glucosinolates are bioactive metabolites mainly of the crucifer family [78]. They are derived from aliphatic (methionine), aromatic (tyrosine and phenylalanine) or indolic (tryptophan) precursors [79]. Their increase can often be stimulated by elicitation with either chemical or biological agents, such as fungi or insects [80, 81]. The production of glucosinolates in hairy root cultures would be attractive, but here it was shown that in many cases the pattern differed significantly from the mother plant [82] and the desired classes were either totally absent or present only in low amounts [83]. Nevertheless, induction of specific pathways by methods described in this chapter could also lead to the increased production of selected glucosinolates (GSL). In intact plants, it was possible to engineer the glucosinolate pathway in various studies (reviewed in [84]). The aim is to improve the glucosinolate levels for either plant protection against pests and/or beneficial health effects for human nutrition. The major studies have been carried out with the model plant *Arabidopsis thaliana*, but also with some other Brassicaceae members, identifying many genes for biosynthetic enzymes, but also transcription factors. Therefore, the genes from *Arabidopsis* have been used in many cases for metabolic engineering. For example, Zang et al. [85] reported the transformation of Chinese cabbage with genes encoding enzymes for the synthesis of aliphatic GSL from *Arabidopsis*. Albeit an accumulation of aliphatic GSL was observed, the patterns were not changed [85]. On the contrary, *Arabidopsis* does not possess aromatic glucosinolates, so, it was possibly to engineer genes from *Sorghum bicolor* into *Arabidopsis*, which indeed resulted in the production of a benzyl-GSL [86]. A different chemotype was generated when a gene *from Manihot esculenta* was expressed in *Arabidopsis*. The resulting enzymatic activity led to the synthesis of valine and isoleucine derived GSL [87]. In addition, strong homologous

overexpression of biosynthetic genes from *Arabidopsis* led to the accumulation of atypic GSL normally not present in this plant [88]. Finally, it was possible to turn a non-GSL synthesizing plant *Nicotiana tabacum* into a GSL producer by expressing a polycistronic message with three genes for GSL biosynthetic enzymes from a single promoter [89]. Upon precursor feeding the transgenic plants accumulated the expected benzyl-GSL, demonstrating that the proteins were synthesised and active [89]. Furthermore, GSL patterns could also be engineered by overexpressing genes for transcription factors (see "Transcriptional Control" in Sect. 21.2.3).

21.3 Alternative Plant Systems in Biotechnology

As discussed above, intact plants, cell cultures and especially hairy root cultures have all their distinct advantages. However, for certain aspects of biotechnology, alternative plant systems need to be investigated. In terms of cultivation procedures there are alternative systems to hairy roots or cell cultures now available. While algae constitute good systems for some products (Chap. 6, this book), moss plants/cultures have received a lot of attention over the last decade [90].

21.3.1 Green Algae

Several products from green algae, which are used today, consists of secondary metabolites which are extracted from the algal biomass (reviewed in [91]). Next to hydrogen production [92] and biofuels [93], the best known examples for secondary metabolites produced in microalgae are the carotenoids astaxanthin and β-carotene. Several microalgae are currently used in bioreactors, e.g. for the production of carotenoids (see Chap. 4; this book). Many species of green algae are able to produce other valuable metabolites such as antioxidants, polyunsaturated fatty acids, vitamins, anticancer and antiviral drugs [91]. Many of these compounds are secondary metabolites which are produced under stress conditions, but other compounds are produced under optimum growth conditions, making the prediction for cultivation yet difficult. An important organism used in biotechnology is *Chlamydomonas reinhardtii*. For *C. reinhardtii* highly efficient transformation systems are available [94]. The two most commonly used involve either glass bead-assisted transformation or biolistic methods [95]. In addition, transformation of *C. reinhardtii* using *A. tumefaciens* is possible [96]. Similarly to higher plants (see above), efficient plastid transformation is possible (reviewed in [97]). Another model system is *Chlorella* sp., which has been used for the production of lipids, even though the green alga is a major producer for hydrogen [98]. Also for this organism several transformation methods are available, among them electroporation [99], *A. tumefaciens* [100] and biolistic transformations [101].

21.3.2 Mosses

The moss *Physcomitrella patens* has been used as a model organism for lower land plants, mainly due to its mostly smaller gene copy number, alternative biosynthetic pathways and last but not least for its high rate of homologous recombination (reviewed in [102]). Like in plastids (see above) this enables a targeted integration, which was initially used for the targeted gene knockout (e.g. [103, 104]). Now other transformation systems for this organism have also been recently developed [105]. An *in vitro* cultivation system for *P. patens* is established basically for all stages of the moss life cycle, but most reports rely on *in vitro* propagation of protonema in photobioreactors [90]. The moss is suitable for the production of many complex biopharmaceuticals, especially glycosylated proteins, because such proteins can be produced safely in various photobioreactors of different sizes without the need for animal-derived medium compounds [106].

More than 400 novel secondary metabolites were isolated from bryophytes during the last decades and structurally elucidated [107]. These compounds encompass the chemical classes of flavonoids, biflavonoids, terpenes and terpenoids. These metabolites most likely are necessary for protection of moss plants in the environment, as for higher plants. In addition, the outer tissues are less well protected in comparison to tracheophytes, therefore a large amount of defense chemicals is most likely needed (reviewed in [108]). Furthermore, most bryophytes grow on forest ground in a close connection to biodegrading microbes. Therefore, a protection against pathogens like fungi or bacteria is essential for survival in this habitat [108]. Antimicrobial and fungicidal activities have been detected in moss extracts. Based on the existence of such antimicrobial chemicals present in mosses, a commercial product was developed and is sold as a natural pesticide [109]. However, due to the risk of allergic reactions bryophyte extracts were not recommended for scientific medicinal use so far [109].

Also, moss plants emit volatiles into their environment to interact with other organisms. Sporophytes of some mosses can be fertilized by insects and the moss plants *Ceratodon purpureus* and *Bryum argentum* attract these by volatiles [110]. This data demonstrate the ability of moss to synthesize a wide range of compounds to adapt in their ecological habitat.

Indicative of its secondary metabolism, *P. patens* possesses a large gene family of polyphenoloxidases, comprising of 13 members [111]. In addition, it releases large amounts of the diterpene 16-hydroxykaurane [112]. Metabolic profiling of moss can result in the identification of novel compounds [113]. Even though *P. patens* produces a large amount of interesting secondary metabolites, its use in biotechnology comes from the ability to produce human glycoproteins in bioreactors [114]. Since heterologous protein production is not the focus of this chapter, the topic will not be touched.

Moss cultures might be suitable in cases where other organisms, also higher plants, are inhibited in growth by the metabolite of interest. For the production of taxol (see above) it was possible to express the gene for taxadiene synthase (see also

Fig. 21.5) from *Taxus brevifolius* in *P. patens* [115]. The authors observed that in stable moss transformants, taxa-4(5),11(12)-diene was produced up to 0.05 % fresh weight of tissue, without significantly affecting the amounts of other endogenous diterpenoids. Unlike higher plants that had been genetically modified to produce taxa-4(5),11(12)-diene, transgenic *P. patens* did not exhibit growth inhibition due to alteration of diterpenoid metabolic pools [115].

Clearly the interest in the exploitation of alternative organisms for the biotechnological production of secondary metabolites will enhance the possible spectrum that can be produced in the future.

21.4 Conclusion

Successful metabolic engineering is always dependent on critical experimental evaluation in the beginning, such as choice of plant material, transformation methods, the ability to detect the product(s) in question and the identification of suitable genes and strategies for transformation (Fig. 21.1). In the future also the potential of sequencing techniques to be used for non-model plants with interesting medicinal components will increase the possibilities for metabolic engineering further.

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Chapter 22
Theoretical Basis of Plant Cell and Tissue Culture for Production of Biomass and Bioactive Compounds

Arturo Lopez-Villalobos, Edward C. Yeung, and Trevor A. Thorpe

Abstract Plant tissue culture is an important biotechnological tool, which involves biochemical and genetic manipulations to onset specific gene programming, which determines an optimal cell differentiation state for the production of bioactive compounds. Indeed, not only the metabolism but also the morphogenetic processes are modified in plant cells to drive the synthesis and accumulation of economically valuable compounds. In this chapter, we provide an overview of the current molecular approaches to control the expression of specific genes encoding putative heterologous and native enzymes as well as transcription factors to enhance the metabolic flow of specific pathways in order that notable bioactive compounds can be accumulated in plant cells at acceptable commercial levels. Such methods vary from single-gene plant transformations to the emerging multi-gene transformation (gene stacking) technologies embraced by private companies focusing primarily in the metabolic engineering of secondary metabolism. The virtues and potential of these molecular methods in their application to tissue culture systems are presented. Additionally, the importance of subcellular targeting of proteins, notably biosynthetic enzymes and pharmaceutical antibodies, for enhancing their activity and stability is also discussed. Finally, the progress in two emerging approaches for the production of bioactive molecules, the manipulation of cell differentiation and cell immortalization are expounded in this article. Thus, we present the molecular basis to control both cell differentiation and cell immortalization in plant tissue culture systems as novel avenues to control and perpetuate the gene programming which in turn creates and regulates cellular microenvironments for the optimal biosynthesis of valuable compounds. Consequently, our objective is to present how basic approaches, including the manipulation of gene expression, are amalgamated to other molecular strategies of higher hierarchy, particularly the manipulation of cell differentiation and immortalization for the synthesis of bioactive molecules in plant tissue culture platforms.

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22.1 Introduction

Plants being sessile organisms have to create a flexible life style in order that they can survive on land. The relative ease for plant cells to regenerate and the production of secondary metabolites as defense compounds can be regarded as a basic mechanism of plant survival on land. The theoretical basis for plant tissue culture was first proposed by Gottlieb Haberlandt in his address to the German Academy of Science in 1902 on his experiments on the culture of single cells [1]. Even though his experiments were unsuccessful, nevertheless he predicted that one could successfully cultivate artificial embryos from vegetative cells. Haberlandt's vision was subsequently proven by the work of White, Gautheret and others. The early findings set the stage for the dramatic increase in the use of *in vitro* cultures in the subsequent decades. Details on the early pioneering events in plant tissue culture could be found in White [2], Bhojwani and Razdan [3], and Gautheret [4] and the general history of plant tissue culture is summarized in the reviews by Vasil [5] and Thorpe [1].

Higher plants produce a large number of diverse organic chemicals, which are of pharmaceutical and industrial interest. Theoretically, plant tissue culture has the potential to produce an infinitive range of bioactive compounds, once the high producing tissue or cell lines are selected and the culture conditions are optimized. The first attempt at the large-scale culture of plant cells for the production of pharmaceuticals took place in the 1950s at the Charles Pfizer Co. The failure of this effort limited research in this area in the United States, but work elsewhere in Germany and Japan in particular, led to the development, so that by 1978 the industrial application of cell cultures was considered feasible [6]. Furthermore, by 1987, there were 30 cell culture systems that were better producers of secondary metabolites than the respective plants [7]. Unfortunately, many of the economically important plant products are either not formed in sufficiently large quantities or not at all by the plant cell cultures. The production of unique products is usually a trait of differentiated cells. In a culture system, as cells can be at different stages of the cell cycle, production of unique metabolite is not a priority for cell growth. Hence, in order to "commend" the cell culture to produce specific metabolites of interest, one needs to understand properties of the culture system and the use of different strategies to elicit a product. The review by Yeoman and Yeoman [8] provides a useful overview for the production of secondary metabolites. In recent years, new strategies such as cell immobilization and elicitation coupled to a bioreactor technology have been developed. Currently, plant cell and tissue culture has successfully driven the large-scale production of several drugs, for instance shikonin, ajmalicine, rosmarinic acid, digoxin, ginsenosides and paclitaxel (reviewed by El Meskaoui [9]).

In order to fully realize the potential of any plant tissue culture system a number of research works have been undertaken to define the most influential factors

controlling the accumulation of bioactive compounds, notably secondary metabolites and recombinant proteins, in plant tissues. In these tasks, recent discoveries have enhanced our knowledge on the involved molecular mechanisms as well as on the plant structure requirements [10–12]. Thus, in intact plants, secondary metabolites and probably other bioactive compounds are synthesized from an assembly of consecutive enzymes regulated by a pool of transcription factors, which are proteins responsible for activation of gene expression. More importantly, these components are produced through intricate networks which constitute complex systems with highly specialized developmental and spatial controls at the cellular, tissue, organ and whole plant levels. In these networks, transcription factors are the actual linkers between specific abiotic and biotic environmental stimuli and developmental programmes to activate genes involved in the synthesis of the bioactive compounds [10]. In contrast, disruption of networks often occurs in plant tissue cultures, as a particular tissue or specific cells are usually excised from various parts of a plant for *in vitro* culture. Failure in the production of the desired bioactive compounds then occurs in many cases when two or more enzymes are required for the synthesis, and they are naturally confined to different plant organs. If enzymes retain organ specificity *in vitro*, the synthesis of the bioactive substances is blocked at intermediate stages resulting in low or no accumulation of targeted compounds [13]. In other cases of arrested accumulation of bioactive compounds, if specific environmental stimuli or a determined cell differentiation conditions are absent, then that will most likely result in failure of specific gene expression necessary for the production of bioactive compounds. All these findings explain to some extent, the causes for the failure in the production of specific bioactive compounds in plant tissue culture. Moreover the experimental findings also provide useful insights of the limiting factors involved and suggest new strategies that should be developed to overcome such hurdles.

In this chapter, we summarize recent findings and provide a theoretical basis for plant cell and tissue culture for the production of biomass and bioactive compounds. We primarily focus on the main strategies that are being applied to plant tissue culture to facilitate the production of bioactive compounds, especially on the molecular biology and other advanced techniques involved in the control of gene expression, cell differentiation and finally, cell immortalization, as an additional cell property to enhance the yields and productivity of the culture systems. These new approaches and technologies promise the generation of bioactive compounds from *in vitro* culture systems.

22.2 Manipulation of Gene Expression to Enhance the Production of Bioactive Compounds

The advent of molecular biology techniques applied to reverse genetics has revealed the pathways for the synthesis of many bioactive compounds, as well as provided an insight on their regulatory molecular mechanisms. These studies make use of knockout mutants or transgenic lines derived from gene silencing platforms

[i.e. antisense RNA (asRNA) and RNA interference (RNAi)] to define primarily the rate-limiting enzymes for a given metabolic pathway [14]. Following the characterization of these enzyme-encoding genes as well as the manipulation of their expression, through various overexpression or post transcriptional gene silencing (PTGS) techniques has led to the accumulation of many types of bioactive compounds in plant tissue cultures [15–17]. Alternatively, an increased synthesis of bioactive compounds has been pursued through the overexpression of the genes encoding key transcription factors responsible for the activation of a group encoding genes involved in such metabolic pathways [18–21]. In other cases, for instance secondary metabolites, the manipulation of a group of genes involved in a specific metabolic pathway has been more advantageous for the synthesis of such substances than those approaches in which the expression of a single gene is controlled [22–24]. Furthermore, in many bioactive compounds, especially recombinant proteins, the targeting of these compounds into subcellular compartments is as important as the levels of gene expression for the reason that their functionality and stability can be manipulated. Therefore, many targeting strategies have been developed to direct the biosynthesis and accumulation of the bioactive molecules to optimal subcellular environments. Here, we are highlighting the progresses in the main genetic strategies used to enhance the production of bioactive compounds in *in vitro* cultured plant tissues through the manipulation of gene expression and compound-subcellular targeting [25–28]. We are presenting key examples to illustrate the progresses of the molecular biology-based platforms without attempting to achieve a comprehensive review.

22.2.1 Manipulation of Enzymes: Single Rate-Limiting Enzymes Versus a Group of Enzymes

The starting point for metabolic engineering is to elucidate the rate-limiting phases of the biosynthetic pathway. Many methods have been applied to dissect these critical metabolic steps but the genetic approaches, including mutant selection, RNAi mediated-gene silencing and gene overexpression through gene engineering have been most effective [23]. These genetic modifications result in transgenics or mutant plants in which the contents of intermediate metabolites are measured to re-assure that the modified gene encodes the most rate-limiting enzyme of the pathway. Therefore, it is not surprising to find a number of research works in which the manipulation of only a single gene, encoding the 'bottleneck' intermediate, was sufficient to shift the entire metabolic pathway and to drive high accumulation of a specific bioactive compound in plant tissues [15, 29].

By applying this strategy, numerous active compounds, including lipids, proteins and carbohydrates have been achieved in the whole intact plant, but the success in *in vitro* cultured plant cells and tissues is limited to a few cases, mainly to secondary metabolites [9, 23, 26, 30]. One of the first reports is that the three to fourfold increase of artemisinin in *Artemisia annua* root hairy cultures when the farnesyl

diphosphate synthase (FPPS) gene from cotton was overexpressed by using the CaMV 35S promoter after *Agrobacterium rhizogenes*-mediated transformation. Such an outcome was possible due to the fact that FPPS produces the precursor farnesyl diphosphate which is critical for tepernoid biosynthesis, including artemisinin [31]. Another economically important secondary metabolite in which its biosynthesis was increased in hairy root culture by gene engineering is the tropane alkaloid scopolamine. Scopolamine is naturally synthesized in many genera of the Solanaceae, for instance *Hyoscyamus*, *Atropa*, *Duboisia*, *Scopolia* and *Datura*, and the enzyme putrescine *N*-methyltransferase (PMT) catalyzes the first committed step, as it produces the metabolite precursor *N*-methylputrescine. Similar to the previous example, the constitutive overexpression of the PMT gene from *Nicotiana tabacum* in hairy root cultures of many Solanaceae species resulted in more than a sixfold increase of scopolamine [29, 32]. However, in many hairy root cultures of the species *Hysoscyamus niger, Atropa belladonna and Duboisia hybrid* the over expression of the PMT tobacco gene had no effect on alkaloid accumulation. These studies demonstrated that the effects of the PMT overexpression are sufficient to increase scopolamine levels in plant cultures, but its action is species-dependent. The failure of PMT in some plant species may be due to the presence of other downstream steps in addition to PMT that would have been more critical for scopolamine biosynthesis [24, 33, 34]. This finding therefore shows that single-gene manipulation is not sufficient in some cases to enhance the levels of a particular economically valuable compound.

Instead of overexpressing a gene to enhance a metabolic pathway, the blocking of the flow of a 'bottleneck' intermediate or precursor to a competitive pathway may result in a more effective means for inducing the accumulation of certain bioactive compounds. Under this genetic approach, this blockage is conducted by any platform of gene silencing in which typically the gene encoding the receiver enzyme responsible for the entrance of such a precursor into the alternative pathway is down-regulated. This strategy has been applied by some research groups in attempting to enhance the biosynthesis of morphinan alkaloids by blocking the metabolic flow of (S)-reticuline into the benzophenanthridine alkaloids, such as sanguinarine, chelirubine, and macarpine. By silencing the genes encoding the berberine bridge enzyme (BBE) in California poppy hairy root and cell cultures, the content of the benzophenanthridine alkaloids was successfully reduced, but still the contents of morphinan alkaloids were unmodified [15–17]. To overcome this problem, research is still going on to detect what other metabolic steps are critical to increase the morphinan alkaloid biosynthesis in hairy root cultures, although the over expression of the gene encoding salutaridinol 7-*O*-acetyltransferase (*SalAT*; [35]) together with the silencing of BBE gene can be a feasible approach.

The previous example illustrates that single-gene manipulations are sometimes not sufficient to promote the accumulation of the final desirable product, even though they are highly effective in driving the accumulation of certain metabolite intermediates in a particular branch within the pathway. Therefore, manipulation of bioactive compounds appears to be a rather complicated task in terms of the complexity of the processes involved in the metabolic pathways. In this view, researchers

have tried to address this scientific problem in order to pursue a more effective way of metabolic engineering. Recent advances on these investigations have shed light on the complexity of the metabolic pathways revealing the presence of multiple crucial steps which are controlled by the coordinated interaction of numerous genes on the same or interconnected pathways under a harmonized regulation [22, 23]. Under this conception, the improvement in biosynthesis of economically important compounds appear to be even more challenging, and researchers have to come up with new strategies in which the manipulation of multiple genes is necessary to achieve a more effective way in the control of the metabolic processes. The simple version of this approach is the co-expression of two genes that are believed to encode enzymes of the most rate-limiting steps of the pathway. A successful example of this strategy is the enhancement of the biosynthesis of scopolamine by the simultaneous constitutive overexpression of the genes encoding the upstream rate-limiting enzyme PMT and the downstream enzyme hyoscyamine 6 β-hydroxylase (H6H) in transgenic henbane (*Hyoscyamus niger*) hairy root cultures. Indeed, these two-gene derived transgenic cultures produced the highest reported contents (411 mg L^{-1}) for scopolamine through the engineering of a plant whereas the contents of scopolamine in cultures harboring a single-gene were very reduced [24].

In the light of the successes with multi-gene transformants in intact plants and tissue cultures [22], metabolic engineers started to develop more sophisticated platforms to integrate numerous genes into the genome. Further encouragement for the application of such techniques came from the discovery that many genes, mainly those involved in the synthesis of secondary metabolites, are assembled in biosynthetic gene clusters for performing multistep specialized metabolism in plants, as well as in other organisms [36]. In fact, these gene clusters are activated as single transcriptional units in an operon fashion in order that all genes are synchronously expressed and intermediate toxic metabolites cannot be accumulated in plant cells. This genetic mechanism undoubtedly guarantees the synthesis of the end products and simultaneously avoids the cell death, as it would likely occur if each gene would independently be transcribed [36–38]. The logical strategy for metabolic engineering to mimic the natural occurrence of gene clusters would be the transfer and integration of a large number of genes. This method is commonly known as "gene stacking" and has been currently embraced by many biotechnological companies (i.e., Mosanto, Chromatin Inc.™, Agrisoma, etc.) which are employing many platforms, including sequential transformations, large conventional vectors with many expression gene cassettes, virus-mediated gene transfer, transformation-competent artificial chromosomes, plant artificial chromosome-assisted gene transfer and transformation with new forms of bacterial artificial chromosome (BAC) vectors, termed binary-BAC vector, capable of transfer large DNA fragments into plant genomes [22, 39–41]. The application of such technologies in plant tissue culture for the production of bioactive compounds is still in its infancy, but a proof-of-concept experiment with BY2-tobacco cells and rice protoplasts have showed its feasibility. In this novel protocol, large DNA fragments (i.e. 100 kb) are transferred and cloned into BAC vectors which are subsequently trapped in bioactive beads that carry the plasmid DNA into the cell for integrated transformation [42]. Undoubtedly,

the flourishing of the gene stacking techniques would enable that many pathways which require the transfer of polycistronic genes, for instance the gene clusters for synthesis of noscapine or benzoxazinones [43, 44], can be easily engineered for a more efficient production of these compounds. However, for the multiple-gene construction of metabolic pathways of bioactive compounds in heterologous plant cell cultures, elimination of endogenous enzyme activities may be crucial to drive metabolite synthesis towards the desired end product [45].

Independently on using either single-gene or multi-gene modifications, metabolic engineers also need to define the type of gene expression, i.e. constitutive or inducible, required to achieve the expected levels of the desired bioactive compound. Due to the complexity of metabolic processes, there is still no available method which can assist researchers to decide what type of expression would be the most suitable for their particular purposes. Instead, the selection of constitutive or inducible promoters for metabolic engineering is a trial-and-error task and needs to be tailored by time and energy-consuming experiments. Currently, the CaMV 35S promoter has been the first option for the constitutive expression of a variety of genes and researchers have successfully produced many recombinant proteins (i.e. human serum albumin, human granulocyte-macrophage colony stimulating factor, Human mAb against HBsAg, etc.; extensively reviewed by Huang and McDonald [11]) and secondary metabolites (i.e. scopolamine and artemisinin as previously detailed) in many types of plant cultures. However, reports have also showed that sometimes the constitutive expression of an enzyme encoding gene generates constantly a final product or intermediate metabolites which are toxic for the plant cells. Therefore modulation of the accumulated quantities of these toxic compounds in plant cultures is indispensable for maintaining both the levels of the bioactive compound and viability of the cultures. In these cases, the use of an inducible promoter to direct the transgene expression has been proven to be a feasible approach. For example, by employing a glucocorticoid-inducible promoter to regulate the expression of the *TRYPTOPHAN DECARBOXYLASE* (*TDC*) gene, the content of the monoterpeneindole alkaloid serpentine was increased as much as 129 % in *Catharanthus roseus* hairy root cultures. The cells may be more competent to detoxify their intermediates [10] leading to the enhanced production of serpentine. The same benefits were obtained by using many chemically inducible, metabolically inducible and physically (i.e. light and temperature) inducible promoters for the expression of genes encoding the pharmaceutical antibodies and proteins in many cell suspension systems (extensively reviewed by Huang and McDonald [11]).

This section discussed significant progresses made in the production of bioactive compounds in plant tissue culture through the constitutive or inducible expression of genes encoding key enzymes of certain metabolic pathways. The emerging high-throughput molecular techniques such as genomics, transcriptomics and metabolomics, will undoubtedly allow researchers in the near future to obtain better understanding of the components and control switches of the metabolic pathways involved in the synthesis of bioactive compounds in *in vitro* culture environments. It can be anticipated that the new genes would surely be discovered and with the advent of novel tools for chemical synthesis and assembly of genes into artificial

chromosomes would allow the stacking of undefined number of genes [10, 12, 46]. Currently, metabolic engineering has started depending more on the gene stacking techniques. However, the efficiency in gene expression needs to be enhanced in order that expected levels of bioactive compounds can meet the expectations of large-scale commercial productions.

22.2.2 Manipulation of the Expression of Key Transcription Factors Regulating the Activation of Biosynthetic Genes

Although the core strategy of metabolic engineering is the control of genes encoding biosynthetic enzymes, currently, an alternative genetic approach based on the manipulation of key transcription factors is rapidly emerging for the production of some bioactive compounds. Transcription factors are specific DNA sequence binding proteins which interact with the promoter regions of the genes to regulate the rate of mRNA synthesis [47]. Hence, it was not surprising to discover that the expression of many genes from a specific pathway are regulated by a common transcription factor which dictates the accumulation of mRNAs in the same type of tissue and developmental stage to guarantee the synthesis of an end-product. Indeed, current research has unveiled that many genes involved in the synthesis of specific branches of secondary metabolism and lipid biosynthesis are regulated by mutual transcription factors [19, 48]. Therefore transcription factors have become a powerful tool for engineering the metabolic pathways. Unfortunately, the use of transcription factors for the production of bioactive compounds in plant tissue culture has been restricted to secondary metabolites, with very few successful examples reported in the literature [19, 47, 49]. One among these secondary metabolites is the terpenoid indole alkaloids (TIAs) which include ajmalicine, catharanthine, serpentine, and vincoline. Studies with TIA synthesizing genes in hairy root cultures of *Catharanthus roseus* demonstrated that ORCA (Octadecanoid-Responsive CatharanthusAP2/ERF-domain) transcription factors are regulated by jasmonic acid (JA) and affect tremendously the activation of several TIA biosynthesis genes, including *ANTHRANILATE SYNTHASE (AS), STRICTOSIDINE SYNTHASE (STR), TRYPTOPHAN DECARBOXYLASE (TDC), DESACETOXYVINDOLINE 4-HYDROXYLASE (D4H), D-1-DEOXYXYLULOSE -5-PHOSPHATE SYNTHASE (DXS) AND CYTOCHROME N P450-REDUCTASE* (*CPR*; [49, 50]). Two ORCA transcription factors (ORCA2 and ORCA3) have been identified and act reluctantly in the transcription of the above TIA genes by binding the JA-cis-element and elicitor-responsive element (JERE). Under an excess supply of the TIA precursor (i.e. liganin) the overexpression of *ORCA3* in *C. roseus* cells promoted the accumulation of many TIAs [18, 19]. It appears that ORCA3 is a key transcription factor to enhance TIA accumulation in plant cells, but its expression alone is still not sufficient to achieve high levels of TIAS. Recent findings suggest that other transcription factors for instance the repressor family of transcription factor IIIA-type, zinc finger proteins (i.e. ZCT1, ZCT2 and ZCT3) and the WRKY

transcriptional activator CrWRKY also affect TIA biosynthesis as assessed by gene overexpression studies in hairy root cultures [19, 51, 52].

In another study working with cultured maize cells, it was demonstrated that anthocyanin biosynthesis can also be engineered using transcription factors. In this case, the ectopic expression of appropriate transcription factors can shift the abundance of metabolites to certain branches of the metabolic pathway. Thus, the overexpression of the transcription factor C1, a c-MYC type activator, and the R transcription factor, a c-MYB type activator, drove the accumulation of two cyanidin derivatives whilst high contents of 3-deoxy flavonoids, a secondary metabolite with insecticide properties were accumulated in transgene cells containing the P transcription factor, which is also a c-MYB type activator [20, 21]. This study illustrates that metabolic engineering can be advantageous for the production of anthocyanin with pharmaceutical, notably anti-carcinogenic or insecticide properties.

In conclusion, the coordinate transcriptional control of gene expression *via* manipulation of transcription factors is emerging as a major tool for metabolic engineering of plant tissue culture for the production of bioactive compounds. The best attribute of this alternative approach is that the expression of many biosynthetic genes can be altered by changing the expression of a single gene encoding a key transcription. In this context, the metabolic engineering through the manipulation of a large number of biosynthetic genes, i.e. gene stacking may not be necessary. Furthermore, with the advent of sophisticated tools for promoter analysis and the designing of artificial promoters and synthetic transcription factors, the metabolic engineering based on transcriptional manipulation appears with unlimited horizon for improvements.

22.3 Genetic Strategies for the Manipulation of Subcellular Targeting of Bioactive Compounds

Manipulation of genes encoding rate-limiting enzymes or key transcription factors appears to be a crucial strategy for metabolic engineering, however, it can easily fail if subcellular targeting for these proteins is not properly controlled. This is because protein synthesis and stability depend on specific redox potential, pH, presence of molecular chaperones to assist protein folding, and the appropriate enzymes needed for further processing such as glycosyltranferases and proteolytic enzymes. These conditions vary greatly between organelles. Therefore proteins are synthesized and stored differentially amongst the cell compartments in accordance to their intrinsic structural and functional characteristics. Thus, either by keeping the native peptide signals or by fusing a specific protein tag signal to the protein of interest, bioactive proteins can effectively target the suitable subcellular compartment to meet optimal conditions for biosynthesis, stability or enzyme activity [25, 26].

Therefore, various organelles have been targeted for the expression of valuable recombinant proteins or enzymes in plant cells. Among them, the endoplasmic

reticulum (ER), a component of the secretory pathway, has been the preferred subcellular compartment to express and accumulate recombinant pharmaceutical proteins in plants due to its advantageous intrinsic molecular and physiochemical characteristics [53–56]. ER appears to generate a favourable niche for biosynthesis of many proteins which requires the formation of disulfide bonds between peptide chains for their proper folding. These bonds do not form in the cytosol due to the prevalence of high reducing conditions that maintains cysteine residues in their reduced state (−SH). In contrast, the ER is an oxidizing environment that facilitates disulfide bond (S-S) formation, chemical reaction that is also catalyzed by the enzyme protein disulfide isomerase which is located in the ER lumen as well [57]. Additionally, the ER also hosts a large collection of molecular chaperones which constitute the quality control machinery that promote the proper folding of a large collection of proteins within the plant cells [53]. Proper protein folding avoids degradation within the ER lumen and therefore large quantities of functional proteins can be accumulated [58]. One can argue that such quantities of proteins can be harmful for the ER but this compartment can also tolerate unusually high accumulation of peptides without compromising cell growth and development [54, 59]. Naturally occurring resident proteins make use of this mechanism for their retention in the ER without causing cell toxicity and such a pathway has been exploited in the accumulation of immunoglobulins, vaccines, and diagnostics in intact transgenic plants [54]. This ER-localized protein expression system is currently gaining grounds to produce valuable recombinant proteins in plant tissue culture. The successful production of phytase, an enzyme used as feed additive to enhance phosphorus uptake in domestic animals in *Medicago truncatula* cell suspension cultures [56, 60] is a good example.

The mechanism by which recombinant proteins are targeted to the ER is well established. A N-terminus signal is added to the recombinant protein to drive it to the secretory pathway in which the ER is the first cell compartment in the route. This leading secretory signal (SSP) sequence encompasses about 20 amino acids, including a stretch of hydrophobic residues which is recognized by the ER co-translational complex, particularly by a peptide-RNA complex named signal recognition particle. This SSP is removed co-translationally while the nascent polypeptide is emerged by a signal peptidase located in the ER lumen [58, 59]. Once the SSP signal is lost, the recombinant protein will flow throughout the secretory pathway, unless an ER-retention signal is added to the peptide at its C-terminus. In plants and, in general, eukaryotes, the C-terminus that confers ER residence is the tetrapeptide K/HDEL which is recognized by a receptor located in the Golgi complex. Upon binding, the receptor retrieves the recombinant protein to the ER. Therefore, this mechanism has a retrieval rather than retention nature and it is very efficient, unless the system is extremely oversaturated by proteins due to a molecularly induced gene over-expression [54, 56]. Beside this latter orthodox signal, other ER-retention signals have also been successfully employed for the accumulation of recombinant proteins in the ER. For instance, a N-terminal ER-retention signal derived from the soybean vegetative storage protein *vspA (*VSPαS) directed efficiently the targeting of the recombinant hepatitis B surface antigen (rHBsAg) to the ER of *Nicotiana*

tabacum cells cultured in suspension. In this case, the rHBs Ag was highly accumulated in the ER as it was kept fused to the VSPαS signal peptide. This is due to the fact that the endogenous signal peptidases were not capable of removing the ER-signal retention signal. The activity of the VSPαS-rHBsAg fusion protein as antigen was surprisingly effective when tested in mice. This work proves that plant cell cultures can be used as biofactories for the production of pharmaceuticals in the near future [61].

It is important to note that one of the main drawbacks of ER-targeting of pharmaceutical proteins is the attachment of glycans to their peptide chains (i.e. glycosylation) and this can subsequently induce adverse immunogenic reactions in patients when they enter to the circulatory system [62]. Protein glycosylation, particularly *N*-glycosylation, initiates in the ER and continues in the endomembrane system of the secretory pathway by the function of many glycosidases and glycosyltransferases [54, 59, 63]. One approach to avoid the ER-derived immunogenic reactions includes precisely the targeting of the bioactive proteins to other cell organelles of the secretory pathway in which the protein attached-glycans can be processed to attain a profile similar to that observed in human proteins [53, 54] An interesting example of this organelle-controlled glycosylation is observed in the production of the recombinant human β-glucocerebrosidase (GCase) in carrot suspension cultures to generate an enzyme replacement therapy for the treatment of Gaucher disease, a rare genetic lysosomal storage disorder [27]. The activity of GCase depends on glycan chains with terminal mannoses in order that macrophage mannose receptors can internalize the enzyme and subsequently perform its function. Therefore, an organelle, the vacuole, which is known to possess enzyme activity to expose mannoses from the glycan complex [64] was targeted in order that an active recombinant GCase could be generated. To direct the *N*-glycosylation to a vacuolar-type glycan profile, a number of modifications were made in the native human GCase gene sequence: (1) the DNA fragment encoding the GCase signal peptide was replaced by that of the *Arabidopsis thaliana* basic endochitinase gene to direct the nascent GCase to the ER and facilitates its co-translation and translocation and (2) a DNA fragment from the gene tobacco chitinase A, encoding the sequence DLLVDTM, was added to the 3′ terminus of the GCase gene to generate a storage vacuole targeting signal in the C-terminus of the GCase recombinant protein [27]. The analysis of the glycan structure contained in the carrot cell-derived GCase demonstrated indeed the success of the above molecular strategy as 90 % of the glycan were rich in mannose with a main core (Man3GlcNAc2) of two *N*-acetylglucosamine (GlcNAc) residues and a β1–4-linked mannose attached to two additional exposed mannose residues in α 1–3 and α 1–6 linkages. Other additional residues included a (β1-2) xylose attached to the bisecting mannose and a (α1-3)-fucose attached to the reducing GlcNAc, which suggested the passage of the GCase through the Golgi complex. More interestingly, this glycan structure was similar to an animal derived-GCase (i.e. GCase produced in CHO cells) following an exoglycosidase digestion, a post-production procedure to expose mannose sugars [27, 28]. Consequently, these results showed that production of GCase in carrot suspension cultures is more advantageous over animal derived-GCase regarding its effectiveness and production

cost. These attributes are currently exploited by the company which created this platform, Protalix Biotherapeutics, and a commercial formulation of the enzyme, named ELELYSO™ is currently being marketed in many countries around the globe.

In other bioactive compounds, for instance secondary metabolites, the subcellular targeting of the biosynthetic enzymes also play a paramount role in obtaining high accumulation of specific economically valuable metabolites in plant cells. Indeed, the flow of the collection of intermediate metabolites can occur properly during biosynthesis only if the native subcellular compartmentalization of enzymes is considered for protein targeting. This is because subcellular compartmentalization of enzymes is necessary for the sequestering of toxic intermediate metabolites that occurs immediately after their biosynthesis, avoiding cell autotoxicity [45]. Cell compartmentalization of biosynthetic enzymes also enables better control of the metabolic flux by reducing extra regulatory level and negative feedback. More importantly, the microenvironments hosted by the cell organelles provide specific requirements to the enzymes to achieve an optimal activity and stability [10, 65]. Therefore, it is not surprising to learn that vast efforts have been invested to elucidate the subcellular localization of the biosynthetic enzymes involved in the production of secondary metabolites (extensively reviewed by Staniek et al. [45] and Ziegler and Facchini [66]). This knowledge has been proven to be advantageous to increase the content of target secondary metabolites in plant cultures without sacrificing cell growth and, ultimately, the biomass. An example for this is the chloroplast targeting of bacterial lysine decarboxylase enzyme in tobacco hairy root cultures. Lysine decarboxylase is natively localized in the chloroplast and represents a rate-limiting enzyme for the synthesis of the precursor (i.e. cadaverine) of the end product anabasine, a nicotine analogue. The over-expression of the bacterial lysine decarboxylase in the plant cells produced higher contents of both alkaloids but the fusion of the gene with the rubisco small subunit transit peptide which drove the enzyme to the chloroplast enhanced even more such accumulation [67, 68]. Undoubtedly, this outcome demonstrates the relevance of directing foreign biosynthetic enzyme to the proper subcellular compartment in the biosynthesis of secondary metabolites.

Subcellular targeting of heterologous enzymes involved in different plant systems poses more challenges. An example for this is the enzyme amorpha-4, 11-diene synthase derived from *Artemisia annua* that generated only minute amounts of amorpha-4, 11-diene, the precursor of the antimalarial drug artermisin, when expressed in tobacco cells [69]. In other cases, the artificial targeting of biosynthetic enzymes overpasses the activity of enzymes with natural targeting, for instance the activity of sesquiterpene synthase with mitochondrial targeting surpassed the activity of this enzyme that was naturally expressed in the cytosol [70]. Clearly, these findings suggest that there are many other factors that mask the effectiveness of the subcellular targeting of heterologous enzymes. In planta, secondary metabolism is limited to specialized cells such as laticifers and glandular trichomes [66] and key transcription factors effectively regulate many of the biosynthetic enzymes within

these cells [71]. Transcription factors may also play an important role in controlling cell differentiation [72]. Consequently, the efficacy of subcellular targeting of biosynthetic enzymes can be substantially enhanced in plant culture systems if a joint molecular strategy can be established to regulate the expression of transcription factors encoding biosynthetic enzymes and genetic mechanism involved in cell differentiation.

22.4 Manipulation of Mechanisms Governing Cell Differentiation in Cell and Organ Culture

Bioactive compounds are differentially synthesized in either undifferentiated or differentiated cells. However, the molecular mechanisms that determine which state of differentiation is optimal for a given compound are not known and cannot be predicted. For example, artemisinin requires a degree of differentiation for its synthesis as differentiated shoot and hairy root cultures to accumulate high quantities, whilst calluses and cell suspension cultures show low or no accumulation [73, 74]. In contrast, high yields of anthraquinones, another secondary metabolite, can be obtained only from dedifferentiated cell cultures of *Morinda citrifolia.* In fact, this work was the first report of the use of undifferentiated cultures for the production of bioactive compounds and set the foundation for the bioreactor culture technique by which many bioactive compounds are currently being produced [75].

Despite its relevance in bioactive compound production, the manipulation of the differentiation state of plant cells is still in its infancy. At present, most of the strategies rely on the selection of a specific type of plant cells which possess a defined differentiation state that appears to be optimal for the production of a particular bioactive compound. This strategy is perfectly illustrated by the use of innately undifferentiated cambial meristematic cells (CMCs) from *Taxus cuspidata*, for the production of paclitaxel (Taxol), an economically important anticancer drug [76]. The attributes of this culture system are that the cambial meristematic cells bypass the negative effects of the dedifferentiation step, therefore, their activity for producing paclitaxel is stable for long periods under a low auxin regime (i.e. 1 mg L^{-1} picloram for establishment of cell suspension culture and 2 mg L^{-1} 1-naphthalene acetic acid for bioreactor subculturing). Additionally, CMCs are more resistant to shear stress and the media and culture condition requirements are less complex [23, 76]. Unfortunately, the molecular mechanisms that regulate and maintain the undifferentiated meristematic state of *T. cuspidata* cells are still not well understood, but the use of a combination of deep sequencing technologies (i.e. massively parallel pyrosequencing and digital gene expression tag profiling) revealed that two kinase receptors which are conspicuously located in CMCs may play a major role: (1) a leucine-rich repeat (LRR) receptor-like kinase (RLK) encoded by the *PHLOEM INTERCALATED WITH XYLEM* (*PXY*) gene and (2) histidine kinase receptor

homolog to that encoded by the *WOODEN LEG* (*WOL*) gene in *Arabidopsis*. The latter is a cytokinin receptor that affects vascular morphogenesis in interaction with many transcription factors and other proteins involved in the signaling pathway of this hormone. Therefore, giving the potential of such a plant culture system, future studies will be required to define the function of such receptors as well as other transcription factors; for instance homeodomain leucine zipper class III proteins, involved in the determination of cambial cells and their relation with the production of paclitaxel and its analogs [77].

Unlike undifferentiated plant cell cultures, the establishment and maintenance of differentiated cell cultures face more hurdles due to specific culture conditions (i.e. hormonal balance) are required to switch on and maintain defined gene programmes. Positional information of cells within an organ is also difficult to duplicate in *in vitro* systems, in order that cells can acquire a specific differentiation state [78–80]. However, manipulation of the differentiation state of cells in plant tissue platforms is still being attempted in view of the benefits that it can provide to the production of bioactive compounds. Instead of starting from undifferentiated cells following the control of culture conditions to onset a specific cell differentiation state, cell transdifferentiation, a lineage gene reprogramming to transform directly a differentiated cell to another type of differentiated cell, appears as a more effective method to manipulate cell differentiation [80, 81]. Transdifferentiation of plant cell *in vitro* cultures has been demonstrated more than three decades ago with the direct transdifferentiation of single mesophyll cells into tracheary elements, mainly in response to the auxin regime [82]. Unfortunately, the application of this discovery has not been explored yet in the production of bioactive compounds. In *Nicotiana sylvestris,* nicotine biosynthesis may be increased by transdifferentiation of tracheary elements from suspension mesophyll cells due to the presence of putrescine *N*-methyltransferase (PMT) which is responsible for the first step in its biosynthesis. And this enzyme is located only in tracheary elements [83]. Clearly, the feasibility of this culture system needs to be demonstrated with formal experimentation, as treachery elements are not the only cells that participate in the biosynthesis of this pyridine alkaloid [66].

The manipulation of cell differentiation for the production of bioactive compounds may appear as an utopic approach; however, the achievement of successful results with transcription factor driven-cell differentiation of maize cells in the accumulation of anthocyanins demonstrated that such strategy is entirely feasible [20, 21]. Indeed, the ectopic expression of the R and C1 genes encoding the c-MYC and c-MYB transcription factors, respectively, drove the differentiation of maize cell suspension culture to a state in which multi lamellar bodies and small vesicles in which anthocyanin accumulates before fusion with the vacuole, are formed in the cytosol. More than 400 genes were up-regulated by these two transcription factors, including a gene encoding a glutathione *S*-transferase (GST) that may be involved in vacuolar uptake of the produced pigments. Furthermore, it seems that all the above genes were associated with cell differentiation and may be responsible for all changes in the ultrastructural organization of cells. More importantly,

such transcription factor dependent-cell differentiation was accompanied by the coordinate up-regulation of many genes that encode enzymes responsible for the metabolization of coumaroyl-CoA into anthocyanins, the end economically valuable product [20, 72]. It is evident that transcription factors become a valuable molecular instrument to drive simultaneously both cell differentiation and expression of genes involved in the biosynthesis of bioactive compounds. Therefore, they may become the major orchestrators to manipulate metabolic pathways in which the activity of biosynthetic enzymes need to be coordinated to specific microenvironments which are hosted by different types of differentiated cells. In this context, complex metabolic pathways, for example monoterpenoidindole alkaloid metabolism, may be efficiently engineered with two or three transcription factors by regulating firstly cell differentiation in hope that the generated microenvironments would later induce the expression of the genes that encode the enzymes involved in the pathway in question. For example, monoterpenoidindole alkaloid metabolism occurs naturally in epidermal cells, mesophyll cells, laticifers and idioblasts [66]. Thus, hypothetically we can drive cell differentiation to artificially promote the formation of epidermal cells, mesophyll cells, laticifers and idioblasts by expressing the genes encoding *AtDEK1*, *TaWRKY71-1, HbEREBP1* and *CrTF12*, respectively [84–87]. Sequential activation of the expression of each gene by different inducible promoters during culture may result in mimicking the stages of biosynthesis and metabolite trafficking as occur *in planta*. High levels of end products may result from the ability of the transcription factors to coordinate the expression of enzyme biosynthetic genes under a proper cell differentiation state (Fig. 22.1).

Although differentiated cell cultures offer many constraints in their application for the production of bioactive compounds, a collection of cells with different state of differentiation packed together as an organ represents the most successful plant tissue platform available at present for the production of secondary metabolites. This is the case of the hairy root culture in which the natural gene programmes of roots are modified in response to two newly introduced set of genes, *aux*and *rol* genes, by the bacteria *Agrobacterium rhizogenes.* The new gene programming, mainly driven by *rol* genes, led to changes in cell differentiation state and physiological processes that result in an increased secondary metabolism [88, 89]. Within the *rol* genes, therol B, a tyrosine-phosphatase which increases auxin perception, has a major role in changing cell differentiation state to generate a mix population of differentiated cells of various nature. This is because the *rol* B protein can stimulate root programming and, simultaneously, activate many meristem inducing-genes (i.e. *ORF13*) to generate meristem-like cells that can later differentiate into various types of organ-specific cells [90–92]. The mechanism by which *rol* genes orchestrate both cell differentiation and secondary metabolism is still unknown, but it seems the increased secondary metabolism is due to the activity of some *rol* B activated-transcription factors that inhibit repressors of secondary metabolism which plant cells normally use to promote metabolic homeostasis [88].

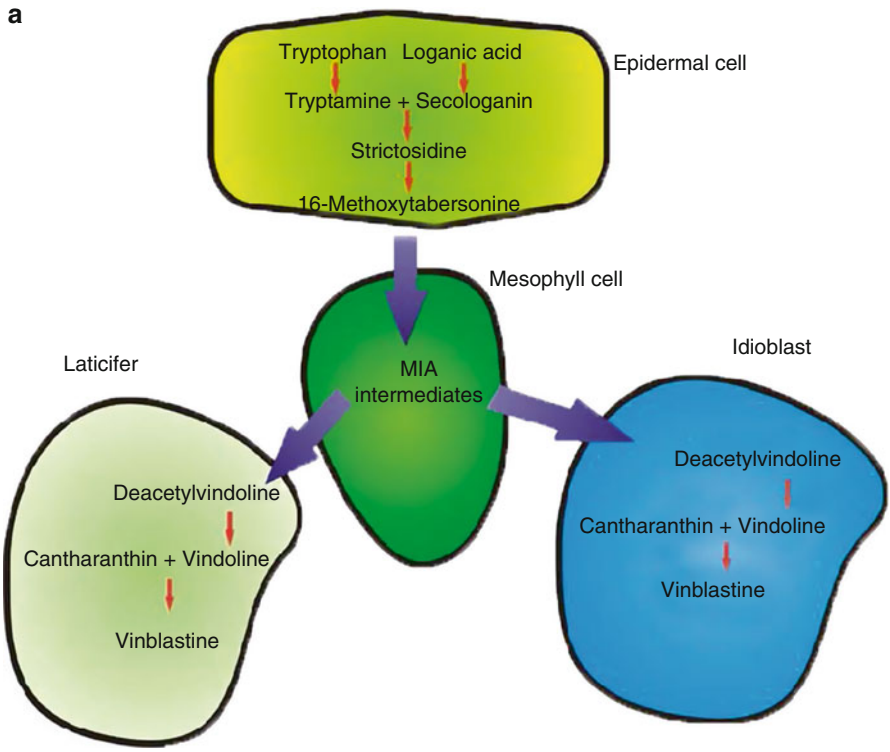

Fig. 22.1 Natural and artificially-induced cell differentiation *in vitro* for the synthesis of monoterpenoidindole alkaloids (MIAs). Natural cell differentiation (**a**) generates four types of cells for MIA biosynthesis: (1) epidermal cells are responsible for the synthesis of the major precursor of MIAs, strictosidine, which is subsequently metabolized into 16-methoxytabersonine by various enzymes, (2) mesophyll cells conduct the biosynthesis of many MIA intermediates, potentially 16-Methoxy-2,3-dihydro-3-hydroxytabersonine, and finally (3) laticifers or (4) idioblasts culminates the biosynthesis of MIAs, notably vinblastine, from deacetylvindoline. **Artificially-induced cell differentiation *in vitro*** (**b**) can hypothetically fuel MIA biosynthesis by the creation of epidermal cells, mesophyll cells, laticifers and idioblasts by a sequential induced expression of genes encoding *AtDEK1*, *TaWRKY71-1, HbEREBP1 and CrTF12* transcription factors, respectively. All these key transcription factors are responsible for cell specification of the above types of cells, respectively. Therefore, we hypothesize that cell differentiation can be manipulated in a timely fashion to mimic the sequential function that the four types of differentiated cells perform in the biosynthesis of MIAs. The likelihood of success would be significantly increased if the source of plant cells (i.e. mesophyll cells) for the establishment of cultures are derived from a species that innately is competent for MIA biosynthesis, such as *Catharanthus roseus*. In our proposed tissue culture system, the *AtDEK1* gene is regulated by sucrose starvation inducible rice amylase RAmy3D promoter, the *TaWRKY71-1 gene by* the light inducible promoter of ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (rbcS3B) from tomato, *HbEREBP1* by the ABA inducible promoter of the *SalT* gene from rice and *CrTF12* by the methyl jasmonate inducible promoter of the potato cathepsin D inhibitor (CDI). Consequently, epidermal cell differentiation (epidermal phase) can be induced by the removal of sucrose in the medium of the *C. roseus* cell cultures, whilst mesophyll cell differentiation (mesophyll phase) is achieved upon the exposure of the generated epidermal cells to light. Subsequently, the laticifer and idioblast phases are generated by the supplementation of ABA and methyl jasmonates, respectively. In this tissue culture model we are assuming that the various induced cell differentiation states would be sufficient to create the cellular microenvironments to activate genes encoding all enzymes involved in MIA biosynthesis to produce the end product vinblastine at high levels (Adapted from Refs. [11, 45, 82–85])

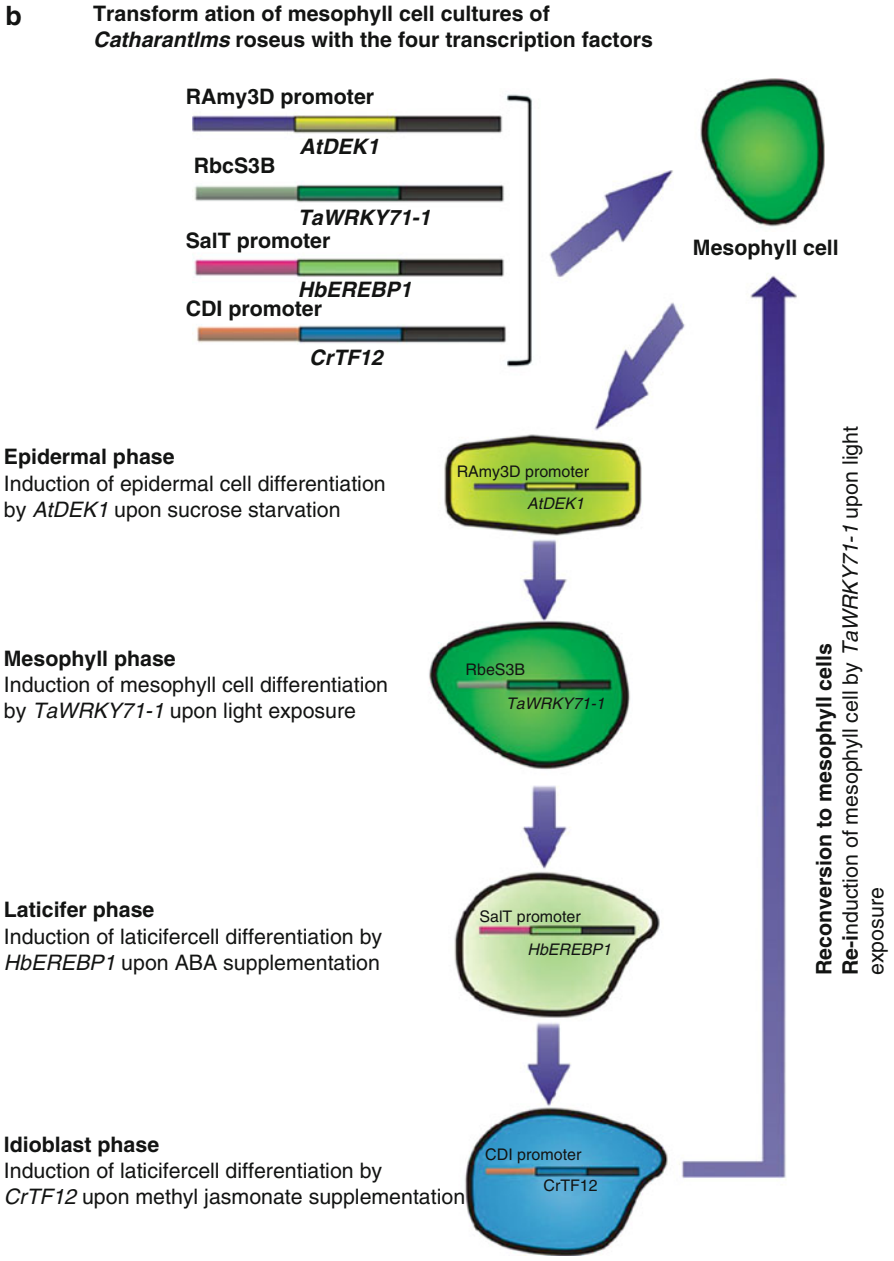

Fig. 22.1 (continued)

22.5 Manipulation of Immortalization of Specifically Differentiated Cell Cultures

The vision of creating immortalized plant cells for the production of bioactive compounds has been embraced for some decades due to its potential benefits [93]. This is because immortalized cells not only can divide indefinitely but also can keep a programme of genes that set the cells to a specific degree of cell differentiation, and this in turn directs them to perform specific physiological and metabolic tasks [94, 95]. Such a condition could be optimal for the production of a particular active compound, therefore it would be advantageous to generate immortalized cell lines from a population of differentiated or undifferentiated cells. Consequently, immortalized cell systems would be able to produce high yields of bioactive compounds in a sustainable fashion. Additionally, the production cost would also be drastically diminished for the reason that the unproductive exponential growth required to create an economically feasible biomass volume is no longer required and practices involved in the preparation of culture media with different composition for each stage are also eliminated [76].

The mechanisms which regulate cell immortality in animals are fairly well understood and, thus, it can be stated that immortalized cells are generated upon a disruption on the cell-cycle checkpoint pathways (i.e. suppression of the cell cycle controllers p53, p16, pRb), hyperactivation of telomerase enzyme, up-regulation of some oncogenes and oncoproteins leading to a higher rate of cell division, or acquisition of epigenetic modifications, such as DNA methylation in CpG islands [94, 96, 97]. Unfortunately for plant cells, the mechanisms that regulate immortality are still obscure [94, 98, 99], although many reports suggest that plant cells share similar pathways to animal cells. In fact, the vast knowledge accrued in immortalization of animal cells is currently propelling the dissecting of pathways occurring in plant cells. In this view, recent reports have pointed out that plant cells, like their animal counterpart, undertakes characteristic epigenetic changes that occur in both euchromatin (i.e. a form of DNA rich in gene concentration under active transcription) and heterochromatin (i.e. a tightly packed form of DNA with high gene silencing) to achieve immortalization. Indeed, an increased DNA methylation is present in euchromatin resulting in gene silencing, reminiscent to immortalized animal cell lines and cancer cells. In contrast, in the heterochromatin portion, transposable elements, major mutagenic factors, become highly activated after DNA demethylation [93]. It is evident that better understanding of the mechanisms that regulate plant epigenetics would provide the foundations to establish tissue culture techniques to induce immortalization in plant cells, as those routinely employed for immortalization of animal cells [94].

Currently, the creation of immortalized plant cells is through empirical knowledge rather than from the application of solid scientific principles. Thus, immortalized cell cultures have been generated spontaneously from callus in few plant species after a differentiated cell state, committed to a developmental gene program, is changed to dedifferentiated state in response to varying concentrations of the two

major plant hormones, cytokinin and auxin [100]. Thus, immortalized cell suspensions are derived from dedifferentiated cells and divide continuously. So that they can be maintained indefinitely without any cell differentiation changes, if specific culture conditions are provided [93].

Another more practical approach has been undertaken to generate immortalized plant cells for production of bioactive compounds. Lee and coworkers [76], for example, isolated cambial meristematic cells (CMCs) of *Taxus cuspidata* to produce paclitaxel (detailed in the previous section). They exploited the natural intrinsic immortalizing properties of CMCs by considering the fact that cambial cells represent the secondary growth meristem which possess stem cell identity that enable them to divide indefinitely and more importantly, the same gene programming is maintained following cell divisions. Indeed, the results of this report show that CMCs are able to sustain high levels of production of paclitaxel for a culture period longer than 4 months. Although the author suggested that two plant receptors may be responsible for the cambial cell identity of the CMCs, the role of other transcription factors, for instance orthologs to the typically procambial specifying factors MONOPTEROS and homeodomain leucine zipper class III AtHB8 [101], in plant cell immortalization needs to be addressed in future research works.

A certain degree of immortalization is also present in hairy root culture which is generated by the effects of the *rol* genes through the regulation of both programmed cell death and cell meristem identity. Apoptosis is inhibited through the rolB protein that enhances the expression of antioxidant genes encoding cytosolic ascorbate peroxidase, catalase, and superoxide dismutase that in turn cause a reduction on the levels of reactive oxygen species [102]. On the other hand, the same gene confers meristem activity of root cells by reactivating many meristem inducing-genes, as described in the previous section of this review [91, 92]. Other genes, including *rolC* and meristem specifying genes, act synergistically to keep ROS homeostasis and promote cell-division dependent immortalization [102]. Consequently, it can be envisioned that more progress can be achieved in the production of bioactive compounds in hairy root culture system, if the expression of the genes that confers cell immortalization is further studied.

Currently, the use of immortalized cells, particularly in the version of *in vitro* propagated- meristematic stem cells, becomes more relevant in virtue of their newly discovered pharmaceutical properties, specifically the beneficial properties for the regeneration of skin and hair [103]. Although the state-of-art research has achieved significant progress in unraveling the gene programs that specify identity of stem cells, precise information is lacking to define the roles of biomolecules (i.e. transcription factors and other proteins, vitamins, essential fatty acids and bioactive compounds that provide defense against free radicals) have in human tissue regeneration. Therefore, the development of techniques to artificially induce plant cell immortalization and the determination of specific plant factors which may have dermal regenerative properties represent a priority research avenue in the near future in the production of bioactive compounds through the utilization of plant tissue culture platforms.

22.6 Conclusions and Future Perspectives

In this overview, we show that manipulation of the expression of a single gene, which affects the most rate-limiting step, can be effective in the metabolic engineering of many compounds, notably secondary metabolites. However, many metabolic pathways possess more than one crucial step. Therefore, the modification of plant metabolism with multiple genes is necessary. This strategy is further justified when metabolic pathways are regulated by polycistronic genes and, therefore, the effective synthesis of an end product requires the application of advanced technologies, for instance "gene stacking". In these cases, such strategies enable the simultaneous expression of multiple genes and, consequently, avoids the accumulation of toxic intermediate metabolites which otherwise may result in cell autotoxicity. However, the manipulation of gene expression can easily fail if the subcellular targeting is not considered. This is because each biosynthetic enzyme and, in general, any protein requires specific micro-environmental conditions, generally provided by their native compartmentalization, for proper folding and functioning. Furthermore, the creation of such subcellular microenvironments is determined by the cell differentiation state. Therefore, the manipulation of gene programming to modulate the degree of cell differentiation is emerging as a powerful molecular tool for the production of bioactive compounds. In this avenue, the selection and *in vitro* propagation of plant cells with specific differentiation state appears as more efficient approach for the production of bioactive compounds, although the application of transdifferentiation technique and the manipulation of key transcription factors also represent promising methods that may become important in the near future for the modulation of plant cell differentiation. Finally, manipulation of cell immortalization has come into sight as an effective approach to perpetuate specific cell differentiation states which are optimal for the production of economically valuable compounds and consequently more significant progress can be obtained when we gain better understanding of the mechanism that regulate such a cell process.

In perspective, manipulation of cell immortalization is an emerging strategy to enhance the efficacy of plant culture systems for the production of pharmaceutical compounds. In fact, many private companies have keenly embraced this technology for further application in regenerative medicine, in which the use of plant-derived stem cells has assisted to overcome ethical issues with animal stem cells. Much progress in plant cell immortalization is likely to be achieved in the near future when key genes, orthologs to animal cells, are discovered in plant cells following the manipulation of their expression with the available repertoire of molecular methods. Furthermore, with the advent of the newly devised molecular methods to control cell differentiation in plant tissue culture systems, it would be possible to generate immortalized cells with specific differentiation states for the optimal synthesis of bioactive compounds. The emerging technologies for the designing and synthesis of artificial genes, transcription factors and promoters have transformed metabolic and protein engineering allowing the expression of multiple genes under the proper subcellular compartment which provides the suitable microenvironment

for protein function and stability. In all these emerging biotechnological methods, transcription factor appears as major orchestrators for the manipulation of cell differentiation and cell immortalization together with the modulation of the expression of genes encoding biosynthetic enzymes or recombinant proteins.

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Chapter 23
Isoprenoid Production *via* Plant Cell Cultures: Biosynthesis, Accumulation and Scaling-Up to Bioreactors

Alexander M. Nosov, Elena V. Popova, and Dmitry V. Kochkin

Abstract Plant cell culture is traditionally viewed as a unique artificially created biological system representing a heterogenous population of dedifferentiated cells. This system undergoes a continuous process of autoselection based on the intensity and stability of cell proliferation. We discuss here the details of formation and regulation of isoprenoid biosynthesis in plant cell *in vitro* based on literature survey and our research. Obviously, secondary metabolism differs in cell culture compared to the plant *per se*, because in cell culture metabolites are synthesized and compartmentalized within a single heterotrophic cell with sparse or underdeveloped vacuoles and plastids. For example, in plant cell cultures isoprenoid biosynthesis *via* MVA pathway was found to be more active than *via* plastid-localized MEP pathway. Also, it was hypothesized that cell cultures preferably produce metabolites, which promote cell proliferation and growth. Indeed, cell cultures of *Dioscorea deltoidea* produced mainly furostanol glycosides, which promoted cell division. Triterpene glycosides (ginsenosides) in the cell cultures of various *Panax* species are represented mainly by Rg- and Rb-groups. Rb ginsenosides are predominantly found as malonyl-esters that may influence their intracellular localization.

Despite the difference in the isoprenoid composition in plant and cell culture the latter became an attractive source of phytochemicals as an alternative to plant harvesting. We provide in this chapter the guidelines to biotechnological production of plant isoprenoids using plant cell cultures and discuss the optimal methods of bioreactor-based cultivation and cryopreservation of plant cell collections.

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23.1 Introduction

Secondary metabolism plays an important role in plant's life. Out of complete genome sequence 15–25 % genes encode enzymes and protein factors involved in biosynthesis of a broad range of so called "secondary products" [1]. These include over 40,000 isoprenoids – or terpenoids, – that play diverse physiological roles in both primary and secondary metabolism [2].

Photosynthesis, respiration, cell organization, compartmentalization and ontogenesis are the common examples of isoprenoid action in primary metabolism [3]. Indeed, carotenoid pigments serve as effective light harvesters and anti-oxidation agents [4]. Sterols are essential components of cell membranes modulating its properties [5]. Side chains of chlorophylls, ubi- and plastoquinones are derived from terpenoids. Phytohormones such as gibberellins, brassinosteroids, abscisic acid and strigolactones as well as cytokinin side chains are of isoprenoid origin [1, 3].

As secondary metabolites isoprenoids are involved in plant – environment interaction including defense against biotic and abiotic stresses, attraction of pollinators etc., suggesting that their physiological functions are even more diverse and complicated [6]. Isoprenoids are the active components in many medicinal plants that have been used for centuries in traditional medicine all over the world. At present they attract commercial interest as potential pharmaceuticals and nutraceuticals [1, 7]. In this chapter, we review in detail the major differences in isoprenoid composition and biosynthesis in *in vitro* plant cell cultures when compared to intact plants and discuss recent advances and challenges of their biotechnological production in bioreactors for pharmacological use.

23.1.1 Introduction to Isoprenoids

Monoterpenes and sesquiterpenes ($\mathbf{C_{10}}$ and $\mathbf{C_{15}}$) is a large family of organic molecules of either 10 or 15 carbon atoms respectively. They are divided into two classes: aliphatic terpenes such as citral from lemons and cyclic terpenes with one or two carbon rings such as menthol, carvacrol, and camphor. In nature mono- and sesquiterpenes are found as highly volatile and strongly scented liquids. They contribute to the scent, flavor and color of plant essential oils and often show pharmaceutical activity. For example, a sub-group called sesquiterpene lactones includes compounds with a bitter taste and is currently being tested on cardiomodulating, anti-bacterial (aucubin) and anti-cancer (arglabin) activities [1, 3].

Diterpenoids **(C_{20})** are the staple components of plant resins from pine, spruce, fir and cedar. Diterpenoids can be classified as linear, bicyclic, tricyclic and tetracyclic diterpenoids depending on their carbon skeletal core. Resin acids, or diterpenoid acids, have the same structure being composed of three hexacarbon rings. Diterpenoids of *ent*-kaurene type have skeleton with four rings. Some diterpenoids have a unique structure such as paclitaxel from yew (*Taxus spp*.) [1, 3]. Diterpenoids have been known for centuries for their antimicrobial qualities. Paclitaxel, or taxol, has become one of the major cytostatic anticancer agents of plant origin. Steviol glycosides produced in *Stevia* leaves is up to 300 times sweeter than sucrose and is extensively used to replace sugar in the diet of patients with diabetes [8].

Triterpenoids **(C_{30})** are derivatives of triterpene molecules and are divided into the following groups:

(i) *Steroidal glycosides (SG)* are glycosides based on C27 steroid-type aglycones with a modifiable side chain, transformed into one (furostanol glycosides) or two (spirostanol glycosides) heterocyclic rings. SG are wide-spread: they have been found at least in 15 plant families including Dioscoreaceae, Liliaceae, Solanaceae, Leguminosae, Costaceae and others. These substances have a broad spectrum of pharmacological activities from anticancer to immune-modulating and sex-stimulating. It is important to note that furostanol and spirostanol type glycosides often show different, even opposite pharmacological activities [9].

(ii) *Triterpene glycosides (TG)* are found in over 30 higher plant families and contribute to unique pharmacological activities of ginseng, aralia, astragalus and glycyrrhiza. Similar to steroid glycosides, TGs are classified based on carbon skeleton of their aglycons. Ginseng glycosides (ginsenosides) found exclusively in *Panax* species are derivatives of two types of tetracyclic aglycons: protopanaxadiols and protopanaxatriols. Pentacyclic compounds are represented by derivatives of ursan, oleanan, lupan and gopan. Glycosides of oleanolic and ursolic acids contribute to biological activities of glycyrrhiza and polyscias [10, 11].

(iii) *Cardiac glycosides* are detected in 13 plant families. Glycoside producing plants include digitalis, lily-of-the-valley, adonis and strofant. Over 400 cardiotonic glycosides have been identified so far. Most common are cardenolides and bufadienolides with additional butenolide or pentadienolide rings, respectively. Cardiac glycosides demonstrate strong heart-beat-modulating and heart-stimulating activity and are irreplaceable with any available synthetic medicines [12].

(iv) *Phytoecdysteroids* are polyhydroxylated steroids found in over 400 plant species from Compositae, Caryophyllaceae and Labiatae families. High phytoecdysteroid content, over 1 % dry weight, was reported in *Serratula spp., Ajuga spp., Rhapontucum spp.* Their environmental function is to protect the plants against insect attack. In humans phytoecdysteroids show well-documented adaptogenic, psychoactive drugs and anti-cancer activities [13].

Tetraterpenoids (C40) in plants are represented mainly by carotenoids: carotenes and xanthophylls. While only 20–30 tetraterpenoids play a role in primary metabolism of the vast majority – over 700 compounds, – are involved in secondary metabolism. They generally function as lipophilic pigments localized in plastids, mainly chromoplasts. Carotenoids demonstrated high and various physiological activities in humans. For example, lycopene and lutein have been recently registered as oncopreventive agents [4].

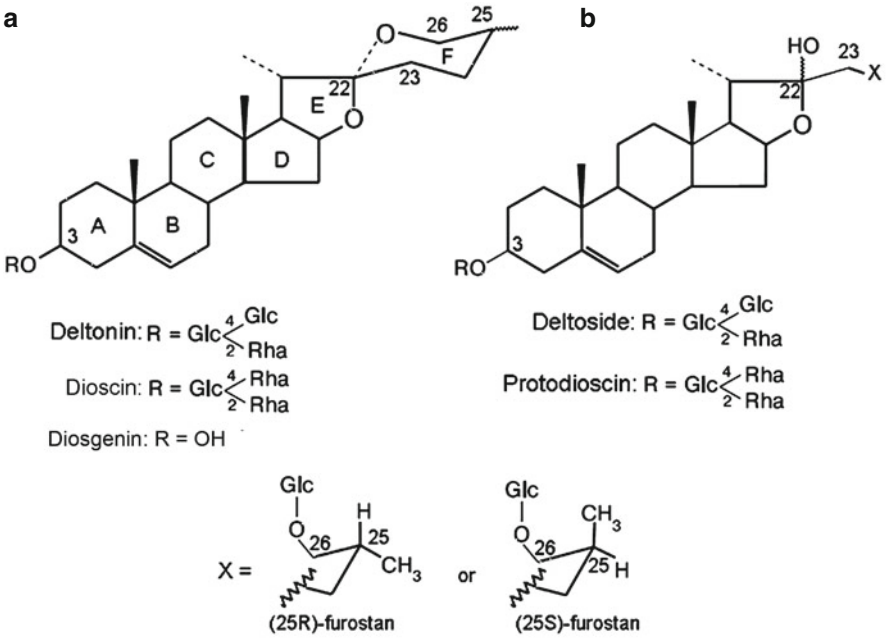
The chemical structures of tri- and diterpenoids discussed in this chapter are shown in Figs. 23.1 and 23.2 respectively.

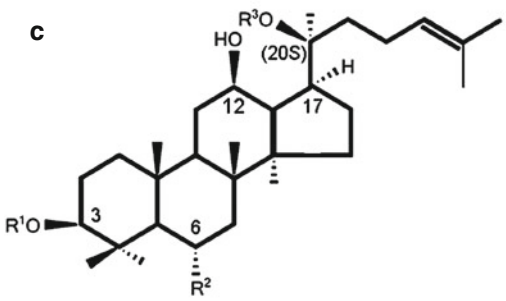
23.1.2 Brief Overview of the Isoprenoid Biosynthesis in Plants

All diversity of isoprenoid structures arises from two isomeric five-carbon (C5) precursors – dimethylallyl diphosphate (DMADP) and isopentenyl diphosphate (IDP) [16]. Assembly of two, three or four C5 units by prenyl transferases (PT) yields geranyl diphosphate (GDP; C10), farnesyl diphosphate (FDP; C15) and geranylgeranyl diphosphate (GGDP; C20), respectively [17]. Pairwise condensation of FDPs or GGDPs produces squalene (C30) or phytoene (C40), respectively. GDP, FDP, GGDP, squalene and phytoene are the substrates for a large family of terpenoid synthases (TPS) [18, 19], and the immediate precursors of all monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids and tetraterpenoids, respectively. TPS catalyze enzyme-specific isomerizations, various rearrangements and cyclizations yielding the vast pool of cyclic and acyclic terpenoid carbon skeletons found in plants. Many plant TPSs are promiscuous, forming multiple products from a single substrate [20, 21]. Subsequent modifications of the basic parent skeletons synthesized by TPS generate numerous different isoprenoids produced by plants. These secondary modifications commonly include oxidation, reduction, isomerization and conjugation that change functional properties of terpenoid molecules.

All living organisms can be classified based on the metabolic pathway used to produce the precursors of isoprenoid biosynthesis. The mevalonate pathway (MVA) is common in archaea, some bacteria, fungi and animals. The non-mevalonate pathway, or 2-*C*-methyl-D-erythritol 4-phosphate pathway (MEP), was discovered in other bacteria and some algae. It is remarkable that plants use both MVA and MEP pathways that occur in the cytosol and plastids, respectively. Prenyl transferases and terpenoid synthases have been also found in both cytosol and plastids. In general mono-, di- and tetraterpenoids are preferentially formed in plastids from the precursors of the MEP pathway, while the majority of sesqui- and triterpenoids is synthesized in the cytosol using precursors from the MVA pathway. It is important to note that the division by biosynthetic origin is not complete, as there is exchange of IPP units between the pathways [22–24].

Since the formation of plant isoprenoids involves several sub-cellular, tissue and organ compartments [25], it requires intra- and possibly intercellular transport of

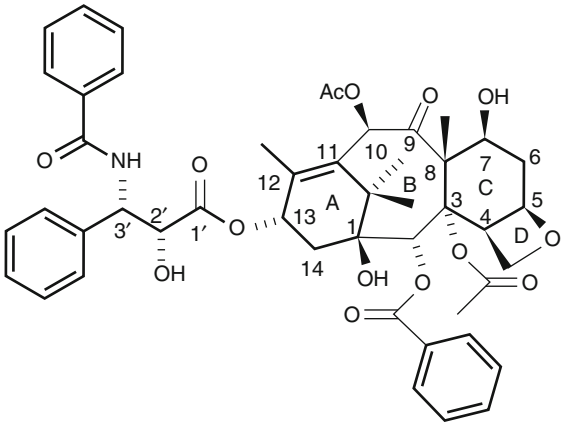




Ginsenoside	R^1	R^2	R^3
Rb_1	Glc(1–2)Glc	H	Glc(1–6)Glc
Rb_2	Glc(1–2)Glc	H	Ara(pyr)(1–6)Glc
Rc	Glc(1–2)Glc	H	Ara(fur)(1–6)Glc
Rd	Glc(1–2)Glc	H	Glc
Re	H	Rha(1–2)Glc -O-	Glc
Rf	H	Glc(1–2)Glc -O-	H
Rg_1	H	Glc -O-	Glc

Fig. 23.1 (**a**–**c**) Chemical structures of triterpenoids discussed in this chapter (Modified from Vasil'eva and Paseshnichenko [9] (**a**, **b**) and Smolenskaya et al. [14] (**c**)). (**a**) Spirostanol steroidal glycosides and aglycon (diosgenin). (**b**) Furoostanol steroidal glycosides. (**c**) Dammarane-type triterpene glycosides – ginsenosides. *Ara(pyr)* arabinopyranose, *Ara(fur)* arabinofuranose, *Glc* glucopyranose, *Rha* rhamnopyranose

a



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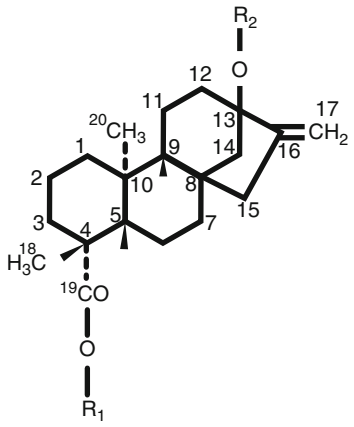

Compound	R_1	R_2
Steviol	H	H
Steviolbioside	H	Glc^{2}-^{1}Glc
Stevioside	Glc	Glc^{2}-^{1}Glc
Rebaudioside A	Glc	Glc^{2}-^{1}Glc |^{3} ^{1}Glc
Rebaudioside B	H	Glc^{2}-^{1}Glc |^{3} ^{1}Glc
Rebaudioside C	Glc	Glc^{2}-^{1} Rha |^{3} ^{1}Glc

Fig. 23.2 (**a**–**b**) Chemical structures of diterpenoids discussed in this chapter. (**a**) Paclitaxel. (**b**) Major steviol glycosides (From Bondarev et al. [15]). *Ac* acetate, *Glc* glucopyranose, *Rha* rhamnopyranose

intermediates, e.g. P450 enzymes involved in the modification of mono-, sesqui-, di- and triterpenoids are associated with the endoplasmic reticulum whereas final products are stored usually in vacuole or periplasmatic space of cells.

23.1.3 In Vitro Culture of Undifferentiated Plant Cells as a Biological System with Unique Secondary Metabolism

In vitro culture of somatic plant cells is a unique artificially created biological system representing a heterogeneous population of dedifferentiated cells. This system undergoes a continuous process of auto-selection, which depends on the intensity and stability of cell proliferation [26]. Both the physiological and genetic studies demonstrated a prominent difference between the cell in such an artificial population and in plant [7, 26, 27]:

- Cells in culture are truly dedifferentiated while in plant they perform specific and predetermined functions;
- Cells in culture are free from the organism control. Without the precise "directives" from plant signaling system cell development is switched to autoselection based on the intensive and stable proliferation;
- Cells in culture are heterogenic morphologically, physiologically, biochemically and genetically heterogenic. This heterogeneity enables flexibility required for the adaptation of proliferating population to *in vitro* conditions.

Figure 23.3 shows microphotographs of cells in suspension cultures obtained from various medicinal plants at the Department of cell biology and biotechnology, Timiryazev Institute of Plant Physiology (Moscow, Russia) as further discussed further in this chapter.

As a result of these unique cell characteristics secondary metabolism in the cell culture undergoes significant changes when compared to intact plant (Table 23.1).

In vivo biosynthesis of secondary metabolites is regulated by the plant signalling system and is not crucial for the survival of the individual cells [28]. In contrast, the auto-selection process in the dedifferentiated cell culture results in preferable production of metabolites that promote intensive and stable proliferation. Thus, biosynthetic pathways leading to the formation of secondary metabolites in cell culture is suppressed or arrested in the due course of the repetitive subcultures. Consequently, cell cultures that demonstrate active biosynthesis and accumulation of secondary products may be highly exclusive. However, there are a few basic principles that may enable intensive production of secondary metabolites in cultures *in vitro* cultures:

Principle 1. Cell cultures produce secondary metabolites that promote cell proliferation. Hundreds of different isoprenoid molecules are involved in plant stress response and adaptation mechanisms and some of them may benefit cell proliferation ability [3]. Between them one can found isoprenoids with remarkable antioxidative, osmoprotective, growth-stimulating and other activities inherited from their stress-defense function in the intact plant.

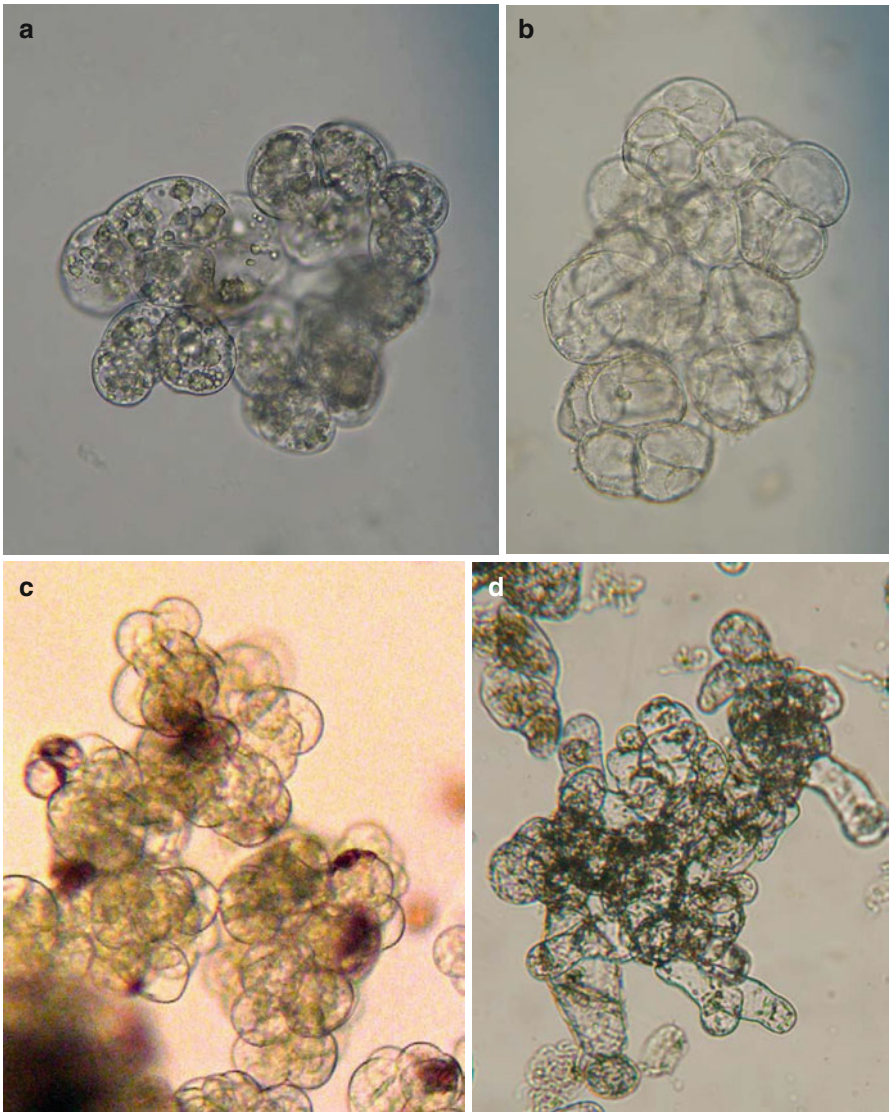

Fig. 23.3 (**a**–**d**) Photographs of dedifferentiated cells of *Dioscorea deltoidea* (**a**), *Panax ginseng* (**b**), *Polyscias filicifolia* (**c**) and *Taxus baccata* (**d**), grown as suspension cultures at the Department of Cell Biology and Biotechnology, Timiryazev Institute of Plant Physiology (Moscow, Russia) and discussed in this chapter

Principle 2. Recently it has been hypothesized that some secondary metabolites perform additional functions beside their main ecological role [29]. For example, on one hand, alkaloids protect plants from herbivore; on the other hand, they may be involved in nitrogen accumulation and storage similar to steroidal glycosides in dioscorea, which serve as sugar reserves. These additional functions may favour accumulation of such metabolites in the cell culture.

Table 23.1 Major differences in the production of secondary metabolites from cultured plant cells when compared to intact plant

Factors affecting secondary metabolite production	*In vitro* culture of undifferentiated cells	Intact plant
Cell differentiation and proliferation	Secondary metabolites are synthesized in continuously proliferating un-differentiated cells	Secondary metabolites are synthesized in differentiated non-proliferating cells
Cell ultrastructure	Scarcity of vacuoles and plastids in the population of meristem-like and/or parenchyma-like heterotrophic cells	Intense intracellular compartmentalization, so numerous organelles available for plastid-associated biosynthesis and storage of secondary metabolites
Cell compartmentalization and tissue-specificity	Both biosynthesis and accumulation of secondary metabolites are limited to either a single cell or 10–30 cell aggregates, or sequestrated to the culture medium	Biosynthesis of secondary metabolites is tissue-specific. Secondary products can be transported and stored in different organs

Principle 3. Production of secondary metabolites in plant cell cultures can be enhanced by the following physiological and biotechnological methods [7, 30]:

(a) Elicitation and/or short-term stress are the most common used techniques. The latter can be only effective if the target metabolite is a part of the inducible defence system. Other approaches include manipulating the plant growth regulators in culture medium, balancing medium nutrient composition and optimization of physical environment such as gas composition, light quality, etc.
(b) Due to high heterogeneity of cell population classical selection aimed at cells with abnormally elevated production of target secondary metabolites have been proved successful.
(c) A dramatic shift in cell metabolism caused by mutagenesis, reaction chain inhibitors, etc. followed by selection of cells with elevated metabolite production.
(d) Metabolic engineering, i.e. overexpression or silencing of genes encoding key enzymes of the specific metabolic pathways.

The vast majority of biotechnological studies utilising plant cell cultures for the production of bioactive organic compounds are focused on the effective methodology of increasing the content of target metabolites in dry cell biomass [7, 31]. This research can be significantly intensified by systematic and detailed investigation of factors affecting the formation of certain groups of secondary metabolites in plant cell cultures *in vitro.*

Below we discuss a systematic analysis of factors that influence the production of two main isoprenoid groups, di- and triterpenoids synthesised through different

pathways (MVA and MEP-associated) in cultures of dedifferentiated cells obtained from various medicinal plant species. Some of the data summarised here have been obtained in the course of 50 years of intensive research in authors' laboratories and is presented in English for the first time.

23.2 Steroid Glycosides in Plant Cell Cultures

23.2.1 Overview of Steroid Glycosides

Steroidal glycosides (SG) is a large group of organic compounds with C27 aglycones composed of cyclopentanoperhydrophenanthrene structure (rings A, D, C and D) and a modified side chain at C-17 position. Steroidal glycosides are classified into three groups based on the structure of their aglycones: (1) Spirostane-type, with a hexacyclic ABCDEF-ring system, (2) Furostane-type having pentacyclic ABCDE-ring system with the sixth open F ring and the least frequent, (3) Pregnane-type, a tetracyclic ABCD-ring system [32]. In spirostanol and pregnane-type glycosides a single carbohydrate chain is attached to a C-3 atom while furostanol glycosides carry two carbohydrate fragments at C-3 and C-26 positions. The additional glucose at C-26 position in furostanol glycosides dramatically changes their biochemical properties and physiological activities compared to other SG types. Furostanol glycosides are hydrophilic substances with pronounced immunostimulating activity and were proved effective in the treatments of patients with sex disorders. Spirostanol glycosides are hydrophobic molecules with well-known antimicrobial, anti-fungal, cytotoxic and anti-tumor activity [33, 34].

In plant cells, steroidal glycosides are synthesised from cholesterol in a series of oxidation reactions yielding furostanol structure, followed by glycosylation of hydroxyl groups at C-26 and C-3 carbon atoms [35, 36]. Interestingly, 25-S and 25-R epimers are formed at early stages of a biosynthetic pathway with no evidence for their subsequent epimerization. All steps of SG biosynthesis are performed in the cytosol in the association with endoplasmic reticulum. Furostanol-type SG are synthesised in leaves, some in acylated forms. The newly formed furostanol glycosides are transported through phloem to different organs, especially to storage, like tubers, to be further converted into spirostanol glycosides in the one-step reaction catalysed by the furostanol glycoside specific 26-O-β-glucosidase (F26G) [37]. Spirostanol glycosides can be accumulated in tubers at high quantities, up to 8 % dry weigh. Several studies suggested that furostanol glycosides could be also transported from leaf mesophyll to stem and leaf epidermis and stored in idioblasts that makes them unavailable for mesophyll-localized specific β-glycosidase [38, 39].

Spirostanol glycosides in underground organs and furostanol glycosides in the upper parts of plant have been shown to carry out protective function in constitutive and semi-inducible plant defence systems, respectively. Furostanol-type glycosides can be considered as non-toxic transport and storage form of SG within the semi-inducible plant defence system. Under pathogen attack the disruption of cell

membranes occurs, so, most furostanol glycosides are converted into spirostanol glycosides caused by mixing with F26G [39]. Spirostanol glycosides are the active and extremely toxic forms of SG and play an important role in constitutive defence mechanism as a "chemical weapon" against the invasive pathogens in storage organs. Non-toxic furostanol glycosides have lots of "positive" properties, such as antioxidant and membrane stabilisation and may show important side-functions in cell metabolism.

23.2.2 Steroid Glycosides (SG) in Cultures of Undifferentiated Plant Cells

First studies of SG in dedifferentiated cultures *in vitro* were presumably focused on their highly valuable aglycones. For decades diosgenin – an aglycone of many SG – was an irreplaceable substrate for pharmacological synthesis of steroid hormones such as cortisone, pregnenolone and progesterone [40]. Tubers of tropical vine, *Dioscorea deltoidea* Wall. were found to be the best natural source of this compound because, they accumulate diosgenin-based glycosides only. Soon after extensive harvesting of *Dioscorea deltoidea* plants in their natural habitats brought the species nearly to extinction and thus promoted the research on cell cultures as an alternative source of diosgenin for pharmacological industry [41]. However in the late 1990s, the interest declined caused by a discovery of economically effective production of steroid hormones by the microbial strains utilizing β-sitosterol, a cheap and available waste product of timber industry. In the twenty-first century research interest to SG production in plant cell cultures was refocused on the production of steroidal glycosides *per se*. SG are commonly used by both Western and traditional medicine to treat hypotension ("Diosponin", "Polysponin"), for strengthening ("Tribestan"), as immunomodulators and adaptogens as well as sex-stimulating drugs. Therefore standardization of SG-containing substrates for pharmacological industry is urgently required and is particularly important because plant material normally contains glycosides of both furostanol- and spirostanol-type that may produce alternative, if not the opposite, physiological effects in humans.

In 1970s–1980s SG and their aglycones have been discovered in cell cultures obtained from various plants including *Dioscorea* spp.: *D. deltoidea, D. tokoro, D. nipponica, D. composita* and *D. spiculiflora* [42–45], other genera like *Solanum* spp. [46, 47]; *Yucca* spp., *Agava* spp. [43], *Licopersicon* spp. [43] and specis *Trigonella foenum-graecum* [48], *Momordica charantia* [49] and *Costus speciosus* [50]. In the majority of studies SG content was estimated by content of their aglycones.

Cell cultures differ from intact plants by both quality and quantity of the produced SG. For example, gitogenin and manogenin were the main SG aglycones found in *Yucca glauca* cell culture while sarsapogenin, neotigogenin, gitogenin, marcogenin, tigogenin and smilagenin in intact plant [51]. Also fractions of various glycosides shifted in cell cultures compared to intact plant. For example, solasodine,

a steroidal pseudoalkaloid, was the major aglycone in *Solanum laciniatum* plants with diosgenin being a minor component. By contrast, cell culture of *S. laciniatum* produced diosgenin as a major compound, while solasodine content was reduced nearly to zero [52].

The effect of medium composition including various combinations of growth regulators, carbohydrate sources, the nitrate and ammonium ratios, phosphate content, etc., on SG content has been thoroughly investigated in plant cell cultures [53–57]. Some authors also suggested that cell differentiation status and morphogenesis changed SG production by plant cell cultures [58]. However despite the remarkable research interest no common pattern of SG formation in cell cultures in response to growth conditions has been found. We assume, based on the literature available for the last 40 years and our experience that regulation of SG biosynthesis in undifferentiated cell cultures is likely to be species- and compound-specific. Below, we describe a systematic approach to step-by-step improvement of diosgenin production in *Dioscorea deltoidea* cell culture. It was based on optimization of culture conditions and enabled the up-scaling of the process from the laboratory to semi-industrial bioreactors.

23.2.3 Steroid Glycosides in Dioscorea deltoidea *Cell Cultures*

The first cell cultures of *D. deltoidea* were obtained independently by Staba (USA) and Butenko (the former USSR) in late 1960s and was followed by detailed analysis of cell growth characteristics and optimization of culture media [27, 57, 59]. Interestingly, in both callus and suspension cultures, sucrose uptake from medium resulted in noticeable starch accumulation in protoplasts [60]. Cultivation of *D. deltoidea* suspension cell cultures in flasks and bioreactors was successful [61, 62]. Kaul and Staba were the first to demonstrate the ability of *D. deltoidea* cell cultures to produce diosgenin [59]. According to their records, diosgenin was synthesized intensively by undifferentiated cells while only trace amounts of the compound have been detected in rhizogenous, i.e. differentiated, tissues [59, 63]. Soon after, the first *in vitro*-produced steroidal glycoside (furostanol-type compound without its carbohydrate chain) was purified from hydrolyzed cell biomass of *D. deltoidea*.

Among pre-screened *Dioscorea* species cell cultures derived from *D. deltoidea* demonstrated the highest diosgenin production [44]. Diosgenin content varied from 3 to 30 $mg \cdot g^{-1}$ dry weight equivalent to 10–100 $mg \cdot g^{-1}$ dry weight SG content, respectively [54, 60]. Some data showed that diosgenin accumulation in cell culture occurred at the later phases of growth cycle [60, 61, 64]. On the contrary, Drapeau [42] reported diosgenin content to remain constant during the cultivation cycle.

Intensive research has been focused on the effect of the environment on diosgenin content in *D. deltoidea* cell culture. The ratio and concentration of ammonia and nitrate in culture medium were proved to be important [61]. Dioscorea cells were able to utilize various carbohydrate sources including sucrose, glucose, galactose, lactose and starch. However, the highest diosgenin content was observed at

4–5 % sucrose [65]. Manipulation of growth regulators in culture medium significantly altered the diosgenin production. It was maximized in presence of 2,4-D alone or in combination with IAA [54]. Supplement of diosgenin precursors such as mevalonic acid and cholesterol into the culture medium also improved diosgenin production [54].

Tal et al. [62] applied a biphasic cultivation to stimulate diosgenin biosynthesis in *Dioscorea deltoidea* cell culture. Increasing phosphate and sucrose concentration in culture medium at the exponential growth phase resulted in eight times increase in diosgenin content by the end of cultivation cycle from 0.4 % initial content. However, the overall productivity of the cell culture remained below 15 $mg \cdot L^{-1}$ medium [66].

Another approach leading to high diosgenin production in *Dioscorea* cell cultures was based on chemical mutagenesis followed by classical selection of cells by their proliferation intensity [67]. Ionizing radiation as mutagenic factor was found less effective. Below, we review the main growth characteristics of the selected *D. deltoidea* cell strains with elevated diosgenin and steroidal glycosides production.

23.2.4 Growth and Biosynthetic Abilities of the Selected Strains of Dioscorea deltoidea *Plant Cell Culture*

The initial cell line D-1 was obtained from *Dioscorea deltoidea* tuber in 1968. New strains were obtained as a result of exposure of D-1 cells to different concentrations of NMM (*N*-nitroso-*N*-methylurea) followed by cell selection by growth intensity [67]. The most promising strains resulted from 0.5; 1.0 and 8.0 $mM \cdot h^{-1}$ NMM treatments were named as DM-0.5, DM-1 and DM-8 respectively. Interestingly, line DM-8 was prototrophic and showed intensive proliferation on the medium without growth regulators.

Cytogenetic and Growth Parameters of the Selected *D. deltoidea* Cell Strains

After 5–7 days of culture chromosome number varied from 8 to 68 for D-1 strain, from 10 to 63 for strain DM-0.5, from 10 to 84 for strain DM-1 and from 9 to 80 for strain DM-8 compared to 20 chromosomes (at 2N = 2C) in cells of intact plant. Thirty to forty percent cells in strains D-1 and DM-0.5 were diploid and triploid while 45–50 % cells in strain DM-8 were diploid. The ploidy of the strains appeared stable under standard conditions, but was altered by changes in composition of culture medium. For example, after eight cycles of subculture in vitamin-free medium 30–40 % cells of strain DM-0.5 became polyploid while 40–45 % cells of strain DM-8 – haploid. When both cultures were transferred back to vitamin-containing medium their ploidy levels retained to the initial values [68]. These data

illustrate the flexibility of undifferentiated cell cultures and their high adaptability based on heterogeneity of the cells in population as well as the stability in constant cultivation conditions.

In all strains, except DM-8, 70–80 % cells formed aggregates of less than 20 cells. In prototrophic strain DM-8 60 % cells formed aggregates of over 20 cells [69].

Proliferation activity was different between strains. For example, in DM-0.5 strain fraction of proliferating cells (assessed by ^{3}H-thymidine accumulation) reached 80 % on the forth day of subculture, while in strain D-1 it remained below 65 % by the end of subculture cycle [70]. Both strains demonstrated two peaks of mitotic activity on the second to third and sixth to seventh day, but the mitotic index (MI) of DM-0.5 was 25–30 % higher than that of D-1 strain [27].

Remarkably, strain DM-0.5 retained the intensive proliferation ability for over 30 years of repeated subculture.

Further analysis revealed significant differences in the duration of mitotic cycles between the initial strain D-1 (26–28 h) and the mutant strain DM-0.5 (24–25 h). Mitotic cycle of DM-0.5 strain was shorter than D-1 due to shortened S and G2 phases (Fig. 23.5). However, the duration of actual mitosis was shorter in D-1 strain [70].

Under batch-cultivation in flasks and bioreactors all strains exhibited classical S-shape time-response curve of dry and fresh weight accumulation and cell count. All strains showed comparable duration of growth phases including the lag-phase from 0 to 6–7 days depending on the inoculum size, 4–6 days in the exponential phase and 1–3 days in the slow growth phase. The total absence of the stationary growth phase in all strains could be a special feature of *Dioscorea* cell culture. Increase in sucrose concentration in culture medium resulted in prolonged exponential growth up to 10–12 days.

The main growth characteristics of individual strains of *Dioscorea deltoidea* plant cell culture are summarised in Table 23.2 (according to [27, 71, 72]).

Steroidal Glycoside Content in Different Strains of *Dioscorea deltoidea* Cell Culture

Analysis of steroidal glycosides in cultivated cells of *D. deltoidea* demonstrated that in all strains overwhelming majority was represented by furostanol forms. Table 23.3 shows how production of SG varied between strains. Note that spirostanol-type glycosides content was less than 3 % of total SG.

To explain near absence of spirostanol-type glycosides in *D. deltoidea* cell lines, the additional experiments were performed such as water extraction enabling autofermentation of each strain were performed. During the procedure, the lyophilized cell biomass was extracted with water at 26 °C for 3 h, thus allowing endogenous β-glycosidases to remain active and convert furostanol glycosides to spirostanol forms. As a control, an extraction with 70 % methanol was performed, which resulted in the inactivation of all types of β-glycosidases. Table 23.4 shows how the content of furostanol glycosides was affected by the selective extraction.

Table 23.2 Growth characteristics of the selected *Dioscorea deltoidea* strains grown in flasks

Strain	Maximum dry mass accumulation, M (g L^{-1})	Growth index, I	Specific growth rate, μ (day^{-1})	Economic coefficient on sucrose, Y	Productivity on biomass, P (g L^{-1} day)	Doubling time, τ (days)
D-1	11.2	8.4	0.18	0.30	0.55	3.9
DM-0.5	11.9	8.4	0.20	0.33	0.66	3.5
DM-1	11.7	7.2	0.17	0.32	0.63	4.1
DM-8	10.9	6.8	0.16	0.35	0.60	4.3

Average of 15 replicates. Coefficient of variation (CV) $\leq$ 25 %

Table 23.3 Content of furostanol-type and spirostanol-type glycosides in *Dioscorea deltoidea* strains [73]

Strain	Glycoside content (% dry weight)		Spirostanol glycosides (% of total)
	Furostanol-type	Spirostanol-type	
D-1	9.3	0.07	0.8
DM-0.5	3.2	0.06	1.9
DM-1	2.0	0.04	2.0
DM-8	2.1	0.06	2.9

Table 23.4 Effect of extraction method on content of furostanol-type glycosides in strains of *Dioscorea deltoidea* cell culture [73]

Strain	Olygofurostanoside content (% dry weight)		Extraction loss (%)
	Extraction with 70 % methanol	Extraction with water (autofermentation)	
DM-0.5	7.8	7.8	0
DM-1	3.5	1.2	70
D-1	0.92	0.15	85
DM-8	1.7	0.24	85

According to the data reported in Table 23.4 the low content of spirostanol glycosides in DM-0.5 strain could be a result of low or inactive β-glycosidase in this strain. In contrast, water extraction of DM-1, D-1 and DM-8 strains resulted in 70–85 % loss of furostanol glycosides caused presumably by high β-glycosidase activity. Thus, total absence of spirostanol glycosides in those strains could be due to intracellular isolation of the enzyme and furostanol glycosides in different compartments.

Analysis of individual SG in all cell strains using RP-HPLC-UV, GS-MS after acid hydrolysis and IR spectroscopy showed that protodioscin and deltoside (aglycone diosgenin, 25R-configuration) and their 25-S-isomers (aglycone yamogenin, 25-S-configuration) were detected as major compounds. Interestingly, 25-S-isomers were not found in *D. deltoidea* intact plants, but have been detected in other *Dioscorea* species [9].

Quantitative analysis of SG content by UV–VIS spectrophotometry, HPLC and GC after acid hydrolysis showed similar results with less than 20 % difference

Table 23.5 Content of furostanol-type glycosides in strains of *Dioscorea deltoidea* cell cultures, $mg \cdot g^{-1}$ dry weight [73]

Strain	Total content	25R-configuration		25S-configuration		25S to 25R ratio (%)
		Protodioscin	Deltoside	S-Protodioscin	S-Deltoside	
D-1	14.2	4.2	1.8	5.5	2.7	58
DM-0.5	92.3	54.3	27.1	7.2	3.7	12
DM-1	57.6	31.2	20.9	3.4	2.1	9
DM-8	29.2	9.1	10.2	5.0	4.9	34

Average of 15 replicates. CV ≤ 35 %

between the methods. Table 23.5 shows SG content in the selected strains at the early-stationary phase of growth (14–18 days of culture).

Highest furostanol glycoside content, about 12 % dry weight, was recorded in DM-0.5 strain followed by 7, 4 and 2 % in DM-1, DM-8 and D-1, respectively. Ratio of 25-R and 25-S isoforms remained constant and specific for each strain. The content of SG in DM-0.5 strain was higher than in tubers of *D. deltoidea* plants.

It is important that furostanol glycoside content remained constant at the initial stages of culture cycle, but increased 1.5–2 folds in transition from the exponential to the stationary phase suggesting the continuous synthesis of SG in cell cultures *in vitro* cell cultures [74].

In each strain SG content and accumulation pattern remained stable for 40 years of maintenance by periodic subcultures. Figure 23.4 shows HPLC profiles of different strains of *D. deltoidea* cell suspension culture maintained in flask in the year 1991 and DM-0.5 strain cultivated in 630 L bioreactor after 20-year interval.

Intracellular Localization of Steroid Ginsenosides in *Dioscorea deltoidea* Cell Cultures

Information on intracellular localization of SG can help to increase the productivity of cell cultures. In the first series of experiments SG content was compared between cells and protoplasts isolated from the same culture to reveal possible SG localization in the cell wall and/or in the periplasmic space. The results showed that over 50 % of total SG accumulated in periplasmic space. In 7-days-old culture, only 7.5 $mg \cdot g^{-1}$ glycosides of total 35 $mg \cdot g^{-1}$ SG were found in protoplasts. With the ageing of culture SG content in protoplasts increased to 20 $mg \cdot g^{-1}$ compared to 50 $mg \cdot g^{-1}$ in periplasmic space, presumably caused by their accumulation in vacuoles [73].

SGs are electron-dense substances and electronic microscopy indicated their intracellular localization. Fixation with glutaraldehyde followed by osmium tetroxide staining was used to prevent SG elution from cells. Electronic microphotographs prepared by this method showed localization of electron-dense substances, which were likely to be SG, in vacuoles, cell walls and inter-cellular spaces. In contrast, other fixations resulted in SG elution from cells and absence of electron-dense compounds in the microphotographs. It is important that distribution of electron-dense

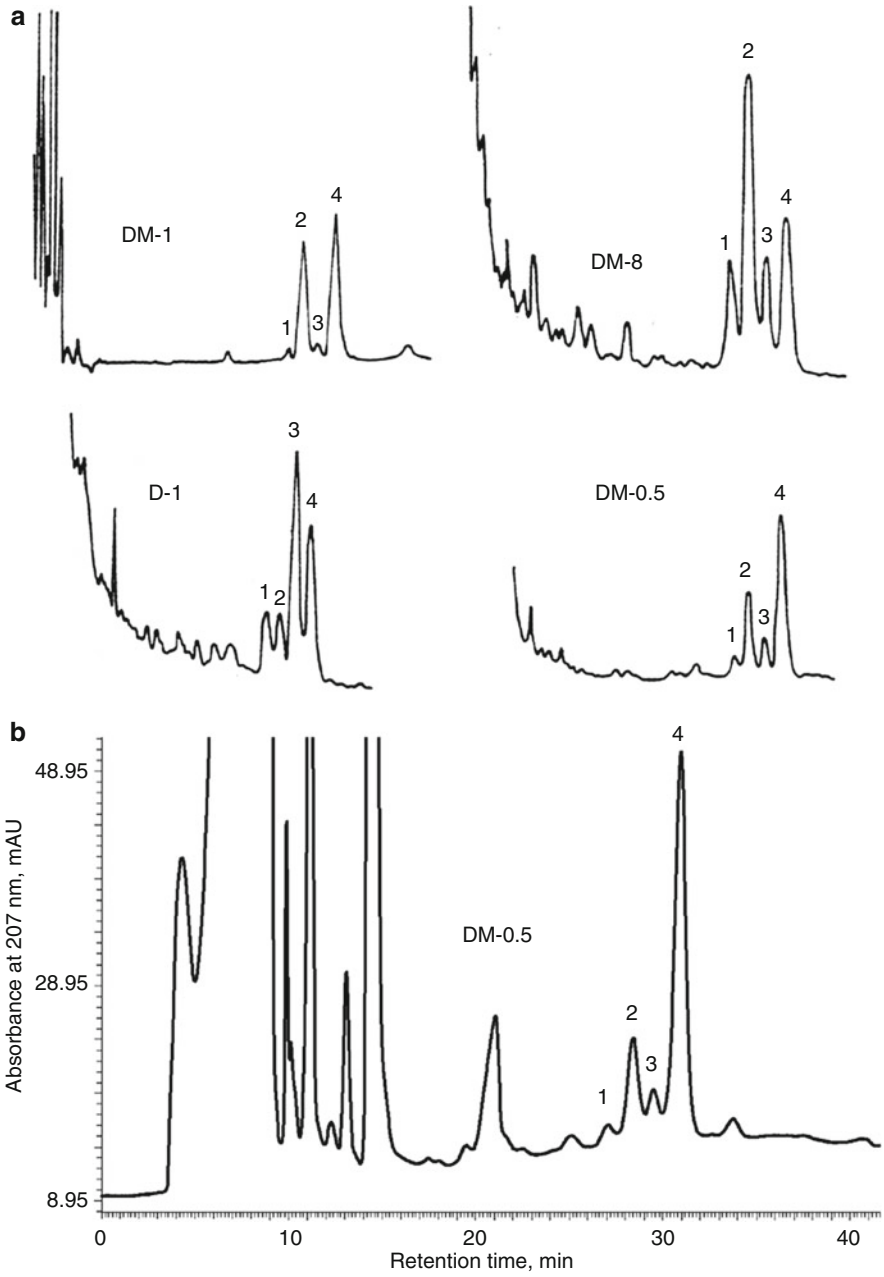

Fig. 23.4 (**a**–**b**) RP-HPLC-UV chromatograms of saponins from *Dioscorea deltoidea* cell suspension cultures. (**a**) Different strains of *D. deltoidea* cell suspension culture, chromatographic analysis made in 1991 [73]. (**b**) *D. deltoidea* cell suspension culture strain DM-0.5, chromatographic analysis made in 2007. *1* 25(*S*)-deltoside, *2* 25(*R*)-deltoside, *3* 25(*S*)-protodioscin, *4* 25(*R*)-protodioscin

compounds between cell compartments was specific for every cell strain. For example, in modest glycoside producers D-1 and DM-8, SGs were detected mainly in vacuoles and cell walls, respectively [75].

Optimization of Culture Medium for Steroidal Glycoside Production in *Dioscorea deltoidea* Cell Cultures

Optimization of culture medium on biomass and SG production in *Dioscorea deltoidea* cell suspension was based on variation in IAA, NAA and 2,4-D concentration, source of carbohydrate and composition of mineral elements. Without growth regulators cell growth was totally arrested at the beginning of the second subculture. Supplementation of auxins into culture medium was essential for the cell growth. Among all auxins studied, addition of 2,4-D to the culture medium was most effective and resulted in 1.5–2.0-fold increase in SG production. The exact mechanism of phytohormone effect on SG biosynthesis in cell suspension remains unknown. The pronounced beneficial effect of 2,4-D may be due to its high ability to intensify the proliferation and dedifferentiation of cultured cells [74, 76, 77].

Biomass accumulation in cell culture increased with the increase in sucrose concentration in the medium from 3 to 5 %, while specific growth rate and sucrose uptake remained unaffected. However at the end of subculture cycle decrease in SG production was recorded for cell suspension in sucrose-enriched medium. Interestingly, in sucrose-enriched medium the portion of 25-S glycoside to total SG increased by 50 %, as well as deltoside to protodioscin ratio. Thus, the increase in sucrose concentration from 3 to 6 % caused a remarkable shift in S-shaped growth curve due to the prolonged exponential phase, elevated accumulation of dry biomass and reduction in SG content; at the same time culture productivity calculated as total SG production remained unchanged [78].

Threefold increase in phosphate in culture medium did not bring any significant changes in productivity of DM-0.5 cell line, and phosphate uptake of 0.4–0.5 mM · day^{-1} was similar to cell culture in standard medium. In contrast to phosphate, nitrogen source affected both cell growth and production of steroidal glycosides. At the standard NH_4^+ to NO_3^- ratio (20:40 mM) nitrogen uptake did not exceed 50 % of total nitrogen content. However, the decrease of both ammonium and nitrate by 50 % resulted in total arrest of culture growth. Decrease only in NO_3^- concentration by 50 % (NH_4^+/NO_3^- = 1:1, 20:20 mM) enabled stable cell growth but resulted in decline of SG production by 4–5 %. At the end the ratio of NH_4^+/NO_3^- was optimized as 1:3 in equivalent of 40 mM total nitrogen [78]. Therefore, optimization of nitrogen source and concentration had the most prominent impact on SG production in *D. deltoidea* cell culture.

Effect of Cultivation Mode on Steroidal Glycoside Production

To investigate the effect of cultivation mode (callus or suspension culture) on SG production 10–12 callus cell lines were obtained from all *D. deltoidea* strains [79]. Table 23.6 summarises the results of SG biochemical analysis in all callus lines of different strains.

Table 23.6 Content of furostanol glycosides in *Dioscorea deltoidea* strains cultured on agar medium ("on-top" cultivation), mg g^{-1} dry weight (summary for all cell lines)

Strain	Total content	25R-configuration			25S-configuration		
		Proto-dioscin	Delto-side	Protodioscin to deltoside ratio	S-proto-dioscin	S-deltoside	Percent of S-configuration
D-1	5.5	1.1	1.4	0.8	1.7	1.3	54
DM-0.5	21.8	13.1	8.7	1.5	0	0	0
DM-1	28.4	13.5	7.5	1.8	4.8	2.6	26
DM-8	6.8	2.3	2.8	0.8	1.1	0.6	25

Mean values, CV ≤ 40 %

Furostanol glycosides varied within the following limits: 0.4–1.0 % DW for D-1 strain, 0.6–3.3 % DW for DM-0.5, 1.2–4.2 % DW for DM-1 and 0.2–1.4 % DW for DM-8. Therefore, total SG production in *D. deltoidea* callus cultured on agar medium was three to ten times lower than that in suspensions in liquid medium. The most significant loss in SG content was recorded for DM-0.5 strain, which has been proved the most productive in suspension cultures. Pattern of biosynthetic productivity of the cell lines cultured on solid medium resembled those of suspension cultures: DM-1 and DM-0.5 strains produced three to four times higher amount of SG than D-1 and DM-8 strains. However, DM-1 cell culture on solid medium was more productive based on SG content of DM-0.5; opposite relations were observed for these lines in suspension.

Protodioscin to deltoside ratio was slightly shifted in callus culture when compared to suspensions. For instance, deltoside became a major glycoside in D-1 strain. The 25-S-glycosides fraction was not detected in DM-0.5 strain but its content increased up to 25 % in DM-1 strain.

Therefore, culture mode affected the SG content in all strains resulting in significant decrease in SG in callus culture when compared to suspension. Also biomass productivity appeared to be two to three times lower on solid medium than in liquid medium, possibly caused by shorter subculture cycle.

Hence we describe below the experiments which show changes in the cell growth related to SG production.

Effect of Cultivation Regime on Growth and Biosynthetic Characteristics of *Dioscorea deltoidea* Suspension Cell Cultures

According to the available literature both growth and biosynthetic traits of cell cultures could be strongly affected by bioreactor mode of operation such as batch, fed-batch and continuous (chemostat, turbidostate and auxostat). This effect is yet to be understood. In our study both D-1 and DM-0.5 strains retained their main growth characteristics when cultured in MF-107 bioreactor (New Brunswick, USA) operated as chemostat. However, maximal dilution rate (D), which equalled maximal specific growth rate (μ) of a strain, was higher for DM-0.5 compared to D-1: at $D = 0.22$ day^{-1} cells of D-1 were gradually eluted from the bioreactor, while DM-0.5 strain retained its growth characteristics [80].

The productivity of DM-0.5 and D-1 strains under the chemostat conditions was 2.3 and 1.8 $g \cdot L^{-1}$ medium per day, respectively, that is two to three times higher than their productivity under the batch conditions. Both strains fully retained their ability to biosynthesise SG under the chemostat conditions. The highest content of furostanol glycosides in DM-0.5 strain was 6 % DW which was lower than under the batch conditions. On the contrary, SG production in D-1 strain cultured under chemostat regime with a low dilution rate was higher than that under the batch regime. The proportion of individual SG such as protodioscin, deltoside and their 25-S-isoforms under chemostat regime remained unchanged when compared to the batch cultivation. Thus, we can conclude that chemostat regime is preferable for DM-0.5 and D-1 strains of *D. deltoidea* when SG productivity is considered: after strain-specific alterations in chemostat regime individual SG content was 1.4–1.6-times higher than that under the batch conditions, possibly due to more intensive cell growth under chemostat conditions [80].

In contrast to microbial cells dedifferentiated plant cells cultured under chemostat regime showed a higher productivity than it was expected based on mathematical models and it was assumed that it may be due to a remarkable change in cell population structure described below. However, the long-term cultivation of plant cell culture in chemostat in a standard medium using high dilution rate is impossible. Dramatic decrease in cell viability has been recorded for both strains after 30–40 days in chemostat with dilution rate $D = 0.20–0.22$ day^{-1}, that was followed by the reduction of growth intensity and subsequent death of the entire population.

Similar to *D. deltoidea* strains the complete loss of viability was reported in tobacco cells after 2 weeks in chemostat. However, it was shown that it was possible to extend duration of cultivation in chemostat up to 70 days by increasing the concentrations of all components of the medium [81].

Our experiments revealed the key role of phosphate in this process. Cell suspension of D-1 strain was cultured in flasks under semi-continuous regime using MS-medium with standard (1.25 mM) and elevated (2.94 mM) phosphate concentration. When cultured in phosphate-enriched medium, the cell suspension showed stable growth for 200 days at specific growth rate (μ) above 0.3 day^{-1}. On the contrary, in the standard MS medium specific growth rate as high as $\mu = 0.23$ day^{-1}, could be maintained only for 40 days, which was equal to 50 cell generations. After this period within several days cell viability was completely lost. Transfer of cell culture to standard medium after 130 days in phosphate-enriched medium caused nearly immediate death of the entire population [82].

The main reason behind the observed instability of cell proliferation and viability under continuous cultivation regime was a dramatic change in the population structure. At a high dilution rate the percentage of intensively dividing cells in the population increased because of rapid elution of cells which stopped growing or which showed slower growth ($\mu < D$). This inevitably resulted in depletion of culture heterogeneity which underpins the population flexibility and stable growth. The rapidly growing and dividing cells that remained in the population were very sensitive and fragile and were unable to survive for long.

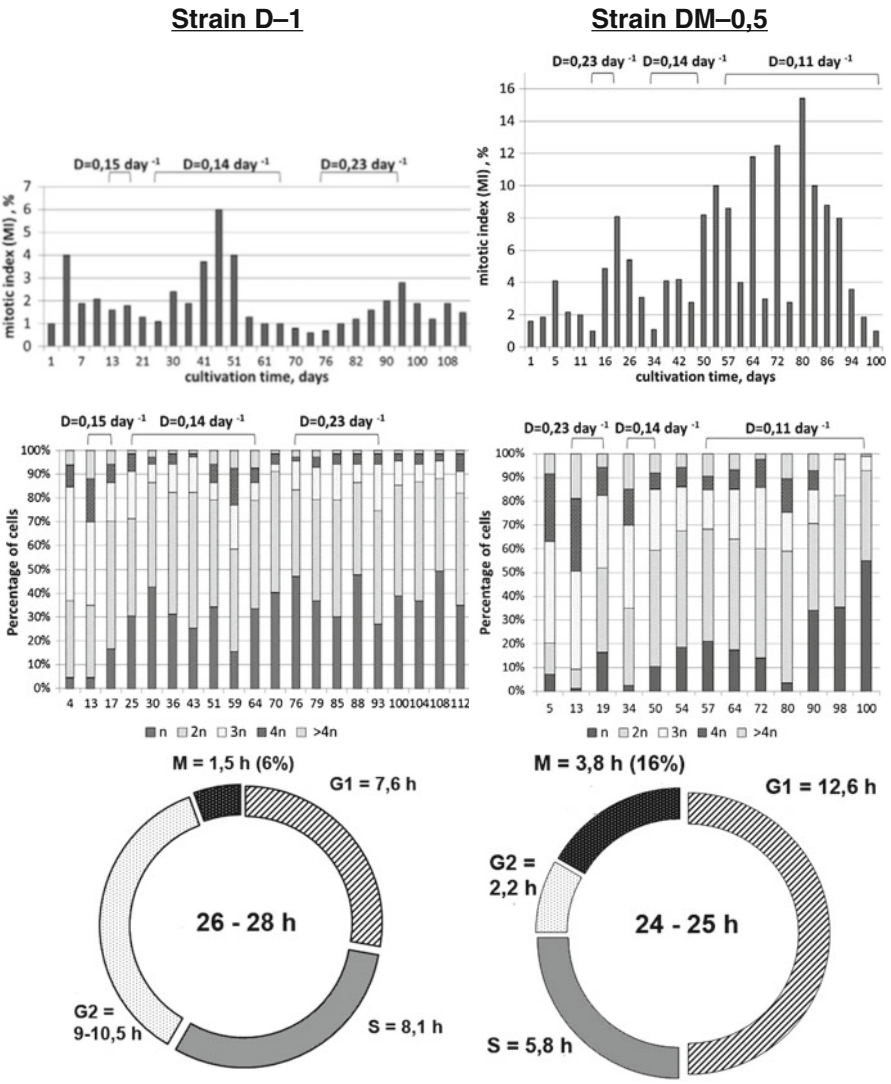

Fig. 23.5 Mitotic index, distribution of cells with different ploidy and duration of mitotic cycle phases in *D. deltoidea* suspension cell culture strains D-1 and DM-0.5 during long-term cultivation in "batch – continuous" regime. *Upper Square brackets* indicate chemostat regime with dilution rate D. n, 2n, 3n, 4n – ploidy of cells

This observation was supported by the results of further experiments with long-term cultivated (over 100 days) D-1 and DM-0.5 strains. These experiments were performed in bioreactors designed to switch between batch and chemostat mode at different dilution rates [83]. It was shown that a step-wise switch from the continuous to batch mode induced significant changes in cell culture (Fig. 23.5). In particular,

a specific growth rate under the batch regime increased due to accumulation of intensively proliferating cells during previous chemostat phase. This was associated with three to fivefold increase in mitotic index in chemostat culture compared to batch. The highest recorded mitotic index for D-1 and DM-0.5 strains under chemostat condition was 6 and 16 %, respectively. Interestingly, mitotic index correlated with the relative phase duration of mitotic cycle: in D-1 strain actual mitosis lasted for 6 % of the cell cycle, in DM-0.5 – 16 %. Assuming that in chemostat culture cell division was not synchronised, equality of mitotic index to the length of mitotic phase indicated that all cells in the population were constantly dividing. As a result, there were no cells in the population which "rested" in between the division circles. This change in cell " cell behaviour" explained the difference of cell cycle parameters in experiment compared to the mathematically modelled ones based on classical chemostat theory. Interestingly, sharp increases in mitotic index were followed by deep decreases down to 1–2 %, confirming our statement that the intensively growing cell population is very unstable. Possibly, cells in cell culture require a rest-period between divisions to produce stable and viable population.

The most dramatic change occurred in cell population under chemostat conditions was the change in ploidy level. In both strains, the proportion of haploid cells increased from 1 to 2 % at the beginning of chemostat conditions to 45–50 % at the end (~100 days). We suggested two possible reasons for that increase. First, it could be due to the lack of phosphate for DNA synthesis and second, that intensive division of cells with a minimal DNA content led to their accumulation in population. It is known that haploid cells are the most sensitive to culture conditions, thus their accumulation may result in significant decrease in culture viability.

Also changes in SG content in cell population under chemostat conditions were likely brought about by changes in cell population. After 30 days in chemostat at $D = 0.14$ days^{-1} furostanol glycosides content in D-1 strain decreased from 1.7 to 1.2 %, but then increased back to 2.7 % [83].

Thus, cell culture in continuous mode bioreactors is a powerful tool to study the unique properties of the cell *in vitro* including biosynthesis of secondary metabolites, because it allows regulation of cell physiology and population structure. For example, our results suggested that a "rest period" between mitotic cycles is necessary for stable cell proliferation and culture growth. Accumulation of intensively proliferating cells caused a deterioration of population heterogeneity, which made it vulnerable in chemostat conditions and led to entire loss of cell viability within a few subculture cycles.

Unfortunately, we have to conclude that chemostat regime despite its higher productivity compared to other culture regimes was not a suitable tool for industrial production of cell biomass.

In general, we found that quantitative and qualitative content of SG in *D. deltoidea* cell cultures under different culture conditions were strain-specific and differed from those in intact plants.

1. Predominant biosynthesis of furostanol-type SG in cell culture was not affected by the presence or changes in activity of endogenous β-glucosidase, but in a

mutant DM-0.5 strain, which showed the highest SG production, the activity of this enzyme was undetectable.
2. Cell cultures of *D. deltoidea* were able to produce glycosides with S-configuration of C-25 carbon atom in their aglycone (yamogenin). These compounds were not found in intact *D. deltoidea* plants, but they have been detected in other *Dioscorea* species
3. All tested cell lines showed similar HPLC profiles of SG, with protodioscin and deltoside found to be the major SG in *D. deltoidea* cell cultures. It is a perfect example for totipotency of plant cells, because in intact plants protodioscin accumulated is seen in leaves and deltoside in tubers, while in cell culture both these SG are co-accumulated.
4. In *in vitro* plant cells SG were accumulated in periplasmic space and vacuoles and the accumulation in vacuoles increased with the ageing of culture.
5. SG content in cell cultures remained stable. The most productive DM-0.5 strain retained the ability to produce SG at the level of 5–12 % during 40 years of observation without any "rejuvenating" treatments.
6. Optimization of culture medium by adjusting the concentrations of growth regulators and mineral nutrition had almost no effect on SG content in *D. deltoidea* cell culture. The maximal increase in SG production due to medium composition was 1.5-fold. On the contrary, change of auxin type and bioreactor culture regime improved SG production suggesting a strong physiological correlation between SG biosynthesis and cell proliferation.

In intact plants SG play a role in protection from stress, so their biosynthesis and function is regulated at the organismal level. Therefore one can expect that SG biosynthesis in cell cultures *in vitro* will be reduced or even stopped. Unexpectedly it remained stable for over 40 years in *D. deltoidea* cell lines. Based on this funding we hypothesize that furostanol-type SG may act alternatively as potential antioxidants and membrane stabilizing agents in dedifferentiated cell population when compared in intact plants, so their biosynthesis is retained to support the culture growth (see "***Principle 1***" in Sect. 23.1).

23.3 Triterpene Glycosides (TG) in Plant Cell Cultures

23.3.1 Overview of Triterpene Glycosides

Tritepenoids are a large group of isoprenoid compounds synthesized from 2,3-oxidosqualene, the common precursor of triterpenoids and steroids. Intact plants contain free triterpenoids, triterpenoid esters of different organic acids and glycosides. The latter can be acylated at different positions of the aglycone and/or carbohydrate chains [11]. Among all triterpenoids triterpene glycosides form the largest group. Apart from aglycone the carbohydrate chains contribute to their vast structural diversity. More than ten different classes of triterpenoids serve as

aglycones in TG. Those from the oleanane, ursane, lupane and dammarane classes are the most widespread in higher plants [10].

Similar to SG formation, biosynthesis of triterpenoids and their glycosides is accomplished *via* a few homotypic stages as following: oxidosqualene cyclization, introduction of a limited number of oxygene-based function groups and attachment of one or more carbohydrate moieties [84]. For instance, the biosynthesis of the ginsenoside Rb_1 includes six sequential steps catalyzed by different enzymes [85].

All enzymes of TG biosynthesis are localized near ER membrane, alongside with the enzymes catalyzing the biosynthetic pathways of their common precursor, squalene [11]. Several reports suggested that individual stages of TG biosynthesis are combined to form metabolic complexes, or metabolomes, which facilitate both spatial and temporal regulation of TG formation [86]. Moreover, as demonstrated for some TG the genes coding the enzymes of TG biosynthesis are organized in clusters within a single chromosome, so they could be expressed in a coordinated manner [87, 88].

Functions of triterpenoids in plants are yet to be elucidated though there is a wealth of evidence pointing out the participation of triterpenoids in ecological physiology of plants, in particular, in the defense system [11, 84].

23.3.2 TG in Cell Cultures of Different Plant Species

Investigation of TG in plant cell cultures started in the middle of the last century. A considerable amount of information about biosynthesis of these compounds in callus and suspension cultures of different plant species was obtained (Table 23.7). The majority of TG found in plant cells cultured *in vitro* belong to the most widespread oleanane and dammarane classes. Together with the glycosides free TG aglycones, including oleanolic, ursolic and betulinic acids and their derivatives, were found [89, 90].

Qualitative Composition

Qualitative composition of TG in cultured cells and intact plants differed significantly. Also novel compounds lacking in the intact plants could be biosynthesized in the cell culture. For example, the callus cultures of *Akebia quinata* and *A. trifoliata* (Lardizabalaceae) were shown to accumulate rare 30-noroleanane-type glycosides which are not found in the intact plants [92, 109]. Furthermore the ratios of different glycoside groups were often altered when compared to the source plants. For example, it was found that the *in vitro* cells of *Glycyrrhiza glabra* (Fabaceae) were incapable of accumulation of glycyrrhizin, the main TG of the licorice tubers [97]. At the same time, these cultures accumulated substantial amount of soyasaponins, which in the intact plant were biosynthesized as a minor component of TG

Table 23.7 Triterpene glycosides (TG) in plant cell cultures obtained from different plant species

Species	Cell culture type	TG found	References
Aesculus hippocastanum	Callus	Escin	[91]
Akebia quinata	Callus	Glycosides of 30-noroleanolic acid and 30-norhederagenin	[92]
A. trifoliata			
Bacopa monnieri	Callus and suspension	Bacosides	[93]
Bupleurum falcatum	Callus and suspension	Saikosaponins	[94]
Calendula officinalis	Suspension	Glucosides and glucuronides of oleanolic acid	[95]
Centella asiatica	Callus and suspension	Asiaticoside, madecassoside	[96]
Glycyrrhiza glabra	Callus and suspension	Soyasaponins I and II, glycyrrhizin	[97]
G. uralensis			[98]
Gymnema sylvestre	Suspension	Gymnemic acid	[99]
Gypsophila paniculata	Suspension	Gypsogenin-3-*O*-glucuronide	[100]
Medicago sativa	Callus and suspension	Glycosides of syasapogenol B and medicagenic acid	[101]
M. truncatula			
Panax ginseng	Callus and suspension	Ginsenosides	[102]
P. notoginseng			[103]
P. japonicus			[104]
Phytolacca americana	Callus	Phytolaccosides A, B, D	[105]
Polygala amarella	Callus	Polygalasaponin XXVIII and other presenegenin glycosides	[106]
Primula veris	Callus and suspension	Primula acid I	[107]
Stauntonia hexaphylla	Callus	Glycosides of 30-noroleanolic acid and 30-norhederagenin	[108]

mixtures only in the underground organs and at certain stages of the ontogenesis. Later studies employing highly sensitive methods such as competitive ISA and HPLC/MS showed that several licorice species were able to biosynthesize glycyrrhizin in callus [98] and suspension [110].

There are also examples of variation in qualitative composition of free triterpenoids in plants and their corresponding cell cultures. Cell culture of *Taraxacum officinale* (Asteraceae) synthesized α- and β-amyrin derivatives found only in the intact plants, but lacked taraxasterol – an essential component of intact plant lactifier [111]. A similar example was reported for *Euphorbia characias* (Euphorbiaceae) cell culture [112], as well as *Eucalyptus perriniana* (Myrtaceae). The latter biosynthesized novel and unique triterpenoids, e.g. C-23-hydroxylated oleananes and ursanes [113]. Changes in tissue specificity of certain reactions in the triterpenoid biosynthesis were also detected (e.g. cell cultures of certain Actinidiaceae species exclusively produced C-24-OH oleananes and ursanes whereas the intact plants

often contained mixtures of C-23/C-24-hydroxylated derivatives of these compounds [89, 90]).

Quantitative Composition

The TG content in cell cultures could vary significantly. Usually TG content is considerably lower in cells cultured *in vitro* compared to intact plants [98, 107, 110]. Often the ability to synthesize TG declined or even disappeared after prolonged cultivation *in vitro* cultivation [107], though exceptions exist. An example of such case could be observed with *Centella asiatica* (Apiaceae) suspension culture, which accumulated asiaticoside in higher amounts than in callus and intact plant [114, 115].

The maximum TG content in plant cell cultures was observed at the end of the exponential/beginning of the stationary phase of the cultivation cycle [98, 107, 110]. Similar pattern was reported for many free triterpenoids [111]. However, there are some exceptions, for example, marigold suspension culture featured two maxima of the accumulation of oleanane-type glycosides: in the beginning of exponential and in the middle-to-the-end of stationary growth phase [116].

Culture medium composition, namely specific phytohormones and their ratio, affected quantitative composition of TG in cell cultures [117]. The same was shown for the precursors of the TG biosynthesis [95], elicitors [99, 118] and stress hormones [117, 119]. Interestingly, the rate of biosynthesis of various triterpenoids by the same cell culture could vary significantly depending on additives to the cultivation medium. For instance, the addition of yeast extract to the cell-suspension culture of *G. glabra* led to the increase in betulinic acid formation and suppression of biosynthesis of soyasaponin, whereas methyl jasmonate had the opposite effect. It is possible these changes occurred due to the differences in regulation of biosynthesis of different triterpenoid groups [97].

Unfortunately, it was impossible so far to generalize on the pattern of TG formation in plant cell cultures *in vitro*. The reason is the scarcity and fragmentation of the knowledge. The only exception is the ginseng cell cultures, in which TG formation has been systematically studied for over 40 years.

23.3.3 Triterpene Glycosides in Ginseng Cell Cultures

The legendary ginseng is a representative of the relic genus *Panax* from Araliaceae family of higher plants [120]. Due to its unique therapeutic properties (adaptogenic, anti-inflammatory, immunomodulatory, neuroprotective, antitumor, etc.) ginseng became one of the most studied medicinal plants in the world [85].

Investigation of ginseng from the phytochemical standpoint lasted for more than 150 years. Different classes of secondary metabolites were isolated from

Panax spp.: polyacetylenes [85], sesquiterpenoids [121], unusual amino acids [122], alkaloids [123], etc., albeit the most typical of ginseng are the TGs of dammarane series – ginsenosides [124]. There is a large body of conclusive evidence supporting the crucial role of ginsenosides in majority of ginseng therapeutic effects [85, 124].

The ginsenosides are traditionally divided into two major classes [125]: glycosides of 20 (S)-protopanaxadiol (the Rb ginsenoside group: Rb_1, Rc, Rb_2 and Rd) and glycosides of 20 (S)-protopanaxatriol (the Rg ginsenoside group: Rg_1, Re and Rf).

The aglycones of these ginsenosides differ by a single hydroxyl group at the sixth carbon atom. The pronounced dissimilarity of the ginsenosides Rg- and Rb-groups in terms of their physicochemical and biological properties stemmed from this hydroxylation. In particular, C-6 hydroxylation with subsequent glycosylation at this position renders the ginsenosides of Rg-group, which are more polar and less toxic in comparison with the ginsenosides from Rb-group [126, 127]. These two groups of ginsenosides also differ significantly in exerting their biological activity, in most cases, the opposite effect. For example, most of the Rg-group ginsenosides show hypertensive and stimulatory effects on central nervous system. By contrast, glycosides from the Rb-group demonstrate sedative and hypotensive effects [128].

Till date, more than 300 different ginsenosides are isolated from different ginseng species [124]. Seven of those (Rg_1, Re, Rf, Rb_1, Rc, Rb_2 and Rd) were first obtained from the roots of *Panax ginseng* and are considered as major ginsenosides [129]. Among the oleanane-type glycosides the ginsenoside R_0 is the most widespread among different ginseng species [85, 125].

Physiological Characteristics of Cell Cultures of Different Ginseng Species

The first callus culture of *P. ginseng* cells was obtained in 1950s from the root of a 4-year-old plant from a plantation at South Sakhalin, USSR [130]. The first suspension cell culture from *P. ginseng* was derived from the callus of a cambial origin in 1970 [131].

After that, numerous callus and suspension culture lines have been obtained from various ginseng species, for example, *P. quinquefolius* [132], *P. japonicus* [104], *P. japonicus* var. *repens* [133], *P. notoginseng* [103], *P. vietnamensis* [134] and *P. sikkimensis* [135].

A vigorous and intensive growth was the basic characteristic of ginseng cell cultures regardless the origin. The optimum cultivation cycle for callus and suspension cultures was 26–30 and 14–21 days respectively, growth index varied from 5 to 12 and specific growth rate at the exponential growth phase varied from 0.12 to 0.23 $days^{-1}$ [136].

Study of ultrastructure of suspension cells showed that in the beginning of the stationary phase *P. ginseng* cells had the structural features of secretory cells, which

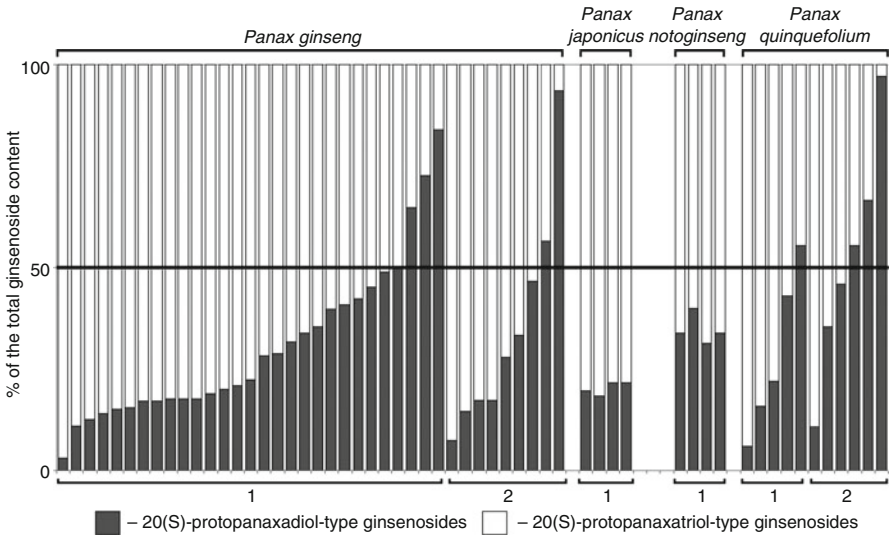

Fig. 23.6 Contribution of the major groups of dammarane-type glycosides in the total ginsenoside content in cell cultures of different ginseng species (according to the literature for the 1973–2013 period). *1* callus cell culture, *2* suspension cell culture

effuse lipophilic compounds to the vacuole. These features included (i) well-developed smooth ER, (ii) plastidial reticular sheath, (iii) osmiophylic depositions in nuclear and plastid envelopes, membranal structures and tonoplast [137]. The osmiophylic matter was deposited in vacuoles as a layer or globules and suggested to be of isoprenoid origin.

Ginseng cell cultures were found to be more sensitive to temperature than other plant species. For a number of *P. ginseng* cell strains increasing the temperature from its optimum by 2.5–4 °C resulted in 30 % decline in culture growth [138].

Ginsenoside Content in Different Ginseng Cell Cultures

The total ginsenoside content and its changes induced by culturing *in vitro* displayed the same relationship as are described above for the cell cultures of other species and TG groups [132, 139–142].

Regarding the ratio of the two major ginsenoside groups ginseng cell cultures *in vitro* were often characterized by predominance of 20 (S)-protopanaxatriol-type ginsenosides (Rg-group) (Fig. 23.6). Accumulation of the 20 (S)-protopanaxadiol-type ginsenosides (Rb-group) was sometimes unstable. Moreover there are indications of a considerable decrease of the ginsenoside diversity during long-term cultivation of *P. ginseng* mainly at the expense of Rb-group [143, 144].

Studies of ginseng cell cultures *in vitro* were focused on the effect of various cultivation conditions and stimuli on qualitative and quantitative composition of

ginsenosides. The chemical stimuli include: variation in cultivation media composition [103, 145, 146], addition of precursors and inhibitors of the isoprenoid biosynthesis pathway [147, 148] and plant stress hormones and different elicitors [149–151]. Among the physical stimuli the effects of aeration [152, 153], light [154] and ultrasound [155] on the ginsenoside composition were described. The majority of the stimuli studied, however, showed no or insignificant effect on culture growth and ginsenoside production. Substantial effects were reported for treatments with certain phytohormone combinations, jasmonates and elicitors [141, 146, 147].

It is difficult to generalize the patterns of TG biosynthesis and accumulation in plant cell culture *in vitro* based on published data, because obtained information comes from different cell lines and strains cultivated in various media and conditions. However, there are a few long-term systemic studies of a particular ginseng culture. Cell cultures of *P. ginseng* and *P. japonicus* var. *repens*, discussed below, serve as an example of such thorough study.

Comparison of Growth and Biosynthesis of Ginsenosides in *P. ginseng* and *P. japonicus* var. *repens* Cell Cultures

A comparative study of growth and ginsenoside production in the suspension cell cultures of *P. ginseng* C.A. Mey. and *P. japonicus* C.A. Mey. var. *repens* Maxim. obtained in 1998 was carried out in the authors' laboratories [133, 143]. Cell culture of *P. japonicus* var. *repens* was obtained from the radix of a 2-year intact plant harvested in Primorsky Krai, Russia; cell culture of *P. ginseng* was obtained from the lateral roots of a 6-year plant from a plantation belonging to 'Ginseng and Tobacco Company', South Korea. Importantly the standard cultivation media (MS with White vitamins) differed in hormone composition: *P. ginseng* was grown on 2,4-D and BAP, but *P. japonicus* var. *repens* on NAA and kinetin.

Growth and Physiology of the Cultures

The growth index and maximum mitotic index of the *P. japonicus* var. *repens* cell culture were found to be 1.5–1.8 folds higher than that of *P. ginseng* (6–7 and 3.5 %, respectively). The peak of mitotic activity was observed on the fifth to sixth day of cultivation in both the species. The number of viable cells was similar in both the cultures ranging between 87 and 90 %. Cell suspension of *P. japonicus* var. *repens* was moderately aggregated: cell clusters consisted of 10–50 cells. In the beginning of subculture cycle the culture contained both meristem- and parenchyma-like cells in equal proportions. At the end of cultivation cycle the proportion of parenchyma-like cells increased twofolds and elongated cells also appeared [14]. Cell suspension of *P. ginseng* contained small aggregates (90 % of aggregates were comprised of 5–20 cells). Majority of cells (80–90 %) in the culture were small meristem-like cells. The fraction of parenchyma-like and elongated cells was insignificant; the ratio of cell types remained constant during the cultivation period [156].

Thus the suspension cultures of the two *Panax* species differed significantly in growth pattern and cytophysiological characteristics. These differences are likely to be caused by species-specific factors and/or different hormonal composition of the media.

Dammarane Ginsenoside Content

The cell cultures of *P. ginseng* and *P. japonicus* var. *repens* differed significantly in total ginsenoside content. Overall biosynthetic capacity of the *P. japonicus* var. *repens* suspension culture was considerably higher when compared to *P. ginseng*: the average ginsenoside content in the *P. japonicus* var. *repens* and *P. ginseng* cell cultures was 3.1 and 0.04 % dry weight, respectively [143, 157]. In both the species compounds from 20 (S)-protopanaxatriol group dominated: their content was five to eight times higher than 20 (S)-protopanaxadiol group [133, 140, 143]. Similar data was obtained from cell cultures of other ginseng species (Fig. 23.6). It is also important to note that biosynthesis of ginsenosides in the *P. ginseng* cell suspension was not stable neither quantitative nor qualitative: sometimes 20 (S)-protopanaxadiol-type ginsenosides were nearly absent [143].

The effect of culture conditions, predominantly the hormone composition was studied for both the ginseng species.

The replacement of 2,4-D to α-NAA in the *P. ginseng* cell culture led to 1.5–2 fold decline in the growth rate within three-to-four cultivation cycles and increase in cell aggregation [156]. In *P. japonicus* var. *repens* cell culture the replacement of α-NAA by 2,4-D did not affect the culture growth [14, 157]. The increase in total auxins content of the medium (2,4-D and α-NAA added at 2 $mg \cdot L^{-1}$ each) resulted in low aggregation in the culture [158].

Use of α-NAA instead of 2,4-D during six to seven cultivation cycles enhanced ginsenoside accumulation in *P. ginseng* cell culture from 0.1–0.3 to 6–8 % dry weight. The increase occurred gradually: from 0.04 to 0.5 % during the first cycle and two to threefold in each of the following cycles. It is likely that such acceleration of ginsenoside production in the cell culture of *P. ginseng* was brought about by α-NAA induced cell differentiation. These processes were manifested by the increase in (i) the proportion of cell aggregates, (ii) cell volume; (iii) number of the cells with doubled nuclear DNA [14, 156, 157]. The recorded changes in cell differentiation pattern were accompanied by altered culture growth rate.

In the *P. japonicus* var. *repens* cell culture the replacement of α-NAA (the usual source of auxins for this culture) with 2,4-D did not lead to significant changes in ginsenoside production. Despite of the decline in cell aggregation, which was induced by replacement of α-NAA by 2,4-D, high level of ginsenoside accumulation (ca. 3 % of dry weight) was retained for six subculture cycles [14, 157].

Even more pronounced effect was observed when *P. japonicus* var. *repens* cell culture was grown in 10-L aerated bioreactor in semi-continuous mode. In the medium containing 2,4-D and α-NAA a decline in ginsenoside content from 3 to 0.5 % was observed at the end of the third cultivation cycle and to 0.3 % at the end of the sixth cultivation cycle. At the same time, in the culture grown on the medium

supplemented with α-NAA high ginsenoside production was retained (at least 1–2 % dry weight) [140, 158].

Malonyl-Ginsenosides in Ginseng Cell Cultures

In the past, ginsenosides of 20 (S)-protopanaxatriol-type have been considered to prevail in ginseng cell cultures. This conclusion was based fully on the detection of seven neutral ginsenosides, Rg_1, Re, Rf (the Rg-group) and Rb_1, Rc, Rb_2, Rd (the Rb-group), for which the commercial pure standards are available [129]. At the same time, the intact plant usually contains not only the free dammarane glycosides but also their esters with different aliphatic acids such as crotonic, acetic or malonic acid. The malonyl derivatives of ginsenosides are the most widespread. Several studies applied modern methods of extraction and analyses and revealed that over 50 % of total dammarane glycosides in fresh roots of *P. ginseng* and *P. quinquefolium* were comprised of malonylated ginsenosides [159, 160]. Thus, the method based on free ginsenoside content was considered to be bias and prone to errors.

The biosynthetic pathway leading to acylated, in particular, malonylated, forms of ginsenosides in cell cultures remains almost unknown. The transformed hairy root cultures of *P. ginseng* were shown to contain significant amounts of malonyl derivatives of the Rb-group ginsenosides [161, 162] though the malonyl-glycosides were not quantified. There are some data suggesting that malonylated derivatives of ginsenosides can be formed in the cell cultures of *P. ginseng* [163, 164].

Considerable amounts of malonyl-ginsenosides Rb_1 (identification was based on ^{1}H- and ^{13}C-NMR data) and Rc, Rb_2 and Rd (RP-HPLC-MS identification) were found in the cell cultures of *P. japonicus* var. *repens* [165, 166].

Variation in the content and composition of the individual ginsenosides biosynthesized in the course of 21-days growth cycle of the cell-suspension culture of *P. japonicus* var. *repens* in flasks was studied by RP-HPLC-UV analysis [166]. Seven ginsenosides were identified: Rg_1, R_0, malonyl-Rb_1, Rb_1, Rc, Rb_2 and Rd. The total amount of ginsenosides usually varied from 30 to 40 mg $\cdot$ g^{-1} dry weight, while the three ginsenosides namely Rg_1 (Rg-type), R_0 (oleanane-type) and malonyl-Rb_1 (Rb-type) accounted for 80–95 % of the total ginsenoside content. Ginsenosides Rb_1, Rc, Rb_2 and Rd (all of Rb-group) were detected at very low levels below 15 % of the total ginsenoside content. These results suggest that in cell suspension culture three ginsenosides (Rg_1, R_0, and malonyl-Rb_1) are the major storage products originating from the ginsenoside biosynthetic pathway. This finding is supported by the ginsenoside profiling data recorded over a 4-year cultivation period (Fig. 23.7).

While the total amount of the ginsenosides varied over a wide range (5.0–49.4 mg g^{-1} dry weight), the ratios of the major groups remained nearly constant: Rg_1, R_0, and malonyl-Rb_1 accounted for 75–93 % of the total ginsenoside content [166]. These findings suggested that both neutral and malonylated ginsenosides should be analyzed to determine actual ginsenoside content in cell-suspension culture.

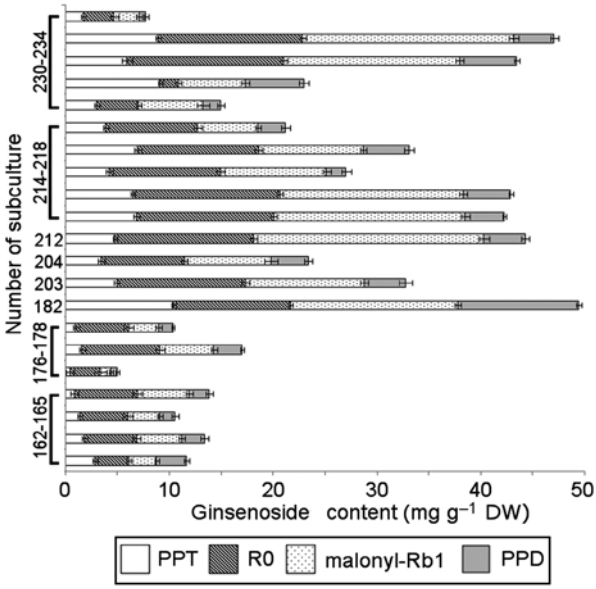

Fig. 23.7 Variations in the amounts of the major groups of ginsenosides in *P. japonicus* var. *repens* cell-suspension culture during 4 years of monitoring (21 independent growing cycles) [166]. PPT = Rg_1; PPD = $Rb_1 + Rc + Rb_2 + Rd$. Analysis was made on day 21 of a subculture

It is well known that secondary metabolic pathways are spatially associated with the compartmentalization of intermediates and end products [29, 167]. Thus, we suggested a tight coordination between ginsenoside accumulation in cell culture of *P. japonicus* var. *repens* and their sequestration into metabolically inactive cellular storage compartment such as the vacuole [168]. This suggestion also takes into account that the triterpene glycosides are a plausible substrate of various glycosidases that produce toxic progenins and aglycones [11, 84]. For example, the Rb-type ginsenosides might undergo non-specific enzymatic cleavage because the *β*-glycosidic bond at the third position of triterpenic aglycones is a common structural motif in nature [169, 170]. Our studies showed that Rb-type ginsenosides (particularly Rb_1) in suspension cell culture of *P. japonicus* var. *repens* were represented mainly by their malonylated forms. The malonylation of various glycosides is a common phenomenon in the plant kingdom [171]. Malonylated glycosides of phytohormones [172], chlorophyll catabolites [173], products of xenobiotic detoxification [174, 175] and several classes of secondary metabolites [171, 176, 177] have been found in different plant species. This modification may alter the molecular properties of parental glycosides in several ways but mainly by preventing the enzymatic degradation of the glucoconjugates and targeting them to specific compartments, such as the vacuole [178–180]. We can speculate that the malonylation of the Rb-type ginsenosides is involved in the regulation of their hydrolysis and cellular compartmentalization in *P. japonicus* var. *repens* cell-suspension culture.

On the contrary to the Rb-type ginsenosides, esterification of Rg-type ginsenosides (particularly malonylation) was not typical [124, 181]. Therefore, we suggest that the specific glycosylation of the Rg-type ginsenosides (attachment of one of the

sugar chains to the α-hydroxyl group at the C-6 position of the dammarane-type aglycone) made them resistant to non-specific glycosyl hydrolases [124, 170], enabling accumulation of ginsenoside Rg_1 without significant disturbance in the metabolic activity of *P. japonicus* var. *repens* cells *in vitro*.

Oleanolic Acid Glycoside Content

Accumulation of the significant amounts of ginsenoside R_0 is observed in suspension cell culture of *P. japonicus* var. *repens*. This finding is consistent with the literature reports [104, 120, 125] emphasizing the predominance of oleanolic acid glycosides as a characteristic trait of this species. Structurally, ginsenoside R_0 belongs to a widespread family of glycosides – glucuronide oleanane-type triterpene carboxylic acid 3,28-bidesmoside, GOTCAB in plant kingdom. These glycosides are characterized by the presence of glucuronic acid residue attached to the hydroxyl at the third position of the aglycone [182]. This structural motif is extremely resistant to non-specific hydrolysis [120]. Attachment of glucuronic acid can play a role in the distribution of metabolites between the cell compartments, for example, molecules tagged with glucuronic moiety are usually targeted to the vacuole [183, 184]. Similar mechanism could be involved in the formation of the ginsenoside R_0 of the *P. japonicus* var. *repens* cell cultures as well as intact plants of other ginseng species.

General Characteristics of TG Accumulation in Ginseng Cell Culture

Summary of TG biosynthesis and accumulation in plant cell cultures, and their differences from SG in general:

1. High ginsenoside content is not necessarily found in every ginseng cell strain. A decline in ginsenoside content occurred sometimes during prolonged cultivation or as a result of change in cultivation medium. However it is possible to obtain strains with robustly high TG content. Optimization of hormonal concentration in culture medium, treatment with stress hormones (e.g. jasmonates) and elicitors often resulted in a considerable improvement of TG production.
2. In many cases alteration in ginsenoside groups in comparison to the intact plants was recorded: in cell culture the protopanaxatriol-type ginsenosides (Rg-group) were often prevailing over the protopanaxadiol-type ginsenosides (Rb-group). The diversity of the glycosides declined in certain strains.
3. In cell culture of *P. japonicus* var. *repens* protopanaxadiol-type glycosides were mainly represented by their malonylated derivatives. This could be explained by (i) necessity of their compartmentalization (vacuole targeting), (ii) lower stability (due to glycosylation at the third C-3-position of dammarane), and (iii) higher toxicity in comparison with protopanaxatriol-type glycosides.

In general we can conclude that ginsenosides do not play a significant role in proliferation of de-differentiated *in vitro* ginseng cell and/or population. Therefore

biosynthesis and accumulation of these compounds is often unstable in cell culture and depends heavily on the cultivation conditions (medium composition, signal molecules, stress, etc.) and physiological state of the population (stages of growth and differentiation).

On the contrary high ginsenoside content observed in certain cell cultures for a long time (e.g. 15 years at the level of 0.5–5 % of dry weight in the case of *P. japonicus* var. *repens* cell cultures) suggests that ginsenosides are essential for cell growth *in vitro*. From this standpoint ginsenosides with different structural groups are not equal. The predominance of the Rg-group ginsenosides frequently observed *in vitro* could suggest they either play a special role in cells *in vitro* or, at least, do not affect their metabolism. Low and unsteady content of neutral Rb-group ginsenosides indicates less significant, if any, role of these compounds in the cell growth *in vitro*. Another possible reason is high toxicity of the Rb-group ginsenosides and/or products of their hydrolysis for vigorously proliferating cells, because carbohydrate moiety at C-3 position of the aglycone of Rb-group can be cleaved off, unlike the carbohydrate chain at C-6 of Rg-group, by non-specific β-glycosidases resulting in the formation of the toxic prosapogenins. Malonylation of the Rb-group alters their properties and targets them to the vacuole. The same is probably true for glucuronide derivatives of oleanolic acid.

Thus, we suggest that ginsenoside biosynthesis in cell culture is closely associated with their compartmentalization which depends on the molecule structure. In particular, the accumulation of the 20 (S)-protopanaxadiol-type ginsenosides depends on malonylation, which likely targets them to the vacuole. This finding provides a new understanding of the ginsenoside accumulation and may help with the rational optimisation of their production in various ginseng cell cultures.

Triterpene Glycosides in the Cell Culture of *Polyscias*

Apart from different ginseng species other representatives of Araliaceae family were introduced to the *in vitro* culture. Recent studies described the *in vitro* cell cultures of different members of the genus *Polyscias*. These plants are broadly used in traditional medicine in South-East Asia. For example, *Polyscias filicifolia* is included in the official Vietnamese pharmacopoeia as an anti-fatigue and cardiac drug [185]. *P. filicifolia*, a relative of *P. fruticosa* is also used for its anti-fatigue, roborant, immune-modifying and anti-dizziness effects [186], but *P. fruticosa* is not as much studied as *P. filicifolia*.

Triterpene glycosides are the essential secondary metabolites of the genus *Polyscias* as well as the other representatives of Araliaceae family. Till date, composition of triterpene glycosides was studied only in 6 out of 130 members of *Polyscias*: *P. scutellaria, P. fruticosa, P. amplifolia, P. guilfoylei, P. fulva* and *P. dichroostachya* [187–190]. All studied species contain triterpene glycosides of the oleanane series.

Studies on cell and tissue culture of *Polyscias* species were initiated in the USSR in the beginning of 1970s. First callus cultures of *P. filicifolia* and *P. balfouriana* were obtained in 1971–1975 [191]. Preliminary phytochemical analysis of the

P. filicifolia callus cultures revealed the presence of significant amounts of starch, free amino acids, reducing sugars, sitosterol and triterpene saponins [192]. The maximum content of the 'saponin fraction' (5.8 %) was recorded on the 5th and 25th day of subculture cycle that corresponded with the peaks of mitotic activity [192]. It should be emphasized that exact structure of the discovered glycosides was not determined; i.e. only 'total glycoside fraction' ('saponin fraction') was studied in these works.

At the end of last century the strain BFT-001-95 of *Polyscias filicifolia* callus and suspension culture was obtained. These cultures were grown in different systems and under different conditions: in flasks (batch mode) and in laboratory-scale bioreactors (continuous and semi-continuous modes). The up-scaled cultivation of *P. filicifolia* cell suspension was performed in the industrial 630 L bioreactor [193, 194]. The analysis of biological activities of the *P. filicifolia* cell culture biomass obtained under diverse cultivation conditions was also performed [195].

Now the cell culture of *P. filicifolia* is used in production of bioactive food additive "Vitagamal" [193, 194]. The authors related bioactivity of the *P. filicifolia* cell cultures to the presence of the triterpene glycosides from the oleanane series in its biomass. The potential presence of triterpene glycosides in the biomass of this suspension culture was studied after nearly 20 years the cell culture was induced. The attempt to find triterpene glycosides or aglycones (of oleanolic acid in particular) in the biomass was unsuccessful [196]. This suggested that high biological activity of *P. filicifolia* cell biomass could be related to other compounds such as polyacetylenes. In 2005, a new line of *P. filicifolia* and – for the first time – callus and suspension culture of *P. fruticosa* [186] were obtained. The new line of *P. filicifolia* suspension cell culture contained almost complete spectrum of oleanolic acid glycosides typical for intact plants. It should be pointed out that this suspension cell cultures was obtained directly from leaves bypassing the stage of callus culture [186].

Triterpene glycosides of oleanolic acid were also found in the suspension cell culture of *P. fruticosa* [197, 198]. Chromatographic (TLC and HPLC) analyses revealed that the major triterpene glycosides of *P. fruticosa* suspension cells are identical to those isolated from leaves of *P. filicifolia*. The major components in the *P. fruticosa* cell cultures were polyscioside E, 28-*O*-*β*-D-glucopyranosyl ester of 3-*O*-*β*-*d*-glucopyranosyl-(1 → 4)-*β*-D-glucuronopyranoside of oleanolic acid (named Pol 3) and ladyginoside A.

To reveal the patterns of individual triterpene glycoside formation in the cell culture of *P. fruticosa* changes in the content of these compounds were studied during a cultivation cycle in flasks. It was found that the total glycoside content increased during the whole cultivation cycle. The maximum glycoside content (0.5 % of dry weight) was recorded in the end of exponential growth phase (14 days). Same was observed for suspension cell culture of *Cyclocarya paliurus* (Juglandaceae), in which the highest accumulation of triterpene acids (oleanolic and ursolic) took place between the end of exponential and the beginning of stationary phase of the culture growth [199].

All found glycosides (polyscioside A, Pol 3, polyscioside E and ladyginoside A) were present in *P. fruticosa* cell culture during the whole cultivation cycle. The major components were polyscioside E and ladyginoside A comprising, respectively, 10–40 and 40–70 % of the total glycosides depending on cultivation period. Of special interest is the finding of significant amounts of monodesmoside ladyginoside A, the most hydrophobic of the glycosides. Its accumulation sustained over the whole cultivation cycle. It is well known that accumulation of monodesmosides is not usually observed in leaves and roots of intact Araliaceae plants, which synthesize saponins of oleanane series [120]. Thus, the biosynthesis of monodesmoside might be unique for *P. fruticosa* and/or can be the result of tissue culture conditions. Additional research is needed to confirm this suggestion.

In general we can conclude that the pattern of triterpene glycoside formation by cell culture strains of two *Panax* and two *Polyscias* species implements the second strategy described under "***Principle 2***" of Sect. 23.1.

23.4 Diterpenoids in Plant Cell Cultures

23.4.1 Taxoids in Cell Cultures of Taxus spp.

Taxol, or paclitaxel, a complex diterpenoid from the bark of yew tree (*Taxus* spp.) is probably the most promising anti-tumor agent of plant origin. Its biological activity is attributed to the unique effect of stabilizing cell microtubules [200]. The evergreen trees and shrubs of *Taxus spp.* are the main natural source of taxan diterpenoids, a group of active molecules of the same pentamethyl tricyclopentadecan structure. However, slow growth of the majority of yew species and relatively low paclitaxel content in the bark and leaves of the plants (0.01 and 0.035 %, respectively) made paclitaxel production from natural sources both time-consuming and economically ineffective [201]. Hence it is important to find the alternative sources of taxol for pharmacological industry.

Since 1997 the Atlantic Forestry Centre of Canadian Forest Service has been engaged in a program for developing ecologically sustainable harvesting protocols of yews in natural stands converting elite cultivars of the wild species into a commercially reared crop [202]. Similarly the Yewcare Company began to plant *T. chinensis* in the nature reserve of Da Huan Mountain in the province of Yunan (China) in 2004. This Taxus plantation covers now more than 30 km^2 and is the largest yew tree provider in the world (http://www.yewcare.com/index.ph.) Another alternative source is semi-synthetic production of taxol, which utilises intermediates such as baccatin III and 10-deacetylbaccatin III, found in needles of *Taxus*. BMS, a leading global supplier of taxol, has a farm of 30 billion yews to supply the bark and needles necessary for the extraction of intermediates [203]. In 2007, Indena developed and patented a protocol of taxol semisynthesis based on 10-deacetylbaccatin III, which is extracted from *T. baccata* trees cultivated in the company plantations (www.Indena.com).

Another possible alternative and environmentally sustainable source of taxol and analogue compounds is plant cell culture. At present it is possibly the most actively developing area of biotechnology [204]. The first report on callus induction and proliferation from gametophytes of *T. baccata* was published in 1973 by Rohr [205]. Further studies have been primarily focused on optimization of culture conditions for better cell growth and taxol production. Within the past 40 years a number of independent research groups performed a broad screening of treatments to increase taxol production in the cell cultures. Different strategies have been applied such as optimization of culture conditions, selection of high-producing cell lines and addition of elicitors and precursors (for the latest reviews [206, 207]). Based on the developed cell lines commercial production of taxoids has been established in many countries. At present Python Biotech is the largest producer of paclitaxel *via* plant tissue culture employing a large-scale bioreactor with a capacity of up to 75,000 L [208]. Another company, Korean Samyang Genex, uses Taxus plant cell cultures to produce paclitaxel under the brand name of Genexol® (http://www.genex.co.kr/Eng/).

On account of a large volume of information available, in this chapter we present a brief overview of the main principles underlying taxoid biosynthesis and its regulation in yew cell cultures *in vitro*.

1. Cell cultures, both callus and suspension, have been obtained so far: *Taxus baccata, T. brevifolia, T. cuspidata, T. canadensis, T. media, T. wallichiana, T. andreanae and T. mairei.* These cultures were reported to be slow growing, which is common feature of cultures of coniferous origin. However, their growth could be speeded-up by optimizing the composition of culture medium or selection methods.
2. The vast majority of cell cultures produced negligible amounts of taxoids, 0.0001–0.01 % dry weight, or no detectable taxoids.
3. Taxoid content in cell cultures exponentially increased under certain treatments. The following treatments were found to be the most effective:

   (a) Selection of the most productive cell lines;
   (b) Two-step cultivation. At first, culture conditions should be optimized to increase biomass production. In the second step, taxol accumulation should be stimulated by changing osmotic potential and growth regulator composition in culture medium.
   (c) Application of elicitors and stress-related hormones to induce taxoid production. Jasmonates (JA and MeJA) were found to be the most effective.
   (d) Paclitaxel is normally accumulated in vacuoles and apoplast of cultured cells. In contrast to triterpenoids, considerable amounts of paclitaxel have been detected in culture medium, presumably caused by its diffusion from apoplast. As a result the amount of paclitaxel in cells could vary from 30 to 100 % depending on the diffusion rate which is found to be species-specific and dependent on culture conditions.
   (e) In gel cell immobilization with or without subsequent MeJA treatment.

4. Metabolic engineering targeting the genes encoding taxadiene synthase, 10-deacetylbaccatin III-10-O-acetyltransferase (dbat) and 3-N-debenzoyl-2-deoxytaxol-N-benzoyltransferase (dbtnbt) has been reported. So far this method had no significant effect on the taxoid production.

Based on the information discussed above and available literature we assumed that the major principles of taxoid production in cultured plant cells differed from those of triterpenoids:

1. Different pathways of biosynthesis. Taxoids are diterpenoid molecules of complex structure, and the first stages of their biosynthesis can be performed *via* plastid-localized MEP-pathway. To support this idea Eisenreich et al. [209] showed that IPP involved in the biosynthesis of the taxane ring was formed *via* the MEP pathway. However, other studies [28] demonstrated the involvement of the cytosolic pathway. A recent study of *T. baccata* cell cultures showed that while taxol biosynthesis was blocked by the addition of fosmidomycin, an inhibitor of the plastid pathway, it was also reduced by mevinolin, an inhibitor of the cytosolic pathway, indicating that both pathways could be involved [210]. Thus, plastids play an important role in taxoid biosynthesis, although their number in cultured cells is scarce.
2. Complexity of biosynthesis localization. While the first steps of taxol biosynthesis are likely to be performed in plastids, a number of the following stages might occur in different compartments of cytosol. For instance, the enzyme cytochrome P450 taxadiene-5-hydroxylase (T5-H), which catalyses hydroxylation at the C-5 position of the taxane ring, is a protein of 56 kDa with an N-terminal of membrane translocation sequence targeting it to the endoplasmic reticulum. A key enzyme of the following stage, a specific taxadiene-5α-ol-O-acetyl transferase (TDAT), is a protein of 50 kDa that bears no N-terminal organellar targeting information [25]. Final products such as paclitaxel and baccatin III are then transported to vacuoles and/or periplasmic space involving yet unknown mechanisms.
3. Fork-branched biosynthesis. Paclitaxel formation involves 19 steps catalyzed by specific enzymes. One of the intermediate products, taxa-4(20),11(12)-dien-5-ol, which serves as a substrate for TDAT, can be also involved in a side reaction catalyzed by Cyt P450-dependent hydroxylase, taxadiene-13α-hydroxylase, yielding taxa-4(20),11(12)-dien-5-13-diol [204]. It was found that this alternative step is especially active in cell cultures elicited with methyl jasmonate [211]. This fact shows that taxol biosynthesis is not a linear pathway and includes branch points, which can lead to other taxoids.
4. Properties of the biosynthate. Taxoids are hydrophobic and toxic molecules. Paclitaxel was involved in apoptosis of *Taxus cuspidata* cell suspension [212]. Correlation between paclitaxel accumulation and increase in number of dead cells was reported for cell cultures of other *Taxus* species [213, 214].

23.4.2 Steviol Glycoside Formation in Cell Culture of Stevia spp.

Steviol glycosides (StG) are the group of molecules incorporating steviol, the tetracyclic diterpenoid of *ent*-kaurane type, as an aglycone. Many compounds of this group are 100–400 times sweeter than sucrose, but low in calories, non-toxic, non-mutagenic and are hardly assimilated by human body [8]. Due to their hypoglycemic effect StG are very promising as sweeteners in the diet of patients with dysfunctions of carbohydrate metabolism, especially for those with diabetes [8].

Steviol glycosides are found in large amounts in leaves of *Stevia rebaudiana* Bertoni, Asteraceae, a perennial shrub native to Northeast Paraguay. Three other species: the Mexican *Stevia phlebophylla* A. Gray, the Chinese blackberry *Rubus suavissimus* S. Lee (Rosaceae) and the Japanese perennial *Angelica keiskei* (Miq.) Koidz. (Apiaceae) also contain steviol glycosides. Thirty four steviol glycosides have been identified in *S. rebaudiana* together with other eight oxidized steviols, including isomers and glycosides.

Stevia cell cultures were obtained by several research teams [215–220]. Most of these cultures demonstrated stable and intensive growth but lacked StG production. The only exception was reported 30 years ago: the callus culture originated from leaf blade showed steviol content as high as 16 % dry weight after 70 days of cultivation [216].

Apart from *S. rebaudiana,* callus cultures of *R. suavissimus* were also obtained with modest rubusoside content, which was promoted by blue light after 28 days in the dark [8]. However, neither blue nor red or white light promoted StG formation in *Stevia rebaudiana* cell cultures.

It was shown that StG content of cultivated *Stevia* plants is five to ten times lower than that of intact plants [8, 221, 222].

Compared to the other cell cultures obtained from, for example, *Taxus spp.*, the literature considering StG formation in cell cultures is rather scarce and fragmented. Therefore it is difficult to generalize on the patterns of StG formation in cells cultured *in vitro*.

In authors' laboratories StG formation was studied in 12 *S. rebaudiana* genotypes originated from Russia, Brazil, Paraguay and Japan. These genotypes had ploidy level from 2n to 5n. Cell cultures were induced from all genotypes and analyzed on StG production. StG content declined in a range from 'outdoor plants (30–80 mg g^{-1} dry weight) to greenhouse plants (15–25 mg g^{-1} dry weight) and finally plants grown *in vitro* (1–6 mg g^{-1} dry weight) [15, 223, 224].

More than 20 callus and suspension lines were obtained from the plants of most productive genotypes and their growth and biosynthetic profiles have been investigated. Both genotype and explant type were found to influence profoundly the morphological and physiological traits of the callus cultures. The ability to produce small amounts of StG (steviol, rebaudioside B, stevioside) was detected in the callus cultures derived from only one genotype. However, even in this cell line, the ability

to produce StG was lost completely after 2-year cultivation. Other callus cultures contained StG only in trace amounts [15, 223, 224].

Growth characteristics of callus cultures sustained upon transition to submerged cultivation, i.e. suspension culture. Although in suspension cultures higher StG formation compared to callus cultures was recorded, yet StG content remained very low (20–120 μg g^{-1} dry weight; productivity of 0.1–0.8 mg L^{-1}). The major glycoside in all cultures was stevioside. Rebaudoside A was absent in some strains. Rebaudiosides C and B and steviolbioside were found only in trace amounts. Optimization of the cultivation conditions on carbohydrate sources including substitution of sucrose to fructose, glucose, maltose, galactose, arabinose, raffinose, rhamnose and sorbitol and changes in sucrose content from 2 to 5 % did not accelerate StG formation. Optimization of mineral salt concentration was turned to be similarly inefficient [15, 223, 224].

Thus, heterotrophic cell cultures of *Stevia rebaudiana* lacked StG completely or produced lower StG amount when compared to intact plants or plants cultured *in vitro*; the diversity of StG also decreased.

Since StG are synthesized *via* MEP pathway and predominantly localized in leaves an effort has been made to obtain mixotrophic cell cultures. Cultivation of callus cultures under illumination (2,000 lx) for several cycles led to the formation of numerous chloroplasts. Light effected callus cultures differently: some cultures intensified growth, some did not show any change. Fortunately in several cultures, in which growth was promoted by light, StG accumulation was also enhanced with its content reaching 30–60 μg g^{-1} dry weight. The composition of StG was strain-dependent with stevioside and rebaudioside A and C being the major components [15, 223, 224].

Organogenesis (gemmogenesis) was induced in several mixotroph callus cultures, and appearance of morphogenic structure and shoot formation led to a considerable intensification of StG biosynthesis. The de-differentiated cells of morphogenic callus contained 70–90 μg · g dry weight StG whereas shoots formed from callus contained tenfold higher amount up to 0.6 mg · g^{-1} StG. The latter was 30 % of StG content of the donor plants *in vitro* [222, 224]. The content of StG in cultured cells and different organs of *S. rebaudiana* is summarized in Table 23.8.

We conclude that effective production of StG in heterotrophic cell cultures was not achieved *via* any tested biotechnological method. Chloroplast formation and/or organogenesis were prerequisite for acceleration of StG biosynthesis. StG production increases as the plant development progresses (compared to greenhouse and *in vitro* plants).

Analysis of the patterns of StG formation in cell cultures demonstrates the following tendencies:

1. Production of the target compound depends not only on the presence of the organelles (plastids), but also on their specialization (must be chloroplasts). In plants StG are synthesized only in green leaves, but not in roots and other heterotrophic organs/tissues because only leaves contain sufficient amount of chloroplasts and certain enzymes for StG formation [8].

Table 23.8 Steviol glycoside (StG) content of *S. rebaudiana* plants and *in vitro* cultures ($mg \cdot g^{-1}$ dry weight) (CV < 30 %) (According to [15, 223, 224])

Culture	Sample	Stevioside	Rebaudioside A	Rebaudioside C	Total StG
Intact plants (2 *n*)	Leaves	24.9	12.0	4.6	41.5
	Stalks	4.5	2.6	0.4	7.5
Plants *in vitro* (2 *n*)	Leaves	3.3	1.91	0.7	5.9
	Stalks	0.8	0.6	0.1	1.5
Etiolated shoots *in vitro* (2 *n*)	Shoots	0.28	0.18	0.07	0.5
Green morphogenic callus[a]	Shoots	0.39	0.11	0.05	0.6
	Cells	0.07	0.02	0	0.09
Mixotrophic[a] callus	Cells	(0.03)	0.02	(0.02)	0.05
Heterotrophic[a] cell suspension	Cells	0.09	(0.01)	0	0.09
Heterotrophic[a] callus	Cells	Traces	0	0	Traces

[a]Data obtained from several cell cultures; compounds, which were not always present are taken in brackets

2. StG and gibberellins in plant leaves are formed from the same precursor. There are evidences [8] suggesting that switching biosynthesis from gibberellins to StG is not due to competition for substrate but leaf development stage. In particular, the enzymes catalyzing StG biosynthesis are vigorously worked in senescing leaves and the enzymes of gibberellin biosynthesis in young leaves. Therefore, temporal 'separation' plays an important role in the regulation of StG biosynthesis.
3. Localization of StG biosynthetic stages could be even more complex, than that of taxoids. It could involve not only cellular compartments, but also specialized morphological structures. There are indications that special leaf glandules are involved in StG formation and storage.

In general we assume that formation of StG in cell cultures of *S. rebaudiana*, similar to taxoid formation, proceeds as outlined in ***Principle 3*** of Sect. 23.1. Still this case is more difficult since no significant StG production have been achieved in de-differentiated cells so far. Formation of morphological structures proved to be necessary for the fully functional StG biosynthesis pathway.

23.5 Biotechnological Aspects of Isoprenoid Production from Plant Cell Cultures

The industrial use of plant cell culture presumes cultivation in large bioreactors up to 75,000 L [208], that imposes a number of prerequisites [225, 226] to support the highest possible culture growth and accumulation of a target compound.

The following traits of plant cell culture should be considered when large-scale cultivation is sought [7, 30, 227]:

- Sensitivity of large cells with vacuole(s) to shear stress caused by mechanical stirring which is thought to be related to fragility of cell walls.
- Rapid increase in sedimentation rate due to cell aggregation and increase in cell suspension viscosity at the end of cultivation cycle often require optimization of stirring process.
- Foamy and highly adhesive above culture broth (so-called 'meringue' formation).
- High importance of aseptic environment, because antibiotics cannot be used and extended batch cycle (ranges from 5 to 20 days to 6 months) under continuous or semi-continuous cultivation.

According to recent reports [31, 228], the adverse effects of shear stress caused by stirring are considerably overestimated in the case of plant cell cultures.

Many of the difficulties that in the past hindered the industrial cultivation of plant cells in bioreactors are successfully resolved [7, 30, 227]. Up-scaling is now technological rather than scientific task. Use of plant cell cultures for industrial production of secondary metabolites has been reviewed, but only a few examples of successful commercial process could be found [7]. Obviously, the main reasons are high cost and demanding technological requirements. To increase the efficiency cell lines with elevated productivity are required as well as innovative methods of biomass production.

Productivity of the cell cultures can be increased by optimization of cultivation conditions. Continuous cultivation in bioreactors is a powerful tool to burst biomass production. Unfortunately, it proved to be unsuitable for commercial cultivation of plant cells.

An attractive alternative is semi-continuous cultivation. This method was implemented in our laboratory using bioreactors with the working volumes ranging from 15 to 550 L and equipped with different stirring systems.

Regardless of the bioreactor capacity the new medium was supplied at the slow-down stage with simultaneous off take of culture. The suspension was diluted with fresh medium to the level which allowed bypassing a lag phase (2.0–4.0 g dry weight per L). The optimal starting density was different for different cultures. The growth curves for the final bioreactor (630 L) are shown in Fig. 23.8. It was shown that plant suspension cultures of *D. deltoidea, Polyscias filicifolia* and *Panax japonicus* retained satisfactory growth and biosynthetic characteristics upon transition to prolonged semi-continuous cultivation in bioreactor. Thus, we concluded that the proposed up-scale scheme is suitable for industrial cultivation.

It is also important that the scheme is universal and may be optimized for different cultures within two or three steps. For this optimization it is necessary to determine the minimal inoculum amount for different cultures to eliminate the lag phase and achieve optimal cycle duration. The biomass should be harvested at the very end of the exponential phase.

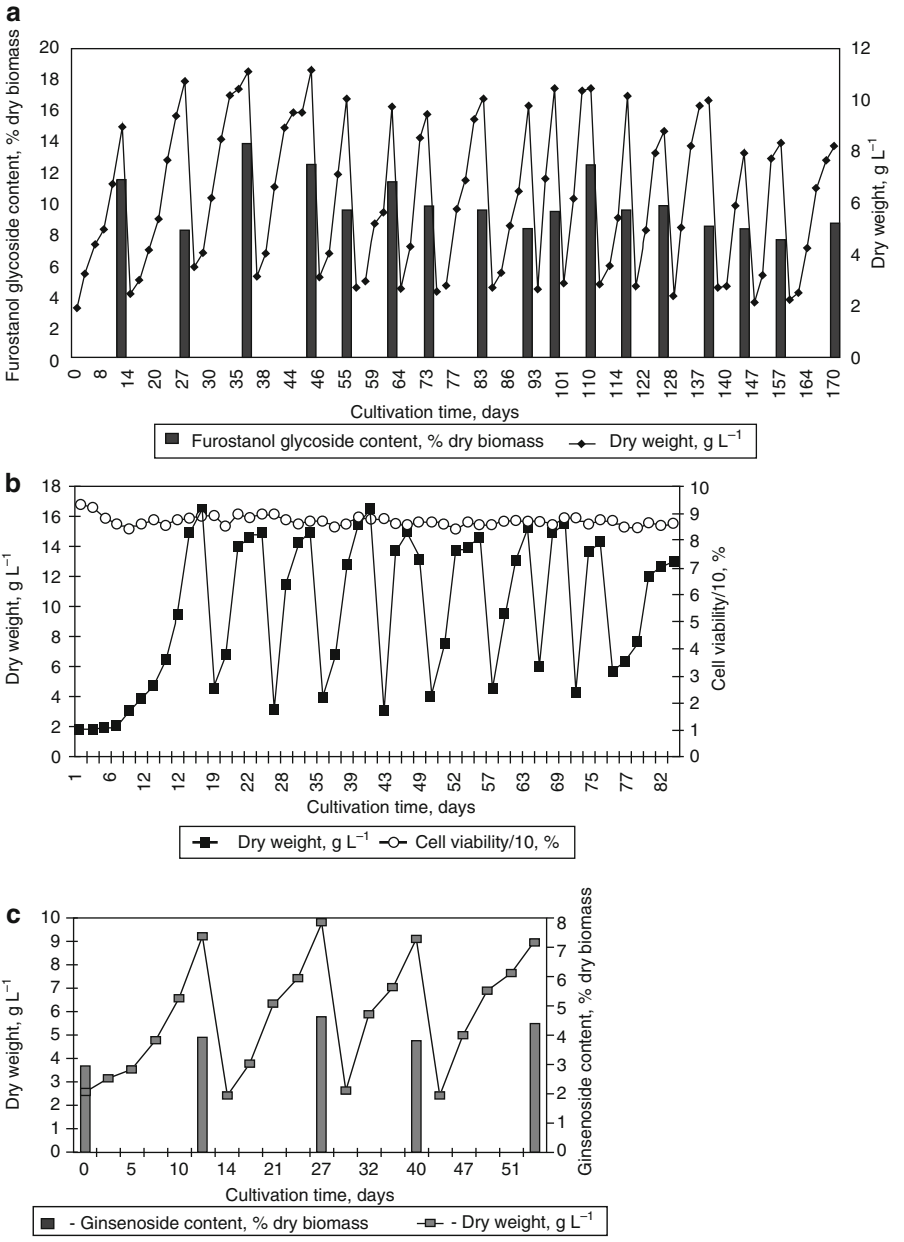

Fig. 23.8 (**a**–**c**) Plant cell cultures growth and secondary metabolites accumulation in 630-L bioreactors, operated in semi-continuous mode. (**a**) *Dioscorea deltoidea.* (**b**) *Polyscias filicifolia.* (**c**) *Panax japonicus* var. *repens*

During such up-scaling both DM-0.5 strain of *D. deltoidea* and *P. japonicus* cell culture retained the capability of sustained production of furostanol glycosides and ginsenosides, respectively, in the amount sufficient for the industrial production.

Generally, the efficiency of biomass production increased by 15–20 % under semi-continuous cultivation due to the absence of lag phase and the technical maintenance in-between-cycles. Hence, semi-continuous mode shall be considered as optimal for the industrial production of plant cell culture biomass.

23.6 Cryopreservation of Plant Cell Cultures with Enhanced Isoprenoid Production

Storage of cell cultures at cryogenic temperatures, below −130 °C, eliminates repetitive subcultures thus reducing the risks of culture loss caused by contamination or technical errors. It also decreases the rate of genetic and epigenetic variations, prevents the loss of regeneration potential and changes in secondary metabolite profile [229, 230].

Building on the classical works of Quatrano [231] and Latta [232], successful cryopreservation has been reported for cell cultures of various medicinal plants which can be potentially used for the production of valuable secondary metabolites. This includes *Digitalis spp*. [233, 234], *Rhaponticum carthamoides* [235], *Artemisia annua* [236], Ginkgo *biloba* [237, 238], and many others (for the most recent reviews see [229, 239–241]).

The majority of cryopreservation protocols resulted from the empirical approaches [242] or have been modified from those applied to cultured animal cells [242, 243]. A classical method of slow, or programmed, freezing was the most applicable so far. With *Panax ginseng* cells suspension, this method resulted in 34–51 % recovery after cryopreservation depending on preliminary treatment [131, 244, 245]. Preculture of *P. ginseng* cells in medium with high sucrose concentrations combined with 3-weeks cold-hardening improved their survival after cryopreservation when compared to the cells grown under standard conditions, assumably due to alteration in intracellular sugar content [131, 245]. Another successful protocol involved pretreatment of *P. ginseng* cell culture on medium supplemented with 20 % sucrose while the temperature of cultivation was gradually reduced from 25 to 4 °C. This pretreatment resulted in 40 % post-freeze cell viability [244]. The maximal regrowth of *P. ginseng* cells after cryopreservation was achieved using 20 % sucrose as a cryoprotector [131, 244]. Combination of 10 % glycerol and 10 % sucrose was found to be less effective [245]. Suspension cultures of *P. ginseng* and *P. quinquefolius* have been cryopreserved by programmed freezing using glycerol and sucrose as cryoprotectors [243]. Maximum post-cryopreservation viability of 55 % was observed for *P. quinquefolius* cells cryoprotected with a combination of 15 % glycerol and 10 % sucrose while pretreatment with 20 % sucrose was detrimental for post-thaw survival. Cell culture of *Polyscias filicifolia* cryopreserved by the same method regenerated at the rate of

45 % [193]. Interestingly, variation in temperature and the way of cryoprotector treatment (at once or with gradual increase of concentration) showed no significant differences in cell viability after cryopreservation.

Maximum viability of 40 % has been reported in *Taxus chinensis* cell culture cryopreserved by slow freezing method using a mixture of 0.5 M DMSO and 0.5 M glycerol [246]. Successful cryopreservation of *Dioscorea deltoidea* cell suspension *via* slow freezing method was accomplished following preculture with 0.02 M asparagine and 0.05 M alanine which induced accumulation of cells with high osmotic- and cryotolerance in the population [131, 247]. Slow-freezing method using 7 % DMSO as cryoprotector was the most effective for cryopreservation of two *D. deltoidea* cell lines [68]. The authors also showed that haploid and polyploid cells of *D. deltoidea* were more sensitive to cryopreservation-induced injuries than di- and triploid cells [68]. Cells of both *P. ginseng* and *D. deltoidea* were found to be more susceptible to cryopreservation at the beginning of the exponential growth phase [246].

Alternative approach to cell cryopreservation was reported by Joshi and Teng [248]. In their study, cells of *Panax ginseng* were exposed to gradually increasing the concentration of glycerol and sucrose followed by direct immersion in liquid nitrogen. The highest viability after cryopreservation achieved by this method was 86.5 %.

Cryopreservation procedure normally involves pretreatment of plant cells with osmotically active and/or toxic chemicals which cause plasmolysis and induce severe and often unrecoverable damages in cell protoplasts. Therefore, the retention of main growth and biosynthetic traits as well as cytological and genetic stability of cell cultures regenerated after cryopreservation should be carefully assessed. It is important that cryopreservation had no effect on ginsenoside profile of *Panax ginseng* cell cultures [244, 249]. Moreover, cultures regenerated after cryopreservation demonstrated higher maximum growth, biomass productivity and yield when compared to non-frozen cells [248]. In contrast, lower accumulation of dry cell weight was recorded for cryopreserved *Taxus chinensis* cell culture when compared with the untreated control in the course of 40-day cultivation, however, paclitaxel production was retained at the same level [246]. Diosgenine, sitosterol and stigmasterols content remained unchanged after cryopreservation of *Dioscorea deltoidea* cell cultures [131]. The same profiles of the relative DNA content have been recorded for cell cultures of *Ginkgo biloba* in the course of 24-month cultivation followed by cryopreservation [237].

Cell culture of *Polyscias filicifolia* showed 25–40 % survival after 5 years of cryogenic storage [193]. This survival was sufficient for regeneration of cell culture following a few consequent steps such as proliferation of callus on solid medium, multiplication of cell suspension in flasks and finally biomass production in semi-continuous mode bioreactors of different volumes, 15 up to 550 L. The main growth and biosynthetic traits of the regenerated culture were retained at levels comparable to those of the initial cell culture (before cryopreservation), regardless of bioreactor volume and type [193]. It is worth to be noticed that the regenerated culture retained the ability for being up-scaled to bioreactors of industrial volume. The same cell

line which has been maintained for 5 years by means of repetitive subcultures showed twofold decrease in productivity when compared to initial cell line and the culture regenerated after 5-year cryogenic storage. To our knowledge, this is the first report of bioreactor cultivation of undifferentiated plant cells after long-term cryopreservation.

23.7 Conclusions

23.7.1 Cell Cultures In Vitro as a Source of Secondary Metabolites and Associated Problems

Formation of secondary metabolites and, in particular, isoprenoids, in cell cultures *in vitro* differs significantly from that in intact plant. The difference resulted from cell dedifferentiation and continuous proliferation and also selection mechanisms being active in cell population. Physiological roles of secondary metabolites in intact plant suggest that their substantial accumulation in cell *in vitro* expected to be exceptional rather than common. Nevertheless, intense biosynthesis of a number of secondary metabolites by cells *in vitro* was reported due to (i) the diversity of functions of secondary metabolites and variety in their effects; (ii) variability of plant cells and their adaptability to *in vitro* conditions and (iii) array of methods available for culture manipulation and stimulation of metabolite production.

Study of secondary metabolism in plant cell cultures are of both fundamental and practical importance. Till date, induction and selection of cell lines with enhanced production of a target metabolite was based on empirical approach, such as optimization of culture conditions and various methods of increasing culture productivity. A profound analysis of the formation of secondary metabolites in cell cultures can make optimization process more efficient and predictable. Since the majority of secondary metabolites are not crucial for vigorous cell growth their biosynthesis in dedifferentiated cell cultures may be inhibited following a peculiar "hierarchies of arrests" which can be of chemical, biochemical and physiological origin and are summarized below:

1. Hierarchy of chemical arrests depends on possible toxic/beneficial effect of compounds and can be visualized as follows: toxic → neutral → beneficial effect on cell growth → stimulation of cell growth and proliferation. Other properties of the compound such as hydrophobicity/hydrophilicity, presence of functional groups, etc. are also important.
2. Hierarchy of biochemical arrests depends on the length and complexity of the biosynthetic pathway. Metabolites with short and unbranched biosynthetic pathway may be easier to obtain than molecules yielded from several unrelated reactions.
3. Hierarchy of physiological arrests depends on compartmentalization and temporal organization of biosynthesis. The most desirable yet the rarest option is

co-localization of both synthesis and storage in one compartment in a single cell. More often biosynthesis and storage occur in two different compartments. The worst case scenario is localization of biosynthesis in different compartments and at a certain stage of plants ontogenesis.

Depending on the position of a target compound in the hierarchy it is possible to predict its probable formation in plant cell culture and even optimal methods of treatment to increase its production. In the worst case scenario (a compound is toxic, biosynthesized in several compartments at a certain stage of morphogenesis) biosynthesis in cultures *in vitro* has not been achieved, e.g. alkaloids of morphine or dimeric indole types. Among isoprenoids diterpenoids including steviol glycosides have the most complicated biosynthesis. Furostanol glycosides have the simplest one.

To stimulate production of "difficult" compounds in plant cell cultures it is helpful to find its precursors and choose the most favorable candidate for the semi-synthesis of a final product, e.g. baccatine III for taxoids and vincamine and catharanthine for dimeric indole alkaloids. Also formation of such compounds in cell cultures could be achieved by mutagenesis and selection of the most productive cells/lines *in vitro*.

23.7.2 Population Engineering Versus Metabolic Engineering

At present two methodologies of secondary metabolite production are widely discussed in the literature: the traditional 'empirical' and the new 'rationalized' approaches. The traditional approach is based on the 'black box' strategy. It involves all manipulations described above: cell selection, optimization of the medium composition, elicitation, use of the biosynthesis precursors, cell immobilization, etc.

The 'rationalized' approach is based on changes in cell metabolism *via* the methods of molecular biology. During the last decade a considerable progress has been made in the study of genes and enzymes involved in secondary metabolism resulting in obtaining the corresponding cDNA [250, 251]. Also the role of transcription factors, promoter and enhancer regions in the regulation of the genes involved in secondary metabolism was revealed [252, 253]. Thus, the novel strategy is based on overexpression or silencing of certain genes involved in biosynthesis/regulation of production of secondary metabolites. Similar approaches are employed, apart from regulation of plant cell culture productivity, to intact plants, plant organ cultures, and, recently, to microbial cultures producing plant-specific compounds [31].

It is accepted that the 'rationalized' approach is more efficient and it should eventually replace the 'empirical' approach.

However, eukaryotic cell, especially plant cell has an array of countermeasures enabling the silencing of foreign genes and segregation of their products. Thus, the immediate success of the molecular approach is questionable, especially if we also take into account the imperfection of the current gene engineering methods and,

most importantly, vast complexity of the problem. It is not enough to obtain the expression of a single gene as such. One has to construct a pipeline of a dozen or more genes including the supply of substrate as well as translocation and compartmentalization of the product, ensuring the temporal coherence of the whole process. It is also not clear whether it is enough to achieve the expression of the biosynthesis pathway genes and/or the regulatory genes and/or transporter genes?

It is also important to note that at present the traditional approach becomes less empirical due to profound knowledge on cell life *in vitro* and peculiarities of the secondary metabolism in such cells. It can be designated as knowledge-based 'population engineering' that harnesses the control over the living and developing of population of somatic cells *in vitro*. The goals of 'population engineering' include creation of new cell populations or conditioning the existing populations to facilitate the production of a target compound. We demonstrated that it is possible to achieve this goal using high variability and adaptability of plant cell populations *in vitro* and a vast array of stimuli affecting these populations in this chapter. Further research is needed to choose the more efficient of the two approaches though we think that the optimal solution is a 'smart' combination of both.

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Chapter 24
Production of Ginsenosides from Adventitious Root Cultures of *Panax ginseng*

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Abstract Ginsenosides are a type of glycosylated triterpenes produced as secondary metabolites by *Panax ginseng* (ginseng). The ginsenosides contain many physiologically and pharmacologically active ones, which possess cardio-protective, immunomodulatory, antifatigue, hepato-protective and anti-tumor properties. Field cultivation of the ginseng plant is a traditional production system to obtain the ginseng bioactive components like ginsenosides, but it is a time-consuming and labour-intensive process. It takes 5–7 years to attain maturity and to reach the harvesting stage, during which a close attention is needed as growth is subjected to several conditions such as soil, climate, pathogens and pests. The use of cell and organ culture has been sought as a potential alternative for efficient production of secondary compounds from ginseng, and various bioprocessing techniques have been developed

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over the past decades. Powerful strategies for adventitious root culture of ginseng have been developed for biomass and ginsenoside accumulation in large-scale bioreactors. The present status and the art of production of ginseng adventitious roots and ginsenosides from bioreactor cultures are described in this chapter.

Keywords Adventitious root cultures • Bioreactor cultures • Ginseng • Ginsenosides • Secondary metabolites • Scale-up • Terpenes

Abbreviations

2, 4 – D	2, 4-Dichlorophenoxy acetic acid
DW	Dry weight
FW	Fresh weight
IBA	Indole-3-butyric acid
MJ	Methyl jasmonate
MS	Murashige and Skoog medium
NAA	α-naphthalene acetic acid
NO	Nitric oxide
PUFAs	Polyunsaturated fatty acids
SA	Salicylic acid
TCMGRs	Tissue culture mountain ginseng adventitious roots
WPM	Woody plant medium

24.1 Introduction

Panax ginseng C. A. Meyer (Araliaceae), commonly known as ginseng is one of the most important medicinal plants, which is widely used in oriental countries such as China, Japan and Korea as a tonic and as an adaptogenic agent. It is also popular in rest of the world especially in North America and Europe as nutraceutical or as functional food. '*Panax*' is derived from a Greek word 'Panacea', which means cure-all diseases. It is used for longevity as well as for improving the physical strength and resistance. The principal ingredients of ginseng are triterpenoid saponins, known as ginsenosides. Ginsenosides are divided into three groups based on their structure i.e., Rb group (protopanaxadiols including Rb1, Rb2, Rc and Rd, etc.), the Rg group (protopanaxatriols including Rg1, Re, Rf, and Rg2, etc.) and the Ro group (Olenolic acid) (Fig. 24.1) [1]. Other than saponins, ginseng roots also contain biophenols, polyacetylenes, sesquiterpernes, polysaccharides, peptidoglycans, fatty acids and vitamins. Pharmacological effects of ginseng have been demonstrated in cancer, diabetes mellitus and in disorders related to cardiovascular system, immune system and central nervous system, including anti-stress and anti-oxidant activities [1]. Due to its entire pharmacological effects ginseng has become a popular tonic and health food in oriental as well as Western countries. Recently, the products of ginseng have also been used in cosmetic industry and in health beverage preparations.

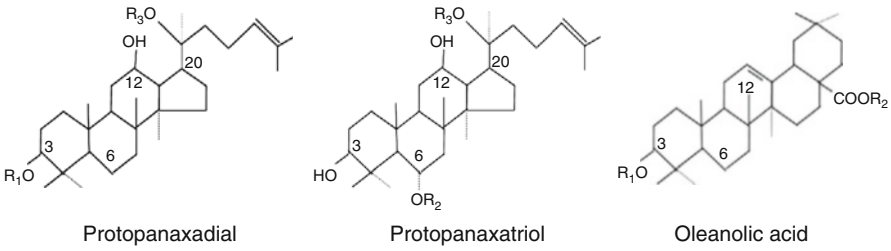

Fig. 24.1 Structure of protopanaxadiol, protopanaxatriol and oleanane ginsenosides

Ginseng plants collected from their natural habitats are highly expensive and become scarce commodity. Ginseng is also cultivated in the fields; however, it usually takes 5–7 years from seedling to the final harvesting stage of the roots, during which a close attention is needed as growth is subjected to several conditions such as soil, climate, pathogens and pests. In addition, the plant has fallen short of supply for a long period. Current advances in plant biotechnology have made it possible to culture plant cells for the production of metabolites. Ginsenoside production by ginseng cell culture has been successful [2–5]. However, the large-scale cultures of plant cells with the objective of commercial production of useful secondary metabolites have been hampered due to the poor productivity and instability of plant cell cultures [6, 7]. In this respect, differentiated organ cultures are seemed to be more promising than undifferentiated cell cultures for the production of useful secondary metabolites [8]. In addition, genetically transformed hairy root culture was recommended for its merit on rapid growing characteristics [9–11], total extracts obtained from hairy root cultures, however, contained opine-like compounds, which has been known to harm potentially the mammalian cells [12]. Therefore, ginseng adventitious root culture is an excellent alternative since the root growth is fast and the ginsenoside production is stable without potential dangers [13]. Adventitious root cultures have been successfully induced and cultured for the production of secondary metabolites in several other plants [14, 15]. There are various reports on ginseng adventitious root cultures focusing on the increase of biomass growth and ginsenoside productivity by the manipulation of culture medium and culture environment. It is also possible to boost the productivity of ginsenosides by applying various strategies such as elicitation, application of suitable bioreactor and bioprocess technologies. This review demonstrates the art of production of ginseng adventitious roots and ginsenosides by bioreactor cultures.

24.2 Induction of Adventitious Roots and Establishment of Suspension Cultures

Adventitious roots were induced from 100 years old mountain ginseng (*Panax ginseng* C. A. Meyer) on Murahige and Skoog (MS) [16] medium supplemented with growth regulators [17, 18]. Root segments developed callus on MS medium supplemented with 4.53 μM 2, 4-D and 0.46 μM kinetin and 3 % sucrose. Adventitious roots were induced from calli masses on MS medium supplemented with 19.68 μM IBA and 3 % sucrose. Furuya et al. [19] found that 2, 4-D was essential for callus

Table 24.1 Effects of salt strength on the growth of ginseng adventitious roots and ginsenoside production. The data were collected after 5 weeks of culture in a 5 L balloon type bubble bioreactor containing 3 L MS medium[a]

MS medium (salt strength)	Biomass		Ginsenosides			Ginsenoside yield (mg L^{-1})
	FW (g)[a]	DW (g)	Rg (mg g^{-1} DW)	Rb	Total	
0.50	366.8 ± 2.22	31.92 ± 0.02	3.05	6.97	10.02 ± 0.77	106.61
0.75	425.6 ± 1.19	34.16 ± 0.03	2.96	5.68	8.64 ± 0.84	98.38
1.00	400.4 ± 4.33	33.32 ± 0.03	2.38	4.12	6.50 ± 0.07	72.19
1.50	330.4 ± 2.13	26.32 ± 0.07	2.96	5.12	8.08 ± 1.15	70.89
2.00	215.6 ± 3.26	15.96 ± 0.08	3.22	7.35	10.57 ± 0.24	56.23

[a]Mean values of three replicates with standard error

induction as well as growth, whereas IBA was found most suitable auxin for adventitious root induction. The adventitious roots were cultured in MS liquid medium supplemented with 19.68 μM IBA and 3 % sucrose in 400 ml conical flasks containing 100 ml of medium until they reached certain biomass. Finally the roots were transferred to 5 L airlift bioreactors containing 3 L of MS medium with 19.68 μM IBA and 3 % sucrose maintained once in 6 weeks.

24.3 Optimization of Medium and Other Culture Conditions

Optimization of culture medium and other culture conditions is very much essential for obtaining the enhanced results during cell and organ cultures. The optimization of medium, the salt strength, growth regulator type, combination and concentration, the type and concentration of carbohydrate, the nitrogen source and concentration, the physical conditions such as light and temperature, agitation and aeration should be standardized. These parameters have been brought in line efficiently in the ginseng adventitious root cultures.

24.3.1 The Effect of Medium Salt Strength

Different salt strengths of MS medium were tested and results showed that the maximum biomass and growth was obtained in 0.75 salt strength MS medium (Table 24.1) and 0.5 salt strength MS medium resulted in higher ginsenoside content. The adventitious root growth was relatively slow in 2.0 salt strength medium, which affected biomass accumulation and overall ginsenoside productivity. However, Son et al. [17] selected Woody plant medium (WPM) [20] for biomass growth and metabolite productivity. Such variations might be due to the selection of different strains of adventitious roots. Therefore, selection of adventitious root clone and selection of suitable medium and salt strength is important during the establishment of cell or organ cultures.

Table 24.2 Effects of macronutrients on the growth of ginseng adventitious roots and ginsenoside production. The data were collected after 5 weeks of culture in a 5 L balloon type bubble bioreactor containing 3 L MS medium[a]

Concentrations of macronutrients		Biomass		Growth ratio[b]	Ginsenosides (mg g^{-1} DW)	Ginsenoside yield (mg L^{-1} DW)
		FW (g)	DW (g)			
NH_4NO_3	0.0	238.6b	23.4c	22.28	10.96 ± 1.12	85.48
	0.5	297.6a	32.0a	30.47	8.62 ± 0.04	91.95
	1.0	274.6a	32.6a	31.05	7.12 ± 0.06	77.37
	1.5	228.6b	27.2a	25.90	6.32 ± 0.18	57.30
	2.0	227.6b	25.2b	24.00	5.65 ± 0.34	47.46
KNO_3	0.0	106.6d	10.6b	10.9	4.24 ± 0.28	14.98
	0.5	263.6b	28.8bc	27.43	8.36 ± 1.12	80.25
	1.0	326.6a	30.8a	29.33	7.04 ± 0.06	72.28
	1.5	254.6b	29.6ab	28.19	8.15 ± 0.22	80.41
	2.0	202.4c	26.8c	25.52	9.25 ± 0.64	82.63
$MgSO_4$	0.0	228.0c	22.8b	21.72	5.56 ± 0.42	42.26
	0.5	312.0ab	30.6a	29.14	6.93 ± 0.03	70.69
	1.0	315.0ab	32.0a	30.48	7.47 ± 0.19	79.68
	1.5	331.2a	32.0a	30.48	7.72 ± 0.22	82.35
	2.0	308.2b	31.8a	30.29	8.89 ± 1.13	94.23
$CaCl_2$	0.0	247.1c	25.0b	23.81	7.26 ± 0.64	60.50
	0.5	317.0b	30.8a	29.33	7.47 ± 1.12	76.69
	1.0	345.0a	32.0a	30.48	7.60 ± 0.72	81.07
	1.5	363.0a	31.8a	30.29	8.27 ± 0.12	87.66
	2.0	365.0a	30.8a	29.33	8.91 ± 0.35	91.47

[a]Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

[b]The growth ratio was determined by the increase in the dry weight after 5 weeks of culture. The values are the quotients of the dry weight after 5 weeks of culture and the dry weight of the inocula

24.3.2 The Effect of Macro and Micro Nutrients

The effect of macronutrients at 0.0, 0.5, 1.0, 1.5 and 2.0 strengths of MS medium were tested on the accumulation of adventitious root biomass and ginsenosides by Sivakumar et al. [21]. It was found that highest ginsenoside content of 10.96 mg g^{-1} DW was obtained when the medium was deprived of NH_4^+ ions (Table 24.2). Half strength KNO_3 in the MS medium was suitable for biomass increase, while 2.0 fold concentrations was responsible for ginsenoside accumulation (9.25 mg g^{-1} DW). 0.5-strength $MgSO_4$ was favourable for increase in root growth and 2.0-strength was favourable for ginsenoside production. In case of $CaCl_2$ 1.0 strengths were favourable for adventitious root growth and 2.0-strength was suitable for ginsenoside accumulation. The results indicated that NH_4^+ inhibited ginsenoside accumulation, while higher concentrations of K^+, Mg^{2+}, and Ca^{2+} increased ginsenoside production.

Table 24.3 Effect of micronutrients on the growth of ginseng adventitious roots and ginsenoside production. The data were collected after 5 weeks of culture in a 5 L balloon type bubble bioreactor containing 3 L MS medium[a]

Concentrations of macronutrients		Biomass		Growth ratio[b]	Ginsenosides (mg g^{-1} DW)	Ginsenoside yield (mg L^{-1} DW)
		FW (g)	DW (g)			
Control	1.0	294.4ab	31.08ab	30.3	1,015 ± 0.07	107.5
$CoCl_2$	5.0	237.4c	22.0d	20.9	9.78 ± 0.27	71.7
	10.0	148.6e	14.8f	14.1	9.95 ± 0.12	49.1
$CuSO_4$	5.0	310.0a	31.0ab	29.5	0.05 ± 0.05	102.8
	10.0	302.8ab	30.0b	28.6	12.42 ± 1.12	124.2
KI	5.0	203.8d	24.4c	23.2	6.52 ± 0.52	53.0
	10.0	143.4e	17.0e	16.2	65.36 ± 0.21	31.3
$MnSO_4$	5.0	309.0a	32.8a	31.2	11.93 ± 0.23	130.4
	10.0	297.6ab	30.4b	29.0	10.88 ± 0.35	110.3
$ZnSO_4$	5.0	287.4b	31.0ab	29.5	9.15 ± 0.22	94.6
	10.0	12.6f	12.4g	11.8	11.66 ± 0.15	48.2

[a]Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

[b]The growth ratio was determined by the increase in the dry weight after 5 weeks of culture. The values are the quotients of the dry weight after 5 weeks of culture and the dry weight of the inocula

The effects of micronutrients on the growth of adventitious roots are shown in Table 24.3. Most of the micronutrients such as $CoCl_2$, $CuSO_4$, KI, $ZnSO_4$ at five or tenfold higher concentrations in the MS medium inhibited the growth of adventitious roots. $MnSO_4$ increased the growth of adventitious roots at a fivefold concentration of the normal medium but decreased the growth at a tenfold higher concentration. $CuSO_4$ at tenfold, $MnSO_4$ at fivefold, and $ZnSO_4$ at tenfold higher concentrations improved the accumulation of ginsenosides. However, KI and $CoCl_2$ inhibited both the adventitious root growth and ginsenoside synthesis at five and tenfold higher concentrations. Thus, the concentration of KI and $CoCl_2$ should be kept at lower levels in the ginseng adventitious root cultures.

24.3.3 The Effect of Ammonium/Nitrate Ratio

The effect of ammonium/nitrate ratio (NH_4^+/NO_3^- – 0.0: 18.5; 7.19: 18.5; 14.38: 18.5; 21.57: 18.5; 28.75: 18.5; 14.38: 0.0; 14.38: 9.4; 14.38: 18.8; 14.38: 26.2; 14.38:37.6 mM) on biomass growth and ginsenoside accumulation has been worked out by Sivakumar et al. [21] and results suggested that nitrate played an important role in biomass increase and ginsenoside production rather than ammonium. A low ammonium concentration combined with a high nitrate concentration was favourable for root growth, showing the largest amount of root biomass at an ammonium/nitrate ratio of 7.19/18.5. Maximum ginsenoside yield was achieved at a ratio of 0 (mM) ammonium to 18.5 (mM) nitrate. Similar experimental observations were reported with cell cultures of *Panax ginseng* and *P. notoginseng* [22, 23] where MS

medium with altered nitrogen concentration was followed for the cultivation of ginseng cells and adventitious roots.

24.3.4 The Effect of Carbohydrate Source

Carbohydrates are important carbon and energy source for plant cell and organ cultures and it has been demonstrated that initial sucrose concentration can affect the culture parameters such as growth and yield of secondary metabolites [24]. Influence of sucrose at a range of 1–9 % was tested on biomass growth and accumulation of ginsenosides in adventitious root cultures [21] and the results showed that 5 % sucrose is suitable for biomass as well as ginsenoside accumulation. Fresh and dry biomass of adventitious roots decreased with the sucrose concentration higher than 5 %. Therefore, 5 % initial sucrose concentration is generally used for cultivation of the adventitious roots in bioreactors.

24.3.5 The Effect of Growth Regulators

Plant growth regulators are one of the key factors influencing the biomass growth and secondary metabolite production. The effect of two exogenous auxins (IBA: 5, 12, 25, 37, and 49 μM and NAA: 5, 11, 16, 25 and 27 μM) were tested by Jeong et al. [25] for multiplication of adventitious roots and they have reported the profuse development of lateral roots from the inoculated roots in the medium supplemented with IBA and NAA whereas development of lateral roots was not observed in auxin free medium. Roots formed on IBA containing medium were slender and elongated, whereas lateral roots formed on NAA supplemented medium were shorter and thicker. Among the different concentrations of IBA tested, 25 μM was found best and in this medium each root explant developed 31.4 lateral roots which are accountable for highest root biomass after 40 days of culture (10.2 g L^{-1} dry weight; Table 24.4).

24.3.6 The Effect of Inoculum Density

There are many reports regarding the effect of inoculum density on biomass and metabolite accumulation [26–29]. There is a critical minimum inoculum size below which cell or root growth will normally fail. With ginseng adventitious roots, various densities of inoculum were tested i.e., 2.5, 5.0, 7.5 and 10.0 g L^{-1}, and the results showed that 5 g L^{-1} was suitable for biomass accumulation (10.5 g L^{-1}) and ginsenoside productivity (5.4 mg g^{-1} DW; Table 24.5) [25]. The length of inoculum is also reported to be critical factor for biomass and metabolites accumulation [30, 31]. The inocula of ginseng adventitious roots chopped to 1–3, 4–6 or 7–10 mm or

Table 24.4 The effect of auxins on number of lateral root development, biomass and ginsenoside content during ginseng adventitious root culture

Auxins	Concentration (μM)	Number of lateral roots	Biomass (g L^{-1}, DW)	Ginsenoside (mg g^{-1} DW)
Control	0	0	3.7 j	2.7 g
IBA	5	6.3 ± 0.5	7.0 I	3.5 e
	12	11.0 ± 0.6	9.0 f	3.8 de
	25	31.4 ± 1.0	10.2 a	5.5 a
	37	30.5 ± 1.0	10.1 b	4.8 bc
	49	30.0 ± 1.0	9.7 d	3.0 f
NAA	5	5.2 ± 0.4	7.6 h	3.8 de
	11	18.0 ± 0.7	8.4 g	4.5 c
	16	15.2 ± 0.7	9.4 e	4.9 b
	25	13.8 ± 0.7	10.1 b	4.7 bc
	27	13.7 ± 0.5	10.0 c	3.9 d

Adventitious roots were cultured in MS liquid medium for 40 days

Table 24.5 The effect of inoculum size on biomass and ginsenoside content during ginseng adventitious root suspension culture for 40 days

Inoculum size (g L^{-1})	Biomass (g L^{-1} DW)	Ginsenoside (mg g^{-1} DW)
2.5	7.1 d	5.1 d
5.0	10.5 a	5.4 a
7.5	10.3 b	5.2 c
10.0	9.7 c	5.3 b

Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

Table 24.6 The effect of root length on biomass and ginsenoside content during ginseng adventitious root suspension culture for 40 days

Length of the roots (mm)	Biomass (g L^{-1} DW)	Ginsenoside (mg g^{-1} DW)
1–3	7.1 d	5.1 d
4–6	8.9 b	5.1 b
7–10	10.0 a	5.5 a
Un-chopped roots	8.4 c	4.1 c

Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

unchopped were used for initiation of cultures and results showed that the adventitious roots chopped to 7–10 mm produced a better yield of biomass (10.0 g L^{-1}) and ginsenoside (5.5 mg g^{-1} DW; Table 24.6).

24.3.7 The Effect of Bioreactor Type, Aeration Rate and Sparger Type

Various types of airlift bioreactors viz. cylinder bioreactor, balloon type bioreactor, cone type bioreactor and bulb type bioreactor were tested for adventitious root growth and ginsenoside accumulation by Kim et al. [32] (Table 24.7) and they reported that bulb type bubble bioreactors were suitable for biomass accumulation (41.92 g DW) as the oxygen transfer capacity (K_La) was optimum with bulb type

Table 24.7 Configuration of various airlift bioreactors and culture conditions used for ginseng adventitious root culture

Configuration	Cylinder type	Balloon type	Cone type	Bulb type
Diameter of bioreactor (cm)	18	22	22	22
Length of bioreactor (cm)	30	30	32	25
Diameter of bubble column (cm)	–	6	–	6
Length of bubble column (cm)	–	5	–	5
Medium volume (L)	4	4	4	4
Air flow rate (vvm)	0.1	0.1	0.1	0.1
Inoculum size (g FW/bioreactor)	20	20	20	20

Table 24.8 Effects of bioreactor on initial oxygen transfer coefficient (K_La) and growth of ginseng adventitious roots cultured in MS medium supplemented with 2.0 mg L^{-1} NAA, 5 % sucrose for 40 days

Bioreactor types	Initial K_La (h^{-1})	Fresh weight (g)	Dry weight (g)
Cylinder	5.24	533.6 b	38.55 c
Bulb	6.98	560.7 a	41.92 a
Balloon	5.49	556.7 a	40.22 b
Cone	5.69	558.8 a	41.52 a

[a]Mean separation within column by Duncan's multiple range test at $P \leq 0.05$

reactors, whereas in cylinder type bioreactors the biomass accumulation was least (38.55 g DW; Table 24.8). Biosynthesis of ginsenosides was not greatly affected the types of bioreactors. Balloon type bubble bioreactors have been selected for further use because of better oxygen transfer, biomass and metabolite accumulation. With balloon type bubble reactors Kim et al. [33] have tested the effect of aeration rate i.e., 0.05, 0.1, 0.2, 0.3 vvm constant air supply and the amount of air supply was increased from 0.05 to 0.3 vvm at 10-days interval on ginseng adventitious root growth and metabolite accumulation (Table 24.9). They found that both the root growth and the ginsenoside accumulation were optimum when the aeration rate was increased gradually at 10-days interval in proportion to root growth. Kim et al. [33] also tested the effect of sparger pore size (15, 30 and 60 µm; Table 24.10) and diameter of sparger (1.5, 3.0, 5.0 and 8.0 cm; Table 24.11) on the adventitious root growth and ginsenoside accumulation and they reported the better root growth (175.9 g DW) with the cultures aerated with sparger of 15 µm pore size. Further, their results revealed that sparger of 8.0 cm diameter and a pore size of 15 µm was suitable for aeration in balloon type bubble bioreactor because the conditions were responsible for production of optimum adventitious root biomass (191.9 g DW) and ginsenosides (4.9 mg g^{-1} DW). These results suggest that selection of suitable bioreactor and modes of aeration are very much critical for cell and organ cultivation.

24.3.8 The Effect of Oxygen, Carbon Dioxide and Ethylene

Biomass growth and accumulation of metabolites in cultures is reported to be influenced by gaseous composition including oxygen, carbon dioxide and ethylene [34, 35] Natural atmospheric sterilized air (N_2-78 %; O_2- 20.8 %; Ar-0.9 %;

Table 24.9 The effect of aeration rate of bulb type balloon bioreactors on the adventitious root biomass growth and ginsenoside productivity after 40 days of culture[a]

Aeration rate (vvm)[b]	Growth of adventitious roots		Total ginsenosides (mg g^{-1} DW)	Ginsenoside productivity (mg L^{-1} day^{-1})[c]
	Biomass (g DW)	Growth rate (fold)		
0.05	149.1 ± 8.2[d]	21.3	3.7 ± 0.1	0.9
0.1	173.6 ± 7.5	24.8	3.9 ± 0.1	1.1
0.2	163.1 ± 9.8	23.3	3.7 ± 0.2	1.0
0.3	151.6 ± 8.4	21.7	4.1 ± 0.2	1.0
0.5/0.1/0.2/0.3[e]	17.58 ± 8.3	25.1	4.3 ± 0.1	1.2

[a]Adventitious roots (7 g DW) were cultured in MS medium supplemented with 2.0 mg L^{-1} NAA, 5 % sucrose
[b]*vvm* volume of gas per volume of aerated liquid per minute
[c]Ginsenoside productivity (mg L^{-1} per day) = total ginsenoside content (mg g^{-1} DW) × dry weight of harvested root (g DW) per volume of culture medium (L) per culture day (d)
[d]Mean values from 3 replicates with standard deviations
[e]Aeration rate increased at every 10 days intervals 0.05–0.3 vvm

Table 24.10 The effect of pore size of the sparger of bulb type balloon bioreactors on the adventitious root biomass growth and ginsenoside productivity after 40 days of culture[a, b, e]

Pore size of sparger (μm)	Growth of adventitious roots		Total ginsenosides (mg g^{-1} DW)	Ginsenoside productivity (mg L^{-1} day^{-1})[c]
	Biomass (g DW)	Growth rate (fold)		
15	175.9 ± 5.6[d]	25.1	3.9 ± 0.3	1.1
30	175.1 ± 4.5	25.0	4.5 ± 0.2	1.3
60	171.9 ± 4.7	24.6	4.9 ± 0.2	1.4

[a]Adventitious roots (7 g DW) were cultured in MS medium supplemented with 2.0 mg L^{-1} NAA, 5 % sucrose
[b]*vvm* volume of gas per volume of aerated liquid per minute
[c]Ginsenoside productivity (mg L^{-1} per day) = total ginsenoside content (mg g^{-1} DW) × dry weight of harvested root (g DW) per volume of culture medium (L) per culture day (d)
[d]Mean values from three replicates with standard deviations
[e]Aeration rate increased at every 10 days intervals (0.05–0.3 vvm)

Table 24.11 The effect of diameter of sparger of bulb type balloon bioreactors on the adventitious root biomass growth and ginsenoside productivity after 40 days of culture[a, b, e]

Diameter of sparger (cm)	Growth of adventitious roots		Total ginsenosides (mg g^{-1} DW)	Ginsenoside productivity (mg L^{-1} day^{-1})[c]
	Biomass (g DW)	Growth rate (fold)		
1.5	175.1 ± 4.3[d]	25.0	4.1 ± 0.2	1.2
3.0	175.9 ± 3.7	25.1	4.0 ± 0.2	1.2
5.0	181.4 ± 4.1	25.9	3.9 ± 0.2	1.2
8.0	191.9 ± 4.4	27.4	4.9 ± 0.1	1.6

[a]Adventitious roots (7 g DW) were cultured in MS medium supplemented with 2.0 mg L^{-1} NAA, 5 % sucrose
[b]*vvm* volume of gas per volume of aerated liquid (min^{-1}) per minute
[c]Ginsenoside productivity (mg L^{-1} per day) = total ginsenoside content (mg g^{-1} DW) × dry weight of harvested root (g DW) per volume of culture medium (L) per culture day (d)
[d]Mean values from three replicates with standard deviations
[e]Aeration rate increased at every 10 days intervals 0.05–0.3 vvm

CO_2- 0.03 %; Ne-, He-) was enriched with pure oxygen (30 and 40 %) or carbon dioxide (2.5 and 5 %) or ethylene (20 and 20 ppm) to promote the ginseng adventitious root growth and accumulation of metabolites by Jeong et al. [36] and only supplementation of 40 % O_2 enhanced the accumulation of root biomass and production of ginsenosides, whereas, enhanced levels of CO_2 and C_2H_5 were unfavourable for the cultures which lead to low accumulation of ginsenosides. Ali et al. [37] reported that the supplementation of higher O_2 levels (above 40 %) induced oxidative stress in the adventitious roots of ginseng as indicated by increased levels of H_2O_2 content, lipoxynase activity and higher activities of antioxidant enzymes. Ali et al. [38] studied the effect of CO_2 (1, 2.5 and 5 %) enrichment on the antioxidant activity and total phenolics in adventitious roots of ginseng. The total phenolics and flavonoids increased over culture duration indicating that these compounds play an important role in protecting the plant from CO_2 toxicity.

24.3.9 The Effect of Light and Temperature

Bioprocess optimization and scale-up of suspension cultures require an understanding of physical parameters for the production of biomass and secondary metabolites [39]. Light and temperature are the two physical parameters which influence the cell and organ cultures and it is observed that optimal temperature and light treatments on suspension cultures is necessary for the accumulation of biomass and production of metabolites [40–43]. Incubation of cultures at 20 °C was most suitable for ginseng adventitious root biomass and ginsenoside accumulation among 10, 15, 20, 25 and 30 °C temperature regimes tested [13]. Reports are also on records that light conditions stimulate the biomass growth of ginseng cells and accumulation of ginsenosides [44]. Yu et al. [18] tested the effect of light quality (red, blue and blue plus red lights) and light intensity (photon flux) on ginseng adventitious root cultures and reported no positive effect of these factors. Therefore, ginseng adventitious root bioreactor cultures are kept in dark and maintained at constant ambient temperature of 20 °C.

24.3.10 The Effect of Polyploid Induction

Polyploidy is responsible for increase in cell size, a characteristic that leads to a larger vegetative and reproductive organs. Polyploids are also responsible for improved secondary metabolite production [45]. Polyploids have been induced in *Chamomilla recutita* [46], *Petunia* [47] and *Salvia miltiorrhiza* [48] which produced more secondary metabolites compared to their diploid counterparts. Octoploid adventitious root lines were induced in ginseng to enhance the biomass and ginsenoside content using colchicine by Kim et al. [49]. They reported that there was improvement in dry biomass accumulation with octoploid lines compared to tetraploid lines (Table 24.12). The total and Rb-group ginsenosides in the octoploid roots were lower (3.3 and 1.1 mg g^{-1} DW respectively) than that of tertaploid roots (3.7 and 1.9 mg g^{-1} DW, respectively), however, Rg-group and Rg1 ginsenosides

Table 24.12 Growth and proliferation of tetraploid (4×) and octoploid (8×) adventitious roots of ginseng after 40 days of culture in MS medium supplemented with 2 mg L^{-1} NAA and 5 % sucrose

Adventitious root lines	FW (mg/vessel)	DW (mg/vessel)	DW/FW (%)	Number of lateral/root explants
4×	106.5 ± 3.9	11.7 ± 0.84	10.9	11.9 ± 0.77
8×	164.8 ± 11.5	13.8 ± 0.69	8.4	4.4 ± 0.61

Data are mean values with standard deviations from 30 adventitious roots

increased by 2 % and 22 % respectively. Hence, octoploid ginseng adventitious root lines are specifically maintained for production of Rg1 ginsenosides (Table 24.13).

24.4 Elicitation

Various types of biotic and abiotic elicitors such as fungal, bacterial and yeast polysaccharides, glycoproteins, xanthan, chitoson, heavy metals, UV radiation, methyl jasmonante and salicylic acid are known to provoke metabolic pathways for enhancing the accumulation of secondary metabolites. Therefore, such elicitors are used in cell and organ cultures for over production of bioactive compounds.

24.4.1 The Effect of Methyl Jasmonate and Salicylic Acid

Jasmonates and salicylic acid are signaling molecules which are used as elicitors to trigger the secondary metabolism in plant cell and organ cultures. Kim [50] tested the efficacy of various jasmonates for elicitation of ginsenoside synthesis in ginseng adventitious root cultures and found methyl epi-jasmonate and methyl jasmonate (MJ) as useful elicitors (Table 24.14). Sevenfold increments in accumulation of ginsenosides with 100 μM MJ treatments compared to control was observed. However, MJ treatments (50, 100, 150 μM) has led to decrease in biomass accumulation. Therefore, two step strategy has been adopted by Yu et al. [51] and Kim et al. [52] by culturing adventitious roots for initial 40 days in elicitor free medium and by adding 100 μM MJ during last ten days of culture. Ali et al. [53, 54] have treated ginseng adventitious root cultures with MJ and salicylic acid (SA) and reported the accumulation of reactive oxygen species and lipid peroxidation in the roots which are responsible for signal transduction of metabolic pathway. They have also reported the accumulation of antioxidant enzymes ascorbate peroxidase, catalase, guaiacol peroxidase and superoxide dismutase enzyme which are responsible for overcoming oxidative stress in the ginseng roots. Tewari and Paek [55] demonstrated that the involvement of nitric oxide (NO) mediation in salicylic acid induced accumulation of ginsenosides.

Treatment of ginseng adventitious roots with copper (5, 10, 25, 50 μM) showed the accumulation of Cu in the roots and resulted in growth inhibition [56]. However, ginsenoside synthesis was triggered by Cu at 5 and 25 μM but decreased with higher

Table 24.13 Ginsenoside accumulation in tetraploid (4×) and octoploid (8×) lines of adventitious roots of ginseng after 40 days of culture in MS medium supplemented with 2 mg L^{-1} NAA and 5 % sucrose

Root lines	Ginsenosides (mg g^{-1} DW)										
	Rb-group				Rg-group						
	Rb1	Rb2	Rc	Rd	Re	Rf	Rg1	TRb	TRg	Total	Rb/Rg
4×	0.6±0.1	0.5±0.1	0.4±0.1	0.4±0	0.6±0.2	0.5±0	0.7±0.2	1.9±0.2	1.8±0.3	3.7±0.3	1.1
8×	0.4±0.1	0.3±0.2	0.3±0.2	0.3±0	0.4±0.1	0.5±0.1	0.3±0.1	1.1±0.3	1.9±0.1	3.3±0.2	0.7

Data represents mean values with standard deviations from three replicates

Table 24.14 Effect of various jasmonates on the concentration of ginsenosides in adventitious roots of ginseng

Jasmonates	Concentration (μM)	Ginsenosdie content (mg g^{-1} DW)[a]
Control	–	6.9f
Methyl dihydro jasmonate	50	11.12e
	100	11.55e
	150	13.90b
Methyl epi-jasmonate	50	44.55b
	100	64.20a
	150	59.70a
Methyl-epi-dihydro jasmonate	50	14.15e
	100	18.30de
	150	30.30c
Jasmonic acid	50	26.80cd
	100	15.50e
	150	16.55e
Methyl jasmonate	50	47.45b
	100	51.50b
	150	49.85b

[a]Mean separation within columns by Duncan's multiple range test at $P \leq 0.05$

Cu treatment of 50 μM. The increase in the ginsenoside content with Cu treatments is attributed to oxidative stress as evidenced by the accumulation of malondialdeyde, reactive oxygen species and hydrogen peroxide.

Tewari et al. [57] tested effect of nitric oxide (NO) elicitation on adventitious root growth, ginsenoside accumulation and antioxidant defense responses. They treated the adventitious roots with 100 μM sodium nitroprusside (SNP). SNP treated root showed enhanced NADPH oxidase (NOX) activity, which is subsequently promoted the root growth and ginsenoside accumulation. They also observed inhibition of NOX activity and decline in dry weight of SNP elicitated adventitious roots in the presence of NOX inhibitor (diphenyl iodonium, DPI), which supports involvement of NOX in root growth.

Among the methyl jasmonate, salicylic acid, nitric oxide and copper stress treatments, methyl jasmonate mediated elicitation seems to be highly beneficial for over production of ginsenosides in the ginseng adventitious cultures. As demonstrated in cell cultures of *Panax notoginseng*, new synthetic elicitors like 2-hydroxyethyl jasmonate had higher inducing activity than conventional jasmonates towards gene expression and ginsenoside biosynthesis [58–60].

24.4.2 The Effect of Organic Germanium

A wide variety of elicitors have been employed to alter cell metabolism in order to enhance the production of secondary metabolites in plant cell and organ cultures.

Table 24.15 Effect of organic germanium on growth of ginseng adventitious roots[a]

Organic germanium (mg L^{-1})	Biomass		Growth yield (L^{-1})	Germanium accumulation (μg g^{-1} DW)	Germanium production (μg g^{-1} DW)
	FW (g L^{-1})	DW (g L^{-1})			
0	470 ± 2	39 ± 1	9.8	–	–
10	497 ± 2	41 ± 2	10.3	180 ± 0	50 ± 1
30	561 ± 3	43 ± 3	10.8	674 ± 6	208 ± 3
60	565 ± 6	44 ± 2	11.1	1,243 ± 5	395 ± 6
90	410 ± 5	34 ± 1	8.5	1,745 ± 9	427 ± 8
120	292 ± 4	25 ± 1	6.2	2,215 ± 25	393 ± 10
150	190 ± 3	17 ± 1	4.1	2,801 ± 18	334 ± 5

[a]Each value represents mean ± SE of three replicates

Table 24.16 Effect of organic germanium on the content of individual ginsenosides[a]

Treatment	Ginsenoside content (mg g^{-1} DW)								
	Rg1	Re	Rf	Rb1	Rb2	Rc	Rd	Total[b]	Rb:Rg[c]
Control	0.7 ± 0.2	1.1 ± 0.3	0.5 ± 0.1	0.4 ± 0.1	0.2 ± 0	0.3 ± 0.1	0.7 ± 0.2	3.9 ± 0.3	1.5
Organic Ge (60 mg L^{-1})	1.1 ± 0.1	1.1 ± 0.1	0.6 ± 0.1	0.5 ± 0	0.4 ± 0.1	0.4 ± 0.1	0.9 ± 0.1	5.0 ± 0.15	1.2

[a]Each value represents mean ± SE of three replicates
[b]Total content = (Rg1 + Re + Rf + Rb1 + Rb2 + Rc + Rd)
[c]Rb:Rg = (Rb1 + Rb2 + Rc + Rd)/(Rg1 + Re + Rf)

Organic germanium, a dietary supplement, was used as an elicitor to enhance the biomass accumulation and ginsenoside production by Yu et al. [61]. When adventitious root cultures were supplemented with organic germanium at 0, 10, 30, 60, 90, 120 or 150 mg L^{-1} concentrations and it was observed that 60 mg L^{-1} organic germanium enhanced both fresh (565 g) and dry (44 g) biomass accumulation (Table 24.15). Table 24.16 presents detailed changes in contents of the different ginsenosides. The contents of Rb and Rg group of ginsenosides were more than the contents found in control treatment. Improvement in the accumulation of protopanaxadiol (Rb, 0.9-fold increments) as well as protopanaxatriol (Rg, 0.3-fold increment) was reported suggesting that it is possible for the production of value added biopharmaceuticals using germanium as an elicitor.

24.4.3 The Effect of Polyunsaturated Fatty Acids

Different polyunsaturated fatty acids (PUFAs) were used as elicitors to enhance biomass accumulation and ginsenoside production in ginseng adventitious root cultures by Dewir et al. [62] and the adventitious root cultures were treated with oleic and linolenic acid at 0, 1, 5, 10 or 50 μmol L^{-1} on the 40th day of culture and roots were harvested on day 47. They observed that except that of 1 μmol L^{-1} linolenic acid, all PUFAs concentrations significantly decreased biomass production in terms of fresh

Table 24.17 Effect of PUFAs elicitation on biomass production of *P. ginseng* roots

PUFAs conc. (μ mol L^{-1})[a]		Biomass production (g/3 L bioreactor)			
		FW	DW	% of DW	Growth ratio
Control	0	369.39a[b]	26.95a	7.30a	29.28a
Oleic acid	1	357.90b	23.61b	6.60b	25.53b
	5	360.91b	23.95b	6.64b	25.91b
	10	355.29b	23.31b	6.56b	25.29b
	50	311.62c	19.87d	6.38c	21.33d
Linolenic acid	1	370.52a	27.71a	7.48a	30.13a
	5	305.45c	22.41c	7.34a	24.18c
	10	295.59d	22.00	7.37a	23.72c
	50	361.75b	24.08	6.66b	26.06b
Significance[c]					
Elicitor type (ET)		***	***	***	***
Elicitor conn. (EC)		***	***	***	***
ETx EC		***	***	***	***

[a]Elicitors were added on the 40th day of culture and roots were harvested on day 47
[b]Mean separation within columns by Duncan's multiple range test at 5 % level
[c]***Significant at $P \leq 0.001$

and dry biomass, percentage of dry biomass and growth ratio (Table 24.17). Cultures supplemented with 1 μmol L^{-1} linolenic acid showed enhancement in ginsenoside accumulation, without the decrease in adventitious root biomass (Fig. 24.2). Linolenic acid enhanced the biosynthesis of both protopanaxatriols (2.95 ± 0.048 mg g^{-1} DW) and protopanaxadiols (5.66 ± 0.043 mg g^{-1} DW) compared to that of control (1.41 ± 0.02 and 1.58 ± 0.006 mg g^{-1} DW respectively). Improvement of ginsenoside accumulation in ginseng adventitious root cultures with the treatment of linoleic and linolenic fatty acid is also reported by Wu et al. [63].

24.5 The Effect of Precursor (Squalene) Feeding and Medium Replenishment Strategies

Various strategies such as feeding the cell and organ cultures with biogenic precursor (precursor of biosynthetic pathways) or with fresh nutrient medium (fed-batch cultures) have been followed [64–70] to improve the productivity of biomass and secondary metabolites. The ginseng adventitious root cultures were fed with biogenic precursor squalene (100, 200, 300, 400 and 500 μM) by Sivakumar et al. [71] and they reported that the cultures which were fed with 300 μM of squalene improved the growth adventitious roots. They also reported the increase in the production of Rg group of ginsenosides i.e., 0.96, 0.09, 1.91 mg g^{-1} DW of Rg1, Rg2 and Re ginsenosides respectively, when compared to control (0.22, 0.02 and 0.86 mg g^{-1} DW of Rg1, Rg2 and Re) with squalene fed cultures. The medium replenishment method was employed by Jeong et al. [72] to enhance 25.5 % increase

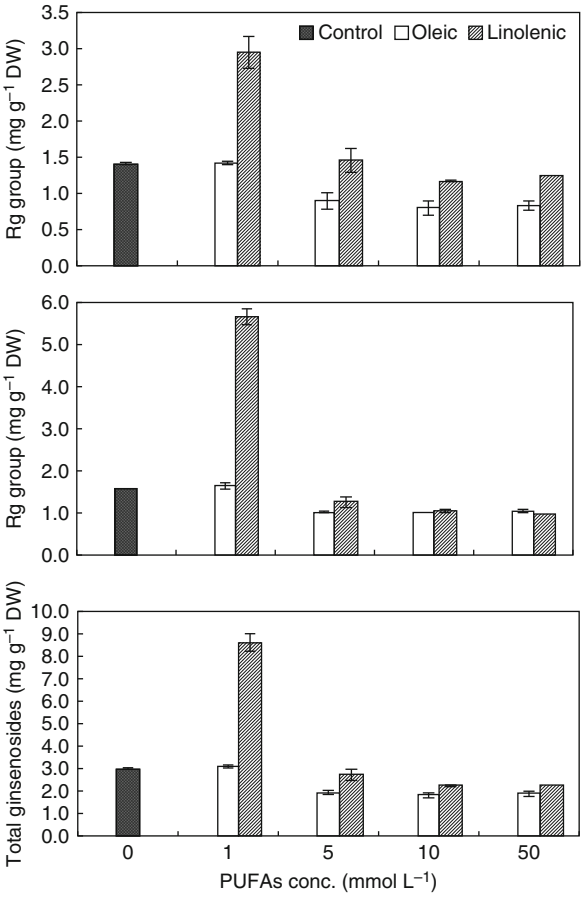

Fig. 24.2 Ginsenoside accumulation (Rg group, Rb group, total ginsenosides) in *P. ginseng* roots as affected by PUFA elicitation (elicitors were added on the 40th day of culture and roots were harvested on 47th day)

in dry biomass (28.7 g L^{-1}) and 8.3 % increase in ginsenoside contents (4.92 mg g^{-1} DW) in culture. These results clearly suggest that precursor feeding and medium replenishment are useful strategies for improvement of secondary metabolites.

24.6 Scale-Up

After optimization of all the chemical and physical parameters for the cultivation of ginseng adventitious roots in small-scale bioreactors (5 L), scale-up of the process was carried out in 20 L (Fig. 24.3a), 500 L (Fig. 24.3b, c) and 10, 000 L (Fig. 24.3d) by CBN Biotech Company, South Korea. Cultures were established using MS medium with 5 mg L^{-1} IBA and 50 g L^{-1} sucrose. Suitable inoculum size has been worked out as 50 kg per for 10,000 L bioreactor (Table 24.18) [73], the root growth pattern was as same as that of small scale cultures and 850 kg of fresh (Fig. 24.3e, f) and 85.4 kg dry biomass (Fig. 24.3g) could be produced in each batch.

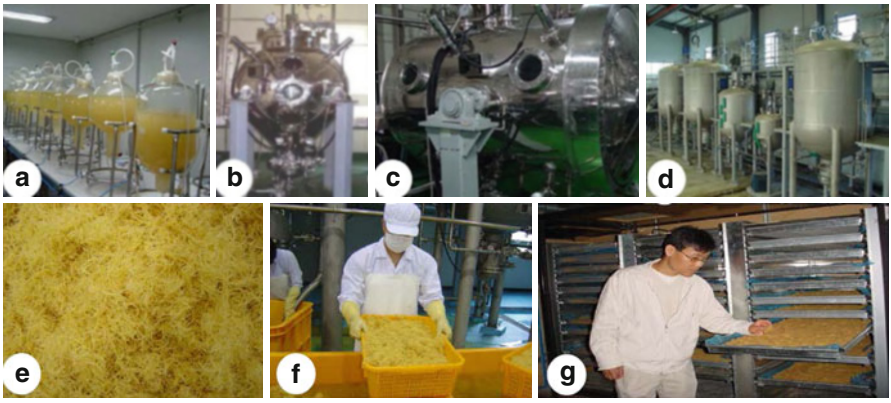

Fig. 24.3 Bioreactor cultures of ginseng adventitious roots (**a**) 20 L airlift bioreactors, (**b**) 500 L balloon type airlift bioreactor, (**c**) 500 L horizontal drum type bioreactor, (**d**) 10,000 L airlift pilot scale bioreactors, (**e**) ginseng adventitious root biomass, (**f**) harvested ginseng adventitious root biomass, (**g**) drying of adventitious roots

Table 24.18 Plant scale (10,000 L) bioreactor culture: effect of inoculum density on accumulation of biomass and ginsenosides

			Ginsenosides (mg g^{-1} DW)		
Inoculum density (Kg)	FW (Kg)	DW (Kg)	Rg-group	Rb-group	Total
50	850	85.4	6.11	20.45	26.56
100	1,100	119.2	3.92	18.98	22.90

Comparative analysis of ginsenosides in cultivated ginseng (which needs minimum 4 year for cultivation and harvesting), red ginseng (needs minimum 6 years for cultivation in field and then it is steam processed), mountain ginseng (~100 years old wild ginseng collected from mountains in Republic of Korea), adventitious root lines induced from mountain ginseng and callus and hairy roots induced from mountain ginseng revealed the differential accumulation of protopanaxatriol, protopanaxadiol in these raw materials (Table 24.19). It was also observed that adventitious root line #4 elicitated with 100 μM methyl jasmonate was superior in accumulation of ginsenoside showing an accumulation of 2.81 mg g^{-1} DW triol and 29.66 mg g^{-1} DW diol and total 32.46 total ginsenosides after 45 days cultivation in bioreactors. Thus, overall three fold increment in accumulation was evident with adventitious root biomass cultivated in bioreactors.

24.7 Economic Feasibility of the Production of Secondary Metabolites from Cell and Organ Cultures

The economic feasibility of secondary metabolite production from cell and organ cultures varies with the plant species, type of culture employed for large-scale cultivation,

Table 24.19 Comparison of ginsenoside contents in various types of ginseng

Various types of ginseng	Ginsenosides (mg g^{-1} DW)			
	Triol	Diol	Ratio of D/T	Total[a]
Cultivated ginseng (4 years needed for harvesting)	5.25 ± 0.15	6.10 ± 0.40	1.16	11.35 ± 0.55
Red ginseng (6 years for harvesting and processing of roots)	4.94 ± 0.20	9.09 ± 0.90	1.83	14.04 ± 1.09
Mountain ginseng (~100 years old)	6.61 ± 0.34	7.57 ± 0.24	1.10	14.19 ± 1.20
Adventitious root line #1	2.75 ± 0.70	1.86 ± 0.30	0.73	4.61 ± 0.98[b]
Adventitious root line #2	3.41 ± 0.0	2.57 ± 0.04	0.75	5.98 ± 0.04[b]
Adventitious root line #3	3.34 ± 0.80	14.74 ± 1.05	4.41	18.09 ± 1.03[b]
Adventitious root line #4	2.81 ± 0.33	29.66 ± 2.30	10.56	32.46 ± 2.28[b]
Callus (from mountain Ginseng)	0.31 ± 0.04	2.54 ± 0.31	8.45	2.85 ± 0.26
Hairy roots (from mountain ginseng)	3.56 ± 0.22	6.26 ± 0.41	1.75	9.83 ± 1.05

[a]Mean values of three replicates with standard error
[b]All adventitious root lines were induced from 100 years old mountain ginseng and are cultured in bioreactors using MS medium and after elicitation with 100 μM methyl jasmonate

Table 24.20 The cost of production of ginseng adventitious roots compared with field cultivated ginseng

Item	Field cultivated ginseng	Adventitious roots obtained from bioreactor cultivation
Yield (kg/0.1 ha)	523[a]	30,000[b]
Production cost (US $/ kg)	35	47

[a]After 5 years of field cultivation (fresh root biomass). Data from 2012 Ginseng statistical yearbook, Ministry of Agriculture, Food and Rural Affairs, Republic of Korea
[b]Ginseng adventitious roots were cultured in four 10,000 L bioreactors for 45 days and bioreactors were operated for 7–8 cycles per year

the kind of bioreactor/s, the method/mode of operation, biomass yield and value of the end product. The cost of production of ginseng adventitious root raw material is illustrated here. In this article, the bioreactor production of ginseng adventitious roots with that of field cultivated ginseng is manifested (Table 24.20). The average yield of Korean ginseng (*Panax ginseng* C. A. Meyer) roots from field cultivation in Republic of Korea is 523 kg per 0.1 ha and cost of production is estimated to be 35 US$ (Table 24.20) [74]. The cost of expenditure for field cultivation includes seedbed preparation, custom seeding, manure, pesticides, fumigation, fertilizer, shade cloth (ginseng is shade loving plant, should be grown under 70–80 % shade and the cost of cloth used for shading will vary depending on the material used) and labour. Whereas, the biomass yield of ginseng adventitious roots cultivated in four 10,000 L bioreactors for 45 days and operated for 7–8 cycles in 1 year (established by CBN Biotech, Cheongju, Republic of Korea) is about 30,000 kg (30 t). The cost of production of ginseng adventitious roots is 47 US$ per kg. The analysis of cost of expenditure is as follows: Chemicals – 13 %, labor – 26 %, electricity/gas/water – 6 %, operation

cost – 11 % and depreciation of machinery – 44 %. So, the quantum of biomass produced by bioreactor cultivation shows that there is an ample scope for commercial application of the plant cell and organ cultures for the production of secondary metabolites when compared to field cultivation.

24.8 Downstream Processing for Biomass and Ginsenosides

Drying is the most common and fundamental method for post-harvest preservation of medicinal plant raw material because it allows quick conservation of bioactive ingredients. In case of mass production, the use of technical drying is indispensable. Drying technology for ginseng raw material has been developed by Kim et al. [75] and roots were dried by forced air drying at 50 °C for 10 h (Fig. 24.3g). The dried roots possessed 1.5 mg g^{-1} DW protopanaxatriols, 15.9 mg g^{-1} DW protopanaxadiols and 17.4 mg g^{-1} DW total ginsenosides. They also tested the far infrared and freeze drying methods and these methods were inferior to forced air dying method. Therefore, forced air drying method is usually followed for drying the ginseng adventitious roots. Kim et al. [76] developed heat reflux method for extraction of ginsenosides by using fresh; air dried and powdered adventitious roots of ginseng. They tested four extraction variables such as nature and concentration of solvent, extraction temperature (water, 10, 30, 50, 70 and 100 % ethanol) and duration (2, 4, 6 and 8 h) and reported that powdered root material, extraction with 70 % ethanol for 6 h at 80 °C were suitable for extraction of ginsenosides. Ultrasonic and microwave extraction methods were also tested by them and reported that heat reflux extraction and heat reflux extraction was found superior to the ultrasonic and microwave extraction.

24.9 Safety and Toxicological Evaluation

Bio-safety and toxicological evaluation was carried out by Sivakumar et al. [77] using tissue cultured mountain ginseng adventitious roots (TCMGRs) produced jointly by Research center for the development of advanced horticultural technology and CBN biotech company, Cheongju, Republic Korea. They observed that the reverse mutation, chromosomal aberration and micronucleus tests did not show much significant mutagenicity of TCMGRs. Furthermore, 13 weeks of repeated dose toxicity of TCMGR oral doses from 300 to 900 mg kg^{-1} did not show any mortality or significant changes in the general behavior and gross appearance of internal organs of experimental rats and Beagle dogs. Histo-pathological examinations of various organs and heamatological tests revealed no difference between the control and the treated experimental animals. These results confirm the bio-safety of tissue

cultured mountain ginseng adventitious roots and based on such evaluation The Korean Food and Drug Administration (KFDA), ISO (9001/2000) and United State of America Food and Drug Administration have approved (2030950, dt. 06/07/2002) commercial production of ginseng adventitious roots and their products.

24.10 Efficacy Tests of Ginseng Adventitious Roots

Continuous evaluation of therapeutic effects of ginseng adventitious roots is in progress. Recently Park et al. [78] evaluated ginseng adventitious root as a fertility agent and the effect of ginseng adventitious roots on spermatogenesis was studied using male rats. The ginseng adventitious root powder was administered orally to 7-week-old rats over a 6-week period. The number of sperms in the testis and epididymis was significantly higher than the control. There were no significant differences in the weights of the heart, spleen, liver, kidney, brain, testes, and epididymis. They induced oligospermia by administering 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) to the rats in order to estimate the feasibility of using ginseng adventitious root powder as treatment for infertility caused by spermatogenic disorders. After exposing the rats to TCDD, the ginsenoside treated (obtained from ginseng adventitious roots) rats showed improvement in the body weight, sperm number and testis morphology and these results reveal that the therapeutic effect of ginseng adventitious roots on spermatogenic disorders. The anti-platelet activity of ginseng adventitious roots was studied by Jeon et al. [79] and they used 70 % ethanol extract of tissue cultured mountain ginseng adventitious roots (TCMGRs), Korean red ginseng (KRG) and *Panax ginseng* (PG) on agonist-induced platelet aggregation and activation in human whole blood. The IC_{50} values for TCMGRs, KRG and PG were 1.159, 3.695 and 4.978 mg ml^{-1} for collagen-induced aggregation, 0.820, 2.030 and 4.473 mg mL^{-1} for arachidonic acid-induced aggregation, and 1.070, 2.617 and 2.954 mg mL^{-1} for ADP-induced aggregation respectively and these results show that ginseng adventitious roots have potent anti-platelet activity. Lee et al. [80] also showed *in vitro* anti-platelet activity of ethyl acetate extract of ginseng adventitious roots. These reported results demonstrate that ginseng adventitious roots and their products have wide therapeutic potential for blood flow disorders. A recent finding has demonstrated that ginseng adventitious roots aid in the prevention of erectile dysfunction [81, 82] symptoms of hyperlipidemia [83], and has anti-fibrotic and anti-oxidant activity [84, 85]. In addition, ginseng adventitious roots stimulate immune cells and inhibit multiplication of cancer cells [86].

After successful evaluation of bio-safety, toxicological, therapeutic evaluation various ginseng products such as ginseng powder, syrup, tonic, wine, soap and ginseng based cosmetics are available in Korean market (Fig. 24.4). Now ginseng health products have also gained the status of functional food and these products are also promoted by Korean commercial organizations.

Fig. 24.4 Ginseng based product made out of ginseng adventitious roots. (**a**) Ginseng based cosmetics, (**b**) Ginseng wine, (**c**) Ginseng soap, (**d**) Ginseng syrup, (**e**) and (**d**) Ginseng tonic

24.11 Conclusions and Perspectives

The *Panax ginseng* is one of the traditional folk medicinal plants which have been used for many therapeutic purposes in oriental countries. Ginsenosides are active ingredients of ginseng, a group of saponins with triterpenoid dammarane structure (Fig. 24.1). Pharmacological effects of ginseng have been demonstrated in cancer, diabetes mellitus, cardio-vascular system, immune system, and central nervous system including anti-stress and anti-oxidant activity [1]. Cultivation of ginseng takes 5–7 years and a close attention is needed since growth is subjected to several conditions such as soil, climate, pathogens and pests. Therefore, biotechnological means of production of ginsenosides have become research focus, and cell and organ culture techniques have been developed by various groups in past decades. Production of ginsenosides through cell culture is successful, but a high fluctuation of ginsenoside content in ginseng cell cultures has been a big problem for commercialization. Production of ginsenosides through transformed hairy root cultures is possible, however, presence of opine like compounds brought various health concerns [12]. Therefore, ginseng adventitious root culture is looked at as an excellent alternative since the growth is fast, ginsenoside production is stable without potential dangers [13]. Large scale adventitious root culture has been established using bioreactors and various chemical and physical parameters of biomass accumulation have been optimized. Productivity of ginsenosides has been also improved over the years by following elicitation technology. Scale up techniques, processing and extraction of ginsenosides from adventitious root biomass have been also well established. Bio-safety, toxicological evaluations are carried out time to time to make ginseng adventitious root biomass as popular raw material for pharmaceutical and nutraceutical industries.

There is still a room for further enhancement of the productivity of ginsenoside with the ginseng adventitious root cultures. There is a necessity for the development in downstream processing of desired products. Metabolic engineering of secondary metabolites and signal transduction engineering have the potentiality to increase the productivity and to improve the product composition [87, 88] and there is a need for research efforts to be focused on these lines.

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Part V
Bio-safety Assessments of Plant Cell and Organ Culture Products

Chapter 25
Food Ingredients from Plant Cell, Tissue and Organ Cultures: Bio-safety and Efficacy Evaluations

Hosakatte Niranjana Murthy and Kee-Yoeup Paek

Abstract Plants are valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, flavors, fragrances, coloring agents and food additives. Various bioactive compounds are produced these days through plant cell, tissue and organ cultures (PCTOC) and the diverse products which are derived from PCTOC are available in the market as pharmaceuticals and food ingredients. Even though PCTOC products are of *in vitro* origin, they possess many components other than targeted compounds, and sometimes these components may be toxic, thus making the biosafety evaluations necessary for the PCTOC raw materials/products. Currently, well framed biosafety evaluation methods/procedures are not available for PCTOC raw materials/products. In this chapter, we have discussed various methods proposed by scientists and we have put forward a general criterion for evaluation of PCTOC products. We have also discussed two specific examples namely, tissue cultured mountain ginseng adventitious roots (TCMGARs) and tissue cultured *Echinacea purpurea* adventitious roots (TCEPARs) to illustrate the various steps involved in the process of safety evaluation.

Keywords Adventitious roots • Biosafety • Blood chemistry • Echinacea • Ginseng • Hematology • Mutagenicity • Toxicology

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Abbreviations

DSHEA	Dietary Supplement Health and Education Act
FDA	Food and Drug Administration of United States of America
FFDCA	Federal, Food, Drug and Cosmetic Act
FEMA	Expert Panel of the Flavor and Extract Manufacturers Association of United States
GRAS	Generally recognized as safe
KFDA	Korean Food and Drug Administration
PCTOC	Plant cell, tissue and organ culture
TCEPARs	Tissue cultured *Echinacea purpurea* adventitious roots
TCMGARs	Tissue cultured mountain ginseng adventitious roots

25.1 Introduction

Plant cell, tissue and organ cultures (PCTOC) have been used to produce a wide range of phytochemicals, including flavors, colorants, essential oils, sweeteners, antioxidants and nutraceuticals [1, 2]. In recent years, various strategies have been developed for the improvement of biomass and phytochemical production. For the large-scale cultivation of plant cell and organs, bioreactor technologies have also been employed and this book includes few reviews based on these aspects.

Various natural and synthetic phytohormones are used in the culture medium for modification of the morphogenetic events to promote growth of the cell/organs and for accumulation of metabolites. Diverse chemicals are also used as elicitors to boost the production of bioactive compounds. Biomass produced through PCTOC is used as a raw material for procuring food and medicinal ingredients. These ingredients are often extracted from raw materials without going through stringent purification. Raw material produced in PCTOC may contain a mixture of many components including toxic byproducts. This has raised concern among food and pharmaceutical industry about the biosafety and efficacy of PCTOC raw material and ingredients therein. This has prompted considerations from regulatory agencies around the world to look at the safety of PCTOC products. A most common approach followed by many regulatory agencies for the safety evaluation of PCTOC-derived products was based on substantial equivalence, that is, if the food/medicinal ingredients derived from cultures are substantially equivalent to their whole plant counterparts [3]. However, when the PCTOC products are commercialized, biosafety regulations come into force. In this review, we have discussed the precautionary measures to be taken for the initiation of cultures till harvesting of the PCTOC raw materials and subsequent methods of biosafety evaluation of PCTOC raw materials.

25.2 Safety Considerations for Plant Cell and Tissue Culture Processes

Fu [3] elaborated the safety considerations for PCTOC manufacturing process in four main steps namely cell line development, scale up process, production and purification. Several types of cultures have been used for the production of food and pharmaceutical ingredients including cell suspension cultures, transformed shoot cultures and hairy root cultures. In recent years, embryo and adventitious root cultures have also been used for the production of food ingredients [2, 4, 5]. Organ cultures are preferred more as they are comparatively more stable than the cell cultures in terms of genetic stability [2, 6]. Following are the safety measures to be taken during the production of PCTOC products:

1. Selection of suitable plant material – which includes a careful selection of source material, providing information on Latin name (genus, species and authority), common name/s, parts used, chemotype, and geographic origin of the plant material.
2. PCTOC method for production of raw material – which include selection of suitable explants for the induction of callus, cell and organ lines.
3. Maintenance of uniform optimized chemical and physical parameters- which is essential for maintaining the consistency of batch-to-batch production
4. Assessment of genetic stability of cultured cells and organs – which can be carried out preferably through molecular biology techniques such as random amplified polymorphic DNA (RAPD) analysis or any such type of refined techniques
5. Analysis of target phytochemicals- which can be carried out by using refined quantitative techniques such as high pressure liquid chromatography (HPLC), gas chromatography (GC) analysis at least by batch by batch basis.

For biomass production, Large-scale bioreactor cultures are used nowadays and the transfer of cultures from shake flasks to bioreactors results in the reduction of productivity [6, 7]. The decrease or shift in the production can be attributed to different physical conditions such as degree of mixing, shear stress, and gas phase composition. Therefore, all these parameters should be assessed thoroughly for small-scale bioreactors before shifting to large-scale bioreactors. Bioreactor design and selection, and regulation of bioprocess parameters are also equally important for obtaining proper yield and maintaining the quality of the product [8].

Techniques that have been used to increase the product yields during the production stage of a cell or organ culture include changes in medium composition, addition of inducers or precursors, elicitation, and *in situ* product removal. In the bioreactor cultivation of ginseng adventitious roots, methyl jasmonate has been used as an elicitor to obtain five to six fold increment in the accumulation of ginsenosides [2, 9]. Various types of chemical and physical elicitors have been tested and used successfully for increased accumulation of bioactive compounds in PCTOC

[10, 11]. Care should be taken to avoid toxic elicitors such as heavy metals, detergents, xenobiochemicals, fungicides, herbicides and other harmful chemicals to maintain the biosafety of the products. However, the elicitors of biological origin and even safe physical elicitors can also be used for the enhancement in the product accumulation.

In situ product removal techniques have been followed in the second phase of PCTOC cultures with an objective to remove the products from cultured cells and organs. By the application of *in situ* product removal the recovery of product can be made easier and it often leads to increased productivity. For example, Buitelaar et al. [12] studied the effect of an organic phase in cell growth and thiophene production in *Tagetes petula* hairy root cultures. They observed a significant improvement in thiophenes productivity (1–70 %) with the addition of hexadecane to the cultures. However, the composition of the thiophenes inside the cells was different from the secreted patterns. Further, such experiments can even lead to the accumulation of toxic substances in the cultures. Therefore, such protocols can be implemented after in-depth positive experimental evidences. Selection of suitable processing and purification procedures have been also suggested by Fu [3] to avoid the accumulation of toxins or undesirable compounds.

25.3 Basic Guidelines for Bio-safety and Toxicological Evaluation of PCTOC Raw Materials/Products

Various measures/criteria have been suggested in the past for biosafety evaluation of PCTOC end products depending upon their nature. These biosafety evaluation methods differ in different countries depending on the type of products and their use. For example, if the PCTOC end product is a food flavor ingredient, FEMA (Expert Panel of the Flavour and Extract Manufacturers Association of United States) safety model should be applied in United States of America as proposed by Hallagan et al. [18]. The FEMA expert panel applies five specific criteria in its evaluation of flavor ingredients.

1. Exposure to the substance in specific food, the total amount in the diet, the total poundage
2. Natural occurrence of food
3. Chemical identity (including purity and method of preparation) and specific chemical structure
4. Metabolic and pharmacokinetic characteristics
5. Animal toxicity.

Whereas in Europe, Council of Europe (CE) and European Union (EU) regulations will apply for bio-safety evaluation of flavorings produced by PCTOC [13]. It involves *general information* such as precise botanical name in Latin together with the common name of the original plant species and part(s) of the plant used; *process*

used if the PCTOC differs significantly from the control plant; *toxicological information* including *in vitro mutagenicity tests* in bacteria, a test for chromosome damage *in vitro* and a 90-days study in a rodent species.

If PCTOC final product is food and dietary supplement then Food and Drug Administration (FDA) implies Federal, Food, Drug, and Cosmetic Act (FFDCA), and product should be declared generally recognized as safe (GRAS) by qualified experts [14]. If the PCTOC products do not fall under GRAS then Dietary Supplement Health and Education Act (DSHEA) regulations will be applied for the approval. The requirements for the approval of PCTOC products under DSHEA are: (1) Identification of the ingredients and the manufacturing process; (2) intended technical effect and used levels in food and (3) Safety studies data are needed. Regulations for the approval of PCTOC products in Canada and Japan are reviewed by McIntyre [15] and Ushiyama [16] respectively and they vary from the regulations of USA or European Union.

As the regulations of bio-safety approval of PCTOC products vary among different countries; here, general criteria of bio-safety approval for PCTOC products based on assessment of all the existing guidelines are projected. These criteria are prepared based on the guidance tool prepared by Kroes and Walker [17] and biosafety considerations proposed for PCTOC product by Fu [3], Hallagan et al. [18], Gry [13], Beru [14], McIntyre [15] and Ushiyama [16] with some modifications and they are open for comprehensive discussion and adoption.

1. Information on botanical source – such as family, genus, species of source plant, and its relevant variety and chemotype, common name, parts used and geographic origin.
2. Method of production of raw materials through PCTOC –
    (i) Parts used for induction of cells (callus) and organ (adventitious roots/hairy roots/embryos/shoots); cell or organ lines used for *in vitro* culture.
    (ii) The parameters optimized for *in vitro* cultivation such as medium, salt strength, type and concentrations of growth regulators, medium pH, temperature, photoperiod, light intensity and quality.
    (iii) Type of culture vessel (bioreactor) used, agitation, aeration, mode of operation, reactor conditions during the growth and production cycles.
    (iv) Elicitation methodology used; type and concentration of elicitor, time of addition and duration of exposure.
    (v) Type of biomass (cells, adventitious roots, hairy roots, embryos or shoots) or harvesting of bioactive ingredients from the medium, method of harvesting/procuring the ingredients.
3. Method of processing the PCTOC raised raw material – drying methods, processing methods, storage conditions.
4. Bio-safety evaluation/toxicology tests –
    (i) Physical, chemical and biological analysis of raw material or product and its nutritional facts.

(ii) Assessment of *in vitro* evaluation of raw material or product by *in vitro* mutagenicity tests in bacteria (e.g., Ames test) and in mammalian cells (e.g., chromosomal aberration test with Chinese hamster lung cells).
(iii) Assessment of *in vivo* evaluation of raw material or products by animal studies (e.g., study on rodent species, hematology, blood chemistry, absolute/relative organ weight, necropsy examination of organ or by histopathological examination).

5. Efficacy tests such as antioxidant assay, anti-diabetic, anti-cancerous, hepatoprotective tests depending upon the usage of product.
6. Approval from competent authorities for commercialization (e.g., FDA in United States of America; KFDA in Korean Food and Drug Administration in South Korea etc.).
7. The safety assessment of PCTOC raw material or ingredients produced by genetically modified organs such as hairy roots, shooty teratomas need thorough toxicological evaluation based on the recommendations proposed by Hallagan et al. [18] or even more stringent guidelines as proposed by Sims [19].

25.4 Case Studies on Biosafety and Toxicological Evaluation of PCTOC Raw Materials/Products

Herein, the two successful examples of PCTOC raw materials that have been approved by FDA and KFDA for commercialization are discussed. They are Tissue cultured mountain ginseng adventitious roots (*Panax ginseng* C. A. Meyer; abbreviated as TCMGARs) and Tissue cultured *Echinacea purpurea* adventitious roots (*Echinacea purpurea* (L.) Moench.; abbreviated as TCEPARs). The various steps involved in the process of bio-safety evaluation are emphasized in the light of these two examples.

25.4.1 Ginseng

Panax ginseng C. A. Meyer (ginseng), a well-known medicinal plant, has been used as a tonic and medicine in oriental countries. The principal bioactive constituents of ginseng are the ginsenosides, a group of glycosylated triterpenes also known as saponins. The physiological and pharmacological effects of ginsenosides include cardioprotection, immunomodulation, antifatigue and hepato-protection. Wild ginseng is a scarce and rare commodity. Field cultivation of the ginseng plant is a time-consuming and labor-intensive process and it take 5–7 years from seedling to final harvesting stage, during which a close attention is needed since growth is subjected to several conditions such as soil, climate, pathogens and pests. The use of cell and organ culture processes has been sought as a potential alternative for an efficient production of ginseng raw materials. TCMGARs were induced from 100 years old wild mountain

Panax ginseng C. A. Meyer collected from Keum Province in Republic of Korea and TCMGARs biomass was produced in 10,000 L bioreactor cultures over 48 days. TCMGARs contain higher concentrations of gisensosides [20], biophenols [21] and antioxidants [22] when compared to field cultivated ginseng.

Biosafety Evaluation of TCMGARs

The chemical analysis of TCMGARs was carried out by RCH Pharmaceutical and Cosmetic Analytical Laboratories, Rancho Dominguez, California (FDA I.D. Code 2030950) and data is presented in Table 25.1. TCMGARs possessed carbohydrates, proteins, fat, vitamin A, vitamin C, sodium and, calcium and free from heavy metals, pesticides and insecticides. *In vitro* reverse mutation test with *Salmonella typhimurium* and *Escherichia coli* strains revealed that the base-pair substitution type and frame shift mutations are on par with control (Tables 25.2 and 25.3). The chromosomal aberration test using mammalian Chinese hamster lung cells (CHL) did not reveal any abnormalities associated with the TCMGARs powder (at dosages up to 600 μg mL^{-1}; Table 25.4). Micronucleus test using mammalian polychromatic erythrocyte cells did not differ significantly from the control group (Table 25.5). A repeated dose toxicity test of 13-weeks duration of TCMGARs powder (up to 900 mg kg^{-1}; Fig. 25.1) did not cause death of rats, absolute body weight (Table 25.6), urine analysis data (Table 25.7), hematology (Tables 25.8 and 25.9), blood chemistry (Tables 25.10 and 25.11), absolute organ weight (Tables 25.12 and 25.13) and histopathological findings (Tables 25.14 and 25.15) revealed that there were no differences between the control and the treated rats. These results confirm that TCMGARs are safe and nontoxic at an average dietary consumption level.

25.4.2 Echinacea

Echinacea purpurea (L.) Moench (Purple cone flower) is one of the top selling medicinal plants widely used to alleviate colds, sore throats and other upper respiratory infections. Various Echinacea products available in the market and are used to stimulate immune system, and their immunostimulating properties are attributed to the bioactive phytochemicals including caffeic acid derivatives, alkamides, polysaccharides, and glycoproteins. Among these phytochemicals, caffeic acid derivatives, especially cichoric acid possesses many bioactive functions including anti-hyaluronidase activity, protection of collagen from free radical degradation, antiviral activity, inhibition of human immunodeficiency virus type-1 integrase and replication, promoting phagocyte activity *in vitro* and *in vivo* and a high free radical scavenging property [23, 24]. *In vitro* adventitious roots were induced in *Echinacea purpurea* and cultured in large-scale bioreactors to meet the market demand [25, 26]. The amounts of bioactive compounds were higher in TCEPARs when compared to natural plants [25, 26].

Table 25.1 Chemical constituents of ginseng adventitious roots

Parameters	Units
pH	5.62 pH units
Calories	353 Cal/100 g
Calories of fat	5.006 Cal/100 g
Fat	0.54 g/100 g
Saturated fatty acid	56.7 g/100 g fat
Cholesterol	0.54 mg/100 g
Carbohydrates	58.8 g/100 g
Total dietary fiber	28.1 g/100 g
Total sugars	3.61 g/100 g
Moisture	1.19 g/100 g
Total ash	11.7 g/100 g
Protein	27.8 g/100 g
Vitamin A	20.0 IU/100 g
Vitamin C	39.6 mg/100 g
Sodium	107 mg/100 g
Calcium	464 mg/100 g
Iron	11.9 mg/100 g

The above analysis is carried out by US FDA (FDA I.D. 2030950 dated 06/07/2002)

Table 25.2 *In vitro* reverse mutation tests on *Salmonella typhimurium* and *Escherichia coli* without S-9 mix treated with ginseng adventitious roots

Dose (μg/plate)	Number of revertants/plate (mean ± S.D. $n = 3$)				
	Base pair substitution type			Frame shift type	
	TA100	TA1535	WP2*uvr*A$^-$	TA98	TA 1357
0	116 ± 6	9 ± 3	34 ± 4	45 ± 2	8 ± 1
312.5	117 ± 17	10 ± 1	28 ± 3	51 ± 7	6 ± 2
625	120 ± 8	10 ± 2	29 ± 5	49 ± 8	7 ± 1
1,250	107 ± 12	12 ± 2	30 ± 9	50 ± 3	8 ± 2
2,500	123 ± 10	10 ± 1	24 ± 3	48 ± 3	8 ± 1
5,000	106 ± 12	11 ± 2	27 ± 5	42 ± 4	7 ± 1
Positive cont.	489 ± 25	242 ± 10	265 ± 5	417 ± 15	286 ± 14

Strain	**Positive control**	**Concentration** (μg/plate)
TA100	2-aminoanthracane (2-AA)	0.01
TA 1535	2-aminoanthracane (2-AA)	1.0
WP2*uvr*A$^-$	2-aminoanthracane (2-AA)	0.01
TA 98	2-aminoanthracane (2-AA)	0.1
TA 1537	2-aminoanthracane (2-AA)	80

Biosafety Evaluation of TCEPARs

The chemical, biological, physical and toxicological analysis of TCEPARs was carried out by Microbac Laboratories, Inc. California, USA (FDA I. D. 2030513) and TCEPARs contained fat, carbohydrates, proteins, vitamin A, Vitamin C, sodium

Table 25.3 *In vitro* reverse mutation tests on *Salmonella typhimurium* and *Escherichia coli* with S-9 mix treated with ginseng adventitious roots

Dose (μg/plate)	Number of revertants/plate (mean ± S.D. $n = 3$)				
	Base pair substitution type			Frame shift type	
	TA100	TA1535	WP2*uvr*A$^-$	TA98	TA 1357
0	116 ± 6	13 ± 2	34 ± 3	45 ± 2	8 ± 1
312.5	109 ± 10	10 ± 1	36 ± 4	51 ± 7	6 ± 2
625	111 ± 9	13 ± 2	25 ± 6	45 ± 3	9 ± 3
1,250	129 ± 12	9 ± 2	31 ± 0	49 ± 5	6 ± 1
2,500	104 ± 6	9 ± 2	28 ± 6	44 ± 4	7 ± 0
5,000	124 ± 6	12 ± 1	25 ± 5	50 ± 3	9 ± 1
Positive cont.	484 ± 26	246 ± 6	286 ± 11	436 ± 7	282 ± 15
Strain		**Positive control**		**Concentration** (μg plate^{-1})	
TA100		2-aminoflurene (AF-2)		1.0	
TA 1535		2-aminoflurene (AF-2)		1.0	
WP2*uvr*A$^-$		2-aminoflurene (AF-2)		10	
TA 98		2-aminoflurene (AF-2)		0.5	
TA 1537		2-aminoanthracane (2-AA)		2.0	

Table 25.4 Chromosome aberration tests on Chinese Hamster Lung (CHL) cultured cells treated with ginseng adventitious roots

S-9 mix	Test item[a]	Dose (μg/plate)	Chromosome aberration/100 metaphase cells (mean with ± S.D.)
S-9 mix (−) 6 + 18 h	CMC	0	1.5 ± 0.7
	TCMGARs	150	1.5 ± 0.0
		300	1.0 ± 0.4
		600	0.5 ± 0.2
	MMC	0.05	26.5 ± 2.1
S-9 mix (+) 6 + 18 h	CMC	0	1.0 ± 0.0
	TCMGARs	150	1.5 ± 0.7
		300	2.0 ± 0.0
		600	1.0 ± 0.0
	B[a]P	20	28.5 ± 0.7
S-9 mix (−) 24 + 0 h	CMC	0	0.5 ± 0.2
	TCMGARs	150	1.0 ± 0.0
		300	1.5 ± 0.7
		600	1.0 ± 0.0
	MMC	0.05	36.0 ± 2.8
S-9 mix (−) 24 + 0 h	CMC	0	0.0 ± 0.0
	TCMGARs	150	1.0 ± 0.0
		300	1.0 ± 0.4
		600	0.5 ± 0.2
	MMC	0.05	27.2 ± 2.1

[a]*CMC* carboxymethylcellulose sodium salt, *MMC* mitomycin C, *B(a)P* benzo(a)pyrene

Table 25.5 Micronucleus test on male ICR mice treated with ginseng adventitious root powder

Test items	Groups	Dose (mg kg^{-1})	Route	Animal number	Sampling time (h)	PCE/ (PCE + NCE)[a] (Mean ± S.D.)	MNPCE[a]/ 1,000PCE (Mean ± S.D.)
Saline	G1	0	PO	6	48	0.483 ± 0.021	0.83 ± 0.41
TCMGARs	G2	500	PO	6	48	0.492 ± 0.016	0.67 ± 0.26
	G3	1000	PO	6	48	0.485 ± 0.019	0.75 ± 0.42
	G4	2,000	PO	6	48	0.495 ± 0.014	0.92 ± 0.38
MMC	G5	2	IP	6	24	0.493 ± 0.006	84.50 ± 6.86[b]

[a]*MNPCE* micronucleated polychromatic erythrocyte, *PCE* polychromaticerythrocyte, *NCE* non-chromatic erythrocyte, *MMC* mitomycin C
[b]Significance $P < 0.01$ by Chi-square test

Table 25.6 Changes in body weight of rats after a 13-week repeated dose toxicity with a 4-week recovery period after ginseng adventitious root powder treatments

TCMGARs powder treatments (mg kg^{-1})		Body weight (g)			Weight gain at week 13
		Day 0	Weak 13	Weak 17	
Male	Control	177.6 ± 4.6	470.7 ± 4.7	468.7 ± 2.4	293.1 ± 4.1
	300	176.2 ± 6.2	436.8 ± 4.9	–	260.6 ± 5.0
	600	174.7 ± 4.2	446.9 ± 4.2	–	272.2 ± 4.2
	900	180.3 ± 3.1	454.7 ± 3.6	454.1 ± 4.2	274.2 ± 3.9
Female	Control	138.4 ± 5.5	267.9 ± 2.9	258.1 ± 3.0	129.5 ± 3.1
	300	141.5 ± 5.1	269.8 ± 2.5	–	128.3 ± 3.4
	600	139.9 ± 3.1	277.8 ± 2.1	–	137.9 ± 3.1
	900	140.0 ± 5.8	276.7 ± 2.3	268.2 ± 2.0	136.7 ± 3.1

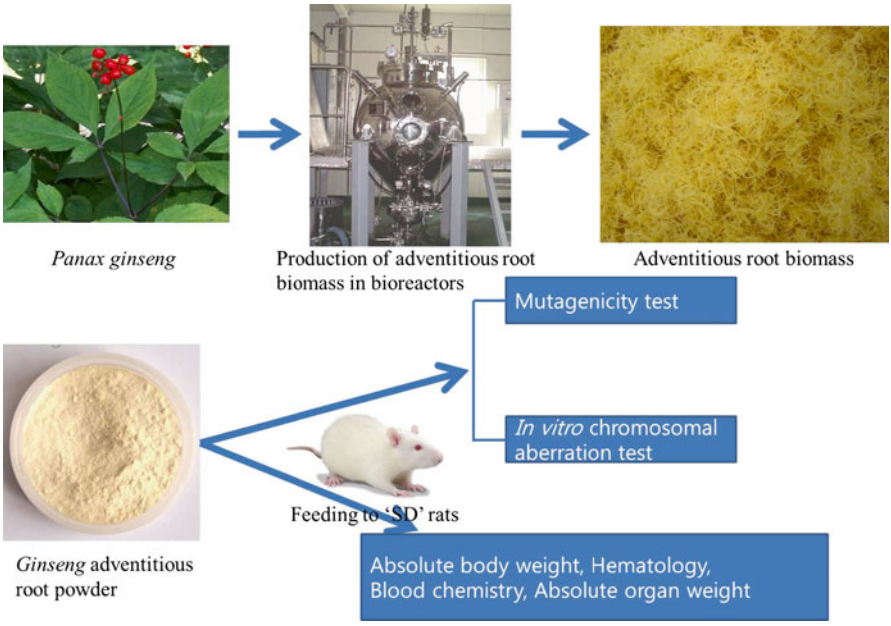

Fig. 25.1 Toxicological evaluation of ginseng adventitious root extract on Spargue Dawley rats

Table 25.7 Urine analysis of a 4-week recovery period for male and female rats

Sex		Male		Female	
Dose (mg kg^{-1})		0	900	0	900
Number of animals		6	6	6	6
Weight (g)		4.6 ± 1.5	4.8 ± 1.8	3.0 ± 1.2	3.1 ± 1.3
Colour	Yellow	6	6	6	6
	Dark yellow				
	Light yellow				
	Light orange				
	Dark orange				
Specific gravity	1,000				1
	1,005		2		
	1,010	2	3		
	1,015	1	1	1	
	1,020	2		2	
	1,025	1		2	
	1,030			1	
pH	5			1	1
	6	1		3	2
	7	2	3	1	2
	8	2	2	1	
	9	1	1		
Leukocyte (Leuko mL^{-1})	0	4	5	6	6
	10–25				
	75	2	1		
	500				
Nitrate	–	6	6	6	6
	+				
Protein (mg dL^{-1})	0	1	2	4	5
	30	4	4	2	1
	100	1			
	500				
Glucose (mg dL^{-1})	0	6	6	6	6
	50				
	100				
	300				
	1,000				
Ketone	0	6	6	6	6
	5				
	15				
	40				
	80				
Urobilinogen (mg dL^{-1})	0	6	6	6	6
	1				
	4				
	8				
	14				

(continued)

Table 25.7 (continued)

Sex		Male		Female	
Bilirubin	0	6	6	6	6
	+				
	++				
	+++				
Blood (Ery mL^{-1})	0	6	6	6	6
	5–10				
	50				
	250				
Hemoglobin (Ery mL^{-1})	0	6	6	6	6
	10				
	50				
	250				

Values are expressed as Mean ± S.D.

and calcium (Table 25.16). However, TCEPARs were devoid of microbes, heavy metals, pesticides and other harmful chemicals. Mutagenicity and toxicological analysis was carried out by Biotoxtech, South Korea, *in vitro* reverse mutations tests were carried out using *Salmonella typhimurium* and *Escherichia coli* strains and the results showed TCEPARs are safe and are not mutagenic (Tables 25.17 and 25.18). *In vitro* chromosomal mutation tests using CHL cells treated with TCEPARs also showed that the samples are safe (Table 25.19). Four-week toxicological studies of TCEPARs on Spargue Dawley rats revealed that TCEPARs were not toxic (Fig. 25.2) and experimental rats showed normal food consumption (Table 25.20), gain in body weight over the period of treatment (Table 25.21). Hematological parameters (Table 25.22), blood chemistry (Table 25.23), gain in absolute weight and relative organ weights (Tables 25.24 and 25.25), necropsy observations with respect to abdominal cavity, adrenal, brain, cranial cavity, oesophagus, heart, intestine, kidney, liver, lung, lymph nodes, mammary glands, ovary, pancreas, pituitary, prostrate, salivary gland, seminal vesicle, skin, spleen, stomach, testis, thoracic cavity, thymus, thyroid, trachea, urinary bladder, uterus and vagina (Table 25.26) showed non-toxicity of TCEPARs.

25.5 Efficacy of PCTOC Products

Efficacy tests were carried out with TCMGARs by many workers and TCMGAR extract aid the prevention of spermatogenic disorder [27], erectile dysfunction [28], inhibition of platelet aggregation in human blood [29, 33], treatment of hyperlipidemia [30]. TCMGARs extract exhibited anti-fibrotic activity [31], anti-oxidant activity [30, 32], augmentation of peripheral blood flow [33] and inhibition of L-DOPA oxide activity of tyrosinase (skin whitening activity). In addition,

Table 25.8 Haemotology (main group summary)

Group/dose (mg kg^{-1})		RBC ($\times 10^6$ µL^{-1})	Hb (g µL^{-1})	Hct (%)	Retics (%)	RBC index			WBC ($\times 10^3$ µL^{-1})	PLT ($\times 10^3$ µL^{-1})	WBC differential counting (%)						Coag. tests	
						MCV (fl)	MCH (pg)	MCHC (g µL^{-1})			Neutrophil		Lymphocyte	Monocyte	Eosinophil	Basophil	PT	APTT
											Seg.	Band						
Male																		
GI	Mean	8.36	15.4	45.5	1.1	54.4	18.4	33.8	5.7	926	24.5	0.0	72.2	1.7	1.6	0.0	16.0	26.3
0	S.D.	0.41	0.5	1.8	0.4	2.5	0.7	0.8	1.7	71	9.4	0.0	9.2	0.8	1.5	0.0	1.4	3.4
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G2	Mean	8.52	15.5	45.3	0.9	53.3	18.2	34.2	5.6	990	28.0	0.0	69.5	1.8	0.7	0.0	16.2	25.9
300	S.D.	0.49	1.0	2.8	0.2	1.9	0.7	0.7	1.7	97	6.1	0.0	6.7	1.0	0.8	0.0	1.3	3.9
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G3	Mean	8.39	15.3	44.6	0.8	53.3	18.2	34.2	4.0	912	23.1	0.0	74.2	1.3	1.4	0.0	14.8	26.4
600	S.D.	0.26	0.5	1.3	0.2	2.1	0.7	0.7	1.6	300	9.4	0.0	9.7	1.2	1.6	0.0	1.7	5.4
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G4	Mean	8.25	15.0	44.0	1.0	53.4	18.2	34.1	5.0	914	25.5	0.0	71.6	1.7	1.2	0.0	16.6	28.1
900	S.D.	0.33	0.7	1.6	0.2	1.5	0.6	0.5	1.8	240	10.3	0.0	11.2	1.6	1.1	0.0	1.9	4.9
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Female																		
GI	Mean	7.72	14.9	44.8	1.3	58.2	19.3	33.2	3.1	945.2	25.9	0.0	71.7	0.8	1.6	0.0	16.0	31.2
0	S.D.	0.50	0.9	1.9	0.4	1.9	0.6	0.9	1.2	71.2	9.3	0.0	9.2	0.8	1.5	0.0	0.8	6.1
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G2	Mean	8.52	15.5	45.3	0.9	53.3	18.2	34.2	5.6	989.6	28.0	0.0	69.5	1.8	0.7	0.0	16.2	25.9
300	S.D.	0.49	1.0	2.8	0.2	1.9	0.7	0.7	1.7	97.4	6.1	0.0	6.7	1.0	0.8	0.0	1.3	3.9
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G3	Mean	7.60	14.7	43.7	1.2	57.5	19.3	33.6	2.5	937.2	28.0	7.3	63.2	1.0	1.5	0.0	16.6	31.2
600	S.D.	0.30	0.6	1.7	0.4	1.3	0.4	0.6	1.0	85.0	9.7	23.1	23.7	1.2	1.6	0.0	1.0	5.9
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G4	Mean	7.78	14.8	44.6	1.6	57.2	19.2	33.3	2.9	1037.9	21.0	0.0	76.0	1.2	1.9	0.0	15.9	30.7
900	S.D.	0.38	0.7	2.0	0.3	1.4	0.7	0.6	0.9	85.7	11.3	0.0	11.4	0.9	2.7	0.0	0.9	6.4
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10

Table 25.9 Haematology (recovery group summary)

Group/dose (mg kg^{-1})		RBC ($\times 10^6$ μL^{-1})	Hb (g μL^{-1})	Hct (%)	Retics (%)	RBC index			WBC ($\times 10^3$ μL^{-1})	PLT ($\times 10^3$ μL^{-1})	WBC differential counting (%)						Coag. tests	
						MCV (fl)	MCH (pg)	MCHC (g dL^{-1})			Neutrophil		Lymphocyte	Monocyte	Eosinophil	Basophil	PT	APTT
											Seg.	Band						
Male																		
GI	Mean	7.64	13.7	40.3	2.1	52.7	18.0	34.1	3.9	873	18.8	0.0	79.2	1.8	0.2	0.0	17.1	27.5
0	S.D.	0.84	1.4	4.2	0.9	2.2	0.8	0.2	1.7	122	7.1	0.0	7.0	1.0	0.4	0.0	1.6	3.6
	N	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
G4	Mean	8.05	14.6	42.6	2.2	53.8	18.1	34.3	4.5	881	20.5	0.0	75.8	3.2	0.5	0.0	16.4	27.9
900	S.D.	0.30	0.7	2.2	0.4	1.2	0.5	0.2	2.4	95	8.7	0.0	8.2	2.5	0.5	0.0	1.4	3.4
	N	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Female																		
GI	Mean	8.08	15.2	46.2	2.3	57.2	18.8	32.9	3.8	794	33.7	0.0	63.8	1.8	0.7	0.0	15.6	20.5
0	S.D.	0.63	1.7	5.3	0.7	3.1	0.9	0.6	0.8	116	18.1	0.0	18.3	0.8	0.5	0.0	0.5	2.3
	N	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
G4	Mean	8.52	15.8	47.7	2.9	55.7	18.6	33.3	2.9	827	3.2	0.0	64.8	2.3	0.8	0.0	15.6	19.5
900	S.D.	1.51	2.7	9.0	1.2	0.8	0.4	0.7	0.8	141	8.9	0.0	9.7	1.8	0.8	0.0	0.3	0.7
	N	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6

Table 25.10 Blood chemistry (main group summary)

Group/ dose (mg kg^{-1})		ALT (UL^{-1})	AST (UL^{-1})	ALP (UL^{-1})	CK (UL^{-1})	Glu (mg dL^{-1})	BUN (mg dL^{-1})	Crea (mg dL^{-1})	T-Bili (mg dL^{-1})	T-Chol (mg dL^{-1})	TG (mg dL^{-1})	TP (g L^{-1})	Alb (g L^{-1})	A/G ratio	γ-GT (UL^{-1})	P (mg dL^{-1})	LDH (UL^{-1})	Ca (mg dL^{-1})	Na	K	Cl
																			(mmol L^{-1})		
Male																					
G1	Mean	37.7	218.0	85.5	2022.1	89.5	16.4	0.4	0.13	69.9	57.0	6.1	2.8	0.8	2.2	6.7	3181.0	8.0	145.0	4.7	106.1
0	S.D.	9.4	18.4	15.7	511.4	18.7	3.8	0.1	0.05	9.4	26.0	0.3	0.3	0.2	1.3	1.4	294.5	1.1	1.2	0.4	1.8
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G2	Mean	43.0	209.5	85.5	1723.5	84.2	16.1	0.4	0.12	70.0	44.1	6.3	2.9	0.9	2.1	6.5	3022.2	7.9	145.1	4.7	105.6
300	S.D.	15.7	31.7	10.8	519.0	18.8	4.3	0.1	0.04	29.7	14.3	0.2	0.4	0.2	0.8	0.5	611.4	0.7	1.4	0.3	1.9
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G3	Mean	39.6	212.2	89.1	1949.1	96.2	16.2	0.4	0.1	75.1	49.8	6.2	3.0	0.9	3.1	7.0	3170.8	8.3	145.2	4.9	106.2
600	S.D.	9.0	36.2	21.8	400.8	18.5	4.4	0.1	0.0	11.1	17.6	0.2	0.5	0.3	2.3	1.1	607.8	0.6	1.1	0.4	0.8
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G4	Mean	48.2	228.1	89.8	2002.5	93.1	13.8	0.4	0.12	74.0	44.7	6.0	3.1	1.0	2.8	6.8	3278.0	8.0	145.3	4.8	105.7
900	S.D.	17.6	31.1	13.6	289.9	21.7	2.9	0.1	0.04	17.4	10.4	0.3	0.4	0.2	0.8	1.0	374.5	0.6	1.4	0.4	0.8
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Female																					
G1	Mean	32.6	219.4	64.0	1866.5	60.8	20.6	0.6	0.13	86.0	27.7	6.4	3.2	1.0	3.0	6.2	2947.6	7.9	145.1	4.2	105.4
0	S.D.	12.8	54.5	14.4	474.0	13.4	2.5	0.1	0.05	12.5	11.3	0.3	0.3	0.2	1.5	1.4	549.7	2.7	0.9	0.4	0.8
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G2	Mean	24.4	214.1	52.2	2050.2	55.9	19.8	0.6	0.14	91.1	25.8	6.4	3.1	1.0	2.6	6.0	3165.0	8.0	145.6	4.3	104.9
300	S.D.	2.4	32.6	10.5	367.2	14.5	2.4	0.1	0.05	17.2	8.9	0.2	0.2	0.1	2.0	1.5	472.6	2.0	1.5	0.4	0.9
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G3	Mean	28.4	274.2	62.4	2234.0	69.6	22.1	0.6	0.13	81.8	27.6	6.3	3.3	1.1	2.2	6.0	3329.5	7.9	146.0	4.1	105.7
600	S.D.	3.4	28.2	17.2	395.2	13.8	4.4	0.1	0.05	17.7	10.7	0.3	0.2	0.2	1.6	1.7	440.9	2.1	1.4	0.4	1.5

(continued)

Table 25.10 (continued)

Group/ dose (mg kg^{-1})		ALT (UL^{-1})	AST (UL^{-1})	ALP (UL^{-1})	CK (UL^{-1})	Glu (mg dL^{-1})	BUN (mg dL^{-1})	Crea (mg dL^{-1})	T-Bili (mg dL^{-1})	T-Chol (mg dL^{-1})	TG (mg dL^{-1})	TP (g L^{-1})	Alb (g L^{-1})	A/G ratio	γ-GT (UL^{-1})	P (mg dL^{-1})	LDH (UL^{-1})	Ca (mg dL^{-1})	Na	K	Cl
																			(mmol L^{-1})		
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G4	Mean	31.6	217.7	65.4	2159.8	62.5	20.6	0.6	0.15	90.6	27.2	6.4	3.3	1.1	2.6	6.5	2995.4	7.4	145.3	4.2	104.5
900	S.D.	6.6	37.5	8.0	552.2	17.4	1.5	0.1	0.06	14.7	13.0	0.3	0.3	0.2	1.9	1.9	663.3	2.0	0.8	0.3	1.4
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10

N number of animals

Table 25.11 Blood chemistry (recovery group summary)

Group/ dose (mg kg^{-1})		ALT (U L^{-1})	AST (U L^{-1})	ALP (U L^{-1})	CK (U L^{-1})	Glu (mg dL^{-1})	BUN (mg dL^{-1})	Crea (mg dL^{-1})	T-Bili (mg dL^{-1})	T-Chol (mg dL^{-1})	TG (mg dL^{-1})	TP (g L^{-1})	Alb (g L^{-1})	A/G ratio	γ-GT (U L^{-1})	P (mg dL^{-1})	LDH (U L^{-1})	Ca (mg dL^{-1})	Na	K	Cl
																			(mmol L^{-1})		
Male																					
G1	Mean	45.9	135.7	82.8	855.0	141.2	17.3	0.6	0.17	85.0	45.5	6.7	3.2	0.9	2.6	4.6	1769.8	8.8	154.8	5.0	112.2
0	S.D.	21.9	26.4	11.5	288.4	14.2	1.0	0.1	0.02	8.4	19.9	0.2	0.1	0.1	1.5	0.8	479.4	0.3	22.7	0.3	11.0
	N	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
G4	Mean	31.6	137.3	87.5	1425.3	128.5	18.4	0.6	0.16	99.3	66.2	6.8	3.3	0.9	2.3	4.6	2128.8	8.5	154.7	5.1	111.0
900	S.D.	6.3	43.0	16.0	958.6	21.1	2.4	0.1	0.01	18.9	17.6	0.1	0.2	0.1	0.9	1.2	887.6	0.6	21.7	0.6	9.6
	N	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Female																					
G1	Mean	23.4	156.5	49.8	1234.3	100.3	16.6	0.7	0.17	93.0	30.0	7.1	3.5	1.0	2.4	4.3	1791.2	8.8	155.7	4.9	112.3
0	S.D.	8.3	51.1	7.1	561.7	15.3	2.4	0.0	0.04	10.9	9.4	0.5	0.3	0.1	0.6	1.4	847.2	0.7	9.9	0.5	4.8
	N	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
G4	Mean	19.4	151.6	57.8	1501.2	107.7	17.9	0.8	0.16	111.0	30.7	6.9	3.4	1.0	2.5	3.6	2110.5	9.0	154.5	4.7	113.8
900	S.D.	4.1	27.6	11.9	725.3	23.2	3.2	0.1	0.04	18.8	10.2	0.3	0.1	0.1	1.7	0.6	845.3	0.4	13.5	0.4	11.4
	N	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6

N number of animals

Table 25.12 Absolute organ weights (main group summary)

Male

Group/dose (mg kg^{-1})		B.W. (g)	Liver (g)	Kidney		Spleen (g)	Adrenal gland		Testis		Brain (g)	Pituitary (mg)	Lung (g)	Heart (g)	Thymus (g)	Thyroid		Epididymis		Prostate (g)
				Lt. (g)	Rt. (g)		Lt. (mg)	Rt. (mg)	Lt. (g)	Rt. (g)						Lt. (mg)	Rt. (mg)	Lt. (g)	Rt. (g)	
G1	Mean	460.1	11.69	1.44	1.47	0.90	22.2	22.3	1.70	1.69	2.13	15.8	1.92	1.40	0.36	8.9	10.0	0.63	0.61	0.70
0	S.D.	48.2	1.64	0.12	0.14	0.15	7.1	6.6	0.14	0.11	0.07	3.1	0.12	0.13	0.11	2.2	1.7	0.04	0.04	0.14
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G4	Mean	412.0	10.13	1.32	1.33	0.80	24.6	23.0	1.64	1.66	2.04	14.5	1.81	1.31	0.29	10.0	9.4	0.59	0.61	0.60
300	S.D.	46.2	1.02	0.09	0.10	0.10	4.1	4.1	0.12	0.12	0.08	1.5	0.34	0.13	0.12	1.6	1.5	0.04	0.05	0.12
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G3	Mean	420.0	10.51	1.31	1.34	0.77	22.2	21.8	1.65	1.66	2.07	15.0	1.78	1.31	0.35	7.9	9.1	0.60	0.60	0.63
600	S.D.	38.4	1.32	0.15	0.17	0.13	3.6	2.7	0.10	0.08	2.3	0.33	0.13	0.05	0.05	1.3	1.5	0.04	0.05	0.12
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G4	Mean	432.6	11.20	1.41	1.42	0.84	23.0	22.8	1.71	1.70	2.09	14.7	1.69	1.36	0.31	9.1	8.9	0.63	0.62	0.64
900	S.D.	24.7	1.46	0.18	0.20	0.11	4.3	3.6	0.11	0.07	0.13	2.2	0.13	0.10	0.05	1.2	1.6	0.05	0.05	0.13
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10

Female

Group/dose (mg kg^{-1})		B.W. (g)	Liver (g)	Kidney		Spleen (g)	Adrenal gland		Ovary		Brain (g)	Pituitary (mg)	Lung (g)	Heart (g)	Thymus (g)	Thyroid		Uterus (g)
				Lt. (g)	Rt. (g)		Lt. (mg)	Rt. (mg)	Lt. (g)	Rt. (g)						Lt. (mg)	Rt. (mg)	
G1	Mean	263.9	66.72	0.84	0.84	0.65	31.9	31.2	47.8	47.7	1.92	17.7	1.54	0.94	0.29	7.0	7.3	0.58
0	S.D.	29.7	0.69	0.09	0.08	0.07	7.5	5.2	12.0	11.9	0.07	2.2	0.39	0.05	0.04	1.4	1.9	0.23
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G4	Mean	254.3	6.67	0.84	0.85	0.64	29.6	30.0	45.4	49.5	1.92	16.8	1.55	0.88	0.26	7.7	8.1	0.52
300	S.D.	24.8	0.65	0.06	0.05	0.09	4.1	4.6	8.0	10.6	0.11	2.7	0.46	0.07	0.09	0.9	1.5	0.22
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G3	Mean	262.0	6.42	0.80	0.82	0.60	30.7	29.6	46.2	44.0	1.97	16.0	1.30	0.90	0.27	6.9	7.8	0.66
600	S.D.	20.7	0.68	0.07	0.09	0.06	3.8	6.9	7.4	7.5	0.06	2.7	0.07	0.07	0.08	0.7	2.3	0.16
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
G4	Mean	269.8	6.88	0.87	0.88	0.64	31.2	34.0	44.4	42.9	1.96	18.2	1.45	0.93	0.27	7.2	7.9	0.56
900	S.D.	18.8	0.60	0.06	0.08	0.05	5.6	7.1	7.6	9.3	0.10	2.7	0.40	0.09	0.08	0.9	0.7	0.21
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10

Significant differences as compared with control $p<0.05$

Table 25.13 Absolute organ weights (recovery group summary)

Male

Group/dose (mg kg^{-1})		B.W. (g)	Liver (g)	Kidney		Spleen (g)	Adrenal gland		Testis		Brain (g)	Pituitary (mg)	Lung (g)	Heart (g)	Thymus (g)	Thyroid		Epididymis		Prostate (g)
				Lt. (g)	Rt. (g)		Lt. (mg)	Rt. (mg)	Lt. (g)	Rt. (g)						Lt. (mg)	Rt. (mg)	Lt. (g)	Rt. (g)	
G1	Mean	449.0	10.89	1.37	1.41	0.74	19.0	20.2	1.74	1.71	2.09	16.0	1.62	1.34	0.25	7.2	8.4	0.62	0.61	0.61
0	S.D.	25.1	0.90	0.10	0.10	0.07	0.07	1.7	2.3	0.19	0.04	2.2	0.11	0.07	0.05	1.7	0.6	0.06	0.07	0.14
	N	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
G4	Mean	432.7	10.41	1.27	1.27	0.76	19.1	18.8	1.68	1.70	2.02	15.6	1.64	1.32	0.22	7.6	8.6	0.65	0.65	0.56
900	S.D.	39.6	1.24	0.14	0.11	0.09	2.9	1.4	0.08	0.11	0.06	1.7	0.14	0.08	0.08	1.5	0.9	0.03	0.05	0.15
	N	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6

Female

Group/dose (mg kg^{-1})		B.W. (g)	Liver (g)	Kidney		Spleen (g)	Adrenal gland		Ovary		Brain (g)	Pituitary (mg)	Lung (g)	Heart (g)	Thymus (g)	Thyroid		Uterus (g)
				Lt. (g)	Rt. (g)		Lt. (mg)	Rt. (mg)	Lt. (g)	Rt. (g)						Lt. (mg)	Rt. mg)	
G1	Mean	224.2	6.04	0.71	0.72	0.51	25.8	26.5	37.5	39.4	1.91	17.0	1.42	0.86	0.19	6.2	6.4	0.78
0	S.D.	28.4	0.53	0.04	0.06	0.07	1.8	1.2	7.3	8.8	0.10	3.6	0.35	0.04	0.05	1.1	1.1	0.38
	N	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
G4	Mean	252.3	6.39	0.76	0.78	0.54	26.5	27.8	38.4	37.9	1.89	16.7	1.20	0.85	0.23	7.4	7.5	0.95
900	S.D.	19.0	0.35	0.06	0.07	0.04	4.1	2.6	7.3	10.4	0.04	1.9	0.14	0.05	0.06	1.1	1.1	0.26
	N	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6

Significant differences as compared with control $p < 0.05$

Table 25.14 Histopathological findings on male and female rats treated with ginseng adventitious roots

Sex		Male		Female	
Dose (mg kg^{-1})		0	900	0	900
Number of animals		10	10	10	10
Organs	Findings				
Liver	Microgranuloma, mild		1		1
	Vacuolization of hepatocytes	2			
Kidney	Cell infiltration, cortex, focal, mild				
	Right	1		1	1
	Left		1		
	Simple tubule hyperplasia of proximal tubule		1		
	Right		1		
	Left				
	Proteinaceous cast, medulla			1	
	Right				1
	Left	1	1	1	
	Bilateral	1	1		
	Hyaline droplet in proximal tubule				
	Left	1			
Lung	Inflammation, mild				
	Bronchi, alveolar septa, left	1	1	1	1
Spleen	Hemosiderin pigmentation				
Small intestine	Congestion in lamina propria, mild				
	Duodenum, jejunum, ileum	1	1		
	Jejunum		1		
Seminal vesicle	Atrophy of epithelium, left	1	1		

The rats were treated for a period of 13 weeks with repeated toxicity doses

TCMGARs extract also showed stimulation of immune cells and inhibition of cancer cell proliferation. All these reports suggest the efficacy of TCMGARs as reported for natural ginseng roots [34]. Based on various bio-safety and efficacy evaluations CBN biotech, South Korea was first to obtain KFDA approval for marketing the TCMGARs and various products such as ginseng syrup, ginseng tablets, ginseng soap, ginseng liquor and many more products in the market.

25.6 Conclusion and Perspectives

Plants are valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, flavors, fragrances, coloring agents and food additives. Herbal industry could not able to meet the increased market demand due to insufficiency of natural resources, varying seasonality, low amount of bioactive components in the available resources. Plant cell, tissue and organ cultures have become alternative for

Table 25.15 Histopathological findings on male and female rats after 4-weeks recovery period

Sex		Male		Female	
Dose (mg kg^{-1})		0	900	0	900
Number of animals		10	10	10	10
Organs	Findings				
Liver	Microgranuloma, mild		1	1	
	Vacuolization of hepatocytes		1	1	
Kidney	Cell infiltration, cortex, focal, mild				
	Right				
	Left				
	Simple tubule hyperplasia of proximal tubule				
	Right				
	Left				
	Proteinaceous cast, medulla				
	Right				
	Left				1
	Bilateral	1	1		
	Hyaline droplet in proximal tubule				
	Left				
Lung	Inflammation, mild				
	Bronchi, alveolar septa, left	1		1	
Spleen	Hemosiderin pigmentation				
Small intestine	Congestion in lamina propria, mild				
	Duodenum, jejunum, ileum				
	Jejunum				
Seminal vesicle	Atrophy of epithelium, left				

Table 25.16 Chemical analysis of tissue cultured *Echinacea purpurea* adventitious roots[a]

Parameters	Results
Calories	345 cal/100 g
Fat	13 cal/100 g
Saturated fatty acid	0.71 g/100 g
Cholesterol	<1 mg/100 g
Carbohydrates	57.9 g/100 g
Total dietary fiber	28.1 g/100 g
Total sugars	4.87 g/100 g
Moisture	7.03 g/100 g
Total ash	8.45 g/100 g
Protein	25.2 g/100 g
Vitamin A	<76 IU/100 g
Vitamin C	8.43 mg/100 g
Sodium	7.84 mg/100 g
Calcium	186 mg/100 g
Iron	ND[b]

[a]Analysis was carried out by US FDA laboratory under the I. D. 2030513 dated 11/04/2007
[b]Not detected

Table 25.17 *In vitro* reverse mutation tests on *S. typhimurium* and *E. coli* without S-9 mix treated with tissue cultured *Echinacea purpurea* adventitious roots powder

	Number of revertants/plate (Mean ± S.D., $n = 3$)				
	Base-pair substitution type			Frame-shift type	
Dose (μg/plate)	TA100	TA1535	WP2*uvr*A (pKM101)	TA98	TA1537
0	109 ± 12	19 ± 3	108 ± 8	27 ± 7	10 ± 4
312.5	106 ± 6	18 ± 4	113 ± 11	28 ± 9	9 ± 1
625	99 ± 7	15 ± 3	118 ± 10	24 ± 4	12 ± 1
1,250	122 ± 7	10 ± 1	120 ± 4	24 ± 2	10 ± 2
2,500	131 ± 14	12 ± 3	116 ± 6	23 ± 1	11 ± 5
5,000	128 ± 18	16 ± 3	116 ± 6	26 ± 7	8 ± 0
Positive control	411 ± 18	410 ± 17	423 ± 16	531 ± 46	447 ± 9

Strain	**Positive control**	**Concentration (μg/plate)**
TA100	Sodium azide (SA)	1.5
TA1535	Sodium azide (SA)	1.5
WP*uvr*A (Pkm101)	4-Nitroquinoline 1-oxide (4-NAO)	5.0
TA98	2-Nitrofluorene (2-NF)	5.0
TA1537	9-Aminoacridine (9-AA)	80.0

Table 25.18 *In vitro* reverse mutation test of tissue cultured *Echinacea purpurea* adventitious roots powder treated *S. typhimurium* and *E. coli* with S-9 mix

	Number of revertants/plate (Mean ± S.D., $n = 3$)				
	Base-pair substitution type			Frame-shift type	
Dose (μg/plate)	TA100	TA1535	WP2*uvr*A (pKM101)	TA98	TA1537
0	122 ± 17	16 ± 2	107 ± 4	26 ± 9	12 ± 1
312.5	118 ± 13	12 ± 3	106 ± 6	27 ± 3	12 ± 4
625	126 ± 12	14 ± 1	101 ± 3	28 ± 5	13 ± 3
1,250	118 ± 5	13 ± 2	113 ± 17	28 ± 6	11 ± 3
2,500	123 ± 8	16 ± 4	114 ± 5	21 ± 9	12 ± 4
5,000	111 ± 12	13 ± 2	107 ± 4	27 ± 3	14 ± 2
Positive control	473 ± 12	183 ± 18	527 ± 20	422 ± 25	123 ± 11

Strain	**Positive control**	**Concentration (μg/plate)**
TA100	2-Aminoanthracane (2-AA)	1.0
TA1535	2-Aminoanthracane (2-AA)	2.0
WP*uvr*A (Pkm101)	2-Aminoanthracane (2-AA)	2.0
TA98	2-Aminoanthracane (2-AA)	1.0
TA1537	2-Aminoanthracane (2-AA)	2.0

Table 25.19 *In vitro* chromosome aberration test in CHL/IU cells treated with tissue cultured *Echinacea purpurea* adventitious roots powder

S9 mix/ time	Test item[a]	Dose (μg mL^{-1})	No. of cell scored	Percentage of cells involved in chromosomal aberrations	Chromosome aberration cells/100 metaphase cells (%) (Mean ± S.D.)
S9 mix (−)/6 + 18 h	Water for injection	0	100	1	1.5 ± 0.7
			100	2	
	Tissue cultured *E. purpurea* adventitious roots extract	275	100	2	1.0 ± 0.4
			100	0	
		550	100	0	2.0 ± 0.8
			100	4	
		1,100	100	2	1.0 ± 0.4
			100	0	
	MMC	0.05	100	22	24.5 ± 3.5
			100	27	
S9 mix (+)/6 + 18 h	Water for injection	0	100	2	2.5 ± 0.7
			100	3	
	Tissue cultured *E. purpurea* adventitious roots extract	275	100	1	1.5 ± 0.7
			100	2	
		550	100	3	3.5 ± 0.7
			100	4	
		1,100	100	0	1.0 ± 0.4
			100	2	
	B[a]P	20	100	24	24.5 ± 0.7
			100	25	
S9 mix (−)/24 + 0 h	Water for injection	0	100	0	1.0 ± 0.4
			100	2	
	Tissue cultured *E. purpurea* adventitious roots extract	275	100	0	1.5 ± 1.1
			100	3	
		550	100	0	0.5 ± 0.7
			100	1	
		1,100	100	3	1.5 ± 1.1
			100	0	
	MMC	0.05	100	27	26.5 ± 0.7
			100	26	

[a]*MMC* mictomycin C, *B[a]P* benzo[a]pyrene

the production of various high value secondary metabolites. Biomass produced through PCTOC is used as a raw material for procuring food and medicinal ingredients. These ingredients are often extracted from raw materials without going through stringent purification. Food and pharmaceutical ingredients derived from PCTOC

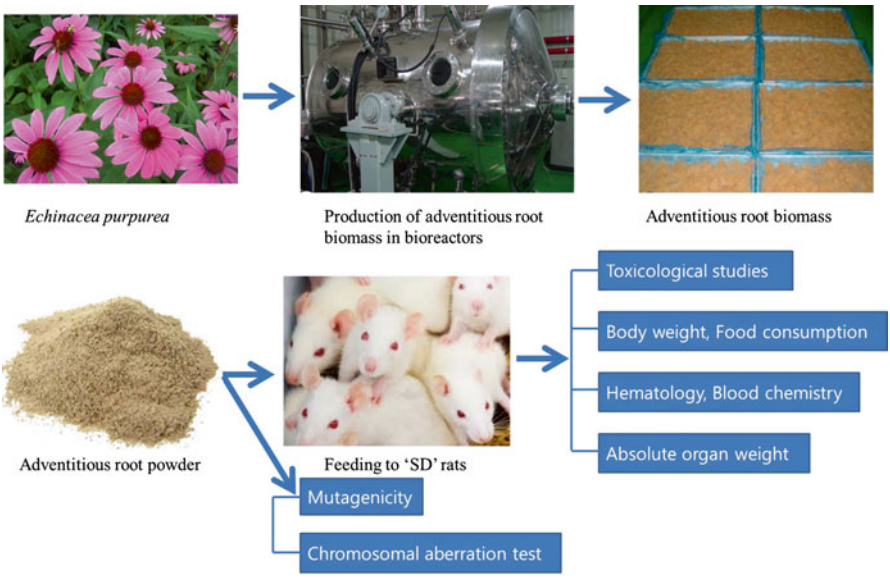

Fig. 25.2 Toxicological evaluation of *Echinacea purpurea* adventitious root extract on Spargue Dawley rats

needs to be evaluated for their bio-safety and depending on the type of product and its use, products of PCTOC fall under number of biosafety schemes [3, 13–16, 18]. Since, common criteria/regulations were not available; we have framed basic guidelines for bio-safety and toxicological evaluation of PCTOC products based on the schemes proposed by Fu [3], Hallagan et al. [18], Gry [13], Beru [14], McIntyre [15], Ushiyama [16].

Even though, several PCTOC experimental trials are carried out for the last three to four decades, there are a very few favorable outcomes of the production of PCTOC raw material/product on commercial scale. Our research institute is involved in research on Ginseng and Echinacea and has developed large-scale plant bioreactors (10,000 L) for the production of Ginseng and Echinacea adventitious roots. The bio-safety of TCMGARs and TCEPARs through *in vitro* mutagenicity tests using bacteria and mammalian system has been assessed by us [20] and even evaluation of TCMGARs and TCEPARs through repeated dose toxicity test on rodents was carried out to prove their bio-safety. Efficacy tests of TCMGARs have been conducted and based on such bio-safety and efficacy tests the KFDA clearance has been gained for marketing of TCMGARs.

Table 25.20 Toxicological studies of tissue cultured adventitious roots of *Echinacea purpurea*: food consumption of 4-week dose range studies in Sprague Dawley rats

		Food consumption in g/animal/day				
		Week				
Group/dose (mg kg^{-1})		0	1	2	3	4
Male						
G1 0	Mean	23.90	27.15	29.00	28.45	28.39
	S.D.	1.60	2.54	4.14	3.31	2.38
	N	5	5	5	5	5
G2 500	Mean	22.84	25.77	27.00	27.31	27.08
	S.D.	1.90	2.95	0.85	3.07	0.69
	N	5	5	5	5	5
G3 1000	Mean	23.97	28.22	29.34	29.31	29.73
	S.D.	2.68	3.43	3.67	3.18	3.53
	N	5	5	5	5	5
G4 2000	Mean	23.32	27.02	28.91	28.96	27.52
	S.D.	1.64	2.43	1.15	3.61	2.91
	N	5	5	5	5	5
Female						
G1 0	Mean	17.07	18.68	19.88	18.75	21.44
	S.D.	1.53	1.26	1.36	3.68	2.15
	N	5	5	5	5	5
G2 500	Mean	16.86	18.28	21.60	21.66	20.72
	S.D.	0.92	1.30	2.29	1.74	3.37
	N	5	5	5	5	5
G3 1000	Mean	17.57	18.56	18.77	18.09	22.23
	S.D.	2.32	0.88	1.87	2.43	1.34
	N	5	5	5	5	5
G4 2000	Mean	18.77	19.55	20.91	21.53	21.18
	S.D.	0.89	0.67	2.35	1.63	1.43
	N	5	5	5	5	5

Table 25.21 Toxicological studies of tissue cultured adventitious roots of *Echinacea purpurea*: changes in body weight of 4-week dose range studies in Sprague Dawley rats with repeated oral dose toxicity study

Group/dose (mg kg^{-1})		Body weight (g) Days of treatment 0	3	7	10	14	17	21	24	27
Male										
G1 0	Mean	188.0	213.8	245.6	267.7	295.2	315.2	337.9	354.6	370.4
	S.D.	9.7	11.8	14.2	20.1	24.5	26.4	32.8	34.7	35.9
	N	5	5	5	5	5	5	5	5	5
G2 500	Mean	187.3	214.4	248.7	271.2	297.3	314.9	336.9	355.3	370.2
	S.D.	9.4	12.1	16.0	18.4	20.6	21.1	23.9	27.0	28.5
	N	5	5	5	5	5	5	5	5	5
G3 1000	Mean	189.4	218.9	255.8	281.3	311.6	334.1	357.6	376.1	394.0
	S.D.	9.8	11.4	15.0	18.9	24.2	25.6	32.4	36.0	36.1
	N	5	5	5	5	5	5	5	5	5
G4 2000	Mean	189.1	217.0	250.5	274.5	302.5	322.6	343.8	359.5	375.4
	S.D.	6.5	8.8	11.6	14.7	16.6	20.3	19.8	21.6	23.4
	N	5	5	5	5	5	5	5	5	5
Female										
G1 0	Mean	147.5	160.0	172.4	184.4	197.4	204.5	213.5	227.0	235.2
	S.D.	7.3	6.8	4.7	4.0	4.1	2.4	3.2	1.2	3.6
	N	5	5	5	5	5	5	5	5	5
G2 500	Mean	146.5	160.6	177.2	192.1	205.2	214.2	224.5	228.8	239.9
	S.D.	9.5	14.3	12.0	10.7	11.6	12.7	13.5	11.2	17.8
	N	5	5	5	5	5	5	5	5	5
G3 1000	Mean	148.2	156.0	172.9	182.1	194.9	201.8	209.8	221.5	228.6
	S.D.	11.1	9.6	9.6	11.0	10.9	11.1	13.3	12.3	14.4
	N	5	5	5	5	5	5	5	5	5
G4 2000	Mean	151.3	162.4	179.1	189.7	201.1	217.3	228.4	237.1	245.7
	S.D.	10.9	11.5	9.5	8.4	10.9	13.9	15.2	13.6	15.7
	N	5	5	5	5	5	5	5	5	5

Table 25.22 Toxicological studies of tissue cultured adventitious roots of *Echinacea purpurea* on Sprague Dawley rats: hematological parameters[a]

Group/dose (mg kg^{-1})		RBC ($\times 10^6$ cells μL^{-1})	HGB (g dL^{-1})	HCT (%)	MCV (f l)	MCH (pg)	MCHC (g dL^{-1})	WBC ($\times 10^3$ cells μL^{-1})	PLT ($\times 10^3$ cells μL^{-1})
Male									
G1 0	Mean	7.70	15.9	44.5	57.7	20.5	35.7	8.56	1,247
	S.D.	0.03	0.5	1.3	1.7	0.7	0.3	1.89	154
	N	5	5	5	5	5	5	5	5
G2 500	Mean	7.88	15.8	44.1	55.9	20.0	35.9	7.53	1,216
	S.D.	0.22	0.4	0.4	1.7	0.6	0.5	1.41	143
	N	5	5	5	5	5	5	5	5
G3 1000	Mean	7.67	15.4	43.3	56.6	20.2	35.7	7.96	1,238
	S.D.	0.54	0.6	1.6	2.3	0.7	0.3	1.64	113
	N	5	5	5	5	5	5	5	5
G4 2000	Mean	7.83	16.2	45.0	57.6	20.7	35.9	7.73	1,240
	S.D.	0.29	0.4	1.2	1.8	0.7	0.2	1.76	105
	N	5	5	5	5	5	5	5	5
Female									
G1 0	Mean	7.68	15.4	42.8	55.8	20.0	35.9	4.53	1,270
	S.D.	0.21	0.4	1.1	0.9	0.4	0.6	0.76	28
	N	5	5	5	5	5	5	5	5
G2 500	Mean	7.82	15.6	43.5	55.8	20.0	35.8	6.31	1,166
	S.D.	0.42	0.5	1.2	1.9	0.6	0.2	1.63	93
	N	5	5	5	5	5	5	5	5
G3 1000	Mean	7.58	15.5	42.5	56.7	20.5	36.5	5.69	1,300
	S.D.	0.32	0.3	0.9	1.5	0.6	0.4	2.05	56
	N	5	5	5	5	5	5	5	5
G4 2000	Mean	7.75	15.4	42.6	55.0	19.9	36.3	6.05	1,332
	S.D.	0.35	0.5	1.4	1.8	0.9	0.6	1.47	106
	N	5	5	5	5	5	5	5	5

[a]Findings of 4-week dose range test

Table 25.23 Toxicological studies of tissue cultured adventitious roots of *Echinacea purpurea* on Sprague Dawley rats: blood chemistry[a]

Group/dose (mg kg^{-1})		ALT (U L^{-1})	AST (U L^{-1})	ALP (U L^{-1})	Glu (mg dL^{-1})	BUN (mg dL^{-1})	Crea (mg dL^{-1})	T-Chol (mg dL^{-1})	TP (g dL^{-1})	Alb (g dL^{-1})	A/G ratio
Male											
G1 0	Mean	32.8	130.5	425.6	118.47	13.58	0.39	81.47	5.9	2.5	0.75
	S.D.	3.7	33.6	42.8	15.26	1.34	0.11	9.27	0.3	0.1	0.08
	N	5	5	5	5	5	5	5	5	5	5
G2 500	Mean	29.4	130.1	428.1	111.64	12.77	0.46	61.81	5.8	2.4	0.70
	S.D.	3.0	35.6	71.3	10.31	1.29	0.08	11.87	0.1	0.0	0.04
	N	5	5	5	5	5	5	5	5	5	5
G3 1000	Mean	30.0	154.5	400.2	115.08	13.15	0.41	79.41	6.0	2.5	0.70
	S.D.	4.9	13.4	51.2	8.02	0.59	0.03	13.58	0.1	0.0	0.03
	N	5	5	5	5	5	5	5	5	5	5
G4 2000	Mean	28.5	165.9	448.3	114.7	14.18	0.47	68.79	5.9	2.4	0.69
	S.D.	3.4	18.2	70.8	4.25	1.39	0.13	15.94	0.1	0.1	0.03
	N	5	5	5	5	5	5	5	5	5	5
Female											
G1 0	Mean	27.3	146.3	281.7	107.94	15.72	0.57	77.78	6.5	2.9	0.82
	S.D.	3.1	15.3	42.3	9.09	2.78	0.03	11.24	0.3	0.2	0.07
	N	5	5	5	5	5	5	5	5	5	5
G2 500	Mean	25.3	134.9	260.2	111.27	15.21	0.44	103.27	6.6	3.0	0.83
	S.D.	3.0	38.5	33.7	14.82	2.22	0.10	0.10	0.4	0.2	0.06
	N	5	5	5	5	5	5	5	5	5	5
G3 1000	Mean	26.4	131.3	303.1	112.48	15.73	0.47	81.15	6.2	2.7	0.79
	S.D.	2.2	33.2	88.9	8.98	1.71	0.07	11.24	0.3	0.2	0.07
	N	5	5	5	5	5	5	5	5	5	5
G4 2000	Mean	26.9	131.8	276.4	104.27	17.04	0.52	71.83	6.3	2.9	0.85
	S.D.	3.0	45.6	43.1	9.34	3.62	0.14	21.50	0.3	0.1	0.09
	N	5	5	5	5	5	5	5	5	5	5

[a]Findings of 4-week dose range test

Table 25.24 Toxicological studies of tissue cultured adventitious roots of *Echinacea purpurea* on Sprague Dawley rats: absolute organ weight[a]

		Organ weight (g)						
							Kidney	
Group/dose (mg kg^{-1})		B.W.	Brain	Heart	Liver	Spleen	Lt	Rt
Male								
G1 0	Mean	344.86	1.81	1.17	9.79	0.69	1.16	1.18
	S.D.	31.93	0.06	0.05	1.25	0.10	0.09	0.09
	N	5	5	5	5	5	5	5
G2 500	Mean	344.38	1.89	1.14	9.31	0.74	1.20	1.22
	S.D.	29.07	0.11	0.09	0.66	0.10	0.11	0.06
	N	5	5	5	5	5	5	5
G3 1000	Mean	363.24	1.89	1.18	10.76	0.72	1.31	1.29
	S.D.	38.86	0.02	0.06	1.64	0.04	0.17	0.14
	N	5	5	5	5	5	5	5
G4 2000	Mean	350.02	1.85	1.11	10.27	0.69	1.27	125
	S.D.	22.85	0.07	0.07	1.04	0.07	0.08	0.14
	N	5	5	5	5	5	5	5
Female								
G1 0	Mean	215.63	1.75	0.82	5.88	0.41	0.75	0.76
	S.D.	1.83	0.10	0.06	0.06	0.18	0.04	0.03
	N	5	5	5	5	5	5	5
G2 500	Mean	222.82	1.80	0.83	6.69	0.46	0.80	0.83
	S.D.	12.83	0.11	0.05	0.75	0.08	0.07	0.06
	N	5	5	5	5	5	5	5
G3 1000	Mean	213.36	1.77	0.79	6.02	0.47	0.78	0.80
	S.D.	12.04	0.12	0.05	0.36	0.05	0.09	0.07
	N	5	5	5	5	5	5	5
G4 2000	Mean	228.17	1.77	0.82	6.58	0.49	0.81	0.83
	S.D.	14.57	0.09	0.02	0.60	0.04	0.08	0.09
	N	5	5	5	5	5	5	5

[a]Findings of 4-week dose range test

Table 25.25 Toxicological studies of tissue cultured adventitious roots of *Echinacea purpurea* on Sprague Dawley rats: relative organ weights[a]

		g/100 g body weight						
							Kidney	
Group/dose (mg kg^{-1})		B.W.	Brain	Heart	Liver	Spleen	Lt.	Rt
Male								
G1 0	Mean	344.86	0.53	0.34	2.83	0.20	0.34	0.34
	S.D.	31.93	0.04	0.02	0.12	0.02	0.03	0.03
	N	5	5	5	5	5	5	5
G2 500	Mean	344.38	0.55	0.33	2.71	0.22	0.35	0.36
	S.D.	29.07	0.04	0.01	0.19	0.01	0.04	0.03
	N	5	5	5	5	5	5	5
G3 1000	Mean	363.24	0.52	0.33	2.95	0.20	0.36	0.36
	S.D.	38.86	0.05	0.03	0.19	0.02	0.03	0.02
	N	5	5	5	5	5	5	5
G4 2000	Mean	350.02	0.53	0.32	2.93	0.20	0.36	0.36
	S.D.	22.85	0.02	0.02	0.19	0.02	0.02	0.02
	N	5	5	5	5	5	5	5
Female								
G1 0	Mean	215.63	0.81	0.38	2.73	0.19	0.35	0.35
	S.D.	1.83	0.05	0.03	0.09	0.02	0.02	0.01
	N	5	5	5	5	5	5	5
G2 500	Mean	222.82	0.81	0.37	3.00	0.21	0.36	0.37
	S.D.	12.83	0.06	0.02	0.21	0.03	0.02	0.02
	N	5	5	5	5	5	5	5
G3 1000	Mean	213.36	0.83	0.37	2.82	0.22	0.36	0.37
	S.D.	12.04	0.05	0.03	0.11	0.02	0.03	0.02
	N	5	5	5	5	5	5	5
G4 2000	Mean	228.17	0.78	0.36	2.88	0.21	0.36	0.37
	S.D.	14.57	0.03	0.02	0.18	0.01	0.02	0.03
	N	5	5	5	5	5	5	5

[a]Findings of 4-week dose range test

Table 25.26 Toxicological studies of tissue cultured adventitious roots of *Echinacea purpurea*: necropsy findings of 4-week dose range toxicity study in Sprague Dawley rats (group summary)

	Sex	Male		Female	
	Group	G1	G2	G1	G2
	Dose (mg kg^{-1})	0	5,000	0	5,000
Organs	No. of animals examined	5	5	5	5
Abdominal cavity	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Adrenal	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Brain	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Cranial cavity	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Esophagus	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Epididymis	Number examined	5	5		
	No remarkable results	5	5		
	Remarkable variants	0	0		
External findings	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Heart	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Intestine	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Kidney	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Liver	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Lung	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Lymphnode, mediastinal	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0

Table 25.26 (continued)

	Sex	Male		Female	
	Group	G1	G2	G1	G2
	Dose (mg kg^{-1})	0	5,000	0	5,000
Organs	No. of animals examined	5	5	5	5
Lymphnode, mesenteric	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Lymphnode, submandibular	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Mammary gland	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Ovary	Number examined		5	5	
	No remarkable results		5	5	
	Remarkable variants		0	0	
Pancreas	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Pitutary	Number examined	5	5		
	No remarkable results	5	5		
	Remarkable variants	0	0		
Prostrate	Number examined	5	5		
	No remarkable results	5	5		
	Remarkable variants	0	0		
Salivary gland, submandibular	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Seminal vesicle	Number examined	5	5		
	No remarkable results	5	5		
	Remarkable variants	0	0		
Skin	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Spleen	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Stomach	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Testis	Number examined	5	5		
	No remarkable results	5	5		
	Remarkable variants	0	0		

(continued)

Table 25.26 (continued)

	Sex	Male		Female	
	Group	G1	G2	G1	G2
	Dose (mg kg^{-1})	0	5,000	0	5,000
Organs	No. of animals examined	5	5	5	5
Thoracic cavity	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Thymus	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Thyroid	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Trachea	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Urinary bladder	Number examined	5	5	5	5
	No remarkable results	5	5	5	5
	Remarkable variants	0	0	0	0
Uterus	Number examined		5	5	
	No remarkable results		5	5	
	Remarkable variants		0	0	
Vagina	Number examined		5	5	
	No remarkable results		5	5	
	Remarkable variants		0	0	

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Part VI
Physiological Disorders in Plants Cultured in Bioreactors

Chapter 26
Biochemical and Physiological Aspects of Hyperhydricity in Liquid Culture System

Yaser Hassan Dewir, Yuvraj Indoliya, Debasis Chakrabarty, and Kee-Yoeup Paek

Abstract Large-scale liquid cultures and automation have proven the potential to resolve manual handling of *in vitro* cultures at various stages and decreases production cost. However, hyperhydricity is a major problem during *in vitro* culture of many crops in liquid culture systems. The environment inside culture vessel normally used in plant micropropagation is characterized by high humidity, limited gaseous exchange between the internal atmosphere of the culture vessel and its surrounding environment, and the accumulation of ethylene, conditions that may induce physiological disorders. Hyperhydricity is a disorder of tissue-cultured plants where leaves become translucent and stems swollen, distorted and brittle. Although numerous hypotheses have been put forward to explain hyperhydricity but there is still a lack of knowledge about the nature of signals responsible for hyperhydricity and the metabolic processes which are affected by its development. The concept of stress in relation to hyperhydricity is not completely established. Therefore, it remains difficult to assume that hyperhydric tissues are stressed. Previous studies argued that abnormal morphology observed in hyperhydricity could be attributed to changes occurring at cellular level due to the modifications of membrane composition or DNA content. In order to understand stress and morphological responses in hyperhydric tissues, in the present article, we are reviewing different biochemical and physiological mechanisms of hyperhydricity in several plant species.

Keywords Bioreactor • Hyperhydricity • Reactive oxygen species • Tissue culture

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Abbreviations

ABA	Abscisic acid
APX	Ascorbate peroxidase (EC 1.11.1.11)
BTBB	Bottom-type bubble bioreactor
CAT	Catalase
DHAR	Dehydroascorbate reductase (EC 1.8.5.1)
GA3	Gibberellic acid
GR	Glutathione reductase (EC 1.6.4.2)
HS	Hyperhydric shoot
LOX	Lipoxygenase (EC 1.13.11.12)
MDA	Malondialdehyde
MDHAR	Monodehydroascorbate reductase (EC 1.6.5.4)
PEG	Polyethylene glycol
PMEs	Pectin methylesterases
POX	Peroxidase (EC 1.11.1.7)
ROS	Reactive oxygen species
SOD	Superoxide dismutase (EC 1.15.1.1)

26.1 Introduction

Liquid culture systems offer many potential advantages over solid cultures like faster growth rates, rapid uptake of nutrients by tissues, dilution of exuded growth inhibitors i.e., phenolics released by explants thus minimizing negative effect on growth [1–4]. About ten times increase in shoot number of *Acacia nilotica* in liquid culture compare with gelled culture was achieved [5]. Automated bioreactors for large-scale production of micropropagated plants are important for the micropropagation industry [6]. Bioreactors are self-contained, sterile environments that capitalize on liquid nutrient or liquid/air inflow and outflow systems, designed for intensive culture and control over micro-environmental conditions such as aeration, agitation, dissolved oxygen, etc. [6] (Fig. 26.1a). Liquid culture systems using bioreactors have proven their potential for large-scale micropropagation in many plant species i.e., oriental lily, chrysanthemum, apple, *Euphorbia millii, Spathiphyllum cannifolium* potato and strawberry by Lian et al. [7]; Chakrabarty et al. [8]; Dewir et al. [9, 10]; Piao et al. [11], and Debnath [12], respectively. However, hyperhydricity is a major problem during *in vitro* culture of many crops in liquid culture systems. It affects shoot multiplication, growth and development impeding the successful transfer of micropropagated plants to *in vivo* conditions. Losses up to 30 % have been reported in strawberry bioreactor culture [12].

Hyperhydricity is a disorder of tissue-cultured plants where leaves become translucent and stems swollen, distorted and brittle [13]. The environment inside culture vessel normally used in plant micropropagation is characterized by high

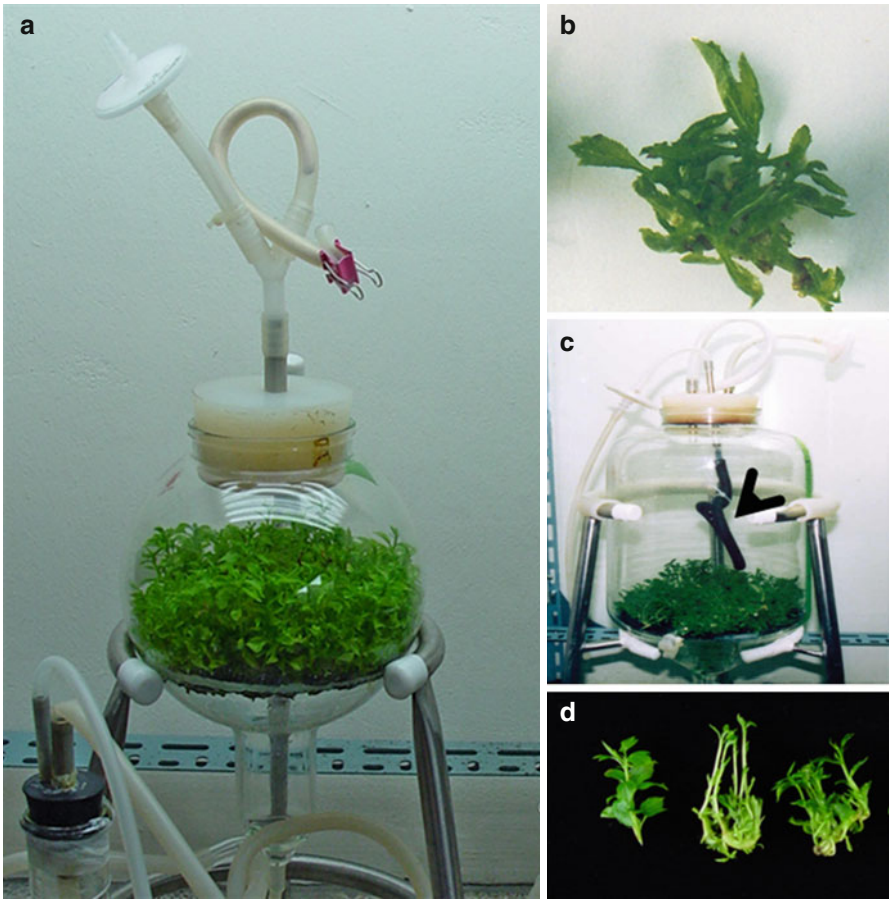

Fig. 26.1 (**a**) Apple plantlets in an immersion air-lift BTBB after 30 days of culture; (**b**) hyperhydric shoots of apple cultured in an immersion air-lift BTBB; (**c**) apple plantlets in an ebb and flood air-lift column type bioreactor after 30 days of culture with forced aeration (*arrow*); (**d**) *in vitro* propagated normal apple shoots cultured in an ebb and flood air-lift column type bioreactor after 30 days of culture with forced aeration

relative humidity, limited gaseous exchange between the internal atmosphere of the culture vessels and its surrounding environment, and the accumulation of ethylene, conditions that may induce physiological disorders. Although numerous hypotheses have been put forward to explain hyperhydricity, but there is still a lack of knowledge about the nature of signals and metabolic processes responsible for hyperhydricity. The concept of stress in relation to hyperhydricity is not completely established. Therefore, it remains difficult to assure that hyperhydric shoots (HS) are stressed. Previous studies argued that abnormal morphology observed in hyperhydricity could be attributed to changes occurring at cellular level due to the modifications of membrane composition or DNA content. In the present article, we

are reviewing different biochemical and physiological mechanisms of hyperhydricity in several plant species.

26.2 Morphological, Anatomical and Ultrastructural Features

Leaves of hyperhydric shoots are thick, translucent, wrinkled and/or curled and brittle. The low dry weight percentage of the hyperhydric leaves indicate that these leaves have chlorophyll deficiency and contain more water as compared to normal leaves. The excessive water accumulation in plant tissue, the most characteristic symptom of hyperhydricity, can generate aeration stress, which depletes oxygen levels in the cells. The anatomy of hyperhydric leaves is also altered, several types of abnormal structures have been defined, such as a reduced number of palisade cell layers, irregular stomata, chloroplast degeneration and the presence of a thin or no cuticle [13–20]. In hyperhydric apple leaves, the pattern of epidermal layer is irregular with several abnormal and malformed structures. Stomata from normal leaves have kidney shaped guard cells, the cell wall bordering the stomatal pore is thickened, well defined and form ridges with a protruding elliptical pore. The stomata from hyperhydric shoots are somewhat abnormal; they are widely open and elevated. We have also observed abnormal stomata in hyperhydric apple leaves that showed irregular guard cells that are more frequently elongated than round and resulted from a deformed cell plate during the division of primary stomata mother cells [13].

Large starch grains are also visible in the guard cells of hyperhydric leaves. It has been reported that the guard cells of hyperhydric plants were larger in size than those of normal plants due to a greater water absorption leading to turgidity and probably to changes in cell-wall structure [21]. The cell wall bordering the stomata pore in guard cells from hyperhydric leaves protruded and appeared to be torn in several places, resulting in some cases in guard-cell deformation. These deformations may result from structural changes in the guard cells followed by changes in cell-wall composition. Similar results were reported by Miguens et al. [22] and Olmos and Hellin [16] for hyperhydric *Datura insignis* and carnation plants, respectively. They also reported that the stomata density was significantly greater in normal than in hyperhydric leaves. The stomata in hyperhydric leaves do not close in response to different signals such as darkness, ABA or Ca^{++} due to modifications in wall elasticity of the guard cells [23].

Anatomical studies demonstrated that hyperhydric leaves only have an unorganized spongy mesophyll as previously described by several workers. Entire ultrastructure of hyperhydric leaf cells of apple is altered with extremely poor, sparse and disorganized cytoplasm. The chloroplasts of hyperhydric plants are very few in number and contain large starch grains. Sometimes one starch grain occupies the whole plastid. Most of the chloroplasts have hypertrophied stroma and the intergranal sacs undergo abnormal compression and expansion so that, frequently, the

whole structure of chloroplasts is deteriorated. In severe hyperhydric leaf, chloroplasts and other subcellular organelles largely disappeared from the cell [13]. Similar observation was reported by Olmos and Hellin [16] for hyperhydric carnation plants. In the hyperhydric pepper plants, the chloroplasts exhibited thylakoid disorganization, low grana number, an accumulation of large starch grains and a low accumulation or absence of plastoglobules. Although the structure of mitochondria and peroxisomes did not change in hyperhydric plants, the number of peroxisomes did increase.

Likewise, several anatomical features were observed in hyperhydric organs such as hypertrophy of cortical and pith parenchyma, enlargement of intercellular spaces, decreased lignification of vascular system [24] and a reduced and/or abnormal vascular system [25]. Picoli et al. [19] have found that the vascular system in hyperhydric plants is reduced. However, it is still unknown whether the vascular system in hyperhydric leaves is also reduced in its function. Decreased lignification has been attributed to the lessening of enzyme activities, as reported for *Prunus avium* [26] and oregano [27].

Saher et al. [28] reported that the total pectins of hyperhydric leaves of three carnation varieties were significantly reduced in comparison with controls. Pectins represent about 35 % of the dry weight of dicot cell walls and are highly heterogeneous group of polymers that includes homogalacturonans and ramnogalactorunans I and II. They contribute both to cell adhesion, *via* their gel-like properties, and to cell wall architecture or cell wall mechanical strength. The degree of esterification of pectins is generally thought to be regulated by the activity of cell wall pectin methylesterases (PMEs). Those group of researcher also reported that the PME activity of hyperhydric leaves was higher (4–10 times) compared to control and suggested that the different PME activities could regulate some of the structural changes related to hyperhydricity in micropropagated carnation plants.

26.3 Physiological State of Hyperhydric Plants

To our knowledge, there is lack of information concerning the physiological state of hyperhydric plants. The morphological abnormalities of hyperhydric plants have shown to be concomitant to their biochemical and physiological characteristics.

26.3.1 Chlorophyll Content

Chlorophyll (a, b) and carotenoid contents were significantly lower in hyperhydric leaves, which may be due to a reduction in the number of chloroplasts in leaves of hyperhydric apple shoots [13]. It was previously reported that oxidative stress induced a reduction of the number of chloroplasts [29], and ultrastructural analysis of hyperhydric cells also showed that chloroplasts are affected with thylakoids.

26.3.2 Photosynthetic and Energetic Capacities

A slight drop of photochemical process yield in hyperhydric shoots suggested that a decrease of pigment content and not a dysfunction of the photosynthetic apparatus were responsible for the lower photosynthetic capacity observed in hyperhydric *Prunus* shoots [30]. However, we measured chlorophyll fluorescence to determine if there is damage to light reaction systems in photosynthetic machinery during hyperhydricity in apple. The inferior intensity of chlorophyll fluorescence transient observed in hyperhydric shoots, suggesting a substantial collapse of photosynthesis in hyperhydric shoots [13]. Reductions of quantum yield of electron transfer (ΦPSII) paralleling to a decrease F_v/F_m in hyperhydric leaves were likely associated with a down-regulation of PSII during steady-state photosynthesis. This suggests additional irreversible damage, perhaps due to a loss of integrity of the thylakoid membrane as observed in TEM photomicrograph [13]. Greater accumulation of non-QB-reducing centers in the hyperhydric leaves inevitably leads to an increase in the fraction of reducing state of QA, thus resulting in a lower photochemical quenching factor (qP) as observed during photoinhibition. This increased fraction of reducing state of QA suggests that these plants were subjected to a higher pressure of excess excitation energy, which could potentially increase the probability of generating reactive radicals which can damage membrane components of PSII.

Franck et al. [30] also reported that there is a general decrease of reduced and oxidized pyridine nucleotides was in hyperhydric shoots as compared to normal shoots but the ratios of the pyridine nucleotides were not altered. These researchers consider that hyperhydric tissues exhibit a typically stress-induced change of physiological state. According to them, the metabolism of hyperhydric tissues can be considered as a temporary state of lower differentiation or a juvenile state with a sufficient activity for their survival and protection. Other major sources of NADPH and NADH are, respectively, oxidative pentose phosphate (OPP) and glycolysis. In hyperhydric shoots, the low activity of some enzymes involved in glycolysis (hexokinase, hexose phosphate isomerase, glycerol-3- phosphate dehydrogenase, phosphofructokinase)andOPP(6-phosphogluconatedehydrogenase,glucose-6-phosphate dehydrogenase) suggests a general decrease of activity of these pathways.

26.3.3 Abnormal DNA Content

An abnormal DNA content was systematically associated with severe hyperhydricity symptoms in grass pea (*Lathyrus sativus*), which hampered the regeneration of rooted, fertile plants [31]. They also suggested that the hyperhydric responses observed were more strongly linked to the presence of auxin in the medium and the hormonal balance between auxins and cytokinins than to the cytokinin level and auxins already have been reported to induce cytogenetic modifications in tissue

cultures of grain legumes [32] and could be responsible for the abnormal DNA content observed in hyperhydric tissues.

26.3.4 Cell Wall Properties and Composition

Cell wall properties and composition can be considered one of the most important factors controlling the development of the anomalous morphology in hyperhydric tissues. Less lignin, associated with low lignin biosynthesis and decreased lignification of the vascular system, has been frequently considered one of the possible causes of hyperhydricity [33]. Different workers have shown modifications in the cell wall constituents mainly cellulose and lignin [28, 34–36] and their mechanical properties [34, 37]. Hypolignification in vascular tissues as consequence of a reduction in cellulose and lignin biosynthesis can alter the mechanical properties of the cell wall. These could lead to reduced cell turgor pressure, changes in the water potential, increased water uptake and as a final result to hyperhydration of tissues.

26.3.5 Polyamine Levels

The changes in polyamine levels and patterns were studied in response to hyperhydric carnation plants *in vitro* [38]. Hyperhydric carnation leaves showed high POD activity, high MDA content and low lignification suggesting oxidative damage. The most predominant fraction of polyamine corresponded to free polymine in hyperhydric leaves as well as in non-hyperhydric leaves. Regarding individual amines, hyperhydricity brought about an almost complex depletion of free 1, 3-diaminopropane, a rise in conjugated form of the amine and a great reduction in bound spermidine in relation to non-hyperhydric leaves. A high percentage up to 80 % of reverted shoots was obtained by lowering the relative humidity inside the culture jars of hyperhydric carnation shoots through bottom cooling. Reversion of hyperhydricity was associated with changes in polyamine patterns. Thus, compared with both non-hyperhydric and hyperhydric leaves, reverted plants showed a drastic reduction in free polyamine, and a major increase in conjugated diamines (especially important in the case of cadaverine, Cad). The polyamine profile in non-hyperhydric and hyperhydric plants could indicate stress condition and a more suitable physiological situation in reverted plants.

26.3.6 Hypoxia Stress and Lipid Peroxidation

In vitro plants are exposed to different stressing conditions. Among them are injuries caused during explantation, high osmoticity of the culture medium (high sucrose and ammonium contents), high relative humidity and gas accumulation in the

atmosphere of the jar, and high levels of growth regulators [39–41]. It was suggested that there may be two kinds of hyperhydricity, one resulting from the passive diffusion of water into tissues, the other from an active phenomenon relating to a disturbance in metabolic processes [42]. Different workers have previously observed that hyperhydric tissues accumulate water in the apoplast creating a water layer around the cells. The excessive water accumulation in plant tissue, the most characteristic symptom of hyperhydricity, can generate aeration stress which depletes oxygen levels and limit its diffusion in the cells [13, 20, 43–49]. It has been observed that hypoxia can induce the generation of H_2O_2 to toxic levels [50]. Therefore, it has been proposed that hyperhydric tissues can be under a hypoxia stress [36, 51]. This hypoxia stress could affect drastically the metabolism of the shoot, affecting energy availability and consequently the respiratory process of the cells. Under these conditions of aeration stress, oxidative injury has been observed by the rise in lipid peroxidation in plant tissues [52]. Lipid peroxidation, determined by accumulation of MDA, is a generally accepted indicator of membrane damage under oxidative stress conditions. Supporting this fact, Le Dily et al. [53] have described lipid peroxidation in membranes of hyperhydric fully habituated callus of *Beta* v*ulgaris* as a consequence of an oxidative stress. Foyer et al. [54] observed a higher rate of solute leakage in hyperhydric leaves compared to controls, indicating marked membrane deterioration. The imposition of stress results in the elevation of activated oxygen levels and causes changes in the redox balance through the oxidation of metabolically active compounds, leading to lipid peroxidation and degradation. Lipid peroxidation may have two origins: enzymatic, due to lipoxygenase (LOX) activity, or autocatalytic, due to activated oxygen species [44]. It has been reported that LOX (EC 1.13.11.12), which is often associated to lipid peroxidation, was activated in hyperhydric shoots compared to normal shoots in *Prunus* and *Euphorbia* by Franck et al. [44] and Dewir et al. [55], respectively. LOX, which is activated in hyperhydric shoots, plays a role in the elimination of damaged plastids and degradation of chloroplast membrane [56]. Some products derived from the LOX pathway can have messenger functions in signal transduction pathway [57] or have, as hydroperoxides, a harmful effect on cell differentiation [58]. The mechanism involved in lipid peroxidation during hypoxia is not clear although it is known that various cellular activities can generate H_2O_2 in plants. Saher et al. [59] found induction of the oxidative pentose phosphate and fermentative pathways in carnation hyperhydric leaves. According to their opinion, hypoxia stress was the main factor affecting metabolism of hyperhydric leaves.

26.3.7 Reactive Oxygen Species (ROS) and Antioxidant Enzyme Activities

Plant survival in the face of the potentially cytotoxic effects of the activated oxygen species depends on the presence of reduced molecules and antioxidant enzymes [54]. Recent evidence suggests that oxidative stress, involving the superoxide ($O2^{*-}$)

and hydroxyl (.OH) free radicals as well as hydrogen peroxide (H_2O_2), is an important damaging factor in hyperhydricity induction for *in vitro* culture tissues [36, 44, 60–62]. The most important sources of ROS are chloroplasts, mitochondria, peroxisomes, and the cytosol [63]. In chloroplasts, one of the sources of ROS production is direct electron flow to oxygen (Mehler reaction). Moreover, during photorespiration H_2O_2 generation occurs at the step of glyoxylate formation from glycolate [64]. In mitochondria, ROS production occurs mainly at two sites of the electron transport chain: NAD(P)H dehydrogenases and the cytochrome *bc*1 complex [65]. Although ROS are inevitable byproducts of aerobic metabolism, a rise in the levels of these molecules may be responsible for many of the observed metabolic changes in hyperhydric tissues such as lipid peroxidation and consequently membrane injuries, protein degradation, enzyme inactivation, damage of DNA. Therefore their production and removal must be controlled [66, 67]. These substances are generally eliminated through a cooperative mediation of the so-called defense enzymes and antioxidants.

The major antioxidant species in shoots are ascorbate, reduced gluthatione, a-tocopherol, carotenoids and flavonoids. The defense enzymes include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) [68, 69]. SODs are distributed in all cellular compartments, FeSOD in chloroplasts, MnSOD in mitochondria and peroxisomes, and Cu/ZnSOD in cytosol and chloroplasts. SODs, which are considered the first line of defense [68], catalyze dismutation of superoxide radical $O^{2\bullet-}$ to H_2O_2 and O_2 at the site of its production. APX, MDHAR, DHAR and GR form so-called ascorbate-glutathione cycle which converts H_2O_2 to water and recycle ascorbate and glutathione. CAT also uses H_2O_2 as a substrate in peroxisomes. PODs catalyse various reactions where H_2O_2 is used as one of their substrates including cell wall lignifications [70].

Several studies have been conducted in order to address the relationship between hyperhydricity and oxidative stress [13, 55]. The time course of H_2O_2 generation in hyperhydric tissues of carnation microshoots confirmed narrow connection between hyperhydricity and oxidative stress in this species [71]. In hyperhydric carnation leaves, increased MDA content and total POX activity was also observed [36, 71]. However, this increase in POX activity was due to increase in the activity of basic isoforms while activity of acidic isoforms and in consequence lignification was reduced [36]. It has been proposed that shoots which are unable to induce their antioxidant defenses (enzymes and soluble reductants) against activated forms of oxygen will become hyperhydric [44, 71]. According to their opinion, hyperhydric tissues exhibit a typically stress-induced change of physiological state.

An oxidative stress characterized by markedly increased content of MDA and activity of lipoxygenase (LOX) were found in hyperhydric shoots of *Euphorbia millii*. At the same time, these plantlets reduced oxidative stress by increased activities of SOD, POX and CAT. The activities of enzymes of ascorbate-glutathione cycle (APX, GR, MDHAR and DHAR) were also increased indicating a crucial role of elimination of H_2O_2 from plant cells [55]. Increased SOD and CAT activities in hyperhydric tobacco leaves were observed by Piqueras et al. [62]. Similarly, higher

activities of SOD, CAT, APX and GR were found in hyperhydric leaves than in healthy leaves of apple regenerants grown in bioreactor [13]. Hyperhydric tissues are able to activate their enzymatic defense system in order to reduce oxidative stress. In contrast, a hyperhydricity in liquid-cultured *Narcissus* induced by growth retardant ancymidol was connected with decreased activities of APX and CAT and increased initiation of meristematic centers [60]. In hyperhydric shoots of *Prunus avium*, H_2O_2 was accumulated due to increased SOD activity and decreased activities of POX, APX, CAT, DHAR, MDHAR and GR [61]. Using bottom cooling to prevent hyperhydricity in micropropagated *Dianthus* decreased H_2O_2 production, lipid peroxidation (MDA content), and SOD and CAT activities [59]. Addition of rare earth elements La, Ce and Nd into medium reduced hyperhydricity in *Lepidium meyeni* shoots and enhanced activities of POD, CAT, APX, SOD, MDHAR, and GR [72].

26.4 Hyperhydricity in Large Scale *In Vitro* Multiplication

Large scale *in vitro* culture using suitable liquid media under controlled conditions in bioreactors is an efficient method for shoot multiplication, production of several pharmaceutical products, therapeutic proteins, and drugs etc. Despite many promising approaches, liquid culture is trickier than growing on agar solidified media. Hyperhydricity is a major difficulty related to the use of liquid media in bioreactors (Table 26.1; Fig. 26.1a). Hyperhydricity is characterized by several morphological and physiological abnormalities including a flat, waterlogged-tissue appearance, abnormal shoot growth more specifically in the leaves [85] (Fig. 26.1b). Submerged plant tissues with the liquid medium, either whole or partial, give rise to hyperhydricity [86–88]. Moreover, Albarran et al. [76] pointed that hyperhydricity increases with increasing immersion frequency. Oxidative stresses expressed by submerged tissues give rise to elevated concentrations of reactive oxygen species (ROS) due to change in anti-oxidant enzymes activity. ROS can affect normal growth and physiology of plants which ultimately give rise to plant malformation and malfunctions. Shoot buds and meristem culture for the organogenic pathway has proved effective as another propagation system for bioreactors and to overcome hyperhydricity, providing a biomass with limited leaf elongation [89]. Likewise, bioreactors with temporary immersion system have also been designed to limit hyperhydricity, based on a principle similar to that of mist bioreactors, prefer contact between the plants and the liquid medium temporarily rather than permanent contact [90]. It has been reported that apple shoots produced in temporary immersion bioreactor showed higher photosynthetic rate, maximum quantum yield of photosystem-II and slow but steady rate of nutrient absorption, indicating the occurrence of higher photomixotrophic metabolism [91]. The authors also reported that hyperhydricity was reduced by 11 % in temporary immersion bioreactor as compared continuous immersion bioreactor [8]. Improved morphological and physiological indicators in *Dioscorea alata* plants cultured in temporary immersion system has been demonstrated [92]. As we have mentioned, leaves and sometime shoots are highly susceptible organs to

Table 26.1 Hyperhydricity in plants propagated in liquid cultures

Name of species	Type of culture	References
Amelanchier grandiflora 'Princess Diana' (Service berry)	Shoots in temporary immersion (5 min/30 min)	Krueger et al. [73]
Apple rootstock M.9 EMLA	Nodal explant in bioreactor (liquid)	Chakrabarty et al. [8]
Begonia × tuberhybrida Voss	Shoots in liquid and solid (gellan gum)	Nakano et al. [74]
Camellia sinensis (L.)	Nodal explants in liquid	Sandal et al. [75]
Coffea arabica	Embryos in liquid (bioreactor)	Albarran et al. [76]
Coffea arabica, C. canephora	Microcuttings in 1 L bioreactor with 2 compartment (RITA)	Berthouly et al. [77]
Cucumis melo L.	Embryos in liquid	Kennedy et al. [78]
Digitalis minor L.	Shoot apices in liquid	Sales et al. [79]
Dioscorea japonica Thunb.	Shoots in solid (agar, gellan gum) and liquid	Kadota and Niimi [1]
Eucalyptus spp	Axillary shoots liquid	Whitehouse et al. [80]
Euphorbia millii	Shoots in bioreactor (liquid)	Dewir et al. [10]
Fragaria × ananassa Duch	Shoots in temporary immersion bioreactor	Debnath [12]
Malus pumila cv M26	Shoots in liquid	Marga et al. [81]
Mangifera indica	Somatic embryo in liquid	Monsalud et al. [82]
Narcissus tazetta	Shoot in liquid	Chen and Ziv [60]
Origanum vulgare L.	Shoots in solid (agar)	Komali et al. [37]
Rubus chamaemorus L	Shoots in plastic airlift bioreactor	Debnath [83]
Zantedeschia aethiopica L. cv. Spreng	Rhizome-bud explants in liquid	Ebrahim [84]

hyperhydricity in bioreactors. To overcome such limitations, alternative approaches such as multiplication of embryogenic tissue and *sensus strict* embryogenesis (globular, heart, torpedo/cotyledon stages) steps should be used as these parts comparatively less exposed to hyperhydricity. However, these alternative approaches overcome hyperhydricity up to certain extent. Further modifications and development are therefore still required for better downstream processing and yield.

26.5 The Control of Hyperhydricity

Methods employed to combat hyperhydricity in gelled cultures have included modifications to the growth medium, such as increasing carbohydrate levels [93], modifying the concentration of gelling agents [86], and adding Bacto-peptone fractions [94] or agar hydrolysates [95, 96]. Although these modifications can often alleviate symptoms they can also cause a simultaneous decrease in multiplication rates [97]. Therefore the addition of a supplement to the medium that would reduce the occurrence, or delay the onset of hyperhydricity, but without detrimentally affecting the multiplication of a culture would be most desirable in a commercial, clonal

propagation programme. Nairn et al. [95] suggested that a constituent of agar, identified as being an agaroid-type xylogalactan bearing pyruvate and sulphate side chains, rather than the physical properties of the gel, were responsible for hydric control of micropropagated shoots of *Pinus radiata*. The anti-hyperhydric effect of EM2 (A0807, Sigma-Aldrich, Poole, Dorset, U.K.) has been reported for *Fragaria ananassa* [98], *Pyrus communis* L. [97] and *Eucalyptus* hybrids [80]. Gelcarin GP812 (Austratec Ltd, Victoria, Australia) is a commercially available, purified form of iota-carrageenan, which is extracted from the cell walls of red algae (*Eucheuma spinosum*). It is reported to be effective in reducing hyperhydricity in plants that would normally display symptoms [99].

In liquid cultures, bioreactors have evolved considerably and numerous models have been proposed to reduce and/or prevent hyperhydricity. Temporary immersion bioreactors have been designed to limit hyperhydricity because it possible to adjust the time spent by the plant material immersed in the liquid nutrient medium. Albarran et al. [76] observed that increasing the frequency for short immersions (1 min) in 1 L RITA bioreactors prevented hyperhydricity, stimulated somatic embryo formation and quality in *C. arabia*. Longer immersion duration (15 min immersions applied 2 or 6 times per day) led to hyperhydric embryo frequencies of 64 and 90 % respectively [100]. It is likely that each plant species as well as each culture step require adaptation of the immersion length and frequency to obtain optimum results. For the organogenic pathway, culturing clusters of buds and meristems has been shown to be an alternative propagation system for bioreactors and to overcome hyperhydricity, providing a biomass with limited leaf elongation [89]. The embryogenic tissue multiplication and *sensus stricto* embryogenesis (globular, heart, torpedo/cotyledon stages) steps can be carried out in conventional bioreactors as this material is less exposed to hyperhydricity [100]. Increased ventilation and/or gas supply may control hyperhydricity [8] (Fig. 26.1c, d). Evaporation of the culture medium is a major problem in airlift type bioreactors. To overcome this problem, addition of a sterile water column or condenser could be helpful. This can also extend the cultivation period [101]. To overcome the problems encountered during the use of air lift and bubble column bioreactors, the bottom-type bubble bioreactor (BTBB) was designed, in which foaming was drastically reduced by the use of cell lifting devices or tube(s) at vessel bottom. In addition, there was the provision of gas recycling system which allowed the examination of different gases in the medium [102]. It has been observed that CO_2 enrichment in a bioreactor culture vessel during the growth phase of shoots of sweet potato, potato and Chrysanthemum and Chinese fox glove enhanced the growth and production of healthy plantlets [103].

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