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Bioprocessing of Plant In Vitro Systems

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Editors

Bioprocessing of Plant In Vitro Systems

With 91 Figures and 29 Tables

Springer

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Preface

Nowadays, because of their valuable pharmaceutical and nutraceutical properties, many plant species have been widely used as ingredients in food, pharmacy, and cosmetic industries. Most of the consumers preferred to use natural products of plant origin, following closely the growing interest to the concept of modern healthy lifestyle, based on prevention by consuming quality foods and natural nutraceutical supplements. Therefore, the market for natural plant products has expanded, and this trend will continue. However, the supply of plant bioactive substances is limited by climatic, environmental, and ecological reasons, which will lead to shortages of various plant products. These problems could be solved by using plant in vitro technologies for producing plant secondary metabolites. There are several important advantages of plant in vitro technologies, mainly the independence from climatic conditions and the sustainable production process under controlled conditions. For the successful industrial implementation of plant in vitro technology for bioactive substances production an integrated approach for process optimization (including selection of productive lines, media optimization, development of suitable elicitation procedures, etc.) must be applied. However, the scale-up of cultivation process in bioreactors still appears among the main problems during development and commercialization of these technologies. This problem remains unsolved, despite the researchers' efforts for more than 55 years in this area.

The aim of this book is to meet the needs of broad range of students, researchers, and specialists working on plant in vitro technologies, and to focus their attention on the current status, advancement, and the prospects for further developments in that field. The book collects chapters written by distinguished scientists and recognized experts in their research fields. It covers several sections, guiding the readers from the historical background of plant biotechnologies (▶ Chaps. 1 and ▶ 2), through phytochemistry of secondary metabolism with focus on the GC-MS based metabolomics (▶ Chap. 3) and microbial transformations of plant secondary metabolites (▶ Chap. 4). Since plant in vitro technologies are considered as the most prospective way for plant secondary metabolites production, all aspects of process development and approaches for yields' improvement (▶ Chaps. 5 to ▶ 14) are discussed in details. Deep reviews are made on bioreactor technologies and process monitoring (▶ Chaps. 15, ▶ 16, and ▶ 17). In this book section, critical overviews are made on the current status of bioreactor cultivations of plant in vitro systems with different

degree of differentiation, and possible decisions to overcome limitations for industrial implementation of plant in vitro technologies were discussed. In the last section (► Chaps. 18 and ► 19), an overview and examples of the current state of plant-based production systems for expression of different recombinant proteins (► Chap. 18) as well as summary for application of hairy roots in phytoremediation (► Chap. 19) are presented.

Finally, we would like to acknowledge all contributing authors for their professionalism and immense efforts in realization of this book. We hope the book *Bioprocessing of Plant In Vitro Systems* will be a base for further development in research and industrial implementation of plant cells and tissue culture technologies.

Atanas Pavlov
Thomas Bley

Contents

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Atanas Pavlov is a Professor at the University of Food Technologies, Plovdiv, Bulgaria, and at the Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences. He graduated his Master's degree in Biotechnology in 1992 at the University of Food Technologies, Plovdiv, Bulgaria. Later, in 1998, he completed his Ph.D. in Biotechnology at the Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, and held position as an Assistant Professor at the same institute. From 2003 to 2005, he did postdoctoral research at the Institute of Food Technologies and Bioprocess Engineering, Technische Universität Dresden, Germany, working on bioprocess engineering of product synthesis with plant in vitro cultures. In 2005, he joined, as an Associate Professor, the scientific group "Bioactive substances from plant in vitro systems" which is a part of Department of Microbial Biosynthesis and Biotechnologies, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences. In 2009, he received his habilitation (DSci). From 2015, Atanas Pavlov is a corresponding member of Bulgarian Academy of Sciences. His research is mainly focused on the production of specific bioactive metabolites by plant cell, tissue, and organ cultures.

Thomas Bley studied mathematics at the Dresden University of Technology (Germany). From 1975 until 1990, he worked as a scientist at the Institute of Biotechnology (IBT) at the Academy of Science of the GDR in Leipzig. He received his doctorate in 1981 and his habilitation in 1990 with theses in the field of modeling microbial population dynamics. In the following years, he was the Head of the Division of Biotechnology at the University of Leipzig. Since 1996, he has held the Chair of bioprocess engineering and is Head of this study course at the Technische Universität Dresden. Prof. Bley is a full member of the German Academy of Science and Engineering (acatech) and of the Saxon Academy of Sciences. He is an active member of numerous organizations and technical committees in the field of Bioprocess Engineering. Prof. Bley's main fields of research are white biotechnology, biomonitoring and biosignals, dynamics of microbial populations, modeling and control of bioprocesses, flow cytometry, and plant cell cultivation (© Photo Hüller).

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Part I

Historical Background

History of Plant Biotechnology Development

1

Ivelin Pantchev, Goritsa Rakleova, Atanas Pavlov, and Atanas Atanassov

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Abstract

It is difficult to write a review on the history of plant biotechnology, especially after the excellent works of Vasil (Plant Cell Rep 27(9):1423–1440, 2008) Thorpe (Mol Biotechnol 37:169–180, 2007), and Sussex (Plant Cell 20 (5):1189–1198, 2008). It is even more difficult to overview the current state of this fast-developing field. Nevertheless, in this review we will make an attempt not only to make a narrative of main stages but also to show the links between plant biotechnology and latest progress in biological science.

Plant biotechnology has its roots deep in human civilization but was established just a century ago. Starting outside the science mainstream of the time period, classical plant biotechnology slowly but steadily grew into a recognized discipline. The explosive growth of biology research at the end of the twentieth century brought plant biotechnology to the fast-track line. The field grew very rapidly and currently turned into a key tool for fundamental research and practical uses. Currently plant biotechnology has been essentially grown, and new disciplines as omics technologies as genome editing have arisen which further intensify both fundamental and practical studies in biology and make a bridge with other scientific areas as informatics, nanotechnology, and so-called digital and intelligent science. As such modern biotechnology speeds up the development of the Fourth Industrial Revolution (Schwab, The fourth industrial revolution. World Economic Forum. ISBN 1944835008, 2016).

Keywords

Plant tissue culture · Transgenic technologies · Omics · Gene editing · Epigenetics · Marker-assisted breeding · Protoplast · Plant transformation · Cell factories · Bioreactors

Abbreviations

2-DE	Two-dimensional electrophoresis
Bt-toxin	Toxic protein from *Bacillus thuringiensis*
CGIAR	Consultative Group for International Agricultural Research
CRISPR/Cas	Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR-associated protein
CYMMIT	Centro Internacional de Mejoramiento de Maíz y Trigo (International Maize and Wheat Improvement Centre)
GC-MS	Gas chromatography-mass spectrometry

GM	Genetic modification
GMO	Genetically modified organism
GWAS	Genome-wide association studies
ICGEB	International Centre for Genetic Engineering and Biotechnology
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
IPR	Intellectual property rights
IRRI	International Rice Research Institute
LC-MS	Liquid chromatography-mass spectrometry
NIRS	Near-infrared spectroscopy
NMR	Nuclear magnetic resonance
RNAi	RNA interference
SNP	Single-nucleotide polymorphism
T-DNA	Transferred DNA from Ti-plasmid (or binary vector) into plant cell
Ti-plasmid	Tumor-inducing plasmid of *Agrobacterium tumefaciens*

1 Introduction

It is difficult to write a review on the history of plant biotechnology, especially after the excellent works of Thorpe [1], Vasil [2], and Sussex [3]. It is even more difficult to overview the current state of this fast-developing field. Nevertheless, in this review we will make an attempt not only to make a narrative of the main stages but also to show the links between plant biotechnology and latest progress in biological science.

Nowadays plant biotechnology turned into a valuable tool for modern biology as predicted by Altman et al. [4]. In vitro cultures served as model systems for studying fundamental processes in cells and whole plants. Transgenic technologies allowed for detailed characterization of gene functions and interplays. Plant tissue cultures were also extensively used for production of complex chemical compounds, either as native metabolites or through enzyme processing of precursors [5–8].

Integration of classic plant biotechnology methods with latest molecular biology approaches (especially DNA marker techniques) allowed the establishment of efficient molecular breeding programs [9].

Currently plant biotechnology has been essentially grown, and new disciplines as omics technologies as genome editing have arisen which further intensify both fundamental and practical studies in biology and make a bridge with other scientific areas as informatics, nanotechnology, and so-called digital and intelligent science. As such, modern biotechnology speeds up the development of the Fourth Industrial Revolution [10].

Revealing the links between early experiments and observations and current developments in the field is tough and challenging task. It is easy to miss some relations or key publications. We are well aware of our weaknesses but are open to any further critics.

2 Brief History Overview

Plant biotechnology from a historic perspective is a challenging but interesting field to overview from the very beginning of plant tissue culture [11]. With roots deep in human civilization, it steadily grew into a contemporary discipline and successfully turned into an integral part of modern plant science with strong impact of all related human activities [12, 13]. The most fascinating feature is that in its present state, plant biotechnology exploits or supplements numerous approaches from chemistry and biology. It also became an indispensable tool for establishment of modern bioeconomy [14–16]. The most notable example is the Netherlands. Successful implementation of state-of-the-art methods with classic techniques boosted the agroeconomy of this small country, bringing it to a second position as agro-food exporter in the world. One of the biotech companies in this country – KeyGene – is an excellent example for integration of latest scientific achievements into breeding programs [17] (http://www.keygene.com/news/). The most important consequence is that this integrated approach is gradually adopted throughout the world and steadily turns into a "golden standard" for modern plant biotech companies and national biotech programs.

Between the early empirical observations and the implementation as a keystone for national success is a story of rise and development of plant biotechnology as a scientific discipline (Fig. 1).

2.1 Prescience Times

Growing plants was one of the prerequisites for establishing the modern civilization. It is anticipated that the earliest agriculture took place some 12,000–14,000 years ago allowing for first complex societies to emerge.

Initially plant growing was simple enough, but soon primitive crop breeding allowed higher yield. Surprisingly, "plant biotechnology" practices also arise in the forms of grafting and vegetative propagation. This was based on observations and practical experience and, obviously, lacks any scientific grounds. Like the use of fermentations processes, these practices receive scientific explanation and deeper explorations only after establishing the modern cell theory and expanded growth of modern bioscience.

2.2 Cell Theory and Early Works in the Field

The principles of modern science were formed during the Renaissance. The invention of the microscope revealed the intrinsic organization of the living matter including plants. Among the morphologic descriptions was the observation of unorganized tissue growth (in fact, callus) at wounds on plants. However, the lack of sufficient scientific knowledge prevented further experimental studies *ex planta*.

The origins of modern plant biotechnology can be traced back to the works of Schleiden [18] and Schwann [19], who postulated that the cell is both the least living

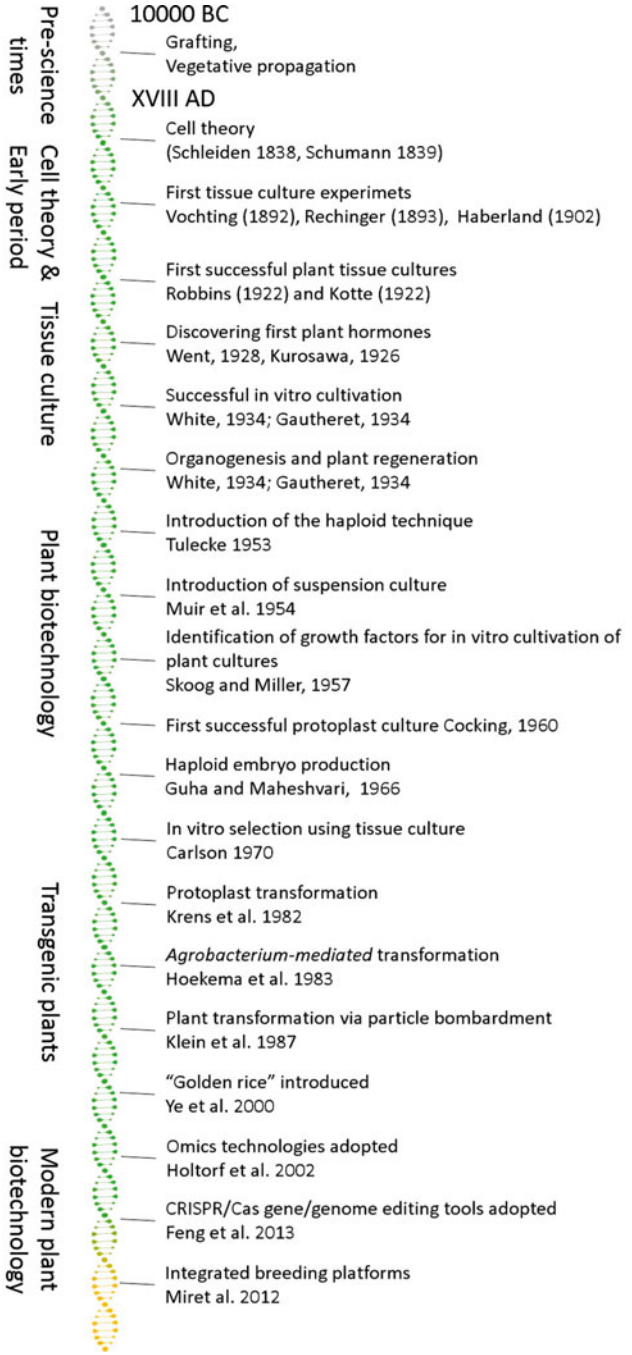

Fig. 1 Milestones in plant biotechnology development – a timeline

structure and the key building part of all complex organisms on Earth. The cell theory thus played central role in the establishment of modern biology in its vast diverse. The key impact for the future of plant biotechnology was the concept of cell totipotency, which actually opened the door of all future developments in the field. However, its validation relatively to the plant cells was achieved only in 1965 [20].

Plant biotechnology would not be possible without the reproductive technologies of plant tissue culture and cloning. During the nineteenth century, the trends in plant sciences were still directed toward the understanding of the basics like plant anatomy and physiology. First attempts to manipulate plants in a "biotechnology manner" have place at the turn of the century, when sufficient knowledge was accumulated. The pioneering works of Vöchting [21], Rechinger [22], and especially Haberlandt [23] had demonstrated that plant cells could be maintained in vitro. Later, Robbins [24] and Kotte [25] successfully grew plant tissue on artificially designed media. Their success as compared to the pioneering works was due to the substantial knowledge on cell biology that has been accumulated at that time.

In the late 1920s of the twentieth century, the first plant hormone – auxin – was identified [26], isolated, and characterized in details [27, 28]. These works explained the earlier observations of Charles Darwin and his son Francis in the 1880s on the possible internal signal factor related to the polarized plant growth [29].

However, it needed another decade and substantial new scientific work in order to provide reliable in vitro growing conditions with more or less defined parameters of the media composition, environmental conditions, and, more important, the role of the nutrition factors and hormones [30, 31].

As a result, in the mid-1930s, there were several excellent demonstrations of the potential of plant tissue culture not only for fundamental research but also for practical purposes. The key achievements were related to callus formation and organ regeneration, paving the road to micropropagation, embryo rescue, and double-haploid techniques – important tools for modern plant breeding.

Saying it simply – the plant biotechnology in its modern form was found.

2.3 The Birth of the Modern Plant Biotechnology

Modern plant biotechnology has arisen from the achievements of plant physiology and biochemistry. During the first half of the twentieth century, all key biochemical pathways were revealed, and substantial knowledge on plant physiology had accumulated. This critical mass of scientific information allowed for clear identification of most appropriate research directions as well as for better experimental designs.

The next two decades (1940–1960) had been a "transition" from proof-of-the-concept stage to a now useful tool for plant research and manipulation. There were several key milestones. First one is the production of haploid cultures from pollen, paving the way for a powerful future tool in plant molecular genetics and plant breeding [32].

Another important technique was the introduction of suspension cell culture by Hildebrandt group in 1954 [33]. The possibility to maintain and manipulate a

culture of single cells outlined the future success in studying cell proliferation and differentiation. The technique was a prerequisite to validate plant cell totipotency (de- and differentiation), thus providing scientific background for future plant transgenesis.

Among the most remarkable achievements during this period were the discovery of key plant hormones and scientific explanation of their role during in vitro cultivation process. The discovery of the role of adenine and its derivative – kinetin – along with the effects of auxin allowed for detailed estimation of the growth parameters [34]. Latest chain of research led to the development of the first "more or less universal" recipe for tissue growing – the famous Murashige and Skoog medium [35]. It is interesting that the discovery of kinetin was related to the interest in the biochemistry of nucleic acids and especially their degradation. This is a nice example how the achievements in a particular discipline boost the progress in a seemingly unrelated field.

In 1960 the next key step in plant biotechnology took place. Cocking [36] successfully applied enzyme treatment to produce first viable protoplast culture which became an important tool for the time being.

From this period are the first records for practical use of plant biotechnology. Morel [37] had used its modified shoot apex technique for orchid propagation.

In its pioneering work, Guha and Maheshwari [38] had successfully cultured anthers and pollen to produce haploid embryos. Their achievement had laid the path to the production of homozygous dihaploid plants within 5 months. Another important tool in modern crop breeding was born.

In 1963 The International Association for Plant Biotechnology was founded. This event symbolizes the transition of the field from juvenile stage to maturity.

During the 1970s plant biotechnology gained significant pace. In vitro cultivation of plant cells and tissue became an important tool for both fundamental research and practical use. First, protoplast technique has showed remarkable potential, and interspecific fusions were obtained between cells of different plant species [39]. Also, during this period, first plant transformation of protoplasts with foreign DNA was demonstrated.

Cell and tissue cultures became indispensable tool for biochemical, genetic, and molecular research. The in vitro selection was applied in order to produce plant material suitable for agriculture [40]. A new topic – somaclonal variation – had been studied [41] but later was abandoned. Further investigations revealed that, in fact, somaclonal variation was a result from stress-induced activation of mobile genetic elements [42].

Modern plant biotechnology was already established, and the fundament for the future fast development was build (Fig. 2).

2.4 Turn of the Century

The end of the twentieth century is characterized with vast expansion in the field of plant biotechnology. Most significant achievement was the establishment of plant

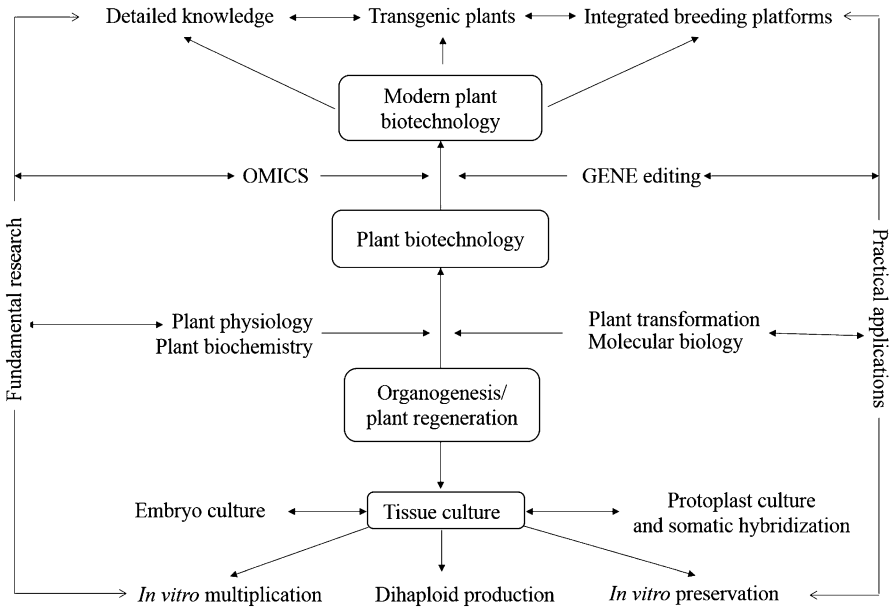

Fig. 2 Developments in plant biotechnology

transformation procedures. First works were on protoplasts but soon after two more practical approaches were introduced. First was the transformation by *Agrobacterium*, and, almost simultaneously, particle bombardment was also demonstrated. Both techniques soon became a standard for the plant biotechnology community, prevailing over the other alternative methods like electroporation, ultrasonic transformation, silicon carbide whiskers, microinjection, etc. Thus, plant transformation methods became an integral part of the modern plant biotechnology and keystone for a whole new research field.

The overall development of molecular biology techniques has reflected on plant biotechnology in number of ways.

In vitro cultivation along with dihaploid plant generation greatly influenced plant molecular genetics by advancing the development of molecular markers [43]. In turn, these techniques resulted in molecular breeding, which effectively supplemented and greatly facilitated crop selection. Nowadays, the integration of the marker-assisted selection with plant biotechnology techniques is the only viable approach to achieve sustainable and competitive crop breeding.

The introduction of in vitro DNA amplification (PCR) and DNA sequencing led to an expansion of our knowledge on plant genes and genetic elements. Genes were cloned and analyzed in their thousands. Slowly but steadily many empirical observations related to in vitro culture and regeneration received their molecular explanations.

2.5 New Millennium

Currently plant biotechnology is recognized as an important tool for both fundamental research as well as practical purposes.

An important feature is that development takes place not only at universities and research institutes but also in numerous private companies. First private entities were found in the 1980s – Calgene, DNA Plant Technology Corp., Phytogene, Plant Genetics Inc., and International Plant Research Institute, to name a few. During that period several large corporations also turned toward the field like Ciba-Geigy, DEKALB AgResearch, DuPont, Monsanto, Pioneer Hi-Bred, and Sandoz. In fact, most multinational corporations realized that investment in plant biotechnology is one of the prerequisites for market success. Not all of the initial players managed to survive for the next three decades when the economic conditions lead to resource concentrations and currently to very few major companies with a strong impact on the field. In the last few years, important mergers occurred among most of them – Bayer with Monsanto, Syngenta with Chem China, and DuPont with Dow Chemical.

The current state in the field has its origins in the explosive development of novel methods and techniques in biological sciences.

There were several key prerequisites.

First breakthrough was the development of improved chromatographic equipment. The introduction of mass spectrometry along with liquid or gas chromatography leads to a new level of chemical characterization which is now known as metabolomics. Several techniques, namely, LC-MS, GC-MS, NMR, and NIRS, allowed detailed analysis of chemical composition in minuscule quantities as well as metabolite dynamics.

Second breakthrough was related to the better understanding in plant molecular genetics. The introduction of the PCR made possible the development of high-density genetic maps. However, the major impact had the introduction of massive parallel sequencing. It revealed the fine organization of the plant genome. Many empirical observations had found its explanation, especially the phenomenon of epigenetics. The discovery of novel regulatory structures and elements in turn led to the development of novel tools for analyzing gene expression and gene interactions.

Genome sequencing was also a prerequisite for the latest state-of-the-art technique – genome editing. This turns to be a method of enormous importance for the next decade or two, allowing for directed manipulation of plant genomes with immense effect on the future of plant sciences.

Third breakthrough was related to the introduction of bioinformatics which leads to the development of omics technologies. The integral omics approaches allowed for efficient analysis of plants as well as for better understanding of all processes and their relations with the environment.

Fourth breakthrough was related to the spread of reproducible transformation technologies. It allowed for detailed characterization of gene interplays and better understanding of plant biology at all levels. Furthermore, it leads to explosive

development of genetically modified plants for numerous practical purposes outside the fundamental research.

2.6 Plant Biotechnology Around the World

Another interesting point is how plant biotechnology was developed in different countries especially in the developing world [44]. The mainstream works were performed in leading scientific centers in Western Europe and the USA. This reflects the fact that these countries were leaders in science during the nineteenth and the first half of the twentieth century. During the second half of the twentieth century, the global political processes allowed formation and fast development of new scientific centers. Some of them had direct links with well-renowned Western science schools (Australia, Canada) or were closely related (Japan, China), while others were formed or developed independently (mainly from former Soviet Bloc). Nevertheless, in all cases the foundation was based on contemporary science, and there were strong interconnections between centers around the world.

Plant biotechnology in Australia and Canada had tight links with the leading universities and research institutes in the UK and USA. The scientists from these countries had demonstrated excellent achievements in all aspects of the field ranging from cell and tissue cultivation to transformation of different crops and wild species.

During the second half of the twentieth century, Japan proved to be the first and fast-developing research center in plant biotechnology. One of the major contributions was the discovery of the gibberellins [45]. For different reasons plant biotechnology research was intense after the WWII. Development was enormous, and currently there are over 150 research centers implementing plant biotechnology methods. Japanese scientists prove their expertise in plant biotechnology by developing a broad scope of research objectives.

China has developed its research capabilities relatively late but was able to exploit and integrate all best practices from the world science. During the last 30 years of the twentieth century, the main focus was on anther cultures and haploid breeding. At the turn of the millennium, the interest included the plant transgenic research, and substantial efforts were made in this direction [46]. Currently there is an explosion of research centers at universities, institutes, and private entities (over 220 key labs) covering all aspects of modern plant biotechnology. As a result, transgenic plants have been elaborated in more than 50 economically important crop varieties (cotton, tomato, tobacco, potato, petunia, and some others that have reached the Chinese market first at beginning of the 1990s) and are ready for commercialization. These efforts are of utmost importance for China, not only for implementation in agriculture but for achieving a more global target – sustainable and competitive bioeconomy.

India had gained independency in 1947. It took nearly three decades to establish a net of national research infrastructure capable of extensive implementation of plant biotechnology approaches for practical purposes. Initial interest was toward the use of haploid cultures and embryo rescue techniques for facilitating plant breeding

programs. Next, there was successful use of protoplast fusion for producing interspecific and intergeneric hybrids. Nowadays India has developed outstanding capacities for plant biotechnology research on broad spectrum of crops and medicinal plants [47], attempting to link ethnomedicine and metabolome [48].

Another interesting example of a country with fast-developing plant biotech research and industry is Israel. Since the independence, gained in 1948, Israel had gradually developed an efficient agriculture sector. Benefitting from a large number of highly educated scientists, trained at leading research centers around the world and with strong administrative support, Israel managed to establish a set of top-class institutes like Volcani Centre (http://www.agri.gov.il/en/pages/1023.aspx), developing and implementing state-of-the-art plant science approaches. Another distinctive feature is the rise of a number of plant biotech companies during the last decades, some of them at a leading position in particular field. More important, there is a strong interaction between the fundamental science and industry, which further strengthen the position of plant biotechnology as a tool for sustainable economic growth.

A country with strong potential in plant biotechnology is Turkey. The existing gene pools, cultivated areas, and human recourses, combined with nearly 200 universities and research centers and under serious governmental support, have resulted in fast development of plant biotechnology and related services. The key prerequisite was the establishment of TUBITAK as a peer to the numerous centers and laboratories. This structure resembles well-known structures in the EU and USA like INRA, NSF, and BBSRC. As a result, Turkey had succeeded in building top-level research infrastructure and education of skilled researchers. Another line of support is provided by seed associations and private companies with interest in molecular breeding and transgenic research. Currently, almost all new techniques could be applied for plant improvement along with the classical breeding and germplasm conservation [49] (Prof. Dr. Nermin Gozukirmizi, personal communication).

On March 18, 2010, Turkey's parliament passed a National Biosafety Law, which regulates the production and import of all products of, containing, or derived from biotechnology, except for drugs and cosmetics. According to Biosafety Law, it is forbidden to cultivate GM crops and animals in Turkey.

The establishment of plant biotechnology in Russia had its roots in the general development of life science at the turn of the twentieth century. Nevertheless, works on plant tissue culture were initiated by Butenko in 1957 at the Institute of Plant Physiology RAS, Moscow [50], in direct collaboration of with the Gautheret laboratory. Under Butenko's strong guidance and coordination, plant tissue culture methods have been successfully implemented in most former Soviet Union countries [51]. Along with the USA, the Soviet Union was pioneering studies on the effect of space environment on different aspects of plant cell and tissue culture. In the 1970s and 1980s, research on protoplasts and plant transformation was also initiated. During the last decades, plant research was concentrated in several institutes and universities, especially those related to the agriculture industry [52].

The development of plant biotechnology in the countries for Central and East Europe began after WWII and was uneven. Poland, former Czechoslovakia,

Hungary, former Yugoslavia, Romania, and Bulgaria had strong scientific traditions and were able to establish high-quality research on plant tissue culture in the late 1960s. Due to historical reasons, most of the research was directly related to agriculture, while fundamental aspects were studied to a lesser extent. The best examples to be mentioned are Biological Research Center, Szeged (Denes Dudits and Pal Maliga), Hungary, [53] and Institute of Genetic Engineering, Kostinbrod, Bulgaria. Since 2000 it has been renamed Agrobioinstitute which in 1999 has been selected by EC as a first Center of Excellence for Plant Biotechnology among all the scientific disciplines and among all the accession countries to EC. Especially for Bulgaria, first works on plant tissue cultures that paved the way to modern plant biotechnology were by Kikindonov and Atanassov [54–56].

An interesting development was the establishment of UN research facilities (CGIAR) throughout the world, most of them in so-called developing countries. These 16 research institutes had broad scientific scope including plant biotechnology. Among the most prominent are CYMMIT (Mexico), IRRI (the Philippines), and ICRISAT (India). Similar role has played ICGEB (India, South Africa). In these institutes plant biotechnology was either used as a key tool or was an independent research subject. The long-term impact of these centers on the regional development proved to be significant. They have successfully served both as a starting platform and a center of excellence for newly build local scientific communities.

3 Plant Biotechnology Interplays

The maturation of plant tissue culture techniques led to their integration in several different scientific and industrial fields (Fig. 3).

3.1 Fundamental Research

The implementation of plant biotechnology tools in fundamental research started very early. Cell and tissue culture was the workhorse of elucidation of plant hormone action. Since the introduction of molecular biology and genetic engineering methods, plant biotechnology became indispensable for analyzing gene functions and relations.

Production of haploid and dihaploid plants has facilitated genetic mapping, thus providing ground for the introduction of marker-assisted breeding.

Detailed knowledge of embryogenesis, cell division and differentiation, organogenesis, etc. could not be possible without the use of well-characterized model in vitro systems.

Interesting field was the use of (transgenic) root cultures for studying secondary metabolism in plants. Plant cultures (cell, tissue, and organ) were successfully used for production of metabolites as well as for biotransformations of organic compounds [57].

Tissue cultures have found their place as a model system to study mechanisms of salt stress in plants. Callus [58, 59] and protoplast-based [60] systems using tobacco

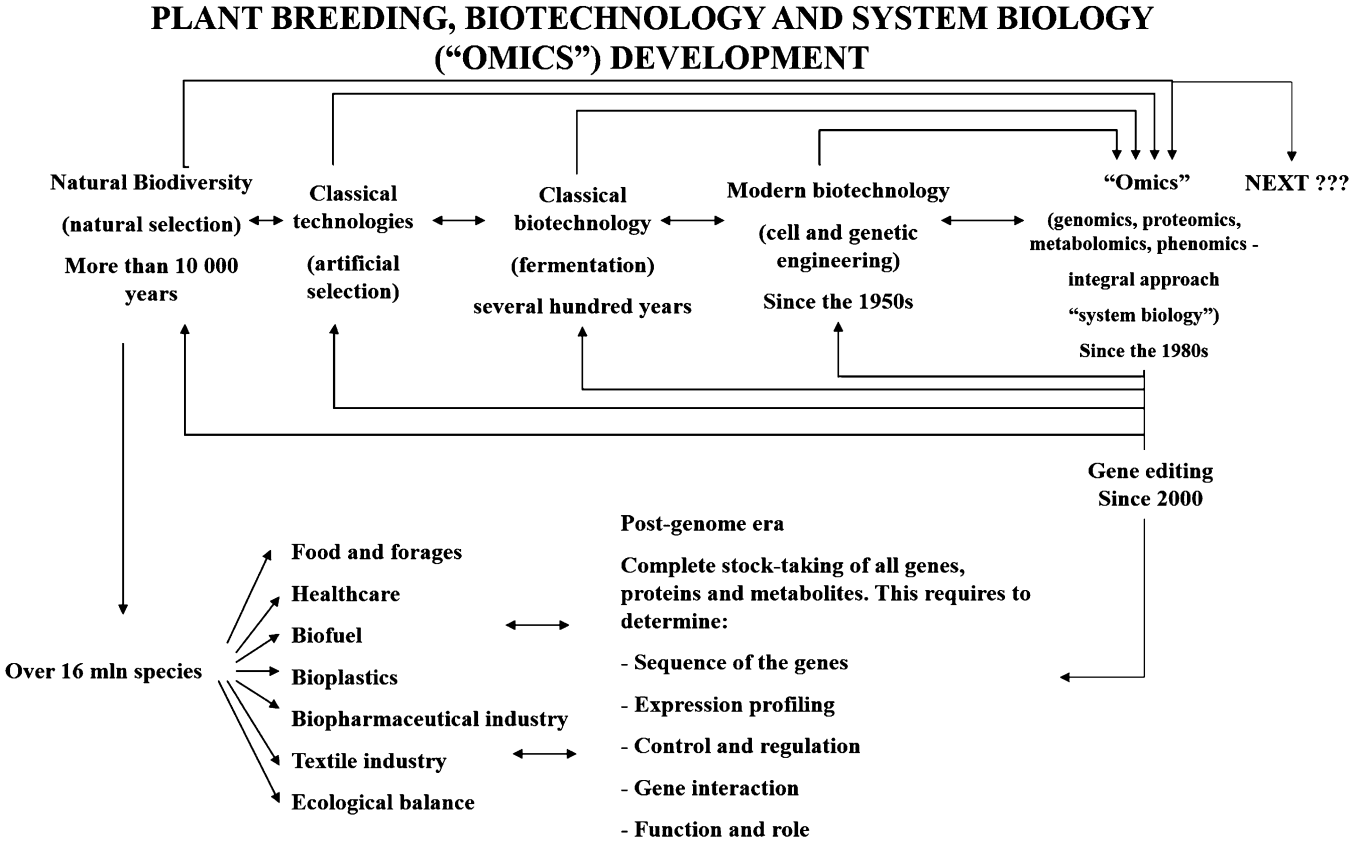

Fig. 3 The role of plant biotechnology for fundamental and practical application

proved to be a useful model. The classical in vitro selection approach is still used for obtaining stress-tolerant plant lines [61]. Particular success was demonstrated by combination of in vitro selection with mutagenesis for producing plant material with novel agronomically important traits [62].

Introduction of transgenic technologies allowed for further understanding of the mechanisms of salt tolerance. Early works include evaluation of the role of proline [63], choline [64], betaine [65], and saccharides [66] as osmoprotectants.

3.2 Agriculture

Agriculture turned toward the plant biotechnology approaches when they became relatively mature. The key techniques were plant regeneration, embryo cultivation, and dihaploid line production (outlined in Fig. 4).

Classic tissue culture followed by regeneration of whole plants is a part of modern breeding process. The possibility to select biological mutants in vitro was demonstrated by Carlson [67]. Later, Gengenbach and Green [68] used maize callus cultures for selecting pest-resistant clones.

Haploidity and especially the production of dihaploid plant lines had a significant impact on crop breeding programs especially in combination with molecular marker techniques. First haploid embryo formation from in vitro culture of *Datura* anthers [69] was successfully repeated in tobacco [70, 71] and rice (*Oryza sativa* L.) [72]. The modern crop breeding benefited from the production of haploid wheat plants anther culture [73, 74], microspore culture [75], or wide hybridization with wild barley (*Hordeum bulbosum* L.) [76] or maize [77–79]. The main impact was achieved by combining dihaploid plant production with molecular marker methods. This integrated approach is the keystone of marker-assisted breeding [80, 81].

The successful implementation of haploid culture strongly depends on excellent tissue culture expertise and deep knowledge of plant biology. The approach is also quite demanding in terms of labor and time. Nevertheless, the advantages for the breeding programs are prevailing, and for many modern breeding entities, DH line production is among the key techniques.

The embryo culture as a technique was introduced relatively early, but the first practical example of its use was for production of monoploids in barley [82]. The method itself still requires empirical optimization for each separate case. In terms of expertise and experience, it is even more demanding than working with haploid culture. On the other hand, embryo culture is the only tool allowing for efficient interspecific and intergeneric hybridization in many species.

Today, the three classic approaches are an integral part of any modern platform for crop breeding.

The link between plant biotechnology and agriculture faces novel challenges. From the very beginning of this intrinsic relation, plant biotechnology was assumed as a tool to provide better foods and guarantee agricultural performance. In April 2016, the United Nations declared the start of an “International Decade on Nutrition” to meet the nutrition-related targets of the Sustainable Development Goals adopted

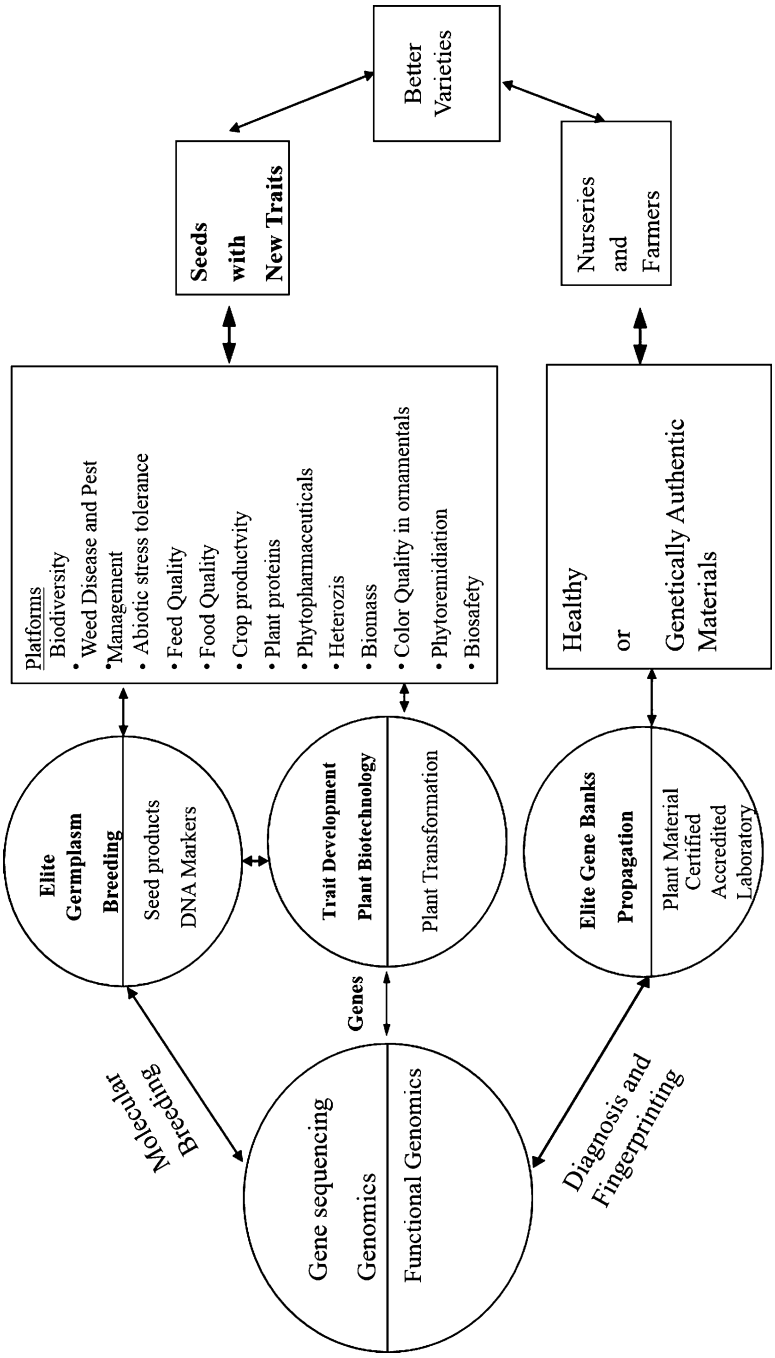

Fig. 4 Plant biotechnology for improvement of the efficiency of plant breeding programs

by its member nations last year [83]. As part of the Sustainable Development Goals, a major aim is to end all forms of malnutrition by 2030, overarching the objectives of ensuring both food and nutritional security. Ending hunger, achieving food security, and improving nutritional quality of crop products will require significant efforts, substantial part of which will rely on state-of-the-art plant biotechnology methods.

Nowadays the calorie supply is not limiting factor in diets, but still phytonutrient deficiencies continue to be prevalent [84]. It appears that the persistence of malnutrition in specific sectors of the population, particularly children, coexists with an increase in obesity and diet-related chronic diseases, such as diabetes. Therefore there is an urgent need to develop a new generation of enhanced crops that can address diet-related chronic diseases. Plant biotechnology along with significant changes in the ways food is transported and processed is among the solutions for coping with this serious problem [85], also providing opportunities to address several of the big challenges associated with the need for high-quality and enough food in the growing world population [86].

One of the key targets are the micronutrient deficiencies – iron, iodine, vitamin, etc. The micronutrient improvement is known as biofortification and can be achieved either through conventional breeding as well as through genetic engineering [87]. Most popular example among the public for biofortified plant product (albeit GM product) is the "golden rice" [88]. "Golden rice" had demonstrated the potential of transgenic technologies to design crops with improved nutrition properties as well as the possibility to feed the rising population in the developing countries.

3.3 Transgenic Technologies and GMO

The ability to produce transgenic plants shaped the current status of plant biotechnology as an important tool of modern science. Transgenic technologies are a blend of plant physiology, molecular biology, molecular genetics, chemistry, and physics. First example of directed plant cell transformation was obtained upon protoplast co-incubation with DNA [89]. The method for direct gene transfer into protoplasts was further optimized [90–92], but the cumbersome procedure forced the development of other methods.

3.3.1 Agrobacterium

In the mid-1970s, research on *Agrobacterium* pathogenesis revealed the role of Ti-plasmid and the mechanisms of DNA delivery. In 1976 a successful integration of the Ti-plasmid DNA from *Agrobacterium tumefaciens* in plants was demonstrated [93]. Soon after the cocultivation, transformation procedure was described, interestingly, for plant protoplasts [94].

While the potential of the system was already demonstrated, the experimental procedures were quite complicated and time-consuming. The requirements for extensive practical experience in microbial genetics also contributed to the limitation of the method to very few laboratories. It took several years of research to realize that T-DNA could be separated from *vir* genes on a different vector [95, 96]. This

discovery simplified the cloning and transformation procedures and made *Agrobacterium*-mediated transformation readily available for a broad range of laboratories, and first practice-oriented works appeared [97].

As a result, numerous transformation procedures were quickly developed and optimized for different species, tissues, and cell types. Some of them were niche methods, while others have received broad recognition. One such method was the use of leaf discs for *Agrobacterium*-mediated transformation followed by plant regeneration [98].

After three decades of development and refinements, *Agrobacterium*-mediated transformation became a rather useful method in plant biotechnology. Simple search with "*Agrobacterium*-mediated plant transformation" at PubMed Central returns nearly 10,000 referred full-text journal publications. Currently, gene transfer was achieved to all economically important crops, all model plant species, and numerous species of medicinal or horticultural interest.

3.3.2 Particle Bombardment

Initial experiments with *Agrobacterium* revealed that not all species were transformed with equal efficiency. For some important crops like wheat, it was impossible to obtain any transgenics despite the efforts. The observed recalcitrance led to the development of several new approaches for transgene delivery. Among them the biolistic gene transfer method [99] has received much appreciation and became de facto standard for generation of transgenic plants. The biolistic further simplified gene cloning and delivery techniques. It made possible to generate transgenic plants with genes cloned in widely available plasmid vectors. The method allowed for gene pyramiding. The method did not require extensive microbiological experience and alleviated the problems with *Agrobacterium* persistence in the transgenic plants. Most important feature was the ability to deliver foreign DNA to any type of cells or tissues without species-related restrictions.

3.3.3 Other Methods

The introduction of foreign DNA in plant cells by electroporation was first demonstrated in 1985 by Fromm et al. [100].

The method was further optimized but never gained the popularity of both leading methods.

Another interesting alternative was based on treatment of plant protoplasts, cells, and tissue fragments with ultrasound [101]. The method was called acoustic permeabilization or sonoporation. It proved to be simple and less demanding in terms of researcher qualifications, equipment, and consumables. Nevertheless, the popularity was rather low.

Plant cell or tissue treatment with silicon carbide whiskers for DNA delivery was first demonstrated by Kaeppler et al. [102]. The method proved to be efficient but was largely abandoned due to safety reasons.

Microinjection was also employed for plant transformation purposes [92, 103], but the need for highly specialized equipment and corresponding skills kept this method out of reach to most research groups.

Similarly, the use of liposomes for transfection was also demonstrated [104] but was not widely applied as compared to the transfection of animal cells.

Ultimately, plant transformation technologies had led to the creation of the agricultural biotechnology industry with annual global market value in the range of USD 20 billion. Part of this global market are the GM crops. Since their introduction in 1996, GM crops are grown in nearly 30 countries. Main grown transgenic crops are corn, soybean, cotton, and canola, while the transgenic traits are limited to either Bt-toxin-based pest resistance or herbicide tolerance.

These limitations are purely for economic and political reasons. The GMO topic is hotly discussed and of constant public interest. While there is no convincing scientific evidence for harmful effect on the environment or human health [105, 106], the public and political opinion in most countries had turned into a critical obstacle for further development of this most promising field of plant biotechnology. Most likely, any further business-oriented development will rely on public perception.

On the other hand, at the laboratory, GM technologies had turned into an efficient tool to study fundamental biological processes. Transgenic technologies were successfully used for analyses of gene functions, different regulatory mechanisms, dynamics of cellular processes, cell and tissue differentiation, etc. Nowadays, gene delivery methods are basic for state-of-the-art gene editing methods.

The tight integration of transgenic technologies, classic plant biotechnology methods, and omics technologies is often called plant engineering [107]. Upon implementation within the breeding programs, plant engineering could become the main area of application and development of classic plant biotechnology.

3.3.4 Applications of GM Plants

GM technologies became one of the main lines of development for plant biotechnology. One direction was the use of transgenic plant cells for production of recombinant proteins. First reports appeared shortly after the introduction and spread of plant transgenic technologies. Interestingly, from the very beginning, plants had attracted attention as protein producers. The reason was the relative simplicity of the experimental design and product processing.

One group of the expressed proteins was of medical interest [108]. Further efforts [109] led to the realization that transgenic plant cultures were a viable and appropriate system for production of compounds with pharmaceutical importance. Soon after, transgenic plants were used for vaccine production. First examples have used modified plant viruses as vectors [110]. The terms “biopharmaceuticals” and “molecular farming” were then coined and soon first reviews on the topic appeared [111]. Several problems had to be solved like the glycosylation pattern [112] before releasing for therapeutic purposes. First commercial application of plant-based vaccine was achieved by Dow AgroSciences in 2006 [113]. Recently, a plant cell-produced recombinant β-glucocerebrosidase for treatment of Gaucher’s disease was approved in the USA by FDA.

Transgenic technologies were further applied for production of novel protein-based materials like the spider silk protein [114]. Nowadays plant-based protein

production is recognized as a viable and sometimes cheaper alternative to microbial systems.

The modification of plant metabolism was also employed for production of ethanol [115, 116], biodiesel [117, 118], as well as precursors for so-called bioplastic [119–121]. The implementation of plant metabolite engineering proved to be more complicated in terms of experimental design. Current developments in the field are described in the recent review by Rusanov et al. [122].

Still however only single or stacked genes responsible for herbicide and insect resistance are dominating in the transgenic market. The GM technology is commercially exploited in four crops – soybean, maize, cotton, and rapeseed – and in the last few years in sugar beet. The attempt to introduce characters related to multigene families (drought, cold, and salinity tolerance; nutrition qualities as fatty acids, vitamins, etc.) turns out not to be so easily regulated in the way their expression and function to be as stable as the single or stacked genes.

Since 1996 until 2014, the cultivation of GM crops has been steadily increased. In 2015 GM crops occupied 179.7 million hectares which is 1% less than in 2014. A year after the second decade of commercialization of biotech/GM crops in 2016, 26 countries grew 185.1 million hectares of biotech crops – an increase of 5.4 million hectares or 3% from 179.7 million hectares in 2015. Except for the 2015 adoption, this is the 20th series of increases every single year; and notably 12 of the 20 years were double-digit growth rates.

3.4 Plant Biotechnology for In Vitro Production in Bioreactors

Numbers of people have preferred to use plant natural products. Therefore, the market for natural plant products has expanded, and this trend will continue. However, the production of plant bioactive substances is limited by climatic, environmental, and ecological reasons. This will lead to shortages of number plant products. These problems could be solved using plant in vitro technologies, which are promising method for producing plant secondary metabolites. There are several important advantages of plant in vitro technologies, mainly the independence from climatic conditions and the sustainable production process under controlled conditions. The main problem appeared during development of these technologies is with cultivation in bioreactors. For the successful industrial implementation of plant in vitro technology for bioactive substances production, an integrated approach for process optimization (including selection of productive lines, media optimization, development of suitable elicitation procedures, etc.) must be applied. This part of the process optimization is intensively studied and well documented [6, 8]. However the question about the process up-scaling is still reminded open, nevertheless, more than 55 years of research in this area. Historical development of large-scale cultivation of plant in vitro systems is very well summarized by Eibl and Eibl [123], Ruffoni and co-workers [124], and Yesil-Celiktas and co-workers [125].

To the best of our knowledge, in 1956 appeared the first patent for cultivation of in vitro plant tissues [125]. The first scientific report about large-scale cultivation of

plant in vitro systems appeared in 1959. Telecke and Nickell successfully cultivated tobacco cell suspension in 10-liter glass carboys [126]. A year later, the same scientists reported about new successful cultivation of plant cells suspension in stainless steel bioreactor [127]. These two pioneer researches have been the fundamental base for the next investigations of large-scale cultivation of plant cell suspensions as the used bioreactor systems with continuously increased working volumes reached 20,000 liters in 1977 [123]. The first industrial process for production of bioactive plant secondary metabolite (shikonin by *Lithospermum erythrorhizon*) has been developed in 1983 [128]. Five years later Professor Westphal [123] reported about successful large-scale process for ginseng biomass production. Based on the promising results received by that time, it is followed by series of successful, large-scale cultivations of various plant cell suspensions in bioreactors with a volume up to 50,000 liters – scopolamine by *Duboisia* sp., podophyllotoxin by *Podophyllum* sp., protoberberine by *Coptis japonica*, rosmarinic acid by *Coleus blumei*, echinacea polysaccharides by *Echinacea purpurea*, geraniol by *Gramineae* sp., arbutin by *Catharanthus roseus*, vanillin by *Vanilla planifolia*, betacyanins by *Beta vulgaris*, etc. [127]. Leaders in the early stages of developing the industrial application of plant biotechnologies are Japan's corporations such as Japan Tobacco Inc., Meiji Seika, Nitto Denko Co., Ajinomoto, and Nippon Shinyaku, which developed their technologies in close collaboration with academic research groups [125]. Industrial plant biotechnology continues its development, and today Phyton Biotech located in Germany operates the world's largest plant cell culture facility with bioreactors with working volumes of up to 75,000 liters, where it produces taxanes (one of their major products) with an annual capacity of 880,000 liters per year [129].

In parallel with industrial research, new constructive designs of bioreactors for the cultivation of plant cell suspensions are intensively developed and/or improved. The aim of these studies was to optimize the mass exchange and minimize the mechanical stresses in the cultivation systems and, on this basis, to optimize the environmental conditions for the cultivation of plant cells. These studies have resulted in the creation of a number of bioreactor systems that can be classified into several groups: stirred tanks, pneumatically agitated bioreactors, as well as disposable and non-agitated bioreactors [123–125, 130].

It is understandable that studies on the bioreactor cultivation of plant in vitro systems have begun with plant cell suspensions, as far as they can use the available microbial biotechnology tools and cultivation algorithms. In the case with differentiated plant in vitro systems (such as hairy roots, shoots, and adventitious roots), it is necessary to develop principally new bioreactor designs in relation to their specific morphology, sensitivity to mechanical stresses, problems with the inoculation processes, gradients created, and subsequent limitation with respect to nutrients and gas-phase components. It seems the stirred tanks are not appropriate for cultivation of these types of in vitro systems, and therefore their industrial application is limited by now [7]. To solve this problem, many investigations on laboratory and semi-industrial levels are reported. Bioreactor designs have been adopted to the specific morphological characteristics of the differentiated plant in vitro systems, and

different scientific groups reported successful cultivations in bioreactors with new or modified constructive designs, such as modified stirred tanks, cone balloon-type airlift bioreactors, rotating drum bioreactors, nutrient mist bioreactors, radial flow bioreactors, wave bioreactors, modified bubble column with internal sections, etc. [7, 131, 132]. To the best of our knowledge, in 2006 for the first time, the temporary immersion cultivation bioreactor has been used for the production of bioactive plant secondary metabolites (betalains by *Beta vulgaris* hairy roots) [133]. Later this technology has been applied to different types of differentiated plant in vitro systems for production of valuable molecules, as well as for solving of some fundamental problems with *Agrobacterium* transformation of plant cells [134–138]. In our opinion, the next direction of the research in the area of bioreactor cultivation of differentiated plant in vitro systems should be development of so-called "low-cost" bioreactor systems, as well as improvement of disposable bioreactors. This will allow significant reduction in the initial investment and the next operating costs, which will open industrial implementation of the differentiated plant in vitro systems for bioactive substance production [139].

4 Future Developments

During the rise and affirmation of plant biotechnology as a defined scientific discipline, several trend lines of development were formed, gradually leading for the amelioration of the DNA marker-assisted breeding and for the new development of such genome editing tools, oligonucleotide-directed mutagenesis, cisgenesis, intragenesis, omics technologies including phenomics (3-D imaging), etc. They are readily entering into the plant breeding programs for speeding up the efficiency in both yield quality and quantity in the agricultural important crops.

The tissue culture techniques in combination with embryo culture or haploid production led directly to plant regeneration approaches, implemented in fundamental research as well as modern crop breeding programs.

The validation of plant cell totipotency ultimately led to the development of plant transgenic technologies. In turn, genetic modifications brought to life a vast number of new practical applications for basic research and commercial applications.

4.1 Integration with the Omics and Marker Technologies

The transition of classic plant biotechnology to its present state can be linked directly to the rise of "omics" technologies and genomics in particular [140, 141]. The general development in chemistry and biology at the end of the twentieth century resulted in novel techniques, methods, and approaches, most of which were capable of generating enormous data. The resulted integration with mathematical and computing approaches (bioinformatics) led to a new evolutionary level which was coined as "omics."

Among the omics technologies applied in plant sciences, genomics was the first one that emerged [142]. Still the genomics field is relatively young. A draft sequence of the human genome was published in 2001 [143]. Ten years after the draft human genome was published, Lander describes the progress since the start of the genome project. Some of this progress is the result of technical innovation. As Lander says, "The per-base cost of DNA sequencing has plummeted by 100,000-fold over the past decade, far outpacing Moore's law of technological advance in the semiconductor industry. The current generation of machines can read 250 billion bases in a week, compared to 25,000 in 1990 and five million in 2000." The speed of DNA sequencing changed drastically from about five million bases per week in 2000 to 250 billion bases in 2010. Dramatic improvements in the sequencing machines have led to plethora of new sequencing projects [144].

The first plant genome that was sequenced in 2000 was of *Arabidopsis thaliana* under the *Arabidopsis* Genome Initiative 2000 [145]. It was chosen because of its relatively small genome and the fact that it has become the model plant for a lot of studies in plant science. In 2002 it was followed by publishing of draft of rice genome [146, 147]. As of May 10, 2017, in NCBI, genome database (https://www.ncbi.nlm.nih.gov/genome/browse/) has reported the following number of entries: 832 animal, 274 plant, 1132 fungal, 16,552 bacterial, and 7379 viral genomes.

The genome sequencing led to identification of huge numbers of molecular markers (e.g., SNPs). Their association with particular genes of interest can be intensively applied for genetic improvement by predicting breeding value [148]. This is an approach called marker-assisted selection [149]. There are different strategies for crop improvement, including the application of DNA markers. Some of them are the use of association mapping or genome-wide association studies (GWAS) [150, 151] and genomic selection [152, 153]. Another approach for genetic improvement is applying of marker-assisted backcrossing [154, 155] when it comes to transfer of important genes from donor to elite variety [153] or into another species, generating in that way GMO.

Another application of genomics in the face of molecular markers, resulting from genome sequencing, is successfully used in the field of authenticity of food and agricultural products. Sequencing of more and more pathogen bacterial strain makes relatively easy and accurate their identification in plant and animal diseases as well as in food contamination.

The genome is quite stable, but the levels of mRNA, proteins, and metabolites can vary considerably depending on cells and tissues analyzed, on time, and on quite different environment factors. In the need for their analysis, other "omics" technologies have also been developed based on different technical innovations. For example, microarrays and RNA sequencing (RNA-Seq) [156] made high-throughput analysis of mRNA expression feasible and led to the emergence of transcriptomics, which is part of the so-called functional genomics. Technological advances since the late 1990s have made transcriptomics a widespread discipline [157]. Today DNA microarray technology is available for many plants [158]. Transcriptomic studies became possible even for plants which genomes are not yet sequenced, through the development of expressed sequence tag (EST) libraries, cDNA collections, high-throughput transcript

profiling, and next-generation sequencing (NGS) [159, 160]. The new sequencing technologies have set up the ground for genome-wide comprehensive transcriptomics and analysis of RNAs [161].

In the post-genomic era of plant biology, the emerging challenge was to determine the functions of all genes in the plant genome. RNA interference (RNAi) is one of the functional genomics tools that have been developed to achieve this goal [162]. The phenomenon of gene silencing process was discovered accidentally in petunia flowers [163]. RNAi also proved that it is a practical tool for plant researchers toward improving crop varieties [164, 165].

RNAi has been used for enhancing the crop yield and productivity, for developing resistance against various biotic and abiotic stresses, and for nutritional improvements of crops. MicroRNAs are key regulators of important plant processes like growth, development, and response to various stresses [166]. RNAi has also been used to generate male sterility, which is valuable in the hybrid seed industry [167, 168].

Another powerful tool for crop improvement is proteomics [169]. The latest improvements in proteomic field as well other advances in plant biotechnology techniques offer new ways to stimulate the application of these technologies by plant scientists for crop improvement. The combination of genomics, proteomics, and other associated omics branches of biotechnology is showing their potential and effectiveness to speed up the crop improvement programs worldwide [170]. Proteomics helps the investigations of abiotic and biotic stress tolerance mechanisms in plants. One of the important applications of the proteomic approaches is useful in the study of the molecular mechanism involved in the interaction between a plant and its pathogens [171].

Before the era of the current proteomic approaches and the advances in the 2-DE and the mass spectrometry machinery, proteins as molecular markers in the plant breeding have been extensively used. Isoenzymes are commonly used protein markers in plant breeding [172]. Practically any enzyme could be used as an isoenzyme genetic marker. As an example, pathogen resistance biochemical markers have been utilized to select salinity stress-tolerant plants [173].

Rapid advances in the technical developments for metabolite analysis have led to the emerging of another omics field, namely, metabolomics [174]. Metabolite profiling is a valuable technology for improving our understanding of plant physiology and biochemistry [175]. The rapid advances in the technologies for metabolite profiling made fact that hundreds and potentially thousands of metabolites be routinely analyzed [176]. With the refining and expanding of these technologies, it will allow analysis of a huge number of important micronutrients that will result in increasingly exhaustive analysis of plant composition at an early stage in breeding programs [177]. In addition metabolite-combined genotyping could be used to accelerate the identification of genetic diversity and its further introduction into plant breeding programs [178].

Major improvements in crop yield are needed to cope with population growth and climate change. Despite that plant breeding benefited a lot from advances in genomics and proteomics, still profiling the crop phenome remains a major technical bottleneck [179]. In continuation of exploration of genomics, among the several

omics technologies that emerged in the recent years is phenomics – the high-throughput analysis of phenotype [180, 181]. Unlike conventional quantitative genetics, phenomics comprises an integrative approach and involves the detailed analyses of physiological parameters and considers the genetic basis of the underlying processes, as well as environmental impacts and agricultural practices.

The effect of "omics" technologies on plant biotechnology was clearly recognized [182], and currently its role is estimated to be of key importance for designing efficient breeding programs [183].

4.2 Integration with Gene and Genome Manipulation Approaches

Modern plant biotechnology successfully incorporated the most advanced methods for genome manipulation. With strong background of using transgenic technologies, the field received a significant boost for further development.

4.2.1 Gene Editing

Transgenic technologies proved useful for both fundamental research and practical purposes but gained negative perception from the public. As a result, the search for other approaches that can change genome without insertion of foreign genes began. In the mid-1990s several possible alternatives were outlined. Most of them relied on intrinsic and fundamental cellular process of genetic recombination [184]. Next step was the identification of systems, capable of targeted induction of recombination at a specific locus. This became possible due to the significant progress in molecular biology. Research on the structure of transcription factors led to the introduction of zinc finger proteins for plant genome editing [185]. Molecular plant pathology provided information about specialized bacterial proteins – TALENs – capable to recognize extended DNA sequences [186]. These systems proved efficient, but difficulties with the experimental design hampered extended adoption from most laboratories.

The situation has changed in 2013 when several groups almost simultaneously published their results on plant gene manipulation using CRISPR/Cas [187–191]. CRISPR/Cas was first discovered in *Escherichia coli* in the late 1980s as a defense system against phage and other elements, but it took almost 20 years to understand its principle of action. Soon after, the system was quickly adapted for targeted manipulation of prokaryotic and animal cells. The simplified experimental design along with the high efficiency made CRISPR/Cas a system of choice for almost all plant biology laboratories.

Currently, there is an explosion of the publications employing CRISPR, which is impossible to review here. Nevertheless, the main tendency is clearly outlined – gene editing by CRISPR/Cas turned into the leading choice for manipulating plant genomes. The method implements, supplements, and sometimes replaces classic transgenic technologies. It has been used not only for fundamental research but also to create plants with new traits, being faster and efficient than classic breeding, mutagenesis, or transgenesis.

4.2.2 Gene Silencing and Epigenetic Manipulations

In the post-genomic era of plant biology, the emerging challenge was to determine the functions of all genes in the plant genome. RNA interference (RNAi) is one of the functional genomics tools that have been developed to achieve this goal. During the decades after its discovery, RNAi proved that it is a practical tool for plant researchers toward improving crop varieties [164, 165] for enhancing the crop yield and productivity, for developing resistance against various biotic and abiotic stresses, and for nutritional improvements of crops.

Manipulation of non-Mendelian inheriting traits is a new fascinating but challenging approach in modern plant biotechnology. Described and studied in the early twenty-first century [192], the role and the mechanisms of DNA methylation on plant gene expression were soon revealed in details [193] and had attracted significant interest as a possible tool for manipulating plant genomes.

Currently, two main approaches were used – RNA interference and modified CRISPR system.

RNA interference in *Arabidopsis* was first demonstrated in 2000 [194]. The phenomenon was extensively studied [195] and was explored for practical applications [196].

A viable and more promising approach to manipulate plant epigenome is the use of modified CRISPR/Cas system. It relies on inactivated Cas9 protein fused to a moiety with histone-modifying activity [197]. Albeit in early stage of development, this method is expected to become an efficient tool for epigenetic manipulation in plants.

4.3 Tool for Integrated Crop Breeding

The new approaches that emerged in the past two decades had demonstrated potential to further enlarge the plant breeder's toolbox. These approaches include techniques allowing for targeted deletion or insertion of genes into plant genomes, site-directed mutagenesis of plant genes, as well as epigenetic modifications of either genome or gene expression. Another innovative trend was the use of transgenes solely as a tool to facilitate the breeding process. To achieve accelerated breeding, transgenes are used in intermediate breeding steps and then selected for removal during later crosses, eliminating them from the final commercial variety [198]. This strategy led to a new breeding approach – the reverse breeding, a technique that produces homozygous parental lines from heterozygous elite plants [199].

As a result, the combined use of multiple research approaches gained popularity and brought the field into a new level of integrated breeding [200].

4.4 Intellectual Property Rights

Another vast and important aspect of modern plant biotechnology is related to the intellectual property rights. It is impossible to cover all issues, related to IP rights and

political or financial regulations. The global tendencies to obtain patents regarding new methods and techniques have their impact mainly on commercial entities while allowing some freedom for fundamental researchers [201, 202].

Initially, most IPRs regarding latest technologies were held by universities and research centers. The involvement of large companies in the research has led to a shift in owning IPRs from public institutions toward the commercial sector. In plant biotech domain, the leader is Monsanto which owns more than 80% of IPR. The top ten organizations with patents in modern plant breeding with reasonable plant biotech involvement are Sangamo Biosciences holding 11 patents; Dow AgroSciences, University of Delaware, J.R. Simplot, and Cornell Research Foundation holding five patents each; KeyGene holding four; Pioneer Hi-Bred, Cibus Genetics, and Wageningen University holding three patents; and Plant Bioscience with two patents. As it can be seen, eight of these organizations are private entities, and only two are public (universities).

It might be expected that this tendency will remain in the near future.

Finding the balance between private interest and revenue and public needs is among the greatest challenges, facing industrial application of plant technology.

5 Conclusions

In 2050 the world should meet a population of nine billion which will require 70% food increase in order to provide food and nutritional quality in a situation when the climatic changes are reality and the resources as land, water, and labor will be decreased two to three times. In 2050 China and India will triple their living standards. The number of elderly people over 80s will increase significantly, which will require further improvements of human diet.

At present the yield increase of the staple crops of wheat and rice is very low (~10%). Similar is the situation with the other important agricultural crops. It shows that conventional breeding needs new tools and innovative approaches to meet and to overcome constrains imposed by changing climate – as a water deficit, tolerance to new disease and insect, salinity, etc.

This review shows that the plant biotechnology, whether they are classical plant biotechnologies (tissue culture methods) or new DNA marker-assisted selection, omics technologies, etc., is not only going to play an essential role for the fundamental development of plant biology and for the enhancement of plant breeding crops. By integrating all available biotechnology methods, we would expect to discover new favorable alleles among the existing cultivated ones as well as in wild species germplasm with different genetic backgrounds.

It has been shown in a number of studies that the genome editing technology (mostly CRISPR) could open the door for enhancing plant improvement by its capacity to change the gene(s) according to the purpose designation without disrupting the genotype background. The next step is probably the synthetic biology and digital and intelligent agriculture which could not be considered any more separately from plant biotechnology.

However the fruitless debate about the regulations and IPR issues of the new methods and techniques should not continue in a chaotic way as Richard Flavell says in its 2016 publication in *Nature Biotechnology* [107]. The principle should be not about GMO versus organic but should be whether we produce more sustainable and healthier food for all – in the right time, place, and price. Strategic planning is urgent.

So the review of history of plant biotechnology development should give us lessons that will help us to be encouraged to follow this principle.

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Plant Tissue Culture Technology: Present and Future Development

2

Svetla Yancheva and Violeta Kondakova

Contents

Abstract

The application of plant tissue cultures in fundamental and applied studies on various biological species has been rapidly growing. The use of in vitro technology for commercial propagation of plant species and for the production of bioactive components from them has become profitable industry worldwide.

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Various regeneration systems (protoplast cultures and somatic embryogenesis) and their importance for the advance of strategically significant priorities in the development of biotechnological science in agriculture, medicine, and pharmacy are treated in the present chapter.

We believe that in the future development of the in vitro technology the major priorities could be conservation of plant genetic resources; restoring the balance between research studies related to genetic transformation of plants with the aim of providing sufficient, quality and safety foods for the world population, on the one hand, and the studies aimed at determining the risk of growing and consuming them, on the other; creating transgenic plants maintaining a constant level of induced protein; and, last but not least, the use of plant resources possessing valuable biologically active substances.

Keywords

Tissue culture · Somatic embryogenesis · Micropropagation · Axillary shoots · Protoplasts · Suspension culture · Resources · Conservation · Genetic transformation

Abbreviations

2, 4-D	2,4-Dichlorophenoxyacetic acid
2-iP	2-Isopentenyladenine
BAP	6-Benzylaminopurin
DKW	DKW/Juglans medium, Driver JA, Kuniyuki AH 1984
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ED	Encapsulation-dehydration
GA3	Gibberellic acid
IBA	Indole-3-butyric acid
IgG	Immunoglobulins
MAT	Multi-auto-transformation
MS	Murashige and Skoog medium,1962
OM	Olive medium
PGRs	Plant growth regulators
PTC	Tissue culture method
PVY	Potato resistant virus Y
TDZ	Thidiazuron
WPM	Woody plant tissue medium, Lioyd and McCown,1980

1 Introduction

Tissue culture is a method for isolation parts of different plant species grown on sterile artificial media. It is an old technology, which is based on totipotensi theory of plant cell. First Matthias Schleiden [1] and Theodor Schwann [2] formulated the cell theory. They put forward the theory of totipotency, which states that the cells

are able to regenerate and to form an intact plant. Cell theory has become the foundation of biology, and it is the most widely accepted explanation of the function of cells [3, 4].

The use of tissue culture techniques started by Gottlieb Haberlandt in 1902 in an attempt to culture hair cells of the monocot plant leaf mesophyll tissue [5]. This first experiment failed because the cells do not divide because of the lack of growth regulators required for cell division. On the basis of his pioneer experiment, Haberlandt is justifiably recognized as the father of plant tissue culture. Later Hanning [6] succeded in planting embryos isolated from several plant crucifers. After 1920s the development of tissue culture techniques continued. Certain conditions have to be fulfilled for their implementation.

The second period of evolution of the plant tissue culture method (PTC) started with discovery and use of auxins. The works of scientists Gautheret [7] and White [8] determined the nutritional and hormonal requirements of the plant tissues cultures. It was observed that the whole plant could be successfully regenerated from undifferentiated tissues.

The period 1940–1960s was marked by the progress of new techniques and the improvements of those already existing. During the process of development of tissue culture technology, the need of basic knowledge in other research areas as botany, plant physiology, biochemistry, physics, and various kinds of analytical work was confirmed. The use of these techniques led to the application of tissue culture to five broad areas, namely cell behavior (including cytology, nutrition, metabolism, morphogenesis, embryogenesis, and pathology), plant modification and improvement, pathogen-free plants and germplasm storage, clonal propagation, and product formation.

Further studies using nucleic acids led to the discovery of the first cytokinin (kinetin), as the breakdown product of herring sperm DNA [9]. Moreover, it led to the recognition that the exogenous balance of auxin and kinetin in the medium influenced the morphogenic fate of tobacco callus [10]. As well, single cells incorporated in a 1-mm layer of solidified medium form somatic embryos from the calluses developed from some cell colonies [11]. Later Kohlenbach [12] managed also to induce embryos from callus. Vasil and Hildebrandt [13] realized Haberlandt's dream for producing an intact plant (tobacco) from single cells and thus demonstrating the totipotency of plant cells. Differentiation of plants from callus cultures has been often suggested as a potential method for rapid propagation. That capacity was rapidly exploited particularly with ornamentals [14]. Early studies had shown that cultured root tips were virus-free [8] and later it was also established for the shoot meristems [15]. This was confirmed by Morel and Martin [16] when virus-free *Dahlia* plants were obtained from infected plants by cultivating their shoot tips. There are different hypotheses about the lack of viruses in meristem tissue. Virus elimination is possible because the vascular tissues, within which the viruses move, do not reach the root or shoot apex. Another hypothesis explains virus elimination in meristem tissue with activation of enzymes during meristem tissue injury that inhibits viral replication.

The method was further developed for commercial application from a large number of researchers [17–19], and nowadays it is a routinely used procedure.

In 1962 Skoog and his student Murashige developed a high salt medium MS [20] and now it is the most widely used nutrient medium for plant tissue culture. The possibilities of the method developed further with the clarification of the requirements of plants and isolated parts of them to the culture medium. Endangered, threatened, and rare species have successfully been grown and conserved by micropropagation because of high efficiency of multiplication and low demands on the number of initial plants and space. The first notice for production of haploid *Datura* plants from pollen grains was made by Guha and Maheshwari [21]. The composition of the medium, particularly the plant hormones and the nitrogen source, has profound effects on the response of the initial explants. Generally, plant growth regulators play an essential role in determining the development pathway of plant cells and tissues in culture medium. The type and concentration of hormones depend mainly on the plant species, the tissue type and the objective of the experiment [22].

2 Micropropagation

Micropropagation is defined as the true-to-type propagation of selected genotypes using in vitro culture techniques. Tissue culture is the most widely used application of the technology for vegetative propagation of plants. It has been used with all classes of plants [23, 24], although some problems still need to be resolved (e.g., hyperhydricity, aberrant plants). Depending on the species and cultural conditions, in vitro propagation can be achieved by the following basic methods:

1. Axillary shoots proliferation (shoot culture)
2. Node culture
3. De novo formation of adventitious shoots through shoot organogenesis
4. Somatic embryogenesis

As a result of increased information, Kane [25] describes in details five main stages (0–IV) for successful micropropagation.

Stage 0: Donor plant selection and preparation
Stage I: Establishment of aseptic cultures
Stage II: Proliferation of axillary shoots
Stage III: Pretransplant (rooting)
Stage IV: Transfer to natural environment

These stages not only illustrate the procedural steps in the micropropagation process, but also represent the altered cultural environment. Such system has been adopted by most commercial and research laboratories as it simplifies production scheduling, accounting, and cost analysis [26].

2.1 Stage 0: Donor Plant Selection and Preparation

Explant quality and subsequent responsiveness in vitro is significantly influenced by the phytosanitary and physiological conditions of the donor plant [27]. Critically important are the selection and maintenance of the pathogen-tested stock plants used as the source of explants and their cultivation under controlled conditions that allow active growth and reduce the risk of disease and insect contamination.

2.2 Stage I: Establishment of Aseptic Cultures

Initiation and aseptic establishment of pathogen eradicated and responsive terminal or lateral shoot meristem explants are the aim of this stage. The primary explants obtained from the stock plants may consist of surface-sterilized shoot apical meristems or meristem tips for pathogen elimination or shoot tips from terminal or lateral buds [25]. The following factors may affect on the successful establishment of in vitro culture: explantation time, position of the explant on the stem, explant size, and sterilization system by treatments with disinfectants and polyphenol oxidation. Approaches used to decrease tissue browning include pretreatment with antioxidants during the sterilization procedure and initial culture in liquid medium with frequent transfer, adding antioxidants such as ascorbic or citric acid, activated charcoal or polyvinylpyrrolidone (PVP) to the medium and culture in low intensity light or darkness.

There is no one universal culture medium for establishment of all the species, but modifications o the MS basal medium [20] are most frequently used [25]. The type and the levels as well as the combinations of growth regulators used in Stage I media are strongly dependent on the genotype and the explant size (Fig. 1a). For many species, particularly herbaceous and woody perennials, consistency in growth rate and shoot multiplication is achieved only after multiple subculture on Stage I medium. Physiological stabilization may require from 3 to 24 months and 4 to 6 subcultures [26].

Plant growth regulators (PGRs) are plant hormones or synthetic compounds that stimulate cell division and regulate the growth and development of shoots and roots of explants and embryos in culture. The major PGRs used are auxins, cytokinin,

Fig. 1 Stages in olive micropropagation: (**a**) Effect of the explant size on the initial development of olive uninodal (*right*) and binodal (*left*) segments, (**b**) Propagation stage of olive after 5 weeks culture (scale bar = 1 cm), (**c**) Elongation of olive shoots, and (**d**) Rooted olive plants

gibberellins, and abscisic acid. Their type, concentration, and balance in the culture medium are the key factors for the subsequent differentiation of specific organs or structure formation.

- Auxins: induce cell division, cell elongation, apical dominance, adventitious root formation, and somatic embryogenesis. Applied in low concentration, auxins promote rhizogenesis and in high the formation of callus. Commonly used synthetic auxins are 1-naphthaleneacetic acid (NAA) and indolebutyric acid (IBA), while 2, 4-dichlorophenoxyacetic acid (2, 4-D) is used for tissue dedifferentiation and somatic embryogenesis induction.
- Cytokinins: promote cell division and stimulate initiation and shoot development in vitro. Zeatin, 6-benzylaminopurine (BAP), kinetin, 2-isopentenyladenine (2-iP), and thidiazuron (TDZ) are the frequently used cytokinins. Applied in high concentration, cytokinins inhibit root formation and induce adventitious shoot formation. The ratio of auxin and cytokinin (A: C) in the culture directs the process of morphogenesis [10].
- Gibberellins and abscisic acid: are less used PGRs. Gibberellic acid (GA3) is mostly used for internode elongation and meristem growth.

Abscisic acid (ABA) has an inhibitory effect and it is used only for somatic embryogenesis and for woody species culturing [28].

2.3 Stage II: Proliferation of Axillary Shoots

It is characterized by shoot proliferation and multiple shoot production. At this stage, each explant has expanded into a cluster of small shoots. Multiple shoots may be separated as single shoots, clusters, and nodal segments and subsequently transplanted to new proliferation medium [29]. The explant type, position on the stem, size, and orientation can affect Stage II axillary shoot proliferation. Depending on the plant species and genotype, the period of subculture may vary between 2 and 8 weeks (Fig. 1b) and the same material could be subcultured several times to new medium to maximize the quantity of shoots produced.

The number of possible subcultures before initiation of new Stage II cultures from the mother block is required depends on the species or cultivar and its inherent ability to maintain acceptable multiplication rates, while exhibiting minimal genetic variation and off-types [26].

Growth regulators selection as type and concentration in Stage II is very specific and is made on the basis of genotype-specific requirements, shoot multiplication rate, shoot length, and frequency of genetic variation. Shoot proliferation increases at higher cytokinin concentrations, but the developing shoots are usually smaller and may exhibit symptoms of hyperhydricity. Depending on the species and particular genotype, exogenous auxins may or may not enhance cytokinin induced axillary shoot proliferation.

In some olive cultivars (Table 1), the application of auxin in the proliferation medium resulted in carryover effect of cytokinin and shoot elongation, thus

Table 1 Summary of the best culture conditions in micropropagation of olive cultivars in vitro by Fabri et al. [30] supplemented

Genotypes	Initial explant	Basal medium[a]	Growth regulators for multiplication	Reference
Aglandau Tanche	Uninodal explants	mOM	Zeatin 4 mg L^{-1}	[34]
Arbequina	Young shoots from grafted plants	DKW	BA 4,4 μM + IBA 0,05 μM	[35]
Arbequina	Uninodal explants	OM	Zeatin 10 mg L^{-1}	[36]
Arbequina Empeltre Picual	Uninodal explants	OM	BA 1 μM + TDZ 1 μM	[37]
Carolea	Uninodal explants	½ OM	Zeatin 4 mg L^{-1}	[38]
Chondrolia Chalkidikis	Uninodal explants	WPM	Zeatin 20 μM	[39]
Dolce Agogia	Uninodal explants from suckers	½ MS	Zeatin 10 mg L^{-1}+ IBA 0,5 mg L^{-1}+ GA_3 0,5 mg L^{-1}	[40]
Ecotype 05300	Uninodal explants	WPM	Zeatin 0,1 mg L^{-1}	[34]
Frantoio	Uninodal explants	OM	Zeatin 4 mg L^{-1} or 2iP 4 mg L^{-1}	[32]
Frontio	Uninodal explants	MS	IBA 1 mg L^{-1} + kinetin 1 mg L^{-1}	[41]
FS-17	Uninodal explants	mOM	Zeatin 2 mg L^{-1} or 2iP 4 mg L^{-1}	[42]
Galega vulgar	Uninodal explants	OM	Coconut water 50 mL L^{-1} + BAP 2,22 μM	[43]
Kalamon	Uninodal explants	mOM	Zeatin 10 mg L^{-1} + crude extract	[44]
Kalamon	Uninodal explants	WPM	BA 1 mg L^{-1} + IBA, 1 mg L^{-1} + GA_3 0,1 mg L^{-1}	[45]
Koroneiki Kalamon	Uninodal explants	MS	IBA 0,5 mg L^{-1} + BAP 10 mg L^{-1}	[46]
Maurino	Three-nodal explants	mMS	Zeatin 0,5 mg L^{-1} or TIBA 4 mg L^{-1}	[47]
Memecik Domat	Nodal explants	mOM	BA 1 mg L^{-1}	[48]
Meski Chemlali de Sfax Chetoui Sig de Sfax	Uni- and binodal explants	mMS	Zeatin 2 mg L^{-1} + kinetin 1–2 mg L^{-1} (or BA, 2 mg L^{-1})	[49]
Meski	Uninodal explants	mOM	Zeatin 1 mg L^{-1}	[50]
Mission	Uninodal explants	½ MS	BA 2,1 mg L^{-1}+ GA_3 1,26 mg L^{-1} + NAA 0,6 mg L^{-1}	[51]
Mission	Nodal explants	½ MS	BA 2,1 mg L^{-1} + GA_3 2.8 mg L^{-1} + NAA 0,6 mg L^{-1}	[52]
Moraiolo	Shoot tips	OM	Zeatin 4 mg L^{-1} or 2iP 4 mg L^{-1}	[32]
Moraiolo	Shoot tips	OM	Zeatin 3,0 mg L^{-1} + BAP 0,5 mg L^{-1}	[53]

(*continued*)

Table 1 (continued)

Genotypes	Initial explant	Basal medium[a]	Growth regulators for multiplication	Reference
Nebbiara	Nodal explants	OM MS	Zeatin 13.68 μM + GA3 4.33 μM + IBA 0.49 μM BAP 8.88 μM + GA3 4.33 μM + IBA 0.49 μM	[54]
Nocellara Etnea	Uninodal explants	mOM	Zeatin 4 mg L^{-1}	[55]
Oueslati	Uninodal explants	mOM	Zeatin 1 or 2 mg L^{-1}	[56]
Rowghani	Uninodal explants	DKW	2iP 4 mg L^{-1}	[57]
ZDH4 Lucques Haouzia Dahbia Amwllau Salonenque Picholine marocaine Picholine du Landuedoc	Uninodal explants	OM	Zeatin Riboside 13,6 μM	[58]

[a]m-modified medium, media: DKW [31], MS [20], OM [32], WPM [33]

increasing the number of usable shoots of sufficient length for rooting. Moreover, shoot elongation in Stage II cultures may be achieved by adding gibberellic acid to the medium [30].

2.4 Stage III: Pretransplant or Rooting

This stage aims to obtaining vital shoots with well-developed stem and leaves, suitable for transfer to ex vitro conditions, and involves a complex of developmental and physiological features: elongation of the Stage II plants, rooting and pre-hardening to increase the percentage of survival cultures in the next stage.

The elongation is an optional additional (not obligatory) step for an efficient rooting procedure and could be performed by transfer to a hormone-free subculture, reduced salt and growth regulators content (Fig. 1c), and application of gibberellic acid to the medium prior to proper rooting medium.

Plant species differ in rooting ability due to their endogenous hormone content and some may form roots in the lack of auxin, but genotypes considered recalcitrant require specific treatments (Table 2). Usually the rooting medium contains auxin to promote root induction, development, and growth within a subculture for 4–6 weeks (Fig. 1d). Among the factors influencing positivelythe prosess of rhizogenesis, the auxin type, concentration, and timing of application play an essential role. Other co-factors are C: N ratio, light/darkness, carbohydrate type, and concentration. An alternative approach used is the pretreatment of elongated shoots in an aqueous

Table 2 Summary of the culture conditions and rooting efficiency of olive cultivars in vitro by Fabri et al. [30] supplemented

Genotype	Rooting (%)	Basal medium[a]	Growth regulators	Reference
Aglandau Tanche	80 62	mOM	IBA 4 mg L^{-1}	[34]
Arbequina	57	½ DKW	IBA 0,5 μM	[35]
Arbequina	NR	OM	IBA 3 mg L^{-1} or NAA 1 mg L^{-1}	[36]
Arbequina Empeltre Picual	75	Compost	IBA 15 μM + IAA 10 μM	[37]
Carolea	NR	½ OM	NAA 4 mg L^{-1} or IBA 2,5 mg L^{-1} + Putrescine 160 mg L^{-1}	[38]
Chondrolia Chalkidikis	70–93	WPM	IBA 12 μM + NAA 3 μM + Putrescine 30 μM	[39]
Dolce Agogia	84	½ Knop + Heller vitamins	NAA 4 mg L^{-1}	[40]
Ecotype 05300	NR	WPM	IBA 1 mg L^{-1} NAA 0,75 mg L^{-1}	[34]
Frantoio	80	½ MS, ½ Knop/ Heller	NAA 1 mg L^{-1}	[32]
Frontio	60	½ MS	IBA 0,2 mg L^{-1} + NAA 0,2 mg L^{-1}	[41]
FS-17	100	½ MS	NAA 2 mg L^{-1}	[42]
Galega vulgar	85	OM	Dipping in IBA 3 mg L^{-1} for 10 sec. and culture in hormone free medium + AC[a]	[43]
Kalamon	82	mOM	NAA 2 mg L^{-1} + crude extract from ovules	[44]
Kalamon	80	WPM	IBA 2 mg L^{-1}	[45]
Koroneiki Kalamon	45 29	Knop macro and Heller micro salts	Putrescine 160 mg L^{-1} + NAA 1 mg L^{-1}	[46]
Maurino	60	mMS	IBA 2 mg L^{-1} ("dip method")	[47]
Meski Chemlali de Sfax Chetoui Sig de Sfax	100	½ mMS	NAA 2 mg L^{-1}	[49]
Meski	100	mOM	IBA 1 mg/L^{-1}+NAA 1 mg L^{-1}	[50]
Mission	80–93	½ MS	IBA 4 mg L^{-1}	[51]
Moraiolo	80	½ MS ½ Knop/ Heller	NAA 1 mg L^{-1}	[32]
Moraiolo	13–87	OM	IBA or NAA, 0,5–3,0 mg L^{-1}	[53]

(*continued*)

Table 2 (continued)

Genotype	Rooting (%)	Basal medium[a]	Growth regulators	Reference
Nebbiara	93–100	½ OM	NAA 3.22 μM	[54]
Nocellara Etnea	100	mOM	NAA 1,5 mg L^{-1}	[55]
Oueslati	45–50	mOM	Dipping in IBA 2000 ppm + Zeatin 1–2 mg L^{-1}	[56]
ZDH4 Lucques Haouzia Dahbia Amellau Salonenque Picholine marocaine Picholine du Landuedoc	52 30 57 54 70 20 65 40	OM	NAA 5,37 μM or IBA 24,6 μM	[58]

[a]*AC*-activated charcoal, m-modified medium, media: DKW [31], MS [20], OM [32], WPM [33], NR not reported

auxin solution prior to culture on hormone-free medium or transplanting directly to peat-perlite mixture. A better understanding of the developmental and molecular basis of rooting and the mode of action of auxin is a prerequisite for more efficient rooting procedures, especially for problematic species [59, 60].

2.5 Stage IV: Transfer to Natural Environment

This stage also known as acclimatization or adaptation to ex vitro conditions is crucial for some plant species due to the anatomical characteristics of in vitro plantlets (small leaf area, less development of palisade tissue, less density or malfunction of the stomata, absence or little development of cuticle wax layer) and their physiology (low transpiration and photosynthetic rate, high respiration, and abnormal morphological development). This requires the cultivation of transplanted to soil substrate plants into greenhouses or mist tunnels under conditions of gradual reduction of atmospheric humidity, prevention of the fungal diseases development, and rapid transition from the heterotrophic to the photoautotrophic state for survival [61].

Tissue culture techniques have been described in many reviews and books [24–26, 28, 29]. Micropropagation is the most reliable and widely used procedure for obtaining of pathogen-free plants through meristem culture. Propagation from preexisting meristems through shoot and node culture is the main approach for commercial rapid multiplication, introducing new cultivars, ornamental foliage plants, and some woody species that are otherwise difficult to propagate. However,

the need for multiple subcultures on different media makes shoot and node culture extremely labor-intensive. Current application of the technology is restricted to high-value horticultural crops. Expansion of the industry to include production of vegetable, plantation, and forest crops depends on the development of more efficient micropropagation systems [25]. Cost-reduction strategies, including the elimination of some production steps, and the development of reliable automated micropropagation systems, including the bioreactor technology, will facilitate this expansion [62].

2.6 Bioreactor: A System for Plant Propagation

Recent decades demonstrated an evolution in the development of plant tissue culture, towards the use of automated systems for micropropagation, as are the bioreactors with liquid medium. Bioreactor cultivation is a fast and effective system for the propagation of a wide range of representatives of the plant kingdom – agricultural, forest, wild species, and pharmaceutical plants [63]. The use of bioreactors for micropropagation was first reported in 1981 for *Begonia* [64]. Initially the intention of scientists using a bioreactor system was to reduce the cost of micropropagated plants. During the development of the system, it was necessary to expand the range of knowledge and to be able to decipher correctly the biochemical and physiological signals of plants propagated in a specific microenvironment. Another important need was to optimize the conditions for each plant species, but also to control the morphogenesis of plants growing in liquid medium [63, 65]. Gradually this system became possible for many types and organs such as shoots, bulbs, microtubers, corms, and somatic embryos [66].

The bioreactor system has several advantages as better contact of the explants with the liquid medium, optimized type and concentration of growth regulators, as well as medium aeration, circulation, and filtration [67]. Among the negatives of the bioreactor micropropagation, the observed hyperhydricity and morphogenic malformations could be noted. Moreover, in a liquid medium some species exhibit oxidative stress, which occurs in the tissues as changes in the anti-oxidative enzyme activity, influencing the anatomy and physiology of the plants and their survival.

Liquid media have been used for plant cells, somatic embryos, and organ cultures in both agitated flasks or various types of bioreactors [68–70]. The environmental conditions in bioreactors as gas flow rates, pH, temperature, dissolved oxygen levels, and agitation speed need a permanent control [71]. Bioreactors used for plant propagation continually improve technological performance. Initially they were divided into two types – the first with constantly, and the second type with partially immersed cultures in the medium [72]. Evolution in the development of this technology has allowed nowadays bioreactors to be classified in the following four categories: mechanically agitated, pneumatically agitated, nonagitated, and temporary-immersion bioreactors (TIB).

TIB were originally presented by Harris and Mason [73] and later reported for the development of effective regeneration protocols in *Fragaria*, *Rubus*, and *Vaccinium*

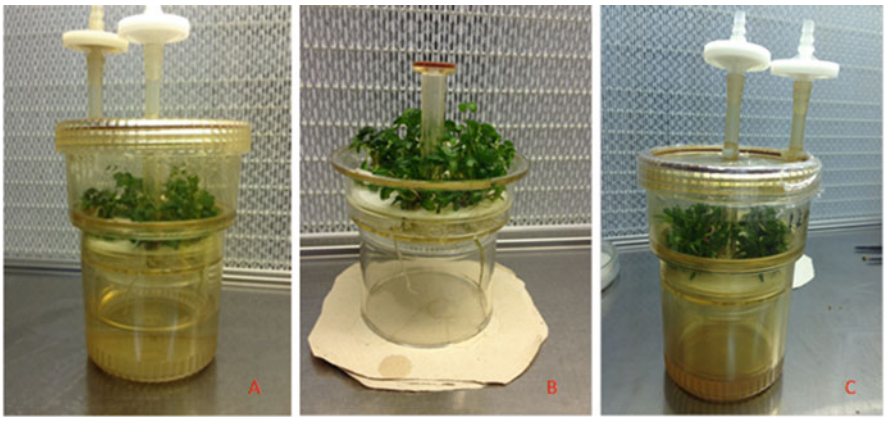

Fig. 2 Propagation of plantlets by RITA temporary immersion system (**a**) *Vaccinium vitis-idaea* L. (**b**) *Strawberry* cv. Elsanta (**c**) *Orchidea* sp.

species [74] (Fig. 2). The cultivation in liquid media using a temporary immersion system with different frequencies of immersion was in effect to improve plant quality and multiplication rates of banana, coffee, and small and wild fruits [75–81]. By means of this system, shoot proliferation increased up to three times in comparison with micropropagation on gelled medium [82].

Plant cell and tissue techniques are used worldwide and their main directions of applying are the commercial micropropagation of different plant species, obtaining of pathogen-free plants; production of haploid plants, and inducing genetic variation. The further development of this technique is related to the facilitation of production through automation of processes and computerization of information. The rapid development of "Omics" technology in recent years, combined with the benefits of plant tissue culture, allows understanding and overcoming challenges of basic biological processes.

3 Somatic Embryogenesis

Somatic embryogenesis and organogenesis are further regeneration pathways of plant tissue culture development. Somatic embryogenesis is unique in vitro method of plant regeneration widely used as an important biotechnological tool for sustained clonal propagation [83]. There are various factors affecting the induction and development of somatic embryos in cultured cells. An efficient protocol was reported for somatic embryogenesis in grapevine that showed higher plant regeneration in liquid medium [84].

The somatic embryogenesis can be initiated directly from the explants or indirectly by the mediation of dedifferentiated cells named callus. Somatic embryogenesis has been reported in many plants including plants of different families (Fig. 3).

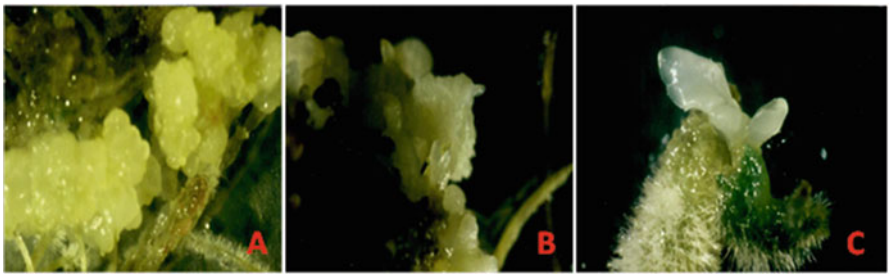

Fig. 3 Induction of somatic embriogenesis from cherry rootstocks of *Prunus incise* × *serrula* (**a**) Callus induction (**b**) Somatic embriogenesis (**c**) Somatic embriogenesis and regeneration

Plant cell cultures are a promising alternative for production of medical compounds of plant origin. Different biotechnological approaches, especially plant tissue cultures, offer additional opportunities to traditional plant breeding and for industrial production of bioactive plant metabolites [85].

4 Cell Suspension Culture

Exploration of the biosynthetic capabilities of various cell cultures has been carried out by plant scientists during the last decade [86]. Cell suspension culture is a suitable system, which can be used for a large scale culturing of plant cells generating the production of secondary metabolites. Cell cultures not only yield defined standard phytochemicals in large volumes but also eliminate the presence of interfering compounds that occur in the field-grown plants [87]. The advantage of this method is that it can ultimately provide a continuous, reliable source of natural products [88]. Another benefit of cell cultures includes synthesis of bioactive secondary metabolites, running in controlled environment, independently from climate and soil conditions [89].

A number of alkaloids important for medicine, anticancer drugs, recombinant proteins, and food additives are produced in various cultures of plant cells and tissues. Until now 20 different recombinant proteins have been produced in plant cell culture, including antibodies, enzymes, edible vaccines, growth factors, and cytokines [90].

The new direction in the development of tissue culture methods is protein synthesis by plants. The significant advantages of this system include the ability to manipulate environmental conditions for better control over protein levels and quality. Usually foreign proteins can be synthesized using microbial cell culture, animal cell culture, plant tissue culture, transgenic animals, and transgenic plants. Many factors can be considered including cost production, market volume, the safety and stability of the product, and the evaluation of biochemical and pharmacological properties.

An alternative but less developed technology for producing foreign proteins is by plant tissue culture. Using this method, plant cells in differentiated or dedifferentiated tissues are grown in nutrient medium in bioreactors under controlled conditions. Suspended plant cells are usually cultured for producing a variety of foreign proteins, including recombinant antibodies and antibody fragments [91–96]. The most commonly used model host species for protein synthesis in suspension cultures is tobacco [97]. Hairy root cultures of transgenic tobacco have also been tested for bioreactor production of recombinant IgG, antibody including in a 2 L sparget bioreactor [98, 99].

Agricultural production of proteins has been reported to be 10–50 times cheaper than *E. coli* fermentation even though product levels in bacteria are higher than those in plants [100]. A new technology should be developed with the aim of improving foreign protein accumulation in culture systems. The time needed for the production of protein from plant cell culture is significantly shorter than the growth cycle of whole plants, and plant cell culture has been shown to produce a wide range of recombinant protein. The potential capacity of plant tissue culture as a means for commercial protein production depends on the ability to express high levels of proteins in vitro by manipulating culture conditions.

5 Somaclonal Variation

The tissue culture method is more than a century old and it has been developing in horizontal and vertical direction, deep, and comprehensive. Biotechnology has been introduced into agricultural practice at an unprecedental rate. Cell and tissue in vitro culture is a useful tool for the induction of somaclonal variation [101]. Genetic variability induced by tissue culture could be used as a source of variability to obtain new genotypes. The term "somaclonal" variation was originally coined by Larkin and Scowfrot [102] considering phenotypic and genotypic variation observed in plants passing through in vitro cultivation. Phenotypic variations in many species are associated with morphological changes. Many of them appear to be epigenetically or physiologically based and are, consequently, reversible. For the purposes of the plant breeding, somaclonal variation can be induced without using sexual hybridization [103].

The occurrence of DNA variation in vegetative propagated plants grown in vitro could be due to the employed propagation specificities [104]. Studies of the researchers confirmed hypothesis that the DNA variation induced by in vitro culture could be lost after the transfer of plants to the greenhouse. This is in accordance with other investigations related to disappearance or maintenance of acquired characteristics in off-type plants [105, 106]. The reduced risk of somaclonal variations in regenerants and transformants can be achieved by regeneration through somatic embryogenesis that recognizes a single-cell origin.

The 1990s showed continued expansion in the application of the in vitro technologies to an increasing number of plant species. Cell cultures have remained an important tool in the study of basic trends in plant biology and biochemistry and have assumed major significance in studies in molecular biology and agricultural biotechnology.

6 Protoplasts Culture

During 1970–1972, protoplasts isolation started developing as a very perspective and promising tissue culture direction [107–109]. The isolation of protoplasts is a perfect model for plant improvement [110]. Protoplasts are able to incorporate naked DNA and consequently, the transformation with isolated DNA is possible [111].

Protoplasts are naked plant cells without the cell wall, but they possess plasma membrane and all other cellular components. They represent the functional plant cells but lack a cell wall as a barrier. The first isolation of protoplasts was achieved by Klercker in 1892 from plasmolysed cells, employing a mechanical method, but no attempt was made to culture them. A real beginning in protoplast research was made by Cocking [112] using an enzymatic method for the removal of the cell wall. Protoplasts have a wide range of applications; more of them have significant scientific value as genetic transformation, which can be achieved through genetic engineering of protoplast DNA (Fig. 4). Protoplasts are excellent model for ultra-structural studies, isolation of cell organelles, and chromosomes and membrane

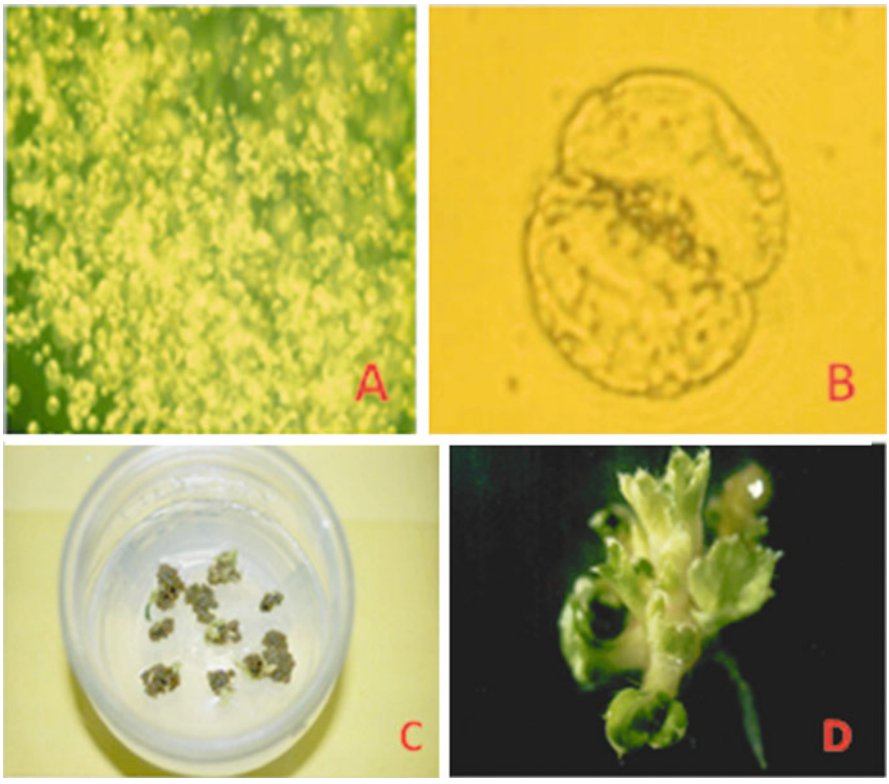

Fig. 4 Protoplasts isolation from cherry rootstock (Prunus incise × serrula). (**a**) Isolation of mesophyll protoplasts (**b**) Cell division (**c**) Regeneration from callus (**d**) Regenerated plantlets

studies, enabling the development of hybrids after protoplasts fusion. Somatic hybridization is an important tool of plant breeding and crop improvement by obtaining interspecific and intergeneric hybrids. The fusion of protoplasts of two different genomes followed by the selection of desired somatic hybrid cells and regeneration of hybrid plants [109]. In vitro fusion of protoplasts opens a way of developing unique hybrid plants by overcoming the barriers of sexual incompatibility. The technique has been applicable in horticultural industry to create new hybrids with increased fruit yield and resistance to diseases [113]. The potential of somatic hybridization in important crop plants is best illustrated by the production of intergeneric hybrid plants among the members of *Brassicaceae* [114].

Contrary to the conventional breeding, it is possible to produce homozygous plants in relatively short time by tissue culture techniques through protoplasts, anther, and microspore cultures [115]. Haploids are sterile plants having single set of chromosomes, which are converted into homozygous diploids by spontaneous or induced chromosome doubling. The doubling of chromosomes restores the fertility of plants, resulting in the production of double haploids with potential to become new pure breeding lines [116]. The technique has a remarkable use in genetic transformation by the production of haploid plants with induced resistance to various biotic and abiotic stress factors. Introduction of genes with desired trait at haploid state followed by chromosome doubling led to the production of double haploids inbred wheat and drought tolerant plants were attained successfully [117–119].

7 In Vitro Conservation of Plant Resources

The preservation of plant genetic resources is of vital importance. The development of biotechnological science offers great opportunities for achieving this goal. It is valuable for different aspects, such as preservation of food security, agro-biodiversity, and for endangered and threatened species. The latest statistics show that more than 15 million hectares of tropical forests disappear every year [120]. It is estimated that up to 100,000 plants, representing more than one third of the entire world's plant species, are currently threatened or face extinction in the wild. For example, biodiversity in Europe is seriously threatened – 64 endemic plants of Europe have become extinct in recent decades and 24% of the species of certain groups of European plants are in danger of being lost. Maintaining the balance in plant biodiversity is of great importance for the development of medical and food industry, which provides a rich source of valuable components. There are many factors that affect biodiversity, the most important being the climate change, globalization, and market economy which contribute indirectly to the loss of diversity [120, 121]. The rapid pace of development and the advantages of in vitro techniques and molecular biology provide some important tools for application of conservation methods and real management of plant genetic resources [122, 123]. In many countries, plant genetic resources conservation has focused on crop species. During the last 20 years, in vitro culture techniques have been extensively developing and they have found application in more than 3000 different species as an alternative to field gene bank, especially for vegetative propagated

species and those which produce recalcitrant seeds (cacao, mango, coconut, and avocado). The seed storage is the most convenient method for long-term conservation of plant resources. Another group of plants are vegetatively propagated species, such as banana, sweet potato, sugarcane, potato, and taro, which due to heterozygocity could be stored by in vitro techniques [124]. Many approaches for storage of vegetative propagated plants and recalcitrant seed are available nowadays. They can be divided into several groups – slow growth procedures, where accessions are kept as sterile plants on nutrient medium and provide short and medium storage, and cryopreservation where plant materials are deposited for long-term storage [125]. Slow growth storage depends on the ability of the shoot culture to grow under conditions reducing the growth rate to a minimum and allow plant material to be health for few years under controlled conditions with periodic subculturing. The younger and more rapidly growing tissues as shoots, leaves, flower pieces, immature embryos, hypocotyl fragments, and cotyledons are a suitable target for this kind of conservation [126]. Some of the species successfully conserved in vitro are *Allium* spp., *Cocos nucifera*, *Vitis, Prunus, Citrus* spp., and *Solanum* spp. [127, 128]. The use of a combination of low light intensity or darkness, low temperature and changes of nutrient medium by reducing the sugar, manitol, or mineral element concentrations are applicable approaches for limiting the grow process [125].

The cryopreservation method is a general strategy for plant genetic conservation. The main advantages of this method are that the material can be stored in a stable way for a long term at relatively low cost and in disease-free conditions [129]. The application of such preservation of germplasm faces the following problems: (1) expensive equipment and (2) specialists with multidisciplinary knowledge in engineering sciences, physics, chemistry, and plant biotechnology are needed. Cryoperservation depends upon the manipulation of meristem, callus, cell cultures, or somatic embryos. Success may depend upon preconditioning, cryoprotection, rate of freezing, and the use of vitrification, and dessication strategies before storage. New cryopreservation methods include ED, vitrification, encapsulation-vitrification, desiccation, pregrowth, and droplet freezing [130]. Classical cryopreservation methods have been successfully applied to undifferentiated culture systems as cell suspensions and calluses [131, 132].

The choice of the cryopreservation method depends largely on plant species. The regeneration rate is an important indicator for the success of the storage method. It usually varies between 0% and 69% depending on the culture. For example, for *Pyrus,* 0.75 M sucrose in preculture and dehydration for 4 h is reported with 80% recovery [133]. Choosing sucrose as an osmotic agent in the preculture medium proved very successful for the survival rate after cryopreservation of palm tissue culture [134]. Widely applicable method of storage is the encapsulation method developed by Redenbauch et al. [135]. It is used in a large number of tropical and temperate species. A successful approach to this method is the use of secondary metabolites as cryoprotectants [136]. That applies most effectively for citrus species, in which 100% survival of somatic embryos was reported [137]. The vitrification-based method is also often used when the water content in the cells is eliminated by a physical or osmotic dehydration of explants. It is characterized by a high rate of recovery, successfully applied to a broad range of plant tissues from various species,

including complex organs like embryos and shoot apices [138–140]. The rapid development of biotechnology, cryopreservation, and molecular markers contribute to a positive and effective management of genetic resources.

8 Genetic Transformation

One of the aspects that determine the importance of cell and tissue cultures is the genetic transformation using the *Agrobacterium* sp. as a vector for transfer of foreign genes with a desirable trait into the host plants and recovery of transgenics. This technology has rapidly developed and it has a great potential for the purposes of plant breeding in many agricultural crops. The objective was to create plants with agronomically valuable properties such as a better quality, higher yields and disease and pest resistance. The list of plant species that have been successfully transformed by *Agrobacterium tumefaciens* is growing very quickly. In many developed countries, a high percentage of economically important species as corn, soybeans, cotton, canola, potatoes, tomatoes, and tobacco are transgenic. Some of them generated by *Agrobacterium*-mediated and others – by direct transformation methods as particle bombardment [141, 142]. The use of virus-based vector as alternative way of stable and rapid transient protein expression in plant cells is providing an efficient mean of recombinant protein production on large scale [143]. There are reports of successful genetic transformation of mature-seed by particle bombardment method [144]. Transformation technology has an important influence on the reduction of toxic substances in seeds [145]. Researchers have achieved successful genetic transformation of potato resistant virus Y (PVY) and transformation of *Petunia hybrida* by multi-auto-transformation (MAT) vector system and the plants obtained exhibit high level of resistance to *Botrytis cinerea*, causal agent of gray mold [146].

The method of genetic transformation of plants by *Agrobacterium* sp. is one of the fastest growing and efficient technologies in the field of biological science. However, there is controvercial discussion on the research studies related to the method on the one hand, and the risk of growing transgenic plants in the field and their impact on human health and the environment, on the other. That was the reason to prohibit experiments with transgenic plants in many countries in Europe. In some research centers investigations still continue in direction of developing strategies to enhance the extent and stability of transgenic expression in plants. One of the important challenges involves genetic transformation of human and animal cells. The recent reports of genetic transformation of human cells suggest the exciting possibility of using *Agrobacterium*, or *Agrobacterium*-like processes, for human and animal gene therapy [147].

9 Conclusion

In vitro propagation of plants is a method with great potential for the development of fundamental and applied biological science. The role of in vitro method in fundamental research has been increasingly growing as a model for comprehensive studies

at the cellular and molecular level. The plants are a tremendous source for the discovery of new products of medicinal value. In recent years, a large number of plants, including wild species, have been registered, as a valuable source of natural products for pharmacy and medicine [148]. This promising area of plant tissue culture technology is rapidly developing. The search of new plant-derived chemicals and development of new strategies for their extraction should be a priority in current and future research efforts.

The utilization of transgenic hairy root cultures has revolutionized the role of plant tissue culture in secondary metabolite production. They are characterized by unique genetic and biosynthetic stability, faster growth, and easier maintenance.

The recent advances in molecular biology, enzymology, and genetic engineering of plant cell cultures suggest that these systems will become a viable source of important secondary metabolites and the obtained transgenic plants can contribute to generating constant levels of protein production.

The technologies for cryopreservation of genetic resources have a great potential and they are rapidly developing worldwide. Deepening of the research in this area is a great challenge but also a great responsibility to future generations.

In the applied aspect, in vitro propagation is highly developed and commercialized area in the entire world. A large number of laboratories produce millions of plants annually, mainly vegetatively propagated ones, such as flowers, ornamentals, fruit trees, grapes, and rootstocks. Challenges to producers are related to the development of innovative in vitro techniques, cutting down the cost of production per plant by applying low-cost tissue culture and ex vitro rooting and acclimatization.

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Part II

Metabolic Phytochemistry

Metabolite Profiling of In Vitro Plant Systems

3

Strahil Berkov, Liliya Georgieva, Borjana Sidjimova, and Milena Nikolova

Contents

Abstract

Gas chromatography-mass spectrometry is one of the base analytical platforms used in plant metabolite profiling. The remarkable recent methodological and technological developments in GC-MS profiling expand the possibilities for its application in different fields of plant science including plant biotechnology. The methods of extraction, fractionation, derivatization, and metabolite identification, associated with GC-MS metabolite profiling, along with examples demonstrating the power and applicability of GC-MS in plant in vitro studies, have been presented in this chapter.

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GC-MS · In vitro · Plant metabolites · Metabolite profiling

Abbreviations

CE	capillary electrophoresis
EI	electron impact
GC	gas chromatography
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
LC	liquid chromatography
LLE	liquid-liquid extraction
MAE	microwave-assisted extraction
MS	mass spectrometry
NMR	nuclear magnetic resonance
RI	retention index
SPE	solid phase extraction
SPME	solid phase microextraction
TOF	time of flight
UPLC	ultrahigh performance liquid chromatography
UV	ultraviolet

1 Introduction

Metabolite profiling is the measurement of hundreds or potentially thousands of metabolites in an extract. In contrast, metabolomics aims the measurement of all metabolites in a given system and requites a platform of complementary technologies. No single technique is comprehensive, selective, and sensitive enough to measure all plant metabolites [1]. The metabolite profiling of plant extracts is a challenging task due to structural diversity of plant metabolites. More than 100,000 secondary metabolites with diverse structure and physical and chemical properties have been found [2] which require advanced analytical platforms for identification and quantification.

In vitro cultivation of plants is widely used in different fields of plant science such as plant physiology, genetics, metabolite engineering, and biotechnology. The in vitro techniques gives an excellent possibility to control the environmental factors facilitating investigations of the effects of one or several factors (e.g., mutation, phytohormones, light, temperature) on the physiological and biochemical characteristics of plants. Due to the sterile conditions of cultivation, the results are not interfered by exogenous metabolites (e.g., microbial) or stress metabolites resulting of plant-insect or plant-microbial interactions. Metabolic profiling of a target metabolite group(s) such as alkaloids, terpenes, phenolics, etc., of an extract is applied more often than metabolomics in the classical plant biotechnology. The metabolomics approach is commonly used in combination with other "omics"

technologies, e.g., proteomics, transcriptomics, and genomics, in plant physiology and genetic studies [3, 4].

In contrast to the intact plants, the samples from in vitro plant material taken for metabolite analyses often have small quantity which requires application of sensitive analytical equipment. Also, plant in vitro cultures release metabolites in the culture medium which have to be analyzed to reveal the biosynthetic potential of a plant in vitro system [5].

Gas chromatography-mass spectrometry (GC-MS) is one of the most used analytical platforms for metabolite profiling of plant extracts including those from in vitro samples. The aim of this chapter is to inspire the application of GC-MS more frequently in plant in vitro studies providing an overview of its application for profiling of different groups of primary and secondary metabolites.

2 Analytical Platforms for Metabolite Profiling

Metabolite profiling requires application of detectors providing structural information. MS and NMR may provide sufficient spectral data for identification of metabolites. Direct metabolite profiling, with minimum sample treatment and without metabolite separation, can be performed by NMR and techniques such as MALDI-MS and DESI-MS [6–9]. The lower sensitivity of NMR-based techniques restricts their application to quantification and identification of the most abundant compounds of an extract [6]. The mayor advantage of NMR, however, is the possibility to measure metabolite levels in vivo [10]. Although MALDI and DESI techniques are rapid, with a high throughput and widely used for image analysis [7, 9, 11], they are semi-quantitative and have also limited application in plant metabolite profiling.

The separation of metabolites in complex plant extracts is achieved by GC, LC (HPLC, UPLC), and CE. GC-MS is a powerful technique for analyzing complex mixtures of volatile compounds from various structural types [12, 13]. The advantages of GC-MS are the high separation ability of capillary columns providing separation of more than 400 analytes within 30–60 min and the application of MS libraries with highly reproducible MS spectra collected under unified standard conditions. The main disadvantages of GC-MS in secondary metabolite analysis, however, are those associated with its limitations: detection of thermostable and volatile compounds with molecular weight up to ca. 800 Da. Structural identification of large, polar, and thermally unstable molecules can be achieved after chromatographic (LC, CE) separation using MS detectors with soft ionization sources (ESI, APCI, etc.).

Conventional HPLC has limited working pressure range and cannot benefit of reducing particle size of stationary phase aiming better separation efficiency. New ultra-performance liquid chromatography (UPLC) instruments working at ultrahigh pressures (up to 1000 bar) allow operation with columns packed with sub-2-μm particles, thus achieving dramatic increases in separation power, resolution, speed, and sensitivity comparable to that of GC [14]. In the recent years, UPLC-HRMS has become the method of choice for analysis of extracts because of its possibility to analyze polar compounds with mass higher than 2000 Da [15, 16].

In contrast to EI-MS libraries, which spectra are collected under standard conditions, the creation of an effective mass spectral library for LC-based metabolite identification is still problematic due to the lack of uniformity of the conditions for mass spectral collection in the different laboratories [17]. Creation of in home mass spectral libraries for a specific group of compounds under specific conditions is a practice for various laboratories [16, 17] thus avoiding the above-mentioned problems for identification of unknown compounds and for rapid structure determination of new metabolites.

3 GC-MS for Plant Metabolite Profiling

Gas chromatography is a commonly used analytical technique in many research laboratories. As mentioned above, it became widely recognized in plant metabolite profiling because of its sensitivity, accuracy, versatility, and relatively lower cost (as compared to LC-MS) of both equipment and maintenance. It is the method of choice for analysis of various metabolite groups such as terpenoids in essential oils, fatty acids, sterols, alkaloids [16–18], Fig. 1. The approaches used for GC-MS metabolite profiling of intact plants can be successfully applied in in vitro plant

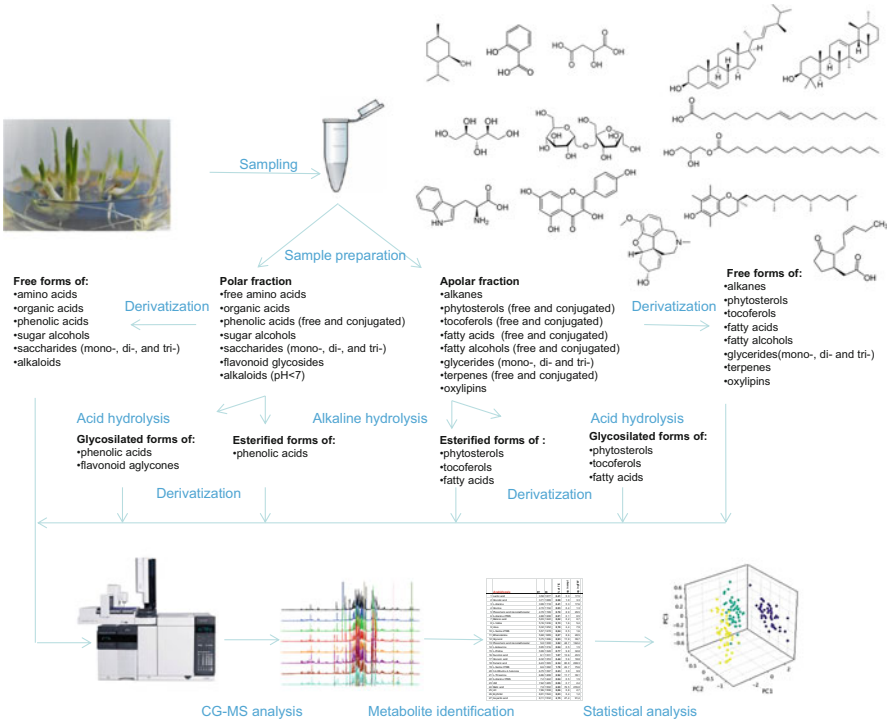

Fig. 1 Workflow of GC-MS metabolite profiling of plants

studies. Prior GC-MS analysis, the samples have to be prepared through metabolite extraction, purification (fractionation), and, when necessary, derivatization. The choice of sample preparation method is important in metabolite profiling because it affects both the observed metabolite content and biological interpretation of the data. An ideal sample-preparation method for metabolite profiling should be as selective as possible, simple, reproducible, fast to prevent metabolite loss and/or degradation during the preparation procedure, enable high-throughput and to represent true metabolite composition at the time of sampling [19].

3.1 Methods of Extraction

The extraction methods are the bottleneck for most of the analytical processes including in vitro plant studies where the experimental designs include several factors and biological replicates for statistically accurate results. Therefore, appropriate fast, reproducible, and efficient extraction steps for plant samples have to be applied.

Fresh or dried plant material may be used for the extraction. The drying process may change the chemical composition of the tissues due to enzymatic degradation of metabolites. It is therefore strongly recommended to freeze-dry the fresh plant and/or to store the plant material in a deep freezer (−80 °C) until analysis. The extraction approach depends on whether we do targeted (e.g., secondary metabolites) or untargeted metabolite profiling. The classical procedures for secondary metabolite extraction are selective and based on the specific physico-chemical properties of the target group(s). In most analytical experiments, metabolites are extracted by decoction, percolation, maceration, ultrasonication, or hot continuous extraction (Soxhlet). However, they suffer from major drawbacks including the consumption of large volumes of polluting solvents and prolonged extraction time, which may lead to the formation of degradation products or artifacts. Furthermore, these conventional methods often suffer from a lack of sufficient selectivity and allow only crude extracts to be obtained, which necessitates further clean-up procedures prior to chromatographic analysis such as liquid-liquid extraction (LLE) or solid-phase extraction (SPE) with conventional sorbents [14].

Metabolite extraction step is much faster applying microwave-assisted extraction (MAE) which uses microwave energy to heat a solid sample immersed in a solvent to extract/desorb analytes from the matrix and depends on the presence of polar molecules or ionic species. Unlike classical conductive heating methods, microwaves heat the whole sample simultaneously, thus leading to very fast and efficient extractions. Different parameters affecting the efficiency of the extraction, such as the nature of the extracting solvent, the particle size distribution, the moisture of the sample, the applied microwave power, and the radiation time, have to be investigated to improve the robustness of the method [19].

Pressurized liquid extraction (PLE), an another fast extraction technique also known as pressurized solvent extraction (PSE), enhanced solvent extraction (ESE), pressurized fluid extraction (PFE), or accelerated solvent extraction (ASE™), is

considered as environmentally friendly because it requires only small volumes of solvents. Elevated temperatures (usually between 50 °C and 200 °C) and pressures (between 10 and 15 MPa) are used in closed vessels, which allow extractions to be completed in a very short time. It is applicable for thermostable metabolites. High pressure allows the solvent to remain in its liquid state even at temperatures above its boiling point and forces it into the matrix pores. High temperatures decrease the solvent viscosity and increase metabolite solubilization, the diffusion rate, and mass transfer kinetics, thus facilitating desorption of the analytes from the plant material. Most PLE applications reported in the literature employ the same organic solvents as those commonly used in conventional solid-liquid extraction techniques. Critical parameters were found to be pressure, temperature, and extraction time. One extraction cycle is generally applied for 5 to 20 min at temperatures ranging from 50 to 140 °C in the vast majority of applications [14].

Solid phase microextraction (SPME) is a straightforward, solvent-free, selective, and fast sample extraction technique. SPME has become a widely used technique in many areas of analytical chemistry, such as food analysis, environmental sampling, forensics/toxicology, and biological analysis. Recent reviews have been published showing the latest development of this versatile extraction method [20, 21]. It is based on the partition of the analyte between the extraction phase and the matrix. The technique can be used to monitor analytes in liquid samples and is basically compatible with GC. SPME is realized with a fiber incorporated into a syringe and coated with a liquid (polymer), a solid (sorbent), or a combination of both. Extraction can be conducted as a direct extraction in which the coated fiber is immersed in the liquid sample or in the headspace configuration by exposing the fiber to the volatile fraction above the liquid sample [14]. Quantitative determination of analytes (e.g., cocaine in leaves of *Erythroxylum coca*) can be achieved in less than 5 min by SPME [22].

Despite the numerous applications of MAE, PLE, and SPME developed for a wide variety of natural compounds from different matrices, surprisingly, they still have quite limited application in chemical studies of in vitro plant material.

In the recent years, GC-MS takes part in metabolite profiling of tissues and biofluids alone or as a complementary technique covering a broad spectrum of primary and secondary metabolites. The developed methodologies are applicable in plant in vitro metabolite studies as they require typically 20–200 mg of fresh plant tissues [15, 23, 24]. The enzymatic processes in the fresh plant tissues used for extraction are quenched typically by liquid nitrogen and precooled methanol after harvesting [25]. Alternatively, the plant material can be freeze-dried [23]. After homogenization (e.g., with vibrating ball micro mill, [26]), the metabolites are extracted with organic solvents (methanol, ethanol, acetonitrile, acetone) or mixtures of water and organic solvent (mainly methanol) with different proportions and polarity, respectively. In these studies, the strategy is to extract exhaustively the metabolites for further metabolite profiling directly or after extract fractionation. The extraction is performed with solvent volumes of ca. 1–2 mL in tightly closed vials (e.g., screw cap Eppendorf tubes) at temperatures of 70 °C for 15 min [23]. When necessary, antioxidants such as 2,6-di-tert-butyl-4-methylphenol (BHT) or ascorbic

acid have to be added to the extragents to prevent oxidation of unstable metabolites, e.g., oxolipins, phytohormones [26]. Aqueous methanol, acidified with formic acid has pronounced pH dependency regarding the precision of metabolite abundance and the number of detectable metabolites. Methanol and acetonitrile have been found to be complementary with respect to extraction efficiency, and their beneficial properties can be combined by the use of a water-methanol-acetonitrile mixture for global metabolite extraction instead of aqueous methanol or aqueous acetonitrile alone [27].

The MS fragmentation and the peak intensity, respectively, of a metabolite depend on its chemical nature. Therefore, internal standards have to be added to the plant material in the beginning of extraction for the generation of reliable quantitative data for the main metabolite groups. Compounds, similar to the analyzed metabolite structure, which naturally do not occur in the samples, are used as internal standards. Nonadecanoic acid for apolar, ribitol for polar metabolites, 3,4-dichloro-4-hidroxy benzoic acid for phenolic compounds, and betuline for phytosterols have been reported as internal standards for relative quantification in a number of articles [18, 23, 28, 29]. For absolute quantification, construction of calibration curves with standard compounds has been reported for a number of metabolites in plant extracts [23] but obviously this approach is not applicable for all metabolites in a studied plant. Ideally, stable isotope labeled metabolites can be used for absolute quantification as they have the same physico-chemical properties as the target plant metabolites [26].

3.2 Methods of Fractionation/Purification and Derivatization

The crude plant extracts consists of hundreds of compounds from different metabolite groups and diverse properties. Essential oils and some alkaloid groups, among others, can be analyzed by GC-MS without prior derivatization [5, 30]. GC-MS profiling of the polar amino acids, organic acids, free phenolic acids, sugar alcohols, mono-, di-, and tri-saccharides, flavonoid aglycones and the apolar phytosterols, tocoferols, fatty acids (saturated and unsaturated), fatty alcohols, mono-, and diglycerides is performed after appropriate derivatization [15, 31, 32]. Fractionation of the crude extract into apolar and polar fractions reduces the number of compounds in the analyzed sample resulting in better chromatographic separation, possibility for concentration and better identification of minor metabolites and different derivatization approaches leading to a significantly higher number of detected and identified metabolites as compared to nonfractionated extracts. The separation of the polar and apolar metabolites in aqueous and organic phases, respectively, is facilitated by addition of water and chloroform to a methanol or methanol:water extract followed by centrifugation and complete drying before derivatization [15, 28].

N,O-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) was found to be the best derivatization(silylation) agent for metabolite profiling. Incubation with BSTFA for 30 min at 37 °C has been found to be sufficient for derivatization of plant metabolites [23]. The carbonyl groups in sugars and sugar derivatives need to be

metoxymated prior silylation of the polar fraction in order to reduce the number isomers. Methoxymation prevents ring formation by reducing sugars and stabilizes carbonyl moieties in the β-position. The best conditions for methoxymation are incubation with methoxymation reagent (20 mg ml^{-1}methoxyamine hydrochloride in pyridine) for 90 min at 30 °C [23]. The TMS derivatization shows poorer reproducibility and instability for amino acids. When the amino acids are in the focus of analysis, methylchloroformate (MCF) derivatives provide better analytical performance [31]. In a number of chapters, the apolar fraction is directly sylilated prior GC-MS analysis. The number of detected and identified fatty acids, however, can be increased if their carbonyl group is transmethylated prior the silylation of apolar fraction due to the higher number of MS spectra of fatty acid methyl ester derivatives (e.g., hydroxy-fatty acids) in the available MS libraries [18, 33]. A GC-MS-based method for the simultaneous quantification of common oxylipins, including jasmonic acid derivatives, along with labile and highly reactive compounds based on in situ derivatization with pentafluorobenzyl hydroxylamine to the corresponding *O*-2,3,4,5,6-pentafluorobenzyl oximes (PFB oximes), has been proposed by Schulze et al. [34]. Useful information on GC analysis and MS identification of lipids can be found at the Lipid library [18].

In case when the target metabolites are a minor compounds (>2–5% of the total ion current, e.g., alkaloids, phytosterols, terpenes, oxylipins, phenolic acids) in the apolar or polar fractions, their further fractionation and concentration is needed before GC-MS analysis. This approach allows the detection of more target compounds, on the one hand, and to apply specific derivatization agents to obtain better reproducibility and sensitivity, on the other.

The alkaloids are extracted as salts or bases and fractionated in the aqueous or apolar solvent, respectively, depending on the pH of the extragent. Their further separation from inert compounds is based on basic-acidic SPE [35] or LLE of the crude extract resulting in pure alkaloid fraction ready for GC-MS analysis. Formation of emulsions during LLE may made the process inefficient, but centrifugation of the mixture or applying prepacked columns with diathomeous earth avoids this problem [36, 37]. A fast separation of alkaloid fractions from in vitro cultures subjected to metabolite profiling is developed using aliquots of the total methanol extract [38]. Equal parts of methanol extract and 2% sulfuric acid in distilled water are mixed and the neutral compounds eliminated by extraction with chloroform applying vortexing for extraction and centrifugation to remove the emulsion. After that the mixture is basified with 25% ammonia and the alkaloids extracted with chloroform which is concentrated before GC-MS.

Free amino acids can be analyzed after derivatization of the polar fraction. For assessment of amino acid patterns, however, water-soluble proteins have to be precipitated by addition of 10% volume of 50% (w/v) trichloroacetic acid, 1 h of incubation on ice, and centrifugation (10,000 g for 10 min). Then the protein pellet is washed with diethyl ether: ethanol (1:1, v/v), dissolved in a small amount of water, and then hydrolyzed in 6 N HCl for 24 h at 100 °C. HCl is removed under reduced pressure, and amino acids dried under vacuum and then derivatized [39].

Direct derivatization (e.g., silylation) of the lipid fraction, often applied in plant metabolite profiling, gives access to the free forms of the lipid compounds and hydrocarbons. The lipid fraction, however, contains also esterified and conjugated fatty acids, phytosterols, fatty alcohols, and terpenes, which need to be converted into their free form before GC-MS analysis. They can be hydrolyzed by heating under reflux with an excess of dilute aqueous ethanolic alkali (e.g., 1 M solution of potassium hydroxide in 95% ethanol) for 1 hour; alternatively, at room temperature overnight. Then the fatty acids, apolar nonsaponificable materials, and any water-soluble hydrolysis products are recovered for further analysis by adding of water and extraction with hexane-diethyl ether (1:1, v/v). The solvent extract is washed with water and dried over anhydrous sodium sulfate, and the nonsaponifiable materials are recovered after solvent evaporation. The nonsaponificable subfraction contains any hydrocarbons, long-chain alcohols, terpenes, tocoferols, carotenoids, and sterols originally present in the lipid sample in the free or esterified form. The water washings are added to the aqueous layer, acidified with 6 M hydrochloric acid, and extracted with diethyl ether-hexane (1:1, v/v; 3 × 5 mL) to recover the free fatty acids after washing the extract with water, drying it over anhydrous sodium sulfate, and removing the solvent by evaporation [18].

Since phytosterols and triterpenes are a minor component with similar structure and properties in the lipid fraction, their subfraction has to be separated converting all conjugated or esterified compounds into their free form prior GC-MS analysis. Free sterols and terpenes and their esters can be fractionated by alkaline hydrolysis (saponification), followed by extraction of the nonsaponifiable material. However, the phytosterols and terpenes may have bonds that are not hydrolyzed by saponification, such as the glycosidic bonds in steryl and terpene glycosides. In this case, acid hydrolysis with hydrochloric acid (~3.5 M) with reflux at 100 °C, followed by lipid extraction using a nonpolar solvent prior to saponification, is necessary [40, 41].

The analysis of plant phytohormones is a challenge due to their low concentration in samples and GC-MS is one of the preferred instrumentation for their determination. Intensive sample preparation leading to separation, purification, and concentration of the phytohormones is needed. Due to instability of jasmonic acid, the time between sample harvesting and sample preparation has to be as short as possible. Different approaches have been used for extraction of phytohormones: with methanol, solution of acetone, and 50 mM citric acid (70:30 v:v,), methanol: water: HCl (6 N); 80: 19.9: 0.1; v/v/v,), 50 mM potassium phosphate buffer (PB buffer, pH 7) [26, 42] etc. SPE (diethyl ether-washed, silica- based aminopropyl matrix) is the most commonly used technique for sample purification and concentration of phytohormones. After column preconditioning with the starting solvent and sample application, the neutral compounds are removed with of $CHCl_3$:2-propanol (2:1, v/v), and the hormone fraction is eluted with diethyl ether containing 2% acetic acid [34]. Acidic phytohormones, including auxins, SAs, JAs, and ABA, can be derivatized with MCF, while cytokinins and GAs can be silylated with MSTFA [42].

Recently, a rapid and convenient derivatization–extraction procedure combining in situ derivatization in aqueous samples with ethyl chloroformate (ECF) with dispersive liquid–liquid microextraction (DLLME) using ethanol–pyridine (4:1, v:v),

both as derivatization catalyst and DLLME dispersant, has been developed for GC-MS analysis of phytohormones [43].

GC-MS is useful for determination of phenolic compounds such as phenolic acids and flavonoid aglycones. Excellent reviews for the analyses of phenolic compounds are provided by [44, 45].

Phenolic acids generally exist in free, esterified, or glycosylated forms which can be extracted sequentially from plant samples. Free phenolic acids are separated by alcohol/water mixtures and analyzed after silylation (BSTFA) of the crude extract or its polar fraction. Subfraction with free phenolic acids can be obtained by extraction of the crude alcohol/water extract with diethyl ether or ethyl acetate at pH 2–3. Hexane can be used to remove lipids before the extraction. Subfraction of bound or esterified phenolic acids is separated after removing the free phenolic acids and lipids and extraction with ethyl acetate. The dried ethyl acetate fractions are treated with 1 M NaOH containing 0.5% sodium borohydride ($NaBH_4$) or in 2 M NaOH for 4 h. After alkaline hydrolysis, the extract is acidified again to pH 2 and the esterified phenolic acids are extracted with diethyl ether. The glycosylated phenolic acids are released by 6 M HCl at 100 °C for 1 h and extracted with diethyl ether [44, 45].

The method of choice for analysis of flavonoids is HPLC. Their aglycones, however, can be analyzed by GC-MS after hydrolysis and derivatization. Flavonoids are often extracted with methanol, ethanol, acetone, water, and their mixtures. After elimination of the lipids by hexane, the free flavonoids are extracted with ethyl ether. The bounded flavonoids are liberated by hydrolysis for 2 h at 80–90 °C of the acidified extract to pH 1 (6 M HCl) in presence of antioxidant (e.g., ascorbic acid, BHT) to prevent oxidation of the mixture. Subfraction of flavonoid aglycones suitable for derivatization and GC-MS analysis is obtained by extraction with diethyl ether or ethyl acetate. BSTFA is commonly used for derivatization of flavonoids prior GC-MS analysis [45, 46].

3.3 Metabolite Identification

The identification of metabolites by GC-MS is based on the match of their mass spectra and retention times with those of standard compounds (co-chromatography). Instead of retention times, the Kovats retention index (RI) is widely used in GC-MS. While retention times vary with the individual chromatographic system (e.g., with regards to column length, film thickness, diameter, carrier gas velocity, and pressure), the derived retention indices are quite independent of these parameters and allow comparing values measured by different analytical laboratories under varying conditions. Mass spectra of stereo isomers and positional isomers often yield nearly identical spectra and are therefore not unique enough for unambiguous compound identification [47]. The MS spectra of many plant metabolites (e.g., unsaturated fatty acids) do not show abundant molecular ion (M^+) and the RI is crucial for their identification. In such cases, chemical ionization may be applied for unequivocal determination of the M^+ [48]. There are many compiled commercial and online-based mass spectral/retention index libraries and softwares to search, match, and

compare the MS spectra [45, 49]. Due to the complexity of the plant extracts, many peaks in a GC chromatogram may be overlapped and the MS spectra of the metabolites must be deconvoluted before their search and match against reference MS spectra in the MS libraries. Deconvolution and analysis programs are freely available, such as AMDIS and Tagfinder [45]. Many EI-MS spectra in the commercial libraries are usually collected by direct inlet, which should be considered in the process of compound identification. Mass fragmentation of some compounds may be changed under GC conditions [50].

Many plant metabolites are not available in the accessible MS libraries and then their identification relays on literature data or application of more sophisticated MS detectors. The structure of many new and low level secondary metabolites has been assigned solely on a base of GC-(EI)MS data [51–53]. Despite that EI-MS spectra are highly specific, coupling GC with high-resolution or tandem detectors provides valuable structural information facilitating the identification of both known and new metabolites [54]. The exact mass of molecular ions and fragments provided by high-resolution mass analyzers working in tandem is crucial for structural determination of new compounds knowing the fragmentation pattern of a set of structurally similar compounds [53]. Stereo isomers and positional isomers can be unequivocally distinguished after preliminary chromatographic separation and MS/MS experiments, respectively [8]. In some cases, the absolute configuration of the compounds, like those of the crinane series of the Amaryllidaceae alkaloids differing in the position (α or β) of the 5,10b-ethano bridge, can only be distinguished by a circular dichroism analysis after isolation of the metabolite [55].

The generated data from metabolite profiling are generally subjected to statistical analysis to discriminate the samples and to reveal their significance. The data pre-treatment and statistics are not a subject of this chapter. Useful information with descriptions of the methods, their advantages and weaknesses, and applications in metabolite analysis can be found in other review articles [56, 57].

4 Application of GC-MS in Plant In Vitro Studies

The application of GC-MS in plant in vitro studies has a long history and a number of articles can be found in the literature. Typically, the GC-MS has been used for quantification of valuable plant metabolites produced in vitro, profiling of lipids and some secondary metabolites, such as alkaloids and terpenes. The range of plant metabolites covered by GC-MS has been expanded with the advance of methodology and GC-MS instrumentation which made possible the application of GC-MS in interdisciplinary studies where the plant in vitro technologies takes part. Generally, the most of plant metabolites with molecular weight up to 800 Da can be analyzed with appropriate derivatization. Despite the methodological advantages demonstrated in the investigations of intact plants, the GC-MS is still rarely used in the analysis of some metabolite groups in in vitro plant cultures such as phenolic compounds, phytohormones, and amino acids.

Here, some selected and useful examples will be presented demonstrating the power of GC-MS for profiling of different metabolite groups in plant genetic, physiology, and biotechnology investigations involving plant in vitro techniques.

The phytosterols and terpenes are important secondary metabolites which can be analyzed by GC-MS. The potato sterol side chain reductase 2 (St SSR2) was identified as a key enzyme in the biosynthesis of cholesterol and related toxic steroidal glycoalkaloids (SGAs) after GC-MS of the sterol fractions of in vitro cultivated genetically transformed potato plants, suggesting that SSR2 is a promising target gene for breeding potatoes with low SGA levels [40]. The samples were extracted with $CHCl_3$:methanol (1:1), and after the addition of Celite and solvent evaporation, the adsorbed samples were placed in columns and eluted hexane:$CHCl_3$ (1:3, v/v), hexane:EtOAc (2:1,v/v), and $CHCl_3$:methanol (1:1, v/v), adding isotope-labeled cholesterol as an internal standard. After saponification of the hexane:$CHCl_3$ eluent (esterified sterol fraction, 4 M KOH in ethanol for 1 h at 80 °C) and hydrolysis of the $CHCl_3$:methanol eluent (steryl glycoside fraction, 4 M HCl for 1 h at 80 °C), the released sterols were fractionated (with hexane) and trimethylsilylated together with the hexane:ethyl acetate eluent (free sterol fraction) before GC-MS analysis. Twelve phytosterols were determined using co-chromatography with authentic compounds and tentative identification with literature data.

Phytosterols and triterpenes production from cell cultures of *Hyssopus officinalis* L were examined by GC-MS. Dried cultures were extracted with dichloromethane, dichloromethane: methanol (1:1, v/v), and methanol and the combined fractions evaporated and partitioned in methanol:water. The sterols and triterpenes were extracted with chloroform and fractionated by column chromatography identifying two sterols and six triterpenes including oleanolic and ursolic acids [58].

The metabolite profiles of polar butanol and apolar ether extracts from in vitro cultivated *Cocconeis scutellum*, a benthic diatom, producing early programmed cell death (apoptosis) of the male gonad and the androgenic gland of the protandric shrimp *Hippolyte inermis* Leach. have been studied by GC-MS [48]. The polar fraction has been metoxymated and then sylilated, while the metabolites of the apolar fraction were analyzed in parallel as sylilated and methylated derivatives. More than 150 metabolites have been detected, 100 of which identified in both fractions, including amino acids and *N*-containing metabolites, organic acids, phosphates, fatty acids (saturated, monoenes, dienes, polyenes, fatty alcohols, alkanes, carbohydrates (monosaccharides and disaccharides), glycerides (monoglycerides and diglycerides), sterols, and isoprenoids. The M^+ ion of long chain polyenes is hardly detectable by EIMS and GC-MS analysis in CI mode was used to display their molecular weight.

A isotope-labeling approach with multiple carbon sources was used to quantitatively reflect fluxes of central carbon metabolism in in vitro developing *Brassica napus* embryos profiling by GC-MS fatty and amino acids (after hydrolysis of the seed proteins) [39]. The amino acids were analyzed as tert-Butyldimethylsilyl (TBDMS) derivatives and fatty acids as TMS derivatives. Analysis of 13C isotope isomers of labeled fatty acids and plastid-derived amino acids indicated that direct glycolysis provides at least 90% of precursors of plastid acetyl-coenzyme A (CoA). Approximately 30% of carbon in seed protein was derived from exogenous amino

acids and as a consequence, the use of amino acids as a carbon source may have significant influence on the total carbon and energy balance in seed metabolism.

GC-MS-based metabolite profiling was used to analyze the response of *Medicago truncatula* cell cultures to elicitation with methyl jasmonate, yeast elicitor, or ultraviolet light [24]. The dried tissues have been extracted with chloroform containing docosanol as internal standard for 45 min at 50 °C. After that water containing ribitol has been added to the chloroform and incubated for a second 45 min period. The biphasic solvent system has been centrifuged to separate the polar and apolar metabolite fractions. The polar fraction has been methoxymated and then sylilated, while the apolar fraction was directly sylilated. Approximately 40% of quantified peaks have been identified (72 out of 169 for polar and nonpolar metabolites). Marked changes in the levels of primary metabolites, including several amino acids, organic acids, and carbohydrates, were observed following elicitation with jasmonate. A similar, but attenuated response was observed following yeast elicitation, whereas little response was observed following UV elicitation.

Alkaloid and lipid dynamics was studied in calli, shoot-clumps, and regenerated plants of *Pancratium maritimum* L [33]. Ninety-eight lipid compounds and sixteen alkaloids were detected by GC-MS. The results demonstrated that alkaloid synthesis in *P. maritimum* is closely related with tissue differentiation. The highest amount of alkaloids and presence of homolycorine and tazettine type compounds (end products of the biosynthetic pathway of the Amaryllidaceae alkaloids) were found in highly differentiated tissues. Saturated fatty acids were found in considerably higher levels in undifferentiated callus cultures and partially differentiated shoot-clumps than in regenerated plants. α-Linolenic acid (a trienoic acid) was found in higher amounts in the photosynthesizing leaves of shoot-clumps and regenerated plants than in bulbs and calli.

Metabolite profiling of crude metabolic extract from *Leucojum aestivum* shoot-clumps revealed 88 metabolites two of which were alkaloids. Further fractionation, purification, and concentration of the alkaloid fraction resulted in detection of other seven alkaloids [38]. GC-MS analysis of *P. maritimum* shoot-clumps and cultivation medium revealed the synthesis of 22 alkaloids, 13 of which were released into the liquid medium [5].

Volatile compounds from plants, callus tissue cultures, and cell suspensions of parsley (*Petroselinum crissum*) were captured during the growth cycle using a dynamic headspace extraction and were identified by GC-MS revealing that the plant produce mainly monoterpenes, and the compound of major abundance is p-1,3,8-menthatriene, followed by beta-phellandrene and apiole. Callus cultures and cell suspensions produced aldehydes (nonanal and decanal) that were also detected in parsley plant. The production of volatiles in plants, callus tissue, and cell suspensions was found to be time-dependent [59].

A GC-MS study on the effects of indole-3-acetic acid, benzyladenine, zeatin, and kinetin on rooting, biomass production, and volatile compounds production in in vitro shoots of thyme (*Thymus vulgaris* L.) revealed quantitative changes of three major terpene compounds, gamma-terpinene, p-cymene, and thymol (extracted by SPME) in response to the effect of varying growth regulators concentrations in

the culture medium [30]. Similarly, the jasmonic acid added to the culture medium affected the terpenoid composition of *Lavandula angustifolia* essential oils depending on the content of jasmonic acid [50].

Terpenoid production in two lines of hairy root culture of *Ageratum conyzoides* L. induced by *Agrobacterium rhizogenes* ATCC 15834 and analyzed by GC-MS revealed that the main terpenes, β-farnesene, precocene I, and β-caryophyllene vary depending on light conditions and age. Qualitative and quantitative differences were found between the volatile oils from the roots of the parent plant and those from the hairy roots [60].

The effects of plant hormones and sucrose on potato tuberization were studied in vitro using phytohormone profiling by GC-MS in 300 mg fresh samples and detecting GA_1, GA_4, GA_9, and GA_{20} and ABA, indicating that GA is a dominant regulator in tuber formation while ABA stimulates tuberization by counteracting GA and Suc regulates tuber formation by influencing GA levels [26].

5 Conclusions and Future Prospects

Gas chromatography-mass spectrometry is a widely used analytical platform in plant metabolite profiling which undergo remarkable methodological and technological developments expanding its possibilities for wider application in plant biotechnology. With the development of improved metabolomics and targeted metabolite profiling methods, the broader measurements of metabolites are now becoming routine approach to characterize plant in vitro systems. In contrast to the technological maturity of GC-MS systems, the identification of unknown or novel compounds is particularly challenging and requires development of advanced commercial and publicly available spectral libraries facilitating data exchange between laboratories.

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Microbial Transformations of Plant Secondary Metabolites

4

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Abstract

The aim of this chapter is to present the authors' view on the place and role of microbial transformation reactions as a perspective means of processing of plant-derived biologically active compounds into metabolites with new and/or increased activity and availability and decreased toxicity. Some microbial transformations providing information regarding metabolism in humans and mammals of plant-derived secondary metabolites applied as drugs and/or food additives are also considered.

Keywords

Plant-derived biologically active compounds · Plant secondary metabolites · Microbial transformation · Microbial metabolism · Drug metabolism

1 Introduction

Microbial transformations are enzyme and/or whole-cell processes of hydroxylation, dehydrogenation, methylation, etc., affecting different positions of the molecule of organic compounds and resulting in formation of metabolites, which retain the basic structure of the parent compound. Bacteria and filamentous fungi are capable of performing these reactions as an adaptive response to their environment at physiological and/or biochemical level [1]. It is considered that microbial transformation reactions comprise the initial steps in processes of foreign for the microbial cells organic compounds, mostly xenobiotics, which are defined as preparative metabolism. In industrial biotechnology microbial transformations provide irreplaceable steps in the preparation of high added value chemicals and/or their key intermediates among which steroid drugs take the central place [2, 3].

From an application point of view, microbial transformation processes enable conversion at positions in the molecule, which are either difficult or impossible by chemical methods or are not economically reasonable. They proceed at mild conditions regarding pH, temperature, and pressure in contrast to the complicated steps required for their chemical synthesis. Finally yet importantly, microbial transformations allow to obtain pure derivatives in good yield.

Here we will discuss microbial transformations as means of processing of plant origin biologically active compounds into compounds with new and/or improved biological activities, enhanced bioavailability, and decreased toxicity. The application of microbial transformations as useful models for predicting metabolism of plant-derived medicines in humans and mammals and preparation of some metabolites for application in kits, tests, etc., as well as structure-activity relationship studies, will be also considered.

2 Place of Microbial Transformation Reactions in Microbial Metabolism of Plant Secondary Metabolites

Plant-microorganism interactions range from antagonism to symbiosis. Together, plants and microorganisms comprise an integral part of the natural cycle of carbon. In any case, the physiological meaning of these interactions comes down to adaptation. In their evolution plants have developed sophisticated biosynthetic pathways one of them being the synthesis of biologically active compounds to assist invading new habitats or self-dependence.

Microorganisms, in turn, have adapted to the presence of plants and their active metabolites in the natural environment. They either transform plant-derived metabolites into more soluble ones as defense against toxicity or completely utilize them as a single or an additional source of carbon and energy. Atilla Szentirmai gave a brief overview on the β-sitosterol sidechain degradation pathway in which nine catabolic enzymes are involved working in 14 consecutive steps and more than 80 molecules of ATP are formed [4].

3 Microbial Transformation Reactions: Process Performance

The number of microbial transformation reactions implemented to industrial scale spreads over a wide array of areas, from traditional starch processing and baking to the production of fine chemicals and biofuels [5, 6]. Different criteria have been suggested for the definition of biotransformation processes as well as for such processes to be considered of commercial relevance. Based on at least 150 processes, these criteria postulate that the biotransformation process should describe a reaction or a set of simultaneous reactions in which a preformed precursor molecule is converted. In other words, microbial transformation processes are strictly opposite to fermentation ones that involve de novo production from a carbon and energy source through primary metabolism. Enzymes and/or whole cells in different formulations are applied to form either a fine chemical or a commodity product that is typically recovered after the reaction [7, 8].

3.1 Whole Cells Versus Isolated Enzymes

The decision on using isolated enzymes of different degrees of purity or whole cells as catalysts for a given biotransformation depends on several factors. These include the type of reaction, requirements for cofactor regenerations, paramount in redox reactions, the scale of the process, etc. [9, 10]. Whole cell based systems are particularly preferred when cofactor regeneration and multienzyme reaction systems, quite common in the transformation of plant metabolites, are considered [3, 9]. However, whole cells often fail to endure concentrations of substrate or product in

excess of 1 g/L, and their use may be limited in the presence on nonconventional reaction media involving organic solvents. Overall, economic reasons favor the use of whole cell systems, provided that side reactions/unwanted follow up reactions are negligible and diffusion of substrate/products is not hampered. Still, should the later occur, cell permeabilization is an option. It has been estimated that for a whole-cell bioconversion process to be cost-effective, a productivity of 100 g of product per liter of broth per day is required, in a typical fine chemistry process [5, 11–13]. Detailed insight on process metrics, including economic and environmental aspects, can be found elsewhere [12, 14].

3.2 Bacteria or Fungi

Whole-cell biotransformations are typically performed by fungi (molds and yeasts) and bacteria, although recently some interest has also been given to archaea [10, 15–17]. Among fungi, some examples of relevant activity on natural products include *Aspergillus* sp. on ginsenosides, terpenes, and steroids; *Coriolus versicolor* on diosgenin; *Cunninghamella* sp. on bufalin, sesquiterpenes, steroids, triterpenoids, and opiate alkaloids; and *Rhizopus* sp. on sesquiterpenes. *Kluyveromyces marxianus* and *Rhodotorula glutinis* have also been reported for activity on sesquiterpenes as well as *Debaryomyces hansenii* on diterpenes. A drawback of the use of filamentous fungi is related to changes in rheology due to the potential formation of mycelial networks [18]. Among bacteria, examples include the use of *Bacillus megaterium* on the triterpenoid betulinic acid; *Mycobacterium* species in the modification of steroids; *Pseudomonas* species in the modification of alkaloids and flavones; *Nocardia* species in the modification of terpenes; or *Streptomyces* species in the modification of flavonoids, opiate alkaloids, polyphenols, and terpenes [3, 19, 20].

3.3 Growing or Resting Cells

Whole cells are used as biocatalysts either as growing cells or nongrowing ones. In the former case, where sterile conditions are mandatory, biotransformation takes place alongside cultivation, therefore providing for metabolic activity for cofactor regeneration and synthesis of the enzyme or enzymatic pathway. Independent substrates are fed for growth and biotransformation, but optimal operational conditions may not be the same for the two processes; thus, a compromise may be required. The second approach involves the use of nongrowing cells, previously produced and typically harvested in the stationary phase of growth, that are resuspended in either buffer or nutrient-limited media, to which the substrate for bioconversion is subsequently added. Decoupling of biotransformation and growth optimizes the use of energy resources and the tuning of each of the two steps. Both approaches have been used in the microbial production of compounds from plant metabolites [3, 21–23].

3.4 Free and Immobilized Cells

In order to ease downstream processing and allow for biocatalyst recycle/continuous use, immobilization is a strategy often implemented, ultimately enhancing productivity. Additionally, when adequately performed, immobilization enhances biocatalyst stability, namely, to heat nonaqueous solvents and to shear stress, the latter being of particular interest when whole cells are considered. On the other hand, immobilization may lead to biocatalyst deactivation; mass transfer resistances often result and become the overall rate-limiting step and the added cost of carrier, immobilization procedure, and carrier disposal once activity is exhausted. In addition, unrestrained growth may occur, leading to matrix burst or cell detachment with concomitant contamination of the product stream and decreased activity. Mass transfer hindrances advise in particular against the use of immobilized cell systems in redox reactions [8, 12, 18, 24]. Among the different methods available for biocatalyst immobilization, viz., carrier cross-linking or flocculation, chemical or physical binding (including biofilms) to a carrier, and entrapment/encapsulation, the latter may be considered the preferred when cell immobilization is considered, although physical (surface) attachment is also common as it occurs naturally [8, 18, 24–28]. Retention of whole cells using membrane systems is less common, yet a renewed interest on this approach has been reported [8, 18, 27, 28]. Microbial transformations of plant metabolites mostly involve the use of free whole cells [3, 19, 20]. Still, there are some examples of the use of immobilized cells, such as entrapment in calcium-alginate beads of *Nocardia iowensis* for the conversion of oleanolic acid into oleanane derivatives, aiming at continuous operation [29] and *Pseudomonas aeruginosa* resting cells for hydroxylation of tyrosol [30]. Alginate was the hydrogel used for the immobilization of *Rhodotorula minuta* for the biotransformation of (L)-citronellal to (L)-citronellol [31].

3.5 Substrate Addition

Most biotransformation processes are performed in hydrophilic aqueous media, as water is easy to handle, widely available at low cost, nontoxic and environmental friendly, a good solvent for polar substrates, and the typical environment of most biocatalysts. Nonetheless this methodology often limits process productivity, due to the poor solubility of substrates and/or toxic nature of substrates/products, as most of those with relevance for drug production are hydrophobic [11, 32, 33]. Several different approaches have been tested to overcome such limitations, as described in more detail in the following subsections [6, 32, 33]. Criteria for the selection of the most adequate strategy for substrate addition, alongside with other critical issues in the design of complex biotransformation systems, have been recently presented [34].

The use of micronized substrate has been suggested in order to improve the contact between poorly soluble substrates and the whole-cell catalyst. This method has been reported for steroids/sterol and digitoxigenin bioconversions, individually or combined with other methods, viz., combination with surfactants/organic

solvents, with various levels of success [35–39]. Surfactants are also used to improve the solubilization of hydrophobic substrates [40]. Care has to be taken, particularly when growing cells are used, as high concentrations of surfactant may interfere with cell growth and activity [41]**.** The use of cyclodextrins and derivatives has also been reported as a tool to improve the solubility of hydrophobic substrates, particularly sterols [39, 42–44].

The use of organic solvents, either water miscible or water immiscible, has become a common strategy to improve the productivity of bioconversions by enhancing solubility of substrate molecules. Moreover, when water-insoluble organic solvents are used, a two-phase system is created, which allows for the controlled delivery of substrate as well and provides a product sink. This approach also overcomes the potential toxic effect of substrate and/or product on the biocatalyst, since whole cells tend to remain in the aqueous phase with a few exceptions. A careful selection of the solvent carrier is required, in order to obtain an adequate compromise among solubilization ability, physical and chemical properties (viz., flammability, melting point/vapor pressure), and toxic effects on the biocatalyst, cost, and environmental impact. As a rule of thumb, the log P criterion (logarithm of the partition coefficient of the solvent in a standard n-octanol-water system) is often used to predict toxicity [32, 33, 45–47].

The use of liquid polymers such as polypropylene glycol and polyethylene glycol as substrate/product carriers, mainly as a less toxic alternative toward both microbial cells and environment when compared to organic solvents, has also been reported, namely, for sterol biotransformations. The use of these carriers has been implemented in either single-phase or two-phase systems, the former involving nongrowing cells [37, 39].

A cloud point system consists of an aqueous micelle solution of a nonionic surfactant exposed to a temperature either above its cloud point or in the presence of certain additives. In those situations, phase separation takes place, leading to the formation of a surfactant-poor phase and a surfactant-rich phase. The two-phase system is reported to provide a mild, nontoxic environment for whole cells [39, 48].

Ionic liquids consist of ions that are liquids at room temperature. They display a set of properties, namely, low vapor pressure and melting point, large electrochemical window, and thermal stability, which mostly depend on the nature of the anion/cation and on the alkyl chain of the anion; hence, they can be tuned according to the envisaged application. They can be used with polar substrates and have been used in reaction systems as sole solvent and cosolvent and in two liquid phase systems [49, 50]. These features make them particularly attractive for use in microbial biotransformations [49, 51, 52]. However, ionic liquids are relatively expensive and data on their toxicity is still scarce [33].

Deep eutectic solvents (DES) consist of mixtures of quaternary ammonium salts, viz., choline chloride, and hydrogen bond donors, viz., alcohols, carboxylic acids, glycerol, and urea derivatives. DES melt at low temperatures as a result of charge delocalization resulting in the hydrogen bonding involving the two different types of molecules [53]. DES share the advantages of ionic liquids, but unlike these, they also display nontoxic and biodegradable nature and can be easily prepared at low cost.

Although quite recent, DES have been already tested with success in whole-cell biotransformations [54–57].

4 Processing of Plant Secondary Metabolites

Plant extracts (water, alcoholic, or fermentative ones) are used both in traditional and conventional medicines as part of cultural traditions of people all around the world, traditional Chinese medicines and Ayurveda being the best-known examples. Many of their active principals are identified and described as plant secondary metabolites and synthesized to become pharmaceutical products. Others became starting material for semisynthesis of valuable medicines. Intensive use of medicinal plants, however, endangered largely their existence and calls into question the protection of biological diversity. In this context, the development of green technologies may contribute to the preservation of plant diversity worldwide simultaneously providing substrates for the sustainable production of food additives and pharmaceuticals with even better characteristics. Microbial transformations of phytosterols reveal the irreplaceable role of microorganisms in the development of steroid-based pharmaceuticals [2, 3]. Due to their unlimited potential for adaptation to different sources of carbon and energy, microorganisms are capable of transforming biologically active plant-derived metabolites, some of them toxic and/or water insoluble, into less toxic and easily available derivatives with higher potential for practical application.

4.1 Microbial Transformations for Improving Qualities of the Plant-Derived Secondary Metabolites

4.1.1 Microbial Transformations of Terpenes

Terpenes are the largest group of plant-derived biologically active secondary metabolites submitted to microbial transformations. Diterpenes with taxane skeleton (presented in *Taxus* plants) may be useful intermediates for the semisynthesis of taxol analogs. Reported was selective microbial hydroxylation of a series of taxoids with 4(20) exocyclic double bond. It was found that 5α,7β,9α,10β,13α-pentaacetoxy-4(20),11-taxadiene could be transformed into more polar C-1 and C-14 hydroxylated derivatives by *Absidia coerulea*, *Cunninghamella echinulata*, *C. elegans*, *C. blakesleeana*, and *Rhizopus arrhizus* [58].

Cunninghamella elegans AS3.2033 and *C. elegans* var. *chibaensis* ATCC 20230 transform 4(20),11(12)-taxadiene derivative with formation of C-1 and C-15 hydroxylated derivatives as main products. Further, reactions of C-14 oxidation and *trans-cis* isomerization of the cynnamoyl were carried out by *C. elegans*, while *C. elegans* var. *chibaensis* achieved an unprecedented hydroxylation at C-17 [59].

Cunninghamella echinulata AS 3.1990 transformed some taxoids with an oxygen substituent at C-14 into their 6α-hydroxylated derivatives as main products [60]. C-14 oxygenated taxanes were region- and stereospecifically hydroxylated

by *Absidia coerulea* IFO 4011 at 7β-position. The presence of β-cyclodextrin (aiming at increasing the yield of the 7β-hydroxylated derivatives), when taxuyunnanine C was used as substrate, led to the formation of three other derivatives. Observed reactions of hydroxylation or acetoxylation of the C-7 or C-13 methylene of taxane derivatives are considered useful as they provide valuable intermediates for the synthesis of paclitaxel or other bioactive taxoids [61].

Fungal strains *Absidia coerulea* and *Mucor genevensis* gave rise to 53 derivatives of 4(20),11(12)-taxadienes sinenxan A and its two derivatives and of yunanxane through an array of reactions including hydroxylation, epoxidation, oxidation, hydrolysis, acylation, *O*-alkylation, *O*-glycosylation, rearrangement, etc. [62].

The candidiol (Fig. 1) 15α,18-dihydroxy-*ent*-kaur-16-ene (present in *Sideritis* plants) was submitted to transformation by *Mucor plumbeus* which led to formation of five hydroxylated products [63].

Diterpenes *ent*-beyer-15-en-19-oic acid and *ent*-kaur-16-en-19-oic acid (Fig. 1) are found in *Viguiera hypargyrea* L. (Asteraceae), a perennial herb growing in Mexico. Submitted to microbial transformation by *Aspergillus niger*, the two substrates were region- and stereoselectively dihydroxylated at 1α,7β-positions. The *ent*-kaur-16-en-19-oic acid was dihydroxylated at 7β,11α-positions as well. The efficiency of the last process was 41% providing a useful tool for preparing new beyerenoic acid derivatives dihydroxylated at these positions [64].

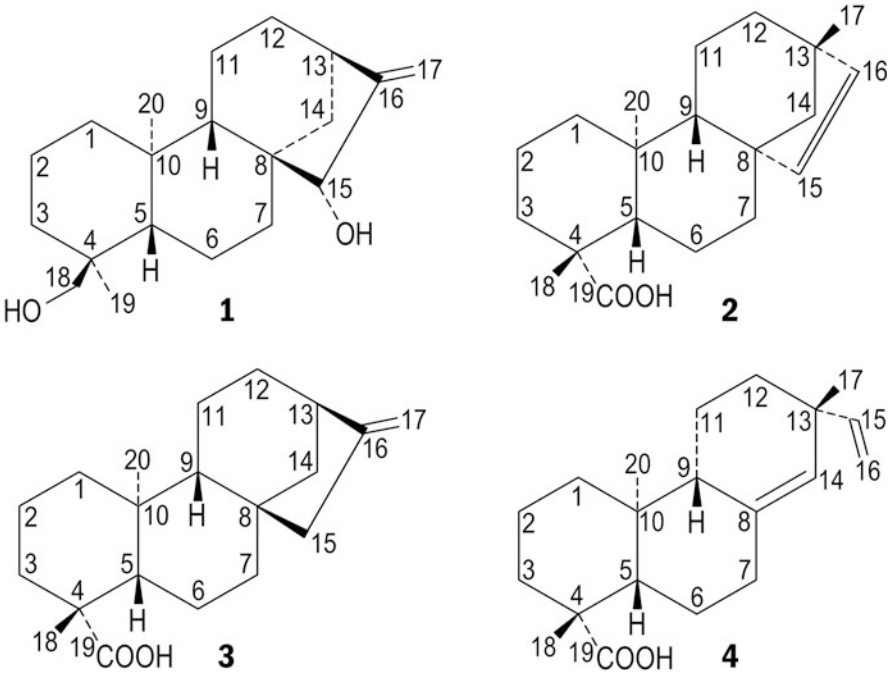

Fig. 1 Structures of kaurene-, beyerane-, and pimarane-type diterpenes: **1**, candilol; **2**, *ent*-beyeranoic acid; **3**, *ent*-kaurenoic acid; **4**, *ent*-pimaradienoic acid

Kauranes are the most abundant component in the traditional Chinese medicinal plants of *Isodon* genus, formerly *Rabdosia* (Lamiaceae). The production of the final, bioactive, *Isodon* diterpenoids, however, clearly requires further decoration of the basic hydrocarbon backbones by downstream tailoring with enzymes such as cytochrome P450 monooxygenases, dehydrogenases, and transferases [65].

Rhizopus nigricans, *Curvularia lunata*, and *Aspergillus niger* transformed *ent*-kaur-15-enones and *ent*-kaur-16-enones affording *ent*-16β,17-epoxy compound (*C. lunata* and *R. nigricans*), *ent*-16α,17- and *ent*-16β,17-glycols (*C. lunata* and *A. niger*), and *ent*-16β-hydroxyl and *ent*-(16*S*),17-hydroxykaurane (*A. niger*) [66].

Results obtained from the transformation of two 15α-hydroxy-*ent*-kaur-9(11),16-diene derivatives with *Fusarium fujikuroi* (*Gibberella fujikuroi*) indicated that the presence of the 9(11)-double bond in the parent compound prevents its 7β-hydroxylation, which is necessary for the formation of gibberellins and *seco*-ring B *ent*-kaurenoids [67].

ent-Pimaradienoic acid (Fig. 1), *ent*-pimara-8(14),15-dien-19-oic acid, present in roots of *Aralia cordata*, was transformed by *Aspergillus niger* strains into four *ent*-pimaradienoic acid derivatives through reactions of hydroxylation, C6–C7 dehydrogenation, and transmigration of the double bond from C8–C14 to C7–C8. The methyl 17α-hydroxy-*ent*-pimara-8(14),15-dien-19-oate obtained was more effective than the substrate [68].

Diterpenes dehydroabietanol, obtained from *Juniperus phoenicea* and *Salvia pomifera*, and teideadiol (Fig. 2), isolated from *Nepeta teydea*, were transformed by *Mucor plumbeus* in six and three metabolites, respectively [69].

Diterpene trachinodiol (Fig. 2), isolated from the endemic to the Canary Isles *Sideritis canariensis* Ait., Labiatae, was transformed by *M. plumbeus* to the corresponding *ent*-kaur-11-ene derivative sicanatriol [70]. Diterpenes triptolide (Fig. 2) and triptonide are active ingredients isolated from Chinese herbal plant *Tripterygium wilfordii* Hook f. Triptolide is effective in the treatment of autoimmune diseases with potent antileukemic and antitumor activities, while triptonide is an effective anti-inflammatory and antiproliferative agent [71]. Li and coauthors reported structure-activity relationships of triptonide, triptolide, and their derivatives as potential selective antitumor agents [72]. Triptolide was transformed by *Cunninghamella blakesleana*, and all seven derivatives obtained exhibited potent in vitro cytotoxicity against human tumor cell lines [73]. Through a two-step chemical process, triptonide can be transformed into 14-deoxy-14-methylene-triptolide in which pharmacodynamic evaluation suggests significant inhibition of human cancer cell growth. The potent application of 14-deoxy-14-methylene-triptolide as an anticancer drug, however, is limited by its significant toxicity. Incubation with resting *Neurospora crassa* CGMCC AS 3.1604 cells afforded a (5R)-5-hydroxy-derivative [71].

Tanshinones (abietane-type diterpene quinones isolated from the roots of *Salvia miltiorrhiza*) are well-known traditional Chinese medicine in the treatment of cardiovascular diseases [74]. They are also potential candidates for tumor therapeutics by enhancing antitumor immune responses as modulators of natural killer (NK) cells

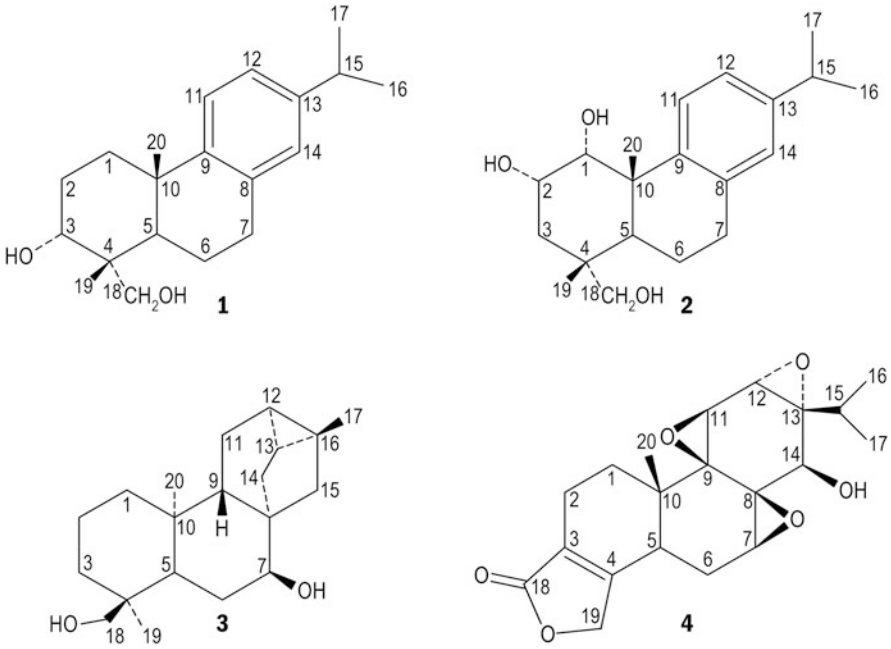

Fig. 2 Structures of diterpenes: **1**, dehydroabietanol; **2**, teideadiol; **3**, trachinodiol; **4**, triptolide

as well [75]. The major lipophilic constituents of *S. miltiorrhiza* are diterpenes tanshinone I, tanshinone IIA, and cryptotanshinone which showed liver protective effects in vivo and in vitro due to its antioxidant effects [76, 77]. It was shown recently that their microbial transformations can lead to derivatives with further improved activities. Thus, the incubation of tanshinone IIA with *Mucor rouxianus* AS 3.3447 leads to formation of two glycosides (tanshinone A and tanshinone B) which anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) activities were significantly increased in comparison with the parent compound [78]. When incubated with *Hypocrea* sp. AS 3.17108, tanshinone IIA afforded tanshisorbicin (an antibacterial agent, which structure is a hybrid of tanshinone IIA and sorbicillinol, the latter known as well as a metabolite produced by *Hypocrea* sp.) [79].

Labdane-type diterpenes are an excellent example of natural products with important therapeutic applications. Being quite abundant in nature and/or commercially available (sclareol, sclareolide, larixol, labdanolic acid, or ozic acid), they are useful starting material for both chemical and biological modifications [80].

Sclareol, labd-14-en-8α,13β-diol (Fig. 3), found in clary sage (*Salvia sclarea* Linn., Labiatae) and a valuable starting material for the hemisynthesis of forskolin, was added to the growing culture of the fungus *Mucor plumbeus* ATCC 4740 and a mixture of triols was obtained. One of the derivatives, labd-14-en-3β,8α,13β-triol was obtained in high yield [81]. Abraham reported microbial transformations of sclareol by different microorganisms leading to formation of 2α-, 3β-, and

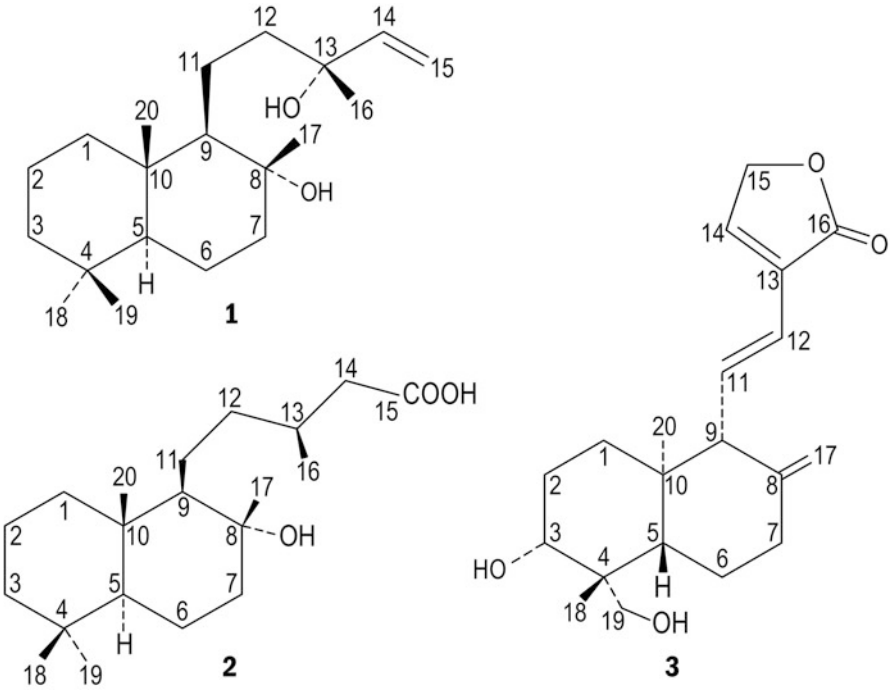

Fig. 3 Structures of diterpenes: **1**, sclareol; **2**, labdanolic acid; **3**, dehydroandrographolide

18-hydroxysclareol, while *Cunninghamella elegans* was shown to form 19-hydroxysclareol additionally [82]. Sclareol was also transformed by the plant pathogenic fungus *Botrytis cinerea* into epoxysclareol and 8-deoxy-14,15-dihydro-15-chloro-14-hydroxy-8,9-dehydrosclareol, the second reaction representing a rare example of microbial halogenation [83].

Labdanolic acid (Fig. 3), the most representative diterpene found in extracts from rockrose (*Cistus ladaniferus*, a wild and persistent plant from the Mediterranean region), was successfully transformed by *Penicillium janczewskii* into 3β-hydroxy-labd-8(17)-en-15-oic acid which in turn is naturally occurring in stems of *Moldenhawera nutans*, Leguminosae [80].

ent-Labdane diterpenoids dehydroandrographolide (Fig. 3) and andrographolide are the principal active constituents of *Andrographis paniculata* leaves, which are widely used to treat acute hepatitis, inflammation, and tumor in China, Southeast Asia, and India. Dehydroandrographolide was transformed by *Cunninghamella echinulata* AS 3.3400 into five derivatives, the major one 9β-hydroxyl-dehydroandrographolide obtained with 72% yield. Two of the metabolites, the 9β-hydroxyl-dehydroandrographolide and 7α-hydroxydehydroandrographolide, exhibited higher cytotoxicity against Bel-7402 and HeLa cells than the parent compound, suggesting that hydroxylation at C-9 or C-7 contributes to the enhanced activity [84].

Boswellic acids are used for the treatment of arthritic diseases. They are the major active component of the gum resin of *Boswellia serrata*, deciduous tree species distributed in some arid zones of Africa and Asia, but unfortunately reveal poor bioavailability [85]. To find derivatives of the 11-keto-β-boswellic acid, KBA (Fig. 4) with enhanced water solubility and bioactivities, it was incubated with *Cunninghamella blakesleana* AS 3.970. Two of the obtained derivatives (7β-hydroxy-11-keto-β-boswellic acid and 7β,22β-dihydroxy-11-11-keto-β-boswellic acid) showed significant inhibitory effect on nitric oxide production in RAW 264.7 macrophage cells. Primary structure-activity relationships indicated that hydroxylation at C-7 and C-22 could reduce toxicity, while retaining the potent inhibitory effects on nitric oxide production. The hydroxylation of C-15, C-16, C-20, C-21, and C-30, however, significantly reduced the bioactivities of nitric oxide inhibition [86].

Cunninghamella blakesleana AS 3.970 transformed the acetyl-11-keto-β-boswellic acid (AKBA) into five metabolites, while *Cunninghamella elegans* AS 3.1207 afforded two. It was assumed from the structural characteristics of the isolated metabolites that the positions of the introduced hydroxyl groups were important for the inhibition effect on lipopolysaccharide-induced nitric oxide production in RAW 264.7 macrophage cells [87].

The pentacyclic triterpene acids possess a wide range of biological activities and are widely distributed in the plant kingdom [88]. The pentacyclic triterpene lupeol (Fig. 4), produced by many plants and more available than betulin, betulinic, and betulonic acids [89], was transformed by each of the filamentous fungi *Aspergillus niger* and *Mucor rouxii* into two products. In this, *A. niger* was good at introducing double bonds in the lupeol structure, whereas *M. rouxii* exhibited the ability to catalyze oxygen insertions [90].

The oleanolic acid (Fig. 4), extracted from different parts of plants like *Rosa woodsii*, *Syzygium claviflorum*, *Prosopis glandulosa*, and *Ternstroemia gymnanthera* as well as from the whole plants *Phoradendron juniperinum* and *Hyptis capitata*, and its structurally related triterpenoids are known for their potent anti-HIV activities [91]. The poor water solubility of the oleanolic acid concerning an intravenous medical treatment is its main disadvantage [92].

The oleanolic acid was transformed by *Nocardia iowensis* into oleanolic and oleanonic acid methyl esters, regarded as potential candidates for novel pharmaceuticals due to their remarkable activities against herpes simplex virus and human immunodeficiency virus reverse transcriptase [29].

Alternaria longipes and *Penicillium adametzi* transformed the oleanolic acid into nine products altogether. Skeletons of two of the derivatives afforded by *Alternaria longipes* (2α,3α,19α-trihydroxy-ursolic acid-28-*O*-β-D-glucopyranoside and 2α,3β,19α-trihydroxy-ursolic acid-28-*O*-β- D-glucopyranoside) were changed from oleanane to uranane ones. The other seven products were glycosidated [93].

One of the four derivatives of the oleanolic acid obtained via biotransformation with *Mucor rouxii* (21β-hydroxy-3-oxo-olean-12-en-28-oic acid) displayed strong activity against the primarily etiological agent of periodontal disease, *Porphyromonas gingivalis* [94].

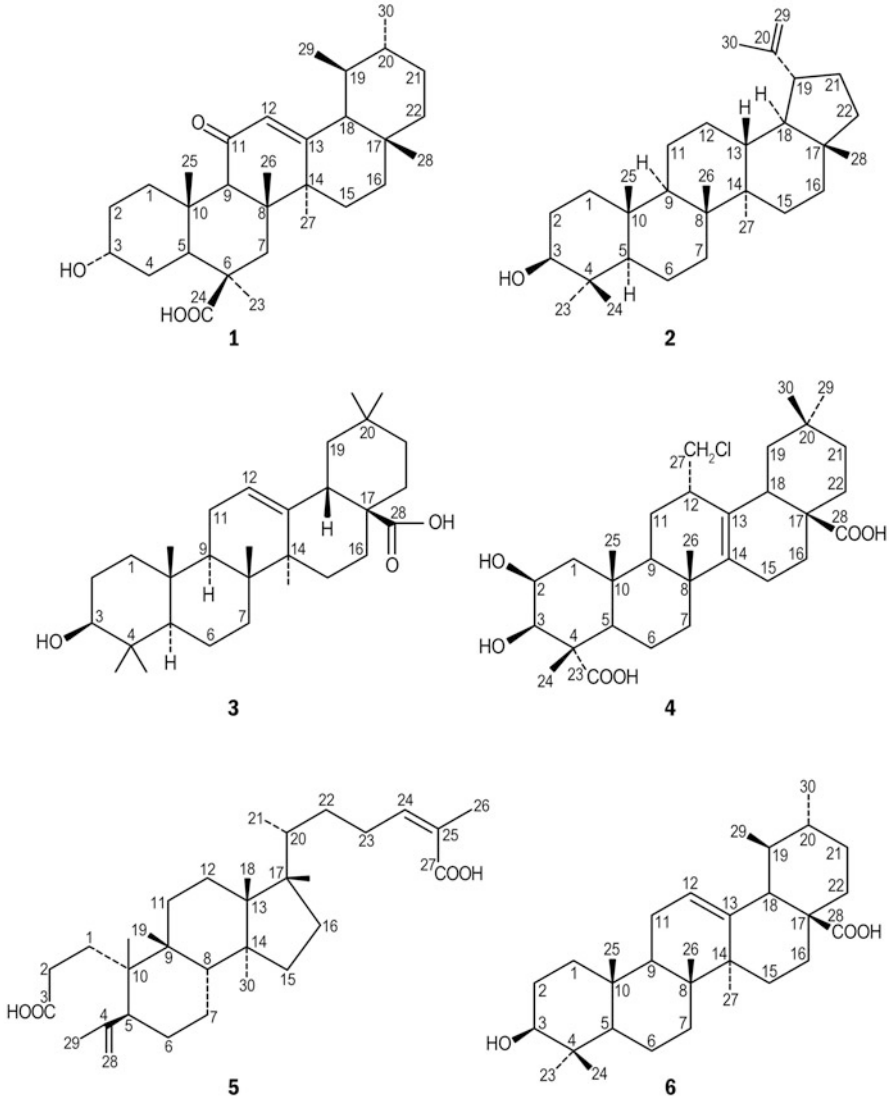

Fig. 4 Structures of pentacyclic triterpenes: **1**, ketoboswellic acid; **2**, lupeol; **3**, oleanolic acid; **4**, senegenin; **5**, nigranoic acid; **6**, ursolic acid

Nocardia sp. NRRL 5646 metabolized senegenin (Fig. 4), ursolic, and oleanolic acids into their corresponding 28-methyl esters. The conversion of ursolic acid to oleanolic acid methyl ester and senegenin to senegenic acid methyl ester, in which skeleton rearrangements occur by methyl migration and elimination of a chloromethylene group, respectively, further exemplified the biocatalytic efficacy of *Nocardia* sp. toward complex natural products, allowing one-step transformations

that are very difficult, if not impossible, to be performed using chemical methods [88].

Nigranoic acid (Fig. 4) is an A ring secocycloartene triterpenoid produced by Schisandraceae plants, possessing a variety of biological activities. It was transformed by *Caryospora callicarpa* YMF1.01026 into 6β-hydroxynigranoic acid [95]. The fungus *Gliocladium roseum* YMF1.00133 hydroxylated the nigranoic acid affording 15β-hydroxynigranoic acid as a major product accompanied by 6α,15β-dihydroxynigranoic acid and 7β,15β-dihydroxynigranoic acid [96].

When *Trichoderma* sp. transformed the nigranoic acid, two new metabolites with weak anti-HIV activity were found. One of these metabolites possessed an unusual 17(20),17(*E*)-ene structure; another one featured an unprecedented 18(13→17β)-abeo-secocycloarta skeleton [97].

The incubation with the freshwater fungus *Dictyosporium heptasporum* afforded two derivatives from the nigranoic acid oxidized at the C-7, C-16, C-17, and C-20 positions. Both metabolites revealed weak anti-HIV activity in vitro [98].

The ursane-type pentacyclic triterpene ursolic acid, 3β-hydroxy-urs-12-en-28-oic acid (Fig. 4), presented in dietary and medicinal herbs shows potent protein tyrosine phosphatase 1B (PTP1B) inhibitory activity. Its biotransformation by the filamentous fungus *Syncephalastrum racemosum* (Cohn) Schroter AS 3.264 yielded five metabolites through reactions of hydroxylation, carbonylation, and condensation. Among them, only 1β,3β-dihydroxy-urs-12-en-21-one-28-oic acid showed moderate PTP1B inhibitory activity in vitro [99].

Ursolic acid derivatives were obtained via its transformation by *Nocardia* sp. strains, and Leipold and coauthors suggested new biosynthetic pathways for their production [100].

The biotransformation of ursolic acid by the filamentous fungus *Syncephalastrum racemosum* CGMCC 3.2500 resulting in the formation of five derivatives was also reported. The structure-activity relationship studies revealed, however, that the anti-hepatitis C virus (anti-HCV) activity of the obtained derivatives has not been affected positively [101].

(−)-Drimenol (Fig. 5), sesquiterpene extracted from the stem bark of a South American tree, *Drimys winteri* Fors., appears to be a valuable starting material for semisynthesis of sesquiterpenoids. It was hydroxylated by *Aspergillus niger* and *Rhizopus arrhizus* at the unactivated C-3 position, while *Mucor plumbeus* afforded an alicyclic product hydroxylated at 6α-position [102].

A mixture of β- and γ-eudesmols (Fig. 5) was transformed by *Gibberella suabinetti* ATCC 20193 into seven eudesmanoidal metabolites identified as carissone, eudesma-4-en-3β,11- diol, and eudesma-4-en-11,15-diol derived from β-eudesmol and eudesma-4(15)-en-8,11-diol, eudesma-4(15)-en-2α,11-diol (pterocarpol), and 1(3) cyclo-eudesma- 4(15)-en-11,12-diol derived from γ-eudesmol [103].

β-Eudesmol, a sesquiterpenoid alcohol isolated from *Teucrium ramosissimum* leaves, may be a novel anticancer agent for treatment of lung and colon cancer either

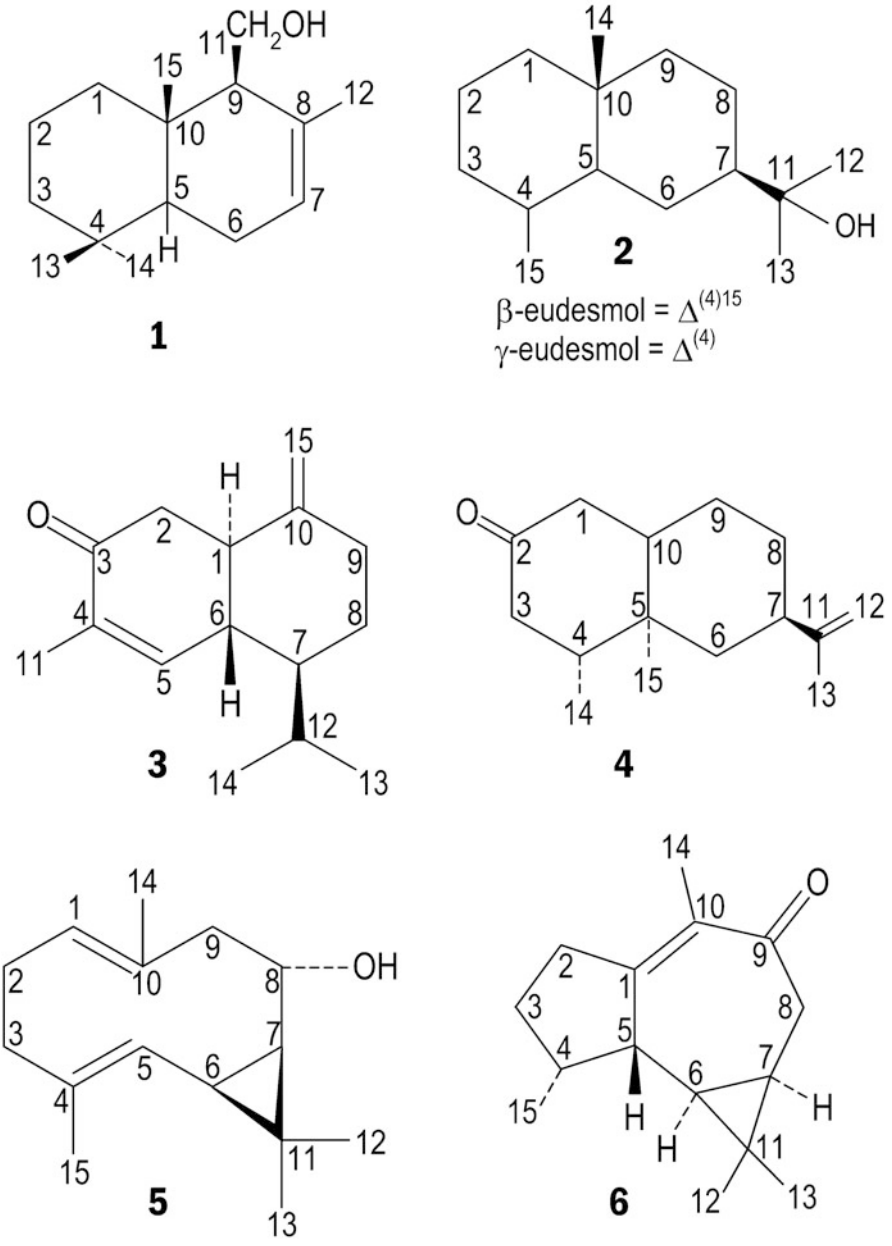

Fig. 5 Structures of sesquiterpenes: **1**, drimenol; **2**, β- and γ-eudesmol; **3**, cadinadienon; **4**, nootkatone; **5**, partheniol; **6**, squamulosone

by inhibition of superoxide production or by blocking proliferation, adhesion, and migration [104].

Amate and co-authors reported a series of biotransformations of 6β-eudesmanolides with functions at C-3 with fungi *Curvularia lunata* and *Rhizopus nigricans*. Some of the obtained products were considered suitable starting material for further chemical or chemical-microbiological processes [105].

Sesquiterpene cadina-4,10(15)-dien-3-one (Fig. 5) isolated in large quantity from the green leaves and twigs of the plant *Hyptis verticillata* was shown to possess insecticidal activity and agaricidal potent. It was subjected to transformation by deuteromycete *Beauveria bassiana* ATCC 7159 in an effort to produce new functionalized analogs with improved biological activities. Nine metabolites were obtained through reactions of reduction, hydroxylation, and elimination. The ketone (4S)-cadin-10(15)-en-3-one showed enhanced activity in comparison with the parent compound [106].

When incubated with *Mucor plumbeus* ATCC 4740, squamulosone, cadina-4,10(15)-dien-3-one, and the triterpenoid methyl ursolate afforded nine new cadinanes. Squamulosone was transformed into five metabolites, reactions being centered on C-2 and C-13 with little stereoselectivity. Methyl ursolate was transformed into a single metabolite, while cadina-4,10(15)-dien-3-one gave rise to seven and nine metabolites, respectively, depending on high or low iron content in the medium [107].

The bicyclic sesquiterpene (+)-nootkatone (Fig. 5), isolated from the heartwood of Alaska yellow cedar, *Chamaecyparis nootkatensis*, and found in trace amount in grapefruit, *Citrus paradise*, is known as a potential insecticide and inhibitor of gastric ulcer formation. Both (+)-nootkatone and one of its metabolites after fungal transformation, 13-hydroxynootkatone, showed strong activity against both human lung adenocarcinoma and human promyelocytic leukemia cells. The authors consider these compounds as good candidates for further studies postulating that the presence of an additional hydroxyl group in the cyclohexane ring contributes to the increased activities [108].

(+)-Nootkatone was transformed also by the plant pathogenic fungus *Macrophomia phaseolina*, giving rise to four main compounds, two of them in stereoisomeric mixture. The substrate and two other products, 13-hydroxynootkatone and 12-hydroxy-11,12-dihydroxynootkatone, revealed significant antiprotozoal activity [109].

Metabolism of partheniol (Fig. 5), sesquiterpenoid from *Parthenium argentatum x P. tomentosa* with fungistatic activity, was studied by *Mucor circinelloides* ATCC 15242. Identified were six partheniol metabolites resulting from microbial hydroxylation, hydration, dehydration, C–C bond cleavage, and transannular cyclizations of the parent compound [110].

The sesquiterpenes squamulosone (Fig. 5), aromadendr-1(10)-en-9-one, and cadin-4-en-1β-ol (isolated from the aerial parts of the plant *Hyptis verticillata* Jacq., Labiatae) were transformed by *Curvularia lunata* to seven novel metabolites, all of them possessing insecticidal activities. Hydroxylation occurred at C-13 methyl group and at C-2 and C-14 allylic positions [111].

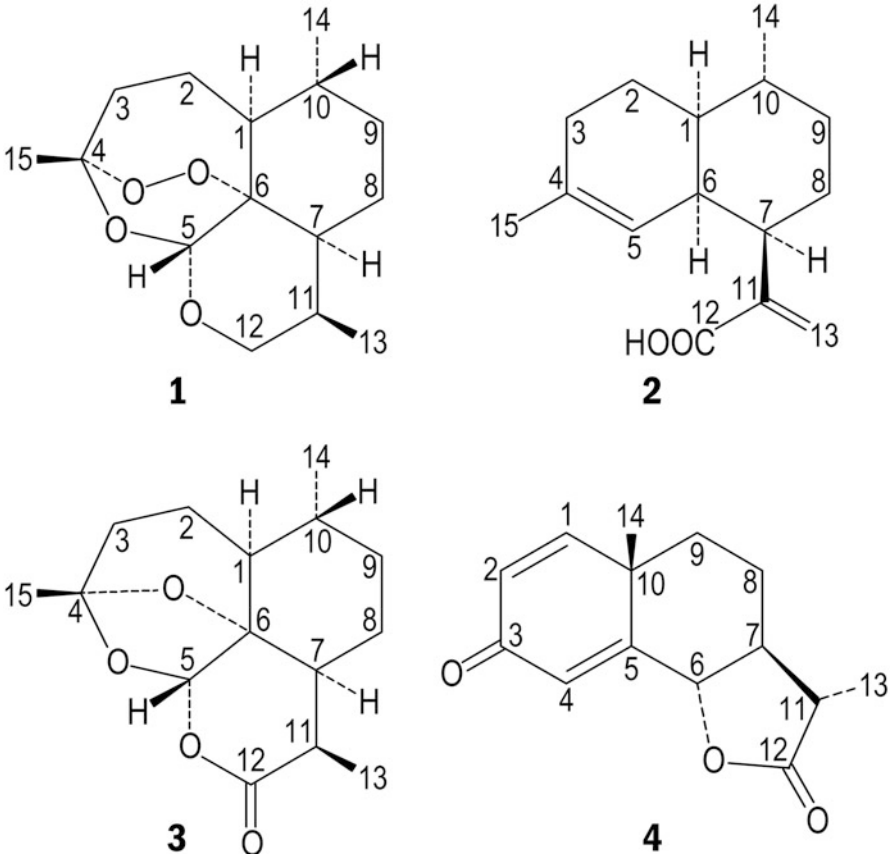

Fig. 6 Structures of sesquiterpene lactones: **1**, artemisinin; **2**, artemisinic acid; **3**, artemisitene; **4**, α-santonin

Artemisinin (Fig. 6) is a sesquiterpene lactone endoperoxide from *Artemisia annua*, an established drug to combat infections with *Plasmodium falciparum* and *Plasmodium vivax*. Most of the structural modifications take place at the lactone moiety of artemisinin; modifications and structure-activity relationship on the two saturated rings remain unexplored due mainly to the difficulty of introducing functionalities on the ring systems by conventional chemical methods [112]. Instead, microbial transformations come to play an important role to overcome the inaccessibility by chemical reactions and to serve as a valuable tool to introduce hydroxyl group(s) on the saturated rings [113]. The fungal strain *Penicillium simplissimum* performed the hydroxylation of artemisinin, which led to the formation of C-9β-acetoxy-artemisinin along with 9α-hydroxy-artemisinin [112]. *Cunninghamella echinulata* AS 4.3400 afforded 10β-hydroxy-artemisinin, while *Aspergillus niger* AS 3.795 afforded 3α-hydroxy-deoxyartemisinin. A key issue is that the hydroxyl group was introduced at C-10 position of artemisinin with the endoperoxide

remaining intact [114]. *Nocardia corallina* ATCC 19070 and *Penicillium chrysogenum* ATCC 9480 transformed artemisinin into two major metabolites, deoxyartemisinin, and 3α-hydroxydeoxyartemisinin [115]. *Mucor polymorphosporus* and *Aspergillus niger* converted artemisinin into five products identified as 9β- and 3β-hydroxyartemisinin, deoxyartemisinin, and 3β- and 1α-hydroxydeoxyartemisinin [116]. *Cunninghamella elegans* converted artemisinin into 7β-hydroxyartemisinin (as main derivative in 78.6% yield) and three other products, 7β-hydroxy-9α-artemisinin, 4α-hydroxy-1-deoxoartemisinin, and 6β-hydroxyartemisinin [117]. *Eurotium amstelodami* and *Aspergillus niger* were reported to transform artemisinin into 5β-hydroxyartemisinin and 7β-hydroxyartemisinin [118]. *Streptomyces griseus* ATCC 13273 produced one major product from artemisinin identified as artemisitone-9 and other three polar metabolites, 9α-hydroxy-artemisinin, 9β-hydroxy-artemisinin, and 3α-hydroxy-deoxyartemisinin. Not surprisingly, 3α-hydroxy-deoxyartemisinin, the metabolite without the endoperoxide bridge, was inactive, whereas other metabolites retained their antimalarial activity, even though they were less active than artemisinin. The decrease of activity by oxidation of artemisinin was expected because oxidative modifications generally are the metabolic reactions of detoxification of drugs in mammals, plants, and microorganisms [113].

Artemisinic acid (Fig. 6), an amorphane sesquiterpene isolated as major compound from the herb *Artemisia annua* L. (Asteraceae), is considered as an immediate precursor of artemisinin. It was transformed by the endophytic fungus *Trichothecium roseum* CIMAPN1 into two major metabolites identified as 3β-hydroxyartemisinic acid and 3β,15-dihydroxyartemisinic acid. The obtained metabolites performed well in comparison with the parent compound both in vivo and in vitro studies revealing their antioxidant and antimicrobial potential. The presence of hydroxyl groups in the metabolites could make them interesting synthones for further modification into new clinically potential molecules [119]. When incubated with *Mucor mucedo* and *Aspergillus flavipes*, artemisinic acid was transformed into epimeric 3-hydroxyartemisinic acids with β- and α-configuration of the hydroxyl, respectively [120].

It was shown recently that artemisinin and its derivatives exert anticancer activity in vitro and in vivo as well. Artemisinin derivatives do not only kill tumor cells by induction of apoptosis, autophagy, or necroptosis as shown in the past but also induce iron-dependent cell death (ferroptosis) in tumor cells. Actually, iron is a crucial determinant of activity of artemisinin-type drugs both in malaria and in cancer [121].

The semisynthetic sesquiterpene artemether was transformed by *Cunninghamella elegans* and *Streptomyces lavendulae* into five metabolites identified as 9β-hydroxyartemether, 9α-hydroxyartemether, ring-rearranged 9β-hydroxyartemether, 3α-hydroxydeoxyartemether, and 14-hydroxartemether [122].

The artemisinin derivative arteether was transformed by *Cunninghamella elegans* ATCC 9245 and *Streptomyces lavendulae* L-105 in large-scale experiments into six metabolites, 2α-, 9α-, 9β-, and 14-hydroxyarteether, 13α-hydroxy-11-epi-deoxyhydroartemisinin, and one ring-rearranged metabolite [123]. From

10-deoxoarthemisinin *C. elegans* afforded three derivatives identified as 5β-hydroxy-10-deoxoarthemisinin, 4α-hydroxy-1,10-deoxoarthemisinin, and 7-β-hydroxy-10-deoxoarthemisinin. The 5β- and 7β-hydroxy-derivatives retained an intact peroxide group which makes them useful scaffolds for synthetic modification in the search for new antimalarial agents [124].

Artemisitene (Fig. 6), a minor constituent of *Artemisia annua* (Asteraceae) and closely related to artemisinin, was metabolized by *Aspergillus niger* NRRL 599 yielding 11-epi-artemisinin, 9β-hydroxydeoxy-11-epi-artemisinin, and 9β-hydroxy-11-epi-artemisinin. Only 9β-hydroxy-11-epi-artemisinin, which retained an intact endoperoxide moiety, showed some activity in in vitro antimalarial test [125].

α-Santonin (Fig. 6) is a sesquiterpene lactone which is commercially available from *Artemisia* spp., possessing cytotoxic, antimicrobial, and antihelmintic activities. Its transformation by *Cyathus africanus* resulted in two dihydroxylated derivatives, 11,13-dihydroxysantonin and 8α,13- dihydroxysantonin [126]. Fungal strains *Rhizopus stolonifer* ATCC 10404, *Cunninghamella echinulata* ATCC 9245, *Cunninghamella bainieri* ATCC 9244, and *Mucor plumbeus* ATCC 4740 reduced regiospecifically the carbon-carbon double bond in ring A of α-santonin. *R. stolonifer* transformed α-santonin into 3,4-epoxy-α-santonin and 4,5-dihydro-α-santonin. *C. echinulata*, *C. bainieri*, and *M. plumbeus* gave rise to 1,2-dihydro-α-santonin [127]. α-Santonin was also transformed by the plant pathogenic fungus *Botrytis cinerea*, leading to 11β-hydroxy-α-santonin in 83% yield [83].

α-Santonin was subjected to combination of chemical, in vitro enzymatic, and microbiological modifications, including C-8 hydroxylation by *Rhizopus nigricans* to afford analogs of natural 8,12-eudesmanolides [128].

α-Santonin and 1,2-dihydro-α-santonin were transformed by the fungal strains *Acremonium chrysogenum* PTCC 5271 and *Rhizomucor pusillus* PTCC 5134 which performed a variety of reactions including C1–C2 and C4–C5 double bond reduction, C-8 hydroxylation, and 15-methyl hydroxylation. *A. chrysogenum* gave four metabolites from α-santonin, including 1,2-dihydro-α-santonin, while *R. pusillus* gave rise to two metabolites. The same strains were incubated also with 1,2-dihydro-α-santonin where both afforded tetrahydro-α-santonin and tetrahydroartemisinin in different ratios [129].

4.1.2 Microbial Transformations of Alkaloids

Alkaloids are a special group of secondary metabolites regarded as part of plant's adaptation to its living environment [130]. Their microbial transformations were thoroughly reviewed [131, 132].

The microbial transformation of piperine (Fig. 7), isolated from species in the genus *Piper*, by *Aspergillus niger* gave a new compound, 5-[3,4-(methylenedioxy) phenyl]-pent-2-ene piperidine [24]. Piperlongumine (isolated from the fruits of *Piper longum* (Piperaceae)) was found to induce apoptosis, and considered as promising strategy for cancer therapy. Its transformation with the filamentous fungus *Beauveria bassiana* ATCC 7159 resulted in formation of 5-hydroxy-

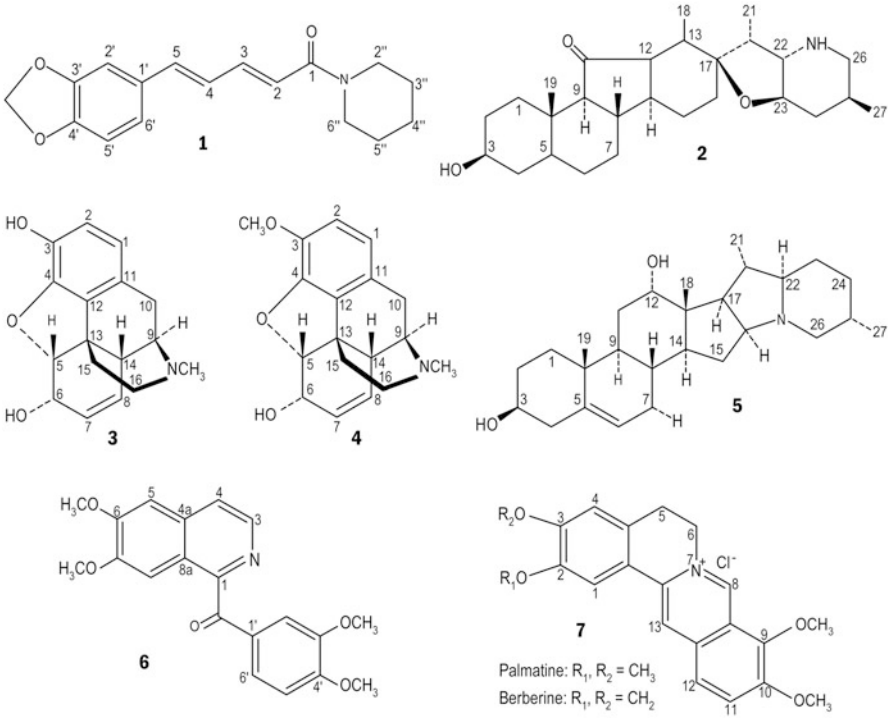

Fig. 7 Structures of alkaloids: **1**, piperine; **2**, jervine; **3**, morphine; **4**, codeine; **5**, rubijervine; **6**, papaveraldine; **7**, palmatine and berberine

piperlongumine. The new hydroxyl group might help introducing other functional groups for further structural modifications [133].

Cunninghamella elegans 9245 transformed the C-nor-D-homosteroidal alkaloid jervine (Fig. 7), found in *Veratrum* plants, into jervinone as the major metabolite and additionally epimerized C-3 position producing 3-*epi*-jervine [134, 135].

Special attention is paid on morphine alkaloids with stress on finding derivatives sharing more potent and less addictive properties. Recombinant *Escherichia coli* was demonstrated to transform morphine and codeine (Fig. 7) into the potent analgesic hydromorphone and the mild analgesic/antitussive hydrocodone, respectively [136]. Fungi were found capable of performing *N*-demethylations and oxidations of morphine, codeine, thebaine, opiravine, hydrocodone, and oxycodone [137]. The cyanobacterium *Nostoc muscorum* was found to convert codeine through reactions of *O*- and *N*-demethylation, C-6 acetylation and oxidation, C-14 hydroxylation, and Δ^7-reduction. In this, the transformation of codeine into oxycodeine, being uncommon for the microbial metabolism, was considered as industrially important [138].

The rubijervine (Fig. 7) was transformed by *Cunninghamella elegans* 9244 into the more polar metabolites 7α-hydroxyrubijervine and solanid-5-ene-3β,12α-diol-1-one [134, 135].

Papaveraldine (Fig. 7) is one of the minor benzylisoquinoline alkaloids of poppy capsules (*Papaver somniferum* L.). *Mucor ramannianus* 1839 entirely depleted and converted papaveraldine to two more polar metabolites, *S*-papaverinol and *S*-papaverinol *N*-oxide [139].

7-*N*-oxide derivatives of palmatine and berberine (Fig. 7) were obtained through their oxidation by the endophytic fungus *Coelomycetes* AFKR-1 [140].

Colchicine, the major alkaloid obtained from *Colchicum autumnale* and *Gloriosa superba* L., is well known with its antimitotic activity but is too toxic to be of value as an antitumor drug in its native form [141]. Colchicine derivatives 3-demethyl colchicine, colchicoside, and thiocolchicoside reveal better therapeutic characteristics but are only minor constituents in the colchicine-producing plants [142].

Bacillus megaterium strain was identified as able to perform double transformation of thiocolchicine into thiocolchicoside under an efficient, industrially green, and cost-effective route to thiocolchicoside [143, 144]. By targeted selection of thiocolchicine-resistant strains of *B. megaterium*, a tenfold improvement of thiocolchicine conversion yield was achieved which made possible thiocolchicoside to be obtained straightly. The process was scaled up and applied for the industrial production of thiocolchicoside [145].

4.1.3 Microbial Transformations of Flavonoids

Flavonoids are secondary metabolites with relatively low bioavailability [146], which limits their practical application due to low solubility, inefficient transport across biological membranes, or low stability. Biotransformation of flavonoids is widely studied as a natural method for modification of their structures [147–149]. The microbial transformation of flavanones is considered as a method of increasing their antioxidant properties [150].

Flavanone (Fig. 8) and 6-hydroxyflavanone were transformed by *Aspergillus niger* strains into a variety of derivatives. The wild strain *A. niger* KB performed reduction of the C-4 carbonyl groups of both substrates with formation of flavan-4-ol and 6-hydroxyflavan-4-ol as racemic compounds. *A. niger* 13/5 afforded three products from flavanone, the major one being flavone, and flavan-4-ol and 7-hydroxyflavan-4-ol as well. *A. niger* SPB afforded four products from flavanone identified as flavone, 3-hydroxyflavone, flavan-4-ol, and 7-hydroxyflavan-4-ol. With flavanone *A. niger* IBR gave 3-hydroxyflavone as a single product. When incubated with 6-hydroxyflavanone, only *A. niger* 13/5 transformed it giving rise to 6-hydroxyflavone as a single product, isolated in high yield [151].

A. niger KB reduced the carbonyl group of 7-hydroxyflavanone affording 7-hydroxyflavan-4-ol, while *A. niger* 13/5 led to the product of C2 and C3 dehydrogenation from the same substrate. *Penicillium chermesinum* 113, apart from its typical reaction of hydroxylation in the ring B, performed *O*-methylation of 7-hydroxyflavanone affording 7-methoxyflavanone and 3′,4′-dihydroxy-7-methoxyflavanone. *Aspergillus ochraceus* 456 performed a nontypical reaction of hydroxylation at C-5 along with carbonyl group reduction. From all metabolites, 3′,4′-dihydroxy-7-methoxyflavanone revealed the highest antioxidant activity possibly arising from the presence of two hydroxyl groups in the ring B [149].

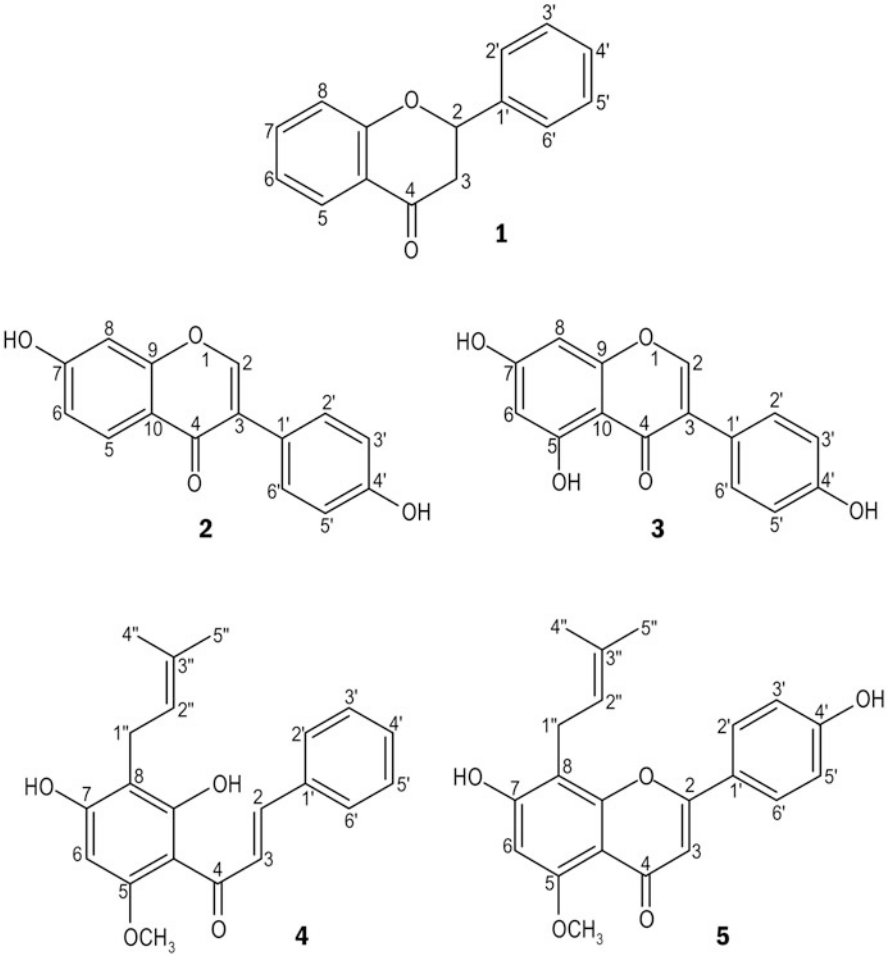

Fig. 8 Structures of flavonoids: **1**, flavanone; **2**, daidzein; **3**, genistein; **4**, xanthohumol; **5**, isoxanthohumol

The biotransformation of the 7-methoxyflavanone resulted in formation of (±)-2,4-*cis*-7-methoxyflavan-4-ol (afforded by *A. niger* KB), (+)-2,4-*trans*-7-methoxyflavan-4-ol and 4′-hydroxy-7-methoxyflavone (afforded by *A. ochraceus* 456), and 4,2′-dihydroxy-4′-methoxydihydrochalcone (afforded by *P. chermesinum* 113). All derivatives revealed higher antioxidant properties than the substrate in a DPPH scavenging test [152].

Flavone was transformed by a panel of fungi and bacteria into 4′-hydroxyflavone, 3′,4′-dihydroxyflavone and two cleavage products identified as *o*-hydroxyphenyl-hydroxtmethyl ketone and 1-(*o*-hydroxyphenyl)-1,2-ethanediol. Isoflavone was transformed into two metabolites, identified as 4′-hydroxyisoflavone and 3′,4′-dihydroxyisoflavone [153].

The introduction of hydroxyl groups into isoflavones by the use of microorganisms presents an attractive alternative to conventional chemical synthesis affording hydroxylated compounds which activity is higher than the original isoflavones. The presence of hydroxyl groups increases the water solubility of the respective derivatives as well [154].

Daidzein (Fig. 8), 4′,7-dihydroxyisoflavone, is a major isoflavone component of soybeans *Glycine max* (L.) Merr., Fabaceae. It was transformed by *Nocardia* sp. NRRL 5646 through reactions of hydroxylation and methylation in two metabolites and by *Mortierella isabellina* ATCC 38063 into the unusual metabolite daidzein-4′-rhamnopyranoside [155].

Streptomyces avermitilis MA-4680 transformed effectively daidzein and genistein (Fig. 8), 4′,5,7-trihydroxyisoflavone, through C-3′ hydroxylation, affording 3′,4′,7-trihydroxyisoflavone and 3′,4′,5,7-tetrahydroxyisoflavone, respectively [154].

Streptomyces griseus ATCC 10137 and *Streptomyces catenulae* ATCC 23893 converted genistein into five derivatives [156]. Further work is needed to assess the feasibility of these compounds for therapeutic applications.

Genistein 7-*O*-phosphate is a promising and effective alternative to genistein due to its improved water solubility, enhanced intestinal permeability in vitro and in situ, and greatly increased plasma exposure after oral administration in rats [157].

Genistein and daidzein 7-*O*-phosphates (water-soluble derivatives of genistein and daidzein) are possible through incubation of the respective isoflavone with *Bacillus subtilis* var. *natto* BCRC 80517. Further work, however, is needed to scale up the production of these metabolites and compare their biological efficacies with those of the parent compounds [158].

The soybean isoflavones daidzein and glycitein were transformed into polyhydroxylated isoflavones by tempe-derived bacterial strains identified as *Micrococcus* or *Arthrobacter* species [159].

Prenylated flavonoid glabratephrin was transformed by *Aspergillus niger* into pseudosemiglabrin [160]. Hop-derived prenylated flavonoid xanthohumol (Fig. 8) is considered as promising starting material for microbial transformations affording compounds, which are difficult to obtain by chemical methods. The transformation of xanthohumol by *Fusarium tricinctum* into α,β-dihydroxanthohumol is a simple way to obtain this active compound in a reasonable amount. Obtained α,β-dihydroxanthohumol showed higher antiproliferative activity against MCF-7 human breast carcinoma cell line than cisplatin (widely used anticancer therapeutic agent) and a comparably high activity against PC-3 human prostate cancer cell line [161]. Through regioselective glycosylation of xanthohumol, fungal strains *Rhizopus nigricans* and *Absidia coerulea* formed 4′-*O*-β-D-glucopyranoside, *Beauveria bassiana* afforded xanthohumol 4′-*O*-β-D-(4″-*O*-methyl)-glucopyranoside, and *Mortierella mutabilis* gave rise to isoxanthohumol 7-*O*-β-glucopyranoside. Compared to xanthohumol, 4′-*O*-β-D-glucopyranoside is a better 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenger, while 4′-*O*-β-D-(4‴-*O*-methyl)-glucopyranoside and 4′-*O*-β-D-glucopyranoside have stronger antiproliferative activity against human HT-29 colon cancer cells [162].

Isoxanthohumol (Fig. 8) has a significantly better solubility than xanthohumol, even though its biological properties are less promising. An often cited feature is its function as a precursor of 8-prenylnaringenin, the most potent phytoestrogen known to date [161]. Isoxanthohumol was transformed by *Beauveria bassiana* AM278 and *Absidia glauca* AM177 into glucoside derivatives and by *Fusarium equiseti* AM15 into a metabolite known to possess antimalarial activity against *Plasmodium falciparum* [163].

The methylated isoprenoid flavones cannflavin A and B (isolated from cannabis, *Cannabis sativa* L., Cannabaceae) were transformed via a panel of 41 microorganisms. *Mucor ramannianus* ATCC 9628 was found to produce three metabolites of cannflavin A, while *Beauveria bassiana* ATCC 13144 produced two metabolites of cannflavin B. Transformation reactions comprised hydroxylation, *O*-glycosylation, and sulfate conjunction as well as combinations of them. *O*-glycosylation occurred at the C-4′ and C-7 of cannaflavins A and B, respectively, while sulfate conjunction occurred exclusively at the C-7 hydroxy [153].

Coumarins are widely distributed in nature and found in all parts of plants. *Glomerella cingulata* was found to transform coumarin and xanthyletin (Fig. 9) into hydrocoumaric acid and into two reduced metabolites, respectively [164].

The application of furanocoumarins imperatorin and isoimperatorin (Fig. 9), abundant in citrus fruits, umbelliferous vegetables, and certain medicinal plants, is limited because of their poor water solubility. *Glomerella cingulata* also transformed imperatorin giving rise to the dealkylated metabolite xanthotoxol, while isomperatorin was transformed into the corresponding reduced acid, 6,7-furano-5-prenyloxy hydrocoumaric acid which revealed slight β-secretase (BACE1) inhibitory activity in vitro [165].

Imperatorin was transformed by *Penicillium janthinellum* AS 3.510 in ten derivatives through hydroxylation of the isopentenyl group, hydrolysis, and reduction at the α/β-unsaturated lactone ring of a furocoumarin skeleton. Some minor derivatives

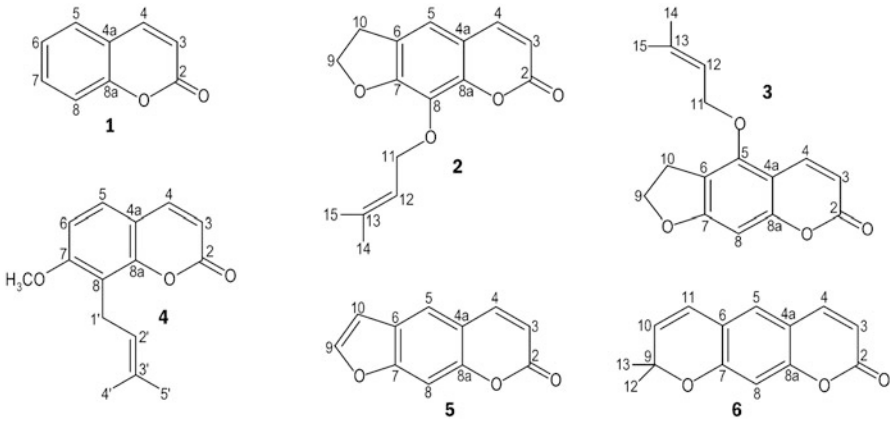

Fig. 9 Structures of coumarins: **1**, coumarin; **2**, imperatorin; **3**, isoimperatorin; **4**, osthole; **5**, psoralen; **6**, xanthyletin

of imperatorin were obtained through reactions of dehydration, glycosylation, methylation, and rearrangement of double bond as well. Two of the metabolites showed potent anti-osteoporosis activities in MC3T3-E1 [166].

Isomperatorin (isolated from *Angelica dahurica* Benth) was transformed by *Cunninghamella blakesleana* AS 3.970 into six products via reactions of hydroxylation, hydrogenation, carbonylation, and glycosylation. Two of the products (14-hydroxyl-isoimperatorin and isoimperatorin-14-*O*-β-D-glucoside) had potent anti-osteoporosis activities and higher water solubility possibly due to their hydroxylation or glycosylation at C-14 [167].

Osthole (Fig. 9), an isopentenoxy-coumarin from *Cnidium monnieri* (L.) Guss., was transformed by *Mucor spinosus* AS 3.3450 through reactions of hydroxylation at C-2′, C-3′, C-4′, and C-5′ of the isopentene group, dehydrogenation at C-3′, and demethylation at C-7 of osthole. Obtained osthole metabolites were considered suitable to serve as the authentic compounds for investigation of osthole metabolism in humans [168].

Psoralen (Fig. 9) gave rise also to furano-hydrocoumaric acid methyl ester with ß-secretase (BACE1) inhibitory activity [164].

4.1.4 Microbial Transformations of Polyphenols

Bioactivities of polypnenols range from antioxidant to anticancer ones, but their potential is seldom fully realized since their solubility and stability are quite low and their bioavailability is hampered due to extensive metabolism in the body. Microbial transformations of polyphenols may provide an effective solution by modifying their structure while maintaining their original bioactivity. Among microorganisms, *Aspergillus*, *Bacillus*, and *Streptomyces* sp. are the most widely used ones [169].

α-Mangostin (tricyclic isoprenylated polyphenol) which is the major secondary metabolite of *Garcinia mangostana* L., the queen of fruits in Thailand, has been the subject of structure modification via transformation by *Colletotrichum* sp. MT02 and *Phomopsis euphorbiae* K12 which performed oxygenation of the prenyl double bond, hydroxylation of the terminal methyl in prenyl group, cyclization of the prenyl group with a free *ortho*-hydroxyl group, and cyclization of the prenyl group with a *peri* carbonyl group. Five mangostin derivatives were isolated, two of which, 12,13,20-trihydroxymangostin and 20-hydroxymangostanin, exhibited weak cytotoxicity against breast cancer MCF-7 cell lines [170].

4.1.5 Microbial Transformations of Saponins and Sapogenins

Saponins occur naturally in plants where they are widely distributed possibly playing defensive role against pathogens, pests, and herbivores [171]. Recently several microorganisms were found capable of transforming saponins. *Arthrobacter nitroguajacolicus* was found to fructosylate furostan saponins adding one or two β-fructosyls at the C6-OH of 26-*O*-β-D-glucopyranosyl of substrates [172]. Total furostanol saponins of *Dioscorea zingiberensis* (a particular plant of the family Dioscoreaceae in China traditional medicine) were fermented with *Absidia coerulea*. Five new steroidal saponins were obtained through biotransformation and they revealed induced platelet aggregation activities [173]. Pseudoprotodioscin was

transformed by *Aspergillus fumigatus* into four metabolites, saponins dioscin and progenin II, and two steroidal metabolites. All four derivatives revealed significant cytotoxicity against cancer cell lines A375, L929, and HeLa [174]. When incubated with *Chaetomium olivaceum*, pseudoprotodioscin was transformed into eight saponins, three of which showed cell-protection activity against H_2O_2-induced H9c2 cardiomyocyte [175]. Data on the microbial transformations of one of the *Centella* plant saponins, the asiatic acid by *Alternaria longipes*, *Penicillium lilacinum*, *Fusarium equiseti*, and *Streptomyces griseus*, were recently reviewed, pointing out that some of the obtained derivatives showed improved characteristics in comparison with the parent compound [176].

In plants steroidal sarsapogenins are precursors of natural defensive chemicals against microbial and predator invasions. Sarsapogenin, isolated from *Yucca schidigera* and *Trigonella foenum-graceum*, was transformed by *Fusarium lini* into two metabolites, 3β-acetoxysarsasapogenin and 7α-hydroxy sarsasapogenin, which showed dose-dependent spasmolytic activity in rat duodenum [177].

Ruscogenin (an important steroidal sapogenin with strong anti-arrhythmia, anti-ischemia, anti-thrombosis, and anti-inflammatory activities) is present in vegetables of genus *Allium* and medicinal plants *Ruscus aculeatus* (Liliaceae) and *Ophiopogon japonicus* [178, 179]. The plant pathogen *Phytophthora cactorum* was found capable of transforming ruscogenin into the unique less polar 4-ene-3-one steroidal sapogenin 1-hydroxy-spirost-4-en-3-one. It was considered that the dehydrogenation and the formation of olefin-ketone moiety in the A ring of ruscogenin contributed to the observed remarkedly increased in vitro anti-tissue factor (anti-TF) procoagulant activity of the obtained derivative [180].

4.2 Microbial Transformations for Providing Information Regarding Metabolism of Plant-Derived Biologically Active Compounds in Humans and Mammals

Since the use of microbial models for studying drug metabolism in humans and mammals was suggested [181], this area of investigations has been significantly developed. The microbial metabolism of a great variety of plant-derived metabolites with biological activity applied in conventional and traditional medicines was studied and comparisons were made between them [182, 183].

Taxol, which is widely used due to its antitumor activities, was incubated with *Pseudomonas aeruginosa* AS 1.860. The transformation process afforded three derivatives, identified as baccatin III, 10-deacetylbaccatin III, and baccatin V, the first two of them known as human metabolites [184].

Silybin A and silybin B are diastereoisomeric compounds, which in a roughly 1:1 mixture form the semipurified, commercially available fraction of silymarin, extracted from *Silybum marianum* (L.) Gaertn., Asteraceae, and known as silybin [185]. Their metabolism is strongly linked to Phase II biotransformations and the respective conjugates are rapidly excreted in bile and urine. Conjugation reactions of both silybins are strictly stereoselective. When incubated with *Trichoderma*

koningii, two pairs of glucosylated derivatives were obtained and identified as silybin A 3-*O*-β-D-glucopiranoside and silybin A 7-*O*-β-D-glucopiranoside as well as silybin B 3-*O*-β-D-glucopiranoside and silybin B 7-*O*-β-D-glucopiranoside [186]. It was found that *Streptomyces* sp. M52104 can transform silybin A and silybin B into silybin A-20-*O*-β-glucuronide, silybin B-20-*O*-β-glucuronide, silybin A-7-*O*-β-glucuronide, and silybin B-5-*O*-β-glucuronide which can be used as invaluable authentic standards in metabolic studies of both silybin diastereomers [187]. Microbial glucuronidation of both silybin A and silybin B by *Streptomyces* sp. M52104 into at least three glucuronides determined to be human metabolites of silybin A has been reported as well [188].

When silybin was submitted to microbial transformation, *Beauveria bassiana* afforded the phase I metabolite 8-hydroxysilybin. *Cunninghamella* species afforded Phase II metabolites identified as 2,3-dehydrosilybin-3-*O*-β-D-glucoside and silybin-7-sulfate and 2,3-dehydrosilybin-7-sulfate. The antioxidant activity of 2,3-dehydrosilybin-7-sulfate more than doubled in comparison with the activity of the parent compound [189].

Cryptotanshinone, one of the major diterpenes isolated from the roots of *Salvia miltiorrhiza* [74], was transformed in a preparative-scale process by *Cunninghamella elegans* AS 3.2082 into three products, which were used for comparison with in vivo metabolites in rat bile sample after intravenous administration. Microbial products were found identical to three of the minor hydroxylated metabolites in vivo, which suggested that microbial biotransformation model was a useful and feasible approach for the preparation of mammalian metabolites in trace [190].

Forskolin (7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one) is the major diterpenoid from the Indian herb *Coleus forskohlii* which is its main source as well [191]. Forskolin is a potential drug for treatment of glaucoma, congestive heart failure, and bronchial astma. Its metabolites were identified through administration of 15-^{14}C-labeled forskolin in rats and pigs as 3β-hydroxyforskolin, its 7-deacetyl derivative and the 7-deacetyl-6-acetyl isomer. 3β-Hydroxyforskolin and its derivatives are especially useful for a rapid confirmation of the metabolites of forskolin formed in animal studies. 7-Deacetylforskolin and 1,9-dideoxyforskolin were hydroxylated by the strain FF 406 at the 3β-position [192, 193]. 7-Deacetylforskolin was prepared from 7-deacetyl-1,9-dideoxyforskolin via its microbial transformation by *Scopuloriopsis* sp. capable of carrying out the 1- and 9-hydroxylation reactions [191].

When incubated with the antimalarial drug artemisinin, *Streptomyces griseus* ATCC 13273 performed oxidative modifications of the substrate, which comprised reactions of detoxification of drugs in mammals, plants, and microorganisms. Produced were one major product, artemisitone-9, and other three polar metabolites, 9α-hydroxy-artemisinin, 9β-hydroxy-artemisinin, and 3α-hydroxy-deoxyartemisinin [113].

Microbial metabolism of artemisinin derivatives makes possible effective predictions regarding their mammalian metabolism as well as mediates production of reference standards and structure elucidation studies [194].

The large-scale fermentations of the antimalarial drug arteether (an ethyl ether derivative of dihydroartemisinin) with *Cunninghamella elegans* ATCC

9245 and *Streptomyces lavendulae* L-105 resulted in the formation of six metabolites, four of which (9β-hydroxyarteether, 9α-hydroxyarteether, 2-α-hydroxyarteether, and 14-hydroxyarteether) are also present in rat liver microsome preparations [123].

Streptomyces lavendulae L-105 and *Rhizopogon* sp. ATCC 36060 metabolized the semisynthetic antimalarial anhydrodihydroartemisinin into four compounds. One of them, 9β-hydroxy anhydrodihydroartemisinin, was the major metabolite in rat plasma which revealed in vitro antimalarial activity as well [195].

Fungal strains *Colletotrichum gloeosporioides* and *Neosartorya spathulata* proved capable of performing Phase II conjugation when incubated with α-mangostin. Both strains produced α-mangostin sulfated regiospecifically at C-3. Sulfate conjugation is an important pathway in mammalian metabolism of xenobiotics which makes obtained products potentially useful in studies on mammalian metabolism of α-mangostin [196].

Curcumenol is an important crude drug frequently listed in prescriptions of traditional Chinese medicine which poor water solubility, however, limits its bioavailability, absorption, and clinical application. When incubated with curcumenol, fungal strains *Mucor spinosus* AS 3.2450, *Penicillium urticae* IFFI 04015, *Cunninghamella echinulata* AS 3.3400, and *Aspergillus carbonarius* IFFI 02087 affforded five hydroxylated derivatives. The C-15 hydroxylated one, identified as 15-hydroxycurcumenol, could serve as standard reference for investigating in vivo metabolism of curcumenol [197].

Marvalin and Azerad developed successfully simple and productive microbial transformation process for preparation of bioavailable, stabilized, and soluble forms of naringenin and quercetin as well as *trans*-resveratrol, rhapontigenin, and deoxyrhapontigenin, starting either from their aglycones or from their corresponding glucosides or rhamnoglucoside extracted from plants. Derivatives glucuronides were in high purity which will certainly help for the in vivo and in vitro elucidation of polyphenol pharmacology and metabolism [198].

Beauveria bassiana ATCC 7159 and ten isolates of *Beauveria* sp. transformed quercetin into four metabolites, which are mammalian metabolites as well [199].

Flavanone glycoside hesperidine (present in citrus fruits) is transformed into its aglycone hesperetin by *Cunninghamella elegans* and the process is used for predicting hesperidine metabolism [200].

Three microbial metabolites of xanthohumol were generated by the fungal strain *Pichia membranifaciens*, which could be used as analytical standards for the detection of metabolites of xanthohumol in biological fluids. One of derivatives was identical to that obtained using rat liver microsomes [201].

The metabolites of 3- and 7-hydroxyflavones from their transformation by *Beauveria bassiana* ATCC 13144, *Nocardia* sp. NRRL 5646, and *Aspergillus alliaceus* ATCC 10060 are results from of Phase I functionalization and Phase II conjugation reactions and are paralleled in mammals [202]. These three fungal strains as well as *Mucore ramannianus* ATCC 9628 are capable of transforming chrysin, 5- and 6-hydroxyflavone, into oxygenation and conjugation products which were paralled in mammals [203].

Studies on microbial transformation of 8-prenylnaringenin are of interest to mimic and predict mammalian metabolism of this phytoestrogen, which occurs naturally in hops and different *Wyethia* species. In a preparative-scale process, *Fusarium equiseti* converted 8-prenylnaringenin into a single product in a high yield, 51.5% after purification. Its 2S isomer, named phellodensin D, occurs naturally in *Phellodendron chinese* var. *glabriusculum* and has been detected as a minor metabolite of 8-prenylnaringenin in human liver microsomes in an in vitro study [204]. Fungal strains *Beauveria bassiana* AM278 and *Absidia coerulea* AM93 formed conjugated products of 8-prenylnaringenin which are analogous to the ones formed in mammalian system during the Phase II metabolism [205].

Shi and coauthors compared metabolites of isoimperatorin, identified in rat urine, plasma, bile, and faeces after its oral administration (19 metabolites) with isoimparatorin derivatives obtained via its transformation by *Cunninghamella blakesleana* AS 3.970 (13 derivatives) and those existed in the in vivo biological samples (11 metabolites). The authors concluded that such an approach may play an important role not only in the process of drug research and development but also in the study of the metabolism of bioactive constituents of foods [206].

The deglycosylated products of timosaponin BII (the most abundant steroidal saponin in *Anemarrhena asphodeloides* Bunge) are part of its in vivo metabolites in rats [207]. They can be effectively and environmental friendly prepared through biotransformation of timosaponin BII with the fungal strain *Colletotrichum gloeosporioides* [208].

5 Closing Remarks and Future Prospects

Microbial biotransformations have been used for centuries in the production and processing of goods in food, pharmacy, and agriculture. This pattern has its roots on the specificity of biological catalysts, mild environment required, and eco-friendly nature. In recent decades, the relevance of microbial biotrasformation within the scope of drug design and production has been increasing, moreover as this approach fits nicely within the growing trend toward sustainable chemical manufacturing processes. Additionally, microbial transformations can mimic mammalian metabolism, therefore contributing to the implementation of fast, effective drug screening methods. Drug design and development strongly anchor on plant secondary metabolites, given their diverse biological action on different organisms. Despite the intrinsic properties of the natural compouds, the need for drugs with improved features, such as improved solubility and pharmacokinetic properties, reduced toxicity, and ability to overcome the emergence of multidrug resistance, is well acknowledged and has led to a second generation of phytopharmaceuticals [209]. One of the strategies to fulfill this goal involves the modification of the complex molecular structure of the natural substrates, namely, through microbial transformations. The implementation of this methodology has actually resulted in a plethora of novel molecules, a significant fraction of which displaying the desired features. Moreover, these efforts are contributing to generate a

structurally diverse chemical library that may also be a factor in gaining further insight in the development of structure-activity relationships. The trend toward a multidisciplinary approach to the lead discovery based on bio- and molecular diversity of plants [210] and the bioprocess design, from novel and/or improved (engineered) microbial catalysts to media engineering and downstream processing, are foreseen to further broaden the number of microbial drug production processes in commercial scale.

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Part III

Secondary Metabolites

Engineering Cell and Organ Cultures from Medicinal and Aromatic Plants Toward Commercial Production of Bioactive Metabolites

5

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Contents

Abstract

Production of secondary metabolites from in vitro cell and hairy root cultures (CHRC) of medicinal and aromatic plants (MAP) is considered a promising alternative to gathering plant material from MAP natural populations, often a reason for their overexploitation and even extinction. However, most of the valuable secondary metabolites extracted from different MAP species are present in very low amounts in the respective CHRC. Plant metabolic engineering offers an attractive opportunity to increase the content of target secondary metabolites in engineered transgenic CHRC for production at feasible levels. Moreover, applying metabolic engineering makes it possible to redirect target metabolic pathway

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(s) in the transgenic CHRC to produce new compounds not present in the wild plant itself. This chapter describes the strategies and experimental toolbox for plant metabolic engineering with examples from engineering secondary metabolite production in CHRC from MAP, as well as a review of these century reported studies on metabolic engineering of CHRC. The directions and prospects for CHRC metabolic engineering applications in production of valuable secondary metabolites are discussed

Keywords
Medicinal and aromatic plants · Metabolic engineering · Secondary metabolite production · Transgenic cell suspension · Transgenic hairy root cultures · Biosynthetic pathway

Abbreviations

CHRC	Cell and hairy root culture
CS	Cell suspension
HR	Hairy roots
MAP	Medicinal and aromatic plants
ME	Metabolic engineering
RNAi	RNA interference
TF	Transcription factor
TIA	Monoterpenoid indole alkaloids

1 Introduction

Medicinal and aromatic plants (MAP) produce a very wide range of secondary metabolites, presenting a rich and untapped source of bioactive compounds. Consumption of MAP-derived products is highly popular, and currently about 80% of the world's population use some herbal medicines for their healthcare needs [1, 2]. This results in steady increase of the herbal market volumes, as recent estimation suggests the global herbal supplements and remedies market to reach US$ 115 billion by 2020 [3]. Besides the wide use of MAP complex extracts for preparation of various herbal products, MAP have been increasingly exploited for purification of selected bioactive compounds used directly as drugs or precursors for production of semisynthetic drugs. Moreover, some of the characterized MAP secondary metabolites have become a source of important lead compounds for new drug development. At present, a remarkable high portion of the contemporary drugs are directly or indirectly derived from MAP secondary metabolites [1, 4, 5]. All this pushes up the demands for plant-derived bioactive compounds, and their availability becomes one of the main bottlenecks to secure and expand their pharmaceutical use. The production of the industry needed volumes for most of the valuable MAP compounds is limited by a number of factors. Many of the high-value compounds are present in low amounts in the raw material of the respective MAP, and some of the widely exploited MAP are also slow growing. Currently, nearly the entire volume of raw MAP material is collected from the wild, and only a very small portion of

the utilized MAP are (semi-)industrially cultivated. This often leads to overexploitation and irreversible damages of the MAP natural populations, and an increasing number of MAP are included in the list of endangered species. The overexploitation is one of the main causes the extinction rate of MAP species to be significantly higher than those of other plants [6, 7]. The content and composition of secondary metabolites produced by MAP often are strongly influenced by the environmental conditions, various stress factors, and stages of plant and plant organ development. Combined with the impact of bulk collection of raw material from natural MAP populations, this results in lower yield and reduced production efficiency of the targeted bioactive compounds. The in vitro plant cell and hairy root cultures (CHRC) developed from selected MAP accessions could address some of the pointed above limitations and provide reliable and continuous source of selected plant bioactive compounds [8, 9]. During the past few decades, significant advances were made in several key directions related to improvement of MAP cell and hairy root cultures toward their use for production of selected secondary metabolite including:

(a) Development and characterization of CHRC for a large number of MAP species, which makes possible the evaluation of their ability and limitations for secondary metabolite production [10]
(b) Characterization of hairy root cultures developed after *Agrobacterium rhizogenes* transformation of various MAP species, providing increased in vitro culture stability and high content of desired secondary metabolites and in some cases production of valuable secondary metabolites not produced by the initial MAP itself [11–13]
(c) Large number of studies on optimization of various culture conditions and accumulation of significant data and knowledge for development of high growth rate and biomass production cultures [14, 15]
(d) Significant improvement of bioreactor design and cultivation, providing culture scale-up efficiency and base for further (semi-)industrial volumes of cultivation [16]
(e) Development of efficient elicitor treatments for a large number of the established MAP cultures, providing increased production of targeted bioactive compounds [17]

In spite of these positive developments, the successful industrial applications of MAP cell and hairy root cultures for production of bioactive compounds are very limited. For example, Hendrawati et al. [1] counted only 14 commercialized plant cell lines used for industrial secondary metabolite production. One of the main constraints of using plant cell cultures for the industrial secondary metabolite production is the low amount of desired compounds produced and accumulated by the undifferentiated cells observed for most of the tested plant cell lines. The plant cell cultures also display relatively high variability and slow growth rates in comparison to microorganisms, which additionally hampers the economic feasibility for wider industrial application. Some of the pointed limitations are partially addressed with the increased use of MAP hairy root (HR) cultures, but their industrial application for secondary metabolite production requires further improvements.

The rapid development and successful applications of plant metabolic engineering (ME) offer attractive reinforcement of the MAP cell and hairy root culture capacity for production of secondary metabolites through both increasing the content of the valuable compounds and producing new ones not present in the initial MAP itself. In this chapter we review the recent research and discuss the prospects for further application of metabolic engineering to plant cell and hairy root cultures for production of valuable secondary metabolites.

2 Plant Metabolic Engineering: General Concept and Toolkit

2.1 Concept

Metabolic engineering is generally defined as targeted alterations of metabolic pathway(s) aiming to improve the yield and/or composition of the produced metabolites or biosynthesis of new metabolite(s) not produced by the engineered organism itself. Plant metabolite engineering developed rapidly at the end of the last century based on the expanding knowledge on plant metabolic pathways and related genes and genomes data, as well as advances of transgenic research and "omics" technologies [18]. The strategies for plant metabolic engineering application were described in details in a number of publications, e.g., [1, 4, 18–20], and in general include:

(a) Increase of the flux into the target metabolic pathway through enhancing the expression and/or activity of the rate-limiting enzyme
(b) Blocking up the feedback inhibition of the pathway key enzyme(s)
(c) Coordinated enhancement of the expression of all genes involved in the pathway
(d) Decrease of the flux to the competitive pathways
(e) Decrease of the catabolism of the target compound
(f) Upgrade of the target pathway(s) for biosynthesis of new compound(s)
(g) Providing efficient compartmentation and transport of the target metabolite(s)

The first choice of the strategy to be applied largely depends on the available information on the biosynthetic pathway and related genes involved in the biosynthesis of the target compound. Plant CHRC are generally a less complex subject for ME than the plant itself, which includes a large number of tissues and cell types often with complex pattern of development. Although in some cases this could simplify the desired metabolite engineering avoiding tissue/cell specificity issues, in many cases the successful CHRC engineering could be additionally complicated by the high tissue and/or cell specificity of the target metabolite biosynthesis in the plant itself.

2.2 Plant Metabolic Engineering Toolkit

CHRC metabolic engineering employs the same toolkit applied in plant metabolic engineering. The choice of the applied experimental approach depends on the

selected strategy for ME and considers the available target pathway and gene information. The methods and techniques employed for plant metabolic engineering were a subject of a number of reviews, e.g., [1, 4, 18, 20]. A brief description of the widely used experimental approaches, techniques, and related issues is described below with examples for CHRC applications.

2.2.1 Gene Silencing

Experimentally induced gene silencing through antisense, co-suppression, or RNA interference (RNAi) techniques is widely used to block or reduce expression of the target gene [21, 22]. Due to the available efficient cloning procedures for RNAi induction vectors [23, 24] and the high specificity and stability of the RNAi-induced gene silencing [25], RNAi induction has become a preferred gene silencing method during the last decade. The experimentally induced gene silencing is an important instrument for plant and CHRC metabolic engineering. Generally silencing of the target gene is applied to increase the yield of desire metabolite(s) or to change and improve the secondary metabolite composition. Gene silencing could be part of different engineering strategies including reduction or prevention of the target compound catabolism, for example, Zhang et al. [26] reported enhanced accumulation of phenolic acids and decreased total flavonoids in hairy root culture of *Salvia miltiorrhiza* following RNAi-mediated silencing of chalcone synthase gene, whereas Li et al. [27] applied antisense-mediated suppression of taxoid 14β-hydroxylase gene in *Taxus* × *media* TM3 cell line to decrease the production of C-14 oxygenated toxoids – side route compounds of the taxol pathway. In another study Rizvi et al. [28] attempted to increase the biosynthesis of terpenoid indole alkaloids (TIAs) in *Catharanthus roseus* through RNAi-mediated silencing of the transcriptional repressor *Zct1* gene. Although analysis of the transgenic hairy root cultures showed reduced expression of the *Zct1* and *Zct2* genes, the expression levels of the third *Zct3* gene remained unchanged, and no increase of the TIA content was observed, suggesting that silencing of all three ZCTs may be required to relieve their repression on TIA biosynthesis. In several other reports, RNAi-mediated gene silencing in CHRC of MAP species was applied to characterize the functionality of the studied genes, for example, EIN3-like protein gene regulating shikonin biosynthesis in *Lithospermum erythrorhizon* [29], A622 and ornithine decarboxylase genes involved in pyridine alkaloid biosynthesis in *Nicotiana* [30–32], copalyl diphosphate synthase gene in *S. miltiorrhiza* [33], and others (Table 1).

2.2.2 Homologous and Heterologous Gene Expression

The overexpression of biosynthetic and other pathway-related homologous genes and expression of heterologous genes originating from other organisms in the target CHRC are the most widely applied tools for their metabolic engineering. So far homologous gene overexpression in MAP cultures is mainly applied to increase the flux into the target pathway through enhancement of the expression and/or activity of the rate-limiting enzyme for the target metabolite biosynthesis. For example, the overexpression of both deacetylbaccatin III-10 β-O-acetyltransferase and 9-cis-epoxycarotenoid dioxygenase genes in *Taxus chinensis* cell suspension

Table 1 Overview of the plant species, techniques, transgenes or target genes, and impact of the metabolic engineering of secondary metabolite production in cell and hairy root cultures of medicinal and aromatic plants (these century reported studies included)

Engineered host plant	Type of culture[a]	Transgene expression strategy[b]	Transgene (source) or target gene[c]	ME impact (class of the targeted compounds)[d]	Ref.
Angelica gigas	HR	OE	Phenylalanine ammonia-lyase (*A. gigas*) Cinnamate 4-hydroxylase (*A. gigas*)	Increased production of decursinol angelate in cinnamate 4-hydroxylase overexpressing lines (P)	[64]
Anisodus acutangulus	HR	OE	Putrescine N-methyltransferase (*A. acutangulus*) Tropinone reductase I (*A. acutangulus*)	Increased tropane alkaloid production in the lines overexpressing both genes (A)	[65]
Anisodus acutangulus	HR	OE	Tropinone reductase I (*A. acutangulus*) Hyoscyamine-6β-hydroxylase (*A. acutangulus*)	Significantly higher increase of tropane alkaloid production in the lines overexpressing both genes (A)	[66]
Atropa belladonna	HR	HE	Putrescine-N-methyltransferase (*N. tabacum*)	Unchanged or lowered alkaloid production (A)	[67]
Atropa belladonna	HR	HE	Tropinone reductases I (TRI) (*Datura stramonium*) Tropinone reductase II (TRII) (*D. stramonium*)	TRI lines – increased tropine, hyoscyamine, and scopolamine and decreased pseudotropine content TRII lines – increased tropinone, tropine, pseudotropine, and calystegine content (A)	[68]
Atropa baetica	HR	HE	Hyoscyamine 6β-hydroxylase (*H. niger*)	Increased scopolamine production (A)	[69]
Atropa belladonna	HR	HE	Putrescine-N-methyltransferase (*N. tabacum*) Hyoscyamine 6β-hydroxylase (*H. niger)*	Improved tropane alkaloid production (A)	[70]
Catharanthus roseus	CS	TF-OE	ORCA3 transcription factor (*C. roseus*)	TF-OE lines – increased tryptamine and tryptophan accumulation TF-OE lines fed with loganin – higher level of increase TIA production (A)	[58]
Catharanthus roseus	HR	HE	3-Hydroxy-3-methylglutaryl-CoA reductase (hamster)	Increased ajmalicine and catharanthine but reduced campesterol content in one of the lines; increased campesterol and serpentine	[71]

				but decreased ajmalicine and no catharanthine in another line (A, T)	
Catharanthus roseus	HR	HE /induc./	Feedback-resistant anthranilate synthase (AS) α subunit (*A. thaliana*)	Increased tryptamine production (A)	[72]
Catharanthus roseus	HR	HE + OE /induc./	Feedback-resistant anthranilate synthase (AS) α subunit (*A. thaliana*) Tryptophan decarboxylase (TDC) (*C. roseus*)	TDC lines – no significant increase of tryptamine TDC + AS α lines – sixfold increase of tryptamine and serpentine (A)	[73]
Catharanthus roseus	HR	HE, OE /induc./	Anthranilate synthase β subunit (*A. thaliana*) Feedback-resistant anthranilate synthase α subunit (*A. thaliana*) Tryptophan decarboxylase (*C. roseus*)	Highest increase in tryptamine production was achieved when all three genes were expressed (A)	[74]
Catharanthus roseus	HR	HE /induc./	Anthranilate synthase (AS) β subunit (*A. thaliana*) (35S promoter) Feedback-resistant AS α subunit (*A. thaliana*) (glucocorticoid-inducible promoter)	5-year maintenance of inducible feedback-resistant AS αβ expression and increased tryptamine and ajmalicine production (A)	[75]
Catharanthus roseus	HR	OE	Deacetylvindoline-4-O-acetyltransferase (*C. roseus*)	Production of horhammericine in the transgenic lines (A)	[76]
Catharanthus roseus	CS	HE	Mammalian *Bax* gene (mouse)	Increased catharanthine and total TIA content (A)	[77]
Catharanthus roseus	HR	TF-OE /induc./	ORCA3 transcription factor (*C. roseus*)	Increased TIA production (A)	[41]
Catharanthus roseus	HR	HE	Tryptophan 5-halogenase (*Streptomyces rugosporus)* Flavin reductase and halogenase (*Lechevalieria aerocolonigenes*)	Production of halogenated (non-plant) TIAs (A)	[51]
Catharanthus roseus	HR	TF-OE	CrWRKY1: WRKY transcription factor (*C. roseus*) CrWRKY1-SRDX: CrWRKY1 with fused SRDX repressor domain (chimeric repressor silencing technology)	CrWRKY1 OE lines – increased tryptamine, serpentine, and ajmalicine production and decreased catharanthine and tabersonine CrWRKY1-SRDX OE lines – increased catharanthine and tabersonine production (A)	[57]

(*continued*)

Table 1 (continued)

Engineered host plant	Type of culture[a]	Transgene expression strategy[b]	Transgene (source) or target gene[c]	ME impact (class of the targeted compounds)[d]	Ref.
Catharanthus roseus	HR	HE + OE	1-Deoxy-D-xylulose synthase (DXS) (*A. thaliana*) Geraniol-10-hydroxylase (G10H) (*C. roseus*) anthranilate synthase α subunit (ASA) (*A. thaliana*)	DXS OE lines – increase of ajmalicine, serpentine, and lochnericine and decrease of tabersonine and horhammericine DXS+G10H OE lines – increase of ajmalicine, lochnericine, and tabersonine DXS+ASA OE lines – increase of horhammericine, lochnericine, and tabersonine (A)	[78]
Catharanthus roseus	HR	OE, GS/RNAi	Apoplastic peroxidase gene CrPrx (*C. roseus*)	Increased ajmalicine and serpentine content in OE lines, changes the expression levels of genes of TIA pathway (A)	[79]
Catharanthus roseus	HR	TF-OE	ORCA2 transcription factor (*C. roseus*)	Increased production of multiple TIAs (tryptamine, ajmalicine, serpentine, catharanthine, 16-hydroxytabersonine, 19-hydroxytabersonine) (T)	[54]
Catharanthus roseus	HR	HE + OE	1-Deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) (*T. chinensis*) 2-C-methylerythritol 2,4-cyclodiphosphate synthase (MECS) (*T. chinensis*) Strictosidine synthase (STR) (*C. roseus*)	Increased ajmalicine production in lines overexpressed DXR, MECS, and STR alone Higher increased ajmalicine production in lines co-overexpressed DXR + STR or MECS + STR genes (A)	[80]
Catharanthus roseus	HR	TF-OE	CrBPF1 transcriptional activator (*C. roseus*)	Little effect on TIA production in the transgenic lines (A)	[55]
Catharanthus roseus	HR	TF-OE + OE	ORCA3 transcription factor (*C. roseus*) Strictosidine glucosidase (SGD) (*C. roseus*)	ORCA3 OE lines – no changes in TIA production ORCA3 + SGD OE lines – increased TIA production (A)	[50]

Catharanthus roseus	HR	GS/RNAi	Transcriptional repressor ZCT1 (*C. roseus*)	Silencing of Zct1 and Zct2 genes, but no increase in TIA production (A)	[28]
Centella asiatica	HR	HE	Farnesyl diphosphate synthase (*P. ginseng*)	Increased squalene and total sterol content (T)	[81]
Coptis japonica	CS	OE	(S)-scoulerine 9-O-methyltransferase (*C. japonica*)	Slight increase of barberine and columbamine production (A)	[47]
Datura metel	HR	HE	Putrescine-N-methyltransferase (*N. tabacum*)	Improved tropane alkaloid (hyoscyamine and scopolamine) production (A)	[82]
Duboisia leichhardtii	HR	HE	Hyoscyamine 6β-hydroxylase (H6H) (*H. niger*) 4-Hydroxycinnamoyl-CoA hydratase/lyase (HCHL) (*Pseudomonas fluorescens*)	H6H line – high conversion (95%) of hyoscyamine to scopolamine (A) HCHL line – no changes in alkaloid production	[83]
*Duboisia myoporoides*_× *D. leichhardtii*	HR	HE	Putrescine-N-methyltransferase (*N. tabacum*)	No significant increase in tropane or pyridine-type alkaloids (A)	[84]
Duboisia myoporoides × *D. leichhardtii*	HR	HE	Hyoscyamine 6-hydroxylase (*H. niger*)	Increased scopolamine production (A)	[85]
Eschscholzia californica	CS	HE	(S)-scoulerine 9-O-methyltransferase (*C. japonica*)	Production of new alkaloid (columbamine) not present in the wild-type *E. californica* cells and decrease of sanguinarine, typical for the wild-type cells (A)	[47]
Glycyrrhiza uralensis	HR	OE	Chalcone isomerase (*G. uralensis*)	Increased flavonoid production (in combination with elicitation) (P)	[43]
Glycyrrhiza uralensis	HR	OE	Squalene synthase (*G. uralensis*)	Increased glycyrrhizic acid production (T)	[86]
Hyoscyamus muticus	HR	HE	Putrescine-N-methyltransferase (*N. tabacum*)	Improved tropane alkaloid production (hyoscyamine) (A)	[82]
Hyoscyamus muticus	HR	HE	Hyoscyamine-6b-hydroxylase (*H. niger*)	No significant increase of scopolamine production after exogenous addition of hyoscyamine (A)	[38]

(*continued*)

Table 1 (continued)

Engineered host plant	Type of culture[a]	Transgene expression strategy[b]	Transgene (source) or target gene[c]	ME impact (class of the targeted compounds)[d]	Ref.
Hyoscyamus muticus	HR	HE	Vitreoscilla hemoglobin	No significant effect on hyoscyamine and cuscohygrine contents (A)	[87]
Hyoscyamus niger	HR	OE, HE	Putrescine-N-methyltransferase (PMT) (*N. tabacum*) Hyoscyamine 6 β-hydroxylase (H6H) (*H. niger)*	Higher increase of the tropane alkaloid production (scopolamine) in the lines co-expressing PMT + H6H (A)	[88]
Hyoscyamus niger	HR	HE	Putrescine N-methyltransferase (*N. tabacum)*	Increased production of methylputrescine but no significant increase in scopolamine production High level of scopolamine production if combined with methyl jasmonate treatment (A)	[89]
Lithospermum erythrorhizon	HR	HE	Chorismate pyruvatelyase (*Escherichia coli*) HMG-CoA reductase (*A. thaliana*)	No effect on shikonin accumulation in (T, P)	[90]
Lithospermum erythrorhizon	HR	OE, GS/RNAi	1-Aminocyclopropane-1-carboxylic acid synthase (*L. erythrorhizon*)	OE lines – increased endogenous ethylene concentration and shikonin production RNAi lines – decreased shikonin production (T, P)	[91]
Lithospermum erythrorhizon	HR	OE, GS/RNAi	EIN3-like protein gene 1 (*Lithospermum erythrorhizon*)	OE lines – increased endogenous ethylene concentration and shikonin production RNAi lines – decreased shikonin production (T, P)	[29]
Mirabilis himalaica	HR	OE	Chalcone synthase CHS (*M. himalaica*) Chalcone isomerase CHI (*M. himalaica*)	Increased rotenoid content in CHS and CHI lines (P) Enhanced rotenoid production in the lines co-expressing CHS + CHI	[92]

Nicotiana glauca	HR	GS/RNAi	A622 gene *(N. glauca)*	Reduced anabasine and nicotine content in the RNAi lines (A)	[32]
Nicotiana glauca	HR	GS/RNAi	Ornithine decarboxylase (*N. glauca*)	Reduced anabasine and nicotine content in the RNAi lines (A)	[31]
Nicotiana tabacum	HR	GS/RNAi	Ornithine decarboxylase (*N. glauca*)	Reduced nicotine and increased anatabine content in the RNAi lines (A)	[30]
Nicotiana tabacum	HR	HE	Δ^1-tetrahydrocannabinolic acid synthase (*Cannabis sativa*)	Production of Δ^1-tetrahydro-cannabinolic acid upon feeding with cannabigerolic acid (T, P)	[37]
Nicotiana tabacum	HR	HE	Hyoscyamine-6b-hydroxylase (*H. niger*)	Increased secretion of scopolamine after culture feeding of hyoscyamine (A)	[39]
Nicotiana tabacum	HR, CS	OE	34 genes, putatively associated with pyridine alkaloid metabolism (*N. tabacum*)	Increased nicotine levels after overexpression of nicotine-enhancing GH3 enzyme 1 (A)	[93]
Nicotiana tabacum	HR	GS/RNAi	Arginine decarboxylase (*N. tabacum*)	Minor changes of nicotine production in RNAi lines (A)	[94]
Nicotiana tabacum	CS	HE	Hyoscyamine-6b-hydroxylase (*H. muticus*)	Increased secretion of scopolamine after culture feeding of hyoscyamine (A)	[39]
Nicotiana tabacum	HR, CS	HE	Lysine/ornithine decarboxylase (*Lupinus angustifolius*)	Increased cadaverine production (A)	[95]
Nicotiana tabacum	CS	TF-OE, TF-GS/RNAi	NtERF32 (Apetala2/ethylene response factor domain) transcription factor (*N. tabacum)*	TF-OE cell lines – increased alkaloid content TF-RNAi cell lines – lower nicotine and total alkaloid contents	[56]
Nicotiana tabacum	CS	HE	Dammarenediol-II synthase (*P. ginseng*)	Production of dammarenediol-II in tobacco (T)	[46]
Nicotiana tabacum	HR	HE	Geraniol synthase (GES) (*Valeriana officinalis*) Geranyl pyrophosphate synthase (GPPS) (*A. thaliana*)	pGES lines – higher geraniol production pGES + pGPPS co-expression lines – in lower geraniol (T)	[96]
Nicotiana tabacum	HR	HE	Geraniol synthase (*V. officinalis* L.)	Geraniol production in tobacco (T)	[97]

(*continued*)

Table 1 (continued)

Engineered host plant	Type of culture[a]	Transgene expression strategy[b]	Transgene (source) or target gene[c]	ME impact (class of the targeted compounds)[d]	Ref.
Nicotiana tabacum	HR, CS	HE	Geraniol synthase (*V. officinalis* L.)	Geraniol production in tobacco (T)	[98]
Nicotiana tabacum	CS	HE	Dammarenediol-II synthase (*P. ginseng)* Cytochrome P450 (*P. ginseng)*	Production of protopanaxadiol in lines co-expressing both genes (T)	[99]
Nicotiana tabacum	HR	HE	Lysine/ornithine decarboxylase (*Lycopodium clavatum*)	Increased production of anabasine in tobacco (A)	[95]
Ophiorrhiza pumila	HR	HE	Strictosidine synthase (*C. roseus*) Geraniol 10-hydroxylase (*C. roseus*)	Increased camptothecin (TIA) production (A)	[99]
Panax ginseng	R	OE	Squalene synthase (*P. ginseng*)	Increased ginsenoside production (T)	[100]
Panax ginseng	R	GS/RNAi	Squalene epoxidase (*P. ginseng*)	Reduced ginsenoside production and increased sterol production in PgSQE1 RNAi lines (T)	[101]
Panax ginseng	R	HE	Taxadiene synthase (*T. brevifolia*)	Production of taxadiene in ginseng roots (T)	[102]
Panax ginseng	HR	OE	Mevalonate-5-pyrophosphate decarboxylase (*P. ginseng*) Farnesyl pyrophosphate synthase (*P. ginseng*)	Increased triterpene production (T)	[103]
Panax ginseng	HR	HE	α-L-rhamnosidase (*Bifidobacterium breve*)	Increased ginsenoside Rg1 production (T)	[104]
Panax quinquefolius	HR	OE, GS/RNAi	Cytochrome P450 /CYP6H/ (*P. quinquefolius*)	OE lines – increased protopanaxatriol-type ginsenosides and decreased protopanaxadiol-type ginsenoside production. RNAi lines – opposite effect (T)	[105]
Panax quinquefolius	HR	OE	Dammarenediol synthase (*P. quinquefolius*)	Increased production of ginsenosides (T)	[106]
Papaver bracteatum	HR	HE	Codeinone reductase (*P. somniferum*)	Increased codeine and morphine production (A)	[107]

Papaver bracteatum	HR	HE	Salutaridinol 7-o-acetyltransferase (*P. somniferum*)	Increased production of morphinan alkaloids (A)	[108]
Platycodon grandiflorum	HR	HE	3-Hydroxy-3-methylglutaryl-CoA reductase (*P. ginseng*)	Increased phytosterol and triterpenoid levels (T)	[109]
Platycodon grandiflorum	HR	TF-HE	AtPAP1 transcription factor (*A. thaliana*)	Increased chlorogenic acid production (P)	[59]
Rauwolfia serpentina	HR	HE	Tryptophan decarboxylase (*C. roseus*)	Increased production of TIAs (A)	[110]
Rhodiola crenulata	HR	OE	Tyrosine decarboxylase (*R. crenulata*)	Increased salidroside production (P)	[111]
Rhodiola sachalinensis	HR	OE	Putative UDP-glycosyltransferase (*R. sachalinensis*)	Increased salidroside production (P)	[112]
Salvia miltiorrhiza	HR	OE, GS/RNAi	Cinnamic acid 4-hydroxylase (*c4h*) (*S. miltiorrhiza*) Tyrosine aminotransferase (*tat*) (*S. miltiorrhiza*) 4-Hydroxyphenylpyruvate reductase (*hppr*) (*S. miltiorrhiza*) 4-Hydroxyphenylpyruvate dioxygenase (*hppd*) (*S. miltiorrhiza*)	Increased rosmarinic acid and lithospermic acid production in lines: c4h-OE, tat-OE, hppr-OE, co-expressing tat+hppr-OE and hppd-RNAi (P)	[113]
Salvia miltiorrhiza	HR	GS/RNAi	Copalyl diphosphate synthase (*S. miltiorrhiza)*	Decreased tanshinone production (T)	[33]
Salvia miltiorrhiza	HR	OE	1-Deoxy-D-xylulose-5-phosphate synthase (dxs) (*S. miltiorrhiza)* Geranylgeranyl diphosphate synthase (ggpps) (*S. miltiorrhiza)* 3-Hydroxy-3-methylglutaryl-CoA reductase (hmgr) (*S. miltiorrhiza)*	Improved tanshinone production in the lines: dxs-OE, ggpps-OE, hmgr-OE Highest tanshinone production in the lines co-expressing hmgr+ggpps-OE (T)	[114]
Salvia miltiorrhiza	HR	OE	3-Hydroxy-3-methylglutaryl CoA reductase (hmgr) (*S. miltiorrhiza)* 1-Deoxy-D-xylulose 5-phosphate reductoisomerase (dxr) (*S. miltiorrhiza)*	Increased tanshinone production in the lines: dxr-OE, hmgr-OE Higher tanshinone production in the lines co-expressing hmgr+dxr-OE (T)	[115]

(*continued*)

Table 1 (continued)

Engineered host plant	Type of culture[a]	Transgene expression strategy[b]	Transgene (source) or target gene[c]	ME impact (class of the targeted compounds)[d]	Ref.
Salvia miltiorrhiza	HR	GS/RNAi	Chalcone synthase (chs) (*S. miltiorrhiza)*	Increased accumulation of phenolic acids in chs-RNAi lines in combination with salicylic acid treatment (P)	[26]
Salvia miltiorrhiza	HR	TF-HE	Maize C1 transcription factor Maize R transcription factor (*Zea mays*)	Decreased salvianolic acid production in C1 and C1+R lines Increased tanshinone production in C1 and C1 +R lines Increased anthocyanin production in C1 and C1+R lines (T, P)	[60]
Salvia miltiorrhiza	HR	GS/RNAi	Cytochrome P450 (*S. miltiorrhiza*)	Increased miltiradiene and decreased tanshinone production (T)	[116]
Salvia sclarea	HR	HE	D-Xylulose 5-phosphate synthase (*dxs*) (*A. thaliana*) 1-Deoxy-D-xylulose 5 phosphate reductoisomerase (*dxr*) (*A. thaliana*)	Increased production of abietanic diterpenes in both dxs-HE and dxr-HE lines (T)	[117]
Saussurea involucrata	HR	HE	Chalcone isomerase (*S. medusa)*	Increased apigenin and total flavonoid content (P)	[118]
Scutellaria baicalensis	HR	OE, GS/RNAi	Chalcone isomerase (*S. baicalensis*)	OE lines – increased production of flavones RNAi lines – decreased production of flavones (P)	[119]
Scutellaria baicalensis	HR	OE	Phenylalanine ammonia-lyase (SbPAL1, SbPAL2, and SbAPL3) (*S. baicalensis*)	Increased flavone content in the lines overexpressing each of the pal genes alone (P)	[120]
Scutellaria lateriflora	HR	TF-HE	AtMYB12 transcription factor (*A. thaliana*)	Increased flavonoid production (P)	[121]

Scutellaria baicalensis	HR	OE	Cinnamate 4-hydroxylase (*S. baicalensis*) 4-Coumaroyl CoA ligase (*S. baicalensis*)	Increased flavone biosynthesis in the c4h-OE and 4 cl-OE lines (P)	[122]
Silybum marianum	HR	HE	Chalcone synthase (*Petunia hybrida)*	Increased silymarin production (P)	[36]
Solanum khasianum	HR	HE	Synthetic construct anti-solamargine single-chain antibody	Increased solasodine glycoside accumulation (A)	[123]
Taxus chinensis	CS	OE	Deacetyl baccatin III-10 β-O-acetyltransferase (*T. chinensis)*	Increased paclitaxel production (T, A)	[35]
Taxus chinensis	CS	OE	9-*cis*-epoxycarotenoid dioxygenase (*T. chinensis)*	Increased paclitaxel (taxol) production (T, A)	[34]
Taxus mairei	CS	OE	10-Deacetyl baccatin III-10-O-acetyl transferase (*T. mairei*)	Low production of baccatin III and taxol in cell cultures Increased baccatin III and taxol production in OE lines only after MJ treatment (T, A)	[40]
Taxus × *media*	CS	HE	Taxadiene synthase (*T. baccata*)	Increased taxane production (T, A)	[124]
Taxus × *media*	CS	GS/antisense	Taxoid 14β-hydroxylase (*T. chinensis*)	Reduced C-14 oxygenated taxoid content (T, A)	[27]
Taxus × *media*	HR	OE	Taxadiene synthase (*T. baccata*)	Increased baccatin III and paclitaxel (taxol) production (T, A)	[125]
Vinca minor	CS	HE	Tryptophan decarboxylase (tds) Strictosidine synthase (str) (*C. roseus*)	Increased TIA production in the *tds+str* co-expressing lines (A)	[126]

[a]*CS* cell suspension, *HR* hairy roots, *R* ginseng roots
[b]*OE* overexpression, *HE* heterologous expression, *TF-OE/TF-HE* transcription factor-overexpression/heterologous expression, *GS/RNAi* gene silencing/RNA interference
[c]"Target gene" for gene silencing application
[d]Class of the target compounds: A alkaloid, P phenolic, T terpenoid

(CS) increased the content of the valuable alkaloid taxol used as anticancer drug [34, 35]. Heterologous expression in CHRC of biosynthetic genes from various sources is mainly employed to upgrade the target pathway for increased production of desired metabolite(s) or to redirect the pathway for production of new compound (s) not present in the host MAP itself. For example, the expression of *Petunia hybrida* chalcone synthase gene into *Silybum marianum* hairy root cultures avoided co-suppression gene silencing and resulted in higher production of flavonolignans and sevenfold increase of the silymarin content [36]. In a similar type of study, the expression of hamster 3-hydroxy-3-methylglutaryl-CoA reductase gene in *C. roseus* hairy root cultures resulted in transgenic clones with different composition and content of the alkaloids and sterols following different levels of transgene expression and possible pathway crosstalk. Some of the studies directed toward production of new compound(s) by CHRC have a straightforward design combining heterologous expression of a biosynthetic gene with precursor feeding. Thus, the hairy root cultures of *Nicotiana tabacum* produced the cannabinoid Δ^1-tetrahydrocannabinolic acid following heterologous expression of Δ^1-tetrahydrocannabinolic acid synthase gene from *Cannabis sativa* and feeding with cannabigerolic acid [37]. Similarly, *N. tabacum* cell suspension and hairy root cultures produced the valuable tropane alkaloid scopolamine after expression of hyoscyamine-6β-hydroxylase genes from *Hyoscyamus niger* and *Hyoscyamus muticus* and feeding with hyoscyamine [38, 39]. In several studies, high production levels of the target metabolites in transgenic lines were reached in combination with their elicitation [40–43], which could be further expanded considering the large volumes of data available on wild-type CHRC elicitation [44, 45]. Depending on the host metabolite network capacity, production of new compounds could be achieved by single transgene expression and redirection of the metabolic pathway, without precursor feeding. For example, the heterologous expression of *Panax ginseng* dammarenediol-II synthase in tobacco cell suspension resulted in production of high amounts of dammarenediol-II, bioactive triterpenoid involved in the dammarane-type ginsenoside biosynthesis [46]. In another study, heterologous expression of (S)-scoulerine 9-O-methyltransferase gene from *Coptis japonica* in *Eschscholzia californica* cell suspension resulted in changes of the alkaloid composition and production of new alkaloid columbamine [47]. Summary of the ME applications on CHRC employing homologous gene overexpression and/or heterologous gene expression is presented in Table 1.

2.2.3 Multigene Transformation and Expression

Metabolic engineering of more than one step in the target metabolic pathways or engineering of an entire biosynthetic pathway requires multiple transgene expressions, which becomes an increasingly important issue in plant and CHRC metabolic engineering [1, 4, 19, 20, 48, 49]. Nearly all reviewed transgenic MAP cell and hairy root cultures were obtained following *Agrobacterium*-mediated transformation directly on the plant cell lines or using simultaneously transformation and hairy root culture initiation via co-transformation with wild-type *A. rhizogenes* strain carrying binary vector(s) with transgene expression cassette(s). This hampers the opportunity to pyramid multiple transgenes using crosses of independently

transformed plants carrying different transgenes and later make cell suspension or hairy root cultures from their seed progeny plants carrying the multiple transgene. Accordingly the reported multigene transformations of CHRC mainly employ the transformation with vector containing stacked expression cassettes of all transgenes of interest or co-transformation with more than one transformation vectors containing the transgenes of interest [48]. For example, Sun and Peebles [50] reported simultaneous glucocorticoid-inducible overexpression of the transcriptional regulator ORCA3 and strictosidine glucosidase (SGD) in hairy root cultures of *C. roseus* generated after seedling transformation with wild-type *A. rhizogenes* containing a single binary vector with T-DNA harboring three tandem arranged expression cassettes, two of which for expression of ORCA3 and SGD under the glucocorticoid-inducible p6xUAX promoter and the third one for expression of GVG chimeric transcription factor providing the glucocorticoid induction of the p6xUAX promoter. In a similar study Runguphan et al. [51] expressed simultaneously two bacterial flavoenzyme PyrH and RebH genes in hairy root cultures of *C. roseus*, which were generated after co-transformation of *C. roseus* seedlings with two wild-type *A. rhizogenes* containing a single binary vector with T-DNA harboring PyrH or RebH gene expression cassette. Although the pointed transformation methods provide the necessary multigene transfer and expression, the transformation and expression of a larger number of genes in CHRC are still challenging, and the efficiency of some recently developed methods for multigene transformation [48, 52] and construction of larger DNA fragments and transformation vectors containing multiple expression cassettes [53] remain to be evaluated.

2.2.4 Transcription Factors

Overexpression or heterologous expression of transcription factors (TF), regulatory genes that control the expression of multiple genes related to the functioning of particular metabolic pathway(s), is a powerful, attractive, and increasingly employed strategy for plant and CHRC metabolic engineering. The main advantage of this engineering approach is the ability to activate simultaneously and at relatively coordinated manner the expression of multiple genes involved into biosynthesis of the target compounds. Moreover, the single transcriptional factor gene transformation and expression make possible to avoid the more laborious and complicated multigene transformation and expression, needed for the complex metabolic engineering tasks. Review of the reported CHRC studies points out two directions of the transcription factor applications. Several studies related to the model MAP species *C. roseus* and *N. tabacum* employed overexpression of endogenous transcription factors [41, 50, 54–58] (Table 1). Overexpression of the ORCA3 transcription factor in *C. roseus* cell suspension [58] and hairy root cultures [41] resulted in increased expression of most of the genes involved into monoterpenoid indole alkaloid (TIA) biosynthesis with the exception of two genes of the important rate-limiting enzymes: G10H (geraniol 10-hydroxylase) and DAT (deacetylvindoline acetyltransferase). Therefore, high levels of TIA production were observed only after feeding of the obtained transgenic ORCA3 cell suspensions with loganin [58] or elicitation of the transgenic ORCA3 hairy root cultures with jasmonic acid [41], providing higher

expression of the entire TIA biosynthetic pathway. In a similar study significantly higher production of TIA compounds was reached after co-expression of ORCA3 and TIA pathway gene SGD (strictosidine glucosidase) [50]. The pointed studies illustrate that the complex expression regulation of the genes involved into the target biosynthetic pathway has to be taken into account in order to realize the full potential of transcription factor expression strategy. Since the molecular basis of secondary metabolite biosynthesis is not well studied in most MAP species, the transcription factor strategy could be applied based on the heterologous expression of respective orthologous TF genes characterized in model plant species. For example, the expression in *Platycodon grandiflorum* hairy roots of *Arabidopsis* AtPAP1 transcription factor, related to the production of anthocyanin pigment in *Arabidopsis*, results in higher production of chlorogenic acid [59]. Heterologous expression of *Z. mays* C1 or C1 and R transcription factors in *S. miltiorrhiza* hairy roots increased the production of tanshinones and anthocyanins [60]. The rapid expanding research and molecular biology data on transcriptional regulation of secondary metabolism and functionality of the isolated transcription factors in both model plant and MAP species, e.g., [61–63], will further fuel ME and production of CHRC secondary metabolites using transcription factor expression.

3 Metabolic Engineering of Plant Cell and Hairy Root Cultures

3.1 General Considerations

Our search reveals 96 studies on metabolic engineering of MAP cell and hairy root cultures related to secondary metabolite production and reported from the beginning of this century (Table 1). Although the parameters of these studies cannot be used to draw general or long-term conclusions, they show some features of the present state of CHRC metabolic engineering research and possible directions for further development. Predominant part (79 out of 96) of the reported studies employed transgenic HR cultures, whereas CS was used in only 17 studies mainly involving the model plant species *N. tabacum*, taxane-producing *Taxus* species, and some earlier studies on metabolic engineering of MAP in vitro cultures from *C. roseus*, *E. californica*, and *C. japonica* (Table 1). The range of MAP species involved in HR metabolic engineering is still smaller in comparison to the large number of MAP species and accessions for which HR culture development and characterization have been reported [44, 127, 128]. The MAP species used within most of the transgenic HR research include the model species in secondary metabolite studies *C. roseus* and *N. tabacum* as well as other well-characterized MAP species like *S. miltiorrhiza*, *A. belladonna*, and others. At the same time to the best of our knowledge, no CHRC metabolic engineering studies have been reported for some valuable MAP species like *Cannabis sativa* [129] in spite of the reported procedures for *Agrobacterium* transformation and HR cultures. Additionally, all transgenic HR lines in the reviewed studies were generated via single co-transformation using wild-type

A. rhizogenes strain carrying binary vector(s) with transgene expression cassette(s). All pointed above gives ground to expect further expanding of the range of MAP species subject of metabolic engineering, taking advantage of the accumulated knowledge and know-how on MAP hairy root cultures.

Further review of the reported CHRC studies shows that they employed all the experimental resources described in the plant metabolic engineering toolkit, earlier. Nearly all studies used constitutive (35S promoter) transgene expression or RNAi induction for metabolic engineering. The inducible transgene expression was engineered using non-plant glucocorticoid-inducible promoter [42, 50, 78]. Further enlarged use of inducible transgene expression in a wider range of studies is expected, considering the needs of more precise evaluation of the impact of CHRC metabolic engineering and the cellular toxicity of some metabolites. The increasing number of characterized MAP genes with elicitor-inducible expression offers additional sources of inducible promoter cloning and utilization. The successful combination of ME and elicitation for high production level of the target compounds in some of the studies [40–43] will further encourage the application of such "hybrid" approach utilizing the available wild-type CHRC elicitation data [44, 45]. Generally, the reviewed studies do not report data on the longer-term stability of the target metabolite production by the engineered CHRC. Long-term transgenic expression stability was rarely studied for transgenic hairy root cultures, e.g., [75]. Therefore, the possible variations in transgene expression and/or target metabolite production, as well as evaluation of the impact of metabolic engineering, is likely to be increasingly included in the ME of CHRC studies.

The aims of ME in the reviewed studies were directed in two main areas: (a) increase of the yield of endogenous compounds which are naturally synthesized by the respective species and (b) synthesis of compounds, which are new for the species. Whereas some of the MAP species (like *C. roseus* and *N. tabacum*) were used as metabolic engineering "platform" for production of both endogenous and new compounds, the ME of other MAP species (like *Taxus* sp.) was directed predominantly toward production of valuable secondary metabolites native for the CHRC species. Given the diversity and complexity of the ME tasks, such ME of CHRC from both "platform" and "unique metabolite" species is likely to continue in parallel.

3.2 Main Groups of Target Secondary Metabolites

Plant secondary metabolites can be roughly classified in three main groups including alkaloids, terpenoids, and phenolics. Compounds from all three groups have been a target for ME in MAP cell and hairy root cultures during these century studies (Table 1). Below is an overview of the main secondary metabolite group subject of the reviewed ME-CHRC studies.

3.2.1 Alkaloids

Alkaloids represent the major groups of compounds which have been a target for ME during the last 15 years. More than 12,000 alkaloids have been identified so far, and a

number of them possess unique pharmaceutical effect including anticancer, analgesic, antiasthma, cardiovascular, sedative, and many others. The amounts of alkaloids in MAP species is generally low which makes them a desired target for biotechnological production.

Terpenoid Indole Alkaloids

The medicinal plant *C. roseus* is well known for the production of pharmaceutical TIAs, and the metabolic pathways and related gene makeup involved in TIA biosynthesis have been extensively studied [130]. *C. roseus* has been a subject of many CHRC metabolic engineering studies in terms of improving the TIA yield. The expression of most of the genes which are part of the TIA biosynthetic pathways is controlled at transcriptional level [131]. Accordingly, a large part of the *C. roseus* CHRC research employed transcription factor overexpression (Table 1). Several studies demonstrated that overexpression of ORCA transcription factors alone or in combination with precursor feeding, elicitation, or biosynthetic gene expression results in increased TIA production by the transgenic CHRC [41, 50, 54, 58]. In another study, the overexpression of WRKY transcription factor had a positive effect on the accumulation of tryptamine, serpentine, and ajmalicine but a negative effect on the amounts of catharanthine and tabersonine [57]. At the same time the overexpression of CrBPF1 transcription activator [55] or the suppression of the transcriptional repressor ZCT1 [28] had a little effect on TIA production. In other group of studies, TIA production was improved via overexpression of key TIA biosynthetic genes including feedback-resistant AS α subunit, anthranilate synthase AS β subunit, and tryptophan decarboxylase alone or in combination [42, 72–74, 78]. In additional study, Peebles et al. [75] showed that transgenic *C. roseus* hairy root line was stable and maintained inducible expression of anthranilate synthase and increase TIA production over 5 years providing attractive perspective for an industrial application. It has to be noted that cloned and characterized TIA biosynthetic genes from *C. roseus* were further used for heterologous expression and increased TIA production in other MAP species, for example, heterologous expression of tryptophan decarboxylase in *R. serpentine* hairy roots and *V. minor* cell suspension cultures [110, 126].

TIA metabolic engineering in *C. roseus* CHRC also employed heterologous expression of genes from non-plant origin. Increased catharanthine and total TIA production were engineered via expression of mouse *Bax* gene in cell suspension of *C. roseus* [77]. In another study, Ayora-Talavera et al. [71] expressed a truncated hamster 3-hydroxy-3-methylglutaryl-CoA reductase gene in *C. roseus* hairy roots and observed substantial variations of the content of different TIA compounds between the individual transgenic lines, for example, one of the lines showed increased ajmalicine and catharanthine and reduced campesterol contents, while another line displayed increased campesterol and serpentine but decreased ajmalicine and no catharanthine content. *C. roseus* hairy root cultures were also used for production of halogenated alkaloids with potentially important pharmacological properties, based on the innovative approach developed by Runguphan et al. [51]. The authors employed a heterologous expression of tryptophan 5-halogenase

from *Streptomyces rugosporus* and halogenase from *Lechevalieria aerocolonigenes* in *C. roseus* hairy root cultures in order to generate 5-chlorotryptophan and 7-chlorotryptophan which were later incorporated in TIA synthesis producing chlorinated alkaloids.

Tropane Alkaloids

A number of reported ME studies using CHRC have been related to the increased production of valuable tropane alkaloids like hyoscyamine and scopolamine. The putrescine N-methyltransferase, hyoscyamine-6β-hydroxylase, and tropinone reductases were the most extensively used genes in these studies (Table 1). The overexpression of the key enzyme putrescine-N-methyltransferase alone resulted in increased production of the high-value scopolamine only in hairy root cultures of *D. metel* [82] but not in the other studied MAP species *A. acutangulus* [65], *A. belladonna* [67], *D. myoporoides x D. leichhardtii* [84], *H. muticus* [82], and *H. niger* [89]. Accordingly expression of the putrescine N-methyltransferase transgene together with either tropinone reductases I [65] or with hyoscyamine 6β-hydroxylase [70, 88] led in all cases to increased amounts of tropane alkaloids including scopolamine. Improved tropane alkaloid production was reported also after the simultaneous overexpression of tropinone reductases I and hyoscyamine 6β-hydroxylase genes in *A. acutangulus* hairy root cultures [66]. From pharmaceutical point of view, scopolamine has a greater value than hyoscyamine. Accordingly several studies reported efficient conversion of hyoscyamine into scopolamine via heterologous expression of hyoscyamine 6β-hydroxylase gene alone or together with putrescine-N-methyltransferase gene in hairy root cultures of the MAP species, naturally producing hyoscyamine and scopolamine [69, 83, 85, 88]. In two similar studies, the heterologous expression of hyoscyamine 6β-hydroxylase in CHRC of the non-hyoscyamine-producing species *N. tabacum* led to high level of bioconversion of culture-fed hyoscyamine into scopolamine [38, 39]. The expanding transcriptomic data and information on genes playing important role in tropane alkaloid biosynthesis [132] provide ground for further improvement of the ME efficiency in this area.

Pyridine Alkaloids

The reported ME studies targeting pyridine alkaloid biosynthesis of MAP cell and hairy root cultures involved predominantly *Nicotiana* species, which are model species in this area of plant ME and also widely used within in vitro culture studies. Employing RNAi gene silencing, DeBoer et al. [32] demonstrate that suppression of A622 gene in *N. glauca* hairy roots resulted in substantial decrease of the levels of both anabasine and nicotine and suggest that the A622 gene is an important component of the enzyme complex responsible for biosynthesis of pyridine alkaloids in *Nicotiana*. In two follow-up studies, DeBoer et al. [30, 31] explored the role of ornithine decarboxylase in pyridine alkaloid biosynthesis using RNAi gene silencing in hairy root cultures of *N. glauca* and *N. tabacum*. The obtained results showed that the silencing of this gene led to reduced nicotine and anabasine contents in *N. glauca* cultures but reduced nicotine and increased anatabine contents in *N. tabacum*

cultures. In confirmation of the observed effects, Bunsupa et al. [95] showed that heterologous expression of lysine/ornithine decarboxylase from *L. clavatum* leads to increased content of anabasine in *N. tabacum* hairy roots. In a comprehensive transcript profiling and functional genomics study using tobacco BY-2 cell suspension, Hakkinen et al. [93] identified 34 genes putatively associated with pyridine alkaloid metabolism and tested the effect of their overexpression in *N. tabacum* hairy root and cell suspension cultures. Increased nicotine content in transgenic tobacco HR line was observed after overexpression of gene designated as nicotine-enhancing GH3 enzyme 1. This and other recent advances in understanding the molecular basis of *Nicotiana* alkaloid biosynthesis [133] pave the road for further efficient ME of pyridine alkaloid production.

Isoquinoline Alkaloids

The ME of isoquinoline alkaloid production in MAP hairy root and cell suspension cultures was subject of only few reported studies. Sato et al. [47] demonstrated that the overexpression of (S)-scoulerine 9-O-methyltransferase in *C. japonica* cell suspension resulted in slight increase of berberine and columbamine production, whereas the heterologous expression of the same gene in *E. californica* cell cultures led to changes in the metabolic flow and production of a new alkaloid columbamine and decreased content of sanguinarine which is typical for the wild-type cells. In another study, Sharafi et al. [107, 108] carried out heterologous expression of codeinone reductase and salutaridinol 7-o-acetyltransferase from *P. somniferum* in *P. bracteatum* HR cultures. The obtained results showed increased codeine and morphine production in codeinone reductase overexpressing lines and increased production of thebaine, codeine, and morphine in salutaridinol 7-o-acetyltransferase overexpressing lines. The further ME of isoquinoline alkaloid production in CHRC will largely benefit from the recent advances in in vitro cultures of MAP producing isoquinoline alkaloids, better understanding of their biosynthesis and results of applications of ME at plant level, reviewed in [134].

Paclitaxel and Taxanes

Paclitaxel is the active substance of taxol (Bristol-Myers Squibb), a drug that has been used for treatment of different types of cancers including lung, ovarian, breast, and pancreatic cancer [135]. Paclitaxel is classified as a diterpenoid alkaloid, part of the taxane family of drugs, which has been originally extracted from the bark of *T. brevifolia*, but its amount is very low, and around 30,000 kg of tree biomass is required to produce 1 kg of refined paclitaxel [136]. The synthetic and semisynthetic production of paclitaxel has been developed; however, the process is complex and expensive [137]. The production of paclitaxel using *Taxus* sp. CHRC is considered promising since the purification of paclitaxel from CHRC is easier than from whole plant material, but the amount of paclitaxel in the culture needs to be increased in order to make it feasible. Several recently reported studies described increased paclitaxel or total taxane contents in CHRC of different *Taxus* species by overexpressing 9-cis-epoxycarotenoid dioxygenase, deacetylbaccatin III-10 β-O-acetyltransferase, and taxadiene synthase genes [34, 35, 124, 125]. In another

study, Cha et al. [102] expressed taxadiene synthase from *T. brevifolia* into root cultures of *P. ginseng* and engineered the transgenic ginseng root cultures to produce taxadiene, a skeletal precursor of paclitaxel. The obtained results open the way toward paclitaxel biosynthesis using transgenic ginseng root cultures and possibly other in vitro cultures of plant species outside of the genus *Taxus*.

3.2.2 Terpenoids

Terpenoids represent a vast group of compounds and valuable natural products with various applications including pharmaceuticals, perfumery, and cosmetics products. The terpenoids could be also used as precursors for synthesis of other valuable secondary metabolites like TIAs. Engineering the production of specific terpenoid precursor by highly productive CHRC could be the first essential step toward further ME production of valuable secondary metabolites. Geraniol is a monoterpenoid which is an intermediate of the TIA pathway and is involved in the synthesis of TIAs. Increasing geraniol synthesis in tobacco CHRC will provide opportunity to use tobacco CHRC as platform for engineering of TIA production in this plant species. In 2014, three separate studies [96–98] showed that the expression of geraniol synthase from *V. officinalis* leads to increased geraniol production in tobacco hairy roots and cell suspension cultures. The results offer a platform to reconstitute TIA synthesis in this species.

Ginsenosides

Ginsenosides are a class of steroid glycosides and triterpene saponins which are almost exclusively found in the *Panax* genus and represent the pharmacologically active component of ginseng. Several research papers have recently been published targeting increased content or improved composition of ginsenosides in *Panax* root and HR cultures. Overexpression of squalene synthase, dammarenediol synthase, or farnesyl pyrophosphate synthase alone led to increased ginsenoside amounts in cultured ginseng roots and hairy root cultures [100, 103, 106], while overexpression of mevalonate-5-pyrophosphate decarboxylase did not result in significant or just in minor changes of the total ginsenoside contents [103], and suppression of the squalene epoxidase resulted in decreased ginsenoside contents [101]. In another study the overexpression of the cytochrome P450 (CYP6H) gene in *P. quinquefolius* HR cultures resulted in increased content of protopanaxatriol-type ginsenosides and decreased protopanaxadiol-type ginsenosides, whereas the RNAi-induced gene silencing of the same gene led to the opposite effect [105]. The attempts to improve the ginsenoside composition also employed heterologous expression of non-plant genes as the expression of α-L-rhamnosidase from *Bifidobacterium breve* in the *P. ginseng* HR cultures led to the selectively increased amounts of the more valuable glycosylated ginsenoside Rg1 at the expense of the less desired non-glycosylated Re form [104]. The ME was successfully applied for production of ginsenosides in CS from plant species outside of the genus *Panax*. Employing the heterologous expression of both dammarenediol-II synthase and cytochrome P450 (716A47) genes from *P. ginseng*, Chun et al. [99] engineered tobacco cell suspension to produce protopanaxadiol, an aglycone of ginsenosides having a wide range of pharmacological

activities. The last opens the way toward ginsenoside production in tobacco and other plant production platforms.

Tanshinones

Tanshinones are a group of active diterpenoids found in low content in *S. miltiorrhiza*, which have been used for treatment of cardiovascular and cerebrovascular disorders, and therefore the increase of their production is of a great interest. Several ME studies managed to successfully increase the tanshinone content in *S. miltiorrhiza* hairy root cultures. Kai et al. [114] overexpressed alone the 1-deoxy-D-xylulose-5-phosphate synthase (*dxs*), geranylgeranyl diphosphate synthase (*ggpps*), and 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgr*) genes in *S. miltiorrhiza* HR cultures. The highest impact on tanshinone content was achieved in the lines overexpressing the *ggpps* gene, followed by *dxs* and *hmgr* lines. In the following two studies, additional increase of the tanshinone content was reached by overexpression of two genes (*hmgr* and 1-deoxy-D-xylulose 5-phosphate reductoisomerase) in combination with culture elicitation [115], as well as by simultaneous overexpression of *ggpps* and *dxs* genes [138]. Applying a different strategy, Zhao et al. [60] achieved an increase in tanshinone content by heterologous expression of maize C1 and maize R transcription factor in *S. miltiorrhiza* hairy roots.

3.2.3 Phenolics and Terpeno-Phenolic Compounds

Shikonin

Shikonin is a naphthoquinone pigment which is extracted from the roots of *Boraginaceae* plants possessing significant anti-inflammatory, antitumor, and antimicrobial activities. In an attempt to increase the amounts of its precursors 4-hydroxybenzoate (aromatic precursor) and geranyl diphosphate (isoprenoid precursor), Koghle et al. [90] expressed simultaneously the chorismate pyruvatelyase (*ubiC*) from *E. coli* and HMG-CoA reductase from *A. thaliana* in hairy root cultures of *L. erythrorhizon.* The results however did not show elevated amounts of shikonin in the obtained transgenic cultures. In recent studies, Fang et al. [29, 91] achieved an increase in shikonin content in the hairy root cultures of *L. erythrorhizon* employing overexpression of the 1-aminocyclopropane-1-carboxylic acid synthase and EIN3-like protein gene 1.

Flavonoids

Flavonoids have been long time known as powerful polyphenolic antioxidants which are ubiquitously found in plants. In an attempt to increase flavonoid content, chalcone isomerase overexpression was carried out in several ME studies including HR cultures of different MAP species [43, 92, 118, 119]. In all cases the overexpression of chalcone isomerase led to increased content of flavonoid compounds. Another successfully employed gene for ME of high flavonoid content was the cinnamate 4-hydroxylase gene. Following the overexpression of this gene, Park et al. [64] observed increased production of decursinol angelate in hairy root cultures of *A. gigas*; however, the overexpression of phenylalanine ammonia-lyase (*pal*) did not

lead to the same effect. In a similar study the overexpression of *pal* gene resulted in higher flavone content in *S. baicalensis* hairy root lines [120].

Phenolic Acids

Together with flavonoids, phenolic acids are the most widespread group of plant phenolics representing powerful antioxidants that could prevent cellular damage due to free-radical oxidation reactions, in which the presence in the human diet is considered health beneficial. To increase the phenolic acid accumulation in HR cultures, the reported ME studies employed mainly blocking the flavonoid biosynthetic pathway. Accordingly, the RNAi-induced silencing of chalcone synthase gene in *S. miltiorrhiza* hairy roots in combination with culture elicitation resulted in suppression of flavonoid biosynthesis and improved production of phenolic acids [26]. In another study, Xiao et al. [113] tested the effect of overexpression and RNAi silencing of cinnamic acid 4-hydroxylase (c4h), tyrosine aminotransferase (tat), 4-hydroxyphenylpyruvate reductase (hppr), and 4-hydroxyphenylpyruvate dioxygenase (hppd) genes in *S. miltiorrhiza* hairy roots. The obtained results showed increased rosmarinic acid and lithospermic acid production in the *c4h*, *tat*, *hppr*, and *tat+hppr* OE lines, as well as in *hppd* RNAi lines. Tuan et al. [59] engineered significant, nearly ten times increase of the chlorogenic acid production in *P. grandiflorum* HR cultures following heterologous expression the AtPAP1 transcription factor from *A. thaliana*.

4 Conclusions and Future Prospects

4.1 New Technologies and Information-Driven CHRC Metabolic Engineering

Emerging at the end of the last century, ME of secondary metabolite production in plant CHRC passed through a period of exiting developments, employing the plant ME and transgenic research toolbox, as well as the current understanding of the molecular biology of secondary metabolite biosynthesis. The reviewed studies demonstrate successful applications of a wide range of experimental methods and engineering strategies for ME of secondary production in CHRC, including a number of MAP species. The obtained results make possible to assess the impact of the metabolic engineering, efficiency of the different strategies, and the overall effect of the metabolite pathway complexity and regulation. The obtained results, accumulated knowledge, and know-how provide a solid base for entering of a next stage of ME applications on CHRC of MAP species related to more precise and complex ME of the target metabolite production. An essential role of such next-stage ME development will be the more effective utilization of the large volume information derived from the application of "omics" technologies in understanding the complexity of plant secondary metabolite biosynthesis and regulation and its use for a better design of ME strategies. The results from genomics studies and NGS data analysis will additionally empower the ME toolbox expanding the range of the genes for various ME applications. It could be expected that the increasing number of

next-stage ME studies will be directed toward realization of more complex ME tasks, as well as further optimization of the parameters of target metabolite production using inducible transgene expression and providing efficient compartmentation and transport of the target metabolite. Further increased implementation in ME of the earlier accumulated data on cultivation and elicitation of the wild-type CHRC is foreseen. The reported so far ME studies pay less attention to the plant accession/genotype used for generation of transgenic CHRC. The expected advance in MAP genetic resources research will possibly offer additional opportunities for improvement of the ME impact through careful selection of the starting MAP genotype.

4.2 Game-Changing Opportunity

The rapid development of plant biotechnology and increased volumes of agriculture production involving genetically modified (GM) plants result in strong and often fierce division in the society (even within the scientific community), in relation to plant biotech research and applications. The public concerns and negative opinion on growing GM plants result in underfunding of plant biotech research, as well as highly restrictive regulations which currently make very difficult the field testing and practically impossible the industrial growing of genetically modified MAP. At the same time, the increasing herbal products and phytopharmaceutical consumption result in overexploitation of natural MAP populations, often endangering their existence. The discussed here rapid advance of the ME of target secondary metabolite production by MAP cell and hairy root cultures and next possible pharmaceutical use of the purified secondary metabolites produced by transgenic CHRC offers a unique "game-changing" opportunity to unite rather diverse groups in society and industry, like plant biotech researchers and supporters, environmentalists, small- and medium-sized biotech companies, etc. Therefore future steady increase in the commercialization of transgenic CHRC producing valuable secondary metabolites at levels passing the economic feasibility thresholds could be expected. Moreover GMO legislation concerning bioreactor cultivated CHRC is generally less restrictive than those for release in the environment and field growing of GM plants. The last could make possible transgenic CHRC commercialization and related secondary metabolite production to be done also by small- and medium-sized biotech companies, which will additionally stimulate the research on ME of secondary metabolite production by MAP cell and hairy root cultures.

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Agrobacterium rhizogenes-Mediated Transformation of Plants for Improvement of Yields of Secondary Metabolites

6

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Abstract

The transgenic hairy root culture has revolutionized the role of tissue culture of plants in the synthesis of secondary metabolites. It was shown that hairy roots in the most cases exhibit higher biosynthetic capacity for secondary metabolite production comparing to the non-transgenic roots. A big number of medicinal compounds have been produced using this approach. However, the mechanism of influence of T-DNA genes on secondary metabolite production is not completely understood. The stimulatory effect of single *rol* genes (*rolA*, *rolB*, *rolC)* on secondary metabolite production was demonstrated for a number of plant species that are widely used in pharmacology. It is interesting to note that these *rol* genes are present in naturally transgenic *Linaria*, *Ipomoea*, and *Nicotiana* plants. Many species from these genera are used as medicinal. Besides, naturally transgenic plants could be a good model for study of possible evolutionary function of *rol* genes in the control of secondary metabolites for plant protection.

Keywords

Agrobacterium rhizogenes · T-DNA · *rol* genes · Secondary metabolism · Naturally transgenic plants · Medicinal plants

1 Introduction

Metabolism is the complex of all biochemical reactions carried out by the organism. Primary metabolic pathways lead to just a few final compounds, while secondary metabolic pathways disperse too many substances. Secondary metabolites are known to play an important role in the plant adaptations to the environmental factors but also are an important source of pharmaceutically active drugs [1, 2]. Biologically active secondary metabolites in plants belong to glycosides, alkaloids, polyphenols (flavonoids, terpenoids, coumarins, saponins), and essential oils. These compounds are essential for communication among various organisms in mutualistic (e.g., pollinators as beneficial organisms' attraction) or antagonistic (e.g., deterrents against herbivores and pathogens) interactions. Besides, they are involved in regulation of resistance to abiotic stress, for example, increased UV radiation. In particular, naphthoquinones exhibit UV sunscreen and antimicrobial properties, triterpenes are biopesticides, polyacetylenes and phenylethanoids demonstrate antiviral activity, and phenolic glycosides, triterpene saponins, and polyines often possess antifungal activity. At the same time identical biological functions can be performed in different groups of plants' families by the compounds of various nature [3].

In order to obtain high yields of secondary metabolites suitable for commercial application, main efforts have been concentrated on increasing of the biosynthetic activities of tissue cultures, achieved by improving of the cultivation conditions, selecting highly productive cell lines, and using precursor feeding and genetic transformation of cultivated tissues [4]. The transgenic hairy root cultures have

revolutionized the role of tissue culture of plants in the production of secondary metabolites. The hairy roots induced by *Agrobacterium rhizogenes* have become popular in the several last decades as a method of secondary metabolite production in plant roots [5, 6]. Hairy roots are characterized by their genetic and biosynthetic stability, fast hormone-independent growth, intensive lateral branching, lack of geotropism, and simplicity of maintaining. A big number of secondary metabolites have been synthesized in hairy roots [7, 8]. These compounds, produced by hairy roots arising from the plant material infected by *A. rhizogenes*, in most cases are the same as those usually synthesized in intact non-transformed roots [9]. This feature, as well as genetic stability and generally rapid growth of hairy roots on hormone free media, makes them especially suitable for different kinds of applications. In this chapter we will try to summarize the present data on *Agrobacterium rhizogenes*-mediated transformation of plants for improvement of yields of secondary metabolites and discuss possible mechanisms of *A. rhizogenes* T-DNA action in this process.

2 *Agrobacterium*: General Information

Agrobacterium is a rod-shaped, Gram-negative soil bacterium [10]. Historically, there are three species of *Agrobacterium*, infecting plants. *Agrobacterium tumefaciens* is the causal agent of so-called crown gall disease (tumorigenesis in the root neck) [11]. *A. rhizogenes* induces hairy root disease (the formation of proliferative multibranched adventitious roots) [12]. *A. vitis* is responsible for crown gall on grapevines and for induction of a hypersensitive response in other plant species [13]. Recent taxonomic studies have reclassified these species as *Rhizobium radiobacter*, *Rhizobium rhizogenes*, and *Rhizobium vitis*, correspondingly [14]. Nevertheless, old names are used as synonyms. We will use the traditional names in the following narrative. *A. tumefaciens*, *A. rhizogenes*, and *A. vitis* are able to insert of fragments of their Ti or Ri plasmids, called T-DNA (for "transferred DNA"), into the plant chromosomal DNA [11].

The T-DNA contains so-called oncogenes and genes, encoding enzymes that cause the plant to create opines (Fig. 1). Opines are modified amino acids that serve as a source of nitrogen for certain strains *of Agrobacterium*, but not for the most other organisms [15]. The oncogenes *of A. tumefaciens* and *A. vitis* are represented by genes *iaaM* and *iaaH* responsible for the production of auxin (indole-3-acetic acid) via the IAM pathway and gene *ipt* for the production of cytokinins [16]. Functions of oncogenes of *A. rhizogenes* are still discussed.

3 Hairy Roots, Induced by *Agrobacterium rhizogenes*

Agrobacterium rhizogenes is able to transfer T-DNA from Ri plasmid (root-inducing plasmid) into the plant through the wound. After integration and T-DNA gene expression, the hairy root phenotype could be observed. It typically includes

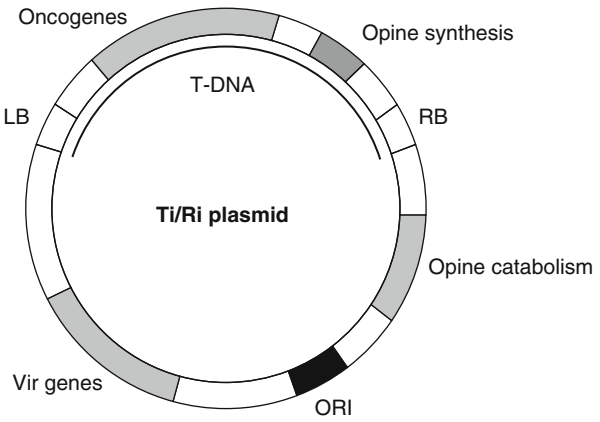

Fig. 1 General structure of Ti/Ri plasmids

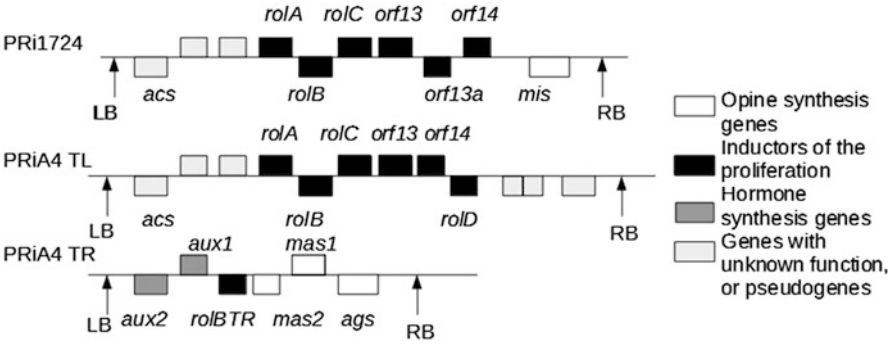

Fig. 2 Structure of T-DNA of pRiA4 and pRi1724

overdevelopment of multibranched adventitious roots at the site of infection. Such roots are characterized by agravitropic growth.

The structure of T-DNA could be different in the different strains. In some strains of *A. rhizogenes* (mannopine-type pRi8196, cucumopine-type pRi2659, and mikimopine-type pRi1724 T-DNA), it is organized as a single fragment; in others (agropine-type plasmid pRiA4), it could be comprised of two parts, TL-DNA and TR-DNA, physically separated by at least 15 kb of a nontransferred T-central DNA (TC-DNA) sequence (Fig. 2) [17]. These two fragments could be transferred into plant chromosome either as complete T-DNA or separately [18]. TL-DNA contains rol genes, *rolA* (*orf10*), *rolB* (*orf11*), *rolC* (*orf 12*), and *rolD* (*orf15*); *orf8*, *orf13*, and *orf14* genes, which are responsible for root formation; and several ORFs with unknown functions [19–21]. TR-DNA contains genes *mas1*, *mas2*, *aux1*, *aux2*, and *ags* encoding the products leading to the biosynthesis of auxin and opines [22, 23]. The T-DNA from Ri plasmids of mannopine, cucumopine, and mikimopine-type

Table 1 *A. rhizogenes* oncogenes

Gene	Characteristics	Possible function	Refs.
rolA	*rolA* is the only T-DNA gene, which is expressed in plant and bacteria, and contains intron. RolA is a membrane-bound (but not the transmembrane) protein	Decrease of auxin concentration and increase sensitivity to it	[27, 28]
rolB	This single gene is sufficient to cause hairy root phenotype; in pRiA4 it is located in plasmalemma and has tyrosine phosphatase activity; in pRi1724 it is nuclear protein without phosphatase activity	Increase sensitivity to auxin	[29–31]
rolC	Expression of *rolC* is triggered by high levels of sucrose	Release of cytokinins from cytokinin glucosides or influence on the cell cycle through the metabolism of sugars	[32, 33]
rolD	*rolD* is present in agropine strains of *A. rhizogenes*	Conversion of ornithine to proline	[34]
orf8	The N-terminal domain shows similarity to the *A. rhizogenes* RolB protein. The C terminus shows similarity to the tryptophan 2-monooxygenases	May modulate the auxin responsiveness of host cells and increase concentration of indole-3-acetamide (IAM)	[35]
orf13	Orf13 binds with the retinoblastoma protein Rb (important regulator of the cell cycle) and releases the transcription factors E2F, causing change of cyclin E to D to transfer cells into S-phase	Induce cell division	[36]
orf14	Overexpression of *orf14* did not produce morphological changes in plants	Act synergistically with the rol genes and *orf13* to improve root induction in carrot and tobacco	[37–39]
aux1	Homologous to *iaaH* of *A. tumefaciens*	Tryptophan 2-monooxygenase that catalyzes the conversion of tryptophan to indole-3-acetamide (IAM)	[40, 41]
aux2	Homologous to *iaaM of A. tumefaciens*	IAM hydrolase that converts IAM to indole-3-acetic acid (IAA)	[42–44]

strains do not carry *aux* genes. Since these strains are still capable to induce a "hairy root" syndrome, it can be concluded that the presence of the aux genes is not necessary for hairy root phenotype. It has been shown that the aux genes are involved in support of the "hairy root" phenotype and extend the host range of the bacterium [9, 24–26]. The general information about *A. rhizogenes* oncogenes is summarized in Table 1.

About 30 years ago it was observed that hairy root cultures have another unusual property: high production of secondary metabolites [45]. Let us focus on this feature.

4 Hairy Root Cultures for Secondary Metabolite Production

Hairy roots often exhibit about the same or higher biosynthetic capacity for secondary metabolite production comparing to the untransgenic roots. This observation gave rise to the development of a new direction associated with the use of hairy roots for the production of these compounds. Normally, secondary metabolites are not essential to plant growth and hence are synthesized in small amounts [46, 47]. Their extraction from field-grown plants has several limitations. Traditional planting methods often require several months to obtain a crop; besides the levels of secondary metabolites from field-grown plants are affected by many biotic and abiotic factors (climate conditions, pathogens, etc.) [4, 48]. In contrast, plant cell culture is not affected by uncontrolled changes of environment. However, synthesis of secondary metabolites in plant cell suspension cultures in some cases is also hindered, because these compounds are usually produced by specialized cells and/or specific to the distinct developmental stages. Some of them are not synthesized in undifferentiated cells [48–52]. Hairy root cultures are deprived of these disadvantages. They are characterized by genetic and biosynthetic stability and fast hormone-independent growth and easily maintained. The hairy roots are widely used as a method of secondary metabolite production in plant roots [5, 6]. Li Tian (2015) [53] in his review has shown that the hairy root cultures, synthesizing various secondary metabolites, were produced by the *Agrobacterium rhizogenes*-mediated transformation of more than 150 species of plants belonging to 33 families.

Here we attempted to supplement his data with new details and organize information based on phylogeny of plants [54] and chemistry of the synthesized compounds (Table 2, Fig. 3).

As we can see from the picture, there are several taxa whose representatives have been actively used for production of secondary metabolites in the hairy roots. Other taxa are not involved in this process. This disproportion could be explained from two points of view: the different plant susceptibilities to the *Agrobacterium rhizogenes* infection and the differences in the content of metabolites of interest in plant tissues. The clade, containing the orders Gentianales, Lamiales, Solanales, and Boraginales, is of special interest. These orders form a monophyletic group and their representatives are actively used in the synthesis of wide range compounds in hairy root cultures. Other taxa, used for the synthesis of secondary metabolites, are distributed across the phylogenetic tree more or less regularly.

Some of biologically active secondary metabolites are found in almost all analyzed plant orders. Others have a wide distribution in closely related families. Let us focus on functions of main groups of secondary metabolites, described in Table 2.

4.1 Alkaloids

Alkaloids are a large group of secondary metabolites with limited taxonomic distribution and diverse biosynthesis pathways [211]. The current classification of

Table 2 Secondary metabolites produced in hairy root cultures of plants of different taxa

Clade	Superorder	Order	Family	Secondary metabolites					Ref.
				Alkaloids	Phenolic compounds and polyphenols	Glycosides	Essential oils	Other organic compounds	
eudicots	Ranunculanae	Ranunculales	Papaveraceae	Quinolizidine **alkaloids**					[55, 56]
			Ranunculaceae	Diterpene **alkaloids**					[57]
core eudicots	Rosanae	Cucurbitales	Cucurbitaceae		Flavonoids, triterpenoids, triterpene saponins, phenolic acids				[58–63]
		Rosales	Cannabaceae	Atropine **alkaloid**				Choline	[64]
			Rosaceae		Flavonoids, tannin				[65]
		Fabales	Fabaceae	Indolizidine **alkaloid**	Triterpene saponins, flavonoids, tannins, anthraquinones, triterpenoids flavones			Steroidal sapogenin, polypeptide pigments	[66–81]
		Malpighiales	Hypericaceae		Phenolic acids, xanthones, flavonoids				[82, 83]
			Linaceae		Lignans				[84]
		Malvales	Bixaceae		Phenolic acids				[85]
			Malvaceae		Disesquiterpene				[86]
		Brassicales	Brassicaceae		Diterpenoids	Glucosinolates		Lipids, fatty acids	[87–89]
			Tropaeolaceae			Glucosinolates			[90]
		Sapindales	Meliaceae		Triterpenes				[91]
			Rutaceae	Benzophenanthridine **alkaloids**	Coumarins, furanocoumarins				[92, 93]
		Myrtales	Lythraceae		Naphthoquinone, tannins				[94, 95]
		Geraniales	Geraniaceae		Tannins				[96]

(*continued*)

Table 2 (continued)

Clade	Superorder	Order	Family	Secondary metabolites					Ref.
				Alkaloids	Phenolic compounds and polyphenols	Glycosides	Essential oils	Other organic compounds	
	Caryophyllanae	Caryophyllales	Droseraceae		Naphthoquinones (ramentaceone)				[102]
			Amaranthaceae					Indole-derived pigments	[103]
			Plumbaginaceae		Naphthoquinones				[104, 105]
			Polygonaceae		Flavonoids, phenol acids, anthraquinones, flavonol **glycosides**				[106, 107, 108, 109, 110]
	Asteranae	Gentianales	Apocynaceae	Terpenoid indole **alkaloid**					[111–116]
			Gentianaceae		Xanthones quiterpenes	Iridoid and secoiridoid **glycosides**			[117–121]
			Rubiaceae	Monoterpenoid indole **alkaloids**					[97–101]
			Asclepiadaceae		Triterpene saponins				[123–127]
			Lamiaceae		Rosmarinic and phenolic acid, diterpenoids flavonoids, phenylethanoids	Phenylpropanoid **glycoside (verbascoside)**		Phytoecdysteroids	[129–142]
			Verbenaceae					Natural sweetener	[122]
			Bignoniaceae			Phenylpropanoid **glycosides**			[135]
			Scrophulariaceae			Phenolic and iridoid **glycosides**			[141]

			Pedaliaceae		Naphtho- and anthraquinones	Phenylpropanoid **glycoside**			[137, 138]
			Acanthaceae		Naphthoquinone esters				[139]
			Orobanchaceae			Phenylpropanoid and iridoid **glycosides**			[140]
			Plantaginaceae		Triterpene saponins, bacosides, flavonoids, anthraquinones	Iridoid **glycosides**			[142–144]
		Solanales	Convolvulaceae	Tropane and quinoline **alkaloids**					[145]
			Solanaceae	Tropane, pyridine, and steroidal glycol-**alkaloids**	Steroidal saponins, triterpene, saponins, steroidal lactones			Polyamines	[146–161]
		Boraginales	Boraginaceae		Naphthoquinones			Polyunsaturated fatty acids	[162, 163]
		Asterales	Asteraceae		Flavonoids, flavones, flavonolignans, triterpenoids, sesquiterpenes, sesquiterpene lactones, phenolic coumarins		**Essential oils**	Polyacetylenes, polyines	128, [164–180]

(*continued*)

Table 2 (continued)

Clade	Superorder	Order	Family	Secondary metabolites					Ref.
				Alkaloids	Phenolic compounds and polyphenols	Glycosides	Essential oils	Other organic compounds	
			Campanulaceae	Piperidine **alkaloids**		Quercetin **glycosides**		Polyacetylenes	[181–187]
		Dipsacales	Caprifoliaceae					Iridoid esters (valepotriate)	[188]
			Valerianaceae					Iridoid esters (valepotriate)	[189–199]
		Apiales	Apiaceae		Triterpene saponins, triterpenoids, sesquiterpene phenolic acid, quinones		**Essential oils**		200, [204–210]
			Araliaceae		Triterpene saponins				[201–203]

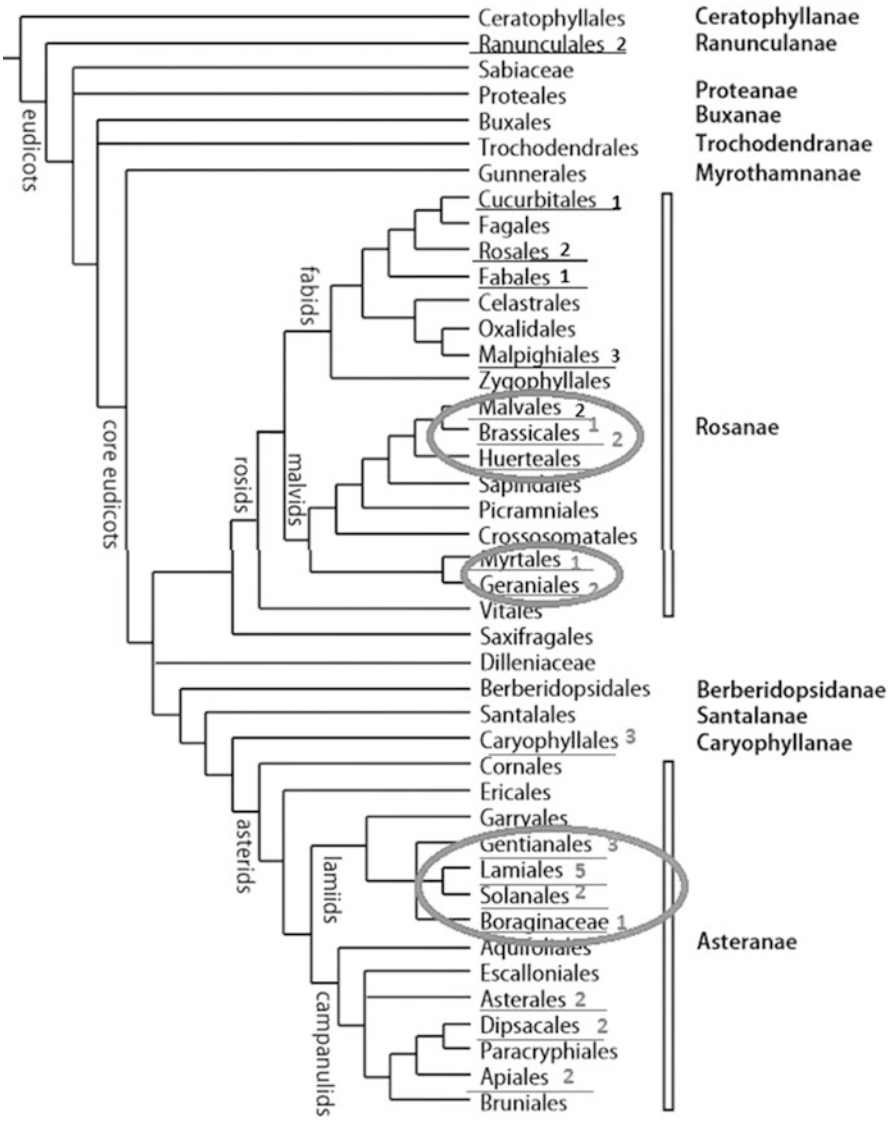

Fig. 3 Relationship of plants, used for secondary metabolite production in hairy roots. (Plant orders are underlined, if they contain at least one plant family, used for secondary metabolite production. A number of such families are shown next to the order name. Monophyletic groups of orders, producing secondary metabolites in hairy roots, are *circled*)

alkaloids is based on the N-containing structures (indole and quinoline alkaloids). The most of the known alkaloids' functions concern plant environmental prostration. Alkaloids were produced in the hairy root cultures of approximately 20% of studied plant families.

Quinoline alkaloids and their derivatives are produced by hairy root cultures of plants, which belong to the Ranunculales, Papaveraceae; Geraniales, Rubiacea; and Solanales, Convolvulaceae (Table 2). They often have high antiviral and fungicidal activities.

Lys-derived indole alkaloids include indolizidine, piperidine, quinolizidine, and lycopodium alkaloids. Indolizidine alkaloids are found in closely related taxa: swainsonine is produced in *Swainsona galegifolia* (Fabales: Fabaceae) hairy root cultures; ajmalicine, ajmaline, serpentine, and vincamine are produced in *Catharanthus roseus*, *C. trichophyllus*, *R. micrantha*, *R. serpentina*, and *Vinca minor* (Gentianales: Apocynaceae) hairy root cultures (Table 2). Hyoscyamine and scopolamine were detected both in the hairy roots and intact plants of this species. At the same time, cadaverine was detected in hairy roots, but not in the intact plants. That is an example of the formation of the secondary metabolites in hairy root cultures *de novo* [147].

Piperidine alkaloid's lobeline is found in *Lobelia inflata* L. hairy root cultures [212]. The hairy root cultures synthesize lobeline in lower quantities than the intact plant [186]. It was found that significant increase in its amount in the hairy root cultures can be achieved by introducing the growth regulators indole-3-acetic acid and α-naphthalene acetic acid into the culture medium [186].

Different classes of alkaloids could be found in hairy root cultures of closely related species. For example, both quinolone (quinine, quinidine) and indole alkaloids (camptothecin) have been identified in the hairy root cultures in *Rubiaceae* species (*Cinchona ledgeriana* and *Ophiorrhiza pumila*, respectively) [97, 100].

4.2 Glycoside Iridoids

Glycoside iridoids are the monoterpenoid glycoside group (monoterpenoid glucosides), which appear in 6–8% of dicotyledonous plants. A number of ecological factors such as photoperiods, plant growth stage, fungal infection, wounding, and insect damage can alter the pattern of glycoside iridoids. According to the most accepted theory, they are involved in defense against herbivores and pathogens. The type of biological activity of iridoid glycosides is often regulated by glucosidases, converting the myrosinase to glucosinolate, antirrhinoside to antirrhide, and aucubin to catalpol. It is interesting that the diversity of these compounds was wider in hairy root cultures than in the intact plants (Table 2).

Iridoids have been used as chemical markers for the Corniflorae, Gentianiflorae, Loasiflorae, and Lamiiflorae superorders. The number and nature of the iridoids detected in plants could be discussed as a measure of the complexity of their biosynthesis pathways. Major iridoid glycosides in Scrophulariaceae and Plantaginaceae plant families are antirrhinoside and aucubin formed by one metabolic pathway [215]. Herewith the aucubin precursor is loganic acid, originally appeared in *Daphniphyllum macropodum* (Daphniphyllaceae) and *Aucuba japonica* (Aucubaceae).

The hairy root culture of *Picrorhiza kurroa* Royle (Plantaginaceae) accumulates kutkoside and picroside, having a broad medical potential (including antiviral effect) [144]. Despite the more complex chemical structure, it is easy to notice that they have the same precursor with antirrinoside.

Thus, in the hairy root cultures of closely related families Plantaginaceae, Orobanchaceae, and Scrophulariaceae (Lamiales), one can find the increase in these iridoid glycosides' outputs demonstrating the plant protective function in vivo (Table 2).

4.3 Polyphenols (Flavonoids, Terpenoids, Coumarins)

There are five groups of phenolic compounds in plants: flavonoids, alcohol phenylpropanoid (lignin precursors), benzoic acid and its derivatives, phenylpropanoids, and phenylpropanoid esters called coumarins (C6–C3). Phenolic compounds are synthesized from phenylalanine by ammonium disengaging accompanied with trans-cinnamic acid buildup. This process is catalyzed by secondary metabolism's key enzyme, called phenylalanine ammonium lyase (PAL). In particular, PAL synthesis occurs in case of fungal infection.

Flavonoid quercetin and its derivatives, including quercetin glycoside – rutin – are found in many plant species. Relevant flavonoids from the hairy root cultures *Polygonaceae* (Caryophyllales) and Campanulaceae (Asterales) are reported [107]. They are the major secondary metabolites in naturally transgenic species *Ipomoea batatas* L. (Solanales*:* Convolvulaceae) [219]. In Asterales (Campanulaceae, Asteraceae) hairy root cultures, one can also find flavonoid anthocyanins – coloring substances, which thus serve the attractants for insects [183, 213]. Betalains are another natural pigments having a strong antiradical and antioxidant activity. They have been obtained from Caryophyllales: Amaranthaceae hairy root cultures [103].

Terpenoids represent the largest and the most diverse class of chemicals among the myriad of compounds, produced by plants. These biologically active compounds are widespread in the hairy root cultures (Ranunculaceae, Cucurbitaceae, Fabaceae, Malvaceae, Meliaceae, Asclepiadaceae, Plantaginaceae, Lamiaceae, Solanaceae, Asteraceae, Apiaceae, Araliaceae) (Table 2). Triterpenoids are a group of compounds that include triterpene saponins, phytosterols, and phytoecdysteroids [276]. For example, azadirachtin and asiaticoside, well known for its insecticidal properties, were found in Meliaceae (Sapindales) and Apiaceae (Apiales*)* hairy root cultures. Phytoecdysteroids were present in Asteraceae (Asterales) and Lamiaceae (Lamiales*)* hairy root cultures [128–131].

Biologically active phenolic compounds, found in the *Apiaceae* hairy root cultures, are coumarins and their derivatives, including glycoside form. Coumarins could be released from glycosides quite easily, for example, during the crop drying. Furanocoumarin accumulated in *Ammi majus* (Apiaceae) hairy root cultures [193] is a compound that protects the plant from insects and fungal infections.

4.4 Naphthoquinones and Anthraquinones

Naphthoquinones and anthraquinones – quinone derivatives – are formed in plants using shikimate pathways. In plants they commonly occur in the reduced and glycosidic forms.

The 1,4-naphthoquinones play an important ecological role in plant–plant, plant–insect, and plant–microbe interactions [270], and they have a wide spectrum of biological actions, including antibiotic, antiviral, anti-inflammatory, antipyretic, antiproliferative, and cytotoxic effects. The major 1,4-naphthoquinones, such as lawsone, plumbagin, isopropenyl naphthazarin-2,3-epoxide, rhinacanthin, and shikonin, were detected in the hairy root cultures of Myrtales (Lythraceae), Caryophyllales (Plumbaginaceae), Lamiales (Bignoniaceae, Acanthaceae), and Boraginales (Boraginaceae), respectively.

The hairy roots of *Ceratotheca triloba* (Lamiales: Pedaliaceae) produced one acridone derivative, one naphthoquinone derivative, and seven anthracenedione derivatives [138]. Also anthraquinones have been found in the hairy root cultures of Fabaceae (Fabales), Plantaginaceae (Lamiales), and Polygonaceae (Caryophyllales). The biological activity of plant anthraquinones is less studied. The pigment indigo was detected in *Ceratotheca triloba* (Caryophyllales: Polygonaceae) hairy root cultures. It's known that in intact plants *Polygonum* indigo is formed from indicant in the case of leaf surface damaging. It is interesting to note that anthraquinones, formed by marine fungi, have the same biological activities as naphthoquinones in plants. Curiously, fungi perform another metabolic pathway for these compounds [220].

4.5 Essential Oils

Essential oils in plants, which belong to the family Lamiaceae, possess antimicrobial and fungicidal activities; the targets for these activities depend on the ratio of various oils in plants. The essential oil of *Salvia nemorosa* has the highest content of sesquiterpene hydrocarbons and oxygenated sesquiterpenes with strong antifungal and low antibacterial activities, while the essential oil of *Origanum vulgare* performs the highest antibacterial activity [221]. Essential oils, obtained from the roots, calli, and the hairy root cultures of *Salvia miltiorrhiza* (Lamiaceae), also demonstrated antimicrobial and antioxidant activity [132]. Besides, these compounds were detected in the hairy root cultures of Asteraceae and Apiaceae families.

Despite the widespread use of hairy root cultures for the production of secondary metabolites, the mechanisms underlying the increase of their levels in hairy roots are poorly understood. In many cases the increase of biomass of transformed tissues results in significant uptake of total yield of all analyzed compounds; at the same time the amounts of particular metabolites vary disproportionately. Thus leaves and roots of pRi-regenerants *Rehmannia glutinosa* (Lamiales: Orobanchaceae) accumulate equal (harpagoside, isoverbascoside), higher (catalposide, aucubin, harpagide, verbascoside), or lower (catalpol, loganin) levels of the metabolites in comparison

with non-transformed *R. glutinosa* plants, and their content varies with plant age and the organ analyzed [139]. These differences in the ratio of substances are explicable: the aucubin can be converted to catalpol (as a response to fungal infection) and has a common precursor with loganin, same way one can explain the lack of catalpol in *Verbascum xanthophoeniceum* (Scrophulariaceae) hairy root cultures [135].

As we mentioned before, T-DNA from the *A. rhizogenes* contains genes for the opine biosynthesis, inductors of growth of hairy roots, genes for auxin biosynthesis (in several strains), and some ORFs with unknown function. It was shown that the absence of TR-DNA (containing auxin synthesis genes) in hairy roots leads to increased transcript accumulation and higher alkaloid concentrations [222]. These data do not contradict with the observations of Robins [223, 224] and Moyano et al. [224, 225]. They demonstrated that the addition of the synthetic auxin to the culture medium had a positive effect on accumulation of callus biomass, although it inhibited alkaloid biosynthesis. The ability of nicotine synthesis is also lost, if root cultures of *N. rustica* are treated with auxin [223]. Opine synthesis genes are different in various strains, used for increasing secondary metabolite production [53]. That is why it is more reasonable to focus on oncogenes from the TL-DNA and their homologues, to discuss mechanisms of their influence on plant morphogenesis and role in the production of different compounds.

5 Influence of Single *rol* Genes and/or Their Combinations on Secondary Metabolites

Several scientific projects were devoted to investigation of influence of single *rol* genes and/or their combinations on secondary metabolites. Let us first consider the individual *rol* genes.

5.1 *rolA*

There are just a few reports concerning function of *rolA* in the regulation of plant secondary metabolism. Palazon et al. [224–225] have shown that the *rolA* gene expression leads to the increasing of synthesis of nicotine in *Nicotiana tabacum*. In the experiments of Shkryl et al. [226, 227], it was shown that the *rolA*-expressing calli of *R. cordifolia* produced 2.8 higher amounts of anthraquinones than control tissues. An interesting feature of *rolA* is that its expression in *R. cordifolia* calli was observed at remarkably stable level over a 7-year period, providing conditions for vigorous callus growth [226].

5.2 *rolB*

According to data of Shkryl et al. [226, 227], among the T-DNA genes *rolB* is the most powerful inducer of anthraquinone biosynthesis and the most powerful

suppressor of cell growth. The highest stimulating activity of anthraquinone biosynthesis was shown for a *R. cordifolia* root culture overproducing RolB protein, where a 15-fold increase of anthraquinone level was demonstrated comparing to untransformed calli. These data are consistent with the observation that in transgenic tissues of *R. cordifolia*, expression of *rolB* positively correlated with increased expression of *ICS* (isochorismate synthase gene) – a key gene for anthraquinones biosynthesis. The *rolB*-induced increase of production of anthraquinones can be abolished by a tyrosine phosphatase inhibitor, indicating the involvement of tyrosine phosphorylation in the rolB-mediated regulation of anthraquinone synthesis. Besides, *rolB* is the most powerful suppressor of cell growth. High levels of *rolB* expression downregulated growth of callus in a manner dependent on the intensity of expression [226].

One of the most remarkable examples of the effectiveness of *rolB*-transgenic tissues was demonstrated for *Vitis amurensis*, where transformation led to more than a 100-fold increase of production of polyphenol compound resveratrol [227].

Bulgakov et al. [227, 228] have shown that *Arabidopsis thaliana rolB*-transgenic calli produced threefold higher levels of indolic glucosinolates, compared with normal calli. Accumulation of these compounds was caused by activation of genes encoding secondary metabolism-specific MYB transcription factors (MYB34, MYB51, and MYB122), and the absence of aliphatic glucosinolates in transformed calli was caused by the inability of rolB to induce MYB29.

5.3 *rolC*

A wide range of compounds were upregulated by *rolC*. By itself, the *rolC* gene is able to positively regulate the production of different classes of secondary metabolites: tropane alkaloids [229], pyridine alkaloids [230], indole alkaloids [225], ginsenosides [231], and anthraquinones [226, 232, 233] in transformed plant tissues (Table 3).

The assessment of the signal transduction pathways affected by the RolC showed that this signal did not interfere with general plant defense pathways leading to synthesis of phytoalexin-type secondary metabolites. In particular, the Ca2+-dependent NADPH oxidase pathway and the jasmonic acid-mediated pathway were not affected in *rolC*-transformed cells of *R. cordifolia* [233, 236]. ROS production and phytoalexin production are most likely dissociated in *rolC*-transformed *R. cordifolia* cells [237].

5.4 *rolABC*

In the experiments of Palazon et al. [225], two transgenic root lines were obtained after the transformation of *N. tabacum* leaf explants with constructs, containing *rol* genes A, B, and C together, under the control of their own promoters, or *rolC* under the CaMV 35S promoter (*rolABC* and *rolC* lines). They were then examined for their

Table 3 Effect of *rolC* expression on accumulation of secondary metabolites

Class of compounds	Subclass	Particular compounds	Plant species	Character of accumulation	Refs.
Alkaloids	Tropane alkaloids	Hyoscyamine and scopolamine	*Atropa belladonna*	*rolC* expression leaded to a 12-fold increase in hyoscyamine and scopolamine accumulation, comparing to untransformed roots	[144]
	Pyridine alkaloids	Nicotine	*Nicotiana tabacum*	Stimulates alkaloid production by stimulating tissue growth or by directly increasing the biosynthetic activity of transformed roots	[5, 225]
	Indole alkaloids		*Catharanthus roseus*	*rolC* increased the production of indole alkaloids, and the amount of the *rolC* transcript correlated with the production of these secondary metabolites	[230]
Polyphenols or phenolic compounds	Triterpenoid saponins	Ginsenosides	*Panax ginseng*	The similar levels of ginsenosides are accumulated in transformed roots and calli, indicating that the activator effect of the *rolC* gene is independent of the cell differentiation. The maximal content of ginsenosides in the rolC-transgenic ginseng roots is very high (6.58% dry wt.) and represented almost the upper limit of the biosynthetic capacity of ginseng cells	[231]
	Triterpenoids	Ecdysteroids	*Ajuga bracteosa*	High rolC expression correlates with accumulation of the ecdysteroids	[234]
	Quinones	Anthraquinones	*Rubia cordifolia*	Stable increase in anthraquinone content up to 4.3 times comparing to the non-transformed cells	[226, 232, 233]
		Naphthoquinone pigments	*Lithospermum erythrorhizon*	Long-lasting inhibitor effect on naphthoquinone pigment accumulation	[235]

nicotine productivity relative to growth rate and amounts of *rolC* gene product [225]. Growth capacities and nicotine production were greatly increased when the *rolA, rolB*, and *rolC* genes were present together, demonstrating their cumulative effect on nicotine production. In *Atropa belladonna* the *rolABC* genes together led to a much higher (75-fold) increase in hairy root growth rate than *rolC* alone [229]. However, the combined action of *rol* genes is not explained by simple summation of their effects. It is interesting to note that the *rolA* and *rolC* genes did not show negative effect on callus growth of *R. cordifolia*. In combination with the rolA and C genes in a *rolABC* construct, *rolB* no longer had an adverse action on growth of rolABC-transformed tissues [226]. However, the stimulatory effect of the *rolB* gene on anthraquinone accumulation is weaker when the gene is combined with the *rolC* and *rolA* genes in a *rolABC* construct.

In general, the accumulation of secondary metabolites in *rolABC*-transformed roots of different plants is similar to that found in wild-type transformed hairy roots [5, 19, 229]. At the same time, Shkryl et al. [226] have shown that at least in case of anthraquinone activation, each of the rol genes appears to have its own individual mechanism. For example, *rolC* (and perhaps *rolA*) has an antagonistic effect on the *rolB*-induced increase in anthraquinones. This observation does not contradict with the effects of the rol genes that have previously been demonstrated. Constitutive *rolB* expression suppressed the growth of tobacco cells, and the *rolC* gene is capable to attenuate this growth inhibition [238]. There are some data demonstrating that the antagonistic effects of *rolB* and *rolC* could be mediated at the level of calcium signaling [233]. Authors propose that *rolB* is a major player in secondary metabolism activation by wild-type transformation, while *rolC* has its own role in secondary metabolism and provides a signal that tunes the action of *rolB*.

6 Mechanism of Modulation of Secondary Metabolites by *rol* Genes

The mechanism of modulation of secondary metabolites by rol genes is poorly understood. However, a few years ago, there was an idea that their products activate secondary metabolism through the upregulation of its key genes [226, 239–241]; according to the emerging role of plant oncoproteins as modulators of RNA silencing, the following mechanism was proposed: T-DNA oncoproteins (such as RolB, RolC, or 6b) interfere with RNA silencing to derepress genes of secondary metabolism normally repressed through miRNA. This process could involve the interaction of the rol gene products with transcription factors, which regulate the biosynthesis of secondary metabolites. The first direct evidence of the participation of Rol proteins in the regulation of transcription factors has been obtained in 2016 [228]. These authors have shown that RolB activates MYB transcription factors (MYB34, MYB51, and MYB122) in *Arabidopsis thaliana* callus tissues. However, the system of regulation of secondary metabolite synthesis is more complicated because there are tissue and organ specificities of this process. It is well known that some secondary metabolites are not synthesized if the cells remain undifferentiated

[48, 49, 242]. Bulgakov et al. [228, 229] have shown that *rolB* stimulates accumulation of secondary metabolites, synthesized in *Arabidopsis* calli, but it cannot overcome the strong suppression block for secondary metabolite production existing there.

There are some data that in cases when a particular secondary metabolite was detected only in the aerial part of an intact plant, hairy root cultures were able to accumulate the same compound [243]. Transformed roots are often able to regenerate whole viable plants. Kayani and co-authors [234] have shown that transgenic plants developed from hairy roots had more phytoecdysteroid content than the parent hairy root lines. These features are determined by yet unknown mechanisms. Understanding these mechanisms may contribute to establishing a new generation of plant cell cultures for practical applications. Besides it could give new information, concerning evolutionary role of *rol* genes.

7 Naturally Transgenic Plants Contain *A. rhizogenes* Oncogenes

One of the interesting models for investigation of evolutionary role of rol genes is naturally transgenic plants, described in genera *Nicotiana*, *Ipomoea*, and *Linaria* [244–249]. Some species from these genera contain T-DNA from *Agrobacterium* in their genomes. It is called cT-DNA (cellular T-DNA). Detailed analysis of these species indicates that there were several independent acts of *Agrobacterium*-mediated transformation in the evolution of these genera [244–250]. One of the discussed functions of cT-DNA is associated somehow with the influence of plants on their environment by changing of the amounts of secondary metabolites [251, 252]. All known naturally transgenic plants contain *rolB* and *rolC* homologs. These genes could be connected to the regulation of secondary metabolism.

Let us take a closer look on secondary metabolite content in naturally transgenic plants.

8 Secondary Metabolites in Naturally Transgenic Plants

8.1 *Nicotiana tabacum*

Over 4000 different compounds have been identified in *Nicotiana tabacum* plants [253]. Phytochemical studies of *Nicotiana tabacum* have shown the presence of alkaloids, sesquiterpene, flavonoids, phenylpropanoids, chromanones, biphenyls, isocoumarins, and the homologous compounds [19]. Most of these compounds (flavones and isoflavones, phenylpropanoids, biphenyls, lignin derivatives) are produced constitutively in the leaves, the stems, and the roots of *Nicotiana tabacum* and demonstrated anti-*Tobacco mosaic virus* activities [254]. A part of them is accumulated as a reaction to wounding, inducing defense of the plants against different insects (nicotine and nicotine derivatives) and diseases (sesquiterpenoid phytoalexins).

Accumulation of alkaloids was detected in the aerial part of the certain "wild" *Nicotiana* spp. as the reaction on leaf damage. Alkaloid pool comprised about 95% of nicotine [255]. Such increase is thought to confer a survival advantage on the plant against herbivory. Two possible biosynthetic pathways of alkaloid in the tobacco plant have been proposed, i.e., biosynthesis from 3,6-dihydronicotine, a key intermediate in nicotine biosynthesis, and from pyridine 3,5-dicarboxylic acid, and for the synthesis of the minor compounds, pyridine derivatives are typically for the root [254, 256]. Evidence has appeared that nicotine production is only part of defensive responses by the genus *Nicotiana*, comprising alkaloids, phenolics, proteinase inhibitors, PR (pathogenesis response) proteins, and sesquiterpenoid phytoalexins.

Many phenolic compounds are accumulated after infection of *Nicotiana tabacum* with *Tobacco mosaic virus* (such as flavonol glycosides; caffeoylquinic, feruloylquinic, and p-coumaroylquinic acids; glucose esters; and glucosides of cinnamic and benzoic acids). The necrotic tissue, as a result of virus infection, usually contains antifungal metabolites capsidiol and glutinosone [257, 258]. Originally capsidiol was isolated from sweet pepper [259]. Capsidiol and glutinosone have a similar function in *Nicotiana tabacum:* they are involved in disease resistance.

8.2 *Ipomoea batatas*

Triterpenes/steroids, alkaloids, anthraquinones, coumarins, flavonoids, saponins, tannins, and phenolic acids have been identified in *Ipomoea batatas*. The total antioxidant activity of the plant extract was 42.94% as compared to ascorbic acid. Sweet potato defends themselves from invasions by pathogens and herbivores based on these compounds with high antioxidant (caffeic acid derivatives, anthocyanosides, and coumarins) and antimicrobial (caffeic acid derivatives, triterpenes) activity [219].

8.3 *Linaria*

On this moment nearly 200 metabolites were identified from the 41 *Linaria* species [209]. Biologically active compounds of species *Linaria* include iridoids (antirrhinoside and its derivatives) [260, 261], flavonoids (pectolinarin, linariin, apigenin, diosmetin, genkwanin, luteolin, scutellarein 40-methyl ether) [262–264], organic acids (palmitic, linoleic, linolenic acid), saponins [amyrin [265], diterpenoids [linarienone [266, 267, 268, 269], and steroids (ergost-7-en-3-ol, stigmasta-5,22-E-dien-3-ol, stigmast-5-en,24S-3-ol) [270]]. The most part of these compounds play a role in plant defenses against pathogens and herbivores. It was important to note that the naturally transgenic plants (sect *Linaria* and *Speciosae*) produce more biologically active metabolites than other species (Table 4).

Table 4 Biologically active compounds from the genus *Linaria* [210, 252]

Compounds	Section								
	Macrocentrum	*Pelisserianae*	*Lectoplectron*	*Versicolores*	*Linaria (cDNA +)*	*Speciosae (cDNA+)*	*Diffusae*	*Repentes*	*Supinae*
Quinazoline **alkaloids**	–	–	–	–	+	+	–	–	–
Polyphenols and phenolic compounds									
Phenolic acids	+	+	+	+	+	+	+	+	+
Phenolic glycosides	–	–	–	–	+	+	–	–	–
Verbascoside	–	–	–	–	–	–	+	–	–
Iridoid glycosides									
6-acyl-antirrhinoside derivatives	+	+	+	+	+	+	+	+	+
5-deoxy-antirrhinoside derivatives	–	–	–	+	–	–	–	+	–
Antirrhinoside	+	+	+	+	+	+	+	+	+
Antirrhide	+	+	+	+	+	+	+	+	+
5-O-glucosyl-antirrhinoside	+	+	+	+	+	+	+	+	+
5-O-beta-allosylantirrhinoside	+	+	+	–	+	+	+	+	+
6-beta-hydroxyantirrhide	–	+	–	–	+	+	+	+	–
6-O-angeloylantirrhinoside	–	–	–	+	–	+	–	–	+
Linarioside and seco-linarioside	–	+	–	+	+	+	+	+	+
Isolinarioside	–	–	–	–	+	+	–	+	–

(*continued*)

Table 4 (continued)

Compounds	Section								
	Macrocentrum	*Pelisserianae*	*Lectoplectron*	*Versicolores*	*Linaria (cDNA +)*	*Speciosae (cDNA+)*	*Diffusae*	*Repentes*	*Supinae*
Iridolinariosides (7-deoxyiridolactonic acid diester of glucopyranose)	–	+	+	–	+	+	–	–	+
Genestifolioside	–	–	–	–	–	+	–	–	–
Arcusangeloside	–	–	–	–	–	–	+	+	–
Procumbide (an isomer of antirrhinoside)	–	–	–	–	+	–	–	–	–
6-O-cis/trans-p-coumaroyl-antirrinoside	–	–	–	–	+	–	–	–	–
3-acetyl-4,6-di-(7-deoxyiridolactonyl)-D-glucopyranose	–	–	+	–	+	–	–	–	–
6′-O-senecioylantirrhinoside, 6′-O-angeloylantirrhinoside	–	–	–	+	–	+	+	–	–
Genistifolin	–	–	–	–	–	+	–	–	–
Flavonoids									
Pectolinarin	–	–	–	–	+	+	+	–	+
Linarin, linariin	–	–	–	–	+	–	+	–	–
Isolinariin A	–	–	–	–	+	–	–	–	–
Isolinariin B	–	–	–	–		–	+	–	–
Diosmin	–	–	–	–	+	–	+	–	–

Apigenin-7-0-β-3-D-glucoside, diosmetin-7-0-β-3-D-glucoside	–	–	–	–	+	–	–	–	–
Apigenin	–	–	+	–	+	–	+	–	–
Genkwanin-41-0-rutinoside	–	–	+	–	–	–	–	–	–
Luteolin, luteolin-7-0-glucuronide, luteolin-7-0-glucosideluteolin-3′,4′-dimethyl ether	–	–	+	+	+	–	–	–	–
Quercetin-7-0-rutinoside	–	–	+	–	–	–	–	–	–
Quercetin	–	–	–	–	+	–	–	–	–
Myricetin	–	–	–	–	+	–	–	–	–
Acacetin	–	–	–	–	+	+	–	–	–
Acacetin-7-0-β-3-D-glucopyranoside	–	–	–	–	+	–	–	–	–
Pectolinarigenin	–	–	–	–	+	–	+	–	–
Hispidulin	–	–	–	–	+	–	–	–	–
Scutellarein-41-methyl ether	–	–	–	–	–	–	–	–	+
Ladanein	–	–	+	–	–	–	–	–	–
Genkwanin, genkwanin-41-0-rutinoside	–	–	+	–	–	–	–	–	–
Chrysin (chrysin derivate, wogonin, Lamiales: Lamiaceae), hesperidin (Dipsacales: Caprifoliaceae *Valeriana officinalis*)	–	–	–	–	+	–	–	–	–

(*continued*)

Table 4 (continued)

Compounds	Section								
	Macrocentrum	*Pelisserianae*	*Lectoplectron*	*Versicolores*	*Linaria (cDNA +)*	*Speciosae (cDNA+)*	*Diffusae*	*Repentes*	*Supinae*
3,5-Di-O-methylkaempferol, 3,7-Di-0-methylkaempferol	–	–	–	–	–	+	–	–	–
Aureusin*, hypolaetin, diosmetin, diosmetin bracteatin-6-glucoside*	–	–	+	+	+	–	–	–	–

The largest groups of secondary metabolites of species from genus *Linaria* are represented by iridoid glycosides (Table 4); their concentrations in plants rank from approximately 1 to 20% (for dry weight) [252]. Iridoids with some neo-clerodane diterpene and triterpene structures and their glycoside are believed to serve as chemotaxonomic markers for the genera of the Scrophularioideae–Antirrhineae tribe of the Plantaginaceae family, including *Linaria*, *Antirrhinum*, *Asarina, Kickxia*, and *Maurandya* [272–275]. Antirrhinoside was initially isolated from *L. vulgaris* [276]. This compound is widely distributed in *Linaria*. Antirrhinoside and antirrhide are structurally and functionally similar to the aucubin and catalpol isolated from *Plantago lanceolata* L. The level and ratio of these compounds are associated with plant's resistance to generalist insects and pathogen [216, 217]. It was concluded that iridoid glycosides aucubin and catalpol can serve as broad-spectrum defenses and that selection for pathogen resistance could potentially result in increased resistance to generalist insect herbivores and vice versa, resulting in diffuse rather than pairwise coevolution. This is supported by the fact that the levels of deterrent iridoid glycosides, aucubin and catalpol, vary geographically, including latitudinally [218]. It should be noted that the antirrhinoside's and aucubin's biological activity appears in two ways. In original form they (similar to nicotine) repel *non*-pollinating insects and leaf beetles. On the other hand, beta-glucosidases can convert the antirrhinosides and aucubins to antirrhide and catalpol, respectively. These compounds possess fungicidal activity [216–218].

Thus, all naturally transgenic plants contain significant amounts of secondary metabolites (Fig. 4). This can be explained by the functioning of the rol gene homologs in their genomes.

9 Conclusions

To date, great success has been achieved in the production of secondary metabolites in hairy root cultures. All major groups of secondary metabolites have already been produced from hairy roots. However, better understanding of the regulation of biosynthesis of valuable secondary metabolites is very important for development and improvement of genetic engineering approaches and fully realizing the biosynthetic potential of hairy roots. For a long time, the function of T-DNA of *A. rhizogenes* was considered in the light of the regulation of sensitivity of the transgenic tissues to hormones. However, in the last decade the situation has been revised, since the involvement of the *rol* genes in secondary metabolism, ROS metabolism, and PR protein expression became clear [277]. Our knowledge of effects of *rol* genes on secondary metabolite production and mechanisms of regulation of secondary metabolism by *rol* genes will contribute to development of highly productive cultures, accumulating commercially valuable compounds. Study of secondary metabolites of naturally transgenic plants may shed light on the evolutionary role of cellular T-DNA in terms of changing of susceptibility of plants to pathogens. Besides, describing new examples of natural pRi T-DNA containing plants will be very useful for pharmaceutical studies. Since it is shown that naturally

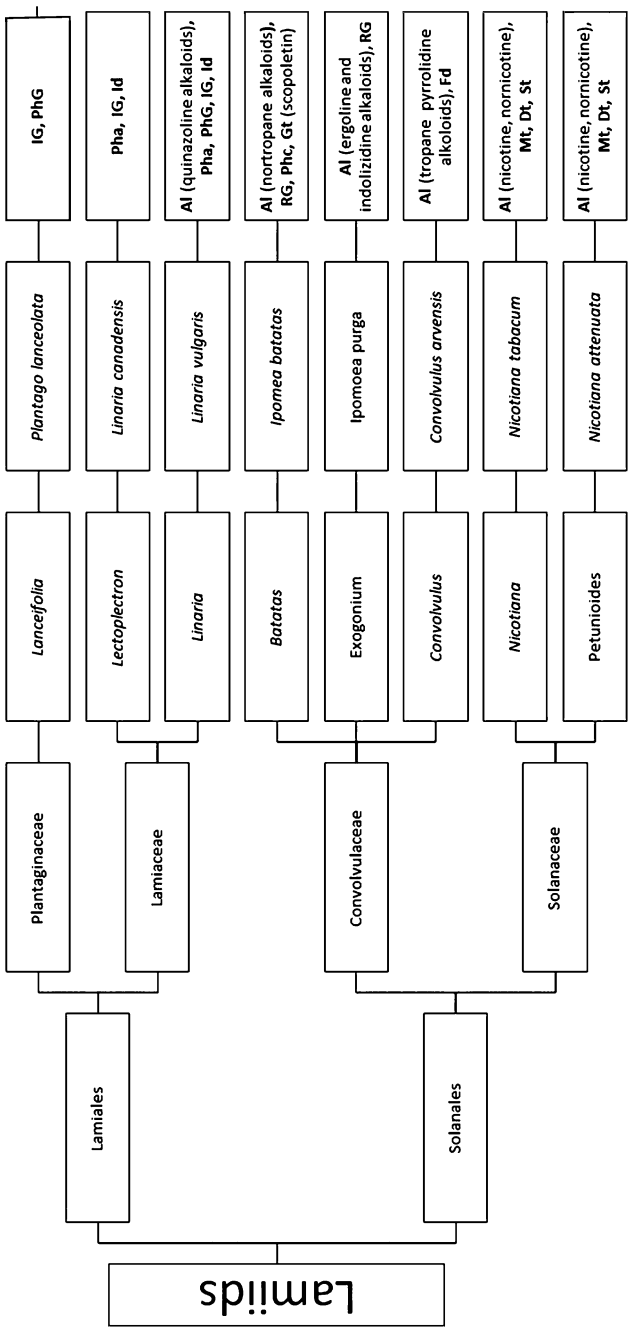

Fig. 4 Main classes of biological activity compounds found in *Lamiales* and *Solanales* species, *IG* iridoid glucosides, *PhG* phenylethanoid glycosides, *Mt.* Monoterpenes, *Dt* diterpenes, *St* sequiterpenes, *Phc* phenolic compounds, *Pha* phenolic acids, *RG* resin glycosides

transgenic plant species contain bigger amounts of secondary metabolites, then their non-transgenic relatives and these findings could help to overcome social prohibits to use transgenic plants for medicinal applications.

From other hand there is an another perspective of hairy root cultures. The increased productivity of secondary metabolites in hairy roots can be achieved by metabolic engineering. Metabolic engineering involves overexpressing the target pathway, overcoming rate-limiting steps, suppressing catabolism of the product of interest, blocking other pathways, competing with the target pathway, or any feasible combination of the above. Metabolic engineering is quite promising, because it can contribute in increasing the yields of known compounds and production of new metabolites for a plant species.

Development of large-scale cultivation methods for hairy root cultures of different plants (design of bioreactors, physical and chemical parameters, etc.) represents an important step on the path from the scientific discovery to the commercial use. We are convinced that this step will be performed very soon for tens or even hundreds of varieties of root cultures.

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Amaryllidaceae Alkaloid Accumulation by Plant In Vitro Systems

7

Dominique Laurain-Mattar and Agata Ptak

Contents

Abstract

Over 300 alkaloids possessing a wide range of biological activities have been isolated from plants belonging to Amaryllidaceae family. Galanthamine, used for the palliative treatment of Alzheimer's disease, is the only one commercialized. The biodisponibility of Amaryllidaceae alkaloids is low. In vitro culture offers an alternative interesting approach for the biotechnological production of these valuable alkaloids. The feeding with different exogenous factors of *Leucojum*

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aestivum, *L. aestivum* 'Gravety Giant,' and *Narcissus* in vitro cultures was reported, and the effects on the biosynthetic pathway of both galanthamine and lycorine were studied.

Keywords
Amaryllidaceae · Alkaloids · Galanthamine · Lycorine · Biosynthetic pathway · In vitro culture

1 Introduction

More than 300 alkaloids with biological activities have been isolated from Amaryllidaceae plants [1]. First unconfirmed reports about galanthamine come from the early 1950s. At that time, the populations of the Caucasian mountains applied bulbs of *Galanthus woronowii* (Caucasian snowdrop) for treatment of young children with developed symptoms of poliomyelitis. The first published work done by Maskovsky and Kruglikova-Lvova in 1951 demonstrated AChE-inhibiting properties of galanthamine. One year later, the isolation and determination of the chemical structure of galanthamine from *Galanthus woronowii* was carried out by Proskurnina and Yakovleva [2]. Until now, only the galanthamine is marketed as a hydrobromide salt for the treatment of Alzheimer's disease in the mild to moderate stages [2–4].

Galanthamine can be obtained either by chemical synthesis or extraction from culture plants. These two methods of production exhibit flaws leading to high costs of this alkaloid. Indeed, the structure of this compound contains three asymmetric carbons, and the respect of the configuration leads to a tedious and complex chemical synthesis [5, 6]. About obtaining this alkaloid from plants, galanthamine has been extracted by Sopharma (Bulgaria) in the early 1960s from *G. nivalis* and *Leucojum aestivum* plants [7]. At the beginning 10–15 tons of plant materials were collected annually from natural populations in Bulgaria. Intensive exploitation led to the inscription of *L. aestivum* in the Red Data Book of Bulgaria as an endangered species [8]. It should be noted that 1 kg of galanthamine is extracted from one ton of bulbs [9]. Currently galanthamine is isolated for industrial application from *Narcissus* spp. in Central and Western Europe, *L. aestivum* in East Europe, *Lycoris radiata* in China, and *Ungernia victoria* in Uzbekistan and Kazakhstan with galanthamine level from 0.1% to 0.52% [7, 10]. This secondary metabolite extracted from crop plants is vulnerable to climate fluctuations, pathogens on crops, and the political situation in producing countries [11]. For all these reasons, it is necessary to find an alternative solution for the production of Amaryllidaceae alkaloids in a commercial and economically viable scale.

The biotechnological approach to obtain galanthamine can be considered as an alternative way for the production of these alkaloids. Efficiency of alkaloid biosynthesis in in vitro cultures is affected by many biological, chemical, and physical factors. The most important of them include the choice of the adequate genotype, degree of tissue and organ differentiation, chemical composition of the medium

(including concentration and source of energy and coal, presence of macro- and microelements, growth regulators, addition of precursors and elicitors), and physical conditions, such as light, temperature, and physical state of the medium. All of these factors have been successfully tested on the production of galanthamine in in vitro cultures. It should be also added that these studies started at the 1990s and were conducted by a group of scientists from several countries like Bulgaria, France, Poland, and Spain. The research works concerned different plant materials (*L. aestivum*, *L. aestivum* 'Gravety Giant,' and *Narcissus* cultures) obtained via organogenesis and somatic embryogenesis. The successful transformation of *L. aestivum* with *Agrobacterium rhizogenes* was also performed [9]. Also the addition in the culture medium of a precursor of the alkaloid biosynthesis pathway in view to enhance the alkaloid accumulation was studied [12–14], while two candidate genes in the galanthamine biosynthetic pathway were identified [15].

2 Amaryllidaceae Family

For centuries, many plants have been used as essential resources of therapeutic agents against various human diseases. Among these traditional sources of drugs, the Amaryllidaceae family contains many plant sources of bioactive molecules [16]. In addition to their pharmacological interest, the ornamental value of Amaryllidaceae has long been recognized and exploited.

This family of monocots includes 65 genera and about 1,100 species spread throughout the world. South America (28 genera) and South Africa (19 genera) are the richest regions in terms of diversity. They are perennial monocots by a bulb. The leaves are flat, ensheathing at the base, and usually glabrous.

The name of Amaryllidaceae family is linked with *Amaryllis*, monospecific genus of South Africa which is widely grown as an ornamental plant. The main genera are *Crinum* with more than 120 species, *Hippeastrum* with 75 species, and *Cyrtanthus*, *Zephyranthes*, and *Hymenocallis* with more than 40 species each. French flora includes ten species of the genus *Narcissus* wild state, as well as *Leucojum* (seven species), *Sternbergia* (seven species), *Pancratium* (two species), and *Galanthus* (one species). Polish flora includes *G. nivalis*, *L. vernum*, *L. vernum* subsp. *carpaticum*, and *L. vernum* subsp. *vernum*. The main genera used for alkaloid extraction with medical use are *Leucojum*, *Narcissus*, and *Galanthus*.

Leucojum genus contains six species:

Leucojum aestivum (summer plant): This plant is spring flowering. It is native to Europe and Asia Minor. Its fragrant flowers, white mottled green to the tip of the petals, are worn in clusters on stems 45 cm in height (Fig. 1). The leaves are bluish green, long, and narrow. *L. aestivum* likes to grow under the cover of deciduous trees.

Leucojum autumnale (autumn plant): This species is distinguished by its white flowers tinged with pink, solitary or grouped by two or three at the top of stems 25 cm in height. The basal leaves are erected, very narrow, and developed after flowering. The flowers bloom in late summer and early autumn.

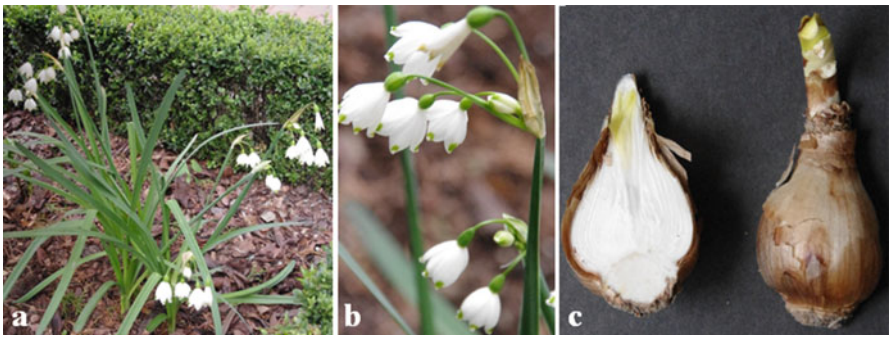

Fig. 1 *Leucojum aestivum* L. (**a**) plant, (**b**) flowers, (**c**) bulb

Leucojum vernum (spring plant): Native to Central Europe, this species of 45 cm in height grows in wet places, in the sun, or in shade and tolerates dry summer. It has flowers with the bell shape; blooming is in late winter and early spring. Grouped in pairs on the stems, flowers have speckled white petals with green or yellow.

The three other species are *L. roseum*, *L. nicaeense*, and *L. trichophyllum* [17].

The genus *Galanthus* includes about 19 species of small bulbous plants, native to Europe and Asia Minor [18]. Announcing the arrival of spring, their small white drooping flowers, sometimes scented, crop up in late winter. The genus is recognized to the three internal petals marked with green, much shorter than the three external petals. Very rustic, snowdrops enjoy a cool climate and bloom in the woods, meadows, and forests. The most studied species are:

Galanthus elwesii: Native to Turkey and the Balkans, this species of 25 cm high thrives in temperate climates and is distinguished by its blue-gray leaves folded over the other at the base. It shows in late winter flowers with white bells.

Galanthus nivalis: This species of 15 cm high, with blue-green leaves, is the most common of this genus.

Galanthus plicatus: Native to Turkey and Eastern Europe, this species of 20 cm high door broad dark green leaves.

Narcissus is a genus of 50 bulb species found mainly in the western Mediterranean, but also in Africa and Asia. These plants bloom in meadows, pastures, and woodlands. The most studied species are:

Narcissus pseudonarcissus: Native to Western Europe, this species shows, in the spring, bright yellow trumpets.

Narcissus poeticus: These plants, with a height of 45 cm, bloom late and are native to southern Europe. Each stem has two flowers with white petals often red or orange curly edge.

Narcissus tazetta: These highly fragrant daffodils exhibit, from autumn to spring, flowers in clusters on stems 40 cm high.

3 Amaryllidaceae Alkaloids

Plants belonging to the Amaryllidaceae family are known to produce an exclusive group of alkaloids called "Amaryllidaceae alkaloids." They are isolated from all genera of this family. Since the isolation of lycorine from *N. pseudonarcissus* in 1877 [19], more than 300 alkaloids were isolated from Amaryllidaceae plants [1]. These alkaloids are known and exploited for their various biological activities.

According to their structure, Amaryllidaceae alkaloids are classified in eight groups, the main representatives of which are lycorine, galanthamine, tazettine, narciclasine, montanine, lycorenine, haemanthamine, and crinine [1] (Fig. 2).

Except for the galanthamine, the most part of researches on the biosynthesis of Amaryllidaceae alkaloids was performed in the 1960s and 1970s [1]. Nevertheless, the biosynthesis pathway remains badly known at biochemical level and especially in molecular level; only two genes have been identified [15, 20]. The intermediates in the pathway have been determined; this knowledge is based on radiolabeling experiments [20]. In 1957, Barton and Cohen explained that the biosynthesis of these alkaloids is the result of several intramolecular oxidative couplings of precursors related to norbelladine [21].

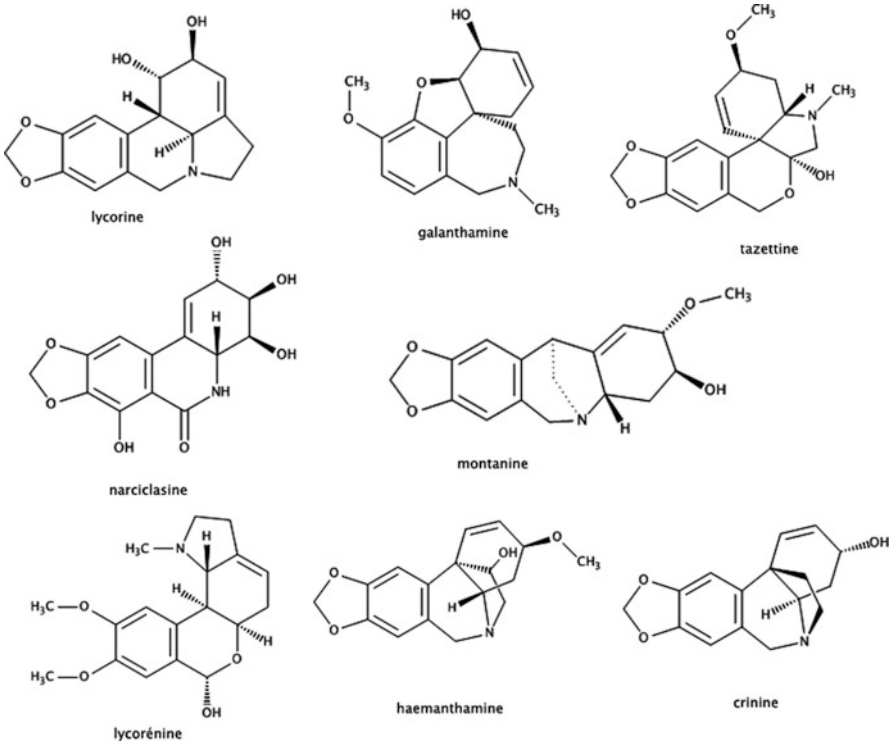

Fig. 2 Amaryllidaceae alkaloid types [19]

Norbelladine is formed through the combination of 3,4-dihydroxybenzaldehyde with tyramine, these two precursors arising from L-phenylalanine (L-phe) and L-tyrosine (L-tyr), respectively [19]. Indeed, the conversion of L-phe into the unit C_6-C_1 of the norbelladine (C_6-C_2-N-C_1-C_6) requests the introduction of two substituents containing oxygen into the aromatic cycle and the subsequent cleavage of two carbon atoms of the secondary chain, via cinnamic acid. L-Phenylalanine ammonia-lyase enzyme eliminates ammonia of L-phenylalanine to give *trans*-cinnamic acid [22] which is later hydroxylated by two P450 cytochromes, cinnamate 4-hydroxylase and coumarate 3-hydroxylase, and leads to caffeic acid. The final compound is 3,4-dihydroxybenzaldehyde [23]. Tyrosine decarboxylase converts tyrosine into tyramine (C_6-C_2-N) before its incorporation in the secondary metabolism. 3,4-Dihydroxybenzaldehyde and tyramine condense into a Schiff base and are reduced to form the first alkaloid in the proposed pathway, norbelladine. After methylation of norbelladine through the 4′-*O*-methyltransferase, 4′-*O*-methylnorbelladine is obtained. This one is considered the key intermediate in the biosynthesis of most alkaloids. 4′-*O*-Methylnorbelladine can undergo three types of oxidative couplings: *ortho-para'*, *para-para'*, and *para-ortho'*. The *para-ortho'* coupling leads to galanthamine, while lycorine is formed through an *ortho-para'* coupling [24]. Crinine and marithidine are formed from a *para-para'* coupling [21] (Fig. 3). A complex network of enzymatic reactions and the three basic skeletons obtained via oxidative couplings lead to more 500 alkaloids [16].

3.1 Galanthamine Skeleton

The *para-ortho'* oxidative coupling gives rise to a dienone which cyclizes spontaneously into demethylnarwedine. Through a stereoselective reduction, this one is transformed into demethylgalanthamine. Then, galanthamine is directly obtained by N-methylation of demethylgalanthamine. Eichhorn et al. (1998) [27] reported that narwedine is not the direct precursor of galanthamine. There exists a balance between the two forms, due to the reversible activity of an oxidoreductase.

3.2 Lycorine Skeleton

Norpluviine is a precursor of lycorine [25]. In this case, norpluviine is oxidized at the benzylic position to yield a cyclic hemiaminal group, which is in equilibrium with the corresponding open form. The rotation of the C-10a-C-10b bond of the amino aldehyde intermediate, followed by hemiacetal formation and methylation, provides the lycorenine core [19].

3.3 Crinine Skeleton

Haemanthamine is formed through a *para-para'* coupling as the enantiomeric skeleton of crinine. The oxidation of haemanthamine to haemanthidine followed

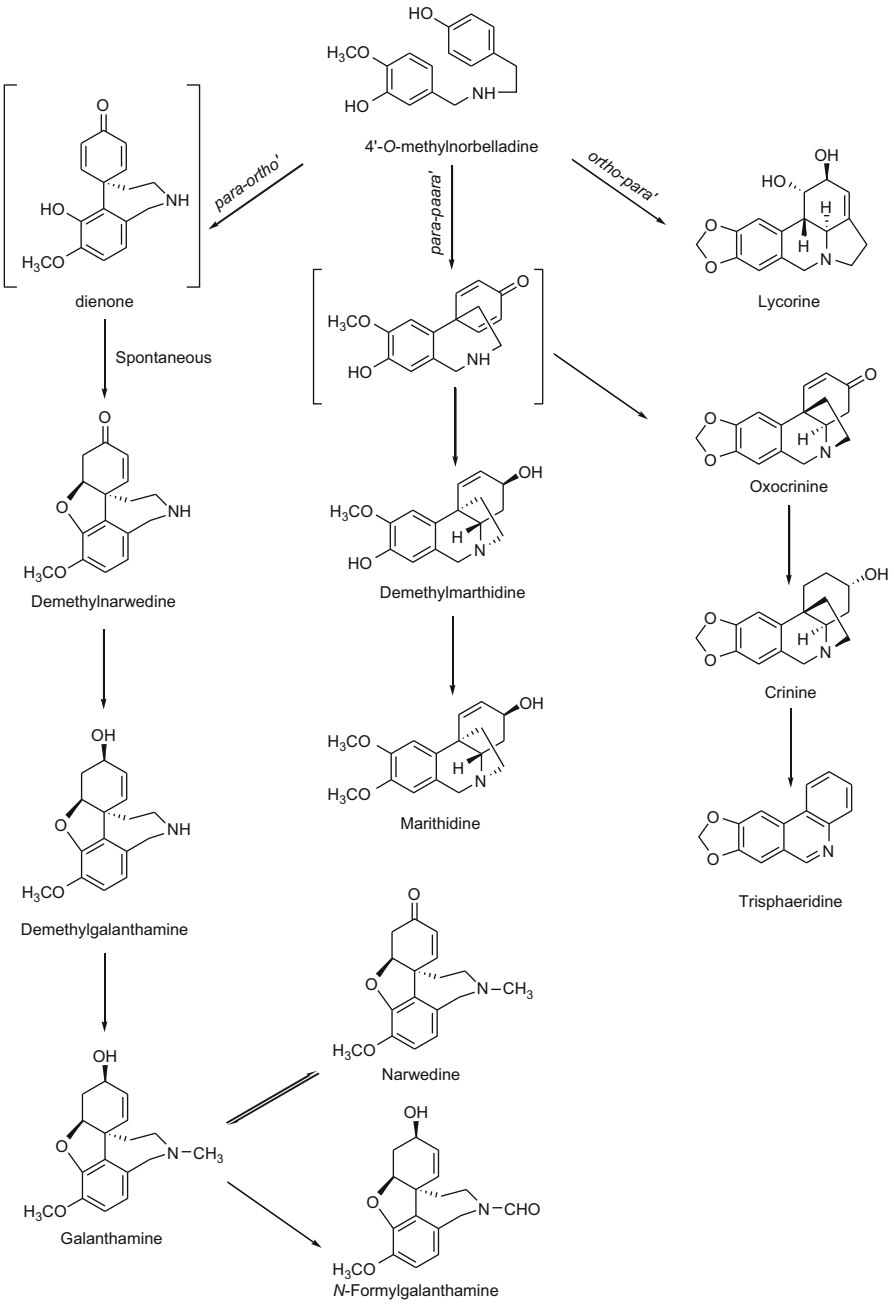

Fig. 3 *Narcissus* alkaloid biosynthesis (Amaryllidaceae) [25, 26]

by ring opening to form the amino aldehyde equilibrium system, after hemiacetal formation and methylation, yields pretazettine [1, 19].

4 Biological Activities of Amaryllidaceae Alkaloids

The remedies made from plants of Amaryllidaceae family have been used for thousands of years. During the last 30 years, many alkaloids have been isolated from plants of this family, tested for different biological activities, and synthesized by several researchers. Nowadays, only the galanthamine is marketed; however, significant pharmacological activities have been reported for other Amaryllidaceae alkaloids. Galanthamine has been isolated for the first time from *Galanthus woronowii* Losinsk [28]. Today, this alkaloid is extracted from other plants of the same family as *Narcissus* spp. and *Leucojum* spp., especially *L. aestivum*. Galanthamine is a selective inhibitor of acetylcholinesterase (AChE), reversible, and competitive with prolonged action. This alkaloid is commonly used for the palliative treatment of Alzheimer's disease. Galanthamine hydrobromide is marketed under the generic names of Reminyl® and Nivalin®. Furthermore, galanthamine acts as a mild analeptic and as a hypotensive. It also has analgesic activity [29].

Lycorine is a potent inhibitor of cell growth, cell division, and organogenesis in higher plants, algae, and yeasts [30–32]. The antitumor activity of lycorine has been demonstrated in vivo and in vitro by inhibiting the growth of various tumor cells such as melanoma BL6 mice, Lewis lung carcinoma, and HeLa cells [33–36]. The molecular mechanism of lycorine against leukemia (HL-60 human strain) shows that it can stop cell growth and reduce cell survival by affecting the cell cycle at the G2/M phase and inducing apoptosis of tumor cell. Studies on KM3 cells show that lycorine induces apoptosis of tumor cells via the intrinsic mitochondrial pathway [37]. Lycorine exerts antiviral effects on several RNA viruses or DNA [38]. These effects were observed against flaviviruses and, to a lesser extent, against bunyaviruses. This alkaloid has pronounced activity against poliovirus, coxsackie virus, and herpes type 1 virus [33, 39]. The mechanism of the antiviral effect is partially explained by blocking viral DNA polymerase [1]. Lycorine is also an analgesic more powerful than aspirin [1]. In addition, this alkaloid has an inhibitory effect on parasite development (*Encephalitozoon intestinalis*) [40], antifungal activity against *Candida albicans* [41, 42], and antimalarial activity (*Plasmodium falciparum*) [42]. In addition, it has anti-inflammatory and antiplatelet activities [43].

5 In Vitro Culture and Amaryllidaceae Alkaloids

As with many natural compounds with high added value, Amaryllidaceae alkaloid accumulation is limited in plants [44]. Although its chemical synthesis is well established, a large amount of galanthamine on the market comes from the extraction of crops, especially of various species of *Leucojum* and *Narcissus* [45]. Indeed,

galanthamine contains three asymmetric carbons, and respect for the configuration of these three centers of asymmetry makes the chemical synthesis complex and costly [5]. Sometimes the crop plants are dying due to contamination by pathogens. In the case of Amaryllidaceae, various diseases caused by fungal pathogens (*Colletotrichum*, *Fusarium*, *Botrytis*), viral pathogens (mosaic virus), and bacterial pathogens (*Serratia plymuthica*, *Stenotrophomonas maltophilia*) are important sources of agricultural losses [46, 47]. Plants of *L. aestivum* used as raw material source contain galanthamine at an average rate of 0.1% dry material (DM). The daily dose of galanthamine prescribed ranges from 30 to 50 mg depending on the patient's weight. For the treatment of a million patients, 50 kg of galanthamine/day is necessary, i.e., 18,000 kg/year. These needs in galanthamine require handling 18,000 tons of bulbs/year [48]. Note also that 24 million people are suffering from Alzheimer's disease in 2001, and that number is increasing [49].

The production of secondary metabolites by plants cannot, by itself, overcome the ever-growing needs of the pharmaceutical industry. In vitro cultures are an interesting and attractive alternative. They control many parameters in contrast to the culture fields or greenhouses. The studies of plant secondary metabolism become easier, and the development of industrial processes in bioreactors to high yields of secondary metabolites becomes possible. In vitro culture has been successfully applied to produce shikonin, the only marketed metabolite produced by cell cultures of *Lithospermum erythrorhizon*, and also to produce paclitaxel from *Taxus baccata*. This method was also successful with the production of saponins from *Panax ginseng* cells, berberine from the cultures of *Coptis japonica*, and sanguinarine from cells of *Papaver somniferum* [50].

The in vitro production of secondary metabolites is an attractive alternative. However, the increase of scale, in order to achieve an industrialization of bioproduction process, is often hampered by the low biosynthetic capacity. Thus, the elucidation of factors ensuring stable alkaloid biosynthesis is essential for the establishment of in vitro cultures to produce large amounts of galanthamine and also other alkaloids with biological interest.

5.1 Effect of Chemical Factors on Amaryllidaceae Alkaloid Accumulation

A detailed list of factors is presented in Table 1.

5.1.1 Plant Growth Regulators

There are several recognized classes of plant growth regulators (PGRs): auxins, cytokinins, gibberellins, ethylene, abscisic acid, and their analogues and inhibitors. PGRs can act and control such process as cell division, cell elongation, cell differentiation, organogenesis, dormancy, and secondary metabolite biosynthesis [50, 68].

The effect of the auxins 2,4-dichlorophenoxyacetic acid (2,4-D), 4-amino-3,5,6-trichloropicolinic acid (picloram), and 3,6-dichloro-*o*-anisic acid (dicamba) at various concentrations (25 and 50 μM) on the capacity of *L. aestivum* callus cultures for

Table 1 Biotechnological strategies for Amaryllidaceae alkaloid production

Factors	Plant species	References
PGRs	*N. confusus*	[51]
	L. aestivum	[52–56]
	G. elwesii	[57]
Sucrose	*N. confusus*	[58]
	N. pseudonarcissus	[59]
	L. aestivum	[57]
	G. elwesii	[57]
Mineral nutrients	*L. aestivum*	[59]
		[60]
Elicitation	*N. confusus*	[61]
	L. chinensis	[62]
	L. aestivum	[63]
	L. aestivum 'Gravety Giant'	[56, 60]
Biotransformation	*L. aestivum*	[12, 64]
	L. aestivum 'Gravety Giant'	[13, 14]
Temperature	*L. aestivum*	[65]
Light	*N. confusus*	[66]
	L. aestivum	[7]
Bioreactor system	*L. aestivum*	[67]
	L. aestivum 'Gravety Giant'	[10, 14, 53, 56, 59, 60]

Amaryllidaceae alkaloid accumulation was studied by Ptak et al. [52]. Galanthamine was detected in callus cultivated in the medium that contained 2,4-D (25, 50 μM), picloram (25 μM), or dicamba (50 μM). The addition of picloram (50 μM) in the medium caused the accumulation of four alkaloids except galanthamine, particularly trisphaeridine, tazettine, and 11-hydroxyvittatine, which are not detected with the other auxin treatments tested. El Tahchy et al. [57] have shown that the enrichment of the medium with 10 μM of 2,4-D enhanced the biosynthesis of: galanthamine, lycorine, crinine and demethylmaritidine in *L. aestivum* shoot cultures. On the other hand, the addition to the medium 10 μM of naphthalene-1-acetic acid (NAA) or picloram led to the highest diversity in alkaloids (galanthamine, lycorine, trispheridine, crinine, demethylmaritidine, and narwedine) in *G. elwesii* shoots cultures. Ten μM of NAA stimulated also biosynthesis of galanthamine, lycorine, trispheridine, crinine, and demethylmaritidine in *N. pseudonarcissus* shoot cultures [57].

The influence of cytokinins 6-benzyladenine (BA), zeatin, kinetin, meta-topolin, and thidiazuron on biosynthesis of galanthamine and lycorine in *L. aestivum* plant cultures regenerated from somatic embryos was tested by Ptak et al. [53]. The highest galanthamine content occurred in the case of plants grown in the RITA® bioreactor with a temporary immersion system (TIS), on medium enriched with 5 μM of thidiazuron (0.05% dry weight, DW). On the contrary, the highest lycorine content was found in plants grown on solid media containing 5 μM of kinetin or BA (0.0074% and 0.0070% DW, respectively). BA (3 mg/L) also increases production

of galanthamine in *N. confusus* shoot cultures, while kinetin (regardless of the concentration) had negative effect on this process in comparison with the control [51].

The same author tested also an inhibitor of gibberellins' biosynthesis: paclobutrazol in liquid cultures of *N. confusus*. It is known that paclobutrazol as a plant growth retardant may affect biosynthesis of secondary metabolites [69]. Codina [51] has proved that 10 mg/L of paclobutrazol increased the biosynthesis of galanthamine, but reduced the total contents of tested alkaloids (*N*-formylnorgalanthamine, galanthamine, haemanthamine, tazettine).

Ethylene, another chemical factor, is usually treated as an unfavorable gaseous growth regulator; however, its stimulating effect on certain morphogenetic processes and biosynthetic pathway has been found as well [70, 71]. The effect of ethylene on Amaryllidaceae alkaloid biosynthesis in *L. aestivum* callus, somatic embryos, and plant cultures was investigated [54–56]. A precursor of ethylene synthesis (1-aminocyclopropane-1-carboxylic acid (ACC)), inhibitors of ethylene synthesis (silver nitrate ($AgNO_3$) and silver thiosulfate (STS)) at concentrations of 1 and 10 μM, and potassium permanganate ($KMnO_4$) for the absorption of ethylene generated by the culture were used. Chromatographic analysis showed that the greatest amount of ethylene was in the dishes in which callus was multiplied on the medium supplemented with ACC and high concentration of auxin. While ethylene action on Amaryllidaceae alkaloid biosynthesis depended on development stage of *L. aestivum* in vitro culture, the presence of ethylene absorbent – $KMnO_4$ – in the dishes in which the young callus was grown caused an increase in the galanthamine content (from trace amounts to 0.002% DW) and the lycorine content (from 0.001% to 0.003% DW). Somewhat smaller amounts of galanthamine and lycorine were found in the callus obtained on the medium enriched with 10 μM STS. However, the addition to the medium of only 1 μM STS or $AgNO_3$, as well as ACC (irrespective of the concentration used), exerted an inhibitory effect on biosynthesis of these alkaloids [54]. It is also worthy of note that callus multiplied in the presence of $KMnO_4$ was characterized by the greatest alkaloid diversity. A similar reaction was observed for callus which showed differentiation capacity. Only the callus multiplied on the medium enriched with 10 μM STS and in the presence of $KMnO_4$ was capable for galanthamine biosynthesis at concentrations of 0.1% and 0.08% DW, respectively [55]. While ethylene was essential for biosynthesis of galanthamine in somatic embryo cultures of *L. aestivum*, the addition to the medium of ACC caused an almost sixfold (up to 2% DW) increase in galanthamine concentration as compared with the control. On the contrary, STS, $AgNO_3$, and $KMnO_4$ exerted an inhibitory effect on galanthamine production [55]. Treatment of *L. aestivum* and *L. aestivum* 'Gravety Giant' plants with 10 μM of ACC stimulated galanthamine biosynthesis in both genotypes (0.10 and 0.60 mg/g DW, respectively) and lycorine in *L. aestivum* 'Gravety Giant' cultures (0.54 mg/g DW) [56]. On the other hand, application of ethephon (2-chloroethylphosphonic acid), a synthetic compound, which is frequently used as an ethylene generator into *L. aestivum* and *L. aestivum* 'Gravety Giant' plant cultures, was not found to induce galanthamine biosynthesis [56].

5.1.2 Sucrose

Plant cell culture is usually grown heterotrophically using simple sugars as an energy and carbon source, as well as an osmotic agent. It is also known that carbohydrate modulates gene expression in plant [72]. Moreover, the level of sucrose has been shown to affect the production of secondary metabolites in in vitro cultures [73]. The effect of sucrose on galanthamine biosynthesis has been studied by Sellés et al. [58], Georgiev et al. [59], and El Tahchy et al. [57]. Addition to the medium of 180 g/L of sucrose resulted in the highest (0.457 mg per culture) production of galanthamine by *N. confusus* shoot cultures [58]. The shoots of *N. pseudonarcissus* had the highest galanthamine content (0.1% DW) when cultured in the presence of 30 g/L of sucrose [57], while 60 or 90 g/L of sucrose improved galanthamine accumulation (0.02% DW) in *G. elwesii* shoot cultures [57]. The study of El Tahchy et al. [57] demonstrated also that shoots of *L. aestivum* grown in the medium enriched with 30 or 60 g/L of sucrose are capable of the highest biosynthesis of galanthamine (0.032–0.074% DW). Similarly, Georgiev et al. [59] proposed modified Murashige and Skoog (MS) medium [74] which contained 60 g/L of sucrose for galanthamine production by *L. aestivum* shoot cultures.

5.1.3 Mineral Nutrients

The most commonly used medium is Murashige and Skoog [74], which was developed for *Nicotiana tabacum* callus growth. Investigations concerning the MS medium modifications (NH_4^+, NO_3^-, KH_2PO_4, and sucrose) for maximal galanthamine production by *L. aestivum* liquid shoot culture were reported by Georgiev et al. [59]. The optimal concentrations of tested components were 4.50 g/L KNO_3, 0.89 g/L NH_4NO_3, 1.25 g/L $(NH_4)_2SO_4$, and 0.10 g/L KH_2PO_4 in combination with 60 g/L sucrose. The presence of galanthamine in the cultures cultivated in modified MS medium reached 98 % total ion current in the shoots and 90 % in the liquid medium, while reducing the macroelements and microelements to half of the standard MS medium or reducing the nitrogen content decreased the growth rate of *L. aestivum* shoot cultures and did not stimulate galanthamine accumulation [60].

5.1.4 Elicitation

Elicitation of in vitro cultures provides a way to achieve higher yield of plant secondary metabolites due to controlled stress conditions. Traditionally, elicitors have been classified into abiotic and biotic type, according to their chemical nature and exogenous or endogenous origin [75]. Within the family Amaryllidaceae, elicitation concerns the following plant materials: *N. confusus* shoots, *Lycoris chinensis* seedlings, *L. aestivum* shoots, *L. aestivum*, and *L. aestivum* 'Gravety Giant' plants [56, 60–63]. It was observed that methyl jasmonate (MeJA) – the most common elicitor – used in high doses in the medium (e.g., 100 μM) inhibited or causes growth compared to the control in *N. confusus* and *L. aestivum* shoots, *L. aestivum*, and *L. aestivum* 'Gravety Giant' plants [56, 60, 61]. On the other hand, the addition of 25 μM MeJA to the medium stimulated the growth of *L. aestivum* shoots [63]. Jasmonates are plant-specific signaling molecules that

activate several important physiological and developmental processes [75]. Studies conducted by Ivanov et al. [63] in *L. aestivum* shoot cultures performed on a rotary shaker revealed that jasmonic acid increased mainly the activity of tyrosine decarboxylase, whereas MeJA stimulates mainly phenylalanine ammonia-lyase enzymes that catalyze the first step in the metabolic pathway of Amaryllidaceae alkaloids [76]. The different levels of both enzymes in the initial step of biosynthetic pathway of Amaryllidaceae alkaloids, caused by MeJA and jasmonic acid, were expressed also in different levels of galanthamine in tested plant materials. In *L. aestivum* shoot cultures grown in a flask on a rotary shaker, galanthamine biosynthesis was stimulated more by jasmonic acid than by MeJA. It should be also noted that in this case shoots produced 1.36 times more galanthamine than in the control. In *L. aestivum* shoot culture grown in a temporary immersion system, galanthamine biosynthesis was elicited with MeJA (total amount of galanthamine in shoots: 2.20 mg/g DW) [60]. On the other hand MeJA was also the best elicitor for galanthamine biosynthesis in *N. confusus* shoot cultured on a rotary shaker, which increased two times the level of galanthamine and three times the total amount of alkaloids [61]. The biosynthesis of galanthamine in *L. aestivum* and *L. aestivum* 'Gravety Giant' plant cultures was stimulated also by MeJA and highly related with genotype. Elicitation of *L. aestivum* 'Gravety Giant' gives better results than in the case of *L. aestivum* plants. Treatment of these cultures with 5 or 50 μM of MeJA for 168 h increased the production of galanthamine from 0.08 (control plants) to 0.4 mg/g DW [56]. The addition of 50 μM of MeJA during 168 h in *L. aestivum* 'Gravety Giant' plant cultures, also strongly promoted lycorine biosynthesis (1.1 mg/g DW) [56], while in *L. aestivum* shoot culture lycorine biosynthesis was stimulated by 25 μM of MeJA added in the early stationary phase (491.4 μg/flask) [63].

Salicylic acid (SA) has been reported as an effective elicitor of secondary metabolites [77]. However, salicylic acid added in *L. aestivum* shoots and plants grown in temporary immersion system did not have a positive effect on the biosynthesis of galanthamine [56, 60]. An opposite reaction was observed in *L. aestivum* 'Gravety Giant' plants which produce eight times more galanthamine (0.8 mg/g DW) in comparison with the control samples. It should be added that in this case the highest lycorine content (1.53 mg/g DW) was observed [56]. Moreover, SA was not cytotoxic for growth of *L. aestivum* 'Gravety Giant' plants.

Among other elicitors, yeast extract (YE) was tested but in *L. chinensis* seedling cultures in [62]. YE (0.15 g/L) increased 1.62 times galanthamine production in comparison with control. In *N. confuses* shoots cultures, the effect of chitosan and arachidonic acid was also studied, however these elicitors did not have any interesting effect on galanthamine biosynthesis [61].

5.1.5 Effect of Precursors of the Alkaloid Biosynthesis Pathway

The use of a precursor of the biosynthesis pathway aims to stimulate the biosynthetic pathway by increasing the overall flow of precursors into the molecule of interest. This aspect has been studied by El Tahchy et al. [12, 64] adding deuterated 4′-*O*-methylnorbelladine (MN) in liquid culture medium of *L. aestivum* calli and bulblets. Previous studies are reported in the literature using other precursors (caranine,

tyrosine, and phenylalanine) labeled with ^{14}C or $^{3}H_3C$ for the study of the biosynthesis pathway in *N. pseudonarcissus* [78] and *L. aestivum* [24]. The deuterium labeling has several advantages; it is widely used as stable and nonradioactive isotopic tracer. Its presence can be revealed by mass spectrometry methods. Deuterated MN added in the culture medium of bulblets of *L. aestivum* was incorporated in the biosynthetic pathway and metabolized in three types of deuterated Amaryllidaceae alkaloids: labeled galanthamine, lycorine, crinine, *N*-demethylnarwedine, demethylgalanthamine, narwedine, *N*-formylgalanthamine, anhydrolycorine, trisphaeridine, and demethylmaritidine [64]. Furthermore, the addition of the deuterated precursor highly stimulated the biosynthesis of both galanthamine (0.5 mg/g DW vs. 0.01 mg/g DW in the control samples) and lycorine (0.2 mg/g DW vs. 0.04 mg/g DW). Also, the non-labeled 4′-*O*-methylnorbelladine feeding enhanced galanthamine and lycorine production by *L. aestivum* shoot cultures [13]. In addition, bulb cultures of *L. aestivum* and *L. aestivum* 'Gravety Giant' were subcultured in medium containing the non-labeled precursor (MN) at various concentrations in bioreactor RITA® [14]. Precursor feeding along with temporary immersion conditions was found to significantly improve the accumulation of both galanthamine and lycorine. The maximal concentrations of galanthamine (0.81 mg/g DW) and lycorine (0.54 mg/g DW) in *L. aestivum* bulblets were reached, respectively, after 40 days of culture with 0.15 g/L of precursor and after 30 days of culture with 0.3 g/L of precursor. In *L. aestivum* 'Gravety Giant' bulb cultures, 0.3 g/L of precursor was the best condition for both galanthamine (0.6 mg/g DW after 50 days) and lycorine (1.13 mg/g DW after 30 days). These combined effects, differentiated tissues, precursor feeding, and temporary immersion conditions, represent an interesting approach for galanthamine and lycorine production by *L. aestivum* bulblets with further possibilities for effective scale-up.

5.2 Effect of Physical Factors on Amaryllidaceae Alkaloid Accumulation

A detailed list of factors is presented in Table 1.

5.2.1 Temperature

A change in the in vitro culture temperature may change the physiology and metabolism of cultured plants and subsequently affect growth and secondary metabolite production [79]. In plant cell, tissue, and organ cultures, the influence of temperature on the regulation of different physiological processes is not sufficiently clear. A temperature range of 17–25 °C is typically used for in vitro culture. However, each plant species may exhibit optimum growth and metabolism under different temperature regimes [73].

Ivanov et al. [65] have shown that the temperature of *L. aestivum* shoot cultivation at temporary immersion conditions had an effect on the activity of enzymes catalyzing phenolic oxidative couplings in the molecule of 4′-*O*-methylnorbelladine which is the precursor of galanthamine and lycorine. At a temperature of 18–22 °C,

para-ortho' couplings were formed, which resulted in the biosynthesis of galanthamine-type alkaloids and inhibition of the formation of crinine-type and lycorine-type alkaloids with *para-para'* and *ortho-para'* couplings. However, at 26 °C the opposite effect was observed. These results demonstrated that temperature of *L. aestivum* in vitro cultivation influences enzyme activities and formation of different groups of Amaryllidaceae alkaloids. It was observed that a decrease in temperature from 26 to 22 °C results in a twofold increase of galanthamine biosynthesis and at the same time decline in lycorine production. It is worth noting that further decrease of the temperature to 18 °C did not have an effect on galanthamine and lycorine biosynthesis.

5.2.2 Light

Plant growth and developmental processes in in vitro culture are regulated by light quality (color, wavelength), quantity, and photoperiod. Light is also often a crucial factor in secondary metabolite accumulation [73, 80].

The research work carried out by Bergoñón et al. [66] has shown that production of galanthamine in *N. confusus* shoot cultures is stimulated by light (long-day photoperiod). The total production of galanthamine under these conditions was 2.50 mg per culture, of which 1.97 mg per culture were released into the medium. Similarly, Berkov et al. [7] demonstrated that light is an important factor affecting the biosynthesis of galanthamine in shoot cultures of *L. aestivum*. The shoots grown under light conditions produced twice as much galanthamine as the cultures grown in the dark (73.8 μg/g DW and 38.5 μg/g DW, respectively). It is worth noting also that more alkaloids were detected in the cultures grown under light as compared to those grown in the dark.

5.2.3 Physical State of the Medium

Plant material can be cultured either in a solid or in a partially solidified with a gelling agent or in liquid medium [81]. Embryogenic callus, somatic embryos, and plants and bulbs of *L. aestivum* were propagated conventionally on solid media [37, 53] (Fig. 4a, b). A semisolid medium containing 6 mg/L of agar was used in *L. aestivum* shoot cultures [60]. Liquid cultures grown on rotary shaker were also applied, for example, for *Narcissus* [61] and *L. aestivum* shoots [13, 82] (Fig. 4c). However, the application of bioreactor system for large-scale cultivation of plant materials for the production of valuable bioactive compounds is a major objective scientific work [83–85]. Different types of bioreactors are available, liquid-phase, gas-phase and hybrid bioreactors, and temporary immersion system (TIS), while liquid-phase bioreactors could be divided into subgroups like mechanically agitated (STR), air agitated (air-lift), and agitated by recirculation pre-enriched medium (convective flow) [85].

The publications of Schumann et al. [60] and Berkov et al. [10] include an extensive study on the use of bioreactors for the production of galanthamine. Schuman et al. [60] investigated the growth of *L. aestivum* shoots and their ability for the biosynthesis of galanthamine during cultivation in different bioreactors: temporary immersion system (TIS) and TIS with air-lift culture vessels and bubble

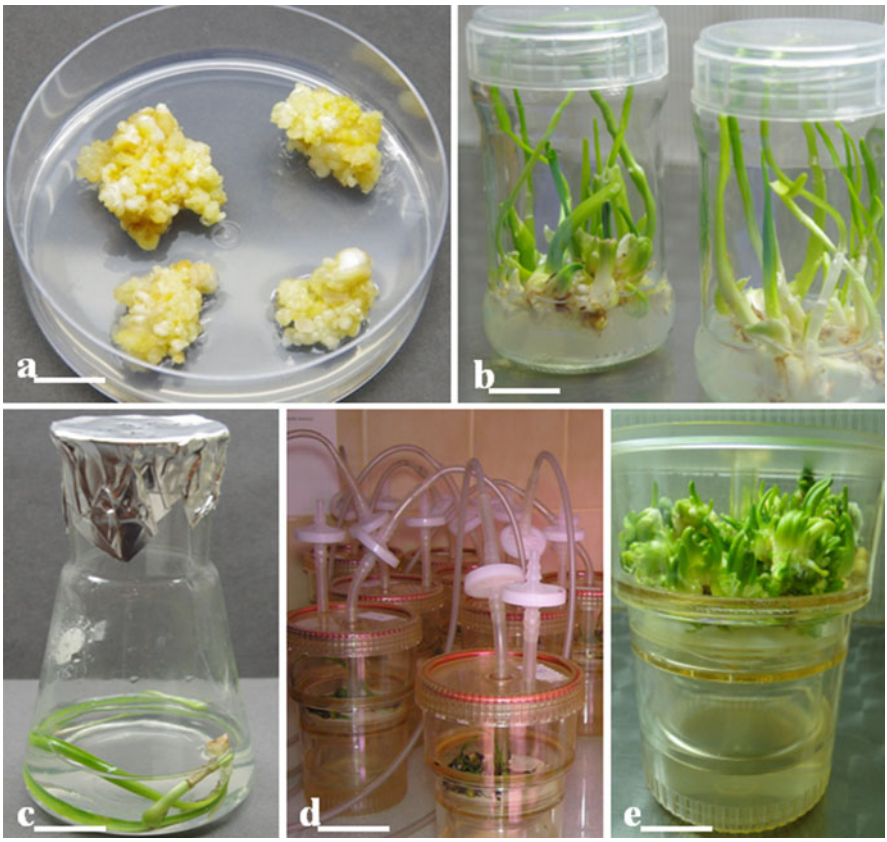

Fig. 4 Different *L. aestivum* culture systems. (**a**) Solid cultures: multiplication of embryogenic callus, *bar* = 10 mm. (**b**) Solid cultures: development of plants, *bar* = 15 mm. (**c**) Liquid cultures on rotary shaker: view of the plants inside the Erlenmeyer flask, *bar* = 19 mm. (**d**) Liquid cultures in temporary immersion system: RITA® bioreactors, *bar* = 37 mm. (**e**) Liquid cultures in temporary immersion system RITA®: view of the plants inside the culture vessel, *bar* = 17 mm

bioreactor. The different volumes of culture vessels and the amount of inoculums on galanthamine content were tested. TIS 1-L bioreactor with air-lift culture vessel, gassing 12 times per day (5 min) used for 10 or 25 g of inoculums, was allowed to obtain 170.71 and 214.7 μg galanthamine/L per day, respectively.

Temporary immersion system with RITA® vessels was also used in the work on the biosynthesis of galanthamine (Fig. 4d, e). Ivanov et al. [67] tested immersion frequencies and temperatures on galanthamine biosynthesis in *L. aestivum* shoot cultures. The highest growth index (2.98) and yield of galanthamine (265 μg/RITA, 1.17 mg/L) were achieved at the cultivation of shoots in bioreactor RITA® with immersion frequency of 15 min flooding and 8 h standby periods, at 26 °C, while the immersion frequency of 5 min every 2 h led to conversion of *L. aestivum* somatic embryos into plants which was twice as high as embryos in solid media [53]. The

growth index achieved at these conditions (2.49) was also higher than growth index (1.03) achieved during the cultivation in solid media (1.03). It should also be noted that the temporary immersion system allowed eliminating the hyperhydricity problem observed very often with continuous contact of plants with liquid medium and high concentrations of cytokinins. In addition, the galanthamine accumulation (0.05 % DW) in plants cultivated in the bioreactor system RITA® was higher than that observed in plants derived from solid medium (0.025% DW).

A new system based on a bubble-column bioreactor was developed by Georgiev et al. [59]. A modified glass-column illuminated bioreactor with internal sections was successfully adapted for *L. aestivum* shoot cultures. The intake airflow rate and cultivation temperature also had an impact on the Amaryllidaceae alkaloid profile. A maximum galanthamine content (1.7 mg/L) was obtained at 22 °C temperature of cultivation and 18 L/(L·h) flow rate of inlet air. Under these conditions over 40% of the biosynthesized galanthamine was extracellular with 45% purity of the total ion current. Moreover, among the seven isolated alkaloids, galanthamine and lycorine were dominant both in intracellular and extracellular alkaloid mixtures.

6 Conclusion

The alkaloids from Amaryllidaceae are an exclusive group of compounds with various biological activities. The best known are galanthamine and lycorine. Alkaloids, products of the secondary metabolism, are synthesized in small amounts by plants. In vitro cultures are an interesting alternative for the production of alkaloids from Amaryllidaceae. However, limited knowledge of the biosynthetic pathway of these alkaloids, both at the biochemical and molecular levels, complicates the task. Several studies reported in the literature have identified several key factors, as the genotype, plant growth regulators, sucrose, elicitors, physical factors, and precursor of the alkaloid biosynthesis pathway, influencing the biosynthesis pathway of Amaryllidaceae alkaloids, particularly in *L. aestivum*. In view to enhancing the production of galanthamine by *L. aestivum* shoots/bulblets, it could be very interesting to combine various parameters which have previously shown positive effects, i.e., bioreactor cultivation in optimized nutrient MS medium containing 4.50 g/L KNO_3, 0.89 g/L NH_4NO_3, 1.25 g/L $(NH_4)_2SO_4$, 0.10 g/L KH_2PO_4 in combination with 60 g/L sucrose, 10 μM of 2,4-D, 5 μM of thidiazuron, 10 μM of ACC, and 0.30 g/L of the precursor 4′-*O*-methylnorbelladine. The elicitor MeJA could be added to these cultures at the level 5 or 50 μM for 168 h. The cultures could be maintained at 18–22 °C under white fluorescent light with a 16 h photoperiod. It is also important to improve Amaryllidaceae extracts' purification, i.e., without losses due to the purification step as reported by Saliba et al. [14]. Highly recommended, validated, and rapid purification of Amaryllidaceae alkaloids can be performed using SPE cartridges with SI and SCX sorbents. The Amaryllidaceae alkaloids are a group of alkaloids with many biosynthetic enzymes yet to be discovered. It is essential to know the enzymes involved in the biosynthetic pathway of these alkaloids in order to better control their production.

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Sustainable Production of Polyphenols and Antioxidants by Plant In Vitro Cultures

8

Iryna Smetanska

Contents

Abstract

Phenolic compounds represent big group of plant secondary metabolites that influence flavor, color, and texture and can be used as food additives, nutraceuticals, and pharmaceuticals.

However, there are some limitations in obtaining sufficient amount of these bioactive compounds from plants, because they are rather seldom or occur

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naturally in plant tissues only at very low concentrations. Alternatively, it is possible to synthesize them chemically, but this way if oft technologically not possible or very sophisticated and economically infeasible.

Plant in vitro cultures provide an attractive route to produce high-value plant-derived products and therefore can be an alternative source of valuable phenolics.

Moreover, compounds synthesized by plant in vitro cultures are natural products and therefore can be more easily accepted by consumers as artificially synthetized substances.

The synthesis of phytochemicals by plant in vitro cultures in contrast to these in plants is not depending on environmental conditions and can be regulated through standard physical and chemical conditions in bioreactor, which helps to avoid qualitative and quantitative fluctuations in product yield.

The process of obtaining valuable phytochemicals can be represented as a multistage technology, each link of which can vary individually in dependence of specific requirements *of* in vitro cultures (e.g., phytohormones, nutrients, light) or properties of end product (e.g., antioxidative potential, stability).

For the establishment of high-producing and fast-growing cell lines, the parent plants should be selected (Murthy et al. Strategies for enhanced production of plant secondary metabolites from cell and organ cultures. In: *Production of biomass and bioactive compounds using bioreactor technology* (pp. 471–509). Springer Plus). The expression of synthetic pathways can be influenced by environmental conditions, the supply of precursors, and the application of elicitors (Schreiner, Eur J Nutr 44(2):85–94, 2005) as well as altered by special treatments like biotransformation and immobilization (Georgiev et al., Appl Microbiol Biotechnol 83:809–823, 2009). The efficiency of bioprocessing can be increased by the simplification of methods for product recovery and afterward its stabilization.

This chapter reviews the recent advances in the optimization of environmental factors for production of phenolics by plant in vitro cultures, new developments in bioprocessing of plant cell, hairy root and organ cultures, and emerging technologies on phytochemical recovery.

Keywords

Plant *in vitro* cultures · Cell cultures · Hairy root cultures · Organ cultures · Phenolic compounds · Antioxidants · Food additives · Cultivation media · Precursors · Elicitors · Exudation · Membrane permeabilization · Fermentation

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BA	Benzyladenine
DMSO	Dimethyl sulfoxide
DW	Dry weight
FW	Fresh weight
IAA	Indole-3-acetic acid
NAA	1-Naphthalene acetic acid

1 Introduction

For centuries, humankind has been very dependent on plants as a source of carbohydrates, proteins, and fats for food and shelter. In addition to essential primary metabolites, higher plants synthesize a wide variety of the secondary metabolites [1]. Plant secondary metabolites can be defined as compounds that have no recognized role in the maintenance of fundamental life processes in plants, but they do have an important role in the interaction of the plant with its environment. They mostly have an ecological role as attractants for pollinating insects or in defense mechanisms against predators. The distribution of secondary metabolites in plants is far more restricted than that of primary metabolites; a compound is often only found in a few species or even within a few varieties within a species. The production of these compounds is often low (less than 1% DW) and depends greatly on plant species and plant physiological and developmental stage [2]. Secondary metabolites often accumulate in the plant in specialized cells or organs.

Many plants, possessing high amount of specific secondary metabolites as, e.g., alkaloids or phenolics, are used in traditional medicine. About 75% of the world's population, especially in developing countries, relies on herbal medicine to prevent and cure diseases, and about 25% of the synthesized drugs are manufactured from medicinal plants [3, 4]. The market for biopharmaceuticals is growing faster than the pharmaceutical market as a whole, and a recent projection suggested the value of this segment could reach $US 278.2 billion by 2020 [5].

Phenolic compounds are important components of the human diet due to their high antioxidant activity [6] and their capacity to diminish oxidative stress and consequently to prevent development of chronic diseases [7] or cancer [8].

Due to increased demand of consumers to obtain food, enriched with natural health-promoting components, the integrating of phenolic compounds into food matrix is an important task of food producers. The problems in obtaining some phenolic compounds from plants include environmental factors, political and labor instabilities in the producing countries, uncontrollable variations in the crop quality, inability of authorities to prevent crop adulteration, and losses in storage and handling [9]. In many cases the chemical synthesis of these is either extremely difficult or economically infeasible [2].

Plant in vitro cultures can be sustainably used as natural sources of phenolic compounds. In contrast to plant tissues, the concentration and individual profile of phenolic compounds in plant in vitro cultures can be controlled, which leads to obtaining of uniform product [10, 11].

During the past four decades, wide range plant in vitro cultures started to be commercially produced for obtaining of phytochemicals in the same way as bacteria and fungi for antibiotic or amino acid production [12]. For example, there has been tremendous success in the production of shikonin from cell cultures of *Lithospermum erythrorhizon* [13]. Such companies as, e.g., BD Biosciences, Vytrus Biotech, Pierre Fabre, Sederma, Protalix BioTherapeutics, Dow AgroSciences, and Greenovation Biotech GmbH produce nutraceuticals and pharmaceuticals using plant cell cultures.

2 Role of Phenolic Compounds

2.1 Chemical Composition and Properties

Phenolic compounds are one of the most common and widespread groups of plant secondary metabolites. The term "phenolic compounds" embrace a broad range of plant substances that possess in common an aromatic ring having one or more hydroxyl substituent. They most frequently occur combined with sugar, as glycosides, and thus make them tend to be water-soluble. There are several classifications of phenolics, but the most spread one is based on the number of phenolic cycles in the molecule [14]. Several subclasses of phenols can be distinguished according to the number of phenol rings and to the structural elements that join these rings. According to this principle, the following compounds are belonging to phenolics: simple phenols and benzoquinones (e.g., catechol), phenolic and hydroxycinnamic acids (e.g., caffeic, ferulic acid), phenylpropenes (e.g., eugenol), coumarins, naphthoquinones, stilbenoids (resveratrol), anthraquinones, flavonoids (e.g., quercetin, genistein), flavanones, isoflavonoids, anthocyanins, lignans, lignins, and polyphenols (e.g., tannins).

2.2 Role of Phenolics for Plants and Humans

Numerous phenolic compounds are responsible for color, taste, and texture of plants and therefore play an important role for plants and for human diet. The most relevant phenolics for the human nutrition are flavonoids, isoflavonoids, anthocyanins, phenolic acids, and polyphenols [15].

2.2.1 Flavonoids

Flavanols as quercetin, rutin, hesperidin, naringin, and tangeritin are found in high concentrations in onions, apples, red wine, broccoli, tea, and *Ginkgo biloba* [16]. The most common in the American diet are quercetin (70%), kaempferol (16%), and myricetin (6%) [17].

Although dietary intake varies considerably among geographic regions and cultures, the average daily consumption of flavonoids by humans is estimated to be 1 g, and epidemiological studies have indicated a relationship between a diet rich in flavonoids and a reduced incidence of heart disease because flavonoids and flavanols possess a wide spectrum of biological activities and indicated having the ability of inhibiting auto-oxidative reactions and scavenging of free radicals. Most of the protective effects of flavonoids in biological systems are ascribed to their antioxidant abilities, capacity to transfer electrons, free radicals, and chelating abilities [18].

Flavonoids have been reported to possess a wide range of activities in the prevention of common diseases, including cancer, neurodegenerative diseases, and gastrointestinal disorders [19]. Quercetin has demonstrated significant anti-inflammatory activity because of direct inhibition of several initial processes of

inflammation. It inhibits the manufacture and the release of histamine and other allergic and inflammatory mediators and might be of therapeutic benefit by treatment of cardiovascular diseases [20].

Numerous studies have shown the accumulation of flavonoids in in vitro cultures, e.g. in callus culture of *Crataegus sinaica* [21] as well as in suspension cultures of *Glycyrrhiza echinata* [22] and *Momordica charantia* [23]. Bandekar and Lele [24] found that suspension culture of banyan (*Ficus benghalensis* L.) synthetized 0.31 mg/g DW of quercetin, which was 20 times more as plant tissue. Arya et al. [25] extracted 0.23 mg/g DW of quercetin from callus culture of *Pluchea lanceolata*.

2.2.2 Isoflavones

Isoflavones are a subclass of flavonoids and also called phytoestrogen compounds due to its structural similarity with human hormone estradiol [26].

In the plant kingdom, isoflavones, contained mainly in Leguminosae, occur primarily in the subfamily Papilionoideae for human diet, like soy and kudzu, or for animal diets, such as clover [27], alfalfa, and beans. Isoflavones also contents in vegetative parts of many legumes, including varieties such as psoralea (*Psoralea corylifolia*), kudzu (*Pueraria lobata*), fava bean (broad bean, *Vicia faba*), lupine (*Lupinus luteus*), soybean (*Glycine max*), clover (*Trifolium* spp.), peas (*Pisum sativum*), chickpea (*Cicer arietinum*), mung bean (*Phaseolus aureus*), and lima bean (*Phaseolus lunatus*).

Isoflavones influence bone health and exhibit weak estrogenic or antiestrogenic activity [28]. Due to the fact that isoflavones have phenolic structure similar to estrogen, thus isoflavones provide a natural alternative to the use of postmenopausal hormone replacement therapy. They have become of immense interest to clinical nutritionists because of their potential to modulate and prevent numerous hormonal and non-hormonal-dependent conditions. The isoflavones daidzein and genistein share identical structures except for an additional hydroxyl group on the A ring of genistein, but genistein may have up to five- or sixfold greater estrogenic activity in some assay systems.

Callus culture of *Pueraria candollei* var. *mirifica* was established by Thanonkeo and Panichajakul [29] with purpose to obtain isoflavones daidzein and genistein. Karuppusamy [30] reported that soy plant suspension culture from batch system can produce genistein and daidzein.

2.2.3 Anthocyanins

Anthocyanins belong to the subgroup of flavonoids and are colored pigments, which are used as natural dyes. Anthocyanins are ubiquitous natural pigments, colors ranging from pink through red, violet, or dark blue, found in plant flowers, fruits, leaves, and storage organs. They change color over the pH range due to the existence of four pH-dependent forms: at low pH they are red and at pH over 6 they turn blue. They are commonly used in acidic solutions in order to impart a red color to soft drinks, sugar confectionary, jams, and bakery toppings [31].

In addition, they also possess antioxidative, anti-inflammatory, and DNA-protective properties. Therefore, they can be used not only as food additives

but also because of their pharmaceutical purpose. Pure anthocyanins are priced \$2000 kg^{-1} [32]; therefore it makes sense to obtain them from bioreactor.

With the great demand of food colorants from natural sources instead of synthetic materials, production of anthocyanins by plant cell culture has been suggested as a feasible technology that has attracted considerable industrial interest [33]. The significant progress in the enhancement of anthocyanin production in plant cell cultures through strain improvement, optimization of media and culture conditions, novel bioreactor design, and intelligent process design has been reported [10].

2.2.4 Resveratrol

Resveratrol (3,4′,5-trihydroxystilbene) is a naturally occurring phytoalexin produced by some spermatophytes, such as *V. vinifera* and other members of Vitaceae as a response to infection, injury, fungal attack, or exposure to UV light.

Resveratrol has been reported to exhibit a wide range of important biological and pharmacological properties [34].

Production of resveratrol has been observed in both grapevine tissue and cell cultures in response to infection or stress [35]. Among the phenolic acids detected in *V. vinifera* cell culture, 3-*O*-glucosyl-resveratrol was predominant, and its content could be increased though application of cobalt, silver, and cadmium [36].

2.2.5 Phenolic Acids

Phenolic acids, particularly chlorogenic and rosmarinic acid, influence aroma and taste [37].

Rosmarinic acid becomes one of the most targeted phenolic compounds in formulating functional foods and supplements due to its antioxidant activities [6]. Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenylacetic acid [38]. The development of functional foods containing rosmarinic acid from medicinal plants such as *Orthosiphon aristatus* that consist of naturally occurring antioxidants is preferable [39]. However, growing *Orthosiphon aristatus*, a native tropic-medicinal plant, in subtropic climate area is considered to be relatively difficult.

Plant cell cultures that accumulate rosmarinic acid have been proposed for production of this phenolic compound [40]. Thus, the characteristic aromas of cocoa and coffee, consisting of the mixture of chlorogenic and rosmarinic acids, have been produced by cell cultures of *Theobroma cacao* and *Coffea arabica*, respectively [41].

2.2.6 Lignans

Lignans are polyphenolic substances derived from phenylalanine via dimerization of substituted cinnamic alcohols. Lignans of some plants as flax possess high content of secoisolariciresinol diglucoside and secoisolariciresinol. These types of lignans can be converted to mammalian lignans, namely, enterodiols and enterolactones, by the action of intestinal microflora [42], which can potentially reduce the risk of certain cancers such as breast, prostate, and colon cancer [43]. Therefore, because of their beneficial effects on human health, lignans are currently well recognized, and many in vitro and in vivo studies are being conducted worldwide [44]. Due to its rich

lignan content, flax is increasingly considered as an important component in functional food and food applications [45].

Gabr et al. [46] (2016) introduced callus and *A. rhizogenes*-transformed hairy roots of flax *Linum usitatissimum* and studied the influence of different factors as cultivation media and phytohormones on accumulation of lignans, namely, secoisolariciresinol diglucoside (SDG), secoisolariciresinol (SECO), and matairesinol (MAT), in in vitro cultures. Antioxidant activity was significantly higher in hairy root cultures than in non-transformed cultures. Furthermore, the extract of hairy root culture showed inhibition of proliferation of human breast cancer cell line (MCF-7).

3 Plant In Vitro Cultures as Source for Phenolic Compounds

3.1 Advantages and Perspectives

Many plants containing high-value phenolic compounds are difficult to cultivate on the large scale due to specific ecological condition.

A corresponded plant cell culture provides an attractive alternative source of phenolics that can overcome the limitations of extracting useful metabolites from limited natural resources.

At the same time, the chemical synthesis of plant-derived compounds is often not economically feasible because of their highly complex structures and specific stereochemical characteristics. Continuous obtaining of valuable secondary metabolites from plant in vitro cultures is an attractive alternative to their extraction from plant material [47].

For example, Yang et al. [48] found that it takes at least 3 years for *Glycyrrhiza inflata* plants to be harvested for medicinal purposes. Flavonoid productivity of the cell suspension cultures which were cultured for 21 days was higher than that of the 3-year-old plant, which suggested that this method is potentially a profitable one.

The application of plant cell culture has three main aspects [49]:

1. Breeding and genetics:
   - **Micropropagation** – using meristem and shoot culture to produce large numbers of identical individuals
   - **Selection** – screening of cells, rather than plants for advantageous characters
   - Crossing distantly related species by **protoplast fusion** and regeneration of the novel hybrid
   - Production of dihaploid plants from **haploid cultures** to achieve homozygous lines more rapidly in breeding programs
   - **Transformation**, followed by either short-term testing of genetic constructs or regeneration of transgenic plants
   - **Removal of viruses** by propagation from meristematic tissues
2. **Model system** for study of plant cell genetics, physiology, biochemistry, and pathology

Table 1 Synthesis of phenolic compounds in plant in vitro cultures compared with the parent plants

Product	Plant	Yield (% DW)		Culture/plant	References
		Plant	Culture		
Anthocyanins	*Vitis* sp.	10	16	1.6	[51]
	Euphorbia milii	0.3	4.2	13.3	[52]
	Perilla frutescens	1.5	24	16	[53]
Anthraquinones	*Morinda citrifolia*	2.2	18	8	[54]
Caffeic acid	*Vanilla planifolia*	0.05	0.02	4	[55]
Rosmarinic acid	*Coleus blumei*	3	27	9	[40]
	Salvia officinalis	3	36	12	[56]

3. **Production of secondary metabolites** – growth in liquid culture as a source of valuable products

As it is known, cultured plant cells often produce different quantities of total and individual phenolics when compared with the intact plant [50].

As represented in Table 1, some phenolic compounds in plant in vitro cultures can be accumulated with a higher titer compared with those in the parent plants.

There are also a number of examples of cultured cells producing phenolic compounds not observed in the plant, so-called de novo synthesis. Thus, *Lithospermum erythrorhizon* cell culture synthesized rosmarinic acid, which was not presented in plant tissues [57].

Since the late 1950s, many food additives have been questioned, mainly by national and international regulatory authorities, about their safety for long-term use and consumption. At the same time, the consumer associations, aware of the inclusion of additives in foodstuffs, have been exerting pressure on governmental bodies to have chemical or artificial additives replaced by natural additives from plant tissues, or these synthesized by plant in vitro cultures [58]. The most valuable food additives from the class of phenolic compounds, which can be obtained from the plant in vitro cultures, are food colorants (anthocyanins), aromas (vanillin), flavors (tannins acid), or antioxidants (chlorogenic and rosmarinic acids). Some food additives of phenolic nature, obtained from plant in vitro cultures, are listed in Table 2.

Some plant phenolic compounds are produced in bioreactors on industrial scale. For example, Nippon Paint Co. Ltd. (Osaka, Japan) obtains anthocyanins from suspension cells of *Aralia cordata* and afterward uses them as food colorant, A. Nattermann and Cie. GmbH (Cologne, Germany) produces rosmarinic acid from cell suspension of *Coleus blumei* and uses them as anti-inflammatory agent, and ROOTec (Witterswil, Switzerland) gets flavonoids from hairy roots of *Carlina acaulis* for cosmetic products [63]. Some more of successfully established and commercialized in vitro cultures producing a high number of phenolic compounds are listed in Table 3.

Table 2 Food additives of phenolic nature, obtained from plant in vitro cultures

Product type	Plant species	References
Colors		
Anthocyanins	*Vitis vinifera*	[32]
	Aralia cordata	[59]
	Perilla frutescens	[49]
Anthraquinones	*Rubia tinctorum*	[60]
	Cinchona ledgeriana	[58]
	Morinda citrifolia	[54]
Flavonoids	*Crataegus sinaica*	[21]
	Glycyrrhiza echinata	[22]
	Glycyrrhiza inflata	[48]
	Momordica charantia	[23]
Naphthoquinones	*Lithospermum erythrorhizon*	[61]
Aromas and fragrances		
Vanillin	*Vanilla planifolia*	[62]
Chlorogenic acid	*Coffea arabica*	[41]
Chlorogenic acid	*Theobroma cacao*	[53]

Table 3 Plant in vitro cultures characterized by high yields of phenolic compounds

Product	Plant species	Yield	References
Anthocyanins	*Perilla frutescens*	8.9% DW	[49]
Anthraquinones	*Morinda citrifolia*	18.0% DW	[54]
	Coleus blumei	21.4% DW	[40]
Flavonoids	*Glycyrrhiza inflata*	95.7 mg/L	[48]
	Glycyrrhiza uralensis	28.38 mg/g DW	[64]
Daidzein	*Psoralea corylifolia*	2.06% DW	[65]
Genistein		0.37% DW	
Resveratrol	*Arachis hypogaea*	4.3 nmol/g DW	[66]
Rosmarinic acid	*Coleus blumei*	78 mg/g DW	[67]
Rutin	*Fagopyrum esculentum*	1.3 mg/g DW	[68]

However, this technology is still being developed, and despite the advantages, there are a variety of problems to be overcome before it can be adopted for the production of useful plant secondary metabolites.

3.2 Obstacles and Problems

The limited success of industrial utilization of plant cell cultures for the production of secondary metabolites is due to various reasons ranging from low yields of the desired compounds, incomplete understanding of the biosynthetic pathways, and the technological processes involved in extraction and purification of these compounds.

Generally, the problems with the plant cell cultures can be classified as biological (like slow growth rate, physiological heterogeneity, genetic instability, low metabolite content, product secretion) and operational (wall adhesion, light requirement, mixing, shear sensitivity, aseptic condition) [49, 69].

In many cases, cell suspension cultures due to undifferentiation of cells are far less efficient than cultured organs in terms of metabolite production, and biosynthetic pathways are often not complete. Some metabolites are stored in specific plant tissues, but in undifferentiated cell suspension cultures, the synthetized product can negatively influence cell growth, or can be degraded by the enzymes, or could be released in the culture media.

Repressed secondary metabolite production in pant cell cultures might also be due to the specific location of some of the key enzymes involved in the biosynthetic pathways. Subcellular localization of various enzymes and transport of intermediates to the respective compartments for further activity of enzymes limits the target of sustainable accumulation of product. This is probably one reason for the low production or release of metabolites in the culture medium for sustainable harvest and downstream processing at the commercial scale [70]. Proper understanding of biosynthetic pathways, exploration of respective enzymes, and investigations regarding their developmental regulation are further needed. Furthermore, strategies to increase metabolite accumulation/secretion in culture medium are often lack of success or accompanied with loss of cell viability [71]. An approach for preserving viability of biomass and its reuse needs to be developed. Optimization of these methods could be explored by utilizing different kinds of bioreactors, combining elicitors and adsorbents, and simultaneous extraction of metabolites from the exhausted medium. The introduction of newer techniques of extraction, stabilization, and purification of metabolites from cells and growing medium is also likely to be a significant step toward making in vitro cultures more generally applicable to the commercial production of secondary metabolites.

4 Regulation of Synthesis of Phenolic Compounds by Plant In Vitro Cultures

The objective of the food and pharmaceutical industry is to develop technology where it is possible to yield valuable metabolites more cheaply from the plant in vitro cultures than to extract them from the whole plant or to synthesize the product. Confronted with having to increase the amount of secondary metabolites in plant in vitro cultures, the need for biochemical and molecular research on the secondary metabolism of plants has been frequently emphasized [9, 72]. The research in this area could lead to the successful regulation of secondary metabolism and significantly increase the amounts of the compounds.

It should be possible to achieve the synthesis of a wide range of valuable phenolic compounds such as flavonoids, phenolic acids, and polyphenols from plant in vitro cultures using multistage technology as it is shown in (Fig. 1). Each link in this

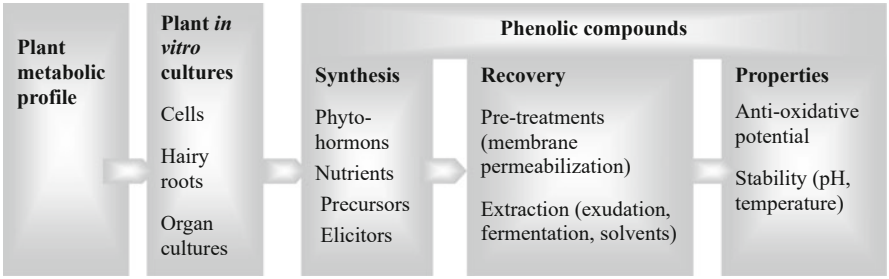


Fig. 1 Multistage technology for obtaining valuable phytochemicals

process may be optimized, separately or in combination, with other processes or treatments.

The main steps in this technology include the following methods:

1. Screening of plant metabolic profile with the aim to find out plants, specifically rich on phenolic compounds
2. Establishing of high-producing and fast-growing in vitro lines and comparison of their productivity to that in intact plants
3. Expression of synthetic pathways of phenolics through the influenced of internal factors (phytohormones) and environmental cultivation conditions (supply with macro- and micronutrients as well as precursors, application of biological, physical, and chemical elicitors)
4. Recovery of phenolics from plant cells or tissues by combining of different methods for permeabilization of cell membranes (via high pressure, electric pulses, ultrasound) and consequently extraction of phenolics from exudates or fermented tissues with or without chemical solvents [73]
5. Analyzing of the properties of the end products in terms of their antioxidative potential and stability during storage [74]

4.1 Selection of Plants According to Individual Profile of Phenolic Compounds

To the strategies for enhancement of phenolic content in plant in vitro cultures belongs screening and selection of plant species and cultivars, rich in phenolics. Plants with high contents of the phenolics should be used for induction of in vitro cultures to obtain high-producing cell lines.

Genetic potential is one of the most important factors influencing the biochemical status of plants and plant cultures. Environmental and physiological factors may modify the expression of genes, participating in phytochemical synthesis, but the genetic background is the major determinate. In order to establish plant in vitro cultures, rich in phenolics, it is necessary to screen phenolic profile of plants containing high concentration of metabolites. Screening over 120 agricultural and

medical plants has indicated [56] that the most promising cell culture for phenolic acid production was *Silybum marianum* with total phenolic acid concentration of 255.8 μmol/g DW, followed by *Artemisia frigida* (173.9 μmol/g DW) (Fig. 2).

Analyzing of individual phenolic acids has shown that *Vitis vinifera* plants as well as cell cultures are especially rich in cinnamic, *Artemisia vulgaris* in isochlorogenic, and *Echinacea angustifolia* in chlorogenic acid [56].

Also, the differences in origin of the same botanical specie can lead to differences in metabolic profile. Hunaefi et al. [75] have identified the profile of individual phenolic acids in in vitro sprouts of red cabbage of different cultivars. All cultivars possessed high amount of phenolic acids as gallic, chlorogenic, caffeic, syringic, and *p*-coumaric acid. The profiles of phenolic acids were similar for all cultivars; however they differ in content. This difference in content of the phenolic acids has been also associated with related antioxidant activities. Moreover, in comparison to in vivo red cabbage sprouts with total phenolic acids 4.27 mg/g DW, the concentration of phenolic acids in in vitro sprouts was considerably higher (Table 4).

In many cases the established cell cultures are characterized by higher concentration of metabolites, synthesized in plants or by de novo synthesis of phytochemicals as Mewis et al. [76] observed it for in vitro cell culture of *Vitis vinifera* L. cv. Gamay Fréaux. Two new compounds were identified in this cell culture: 3-*O*-glucosyl-resveratrol – a stilbene derivative, abundant in cell suspension culture – and a hydroxyphenol, 4-(3,5-dihydroxyphenyl)-phenol, abundant in callus culture, but not presented in related plant.

After the establishment of plant cell culture, rich in phenolics, it becomes important to select high-producing and fast-growing in vitro cell line [77]. Cell lines, selected for further cultivation, should be genetically stable and produce constant yield of metabolites [78].

Genetical modifications can be also an important tool to influence phenolic content in in vitro cultures. Manela Neta et al. [79] reported that transgenic *Vitis vinifera* cv. *Gamay Red* cell cultures containing bacterial enzyme of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (AroG*) had modified shikimate and aromatic amino acid pathways. The AroG* transformed lines with modified phenylalanine and tyrosine pathways accumulated up to 20- and 150-fold higher levels of resveratrol and dihydroquercetin, respectively. In contrast, increased

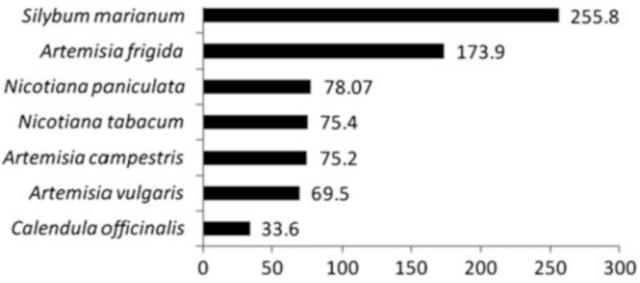
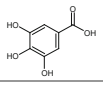
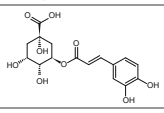
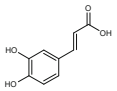
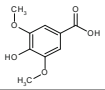
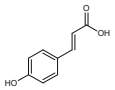
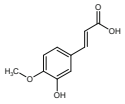


Fig. 2 Total phenolic acid content in studied plant cell cultures (μmol/g DW) [56]

Table 4 Concentration of predominant phenolic acids in in vitro sprouts of red cabbage of different cultivars, μmol/g DW [75]

Phenolic acids	Chemical structure	Cultivars of red cabbage				
		Marner Lagerrot	Kalibos	Frührot fruh	Schwarzkopf	Purple
Gallic		0.06	0.08	0.08	0.06	0.04
Chlorogenic		1.74	1.18	1.82	1.37	1.35
Caffeic		0.97	0.92	1.43	1.44	1.08
Syringic		1.14	0.81	0.72	0.58	0.86
p-Coumaric		1.03	0.85	1.12	0.93	1.21
Trans-3-hydroxy-4-methoxy-cinnamic acid		2.15	1.97	2.24	2.08	1.79
Total phenolic acid		7.85	6.05	8.05	7.38	6.88

phenylalanine production did not lead to elevated concentrations of anthocyanins, even though they are also phenylpropanoid metabolites.

The next aspect, important for the obtaining of substances from the in vitro culture, is to select whether **cell, hairy root, or organ in vitro culture** is the most suitable one.

According to Shetty [80], in vitro shoot cultures obtained from direct shoot organogenesis are generally preferred for mass production of the targeted compound as they are more genetically stable and have metabolite profile, very similar to intact plants. Hunaefi et al. [81] confirmed that phenolic profile in shoot culture of *Orthosiphon aristatus* was similar to in vivo plant but delivered higher amount of phenolic acids. Shevchenko et al. [82] reported that the biomass production of shoot culture of *Stevia rebaudiana Bertoni* exceeded the plant production by a factor of four. In this study shoot cultures have shown, in contrast to cell cultures of *Stevia rebaudiana*, permanent genetic stability, constant profile of phenolics and antioxidants, as well as high growth intensity.

Hairy root cultures are established by transformation of plants with *Agrobacterium rhizogenes*. Hairy roots are characterized by high growth rate, genetic stability, and growth in hormone-free media [83, 84]. Gabr et al. [85] observed rapid growth of transformed hairy roots of *Linum usitatissimum* in

comparison to non-transformed roots and intensive accumulation of phenolic acids (Table 5).

Hairy roots of *Fagopyrum esculentum* Moench accumulated phenolic compounds [86]. Artemisinin has been shown to be accumulated in hairy roots of *Artemisia annua*, although this compound can be accumulated only in the aerial part of plant [87].

Lignan secoisolariciresinol diglucoside (SDG) was found to be twofold higher in hairy roots than in non-transformed callus culture of *Linum usitatissimum* (Fig. 3) [85].

The reason for the enhanced production of lignans in transformed hairy root cultures compared with non-transformed cultures of the plant might be due to the alteration of hormonal metabolism and transport which generally happens during transformation with rol genes [88].

RolA, rolB, and rolC genes are essential for the induction of the hairy root phenotype. Despite most of the research performed over the years, the biochemical and molecular functions of these genes in modifying plant's development remain poorly understood [88].

Table 5 Phenolic acids content in flax roots, callus culture, and hairy root culture, μmol/g DM [85]

Phenolic acid, μmol/g DM	Root	Callus culture	Hairy root culture
4-Hydroxybenzoic	3.21	5.05	8.09
Vanillic	1.10	1.14	1.88
Chlorogenic	0.93	1.55	2.39
Sinapic	4.16	2.21	8.86
Ferulic acid	0.36	0.39	0.62
Total	11.44	11.84	22.92

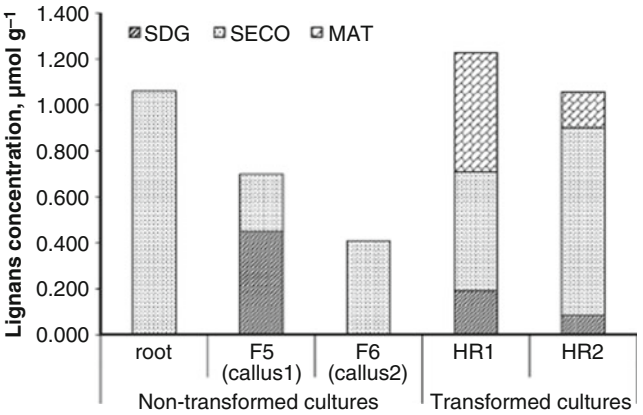


Fig. 3 Lignan SDG (secoisolariciresinol diglucoside) and SECO (secoisolariciresinol) accumulation in transformed and non-transformed cultures of *Linum usitatissimum* [85], where F5, B5 + 1.0 mg/L 2,4-D + 0.5 mg/L GA3; F6, B5 + 1.0 mg/L 2,4-D + 1.0 mg/L GA3; HR 1, hairy root culture 1 from F5 callus; and HR 2, hairy root culture 2 from F6 callus culture

Bulgakov et al. [89] reported that rolC is the most interesting gene for biotechnological studies because it is capable of stimulating both the growth of transformed cells and the biosynthesis of secondary metabolites. In transformed plant cell cultures, rolC gene induces the production of high levels of anthraquinone phytoalexins [90], chlorogenic acid, caffeoylquinic acids, and dicaffeoylquinic acids [91]. The rolB expression positively correlated with increased expression of a key gene for anthraquinone biosynthesis, leading to high levels of anthraquinone production [90]. Furthermore, rolB stimulated the biosynthesis of resveratrol in cell cultures of *Vitis amurensis* for more than 100-fold [34]. Gabr et al. [92] observed a high production of phenolic compounds in transgenic hairy root cultures of buckwheat roots, leaves, and stems and confirmed the presence of rolB gene in the transgenic cultures.

4.2 Targeting Metabolism

A number of chemical and physical factors strongly affect the production of phenolic compounds. The expression of metabolite pathways can be altered by external factors such as environmental conditions (e.g., light, nutrients) and special treatments (e.g., precursors, elicitors).

4.2.1 Nutrient Media

Although almost all intact higher plants are able to grow autotrophically in light under atmospheric air conditions and sufficient supply of water and mineral nutrients, the requirements of plant in vitro cultures differ from that for intact plants [93]. For example, roots of intact plants require the import of phytohormones and sugars from shoot tissue, but undifferentiated cells in in vitro cultures become necessary for growth components from nutrient medium [94].

Nutrient medium for in vitro cultures includes organic components as source of carbon, inorganic components as nitrogen and phosphate, and phytohormones. Changing of medium components (concentration, proportion, and form) is a very powerful way of enhancing growth of plant in vitro cultures and synthesis of metabolites as phenolics. Ananga et al. [93] (2013) reported that transferring of anthocyanin producing cell suspension of *Vitis vinifera* from the original B5 medium into LS medium completely changed the color pattern from red to yellow and the cell growth pattern from small colored aggregates to single colorless cells.

Carbohydrates, especially sucrose, are important carbon and energy sources for most plant in vitro lines. It has been demonstrated that 10 g/L sucrose in MS medium led to synthesis of 18.8 mg/L flavonoids in cell suspension culture of *Glycyrrhiza inflate*, while 50 g/L sucrose increased flavonoid content to 72.3 g/L [48].

Nitrogen. Plant tissue culture media such as MS, LS, or B5 have both nitrate (NO_3^-) and ammonium (NH_4^+) as sources of nitrogen. For example, nitrogen source is very important for plant suspension cultures of *Vitis vinifera* for anthocyanin formation [95]. Yang et al. [48] reported that cell suspension culture of *Glycyrrhiza*

inflata produced 36.4 mg/L flavonoids under 10 mmol/L of nitrogen in MS medium, while under 120 mmol/L of nitrogen, flavonoid content reached 73.1 mg/L.

The ratio of NH_4^+/NO_3^- and overall levels of total nitrogen have been shown to markedly affect the production of flavonoids and phenolic acids in cell culture of *Coleus blumei* [96]. Reduction of nitrate ions in the cultivation medium to a critical level and increasing of ammonium enhanced the anthocyanin production in cell suspension of *Vitis* Bailey Alicante [97].

Phosphate. It has been reported that phosphate levels induced the production of phenolics in *Catharanthus roseus* [11].

Carbon source. Plant cell cultures are usually grown heterotrophically using simple sugars as carbon source and inorganic supply of other nutrients. Sucrose or glucose at 2 to 4% is suitable carbon sources, which are added to the basal medium. Fructose, maltose, and other sugars also support the growth of various plant cells. The choice of the most suitable carbon source and its optimal concentration depends on the plant species and products [49]. The concentration of the carbon source affects cell growth and yield of secondary metabolites in many cases. Sucrose concentrations of 2.5% and 7.5% in *Coleus blumei* media brought about rosmarinic acid yields of 0.8 and 3.3 g/L, respectively [97].

The osmotic stress created by sucrose alone and with other osmotic agents was found to regulate anthocyanin production in *Vitis vinifera* [98]. However, higher concentrations of sucrose at 5% reduced the anthocyanin production in cell suspension cultures of *Aralia cordata*, where 3% favored the anthocyanin accumulation [59].

Addition of sucrose or mannitol in the medium enhances the osmotic pressure and found the level of anthocyanins accumulated in *Vitis vinifera* culture was increased to 1.5 times and reached 55 μg/cell [96].

4.2.2 Phytohormones

Phytohormones or growth regulators are required to induce callus tissues and to promote the growth of many cell lines. Since each plant species requires different kinds and levels of phytohormones for callus induction, its growth and metabolites production, it is important to select the most appropriate growth regulators and to determine their optimal concentrations [99]. Auxins and cytokinins have shown the most remarkable effects on growth and productivity of plant metabolites. The type and concentration of auxin or cytokinin or the auxin/cytokinin ratio alters dramatically both the growth and the product formation in cultured plant cells.

Auxins are generally used in plant cell culture at a concentration range between 0.1 and 50 μM. An increase of auxin levels in the medium stimulates dedifferentiation of the cells, cell division, callus formation, and growth. That is the reason why auxins are commonly added to the medium for callus induction, but they are added at a low concentration for production of metabolites. As an auxin, 2,4-D or NAA is frequently used. The growth regulator 2,4-D has been shown to inhibit the production of secondary metabolites in a large number of cases. The elimination of 2,4-D or its replacement by NAA or IAA enhanced the production of anthocyanins in suspension of *Daucus carota* and anthraquinones in *Morinda citrifolia*

[100, 101]. However, 2,4-D stimulated anthocyanin production in *Oxalis linearis* [102]. 2,4-D had been used to induce rosmarinic acid accumulation in *Salvia chamelaeagnea* [8]. Gan et al. [103] found that supplementation of suspension culture *Agastache rugosa* Kuntze with plant growth regulators, 2,4-D 2 mg/L and 0.1 mg/L 6-benzylaminopurine (BAP), was the best combination for producing rosmarinic acid.

Cytokinins are used in plant cell culture at a concentration range of 0.1 to 10 μM. They promote cell division and modulate callus initiation and growth. Cytokinins have different effects depending on the type of metabolite and species concerned. Kinetin stimulated the production of anthocyanin in *Haplopappus gracilis* but inhibited the formation of anthocyanins in *Populus* cell cultures [53].

Gibberellins are represented by over 90 forms, but gibberellic acid is the most commonly used for plant cell cultures. Dicosmo and Misawa [77] reported that the growth of *Taxus cuspidata* callus was significantly promoted by the addition of gibberellic acid into the solid medium. However, gibberellic acid suppresses production of anthocyanins in a number of cultures [59].

Gabr et al. [46] investigated the effect of different growth regulators on callus formation and lignans accumulation in callus culture of *Linum usitatissimum* (Table 6).

The maximum proliferation of callus (1.31 g FW) was recorded on the medium containing 2.0 mg 2,4-D/L. However, the callus grown on 1.0 mg 2,4-D/L in combination with two concentrations of GA_3 (0.5 and 1.0 mg/L) showed significantly higher growth (1.46 and 1.39 g FW) than the callus grown on 2,4-D in combination with different concentrations of BA. The callus grown on media supplemented with 1.0 mg 2,4-D/L and 0.5 or 1.0 mg GA_3/L was light-white and

Table 6 The effect of different growth regulators on callus formation and lignan accumulation in callus culture of *Linum usitatissimum* after 30 days of cultivation [46]

Callus treatment	FW, g	Morphology	Secoisolariciresinol diglucoside, mkmol/g	Secoisolariciresinol, mkmol/g
B5 + 1.0 mg/L 2,4-D	0.97	Yellowish, friable	Nd	0.66
B5 + 2.0 mg/L 2,4-D	1.31	Yellowish, friable	Nd	0.67
B5 + 1.0 mg/L 2,4-D + 0.1 mg/L BA	0.76	Dark yellowish, compact	Nd	0.16
B5 + 1.0 mg/L 2,4-D + 0.2 mg/L BA	0.65	Dark yellowish, compact	Nd	0.24
B5 + 1.0 mg/L 2,4-D + 0.5 mg/L GA3	1.46	Light-white, very friable	0.45	0.25
B5 + 1.0 mg/L 2,4-D + 1.0 mg/L GA3	1.39	Light-white, very friable	Nd	0.41

more friable. However, the callus grown on 2,4-D-fortified media was yellowish and less friable compared with other treatments. The use of BA at different concentrations (0.1 or 0.2 mg/L) with 1.0 mg 2,4-D/L produced dark yellowish and compact callus.

Lignan secoisolariciresinol diglucoside (SDG) was found to be twofold higher in hairy roots than in non-transformed callus culture. The accumulation of SECO was higher in the presence of 2,4-D with values of 0.66 and 0.67 mkmol/g, respectively. However, lignin secoisolariciresinol (SECO) was detected only in the callus obtained on 1.0 mg 2,4-D/L and 0.5 mg GA_3/L.

Szopa and Ekiert [104] reported that plant growth regulators, cytokinin (BA) and auxin (NAA), stimulated the production of lignans (schisandrol A and B) in in vitro cultures of *Schisandra chinensis*.

4.2.3 Precursor Feeding

The production of the desired metabolites is often limited by the lack of particular precursors; biotransformation using an exogenous supply of biosynthetic precursors may improve the synthesis of desired compounds. Biotransformation is a process through which the functional groups of compounds are modified by cell cultures to chemically different products [53]. In biotransformation, plant cells transform compounds introduced into the cultures, natural or artificial, through a variety of reactions, e.g., hydrogenation, dehydrogenation, isomerization, glycosylation, and hydroxylation.

The concept of feeding with **precursors** is based upon the idea that supply with compounds, which are intermediate or at the beginning of biosynthetic route, gives a good chance of increasing the yield of the final product. Precursor feeding is an obvious and popular approach to increase production of phenolic compounds in plant in vitro cultures.

Feeding ferulic acid to cultures of *Vanilla planifolia* resulted in an increase in vanillin accumulation [105]. Addition of phenylalanine as one of the biosynthetic precursors of rosmarinic acid to *Salvia officinalis* suspension cultures stimulated the production of rosmarinic acid [40]. Phenylalanine can also be used as the precursor of anthocyanins [106].

4.2.4 Elicitors

Secondary metabolites represent the adaptations of plants to environmental stress, or they may serve as defensive, protective, or offensive chemicals against microorganisms, insects, and higher herbivorous predators. When infected by pathogenic microorganism, plants respond with rapid activation of various spatially and temporally regulated defense reactions. These responses include oxidative cross-linking of cell wall proteins, production of phytoalexins, hydrolytic enzymes, and incrustation of cell wall proteins with phenolics and finally hypersensitive death of plant cell.

Plant in vitro cultures show physiological and morphological responses to microbial, physical, or chemical factors, known as elicitors. Elicitor may be defined as a substance which, when introduced in small concentrations to a living cell system, initiates or improves the biosynthesis of specific compounds. Elicitation can be

considered as one of the most efficient strategies that lead to the highest enhancement in many secondary metabolites production among the strategies for the improvement of the biosynthesis in plant cell cultures [70].

Since phenolic compounds protect plants from the environmental changes, the way to induce their synthesis is to apply unfavorable factors, i.e., simulate pathogen attack, herbivores, heavy metals, etc. The type and structure of elicitors varies greatly. Depending on their origin, they are classified as biotic or abiotic.

Abiotic elicitors are the substances of non-biological origin. The causes of the abiotic stress can be of chemical of physical nature; among them are:

- Chemicals like inorganic salts, heavy metals, and some chemicals that disturb membrane integrity
- Physical factors like mechanical wounding; UV light; high salinity; high or low osmolarity; extreme temperature; inorganic ions such as Ca^{2+}, Mn^{2+}, and Zn^{2+}; freezing; and thawing [107]

Plants adapt themselves for growing under suboptimal conditions, and one of the strategies to survive is to increase the concentration of secondary metabolites as phenolics in tissues.

It has been found that garden cress plants protect themselves against drought and salinity stress by increased synthesis of total phenolics, flavonoids, and flavanols [108].

Table 7 illustrates influence of abiotic elicitors on synthesis of phenolic compounds in plant in vitro cultures.

Ultraviolet (UV) irradiation is necessary for in vivo plants but can be also used as abiotic stress factor. Lavola [111] explained this phenomena antioxidant protective

Table 7 Abiotic elicitors and production of phenolic compounds in plant in vitro cultures

Abiotic elicitor	Product	In vitro culture	References
Drought (polyethylene glycol, mannitol)	Phenolics, flavonoids, and flavonols	*Lepidium sativum*	[109]
Salt (NaCl)			
High hydrostatic pressure	Anthraquinones	*Morinda citrifolia*	[62]
Metal ions: Cu^{2+}, Cd^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+}, Va^{2+}	Isoflavonoids	*Vigna angularis*	[2]
	Resveratrol	*Vitis vinifera*	[36]
	Phenolic acid	*Fagopyrum esculentum*	[86]
Ultraviolet	Total phenolics, flavonoids, flavonols	*Orthosiphon aristatus*	[110]
Ultrasound	Anthraquinones	*Morinda citrifolia*	[62]
	Total phenolics, flavonoids, flavonols	*Orthosiphon aristatus*	[110]

function of plants against ultraviolet, which causes accumulation of phenolic compounds in plant tissues.

UV-A light significantly stimulate the expression of structural genes, encoding the entry enzymes of the shikimate pathway, whereas only the UV-B and UV-C irradiation triggers the production of anthocyanins in *V. vinifera* L. *cv.* Cabernet Sauvignon [95].

Ultraviolet in combination with ultrasonic acts as abiotic elicitor of plant defense systems [112], and the treatment induces phenolic production as part of the defense response to the stresses applied to *Panax ginseng* cell culture. The combined treatment with ultraviolet and ultrasonic resulted in the highest level of flavonoids and flavanols in in vitro sprout culture of *Orthosiphon aristatus*, 8.02 and 7.12 mg QE/g DW, as compared to 6.09 and 4.86 mg QE/DW in control cultures at day 14 postexposure [75].

It has been shown that drought and salt stress factors are physical elicitors that influence accumulation of phenolics, flavonoids, and flavanols in in vitro cultured sprouts of garden cress (*Lepidium sativum)* [109]. Drought stress has been induced by supplementation of culture medium with polyethylene glycol (PEG) and mannitol, while NaCl was used in various concentrations as salt stress. Extraction yield was clearly influenced by different concentrations of PEG, mannitol, and NaCl added to culture medium using methanolic extract. The highest extraction yield from the cultured in vitro sprouts (48.4%) was observed with 40 g/L PEG. The amount of total phenolic varied in the different treatments, ranging from 1.66 mg/g DW with 8% mannitol to 2.63 mg/g DW weigh with 20 g/L PEG. *Lepidium sativum* sprouts showed best results of flavonoid contents of methanolic extract at level of 60 mM NaCl followed by addition of mannitol at levels 6 and 8%.

Biotic stress can be caused by bacterial, viral, or fungal attack as well as by **biotic elicitors**. They include:

- Enzymes, cell wall fragments of microorganisms, polysaccharides derived from microorganisms (chitin or glucans), and glycoproteins
- Phytochemicals produced by plants in response to physical damage, fungi or bacteria attack, polysaccharides derived from plant cell walls (pectin or cellulose), and fragments of pectin, formed by action of microorganisms on plant cell wall
- Chitosan, glucans, salicylic acid, and methyl jasmonate (formed by the action of plant on microbial cell walls) [113]

Some researches on the influence of biotic elicitors on phenolic substances in plant in vitro cultures are summarized in Table 8.

Ethephon is a plant phytohormone, which can be metabolized into ethylene, a gaseous ripening agent, controlling senescence. The biosynthesis of ethylene in plants is induced by the plants' stage of development, phytohormonal activity, and environmental changes, e.g., injury or red light [120]. Cai et al. [121] reported that cell suspension cultures of *Vitis vinifera* produced high level of phenolic acids with ethephon treatments than untreated culture. The same result was reported in *V. vinifera*, a common grapevine, in which ethephon increased anthocyanin content over twofold [114].

Table 8 Biotic elicitors and production of phenolic substances

Biotic elicitors	Substances	Cell cultures	References
Ethephon	Total phenolics	*Cynara scolymus* L.	[85]
	Anthocyanins	*Vitis vinifera*	[114]
Chitosan	Anthraquinones	*Rubia tinctorum*	[60]
	Anthraquinones	*Morinda citrifolia*	[62]
	Silymarin	*Silybum marianum*	[85]
Fungal elicitor	Anthraquinones	*Morinda citrifolia*	[115]
	Rosmarinic acid	*Coleus blumei*	[57]
Sodium acetate	Resveratrol	*Arachis hypogaea*	[35]
Jasmonic acid	Anthocyanins	*Vitis vinifera*	[32]
	Resveratrol	*Vitis vinifera*	[65]
Methyl jasmonate	Isoflavonoid	*Glycine max*	[116]
	Rosmarinic acid	*Coleus blumei*	[57]
Salicylic acid	Daidzein	*Psoralea corylifolia*	[65]
	Phenolic acids	*Salvia miltiorrhiza*	[117]
Yeast extract	Anthraquinones	*Morinda citrifolia*	[115]
	Rosmarinic acid	*Coleus blumei*	[40]
	Flavonoids	*Glycyrrhiza uralensis*	[118]
	Isoflavonoid	*Pueraria candollei*	[119]

The total phenol content in the callus culture of *Cynara scolymus* L. treated with 45 and 90 µl ethephon was significantly higher than in the untreated callus cultures [85]. However, treatment with 180 µl/l ethephon decreased the total phenol content as compared with the untreated cultures. The highest value of total phenol was reached with callus cultures treated with 90 µl/l ethephon (15.2 mg/g DW) on day 12. The lowest total phenol content was observed on day 21 with all ethephon concentrations (3.2, 4.4, and 1.6 mg/g DW), respectively.

Increasing of isoflavonoid biosynthesis by five to six times was observed by methyl jasmonate addition. Isoflavonoid production in the 7-day-old culture increased with pulsed electric field application, and aglycone forms were influenced to a greater extent [116].

Microbial infections of intact plants often elicit the synthesis of specific secondary metabolites. Most of the strategies employing fungal elicitors utilize undefined mixtures such as autoclaved fungal homogenate or fungal culture filtrates. With the consideration of several parameters such as elicitor specificity and concentration, duration of contact, and quality of cell wall materials, substantial enhancement of total phenolics, particularly isoflavonoids, has been reported by Hunaefi et al. [110] for in vitro sprout culture of *Orthosiphon aristatus*.

Balasa [122] explained that the main physiological effect of a number of different external stressors is destabilization of the cell membrane, which can be achieved by the application of low intensity pulsed electric field treatments.

The enhancement of production of secondary metabolites after elicitation is compared with that of the non-treated cultures is represented in Table 9.

Table 9 Comparison of production of secondary metabolite after elicitation

In vitro cultures	Elicitors	Substances	Product concentration		Yield	References
			Control	Elicitation		
Arachis hypogaea	Sodium acetate	Resveratrol	0.42	12.621	Mmol/g DW	[35]
Echinacea purpurea	Light	Caffeic acid derivatives	35.0	56.0	Mg/g DW	[123]
Glycyrrhiza uralensis	Yeast extract	Flavonoids	28.38	45.40	Mg/g DW	[118]
Glycine max	Pulsed electric fields	Isoflavonoid	0.80	1.52	Mg/g DW	[116]
Cynara scolymus	Ethephon	Total phenolics	3.86	15.19	Mg/g DW	[85]
Lepidium sativum	Salt	Flavonoids	0.35	0.60	Mg/g DW	[109]
Morinda citrifolia	Chitin	Anthraquinones	3.0	7.0	μg/g FW	[115]
Morinda citrifolia	Ultrasound	Anthraquinone	16.74	41.85	Mg/g DW	[124]
Orthosiphon aristatus	Ultraviolet and ultrasound	Flavonoids	6.09	8.02	Mg/g DW	[75]
		Flavonols	4.86	7.12		
Orthosiphon aristatus	Yeast extract	Total phenolics	151.2	324.5	Mg/g DW	[81]
Pueraria candollei	Yeast extract	Isoflavonoid	60.0	270.0	Mg/g DW	[119]
Rubia tinctorum	Chitosan	Anthraquinone	58.0	128.9	μmol/g FW	[60]
Vitis vinifera	High hydrostatic pressure	Anthocyanins	0.95	3.4	μmol/l	[106]
	Ethephon	Anthocyanins	0.89	1.99	Mg/g DW	[114]
	Jasmonic acid	Anthocyanins	9.2	20.7	Mg/g DW	[32]
		Resveratrol	1.6	6.4	Mg/l	[125]

Ogata et al. [126] reported that yeast extracts 5 g/L as an elicitor induced an increase of rosmarinic acid content in *Lithospermum erythrorhizon* cell suspension cultures of fourfold compared to the group of control. Similarly, Kim et al. [61] reported that the stimulation of rosmarinic acid biosynthesis in *Agastache rugosa O. Kuntze* in response to the addition of yeast extract could elevate the rosmarinic acid content up to 5.7-fold compared to non-elicited suspension cells. Sevenfold increase in rosmarinic acid accumulation in cell suspension culture system of Orthosiphon aristatus in comparison to the same plant plant had been reported by

Sumaryono et al. [127]. Hunaefi et al. [81] reported that among several treatments with stress substances, elicitation with yeast extract resulted in the highest amount of total phenolic compounds, particularly in 324.5 mg/g DW, in shoot cultures of *Orthosiphon aristatus* as compared to 151.2 mg/g DW for the non-treated cultures. The content of rosmarinic acid in cultured cells of *Lithospermum erythrorhizon* increased after addition of yeast extract: a maximum was reached in 24 h [40]. When the plant cells were treated with yeast extract on the sixth day of the cultivation, the level of rosmarinic acid increased 2.5 times.

However, the use of microbial elicitors may not be economical since an elicitor-producing microorganism should be cultivated separately from cultivation of plant cells. The fermentation cost for an elicitor-producing microorganism is not always inexpensive. Elicitors do not function equally in all species. They are most effective at optimum concentrations and at right stage of culture.

5 Technologies for Recovery of Phenolic Compounds

5.1 Pretreatments

Cultivated plant in vitro cultures accumulate phenolic compounds mostly intracellularly in vacuoles; therefore, efforts have been made to develop procedures for induced release of such products into extract. In order to release metabolites from vacuoles, two membrane barriers, plasma membrane and tonoplast, should be penetrated. Cell permeabilization depends on the pore formation in one or more of the membrane systems, enabling the passage of molecules into and out of plant cell [128]. A wide variety of methods and agents have been used to increase membrane permeability, including chemical treatments, e.g., solutions of high ionic strength, external pH change, dimethyl sulfoxide (DMSO), TWEEN 20 (polyoxyethylene sorbitan monolaurate), and chitosan addition, and physical treatments, e.g., pulsed electric fields, ultrasound, and high hydrostatic pressure [129].

Attempts have been made to permeabilize the plant cells transiently, to maintain the cell viability, and to have short-time periods of increased mass transfer of substrate and metabolites to and from the cell [122]. Permeabilization of plant membranes for the release of secondary metabolites is often connected with the loss of viability of the plant cells treated with permeabilizing agents and methods [130].

5.1.1 Physical Methods

Physical treatments such as pulsed electric fields (PEF), high hydrostatic pressure (HHP), and ultrasound (US) can cause reversible or irreversible membrane permeabilization and thus increase transfer of phenolics from cells of in vitro cultures into extracting solution or into cultivation media.

Pulsed Electric Fields

Application of PEF is based on the principle of development of membrane pores under external electric fields. PEF enhances release of intracellular molecules from permeabilized tissue as well as improves uptake of low molecular substances into the cells [131]. Depending on electric field strength or pulse number, the pore formation can be reversible or irreversible, depending on electric field strength and pulse number [132].

PEF induces a defense response in plant cells, and it may also alter the dielectric properties of cells and/or cell membranes and can serve as a viable elicitor of secondary metabolites in plant cell cultures. The use of PEF with very low intensity as an external stress source induces a stress reaction of the cells with a possible stimulation of secondary metabolite production. Knorr et al. [55] concluded that application of electric field pulses or high pressure has only limited potential for cell permeabilization with concurrent retention of cell viability.

Gueven and Knorr [116] reported that application of PEF between 1.6 and 2.0 kV/cm (5 Hz, 36 s) induced reversible membrane permeability and increased isoflavonoid concentrations in *Glycine max* cell culture, but further increase in voltage resulted in decrease in isoflavonoid concentration.

Cai et al. [133] showed that application of PEF increased the phenolic acid accumulation in *V. vinifera* suspension culture medium; the total extracellular phenolic acids were 11% higher than that of the control. PEF elicited defense response and stimulated the accumulation of anthocyanins, resveratrol, and phenolic acids in plant cell cultures and exudates of *Vitis vinifera*.

Saw et al. [114] found that after the treatment of cell suspension culture of *Vitis vinifera* at day 14 with PEF, the production of anthocyanins increased 1.7-fold (1.42 mg/g DW) when compared to the control cells, while, treatment with ethephon resulted in 2.3-fold increase (1.99 mg/g DW). When cells were treated with both ethephon and PEF, 2.5-fold increase in anthocyanin content (2.2 mg/g DW) was measured.

High Hydrostatic Pressure

HHP is an elicitor-like physical stress factor, which can cause membrane permeabilization. HHP was reported to inactivate enzymes in plant tissue [134], permeabilize membrane [55], and induce stress responses in the plant cells [131]. It is assumed that the pressure-dependent destruction of the tonoplast, the loss of compartmentation, and subsequent release of the content of the vacuoles may cause the pH change in the medium and the resultant cell death. Although the application of HPP has only limited potential for cell permeabilization with concurrent retention of cell viability, the treatment can be used as a tool for product recovery from plant cells and tissues with minimum effects on cell viability [135].

HHP of 50 MPa increased the production of anthraquinones in *Morinda citrifolia* cell culture, but 250 MPa caused loss of cell viability [115]. HHP of 50 MPa resulted in a 10–30% increase in isoflavonoid biosynthesis in *Glycine max* cell culture, but 75,100 MPa did not cause further increase of metabolites [116]. It was also reported that HHP of 40 MPa increased the phenolic acid production up to ninefold in exudates from *Vitis vinifera* cell culture [106].

HHP causes the loss of cell viability, most likely because of permeabilization of tonoplast. It has been assumed that the pressure-dependent destruction of the tonoplast, the loss of compartmentation, and subsequent release of the content of the vacuoles may have caused the pH change in the medium and the resultant cell death. HHP at high levels may also cause denaturation of enzymes which are necessary for metabolite biosynthesis and influence membrane integrity causing irreversible permeability. Although cell permeabilization was coupled with concurrent loss of cell viability, recent researches have demonstrated that application of both PEF and HHP could become useful tools for product recovery from plant cells and tissues with minimum effect on cell viability and product composition [136].

Ultrasound

Another efficient permeabilization method is ultrasound (US). Studies have shown that exposure of plant cells to low-energy US enhances the biosynthesis of phenolic substances, particularly anthraquinones from *Morinda citrifolia* [124]. Exposure to US can also enhance exudation of metabolites into medium.

The enhanced secondary metabolite biosynthesis of plant cells by US has been proved to be a physiological activity of the cells stimulated by US rather than the mass transfer effects proposed for other biological systems [137]. The unique effect of US on secondary metabolite production in plant cell cultures is of both fundamental and applied significance.

5.1.2 Chemical Methods

The chemical permeabilization includes the change of nutritious status, addition of chemical agents, etc. Permeabilizing agents are those chemical compounds which do not inhibit cell growth and at the same time have the ability to reversibly increase the pore size of the cell wall [115]. The normal pore size of the cell should be restored upon removal of the ideal permeabilizing agent.

Permeabilization of plant membranes for the release of secondary metabolites is often connected with the loss of viability of the plant cells, but there are exceptions, for example, DMSO and Triton X-100 applied to *Catharanthus roseus* cells for improving extraction of rosmarinic acid [11].

TWEEN 80 increased licochalcone A ninefold as compared to non-treated hairy root culture of *Glycyrrhiza uralensis* and total flavonoid elevenfold, respectively. Moreover, TWEEN 80 caused the secretion of 98% of licochalcone A and 94% of total flavonoids from the cells into the culture medium, without loss of cell viability [64].

5.2 Extraction

5.2.1 Exudation

An important advantage of plant cells is the ease of product isolation and purification, especially when the product is secreted into the medium. The chemicals secreted by plant hairy roots, suspension cells, organ cultures, and callus into the surrounding growth medium are broadly referred to as exudates [3, 138].

Plant in vitro cultured cells actively secrete a variety of metabolites into the growth medium. Thus, the medium also functions as an external storage compartment to some degree. The growth medium composition of organic acids, amino acids, and enzymes is very similar to that of vacuoles [139].

Exudates are relatively simple mixtures, in comparison to solvent extracts of plant tissue, which makes the isolation of chemicals an easier task. Exudation can be operated continuously without destroying the plant cells in vitro, thus producing a higher total yield of the phytochemicals over the lifetime of the culture.

Ye et al. [140] reported that up to 25% of phenolics in *Taxus chinensis* cell culture were released into the liquid medium. Zamboni et al. [141] reported that resveratrol is much more localized in the medium than within the cell of suspension cultures. The secretion of resveratrol in growth medium of *Vitis vinifera* cell suspension cultures could be related to active transport mechanisms involving ABC transporters or H^+-gradient-dependent mechanisms [142]. It has been suggested that the localization of the stilbene synthase enzyme close to the cell wall in grape berries of *Vitis* sp. is linked to an excretion mechanism of resveratrol [143]. In *Vitis vinifera cv. Barbera* cell suspension cultures, the release of trans-resveratrol into the culture medium was about 60% of its content in suspended cells in 100 ml cultivation vessels [144] until 95% in 1 L bioreactor [145]. Also results obtained by Donnez et al. [142] reported that cell suspension culture of *Vitis vinifera cv. Chasselas* cultivated in 2 l stirred bioreactor secreted 90% of the total resveratrol into the liquid medium.

There are also numerous studies on phenolic compounds in hairy root exudates. Zhang et al. [64] found that up to 98% of total flavonoids were secreted into the culture medium by *Glycyrrhiza uralensis* hairy root culture. In *Arachis hypogaea* hairy root culture, over 90% of the total resveratrol was accumulated in the medium [66]. Abbott et al. [35] confirmed that majority of resveratrol produced in hairy root cultures of peanut (*Arachis hypogaea*) can be released and recovered from the growth medium.

Therefore, one of the most fruitful areas of research for the production of phenolic compounds may be the study of methods to induce product leakage from cells. Enhancing transfer of compounds from the cell into the culture medium through the additional application of chemical or environmental agents can be very useful in terms of costs for product recovery.

5.2.2 Fermentation

Fermentation has long been used to preserve and improve shelf life and nutritional quality of the plant food. However, scarce attention is given toward its impact on plant secondary metabolites [146]. Fermentation may induce structural breakdown of plant cells through the decreasing of pH, increasing of turgor, and tissue softening. These conditions lead to the decomposition, resynthesis, synthesis, or leakage of various compounds.

The impurities presented in extracts of metabolites from in vitro cultures are mostly carbohydrate compounds. Several different techniques can be used to purify metabolites, an example is membrane filtration. However, this method is limited by

operational problems and small differences in the molecular weights of the compounds to be separated. Another commonly used method is a column filled with an adequate resin; the Amberlite XAD-16 is used to retain anthocyanins. Anyway, the drawback of the column technique is at times the high content of carbohydrates. Application of fermentation can reduce carbohydrates and simplify processes for purification of phenolic compounds [147].

The fermentation process significantly increased the total phenol content of *Codonopsis lanceolata* when compared to the conventional extraction without fermentation. The total phenol content of *Codonopsis lanceolata* was the highest for high-pressure assisted extraction from *Lactobacillus rhamnosus* fermented (8.45 mg GAE/g), followed by *Bifidobacterium longum* fermented samples (8.25 mg GAE/g), non-fermented (7.38 mg GAE/g), and conventional extraction without fermentation (6.69 mg GAE/g) [135].

After the fermentation of *Glycine max* with *Trichoderma harzianum* NBRI-1055, the total phenolic content increased significantly, from 61.16 mgGAE/gDW in water extracts of non-fermented tissue until 181.32 mgGAE/gDW, but in fermented tissue and in methanol extract from 67.14 to 124.61 mgGAE/gDW, respectively [148].

Ng et al. [149] found that fermentation of *Anoectochilus formosanus* Hayata inoculated with 5×10^6 CFU/ml *L. acidophilus* BCRC 17002 elevates the level of total phenolic compounds more than twofold, from 6.07 mg/g DW to 14.05 g/g DW, after 35 h of fermentation. Another example of 30% increasing total phenolic compounds of *Graptopetalum paraguayense* E. Walther by *L. plantarum* BCRC10357 fermentation has been reported [148]. These increasing facts are based on the observation that fermentation may induce structural breakdown of plant cell walls through the decreasing of pH that create condition for bound phenolic constituents to be released through enzymatic processes [149]. During plant fermentation, enzymes such as amylases, xylanases, and proteases derived from the plant and fermented microbes contribute to the modification of plant composition [150]. The bound phenolics are very important in estimation of total phenolic compounds. Since they can be released by alkali, acid, and enzymatic treatments of samples prior to extraction, this condition, thus, can be used to explain fermentation-induced increase in the total phenolic content.

Hunaefi et al. [146] reported that during fermentation, the content of total phenolic compounds and anthocyanins in tissue of in vitro culture has been reduced, but the concentration of phenolic acids, total flavonoids, and flavonols in extracts increased. Fermentation of *Orthosiphon aristatus* shoot culture with lactic acid bacteria, acetic acid bacteria, and yeast increased the yield of phenolic compounds in extracts [75].

5.2.3 Extraction with Solvents

The yield of valuable secondary metabolites after extraction of them from the plant cells or tissues depends also from the solvent, particularly on the polarity and concentration of the solvents used.

Lee et al. [151] investigated the influence of different solvents on extraction. They found out that methanol extract showed the highest yield of phenolic compounds and

the strongest antioxidant activity. Methanol was the most efficient solvent for extracting phenolic compounds in comparison with ethanol and water which has highest yield and total phenolic recovery. They further explained that the highest yield of phenolics in methanol extracts is due to methanol ability to inhibit the polyphenol oxidase that causes the oxidation of phenolics and its ease of evaporation compared to ethanol and water.

Hunaefi et al. [110] have shown the dependence of the recovery of total phenolics, flavonoids, flavonols, and rosmarinic acid from the in vitro shoot culture of *Orthosiphon aristatus* after extraction with water, 70% methanol, and 80% ethanol (Fig. 4).

In this experiment shoot cultures were treated with phytohormone (kinetin), precursors (proline and L-Glu-*L*-glutamine), and elicitors (jasmonic acid and yeast extract). The strongest antioxidant activities and highest antioxidant components were obtained in methanol extracts for all samples either control or supplemented. In association with antioxidant component results, yeast extract elicitation resulted in the highest percentage of antioxidant activity (32.2 for 70% methanol extract).

6 Product Properties: Antioxidant Potential

Antioxidant potential of the product is an important characteristic for estimation of stability during storage as well as content of health-relevant free radical reducing substances [152].

There are several definitions of antioxidants. According to Moreno et al. [11], they are compounds that inhibit or delay the oxidation of substrates even if they are present in significantly lower concentration as the oxidized substrate. They can be also defined as substances that delay, prevent, or remove oxidative damage to target molecules [153] or substances that directly scavenge reactive oxygen species (ROS) or indirectly act to upregulate antioxidant defenses or inhibit ROS production [154]. Another characteristic that compounds should have to be considered as antioxidants are the ability, after scavenging the radical, to form new radicals that are stable through intramolecular hydrogen bonding on further oxidation [6].

Basically, in the biological system, antioxidants are divided into two major groups, enzymatic antioxidants and nonenzymatic antioxidants [155] (Fig. 5), while in relation to their mechanisms, they are divided into two categories, primary and secondary antioxidants.

Primary antioxidants are free radical scavengers that inhibit oxidation through chain terminating reactions. They normally have reactive OH or NH groups that the inhibition occurs by transferring of proton to the free radical species. Meanwhile, secondary antioxidants, often referred to as hydroperoxide decomposers, decompose hyroperoxides into non-radical stable products (Fig. 6).

As increasing demand in finding naturally occurring antioxidants to replace synthetic antioxidants in foods and medicine, many studies have suggested to use the natural antioxidants from plants and plant in vitro cultures [156]. Many plant-

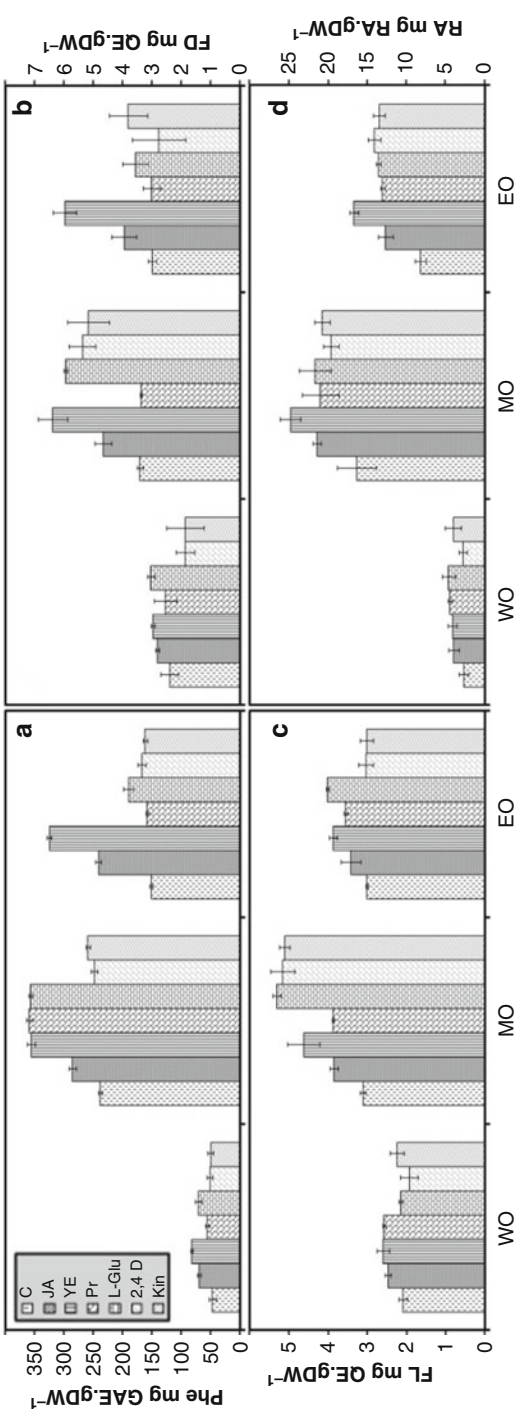


Fig. 4 Influence of different solvents (*WO* water, *MO* methanol, and *EO* ethanol) on the yield of (**a**) total phenolic compounds, (**b**) flavonols, (**c**) flavonols, and (**d**) rosmarinic acid from in vitro shoot culture of *Orthosiphon aristatus* (*C* control, *JA* jasmonic acid, *YE* yeast extract, *Pr* proline, *L-Glu L*-glutamine, *Kin* kinetin)

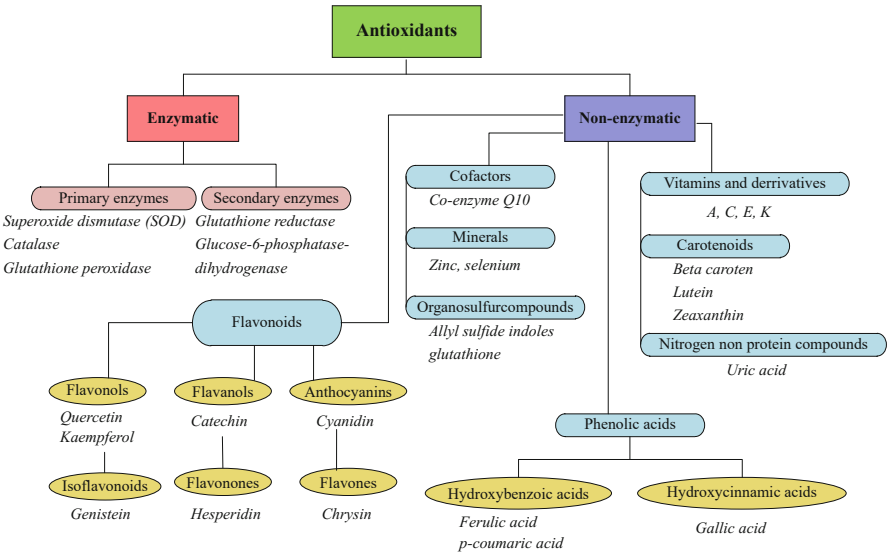


Fig. 5 Classification of antioxidants (Adapted from Refs. [146, 154, 155])

derived secondary metabolite antioxidants have constituted one of the major groups of compounds acting as primary antioxidants [157].

For instance, the protective effect of phenolic compounds as antioxidants by direct trapping of the radicals, thus the phenolic compounds are oxidized by the radicals (ROO•) leading to less reactive species. The formed phenolic compound radical (O•) is stabilized by resonance. The non-paired electron can be delocalized on the whole of the aromatic cycle. But it can continue to evolve according to several processes (dimerization, dismutation, recombination with other radicals, oxidation in quinone) either while reacting radicals and other antioxidants or with biomolecules (semi-quinone). The semi-quinone can react with another radical to form stable quinine [158] (Fig. 7).

In terms of analytical technique to measure activities of plant secondary metabolites as antioxidants, there are several methods to measure the effectiveness of plant-derived antioxidants, which are generally divided into two groups, in vivo and in vitro method [159]. However, this chapter will just give a glance of several in vitro models to determine antioxidant activities.

Two of the most used in vitro methods are (1,1-diphenyl-2- picrylhydrazyl) DPPH scavenging activity and Trolox equivalent antioxidant capacity (TEAC) assays.

DPPH free radical-scavenging activity is based on the ability of the presence of antioxidant compounds to decolorize DPPH reagent. DPPH is considered to be a model of a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The presence of antioxidants in the mixed solution

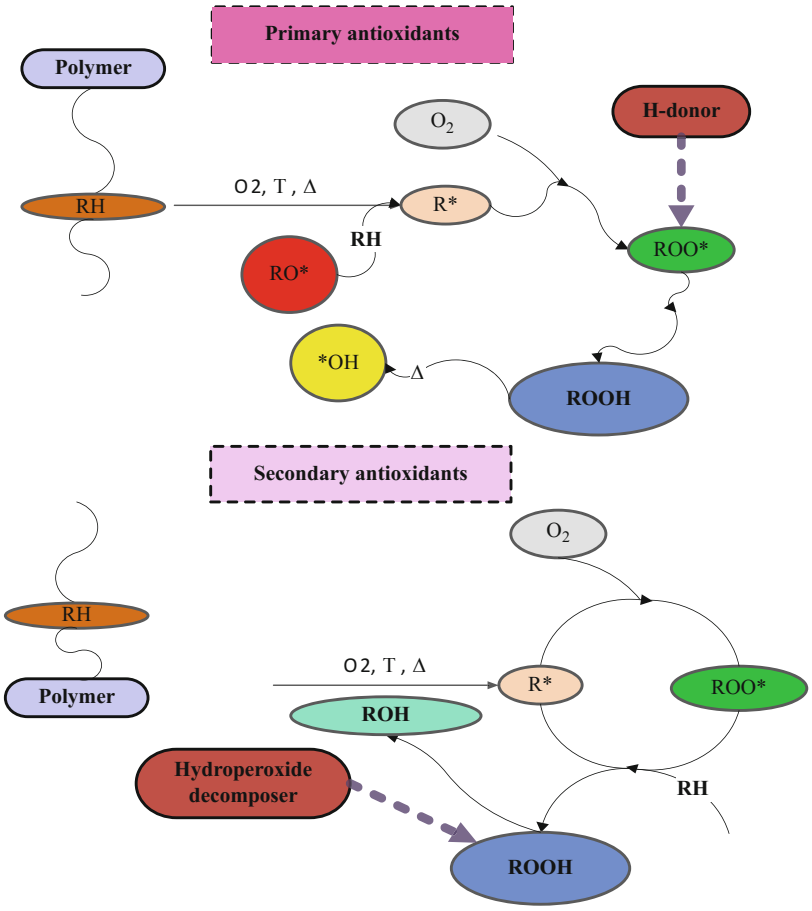


Fig. 6 Illustration mechanism of primary and secondary antioxidants

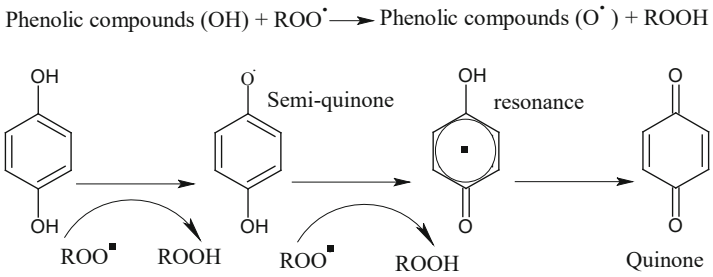


Fig. 7 Free radical-scavenging mechanism of plant phenolic compounds (Adapted from Ref. [158])

reacts with DPPH and reduces the number of DPPH free radicals to the number of their available hydroxyl groups indicated by discoloration of the solution.

TEAC method is based on the extent of decoloration as the indicator of inhibition of 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) **(ABTS)** by the availability of antioxidant compounds. Moreover, it is calculated relative to the reactivity of Trolox as standard under the same conditions [158]; whereas, the determination of **superoxide dismutase (SOD)-**like activity permits the assessment of superoxide anion scavenging activity when a xanthine and xanthine oxidase system is used to originate superoxide radicals [159].

To complete those antioxidant activity assessments, it was considered important to use a food or biologically relevant oxidize-able substrates such as ***β*-carotene/ linoleic acid lipid–water** emulsion known as *β*-carotene/linoleic acid bleaching antioxidant activity. In this assay, oxidation of linoleic acid produces hydroperoxide-derived free radicals that attack the chromophore of β-carotene, resulting in bleaching of the reaction emulsion. Furthermore, the availability of antioxidant compounds is able to retard or to inhibit the oxidation of β-carotene and work as primary antioxidants [160]. Thus, by in vitro method, it can generally draw a rule that the measurement of antioxidant activities of plant secondary metabolites was based on how many chains may be terminated by those compounds to estimate the effectiveness and the capacity of the compounds as antioxidants.

One method of antioxidant activity analysis is not sufficient for the complete assessment of plant extract antioxidant potential due to the fact that antioxidant component in the plant extract may react differently to different antioxidant activity assay [161]. As stressed by Huang et al. [162], no single method is adequate for evaluating the antioxidant capacity, since different methods can yield widely diverging results. Several methods based on different mechanisms must be used. Most common assays for estimation of antioxidative potential are DPPH radical-scavenging activity and ABTS radical-scavenging activity.

For example, Wang et al. [163] found that some compounds, which have ABTS radical-scavenging activity, may not show 1,1-diphenyl-2- picrylhydrazyl (DPPH) radical-scavenging activity. Considering the limitation on using one antioxidant activity assay, thereby, determination of antioxidant activity can be out using three different methods: DPPH free radical-scavenging, Trolox equivalent antioxidant capacity (TEAC), and superoxide dismutase (**SOD**)-like antioxidant activity methods.

6.1 DPPH Radical-Scavenging Activity

Free radicals involved in the process of lipid peroxidation are considered to play a major role in numerous chronic pathologies such as cancer and cardiovascular diseases [164]. DPPH is a stable radical, which could be easily used for the detection of antioxidant properties of different compounds in terms of hydrogen donating ability. The advantages of the method are expressed mainly in its rapidity and

selectivity. Mohdaly et al. [165] pointed that antioxidant activity of plant extracts is primarily carried out by phenolic compounds.

The scavenging activity of hairy root cultures of *Fagopyrum esculentum* Moench against DPPH• is represented in Fig. 8 [166]. Significant differences between samples were observed, but the results clearly indicate that all lines of hairy root of *Fagopyrum esculentum* exhibited higher antioxidant activity as corresponding explant sources (roots, leaves, and stems of intact plants).

6.2 ABTS Radical-Scavenging Activity

Although the DPPH• free radical is ubiquitously used to estimate the potential free radical-scavenging activity, the ABTS• + free radical is an alternative method, commonly used when solubility arise, and the use of DPPH•-based assays becomes inappropriate. Phenolic extracts from hairy roots *Fagopyrum esculentum* demonstrated a range of ABTS• + −scavenging activities from 83.7 to 98% (Fig. 8, [166]). In this research work, the scavenging of the ABTS+ radical by the hairy root cultures of *Fagopyrum esculentum* was found to be much higher than that of DPPH radical.

Factors like stereoselectivity of the radicals or the solubility of the extract in different testing systems have been reported to affect the capacity of extracts to react and quench different radicals [167]. Therefore, some compounds which have ABTS + scavenging activity did not show DPPH scavenging activity.

Gabr et al. [85] found that $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ free radical-scavenging assay had the same trend as total phenol concentration in callus cultures of *Cynara scolymus* treated with ethephon and was significantly higher (93.36 and 98.18%, respectively) than in the untreated callus cultures. Also, the comparison of hairy roots of *Linum usitatissimum* in comparison to non-transformed culture possessed significantly higher antioxidant capacities according to $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ free radical-scavenging assays.

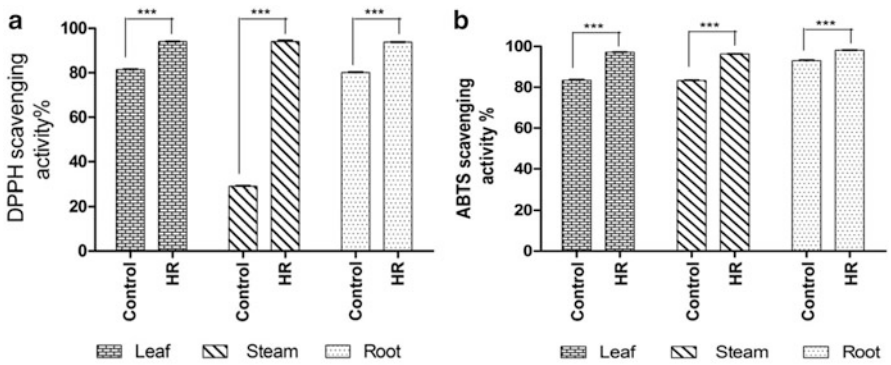


Fig. 8 DPPH• (**a**) and ABTS• (**b**) radical-scavenging activity of methanolic extracts of transgenic root and wild type cultures of *Fagopyrum esculentum* [166]

6.3 POD Activity

Peroxidase (POD, donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7) catalysis the oxidation reactions using either peroxides or oxygen as hydrogen acceptor. POD activity in plants increases in response to various biotic and abiotic stresses [74]. Presumably plant cultures are considered to be grown under stress conditions, particularly oxidative stress, which suggests that plant cell culture is an ideal system for the production of antioxidant enzymes including POD.

To better understand the influence of the used elicitors on plant defense and cell culture secondary metabolism, antioxidant activity and POD were investigated by Cai et al. (2013). It has been found that heavy metal elicitors, except Ag at high concentrations, increase POD activity in cell cultures of *Vitis vinifera* (Fig. 9).

Cd at concentration of 50 μM had a remarkable elevation effect of the POD activity, 24 h after the treatments, which was 1.6-fold of that of the control level. DPPH radical-scavenging activity remained unaffected by heavy metal elicitors, except of Ag at concentration of 50 μM, which significantly reduced the antioxidant activity 24 h after the treatment.

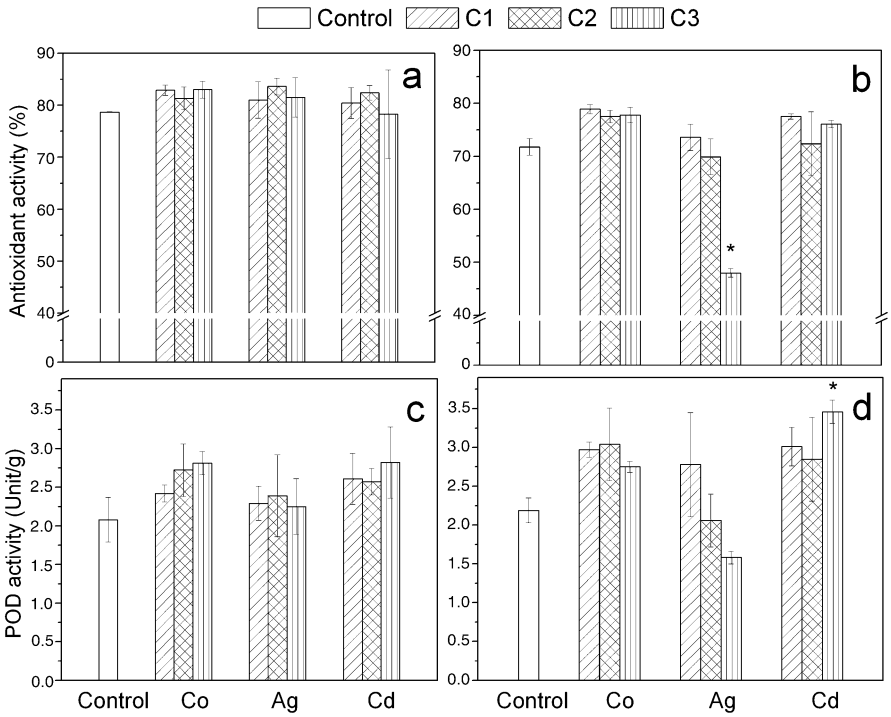


Fig. 9 Effects of heavy metals on antioxidant activity in *Vitis vinifera* cell culture at concentrations C1, C2, and C3 as shown in Table 1 and cultures harvested 4 h (**a**) and 24 h (**b**) after the treatments; on POD activity harvested 4 h (**c**) and 24 h (**d**) after the treatments [36]

Also, Mohdaly et al. [160] explained that solvents with different polarities, used for the extraction of phytochemicals, have significant influence on antioxidant activity (Fig. 10). The extracts obtained by the use of higher polarity solvents were more effective radical scavengers than those obtained using less polar solvents. Thus, phenolics extracted with methanol and ethanol tended to possess much higher antioxidant activity in comparison to these extracted with BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), and TBHQ (tert-butyl hydroquinone).

Hunaefi et al. [75] have also observed that the amount of antioxidant compounds extracted largely depends on the polarity of the solvents. Among many of the available solvent varieties, 70% methanol has been reported to have best extractability (as compared to water, 30–60% methanol, and 30–80% ethanol) for antioxidant compounds, especially, phenolics from the sprout cultures of *Orthosiphon aristatus*.

Hunaefi et al. [168] have compared the efficiency of three solvents by the extraction of antioxidants from in vitro sprouts of red cabbage (Fig. 10). The results have shown that 70% methanol extracts of phenolic acids had the highest percentage of DPPH antioxidant activity. In terms of different cultivars of red cabbage, significant differences in antioxidant activity of in vitro sprouts were observed. When utilized water and 80% ethanol for extraction, sprouts of cultivar *Marner Lagerrot* had higher antioxidant activity than other cultivars, but after application of 70% ethanol, *Frührot fruh* sprouts exhibited the highest percentage of antioxidant activity (45.63%).

By using TEAC method, similar trend to DPPH method was observed. Application of 70% methanol as solvent for extraction of antioxidants from in vitro sprouts *Frühkohl* led to the highest antioxidant activity of 1.27 mM/g DW Trolox equivalent. It was considered that antioxidant activities of plant extract are closely linked to their secondary metabolites, in particular phenolics.

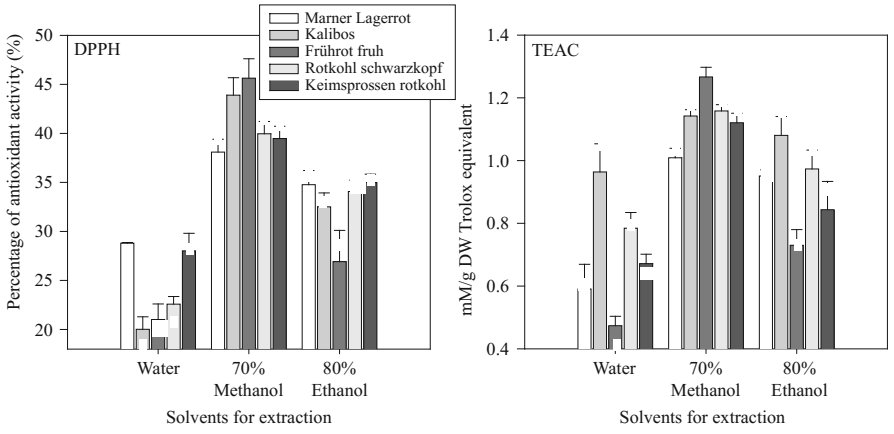


Fig. 10 Influence of different solvents for extraction on antioxidant activities of in vitro sprouts of red cabbage [168]

7 Conclusions and Outlook

In recent years, the market for plant products has expanded rapidly, and this trend will continue because more and more people prefer to use natural products as colorant, taste, and flavor substances. However, because of the seasonal fluctuations, weather conditions, soil properties, nutrient supply, and processing techniques, it is not always possible to get plant material in necessary quantity and quality.

Plant in vitro cultures offer promise for high production and collection of valuable secondary metabolites as phenolics. However, nowadays, because of the marginally developed cultivating technologies and economic feasibility, only few in vitro cultures are commercially used as a source of anthocyanins, phenolic acids, and polyphenolics. To improve this situation, it is important to develop multistage technology that would allow obtaining yields and quality of particular valuable phenolic compounds.

First of all, the selection of productive in vitro cell, hairy root or organ culture, and fast-growing lines can result in accumulation of phenolics.

Knowledge of biosynthetic pathways of phenolic compounds in in vitro cultures opens new possibilities to regulate their production through selection of cultivation media, phytohormone composition, feeding with precursors, and application of elicitors.

Because of the complexity of biochemical and physiological processes in in vitro cultures, case-by-case studies can solve the problems occurring in the production of anthocyanins, isoflavonoids, phenolic acids, and polyphenolics. Production of phytochemicals by in vitro cultures must be economically competitive with other conventional strategies for production as field cultivation of plants, chemical synthesis, or using of genetically modified microorganisms.

The combined efforts of experts in the fields of plant science, food biotechnology, engineering, biochemistry, molecular biology, food technology, and pharmacology can exploit the potential of plant in vitro systems for sustainable production of valuable phenolic compounds.

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9 Production of Iridoid and Phenylethanoid Glycosides by In Vitro Systems of Plants from the Buddlejaceae, Orobanchaceae, and Scrophulariaceae Families

Ewelina Piątczak, Renata Grąbkowska, and Halina Wysokińska

Contents

Abstract

The plants belonging to Buddlejaceae, Orobanchaceae, and Scrophulariaceae families are rich sources of iridoid and phenylethanoid glycosides, which are widely used as anti-inflammatory, hypoglycemic, and nourishing agents. Recent years have seen the application of various in vitro culture systems as alternative source of these metabolites. We discuss the use of callus, cell suspension cultures, shoot cultures, and the whole regenerated plants as possible approaches for production of the compounds. Additionally, methods of efficiently improving metabolite accumulation in in vitro cultures through elicitation, precursor feeding, and both *Agrobacterium rhizogenes*- *and A. tumefaciens*-mediated genetic

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transformations (hairy roots, transformed plants) among the plant families are also presented.

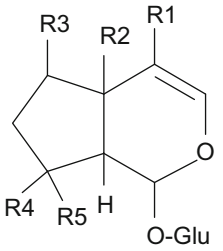
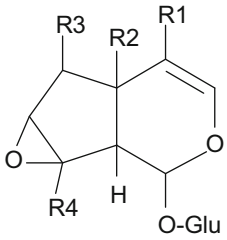
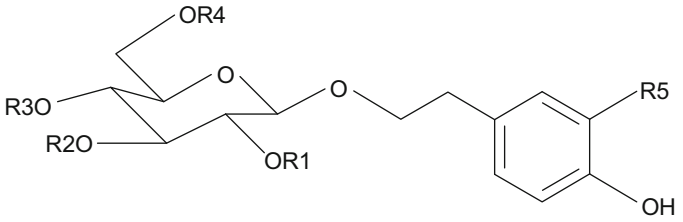
Keywords
Buddlejaceae · Bioreactor · Callus · Cell suspension culture · Elicitation · Hairy roots · Iridoid glycosides · Orobanchaceae · Phenylethanoid glycosides (PeGs) · Scrophulariaceae

1 Introduction

Plant secondary metabolites are bioactive compounds of low molecular weight which play many roles in plant interactions with the environment. Since ancient times, human beings have exploited many plants as sources of secondary metabolites for therapeutic purposes. Plants belonging to the Buddlejaceae, Orobanchaceae, and Scrophulariaceae are widely known sources of iridoid and phenylethanoid glycosides (PeGs).

Iridoid glycosides are monoterpenes based on the cyclopentano [c] pyranoid skeleton ring represented by iridane (*cis*-2-oxabicyclo [4.3.0] nonane) [1]. The term is also used for a number of other structural types which can be considered to arise from iridane. The iridoid chemical structure was identified in the mid – 1950s on iridomyrmecin and iridodial isolated from Australian ants, *Iridomyrmex* spp. [2]. According to data reported by Ghisalberti [2], by 1998 over 600 iridoid glycosides have been isolated from plants belonging to the superorders Cornanae, Ericanae, Gentiananae, Lamianae, and Loasanae. Among the iridoids, four main groups can be distinguished: (1) non-glycosidic iridoids, (2) iridoid glycosides, (3) iridoid acetal esters, and (4) secoiridoid glycosides [3]. In each group, some modifications (epoxidation, hydroxylation, esterification of the hydroxyl groups with aromatic acids) of the basic iridoid skeleton are possible. Although the biosynthetic pathways of iridoids have been investigated, a number of stages still remain unclear [3, 4]. Plants synthesize iridoids from 9-hydroxy nerol by phosphorylation followed by several steps including cyclization, oxidation, and glycosidation [5]. While iridoids were not initially considered pharmacologically important compounds, they are now known to be present in many medicinal plants used as sedatives, cough medicines, remedies for wounds and skin disorders, hypertensive, and bitter tonics in folk medicine [2]. The chemical structure of iridoids found in plants from the Buddlejaceae, Orobanchaceae, and Scrophulariaceae, i.e., catalpol, aucubin, catalposide, harpagide, and harpagoside, is presented in Table 1. All possess interesting biological activity. Aucubin shows hepatoprotective properties after α-amanitin poisoning and hinders replication of the DNA of the hepatitis B virus [6]. The compound could also be considered a promising anticancer drug [7, 8]. Catalpol stimulates angiogenesis in the brain [9] and was found to stimulate weakened cell apoptosis in the brain after an ischemic stroke in mice [10]. Moreover, the compound also exhibits diuretic, laxative, hypoglycemic, antihyperglycemic, and immunomodulatory properties [11, 12]. An ester of catalpol

Table 1 Chemical structure of main iridoid and phenylethanoid glycosides (PeGs) in plants of Buddlejaceae, Orobanchaceae, and Scrophulariaceae families

Iridoids	 Compounds: 1-3			 Compounds: 4-5	
Compound name	R1	R2	R3	R4	R5
1. Aucubin	–	H	OH	CH_2OH	–
2. Harpagide	–	OH	OH	OH	CH_3
3. Harpagoside	–	OH	OH	Cin	CH_3
4. Catalpol	–	H	OH	CH_2OH	–
5. Catalposide	$COOCH_3$	H	Hyd	CH_2OH	–
Phenylethanoid glycosides	 Compounds: 6-10				
Compound name	R1	R2	R3	R4	R5
6. Acteoside	H	Rha	Caf	H	OH
7. Isoacteoside	H	Rha	H	Caf	OH
8. Cistanoside A	H	Rha	Caf	Glc	OCH_3
9. Echinacoside	H	Rha	Caf	Glc	OH
10. Salidroside	H	H	H	H	H

Caf, *trans*-caffeoyl; Cin, *trans*-cinnamoyl; Glc, *β-D*-glucopyranosyl; Hyd, *p*-hydroxybenzoyl; Rha, *α-L*-rhamnopyranosyl

with p-hydroxybenzoic acid is catalposide which possesses significant anti-nociceptive and anti-inflammatory activities [13]. It was also found that catalposide inhibits the synthesis of nitrogen oxide [14]; the production of proinflammatory agents such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6); as well as the activation of the nuclear factor κB (NF-κB) in cell lines of mouse macrophages RAW 264.7 activated by means of lipopolysaccharide [15].

Strong anti-inflammatory properties are also demonstrated by two other iridoid glycosides: harpagide and harpagoside. The compounds inhibit both arachidonic acid metabolic pathways and the activity of three enzymes: cyclooxygenase-2

(COX), lipoxygenase (LOX), and nitric oxide synthase (NOS) induced by means of lipopolysaccharide by inhibiting the activation of one type of NF-κB [16, 17]. Moreover, harpagoside exhibits antiviral properties against vesicular stomatitis virus [18]; antiprotozoal properties against *Leishmania donovani* [19]; cytotoxic activity on the A431, HeLa, and MCF7 cell lines [8]; and neuroprotective activity by inhibiting the release of lactate dehydrogenase (LDH) [20].

Another main class of metabolites found in plants from Buddlejaceae, Orobanchaceae, and Scrophulariaceae families are phenylethanoid glycosides (PeGs). The metabolites are water-soluble, naturally occurring compounds, characterized by dihydroxyphenyl ethyl (C6–C2) moietes (aglycone) attached to a β-D-glucopyranose (such as apiose, galactose, rhamnose, or xylose) via a glycosidic bond [21, 22]. These compounds often comprise a number of substituents such as aromatic acids (e.g., cinnamic acid, coumaric acid, caffeic acid, ferulic acid, or isoferulic acid) [23, 24].

Caffeic acid is thought to be a critical compound for the PeG biosynthetic pathway. The pathway involves the metabolism of phenylalanine (Phe), which is converted into *trans* cynnamic acid by phenylalanine ammonia-lyase (PAL), followed by hydroxylation at the 4-position of the aromatic ring, forming 4-hydroxycinnamic acid or p-coumaric acid (Fig. 1). Another amino acid – tyrosine (Tyr) – can be also directly transformed into p-coumaric acid by tyrosine ammonia-lyase (TAL). Further hydroxylation at the 3-position of the aromatic ring yields caffeic acid, which is subject to subsequent glycosylation, resulting in the formation of PeGs [23, 25]. The metabolites are absorbable by human intestinal cells [26] and exhibit wide biological activities, including antifungal [27], anti-inflammatory [28], antiradical [29], cytotoxic [30], and gastroprotective [31] properties and have been found to also inhibit cholinesterases [32]. The main PeGs found in the Buddlejaceae, Orobanchaceae, and Scrophulariaceae are acteoside, isoacteoside, cistanoside A, echinacoside, and salidroside (Table 1). Acteoside, also known as verbascoside, orobanchin, or kutcoside, appears often with isoacteoside (isoverbascoside). The compounds have been found to possess antibacterial, hepatoprotective, and antioxidant properties [33–35]. They have also found to have anti-inflammatory activity, which is probably connected with the inhibition of COX-2 and its complement in human serum, as well as with the inhibition of proinflammatory chemokine release [36]. Xiong et al. [33] reported that acteoside significantly suppresses NADPH/CCl4-induced lipid peroxidation in rat liver microsomes. Moreover, isoacteoside inhibits cell proliferation of the human gastric cancer cell line MGC803 [37].

Echinacoside, which is, together with salidroside, the major bioactive PeGs in plants from *Cistanche* sp. (Orobanchaceae), exhibits a broad range of therapeutic applications as an antioxidative, anti-inflammatory, antivirus, cardioactive, and neuroprotective agent [38–41]. Echinacoside also exhibits bactericidal activity with respect to *Staphylococcus aureus* and *Streptococcus* sp. Echinacoside together with acteoside has been found to inhibit the increase in postprandial blood glucose levels and significantly improve glucose tolerance in starch-loaded mice [42]. Salidroside exhibits a neuroprotective effect against H_2O_2-induced cell apoptosis in primary cultured rat hippocampal neurons [43, 44]. It is a broad-spectrum

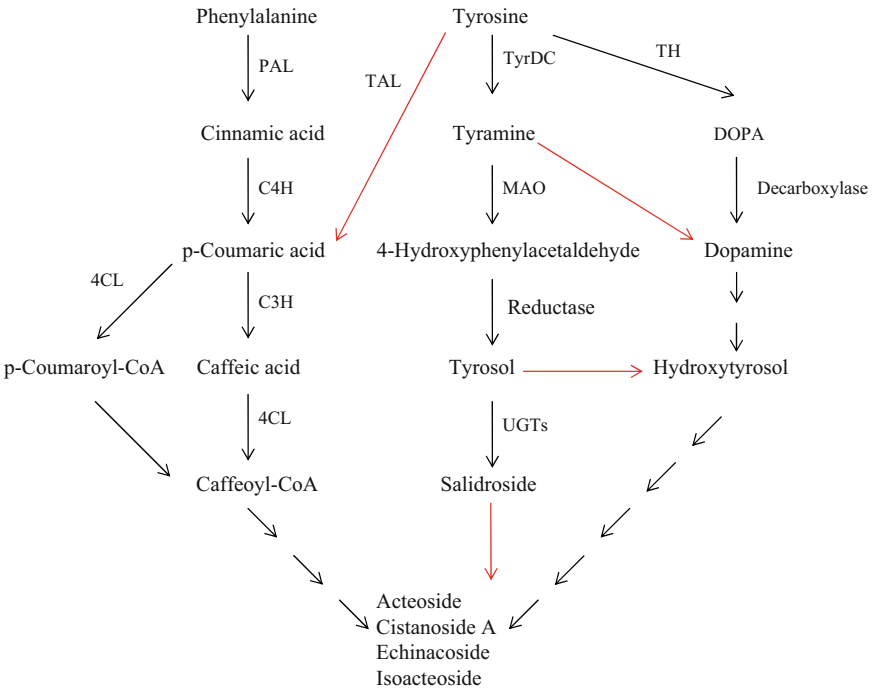


Fig. 1 Tentative pathway of phenylethanoid glycoside biosynthesis (Modified after Alipieva et al. [25]). Biosynthesis step: confirmed (→) or probable (→). *C3H* coumarate 3-hydroxylase, *C4H* cinnamate 4-hydroxylase, *4CL* 4-coumarate CoA ligase, *MAO* monoamine oxidase, *PAL* phenylalanine ammonia-lyase, *TAL* tyrosine ammonia-lyase, *TH* tyrosine hydroxylase, *TyrDC* tyrosine decarboxylase, *UGTs* uridine diphosphate glucuronosyltransferases

agent for preventing and/or treating neuronal damage in disorders of the central nervous system including Alzheimer's, Parkinson's, and Huntington's diseases [44–46]. Tang et al. [47] report that salidroside has protective effects against fibrotic lung injuries which could be correlated to anti-inflammatory, antioxidative, and antifibrotic properties of the compound. Zheng et al. [48] demonstrated that salidroside could attenuate ischemic heart disease. Besides these previously reported effects, it has recently been noted that pretreatment with salidroside can mitigate acute lung injuries in lipopolysaccharide- or paraquat-challenged animals [49, 50].

For the production of abovementioned metabolites, wild-grown or field-cultivated plant materials are usually used, but their levels are affected by the physiological and developmental stage of the plant as well as by changes in the environmental conditions. In vitro cell, tissue, and organ culture systems could become a viable source of biologically valuable compounds. The major advantages of in vitro culture systems over the conventional cultivation of whole plants are:

Metabolites can be produced under controlled conditions independent of climatic changes and soil conditions

In vitro cultures are free of microbes and insects
In vitro cultures may grow in bioreactor
Metabolites are easily extractable
Novel compounds which have not been found in parent plants could be produced

The following describes the production of iridoids and PeGs by various types of in vitro cultures (callus, cell suspension, organ cultures) using members of the Buddlejaceae, Orobanchaceae, and Scrophulariaceae families. The strategies used for improving metabolite accumulation in these cultures, such as elicitation, precursor feeding, as well as genetic transformation with *Agrobacterium rhizogenes*, are also described.

2 In Vitro Culture Types

The iridoids and PeGs accumulated in various types of plant cultures from Buddlejaceae, Orobanchaceae, and Scrophulariaceae families are summarized in Table 2.

2.1 Metabolite Production in Callus and Suspension Cell Cultures

Callus and suspension cultures could be effective sources of some secondary metabolites, including PeGs [51–56]. Callus culture of *Cistanche deserticola* (Orobanchaceae) – a desert Chinese medicinal, perennial, parasitic plant – was obtained by Song et al. [53] and Ouyang et al. [52]. Song et al. [53] cultured the callus tissue on B5 medium [57] supplemented with 0.5–2 mg L^{-1} 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA) in the concentration range 0.5–1.5 mg L^{-1}. They found that the content of acteoside in the callus tissue reached 4.4% of dry weight (DW). Callus culture was also obtained for another member of the Orobanchaceae (*Rehmannia glutinosa*) by Inagaki et al. [51]. The species is a Chinese medicinal plant with antisenescence, hypoglycemic, and anti-inflammatory activities. HPLC analysis showed that the production of acteoside in the undifferentiated callus of *R. glutinosa* var. *purpurea* cultured on Murashige and Skoog (MS) medium [58] containing 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 3% sucrose amounted to 1.1% DW. The compound was also accumulated in callus culture of *Scrophularia striata* (Scrophulariaceae), which is an endangered plant species native to Iran, used traditionally to treat ulcers [59], kidney disease [60], and eye and ear infection [61]. The callus produced 1.6 μg per g fresh weight of acteoside when it was cultured on MS medium supplemented with 0.5 mg L^{-1} of naphthalene acetic acid (NAA) and 2.0 mg L^{-1} BAP [55]. Very high acteoside level (86.3 mg g^{-1} DW) was found to be in the callus tissue of *Buddleja cordata* (Buddlejaceae) grown on MS medium containing 2,4-D (0.45 μM) and kinetin (KIN) at the concentration 2.32 μM [54]. *B. cordata* is a shrub or tree, whose leaves, bark, and roots are used in Mexican traditional medicine to treat skin abscesses,

Table 2 Distribution of iridoid and phenylethanoid glycosides (PeGs) in in vitro cultures and regenerated plants from Buddlejaceae, Orobanchaceae, and Scrophulariaceae families

In vitro culture	Plant species	Type of metabolite	References
Callus	*Buddleja cordata*	Acteoside	[54]
	Cistanche deserticola	Acteoside, PeGs	[53], [105], [106]
	Rehmannia glutinosa var. *purpurea*	Acteoside	[51]
	Scrophularia striata	Acteoside	[55]
Suspension culture	*Buddleja cordata*	Acteoside	[54]
	Cistanche deserticola	Acteoside, cistanoside A, echinacoside, PeGs, salidroside, tubuloside A	[52], [63], [89], [92–99], [103], [104], [110]
	Cistanche salsa	Acteoside, 2′-acetylacteoside, echinacoside,	[102]
	Scrophularia striata	Acteoside, echinacoside	[55], [109]
Shoot culture	*Castilleja tenuiflora*	Acteoside, isoacteoside, PeGs	[111]
	Rehmannia elata	Acteoside, harpagide, isoacteoside	[64]
	Rehmannia glutinosa	Acteoside, aucubin, catalpol, harpagide, harpagoside, isoacteoside	[56]
	Scrophularia takesimensis	Harpagoside	[107]
Regenerated plants	*Castilleja tenuiflora*	Aucubin	[68]
	Rehmannia elata	Acteoside, catalpol, harpagide, isoacteoside	[64]
	Rehmannia glutinosa	Acteoside, aucubin, catalpol, catalposide, harpagide, isoacteoside, loganin	[56]
	Scrophularia nodosa	Aucubin, catalpol, harpagoside	[66]
	Scrophularia takesimensis	Harpagoside	[67], [107]
	Scrophularia yoshimurae	Harpagoside	[65]
Untransformed root	*Rehmannia glutinosa*	Acteoside, aucubin, catalpol, catalposide, isoacteoside, loganin	[78]
Hairy roots	*Rehmannia glutinosa*	Acteoside, catalpol, catalposide, harpagide, isoacteoside, loganin	[78], [80], [100]
	Verbascum xanthophoeniceum	Acteoside	[75]
Transformed multiple shoots	*Rehmannia glutinosa*	Acteoside, aucubin, catalpol, isoacteoside, loganin	[82]
Transformed plants	*Rehmannia glutinosa*	Acteoside, aucubin, catalpol, catalposide, harpagide, harpagoside, isoacteoside, loganin	[82]

burns and wounds, gastrointestinal infections, liver disorders, nasal hemorrhages, muscle cramps, and rheumatism, and it is also known to possess antipyretic and diuretic properties [62].

Cell suspension cultures often represent as better system for biomass accumulation and metabolite production than calli because of their fast growth rate and shorter growth period (2–3 weeks compared with 5–6 weeks for callus). For example, in cell suspension culture of *B. cordata,* acteoside content (116.4 mg g^{-1}) after 3 weeks was 1.7 times greater than observed in callus [54]. Similar results were achieved by Khanpour-Ardestani et al. [55] for *Scrophularia striata* cell suspension cultured in liquid MS medium with 0.5 mg L^{-1} NAA and 2.0 mg L^{-1} BAP. The cells produced almost nine times as much acteoside than callus grown in the same medium composition (14.25 vs. 1.6 μg g^{-1} fresh weight). A high concentration of PeGs was found in the cell suspension culture of *C. deserticola* (Orobanchaceae) when it was grown in liquid B5 medium supplemented with 1 mg L^{-1} 2,4-D, 800 mg L^{-1} casein hydrolysate, and 10 g L^{-1} sucrose [52]. The cells grew fast and the biomass reached the highest level of 6.2 g L^{-1} DW on day 20. At the time, the total PeG content, assayed by UV-visible spectroscopy, was the highest (10.2%), and total PeG production, calculated in respect to biomass dry weight, was 0.63 g L^{-1} [52]. Li et al. [63] reported that the main factor affecting the growth of the cell suspension cultures of *C. deserticola* is sucrose concentration. When used at a concentration of 30 g L^{-1}, the sugar promoted cell growth and increased PeG content and their production (10.8 g L^{-1}, 16.3%, and 1296.0 mg L^{-1} DW, respectively) [63].

2.2 Metabolite Production in Organ Cultures and Regenerated Plants

The production of some metabolites demands the use of differentiated tissues such as shoots or roots. For the production of these metabolites, the most appropriate systems may be in vitro organ cultures. The use of shoot cultures as a source of iridoids and PeGs was studied in *R. glutinosa* [56] and *R. elata* [64]. Moreover, the formation of multiple shoots is one of the steps in micropropagation which allows the whole regenerated plants to be obtained and used as an alternative system for the production of pharmacological important metabolites in the particular organs (leaves, roots, flowers, fruit). In respect to the biosynthesis of iridoids and PeGs, whole in vitro-regenerated plants of *R. glutinosa* [56], *R. elata* [64], *Scrophularia yoshimurae* [65], *S. nodosa* [66], *S. takesimensis* [67], and *Castilleja tenuiflora* [68], which are limited in production under natural conditions, were investigated. A shoot culture of *R. glutinosa* from the Orobanchaceae family was established through indirect organogenesis from callus tissue [56]. The frequency of adventitious shoot induction and the number of shoots corresponding to one explant were assessed on MS medium supplemented with 0.2–3.0 mg L^{-1} BAP singly or in combination with auxin (0.1 mg L^{-1} IAA or NAA). The highest response was obtained from hypocotyls cultivated on MS medium enriched with 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} IAA after 6 weeks of growth. Under these conditions, 90% of explants developed

organogenic callus with an average of nine adventitious shoots. The callus-forming shoots accumulated both the: – catalpol (~3.6 mg g^{-1} DW), aucubin (~0.9 mg g^{-1} DW), harpagide (~0.5 mg g^{-1} DW), and harpagoside (0.03 mg g^{-1} DW) and the PeGs: acteoside (~12.8 mg g^{-1} DW) and isoacteoside (~3.3 mg g^{-1} DW). The contents of harpagide, acteoside, and isoacteoside in the culture were two to six times higher, and those of aucubin even 26 times higher, than found in shoots and roots of *R. glutinosa* plants obtained from seeds. Trace amounts of loganin and catalposide in the in vitro culture were also detected [56]. The organogenic callus could be maintained for over 4 years without losing morphogenetic potential and the ability for metabolite production. The obtained adventitious shoots of *R. glutinosa* were rooted in vitro, giving fully grown plants, which were acclimatized into ex vitro conditions in a greenhouse, and then transferred to a field [56]. The in vitro-regenerated plants were characterized by high levels of catalpol (45 mg g^{-1} DW) and harpagoside (0.1 mg g^{-1} DW), but only trace amounts of loganin and catalposide were produced. Among the PeGs, high content of acteoside (4.8 mg g^{-1} DW) was found in the leaves of the regenerated plants [56]. Another *Rehmannia* species which is able to produce iridoid glycosides and PeGs under in vitro conditions is *R. elata*. Axillary shoot culture of *R. elata* was obtained through the proliferation of apical parts of shoots on MS agar medium supplemented with 0.57 μM IAA and various concentrations (2–8 μM) of BAP, KIN, or 2-isopentenyladenine (2iP) [64]. The best shoot multiplication rate was found for 2iP at a concentration of 6 μM: nine axillary shoots and buds were formed on single explant after 4 weeks of cultivation. UHPLC examination was used to determine the influence of cytokinins (BAP, 2iP, KIN) on the accumulation of an iridoid glycoside, harpagide, and two PeGs, acteoside and isoacteoside, in shoot culture of *R. elata* [64]. The acteoside contents in the culture were as much as 9.7 times higher in BAP-treated shoots, 8.9 times higher in 2iP-treated shoots, and 6.4 times higher in kinetin-treated shoots compared to the control, i.e., *R. elata* shoots grown on PGR-free MS medium. Isoacteoside biosynthesis was less affected by these growth regulators. The presence of cytokinin in the medium was also found to stimulate the biosynthesis of harpagide. The best results in terms of harpagide were obtained in *R. elata* shoots from the combination of 0.57 μM IAA and 4 μM 2iP. Under these conditions, the harpagide content was twice than of the control (1.1 mg g^{-1} DW vs. 0.6 mg g^{-1} DW, respectively) [64]. After rooting, *R. elata* plants were acclimatized in flowerpots with soil for 4 weeks and then transferred to a field. This micropropagation procedure permits as many as 750 plants of *R. elata* to be obtained from one shoot tip explant after three cycles of multiplication and three cycles of rooting [64]. The 4-month-old in vitro-regenerated *R. elata* plants produced 12.2 times less harpagide (0.09 vs. 1.1 mg g^{-1} DW) and 2.7 times more of acteoside (34.6 vs. 12.8 mg g^{-1} DW) and isoacteoside (1.7 vs. 0.6 mg g^{-1} DW) than the shoot culture. Catalpol was found only in the leaves of seed-derived *R. elata* plants [64]. An efficient protocol for in vitro shoot regeneration from nodal explants of *Castilleja tenuiflora*, a Mexican hemiparasitic, medicinal plant [69] which exhibits anti-inflammatory, antioxidant, antiulcerogenic, and cytotoxic activities, was described by Martínez-Bonfil et al. [68]. For shoot multiplication, MS medium was used, either without auxins or supplemented with 0.5 μM of indole-3-butyric acid (IBA), together

with various concentrations of BAP, thidiazuron (TDZ), or kinetin (KIN) (0–20 μM). The highest proliferation rates (four axillary shoots per explant) were achieved when 5 μM KIN was added to MS medium without IBA. Proliferated shoots were rooted in vitro and ex vitro and intact plants were regenerated and acclimatized in soil. Colorimetric method showed that the highest levels of the iridoids (average 24.2–26.3 mg of aucubin equivalents per g dry weight) were found in leaves and stems, and the in vitro plants contained more iridoids than those derived in vivo [68].

Harpagoside production was examined using root and shoot cultures as well as different organs of in vitro-regenerated plantlets of *Scrophularia nodosa* [66]. The species is a perennial herb native to the forests of Central Europe, Central Asia, and North America used in the treatment of eczema, wounds, and eye complaints as well as a diuretic and anthelmintic agent [70]. The highest concentration of harpagoside (1.1% DW), similar to that found in leaves of field-grown plants, was determined in flowers of in vitro plantlets. The field-grown plants were also characterized by the highest concentration of aucubin (1.7% DW). While catalpol was produced in the all examined plant organs, only trace amounts were found [66]. In order to obtain a shoot culture of *Scrophularia yoshimurae*, different explants (shoot tips, leaf base, stem node, and stem internode) incubated on MS medium with 4.44 μM BAP and 1.07 μM NAA were studied [65]. The stem node explants showed the highest response for shoot proliferation (100%). The multiple shoots gave healthy plants after the rooting stage. The HPLC analysis demonstrated that the underground parts of the in vitro-regenerated *S. yoshimurae* plants produced 3.4 mg g^{-1} DW of harpagoside. This amount was 1.4–1.6 times lower than found in underground parts of wild plants of the species but it was significantly higher than in crude drug (roots of *Scrophularia ningpoensis*) [65] used in the traditional Chinese medicine to treat inflammation, laryngitis, and tonsillitis [70]. Harpagoside was also detected in different organs (fruit, leaf, root, stem) of micropropagated *S. takesimensis* plants derived from shoot tips by Sivanesan et al. [67]. After 6 months of growth in the greenhouse, concentration of the iridoid ranged from 1.1 to 2.7 mg g^{-1} DW depending on the plant organ. The highest harpagoside level was determined in fruits [67].

3 Approaches for Enhancing Iridoid and Phenylethanoid Glycosides (PeGs) Content in In Vitro Cultures

3.1 Genetic Modifications

Genetic *Agrobacterium tumefaciens*- and *A. rhizogenes*-mediated transformation represents promising approach for inducing new genes and obtaining transgenic plants or hairy root cultures. Among plants belonging to the Orobanchaceae, Scrophulariaceae, and Buddlejaceae, the experiments with *A. tumefaciens* to generate transgenic plants have been very limited. Tomilov et al. [71] have used three various strains of *A. tumefaciens* (A281, C58, GV3101) to transform *Triphysaria versicolor* (Orobanchaceae) seedlings, but they obtained only root-forming callus with GUS activity [71]. There are several reports using *A.*

rhizogenes for genetic transformation and transformed root production in the plant from Orobanchaceae and Scrophulariaceae. Transformed roots, also known as hairy roots, develop following the infection of plant fragments with the soil bacterium – *A. rhizogenes*. The Gram-negative strain has a natural ability to colonize plant tissues and incorporate a fragment of its plasmid Ri (T-DNA) to the plant genome [72]. The expression of *rol* genes (A, B, C, D) contained in this DNA fragment leads to the development of adventitious roots on the plant explant. Simultaneously, the expression of *rol* genes changes the plant metabolism, which may result in changes in the qualitative and quantitative profiles of synthesized compounds by the plant. The fast growth, easy maintenance, high genetic stability, and the ability to synthesize a range of metabolites of hairy root cultures are some of the advantages they offer over plant cell suspension cultures as a continuous source for the production of valuable secondary metabolites, especially those biosynthesized or accumulated in roots. According to Georgiev et al. [73], hairy root cultures of more than 450 different plant species had been induced in 2008. For obtaining hairy root lines, various techniques of T-DNA delivery from *Agrobacterium* plasmid to a plant cell have been applied. One of them is the technique called sonication-assisted *A. rhizogenes*-mediated transformation (SAART), which was applied by Ishida et al. [74] for transformation of *Phtheirospermum japonicum* (Orobanchaceae) seedlings with four *A. rhizogenes* strains and by Georgiev et al. [75] for the establishment of hairy root cultures of *Verbascum xanthophoeniceum* (Scrophulariaceae). The technique, developed by Trick and Finer [76], is a potentially more effective method of delivering the fragment of *Agrobacterium* plasmid to target plant tissues than direct infection methods or even cocultivation. SAART involves subjecting plant explants to short periods of ultrasound in the presence of *Agrobacterium*. The ultrasound treatment produces small uniform fissures and channels throughout the plant tissue which allows *Agrobacterium* better access to internal plant tissues [76]. When the technique was used for inoculation of *V. xanthophoeniceum* leaves with *A. rhizogenes* (ATCC 15834) following 45 s of ultrasound exposure, hairy roots appeared on 75% of them. Hairy roots were not formed when over 600 explants of the species were wounded and treated with the same strain of *A. rhizogenes* by cocultivation/direct infection methods [75]. The selected hairy root lines of *V. xanthophoeniceum* accumulated over six times more acteoside than mother plant leaves. Apart from acteoside, the presence of the other PeGs (forsythoside B, leucosceptoside B, and martynoside) and an iridoid glycoside, aucubin, in these hairy root lines by LC-APCI-MS was found [75]. The SAART method and ATCC 15834 *A. rhizogenes* strain were also suitable for the transformation of another *Verbascum* species – *V. nigrum* [77]. Under these conditions, hairy roots with high frequency (83%) were obtained, which did not produce harpagide nor harpagoside. No hairy roots of *V. nigrum* were induced when direct infection or cocultivation methods for *A. rhizogenes* transformation were applied [77].

The method of direct infection was suitable for the transformation of fragments of shoots and leaves of *Rehmannia glutinosa* (Orobanchaceae) by A4 strain of *A.*

rhizogenes [78]. As a result, 40 lines of roots were induced and 10 lines were selected based on the morphology of roots and biomass increase over a course of passages. The roots were then cultivated in liquid woody plant medium (WPM) [79] and half-strength B5 medium (½ B5). Both tested media affected growth and metabolite production. After 4 weeks of cultivation in WPM medium, the roots showed 1.8–4.5 times fresh mass increase and 2–4.4 times dry mass increase, depending on line tested, in comparison with roots cultured in half-strength B5 medium [78]. *R. glutinosa* hairy roots produced four iridoid glycosides (catalpol, aucubin, loganin, and catalposide) and two phenylethanoid glycosides (acteoside and isoacteoside) [78]. Highly productive lines contained four times higher amounts of loganin (up to 4.7 mg g^{-1} DW), 3.2–4 times more catalposide (up to 4.5 mg g^{-1} DW), and 2.6–3.5 times more acteoside (up to 16.9 mg g^{-1} DW) and isoacteoside (up to 3.5 mg g^{-1} DW) in comparison with roots of 1-year-old seed-derived *R. glutinosa* plants growing in soil and non-transformed roots cultured in vitro in liquid WPM medium supplemented with an auxin – IBA (0.5 mg L^{-1}). The data indicated that the process of *Agrobacterium*-mediated transformation of *R. glutinosa* facilitated increased production of several analyzed secondary metabolites [78]. Trace amounts of aucubin and catalpol were also detected in some *R. glutinosa* lines of transformed roots, but their levels were much lower than in non-transformed roots. Higher level of catalpol (0.54% DW) was found in *R. glutinosa* hairy roots induced by *A. rhizogenes* strain ATCC15834 by Hwang [80] when the roots were cultured in Schenk and Hildebrandt (SH) medium [81] containing 4% of sucrose [80].

Hairy roots of *R. glutinosa* and *R. elata* were characterized by spontaneous ability to regenerate of adventitious shoots in darkness [82–84]. There is only one report [82] where the production of metabolites in the shoots and regenerated pRi-transformed plants was determined. The shoots of *R. glutinosa* were multiplied on agar MS medium enriched with 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} IAA in 300 mL glass jars, giving proliferation index of 17 axillary shoots per explant. They were able to synthesize catalpol, acteoside, and isoacteoside, but the contents of the compounds were low (max. 4.9, 10.5, and 0.6 mg g^{-1} DW, respectively). In order to obtain transgenic plants, multiple shoots of *R. glutinosa* were rooted, transferred to flowerpots with soil, and acclimatized in a greenhouse. The transformation process in the regenerated plants was confirmed by means of PCR and RT-PCR reactions. The transformed plants obtained after transformation with *A. rhizogenes*, also called pRi-transformed plants, often demonstrate phenotypical changes defined as the hairy root syndrome, i.e., shortened internodes, dwarf structure, and wrinkled leaves. Such morphological changes were not observed in the transformed plants of *R. glutinosa*. The plants were distinguished by higher biomass of the aboveground part and a root system with numerous and longer branches than non-transformed plants [82]. Because of higher biomass of shoots and the strong root system, production of all analyzed compounds (catalposide, aucubin, catalpol, harpagide, harpagoside, loganin, acteoside, and isoacteoside) in transformed *R. glutinosa* plants was higher than in the non-transformed ones when amounted per single plant [82].

3.2 Elicitation

The biosynthesis of many secondary metabolites in plants is usually a defense reaction to various stressful conditions. Many biotic (mainly microbial origin) and abiotic (nonbiological origin) elicitors act as plant particles causing transduction of signals from the surface of the plasma membrane [85, 86]. This consequently stimulates biochemical responses involved in the development of low molecular defense components in plants in response to stresses such as attack by pathogens [87, 88]. Exogenous addition of such elicitors to in vitro plant cultures induced the expression of genes often associated with the enzymes responsible for synthesis of secondary metabolites, resulting in the improvement of their concentrations [89, 90]. The efficiency of elicitation depends on many factors such as the type of the elicitor, its concentration, the stage of culture growth, and time of exposure [91]. The parameters must be appropriately selected according to plant species and type of metabolite.

Several studies have demonstrated improvements in the phenylethanoid pathway in in vitro cultures of some species from Orobanchaceae by inducing a defense reaction with a variety of elicitors [89, 92–100]. Such experiments were performed in cell suspension culture of *Cistanche deserticola* using autoclaved homogenate of *Fusarium solani* culture as elicitor [92]. The elicitor used at optimal concentration (40 mg L^{-1}) did not significantly affect the biomass of culture, but increased the content of echinacoside by threefold (up to 16 mg g^{-1} DW) and acteoside (up to 9.6 mg g^{-1} DW) in comparison with non-elicited control culture [92]. Cheng et al. [89] attempted to promote the biosynthesis of PeGs in cell cultures of *C. deserticola* by repeated addition of chitosan. It was found that the elicitor at a concentration of 10 mg L^{-1} added on days 15 and 17 increased the production of PeGs almost threefold (from 15.6 to 43.6 mg g^{-1} DW). A strategy of repeated addition of yeast elicitor to cell suspension cultures of *C. deserticola* was employed by Cheng et al. [96]. Carbohydrate fraction of the elicitor (added at days 15, 17, and 19) improved the production of PeGs by 2.5-fold compared to single treatment with the elicitor.

Various abiotic elicitors (putrescine, silver nitrate, rare earth elements, hydrogen peroxide, methyl jasmonate, and salicylic acid) were used for elicitation of *Cistanche* sp. cell suspension cultures. The addition of putrescine (25 μM) on day 8 and Ag^+ (10 μM) on day 16 led to the highest improvement of echinacoside (1.7 g L^{-1}) and acteoside (0.4 g L^{-1}) [95]. These levels were significantly higher than those observed after elicitation with putrescine or Ag^+ alone [95]. Also, treatment of cell suspension cultures of *C. deserticola* with mixture of rare earth elements (La_2O_3/CeO_2/Pr_6O_{11}/Sm_2O_3 = 255:175:3:1, mol/mol) increased both cell growth and production of PeGs, these being 26% and 167% higher than those of controls, respectively [93]. Hyperosmotic stress has also been used and proven to be an effective factor for increasing accumulation of PeGs in *C. deserticola* suspension culture [97]. The stress conditions were obtained by combination of 87.6 mM sucrose and 164.7 mM mannitol (303 mOsm kg^{-1} osmolarity). When the cells were grown under these conditions, the maximum PeG content (26.9 mg g^{-1} DW) was achieved, which was 29% higher in comparison with the control conditions (300 mOsm kg^{-1}) [97].

Another effective abiotic elicitor which enhanced PeG production in *Cistanche* cell suspension cultures was hydrogen peroxide [98]. The elicitor added at concentrations 40–80 μg L^{-1} to *C. salsa* cell suspension cultures enhanced the production of echinacoside, acteoside, 2′-acetylacteoside, and total PeGs 1.2-fold to 1.8-fold when compared with an untreated suspension culture of the species. Greater biomass accumulation and PeG biosynthesis was also observed in *C. deserticola* suspension cultures elicited with methyl jasmonate (MeJa) or salicylic acid (SA) [94]. Addition of MeJa at a concentration of 5 μM to 2-week-old culture resulted in 2.6-fold and 3.8-fold greater production of total PeGs and echinacoside, respectively. Similar results were obtained when 50 μM SA was added to a 28-day-old culture [94]. In a study conducted by Cao and Jia [99], the synergistic effect of MeJa, SA, and sodium nitroprusside (as NO donor) on accumulation of echinacoside and other PeGs was observed in cell suspension cultures of *C. deserticola*. The elicitors were added to culture on day 12 (0.05 mmol L^{-1} of sodium nitroprusside and 0.50 μM SA) and on day 16 (20 μM MeJa). The elicited cells produced four times more echinacoside and other PeGs than untreated ones. The biomass of the culture also increased (1.4 times compared to control) [99]. MeJa and SA appeared to be effective in enhancement of both biomass and secondary metabolite accumulation in hairy root cultures of *Rehmannia glutinosa* (Orobanchaceae) [100]. They were incubated with MeJa and SA added either alone or in combination for the production of PeGs (acteoside and isoacteoside) and iridoids (catalpol, harpagide, and catalposide) [100]. The elicitors were added to 23-day-old culture, and its biomass and metabolite production were analyzed 72 and 120 h after elicitation. It was found that elicitation by MeJa was significantly higher than that by SA, suggesting that MeJa and SA have different modes of action on *R. glutinosa* hairy root culture. Hairy roots of *R. glutinosa* grown in WPM liquid medium and elicited with 200 μM MeJa for 72 h were characterized by the greatest content of acteoside (ten times more than in roots not subjected to elicitation) and isoacteoside (6.4 times more). For the biosynthesis of harpagoside, the best results were achieved when the hairy roots were treated with 150 μM MeJa for 72 h. The addition of the same concentration of MeJa, but with a longer elicitation period (120 h), was found to be the most favorable for catalpol level; under these conditions, transformed roots produced twice the amount of the iridoid than control [98]. MeJa and SA applied in combination also increased the levels of all analyzed metabolites in *R. glutinosa* hairy roots in comparison to control, but their contents were lower than those observed when MeJa was added separately at the optimum concentration [100].

3.3 Precursor Feeding

It is possible to increase the biosynthesis of secondary metabolites, particularly PeGs, in suspension culture of *Cistanche* species, by the addition of precursors to culture media. The precursor may be incorporated directly into the product, or the precursor may enter a specific product indirectly through degradative metabolism and entering into interrelated pathways [101]. The amino acids tyrosine (Tyr) and

phenylalanine (Phe) and caffeic acid, as the precursors of PeGs, were added to cell *C. salsa* at different concentrations with or without the addition of cucumber juice [102]. The highest accumulations of echinacoside (640 mg L^{-1}), acteoside (689.4 mg L^{-1}), and 2′-acetylacteoside (54.9 mg L^{-1}) were achieved after treatment of the cells with the combination of Phe (400 mg L^{-1}), Tyr (500 mg L^{-1}), and cucumber juice (50 mL). Phe was also found to have a positive effect on PeG biosynthesis in a cell culture of *C. deserticola* [52]. The application of 0.2 mmol L^{-1} Phe on day 8 of culture increased PeG concentrations by 143% in shake flasks and 160% in 2 L bubble column bioreactor, as compared to cell suspension cultured without the amino acid [52]. Also, Tyr acted as a precursor for PeG production in cell suspension culture of *C. deserticola* [103]. Feeding the precursor has an especially beneficial effect on salidroside biosynthesis; its level increased fourfold in the presence of 5 mmol L^{-1} of Tyr in the culture medium [103]. Cheng et al. [104] reported that also a combination of 0.1 mM Phe and 1.0 g L^{-1} casein hydrolysate increased the PeG production in the suspension culture of *C. deserticola* to levels 1.5-fold of those in the control.

3.4 Physical Factors

Important physical factors affecting the production of secondary metabolites in in vitro cultures are temperature, light intensity, and its spectrum [105–107]. Ouyang et al. [105] report that *Cistanche deserticola* callus culture was sensitive to temperature. The optimal PeG production (10.7% DW) was achieved at 25 °C with a light irradiation intensity of 24 μmol $m^{-2}s^{-1}$ [105]. Ouyang et al. [106] report that blue light (435 nm) gave the highest biomass growth and biosynthesis of PeGs (19% and 41% higher than observed under white light, respectively) in the *C. deserticola* callus. The blue LED light was also found to be the best for iridoid production in *Scrophularia takesimensis* shoot culture. In the culture, the harpagoside content was 4.9 mg g^{-1} DW followed by 3.3 and 2.7 mg g^{-1} DW in white and red LED light, respectively [107]. Light spectrum can affect the accumulation of secondary metabolites in two ways: by supplying energy for photosynthesis via carbohydrates or by its signaling effects in photomorphogenetic processes [108].

4 Bioreactor Systems

The application of bioreactor systems could increase the scale of metabolite production and give rise to the commercial production of these compounds. The bioreactor types used for plant culture systems differ with respect to how the cultures are mixed, how the medium is supplied to the culture, and how gas exchange is controlled. Dedifferentiated cell suspension cultures are most extensively studied for their growth in various bioreactor systems. Such cultures are easier for growth in bioreactors than organ cultures, which are more sensitive for shear forces appearing during shaking of the medium. The forces often seriously damage plant tissues,

cause callus induction, and as the consequence, caused both the worse growth and metabolite production in the culture. A 10 L column bioreactor was used for cell suspension culture of *Scrophularia striata* by Ahmadi-Sakha et al. [109]. Under optimal conditions, i.e., in darkness at pH of 4.8, air flow rate of 0.5–1.5 L min^{-1}, mixing speed of 110–170 rpm at 25 ± 1 °C, cell biomass (15.6 g L^{-1} DW), and acteoside content (1.4 mg g^{-1} DW) were higher than those in shake flasks (14.2 g L^{-1} DW and 0.5 mg g^{-1} DW, respectively). However, the cells grown in the bioreactor accumulated 1.4-fold less echinacoside than in shake flasks [109]. Two types of bioreactors: a 2 L internal loop airlift bioreactor with sifter riser and 2 L bubble column bioreactor were used to improve total PeG content in cell suspension cultures of *Cistanche deserticola* [110]. The best results were obtained in 2 L internal loop airlift bioreactor at the air flow rate of 0.075 m^3 h^{-1} and the inoculation size of 4.7% [110]. Under these culture conditions, the PeG content and production were two times higher than those in 2 L bubble column bioreactor and shake flasks [110].

Shoot cultures of two members of Orobanchaceae, *Castilleja tenuiflora* and *Rehmannia glutinosa* (Orobanchaceae) [82, 111], were cultured in bioreactors to evaluate their growth and metabolite accumulations. *C. tenuiflora* shoot culture grown in 200 mL RITA® temporary immersion bioreactor in liquid B5 medium without plant growth regulators under nitrogen-deficient conditions for 21 days produced two times more acteoside and isoacteoside than shoots cultured in shake flasks. The physiologically most important advantage of the system is the efficient gaseous exchange between plant tissue and gas phase inside the vessel [111]. For the culture of *R. glutinosa*, pRi-transformed shoots were cultured in liquid MS medium enriched with 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} IAA in a 5 L nutrient sprinkle bioreactor. In the type of bioreactor, shoots are placed in the growth container on the net situated 18 cm above the bottom. The medium was administered to the culture by a peristaltic pump through a nozzle situated at the bottom of the growth container. The circulation rate of the medium was 60 mL per each 40 s delivery, with 3.0 min breaks between each delivery. The multiplication of the *R. glutinosa* shoots resulted in a proliferation index of 28 axillary shoots per explants after 4 weeks of culture [82]. The transformed shoots produced 10.5 mg g^{-1} DW of acteoside (1.4 times more than shoots proliferated in glass jars) and 0.6 mg g^{-1} DW of isoacteoside (comparable as in glass jars). Among iridoids, *R. glutinosa* multiple shoots accumulated 4.9 mg g^{-1} DW of catalpol (2.8 times more than in glass jars). Aucubin and loganin were detected in trace amounts and catalposide, harpagide, and harpagoside were not presented in the tested plant material [82].

5 Conclusions

Phenylethanoid and iridoid glycosides are members of a group of natural compounds which will be undoubtedly used for the improvement of human health in the future. Therefore, it is important to develop biotechnological methods for obtaining high amounts of the metabolites. The data presented in the chapter indicates that it is

possible to achieve successful and controllable production of phenylethanoids and/or iridoids using in vitro cultures of various plant species belonging to the Buddlejaceae, Orobanchaceae, or Scrophulariaceae. Phenylethanoids can be produced at high levels even in undifferentiated cultures such as callus and suspension cell cultures. For example, the acteoside content in cell suspension culture of *Buddleja cordata* was 11.5 times higher than that observed in the leaves of intact plants. However, dedifferentiated cells usually do not accumulate iridoid glycosides in sufficient quantities. To increase the production of these compounds, other culture systems, such as cultures of highly differentiated organs (shoots or roots, especially hairy roots) or in vitro-regenerated plants, should be used. For example, roots of regenerated *Scrophularia yoshimurae* plants are able to produce high amounts of harpagoside, and they can be used as a substitute for the roots of *Scrophularia ningpoensis*, which are currently used in traditional medicine for the treatment of inflammation, laryngitis, tonsillitis, and constipation. The leaves of in vitro-developed plants of *Rehmannia glutinosa* offer a great potential for catalpol production. In addition, the elicitor approach is an effective method of improving iridoid biosynthesis, as demonstrated in *R. glutinosa* hairy root culture.

However, although the use of an appropriate strategy and culture system greatly improved iridoid yields, it is likely that the maximum yield was still not achieved. Further greater efficiency in metabolite biosynthesis might be achieved by metabolic engineering: perhaps by the incorporation and overexpression of genes coding the key enzymes of a biosynthetic pathway or decreasing the expression of genes in a competitive pathway branch by antisense technology. To date, no such studies on iridoid production by in vitro cultures have been reported.

One key to improving productivity is by application of better large-scale production systems by optimizing the bioreactor type and the culture parameters, such as inoculum size, duration of subcultures, product recovery, data relating to oxygen, and nutrient transfer. It is likely that in the future, bioreactors in which the plant material has a short time contact with liquid medium during the culture period (temporary immersion culture systems) will predominate for organ cultures, especially shoot cultures, to avoid their hyperhydricity in the liquid medium.

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Taxus Cell Cultures: An Effective Biotechnological Tool to Enhance and Gain New Biosynthetic Insights into Taxane Production

10

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Contents

Abstract

Mass phytochemical production in biotechnological platforms based on plant cell and organ cultures provides an alternative to the field cultivation of plants. The system is being successfully applied to produce plant bioactive compounds scarce in nature, including taxol, a potent chemotherapeutic agent, and its analogs. Additionally, plant cell cultures are a potent tool to shed light on the biosynthesis of phytochemicals and its control. Several studies with *Taxus* spp. cell cultures, focused on increasing taxane production, have gained considerable molecular understanding of how these compounds are metabolized in the target cell cultures, particularly by the application of omics tools. This chapter summarizes the state of the art in the biotechnological production of taxol and related taxanes used for

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the semisynthesis of a new taxane generation. Special emphasis is given to the application of the latest cutting-edge technologies that reveal the molecular changes taking place in plant cells subjected to optimized conditions for taxane biosynthesis and accumulation.

Keywords
Coronatine · Elicitors · Methyl jasmonate · Plant cell cultures · Taxanes, taximin · *Taxus* spp. · Taxol

1 Introduction

The demand for natural products in the pharmaceutical and cosmetic fields or in the food industry for use as flavorings, preservatives, etc., is growing. While the consumption of these predominantly plant-derived products is increasing, the number of plant-cultivated hectares per capita in the world has decreased significantly, due to population growth, problems of desertification related with climate change, and also because considerable land previously dedicated to the cultivation of food or medicinal plant species is today taken up with biodiesel crops [1]. There is therefore an urgent need to find alternative means for the production of bioactive plant compounds.

When the market demand for a phytochemical can no longer be met from its natural source, which may have deteriorated from over-exploitation or degradation of the natural habitat, a possible solution is the domestication of the species for extensive cultivation. However, when this is not possible, the development of plant cell biofactories can provide an alternative production system [2]. The advantages of a biotechnological system over conventional field cultivation of plants include:

(a) The production of bioactive compounds using a low number of plants.
(b) Avoidance of problematic issues related to climate and geopolitics.
(c) The conservation of endangered plant species, helping to ensure their availability for further generations.
(d) A guarantee of uncontaminated plant material, since in vitro cultures are free of microorganisms, herbicides, pesticides, and fungicides.
(e) Agricultural systems are not necessary, thus freeing up land for food-plant production.
(f) A drastic reduction in use of water and organic solvents due to simplified extraction processes.
(g) In the case of GMOs, there is no risk of transgene dissemination [3].

Overall, plant cell factories could be considered as bio-sustainable and ecological processes (Fig. 1).

However, despite these advantages, so far only a few production processes based on plant biofactories have been developed at an industrial level. Among them is the production of shikonin by Mitsui Chemicals since 1984. Another milestone was the production of taxol by Phyton Biotech, which began in 2002, and more recently, in

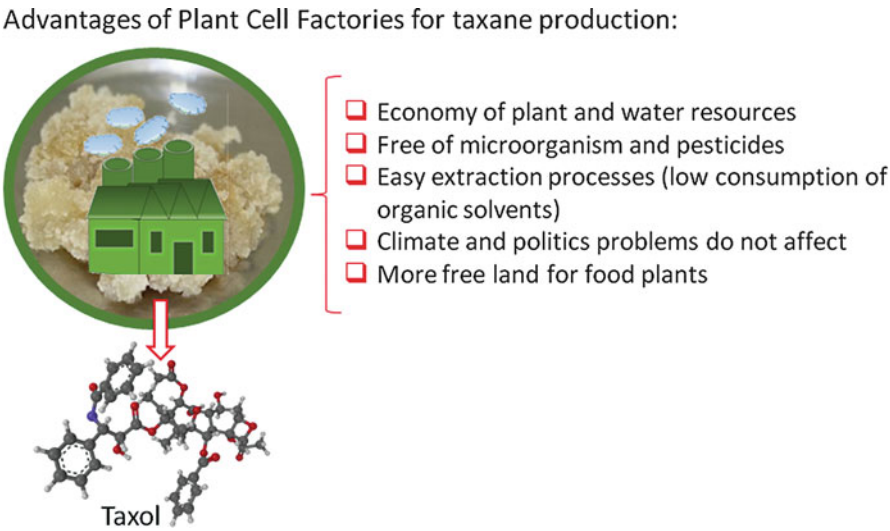

Fig. 1 Schematic representation of plant cell biofactories and some of their advantages

2012, the first recombinant protein, glucocerebrosidase, was produced in carrot cell cultures by Protelix in Israel [4, 5]. It is striking that major progress in this field has occurred only very recently, and no compounds have been marketed to date.

When the production of plant bioactive compounds needs to be increased or recombinant proteins produced, in vitro cultures and genetic engineering techniques form an essential part of the process (Fig. 2). Cell cultures, hairy roots, or other organ cultures can be used for the production of secondary compounds and therapeutic proteins at a bioreactor level, but a previous optimization step in flask cultures is generally required. Key process accelerators include elicitation and the application of advanced techniques such as metabolic engineering that can promote the flow of precursors to the formation of target compounds [6, 7].

Nevertheless, as previously mentioned, the production of biomedical compounds in plant cell factories has had little commercial success to date, due to retarding factors, many of which have little to do with the technologies in use. They include concern about the current regulations of biofactories, intellectual property rights, conflicting attitudes toward new technologies between researchers and the general public, as well as the limited synergism between academic research and industry in most countries [7].

In the optimization step prior to establishing phytochemical production in a plant cell biofactory, empirical factors are considered, such as the selection of highly productive cell lines, variations in culture conditions and media, or the use of elicitors to improve the yield and performance of the system. However, such an approach does not provide information about the changes that occur in plant cells at the molecular level [8].

In contrast, a rational approach will study how the input factors affect the physiology of cells, for example, if transcriptomic and proteomic profiles are altered

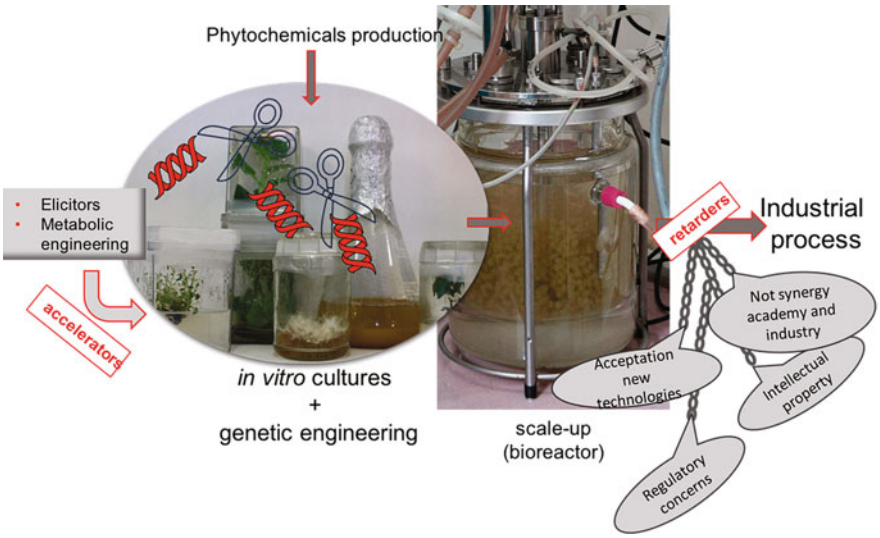

Fig. 2 The fundamentals of a plant cell biofactory, including examples of process accelerators as well as the retarders that hamper implementation at an industrial level (Modified from Sharma et al. [7])

and how the metabolome is affected. This leads to a better overall understanding of secondary metabolic pathways and their control [9]. Thus, rational studies have shown that plant biofactories are also a powerful tool for the study of plant metabolism and its regulation (Fig. 3).

A notable phytochemical currently in commercial production is taxol, a complex diterpene alkaloid with a very limited natural supply, as it accumulates mainly in yew bark in concentrations below 0.02%. The use of taxol for the treatment of cancer was approved by the FDA in 1992 [10], and together with its derivatives, it is included in the Anatomical Therapeutic Chemical (ATC) code L01CD. Today taxol remains a drug of choice for many types of cancers such as non-small cell lung tumors, metastatic carcinoma of the ovary, metastatic breast cancer, and tumors of the colon, skin, kidneys, brain, and prostate, as well as against the Kaposi sarcoma associated with AIDS and some leukemias [10]. Taxol is also currently being studied for the treatment of diseases not related with cancer that require microtubule stabilization and the avoidance of cell proliferation and angiogenesis, for example, psoriasis and Alzheimer [10]. Taxol and its drawbacks, such as a low solubility or side effects, have been partly resolved with the commercialization of semisynthetic derivatives. The market for taxanes continues to grow and new sources of taxanes or the enhancement of existing systems is required [11].

Although several systems have been described for the total synthesis of taxol, low yields impede their application at an industrial level [12]. However, commercial success has been achieved with the semisynthesis of taxol derivatives from baccatin III or other precursors extracted from the needles of *Taxus baccata*. Agronomic crops of *Taxus chinensis* have also been developed in the Yunnan Province in China, but at

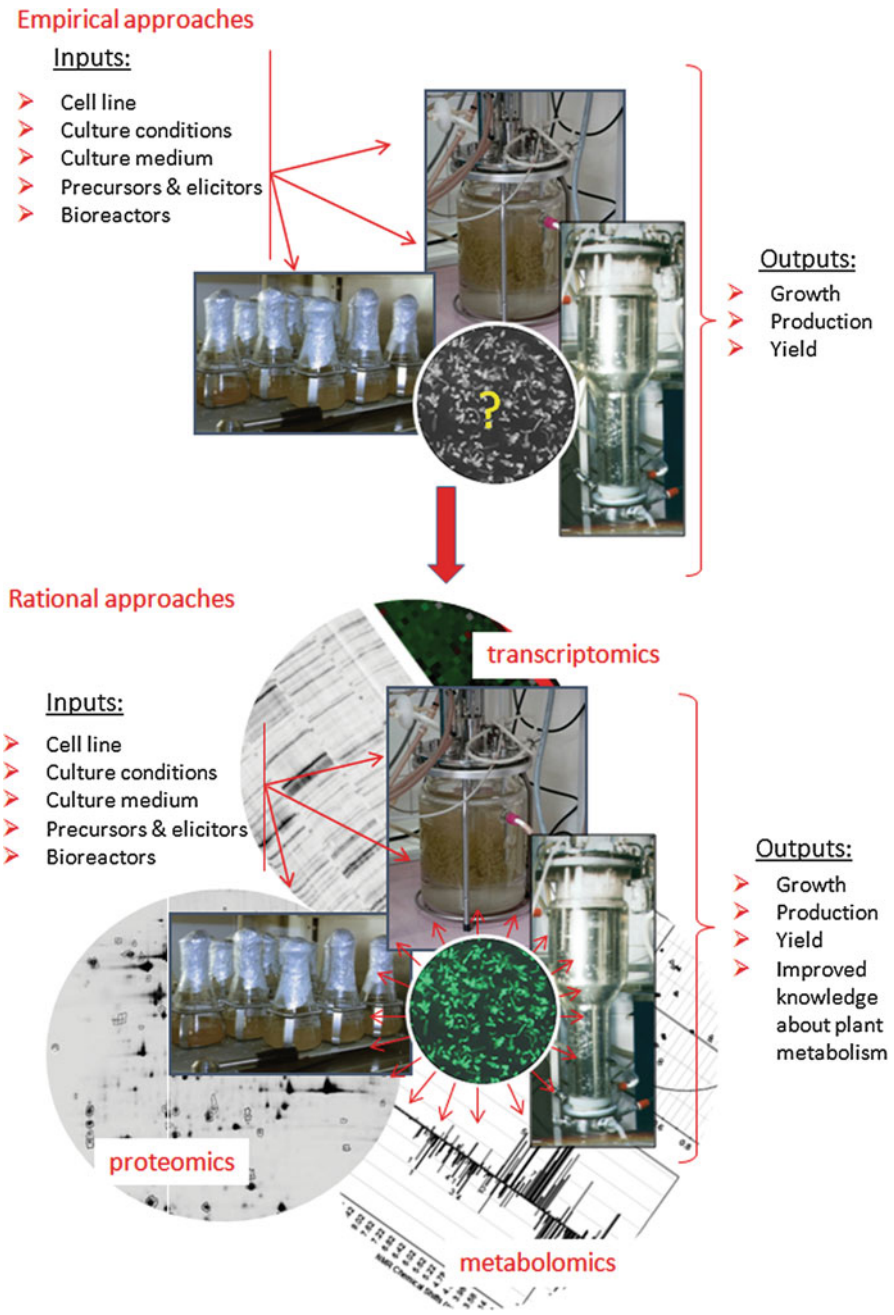

Fig. 3 Moving from an empirical to a rational approach for a better understanding of the molecular changes in target plant cells in a biofactory

a huge environmental cost. Therefore, the main current source of taxol and its precursors is biotechnological production using different species of *Taxus* by Phyton Biotech or Abraxis [9].

This chapter will describe the plant cell cultures of *Taxus* spp. established to date and examine their application to improve taxane production as well as gain insights into taxane biosynthesis and its control. Emphasis will be given to the advances achieved in this field by the application of new biotechnological tools such as metabolic engineering and omics technologies.

2 Improving the Biotechnological Production of Taxanes in *Taxus* Cell Cultures by an Empirical Approach

As already mentioned, to improve taxane production in biotechnological platforms based on *Taxus* cell cultures, both empirical and rational approaches can be useful (Fig. 3). The former considers input factors, such as the selection of highly productive cell lines, composition of the basic nutrient media, sugar supplements, plant growth regulator combinations, or the addition of taxane precursors and elicitors to the culture medium, and as output factors the biomass and taxane productivity [10]. In contrast, a rational approach focuses on the cellular changes in the transcriptome, proteome, and metabolome that lie behind the enhanced taxane production and has been successfully applied to gain further understanding of primary and secondary metabolic pathways and their regulation [13].

During the last two decades, our research group, as well as several other groups around the world, has developed different cell cultures of *Taxus* species, including *T. baccata*, *T. wallichiana* or *T. media*, and most recently Mexican yew (*T. globosa*), scaling up from shake flasks to different types of bioreactor. All these processes were optimized by testing several culture media (e.g., WPM, B5, MS) with variable proportions of a range of growth regulators and by adding precursors and elicitors, among other factors. Our results confirmed that taxane production does not depend on cell growth and that taxanes accumulate mainly during the stationary phase of the culture [14, 15]. It was also found that the culture media optimized to achieve high amounts of biomass differs from the optimum media for taxane biosynthesis and accumulation, and each of the *Taxus* species assayed have different nutritional and other culture requirements for growth and production in optimized conditions (Table 1).

These empirical studies led to the establishment of two-phase cell cultures as an optimal system for the production of biomass and taxanes. In a first step, plant cells are placed in an optimal growth medium and are subsequently transferred to an optimized taxane production medium. This system has the added advantage of permitting the addition of biosynthetic precursors and elicitors when the taxane production is at its highest, and the maximum taxol yield is normally achieved with elicitation [16, 17].

Elicitors are compounds that stimulate plant defense systems, promoting secondary metabolism to protect the cell and the whole plant, and they are commonly used

Table 1 Examples of the composition of optimized culture media for growth and taxane production in several *Taxus* species

Plant species	Optimized medium	Basal nutrients	Auxins	Cytokinin	References
T. baccata	Growth[a]	WPM	2 mg/L NAA	0.1 mg/L BAP	[16]
	Production[a]	B5	2 mg/L Pic	0.1 mg/L Kin	
T. media	Growth[a]	B5	2 mg/l Pic	0.1 mg/L Kin	[17]
	Production[a]	B5	2 mg/l 2,4-D	0.1 mg/L BAP	
T. globosa	Growth	WPM	2 mg/L Pic	Kin 0.1 mg/L	[18]
	Production[a]	B5	2 mg/l 2,4-D	0.1 mg/L BAP	

[a]All the media were also supplemented with 0.1 mg/L of gibberellic acid
WPM woody plant medium, *B5* Gamborg's medium, *NAA* naphtalenacetic acid, *Pic* picloram, *2,4-D* 2,4-dichlorophenoxyacetic acid, *BAP* bencylaminopurine, *Kin* kinetin

to induce plant secondary metabolite production in plant cell factories [19]. Several elicitors have been applied to increase taxane production in plant cell cultures of *Taxus* spp. (for details, see the reviews by Malic et al. [10]; Onrubia et al. [11]), above all methyl jasmonate (MeJA). Jasmonates (JAs) are specific signaling molecules able to activate important physiological and developmental processes in plants [20]. The biosynthesis of these hormones is induced by pathogen attack and wounding and triggers defense responses, both locally and systemically. MeJA in particular plays an important role in signal transduction processes by regulating several plant defense genes [21]. Treatment of *Taxus* cell cultures with MeJA is one of the most effective strategies for boosting taxane production [11], although it can limit biomass formation. In fact, *Taxus* cell cultures were one of the first in vitro systems to give positive results after the addition of MeJA to the culture medium [22].

Coronatine (CORO) is a pathogenic toxin produced by *Pseudomonas syringae* that has been tested in plant cell cultures of *T. media* and *T. globosa* [23, 24]. It acts as a molecular mimic of the isoleucine-conjugated form of jasmonic acid (JA–Ile) [25], while being more stable [23], and thus has a similar mechanism of action to the elicitor MeJA. When CORO was added to *T. media* and *T. globosa* cell cultures, it was found to be more effective than MeJA in enhancing the taxane production, even when used at lower concentrations. Taxane yields reported in *Taxus* spp. cell cultures treated with this elicitor have been up to 5.3-fold higher [23, 24].

Another successful strategy used in *Taxus* cell cultures is treatment with permeabilizing agents that promote the release of taxanes to the culture medium. This could unblock metabolic pathway steps controlled by feedback inhibition mechanisms, leading to a higher taxane production [10]. In this context, cyclodextrins (CDs), which are cyclic oligosaccharides, were recently tested as elicitor/

Table 2 Comparative study of the effect of classical (MeJA) and new elicitors (CORO)/permeabilizing agents (CDs) on taxane production in *Taxus* spp. cultures

Species	Elicitor	Increased production	Release to the culture medium (%)	References
T. media	MeJA	x10	45	[23]
T. baccata	MeJA	x9	40	[27]
T. media	CORO	x20	52	[23]
T. media	CD	x6	90	[26]
T. media	MeJA +CD	x83	90	[26]
T. media	CORO +CD	x18	90	[24]
T. globosa	CORO +CD	x40	100	[24]

MeJA methyl jasmonate, *CORO* coronatine, *CD* cyclodextrin

permeabilizing agents in cell cultures of *T. globosa* and *T. media* [24, 26]. β-Methyl cyclodextrin was found not to have a strong effect on taxane production, but it acted as an effective permeabilizing agent, increasing the release of taxanes to the culture medium (Table 2). However, when combined with other elicitors, such as MeJA or CORO, a synergic elicitor effect achieved significant increases of taxol and other taxanes.

3 Taxane Biosynthesis

The taxol biosynthetic path has not been completely elucidated (Fig. 4), although it is hypothesized that 19 enzymes are involved, for which 13 genes and the corresponding proteins are known, and six steps have been postulated according to known intermediates and the modified positions in the taxol molecule. In brief, the biosynthesis begins in plant plastids by the action of the taxadiene synthase enzyme, which produces the tricyclic structure, taxadiene, from geranylgeranyl diphosphate (GGPP). Following a number of hydroxylation, benzoylation, and transacylation steps, baccatin III is formed, and after the binding of a β-phenylalanine-derived phenylisoserine chain, and two other steps, taxol is obtained [28, 29] (Fig. 4).

Most of the research to elucidate the taxane biosynthetic pathway has been carried out in *Taxus* spp. plant cell cultures. Once GGPP has been cyclized into taxadiene, this intermediate is hydroxylated at C5. In the subsequent pathway to 10-deacetyl baccatin III, the order of steps and proteins involved is uncertain, but the following has been postulated [28]. The first bifurcation of the pathway occurs with C13 hydroxylation on one side and C5 acetylation on the other. After the C5 acetylation, there is a C10 hydroxylation, followed by an active taxoid-14β-hydroxylase, which yields to an alternative pathway. Once taxane-5,10,13-ol acetate is obtained, it is hydroxylated at C1, C2, C7, and C9, then acetyl/benzoyl groups are added at C2 and C4, an oxetane ring (D-ring) is formed, and, finally, there is a C9 oxidation. An

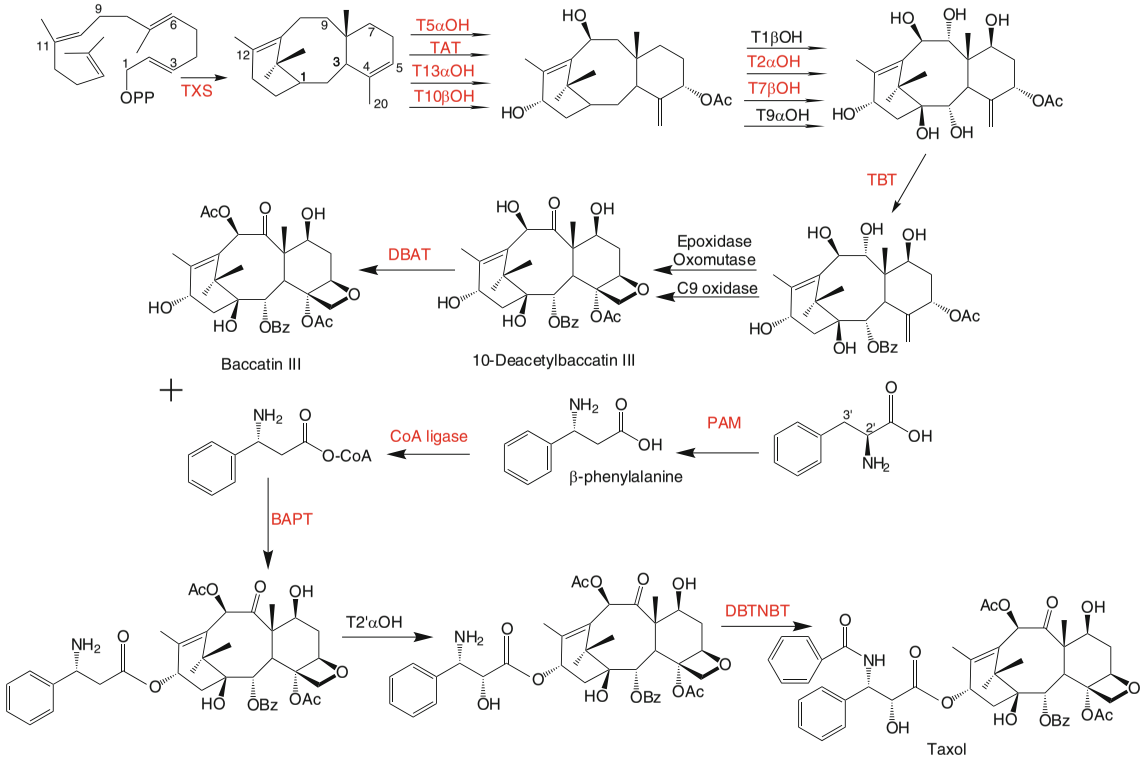

Fig. 4 Taxane biosynthesis from GGPP. *TS*, taxadiene synthase; *T5αOH*, taxane 5α-hydroxylase; *TAT*, taxadiene-5α-ol-*O*-acetyltransferase; *T13αOH*, taxane 13α-hydroxylase; *T10βOH*, taxane 10β-hydroxylase; *T1βOH*, taxane 1β-hydroxylase; *T9αOH*, taxane 9α-hydroxylase; *TBT*, taxane-2α-*O*-benzoyl transferase; *DBTA*, 10-deacetylbaccatin III-10-*O*-acetyltransferase; *BAPT*, baccatin III-3-amino, 13-phenylpropanoyl-CoA transferase; *T2'αOH*, taxane 2'α-hydroxylase; *DBTNBT*, debenzoyl taxol *N*-benzoyl transferase. In *red*, known genes. *Red arrows*, strong gene activation induced by elicitors. *Grey arrows*, slow gene activation induced by elicitors

acetyl group is then bound to C10 of 10-deacetyl baccatin III, yielding baccatin III. The remaining step to reach taxol is the addition of the lateral chain. A β-phenylalanine-CoA is bound to C13 from the taxane core, hydroxylated at C2 and *N*-benzoylated at C3 (Fig. 4). Recently, a CoA ligase from *T. baccata* has been cloned and its functionality in taxol biosynthesis has been demonstrated [30].

4 Rational Approaches

As mentioned above, rational approaches (Fig. 3), especially if supported by omics technologies, have allowed a greater understanding of plant secondary metabolism, frequently highly complex as in the case of taxanes. In *Taxus* sp. cell cultures treated with the elicitors MeJA, CORO, and CDs, the expression level of the following genes has been determined by qPCR: the TXS gene; genes encoding different hydroxylases involved in the hydroxylation of the taxane ring; the phenylalanine aminomutase (PAM) gene, which controls the conversion of α-L-phenylalanine to β-phenylalanine; the 10-deacetylbaccatin III-10β-*O*-acetyltransferase (DBAT) gene, responsible for forming baccatin III; the baccatin III 13-*O*-(3-amino-3-phenylpropanoyl) transferase (BAPT) gene, involved in the attachment of the side chain to baccatin III; and, finally, the 3′-*N*-debenzoyl-2′-deoxytaxol-*N*-benzoyltransferase (DBNBT) gene, which encodes the last benzoyl transferase leading to taxol [9, 23, 24, 26, 31, 32]. These studies revealed that elicitors generally increase the expression level of the genes involved in taxane biosynthesis, particularly those acting in the first steps of the pathway (above all hydroxylases), while those involved in the last steps are less induced (DBAT, BAPT, and DBNBT) (Fig. 4). So, in terms of elicitation, it can be inferred that the flow of carbon to the formation of taxol is limited downstream, and the genes/enzymes involved in the last steps of taxane biosynthesis could be excellent targets for metabolic engineering studies, which would attempt their overexpression in *Taxus* sp. transgenic cell lines (Fig. 4).

The aforementioned studies in *Taxus* also revealed that CDs activate the expression of an ABC transporter whose effect could be partly responsible for the high levels of extracellular taxol found in cultures treated with the elicitor [26]. Altogether, our results confirm that *Taxus* cell cultures not only provide a biotechnology platform for taxane production but also constitute a powerful tool for the elucidation of the taxane biosynthetic pathway and its control.

5 Omics Studies

Genome-wide transcript profiling studies have investigated if the metabolic changes induced by elicitors are correlated with differential expression of the genes involved in plant cell metabolism [33]. Several transcriptome studies on *Taxus* cell cultures have explored taxane metabolism and the effects of elicitors at a molecular level. Most of these studies have been performed with MeJA-elicited cells and different *Taxus* plant species such as *T. chinensis* [34–37], *T. cuspidata* [38], and *T. media*

[39]. The profiling methods used are Illumina deep sequencing [34–36, 39], random Sanger sequencing of a cDNA library [37], and Sanger sequencing of a substractive hybridization library [38]. In these studies, the transcriptional reprogramming under MeJA elicitation was confirmed, and several transcripts corresponding to genes involved in taxane biosynthesis have been identified, although without leading to the discovery of new genes.

In this scenario, the use of cDNA-amplified fragment length polymorphism technology (cDNA-AFLP) has revealed that MeJA elicitation of *T. baccata* cell cultures results in a complete transcriptome reprogramming [27, 30]. A comparative study of the transcriptome of MeJA-elicited cells and unelicited control cell cultures allowed the identification and sequencing of more than 650 tags corresponding to genes whose expression was modified by the elicitor. Of these, 27% are involved in plant metabolism, including several genes known to be active in taxane biosynthesis, together with other tags that may correspond to putative candidate genes for unknown steps of the metabolic pathway (Fig. 5).

The full lengths of the putative candidates were obtained, and their AA sequences were submitted to an in silico study to highlight those most likely to correspond to the unknown steps. This approach was based on determining only the positions of the candidate enzymes that could be directly involved in the enzymatic action and evaluating the similarity between our candidates and known proteins with the same function that act on the same or similar substrates. Several tools, including protein databases (MACiE, CSA, PDB, Uniprot), T-Coffee, multiple sequence alignment software, and PyMOL, a graphical resource, were used for the in silico approach (Fig. 6).

Among 15 candidates for hydroxylases (at C1 and C9 on the taxane core and C2 on the lateral chain), the oxidase at the C9 position, the epoxidase and oxomutase of the oxetane ring (D-ring) formation, and also the CoA ligase (Fig. 5), we highlighted three candidates most likely to be involved in taxol biosynthesis: a hydroxylase, epoxidase, and CoA ligase [30]. Along with these genes, tags with a possible regulatory function were also detected, including some transcription factors and hypothetical proteins with unknown functions (Fig. 5). A homology search revealed that the Tb595 gene encodes a small peptide of 73 AAs, highly conserved in the plant kingdom, but with an unknown function. It was therefore selected for further functional analysis [27].

6 Functional Analyses of the Candidate Genes

The peptide encoded by the Tb595 gene, named Taximin, is highly conserved and found in all studied species of Dicotyledoneae plants, monocots, and gymnosperms ever studied. It is characterized by having an identical length regardless of species and a large number of conserved cysteine and proline residues, which give a strongly hydrophobic character [27].

Taximin is directed by a signal peptide of 27 amino acids at its N-terminal secretory end toward the vacuoles or extracellular plasma membrane (Fig. 7a). To know its exact subcellular localization, the Taximin gene was fused at its C-terminal

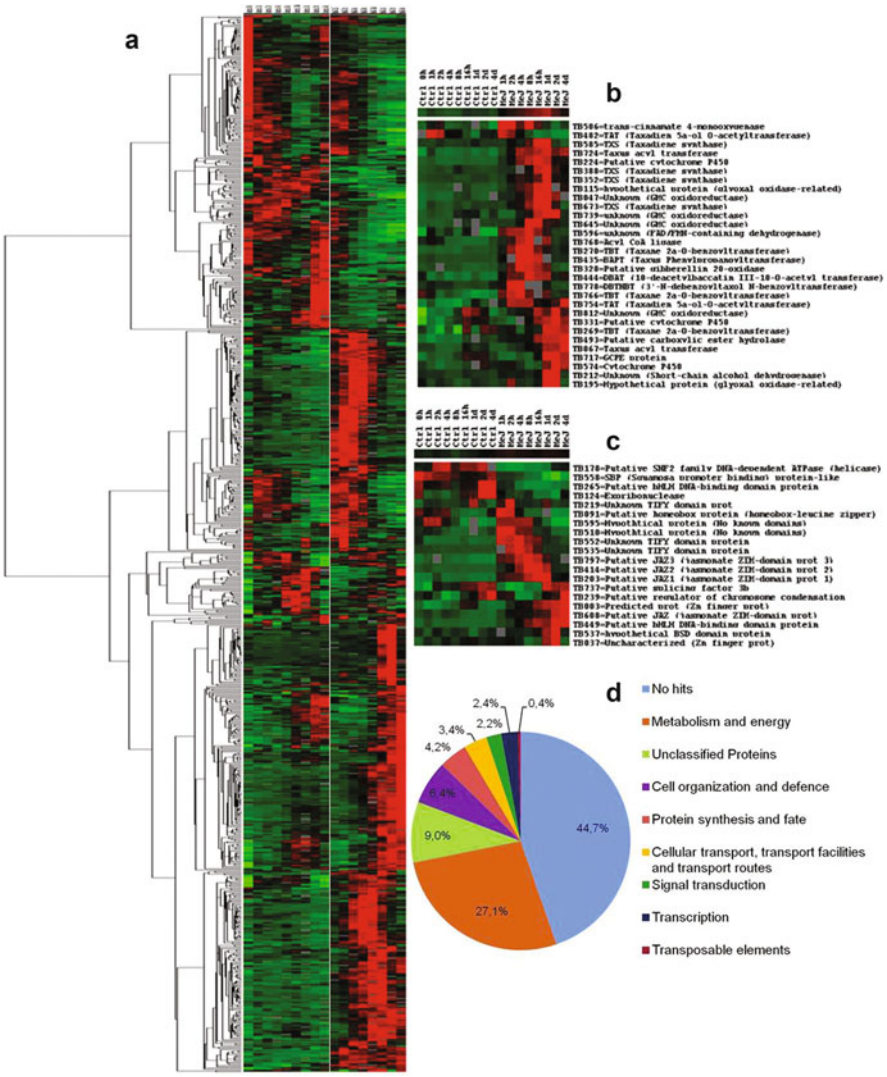

Fig. 5 Average linkage hierarchical clustering of the transcriptome of selected gene tags from MeJA-elicited *T. baccata* cell cultures. (**a**) Complete transcriptome, (**b**) tags related to taxol biosynthesis and candidates for unknown steps, (**c**) tags with homology to transcription factors. The time points for the cluster analysis are indicated at the top: 12 days in GM, and 1 h, 2 h, 4 h, 8 h, 16 h, 1 day, 2 days, 4 days in PM in control and MeJA-elicited conditions. *Red* and *green boxes* reflect transcriptional activation and repression, respectively, relative to the average expression level in control cells. *Grey boxes* correspond to missing time points. The dendrogram at the *left* side of the transcriptome indicates the clusters of activated and repressed genes. (**d**) Sequenced tags classified in functional categories according to the FunCat classification

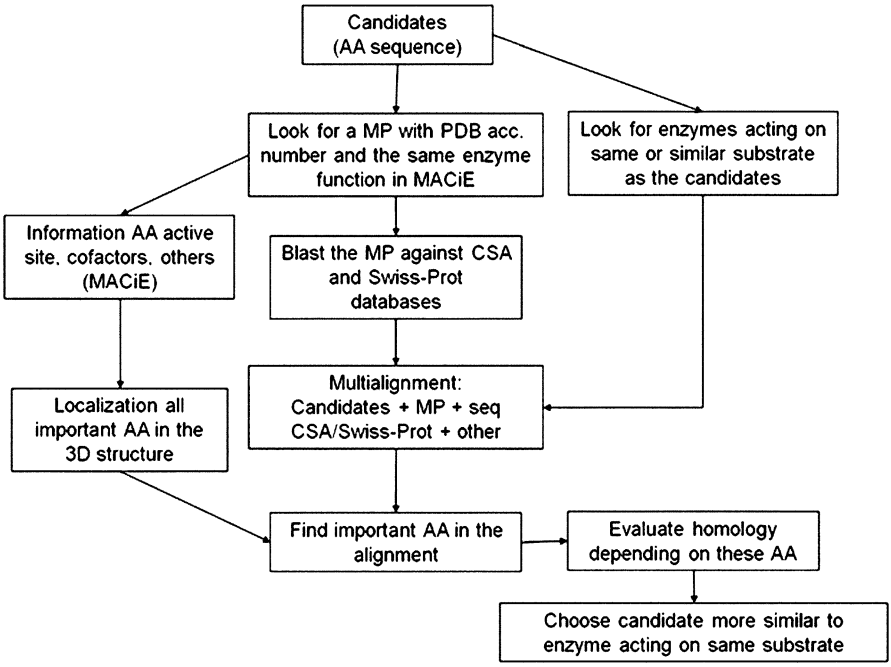

Fig. 6 A flow chart of the procedure followed in the search for candidate genes for the unknown steps of taxol biosynthesis and its control. *MP* model protein, *PDB* Protein Data Bank, *MACiE* Mechanism, Annotation and Classification in Enzymes, *CSA* Catalytic Site Atlas

with the protein yellow fluorescent protein (YFP) Venus, and the construction was transiently expressed in *N. benthamiana*. Confocal microscopy revealed a band similar to that of the free Venus located in the peripheral cytoplasm of the cell. The problem was that the epidermal cells of *Nicotiana* have a large vacuole, and the cytoplasm is completely attached to the membrane, so Taximin could be located in the membrane itself or in the cytoplasm. To clarify this, the FRAP (fluorescence recovery after photobleaching) technique was performed, in which part of the cell is treated with a fluorescence-destroying laser beam. If fluorescence is recovered a few seconds after the treatment, the protein must be mobile and free in the cytoplasm, while a slow recovery indicates it is anchored in the membrane [40, 41]. In this experiment, a rapid recovery of fluorescence was observed for Venus, but in the case of Taximin it failed to recover, showing that the peptide is located in the plasma membrane (Fig. 7b).

Regarding the biological actions of Taximin, HyproTaximin, a synthetic derivative more soluble than Taximin, in which proline residues have been changed by hydroxyproline, caused a transient activation of taxane biosynthesis when added to MeJA-treated *T. baccata* cell cultures. This activating action of secondary metabolism is not limited to taxanes, and ectopic expression of the Taximin gene in tobacco root cultures, also elicited with MeJA, significantly increased the production of nicotine and other tobacco alkaloids [27].

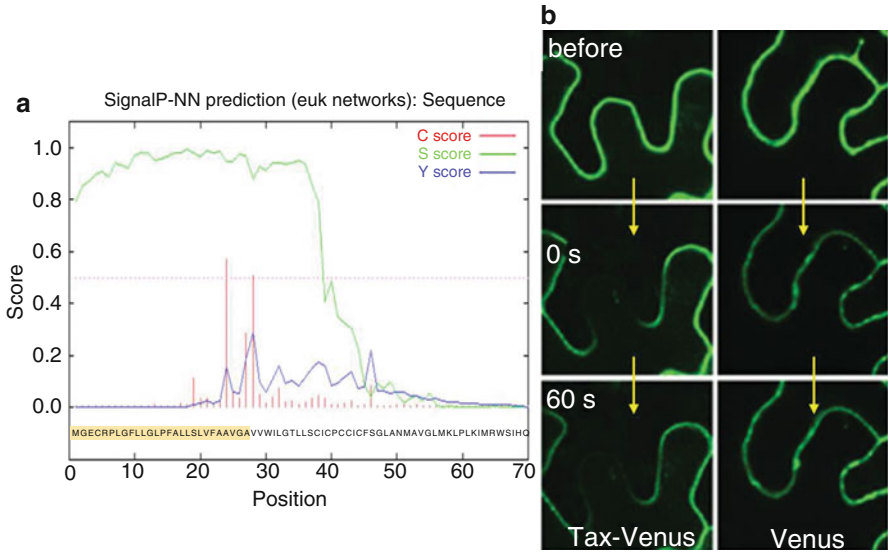

Fig. 7 (**a**) Figure obtained by signal peptide 4.0, showing the different parameters used to evaluate the presence of signal peptides in peptide sequences. The predicted signal peptide is highlighted in yellow. The amino acids of Taximin are represented in 1 letter code. (**b**) Fluorescence recovery after photobleaching (FRAP analysis) in *N. benthamiana* leaves over 60 sec

Recently, overexpression of the Taximin gene in *Arabidopsis thaliana* resulted in minor alterations in the primary shoot and root metabolome, abnormal fruit morphology, and fusion of the base of cauline leaves to stems, forming a decurrent leaf attachment. It was demonstrated that this plant signaling peptide can influence lateral organ separation, thus implying the existence of a peptide signal cascade regulating this process in *A. thaliana* [42]. More recently, the same authors reported that overexpression of the Taximin gene in *Arabidopsis* seedlings leads to a decrease of sinapoyl malate with concomitant hypersensitivity to continuous light, and the effect was reverted by cefotaxime [43]. These results point to another role of Taximin in plant development and primary metabolism.

In addition to the regulatory genes and most of the known genes encoding enzymes involved in taxane biosynthesis, cDNA-AFLP studies have revealed certain gene tags whose expression is also modulated by MeJA and might be involved in biosynthetic pathway steps without assigned genes/enzymes (Fig. 5). A comprehensive bioinformatics study identified a total of 15 genes that could potentially participate in the route [30]. From these, the gene TB768 was selected as potentially involved in the formation of the taxol side chain and a likely candidate for functional studies. This gene has a high homology with other genes of the CoA ligase family and could act on β-phenylalanine, transforming it into its corresponding coenzyme ester for incorporation into the taxane core, giving rise to the side chain in the taxol molecule (Fig. 4).

The protein encoded by TB768 has a hypothetical 3D structure very similar to that of other CoA ligases such as the protein 3a9v, a CoA ligase from *Populus tomentosa*

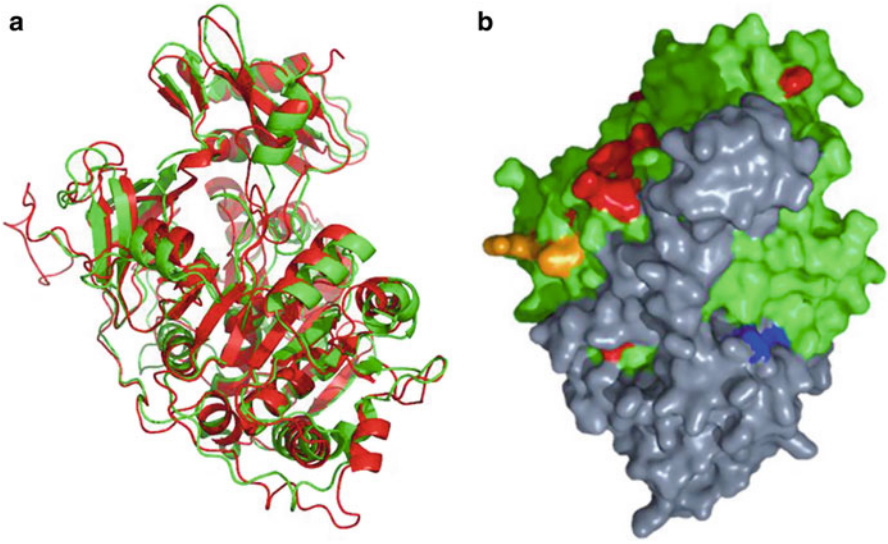

Fig. 8 Phyre2 model of the TB768 protein (*red*) and the 3A9U PDB file of 4-coumarate-CoA ligase (*green*). (**b**) Predicted β-phenylalanine-CoA ligase catalytic domains. Active site colored in *green*, AMP binding in *red*, putative CoA binding site in *orange*, and the acyl activating site in *blue*

[44] (Fig. 8), with a highly conserved region corresponding to the catalytic domain with binding sites ATP and CoA and acyl activator site CoA ligase [30].

To confirm the in vitro activity of CoA ligase, the purified protein TB768 was added to a reaction mixture containing β-phenylalanine and CoA as substrates, and the reaction product was detected by HPLC-mass spectrometry (MS/MS). The CoA ester formed was identified by comparing its retention time and molecule fragmentation pattern with pure standards. However, like other CoA ligases, this enzyme is not specific to β-phenylalanine and can bioconvert other substrates such as 4-coumaric acid (Fig. 9). Overall, the results indicate that the TB768 gene encodes a protein capable of forming CoA esters with β-phenylalanine and 4-coumarate; this protein has been named β-phenylalanine-CoA ligase (TBPCCL) and is the first acyl-CoA ligase characterized in *Taxus* spp. (Fig. 9).

Like other secondary metabolic pathways, taxane formation is compartmentalized at the subcellular level and different organelles could be involved. So far, it is known that the first steps of the pathway take place in the plastids leading to the formation of the taxadiene nucleus from GGPP [45], hydroxylation steps are catalyzed by the group of P450 enzymes associated with the endoplasmic reticulum, and bioinformatics studies indicate the last steps toward taxol are catalyzed by free cytosolic enzymes [46].

Regarding the subcellular location of CoA ligase, bioinformatics analyses predicted that this protein lacks a localization signal peptide (Fig. 10), suggesting that the enzyme is found in the cytosol. To experimentally confirm these results, the gene TB768 was fused to the C-terminus of the yellow fluorescent protein (YFP), and the

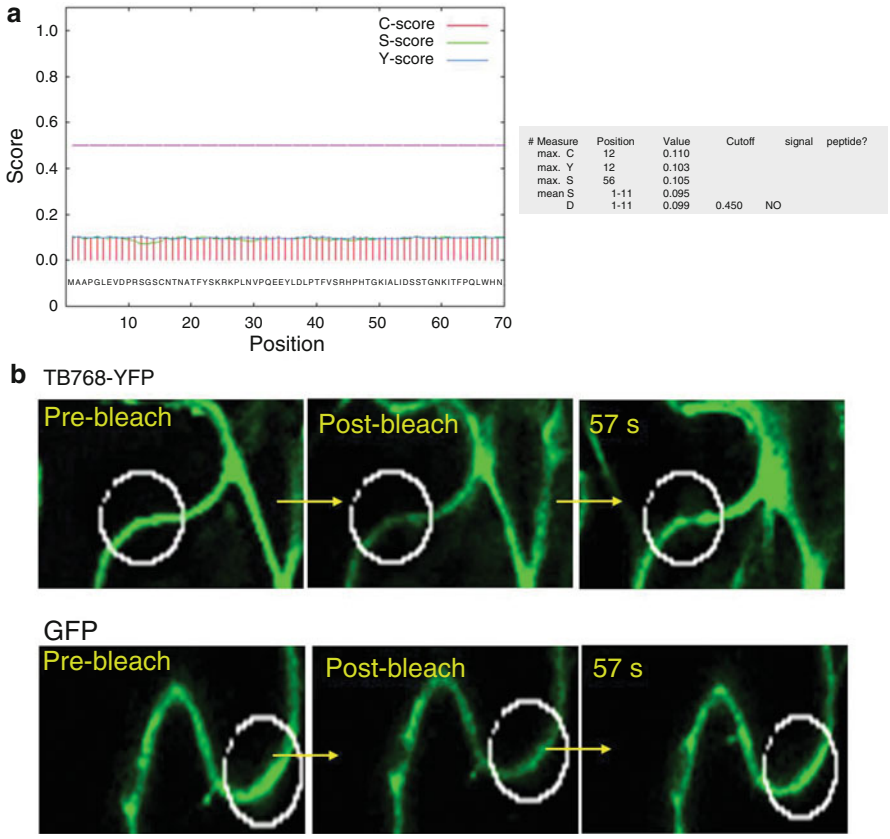

Fig. 9 MRM analysis of 4-coumaric acid and β-phenylalanine-CoA ligase activity. The arrows indicate the peak at the corresponding retention time of the two specific ion fragments analyzed for each molecule used to confirm their identity (914.3/407.3 and 914.3/428.2 for 4-coumarate-CoA with an RT of 3.87; 916.2/409.3 and 916.2/307.3 with an RT of 3.89 for β-phenylalanine-CoA)

resulting plasmid was transiently expressed in *N. benthamiana* leaves. Microscopy analysis showed the TB768 signal-YFP as a thick band at the periphery of the cells, indicating that CoA ligase could be located in both the plasma membrane and cytosol. However, in this case, FRAP showed almost complete recovery of fluorescence after a few seconds, thus confirming a cytosolic location of the enzyme [30] (Fig. 10).

7 Metabolic Engineering for Improving Taxane Production

Although an optimized method based on the *Agrobacterium* system [47] has been recently reported for the genetic transformation of *Taxus* cell cultures, one of the main problems when applying metabolic engineering techniques to *Taxus* cell cultures has always been the difficulty of genetically transforming a gymnosperm

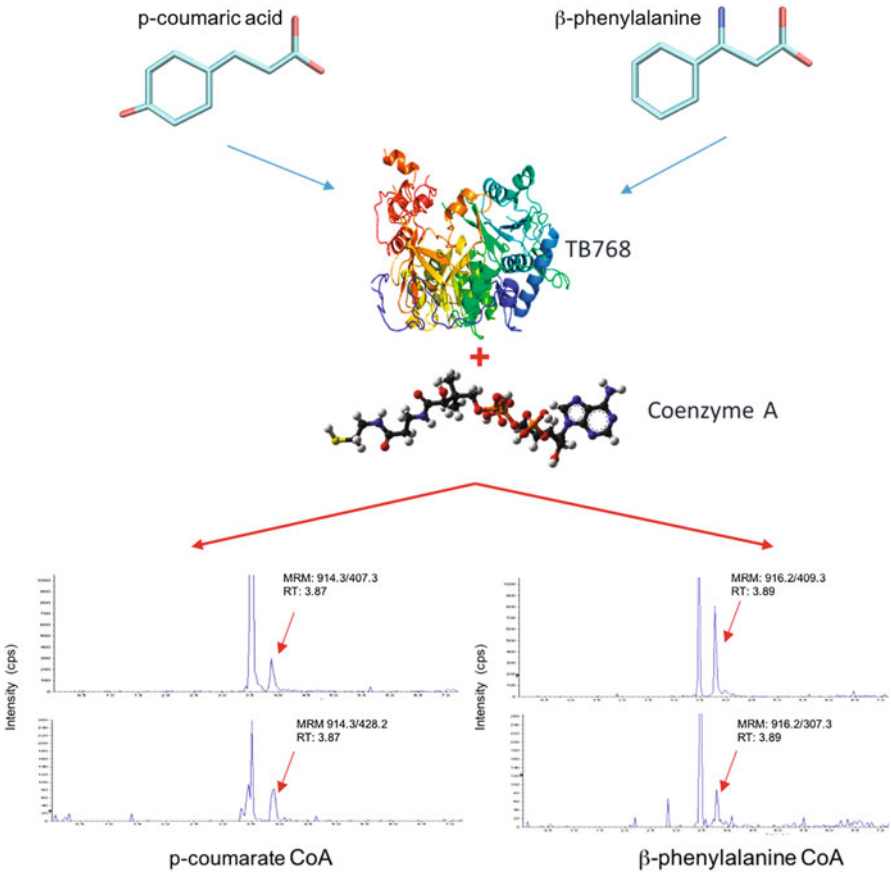

Fig. 10 (**a**) Signal peptide screenshot showing there is no signal peptide in protein TB768. (**b**) Fluorescence recovery after photobleaching (*FRAP*) of TB768-YPF and GFP in *Nicotiana benthamiana* leaves. Fluorescent images were recorded prior to, immediately following, and 57 sec after photobleaching in the indicated region of interest (ROI: *white circle*). The color of TB768-YFP in the microscopy confocal photos has been changed to *green* for better visualization

plant and the slow growth capacity of the transformed plant material in vitro conditions. Nevertheless, *T. brevifolia* and *T. baccata* were successfully genetically transformed using wild strains of *Agrobacterium tumefaciens* [48], and 2 years later, Luan et al. [49] reported transient expression of the GUS gene in *T. brevifolia* embryos. In the same way, hairy root cultures of *Taxus x media* were obtained by transformation with *A. rhizogenes* [50]. The problem of these materials is that although hairy root cultures can biosynthesize taxanes, their growth capacity is very slow. The same authors [51] reported that the addition of the precursors L-phenylalanine and p-aminobenzoic acid to the culture medium significantly increased taxane production. The low growth capacity of *Taxus* hairy root cultures has also been confirmed by Kim et al. [52].

For the first time, Ho et al. [53] applied metabolic engineering tools to increase the biotechnological production of taxanes, obtaining transgenic *T. marei* cell cultures constitutively overexpressing the DBAT gene, but the production of taxanes even in the transgenic root lines was dependent on MeJA, and high taxol levels were only achieved in a high-producing line. More recently, Exposito et al. [32] obtained *T. x media* hairy root cultures overexpressing the TXS gene from *T. baccata*, which encodes one of the first committed steps in taxane biosynthesis (Fig. 4). As previously reported, *T. x media* hairy root lines have a low growth capacity. To overcome this problem, two *T. x media* hairy root lines, one of them harboring only the *rol* genes from *A. rhizogenes* [54] and the other the *rol* genes together with the TXS gene, were selected and treated with plant growth regulators for hairy root dedifferentiation. The resulting calli were used to establish cell suspension cultures. Three cell lines were developed: a control line without added genes, the RolC line (carrying *rol* genes), and the TXS line (carrying *rol* genes together with TXS gene). A two-stage culture system was established with these three *T. x media* cell lines (see Sect. 3). In the first stage, cells were cultured in a growth medium optimized for biomass production for 12 days and then transferred to an optimized production medium for taxane production, with or without the elicitor MeJA for 28 days. In the elicited production medium, the highest taxane production was observed in the TXS line, being 2.64-fold greater than in the control line and 1.55-fold greater than in the RolC line [32].

Another metabolic strategy used in *Taxus* cell cultures was to block branching points in taxol biosynthesis [55] by the antisense-induced suppression of taxoid 14β-hydroxylase which catalyzes C-14 oxygenated taxane biosynthesis. These compounds compete with taxol for the same initial precursors, and this strategy led to an enhanced taxol production in the transgenic cell lines. On the other hand, it is known that ozone induces taxane production in *T. chinensis* cell cultures, and the response is at least partially dependent on abscisic acid (ABA) signaling [56]. Applying this strategy, Li et al. [35] obtained *Taxus* transgenic cell lines overexpressing a 9-cis-epoxycarotenoid dioxygenase, which is responsible for the cleavage of 9-cis-epoxycarotenoid, a rate-limiting step in the biosynthesis of ABA. The corresponding transgenic cell lines increased ABA production and taxol accumulation. These results confirmed the effectiveness of metabolic engineering approaches for increasing the production of taxol and related taxanes in *Taxus* sp. cell cultures.

8 Conclusions and Future Prospects

Although the milestone of taxol biotechnological production in industrial-level plant factories was reached in 2002, the commercialization of new taxane derivatives with improved chemotherapeutic properties has increased the demand for natural taxanes for use in semisynthetic processes. Meeting this growing demand requires a greater understanding of taxane biosynthesis and metabolic control to improve taxane biotechnological production. Several researchers worldwide are currently working in *Taxus* cell cultures to achieve this goal.

The progress in this field summarized here confirms that target cell cultures of *Taxus* spp. constitute an excellent biotechnology platform for taxane production. Moreover, the use of this system in basic research, with the support of omics technologies coupled with bioinformatics studies, is advancing our knowledge not only of taxane biosynthesis but plant secondary metabolism in general.

In new holistic approaches, instead of manipulating single genes from the metabolic pathway, researchers are aiming to overexpress transcription factors that activate several biosynthetic steps, but insufficient understanding of the biosynthetic control calls for further rational investigation. The recent discovery of Taximin and its role in taxane metabolism could open a new door to develop holistic metabolic engineering approaches in *Taxus* cell cultures.

On the other hand, due to the high metabolic complexity of plant systems and the difficulty to obtain transgenic material from *Taxus* spp., other strategies could be assayed. The discovery of several fungal endophytes from *Taxus* spp. able to produce taxol was important, although subsequent more in-depth studies revealed that no independent taxol biosynthesis took place in the endophytes [57]. The generation of plant cultures harboring an endophyte might be possible, but there has been little success so far. However, the total or partial transfer of the plant biosynthetic system to other organisms that are easier to manipulate genetically could be a very promising strategy. Thus, developing transgenic bacteria or yeast carrying taxane biosynthetic genes may have several advantages over transgenic plant cell cultures, such as a higher growth index or easier scale-up to industrial bioreactor level. Accordingly, a pioneer study to overexpress genes encoding initial steps in terpene biosynthesis together with TXS and taxadiene-5-hydroxylase genes in an engineered *E. coli* strain led to a production of 1 g L^{-1} of taxadiene and 60 mg/L of taxadiene-5-ol [58]. Both compounds could be used in the semisynthesis of taxol as well as its derivatives.

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Challenges for the Cultivation of Plant Cells on the Example of *Hypericum perforatum* and *Taxus chinensis*

11

Mariam Gaid, Thomas Wucherpfennig, Stephan Scholl, Ludger Beerhues, and Rainer Krull

Contents

Abstract

Medicinal plants are sustainable bio-factories for valuable active pharmaceutical ingredients (API). They are commonly grown in the field and their extracts have a given combination of constituents. There is some variation due to climate

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fluctuations and plant diseases (microbial infections), genotypic changes, soil differences, etc. Additionally, fertile agricultural areas are increasingly limited. However, these variations are undesired because they are non-controllable and can affect the batch conformity of a drug significantly. This is a challenge for producers of phyto-pharmaceuticals, and the variations in the API composition are compensated by mixing extracts from various batches to achieve the required continuous quality of an authorized drug. These drawbacks of field cultivation are overcome by well-defined bioreactor-based cultivation. Biomass growth and API production take place under variable but controllable cultivation conditions, resulting in customized extracts. Variation of the cultivation conditions leads to qualitative and/or quantitative changes in the metabolome. During bioreactor cultivation, plant cells tend to stay connected after division, which leads to the formation of aggregates. The size of shear-sensitive plant cell aggregates influenced by hydrodynamic forces resulting from mechanical agitation was often recognized as an intangible parameter, which might be responsible for general variability in plant cell culture processes. To date, however, the bioreactor approach is not often industrially implemented. This chapter provides an overview of the challenges in the cultivation of plant cell systems, briefly illustrated by (i) research on *Hypericum perforatum* tissue cultures into up-to-date approaches for production of hyperforin and hypericin, possibly functional at a pre-commercial level in the future, and (ii) effects of hydrodynamic mechanical forces on *Taxus chinensis* submerged cultures for production of paclitaxel.

Keywords
Hypericum perforatum · Hyperforin · Hypericin · Biodiversity · In vitro cultures · *Taxus chinensis* · Plant cell aggregates · Hydromechanical stress · Cell viability

1 Introduction – Cultivation of Plant Cells in Suspension: Impact, Issues, and Relevance

Higher plants are an abundant source of bioactive and pharmaceutically important chemicals including drugs such as morphine, codeine, reserpine, and several alkaloids and steroids [1]. The world market for herbal medicines has reached US $ 60 billion, with annual growth rates of 5–15% [2]. Over 60% of anticancer drugs and 75% of drugs for infectious diseases currently used are made or extracted from natural sources [3]. The increasing demand for tailored phyto-pharmaceuticals with innovative active pharmaceutical ingredients (APIs) and activity profiles, produced by ecologically more sustainable bio-factories, and the significant reductions in biodiversity are driving efforts to find alternative ways of producing high-value plant-derived metabolites under well-defined cultivation conditions [4].

The development of plant cell cultures and their cultivation under constant environmental conditions for biotechnological production of valuable secondary metabolites hence results in a constant profile of constituents. This is an attractive economic alternative to the extraction of low amounts, which often occur in whole plant material from field cultivation [5]. Further, advantages of plant cell suspension cultures include inexpensive defined cultivation media, their capability to form complex glycosylation, and finally product safety [6].

Haberlandt undertook the first attempt to cultivate plant cells in 1902 and is recognized as the founder of plant cell cultures [7, 8]. In 1956, the first patent for submerged cultivation of numerous tissues isolated from several plant species, and the possibility of producing oxalic acid and coumarin by such cultures, was filed [7]. Nowadays, plant cell cultures can be generated from virtually any plant through the isolation of plant tissue [9]. Explants are sterilized using chemical treatment and plated on solid growth media containing growth hormones and nutrients necessary for proliferation [10]. Assuming an adequate growth medium composition, the explant proliferates into a callus consisting of dedifferentiated cells, which can be screened and transferred to liquid cultivation medium for the generation of suspension cultures [9]. In this way, plant cell suspension cultures often produce similar concentrations of secondary metabolites as the native plant [11]. Hairy root cultures, which are generated through infection of root cultures with *Agrobacterium rhizogenes,* are an attractive alternative to regular plant cell cultures, as they can stably produce high levels of secondary metabolites and do not require growth hormones [9, 12].

Commercial systems for cultivation of plant cells were established more than 25 years ago; still there has been little progress toward wide application, and only a limited number of compounds are produced on a large scale [13]. A more widespread application seems prevented by low metabolite yields, biochemical and genetic instabilities, low specific growth rates, and difficulties associated with scale-up [7, 14]. For plant cell cultures, the transfer and scale-up from shake flask to bioreactor cultivation is still a big challenge [9]. A unique and often frustrating characteristic of plant cell cultures, in comparison to microbial host systems, is, moreover, their disposition to grow in cell aggregates. After division, plant cells often remain connected and, as a result, form aggregates up to several hundred cells [15]. Therefore, these aggregates can reach sizes up to several millimeters, leading to the formation of heterogeneities due to limitation of the carbon source fed or oxygen within the inner parts of the cell aggregates [16]. During the past years, aggregate size was identified as a key parameter for production performance.

However, despite diffusion limitations, large aggregates were found to be favorable for the production of secondary metabolites [16–18]. Other authors reported beneficial effects of larger aggregates only up to a certain critical size [19, 20]. Large aggregates were also found to be disadvantageous for production of secondary metabolites [15, 21, 22]. The optimal production window to produce valuable secondary metabolites by particular sizes of cell aggregates is apparently a characteristic of a species or a cell line [23]. The average aggregate size of a certain

population also varies over the cultivation time and can affect secondary metabolite formulation. A current research focus is the elucidation of the link between aggregate size and secondary metabolite accumulation. A better understanding of this link and the associated heterogeneities might allow product yields to be maximized by adjusting process conditions [9].

These requirements show the great challenges in the development of scalable biotechnological processes for the cultivation of plant cell and tissue cultures including genetic tailoring approaches. Here, they are illustrated by two examples of phyto-pharmaceutical systems: *Hypericum perforatum* (Sect. 2) as an efficient biotechnological tool to realize a key step for commercialization and *Taxus chinensis* (Sect. 3) as a successful commercial application for paclitaxel production via plant cell cultivation. Section 4 summarizes the main challenges of these promising cell culture systems for producing secondary metabolites of high pharmaceutical potential.

2 *Hypericum*: An Ancient Healer

Among the physicians of ancient times, *Hippocrates* (approx. 400 BCE) was the first one to prescribe *H. perforatum* as herbal remedy for the treatment of depression and infected wounds [24]. Since then, the genus *Hypericum*, which comprises approx. 500 species [25], continuously enriched the worlds of pharmacy and chemistry with diverse collections of extracts and compounds. In recent times, the medicinal value of *Hypericum* has been assured by innumerable researchers. Experiments conducted on isolated animal tissues and animal models as well as clinical trials proved an array of biological activities ranging from antidepressant, neuroprotective, anti-inflammatory, antimicrobial, antitumor, and antiviral activities [26–29]. The most intensively investigated species of *Hypericum* is *H. perforatum* (St. John's Wort; Fig. 1).

Extracts from *H. perforatum* were shown to have antidepressant activity by inhibiting the reuptake of neurotransmitters in synapses [30]. Currently, they are considered as the only herbal remedy that can be used as substituent in the therapy of mild-to-moderate depression [31]. The first commercialization of an extract was in 1992 when *H. perforatum* pills were produced by *Lichtwer Pharma*, Berlin, Germany [32]. The product then shared 27% of the total German market of antidepressant drugs. Since 1997, the aerial parts of the plant are approved by ESCOP as a non-prescribed drug [33, 34]. The herb is the main source of the interesting bioactive compounds such as hyperforin and hypericin (Fig. 2a, b). Hyperforin mainly accumulates in flowers (Fig. 1b) and unripe fruits (6–8%), while hypericin is predominantly present in small buds and just opened flowers, especially stamens (0.3–1%) [35, 36]. The name of the plant refers to the perforated appearance of its leaves and flowers due to characteristic translucent oil glands, the main storage organelles for hyperforin (Fig. 2a, c) [37]. Hyperforin is the prominent component for the elevated

Fig. 1 *Hypericum perforatum*. (**a**) Entire plant; (**b**) blossom

mood effect and the antidepressant activity of the extracts, as demonstrated by controlled clinical trials [38]. It is also an attractive API for the treatment of atopic skin [39]. Hypericin is a naphthodianthrone, which results from photoconversion of protohypericin and occurs in dark nodules of leaves and flowers (Figs. 1 and 2b) [40]. It is considered as attractive adjuvant in photodynamic diagnosis and therapy, commonly used to treat precancerous cells and sun-damaged skin [41]. Hypericin was commercially introduced in the global pipeline as a photosensitizer in cutaneous oncologic diseases [42]. Although *H. perforatum* extracts are popular in the German market and have been used for decades as a safe remedy, treatment should be done under qualified medical guidance due to the reported drug interference potential [43]. Drug-drug interactions are most likely due to the ability of *H. perforatum* extract to induce certain isoenzymes of the CYP450 and P-glycoprotein families, which are responsible for the metabolism and excretion of a series of pharmaceutical compounds [44], resulting in reduced efficacy of the co-medication. Furthermore, phototoxicity was reported after topical application of hypericin-containing cream to fair-skinned individuals and unhealthy skin and after extended solar irradiation [45].

2.1 Variability Among Commercial Preparations

Hypericum extracts are natural sources of medicines with various bioactivities [46]. The contents of hypericin and hyperforin (Fig. 2a, b) were extensively studied as biomarkers of the extracts. The quantity and quality of protohypericins and hyperforins are affected by biological and environmental factors, such as age, plant tissue, and harvest season [47–49]. Insects and herbivory were reported to affect both the hypericin and hyperforin levels by 30–100% variation [50]. Similar fluctuation was observed for the hyperforin content among various plant lines (0.3–1.3%) [51]. However, dramatic decreases in temperature and light intensity

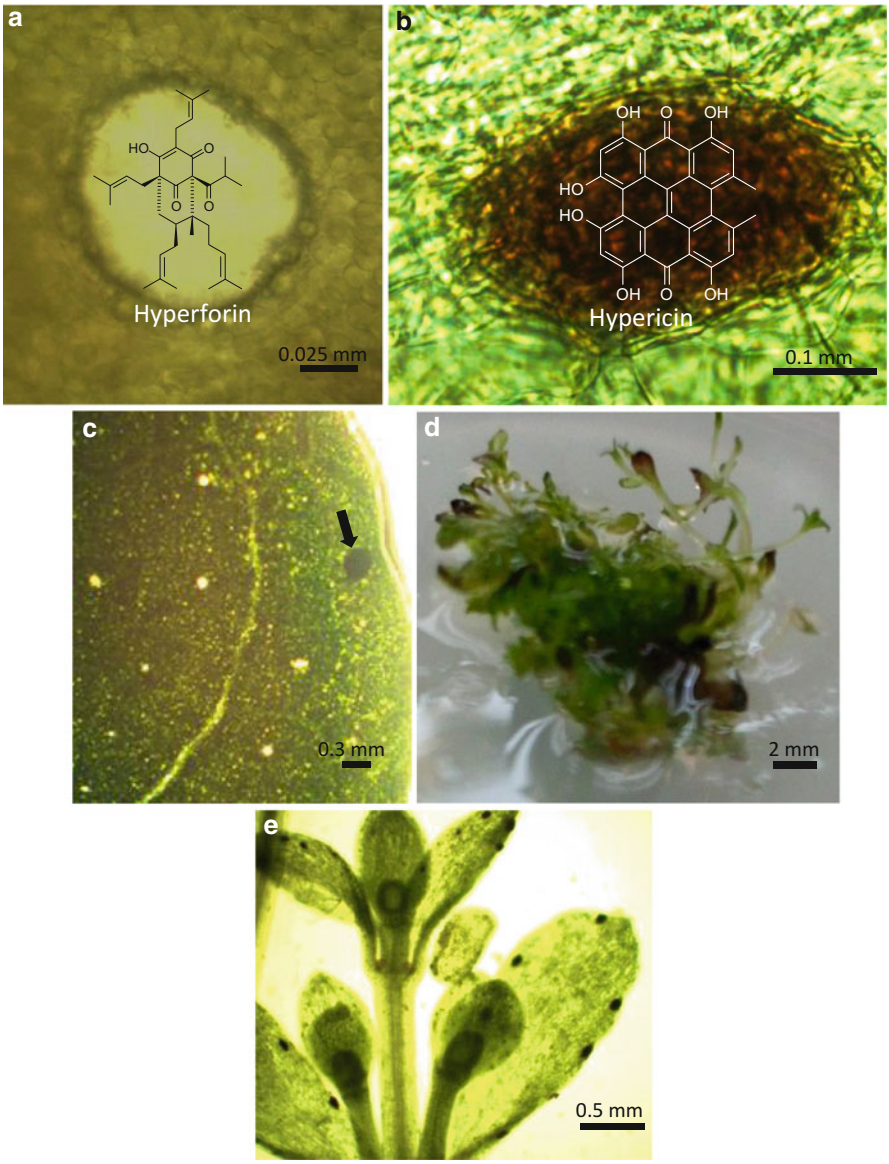

Fig. 2 Details of *H. perforatum* leaves. (**a**) Translucent gland containing hyperforin, (**b**) dark nodule containing hypericin, (**c**) leaf section with dark nodule (*black arrow*) and translucent glands spread over the lamina (3 weeks after transfer to new medium), (**d**) *H. perforatum* in vitro shoot cultures, and (**e**) shoot with dark nodules in the leaf margin (3 days after transfer to new medium)

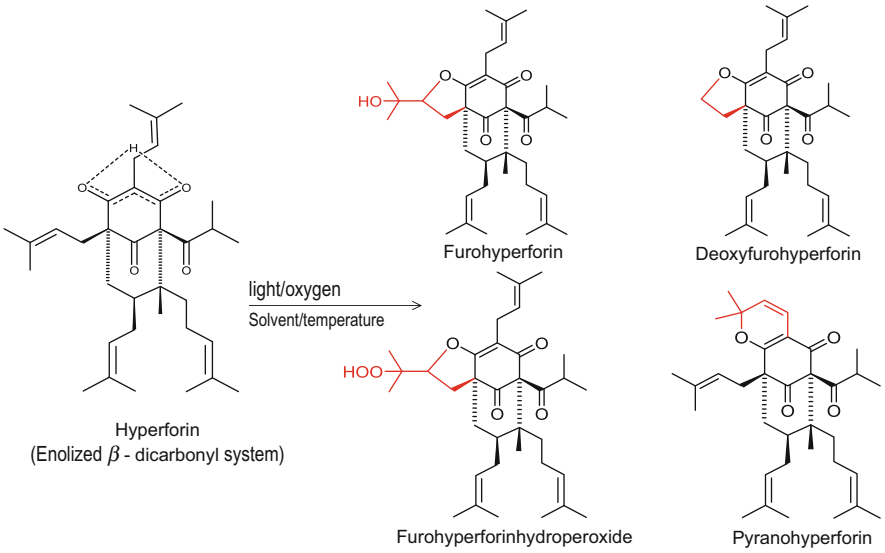

Fig. 3 Hyperforin and its oxidation analogues [59, 62, 68, 69]

negatively affected both hypericin and hyperforin accumulation [52, 49]. Moreover, hypericin accumulation was influenced by the nitrogen availability [52]. The variability of the metabolites contents in field-grown plants affects the standardization process of the extracts produced. Extraction conditions and downstream purification processes play an important role in the final phytochemical profile of the biomarkers among commercial preparations [53–58]. Naturally occurring protopigments (protohypericins) in the extracts are totally photoconverted to their corresponding hypericins. Furthermore, hyperforins are sensitive to oxygen, light, and temperature (Fig. 3) [59–61]. These drawbacks dramatically affect the bioactivities of the extracts and make standardization an obvious hurdle [58, 62]. The current quality requirements for the internal use of *H. perforatum* extracts as antidepressants apply to hypericins (0.1–0.3%), hyperforins (up to 2%), and flavonol glycosides (6–12%) [58]. Many studies confirmed that most labels overestimated the hypericin content (7.7–38.6% of the labeled claim) in commercial *H. perforatum* products, which were standardized to contain 0.3% hypericin [57, 63]. Furthermore, batch-to-batch variation in the hyperforin content was observed for some, but not all, products on the German market [64]. A dicyclohexylamine (DCHA)-derived salt strategy was used to stabilize pure hyperforin, and the weak salt was reported to retain the same pharmacological activities by release of hyperforin in solution [65, 66]. Recently, the DCHA stabilization strategy was also used to enrich and stabilize the hyperforin-rich extract prepared from *H. perforatum* root cultures, and DCHA-treated extracts were fairly stable and storable as dried residue for months [67].

2.2 Conservation of Biodiversity via In Vitro Cultures

The need of standardized extracts on the one hand and biodiversity, variability in quality, and contamination issues of field-grown plants on the other hand prompted researchers to undertake numerous in vitro trials [70–75]. Plant cell and organ cultures provide valuable and renewable sources of API-containing extracts. The possibility of quantitative and qualitative control of their metabolic profile is an important aspect for the production of standardized formulations making plant tissue cultures a promising alternative to the conventional field production. Due to the medicinal value of *H. perforatum* constituents, various attempts were made to establish in vitro cultures with confirmed production of hypericins and hyperforins. Unorganized cell suspension cultures (Fig. 4) were generally inferior in the accumulation of these metabolites, compared to differentiated tissue such as shoot (Fig. 2d, e) and root cultures (Fig. 5e). In some studies, the application of elicitors influenced the production of API. For example, the hypericin content of methyl jasmonate-treated adventitious roots was 1.68 mg g^{-1} DW, which is six times that of 1-year-old field-grown plants [76]. Non-treated root cultures accumulated only 0.04 mg g^{-1} hypericin [77]. Due to the lack of differentiation in cell suspension cultures, the treatment with pathogenic *Colletotrichum gloeosporioides* failed to enhance the accumulation of hyperforin and hypericin, while its application to stem and leaf tissues led to detectable accumulation of hyperforin (4 mg g^{-1}), hypericin (0.018 mg g^{-1}), and pseudohypericin (0.035 mg g^{-1}) [71, 78]. The developmental stage of shoot cultures has an effect on the metabolite profile. For instance, young shoots (3 days old) show tiny dark glands with measurable hypericin content (Fig. 2e), while the translucent glands differentiate later (Fig. 2c). Many studies used *H. perforatum* roots to regenerate a constant source of plant elite material for micropropagation and establishment of stable cultures [79, 80]. Recently, hyperforin was detected in auxin-induced root cultures [67]. The extraction yield at the flask scale was 5 mg g^{-1} DW with a productivity of 50 mg L^{-1} culture (Fig. 5). In contrast to hyperforin, seco- and adsecohyperforins are rare analogues, which exclusively accumulated in in vitro cultivated shoots [81]. Detection of these analogues during the cultivation period of root cultures is a promising finding

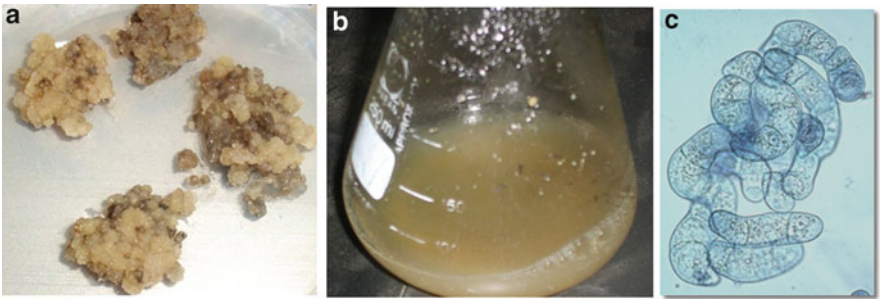

Fig. 4 Undifferentiated cell cultures of *H. perforatum*: (**a**) callus, (**b**) cell suspension culture, (**c**) cell cluster

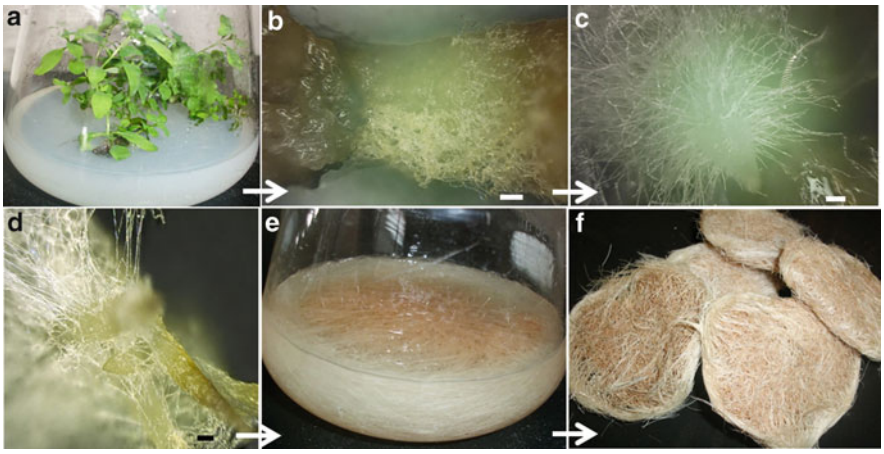

Fig. 5 Establishment of auxin-induced root cultures of *H. perforatum*: (**a**) in vitro plantlets, (**b**, **d**) callus and root formation in auxin-containing medium, (**e**) root cultures, (**f**) freeze-dried root biomass (bars = 100 μm)

to shed light on their biological activities [67]. In these root cultures, all prenylated acylphloroglucinols accumulated concomitantly to growth, suggesting that continuous extraction from actively growing cultures is possible. Beside elicitation, feeding of precursors is a well-studied strategy to enhance the production of specific metabolites if the endogenous level of the precursor is the limiting factor for the respective biosynthesis. The level of a hyperforin analogue, adhyperforin, was increased 3.7-fold after feeding of the precursor amino acid isoleucine to *H. perforatum* shoot cultures [82]. However, valine as a precursor of hyperforin showed no stimulating effect, suggesting that this amino acid is no limiting factor in hyperforin biosynthesis.

Bioreactors represent a key element toward the commercial production of in vitro-produced phyto-pharmaceuticals. Many reactor types were reported as tools to enhance the economic production of the target product from plants including mechanically stirred bioreactors, pneumatic homogenized bubble column and airlift reactors, fluidized bed, biowave devices, and disposable bioreactors [83]. The choice of the bioreactor mainly depends on the type of the in vitro culture, the required light conditions, and the pattern of target product accumulation [84]. A 500 L airlift bioreactor has recently been used to cultivate *H. perforatum* roots for the production of hypericin; the presence of hyperforin was not reported [77]. For biomass acquisition, balloon-type airlift bioreactors were superior to horizontal drum type. Among metabolites detected in *H. perforatum* in vitro cultures, only small portions were secreted to the extracellular medium [85, 86]. For instance, no hyperforins were detected in the culture medium of *H. calycinum* cells that primarily accumulate adhyperforin (0.25 mg g^{-1} DW) [87]. Metabolite accumulation inside the cells requires harvesting and cell rupturing followed by extraction. An alternative to cell rupture is the in situ product removal from the cells or culture medium through a two-phase culture technique [88].

2.3 Cryopreservation

In general, cell totipotency is sufficient for whole plant regeneration and in vitro propagation. However, some species were reported to be recalcitrant to regeneration and growth in vitro. Cryopreservation provides a vital alternative to maintain the biodiversity among species and populations and to reduce the individual phytochemical variability. Urbanová et al. [89] developed a cryopreservation protocol for in vitro grown *H. perforatum* meristems. Using a modified protocol, the post-cryogenic survival rate (34%) was genotype dependent [90]. In the same year, 59–71% recovery rate was recorded by *Bruňáková* et al. [91], which was proportionally related to the incubation time of abscisic acid-treated explants in a specific plant vitrification solution. Long-term elite germplasm preservation should be confirmed through the stability of both genetic and phytochemical features. For instance, chromosomal inspection and hypericin analysis in cryopreserved *H. perforatum* meristems revealed the same features as unfrozen control [89, 92]. In general, cryopreservation offers a chance of selecting target genotypes with desired properties and in vitro culture material for biotechnological studies and stable metabolite production [93].

2.4 Transgenic *Hypericum* as Stable Source of Phytomedicine

The pharmacological value of *H. perforatum* extracts is mainly due to the biosynthesis of hyperforins and hypericins. Genes involved in the biosynthesis of these therapeutically valuable compounds are important targets for genetic transformation [94]. Although *H. perforatum* is highly recalcitrant to *Agrobacterium tumefaciens*-mediated genetic transformation [95], hairy roots were successfully induced by *A. rhizogenes* T-DNA transfer. The ability of hairy roots to grow without the application of exogenous phytohormones makes the upscaling in bioreactor systems cost-effective. Many researchers have issued *H. perforatum* hairy root cultures; however, neither hypericin nor hyperforin was traced in these records [96, 97]. Since hyperforin and hypericins mainly accumulate in the aerial parts of the plant, many reports focused on shoot regeneration from established transgenic hairy root cultures [72, 98–100]. However, the main objective is not yet completely realized due to the low transformation efficiency and the poor information about the genes involved in the metabolic pathways.

3 Production of Paclitaxel by Submerse Cultivation of *Taxus chinensis*

The yew is one of the most fascinating trees, botanically notable, and culturally with rich references in our social and historical life [101]. *Taxus* originates from the greek term *tóxo* for bow [102], which gives an indication of the most common uses of this tree's timber. Because of the use of its wood in long- and crossbows, the yew is predominantly associated with England [102]. The heavy use of yew wood for

purposes of war soon led to a serious shortage. A vast network of trade relations for extraction and supply of yew wood was established all over Europe, to satisfy the high demand for this resource [101, 102]. As a result, the yew tree became almost extinct in central Europe, and up to this date, yew trees are scarce.

Even *Julius Caesar* describes in *de Bello Gallico* a local chieftain who avoids capture through suicide by consumption of *Taxus* needles [103]. However, ingredients from the yew were not only used as poison but also as medication. Historical literature shows that the medieval physician *Avicenna* (980–1037) used a herbal drug extracted from *Taxus baccata* as a cardiac remedy [104]. Recently, alkaloid mixtures (taxines) from yew trees have been demonstrated to possess calcium channel blocking activity. So, it is evident that *Avicenna* used a drug with calcium channel blocking activity much earlier than the arrival of commercial drugs belonging to the same pharmacological group [104]. In the Central Himalayas, the plant has been used as a treatment for breast and ovarian cancer for a very long time [105]. In most European countries, yew leaves had a popular use as an abortifacient [104]. Additionally, extracts from *T. baccata* were used in the treatment of asthma [106]. It was not until 1962 that *Taxus* samples were tested for their biological activity by *Barclay*. By 1964, it was discovered that yew extract inhibited cancer cells [107]. The inhibiting substance was isolated; however, purification of 0.5 g of pure paclitaxel from the pacific yew *Taxus brevifolia* took about 2 years [108].

T. chinensis plant cells are a major source for the antitumor agent paclitaxel with a broad spectrum of antitumor activities [107]. The sophisticated structure of this diterpenoid was elucidated and published in 1971 [109]. In 1979, *Horowitz* explained the mechanism of action of the drug on cancer cells [107]. Paclitaxel stabilizes microtubules and thus blocks the necessary reorganization for mitosis [107]. It prevents cell reproduction and works predominantly on quick-dividing cells, like tumor cells [107]. Paclitaxel was released in 1984 by the FDA for clinical phase I tests [107]. It was approved for marketing as an anticancer agent in 1992 by *Bristol-Myers Squibb* and in 1998 was the best-selling anticancer drug in history with $1 billion in commercial sales [9, 110]. Paclitaxel is used for the treatment of ovarian, breast, and lung cancers as well as AIDS-related Kaposi's sarcoma [9]. A quantity of 340,000 kg of *Taxus* bark, equivalent to 38,000 trees, is required to meet the 25 kg per year demand for the anticancer drug paclitaxel [111]. The growing demand for the drug could potentially exceed 200 to 300 kg per year as applications are being developed for paclitaxel in the treatment of Alzheimer's and post-heart surgery patients [9, 112].

An obstacle to overcome is the generally low concentration (0.001–0.05%) of paclitaxel found even in the most productive species. Thus, considerable energy was invested in trying to increase the extraction yield [113]. Extraction requires a complex system and specific purification techniques using advanced and expensive technology [113]. The harvest depends upon seasonal variability and the slow growth rate of the tree, making it nearly impossible to keep up with demands [13]. Some effort has been undertaken to establish ecologically sustainable harvesting protocols of yews in natural habitats [113, 114]. Likewise, a plantation of yew trees has been established in Yunnan, China, by the company *Yewcare*,

making it the largest yew tree producer in the world [113]. Since yew trees take over a 100 years to yield good amounts of paclitaxel [107], the planting of trees seems to be a far-fetched and sustainable investment.

To prevent the extinction of *Taxus* sp. and decrease processing costs, alternative paclitaxel production methods were investigated [9]. Chemical synthesis is possible, but very complex, as it requires 40 synthesis steps with only 2% yield [107, 115]. Chemical semi-synthesis can be applied to use paclitaxel precursors, which are present in amounts up to 0.1% in *T. baccata* [116]. Semi-synthesis requires 11 chemical transformations using 13 solvents and 13 organic reagents, making it expensive and environmentally unfavorable [117]. The cost of paclitaxel through semi-synthetic means can be decreased to 25% of that for natural harvest [118].

Nevertheless, the production in plant cell cultures provides an alternative and environmentally sustainable source of paclitaxel. This methodology offers several advantages, not being subject to weather, season, and contamination, and the material can be grown independently of its original, potentially remote location [113, 117]. Plant cell cultivation of *Taxus* cells is not only environmentally sustainable but also economically reasonable, as it reduces process costs to just 20% of that for natural harvest and is therefore even cheaper than semi-synthesis of paclitaxel [118]. Currently, *Phyton Biotech GmbH*, Ahrensburg, Germany, is the largest producer of paclitaxel via plant cell cultivation.

3.1 Particle Size Measurement of Suspended Plant Cells and Aggregates

A more widespread application of plant cell cultivation seems to be prevented by low growth rates and difficulties associated with scale-up, which are related to the disposition of plant cells in submerse cultivation to form aggregates of up to several hundred cells. Even if it remains unclear whether large aggregates or small plant cell aggregates are desirable for an optimal process, and this might very well depend on the scale, the cell line and the product, aggregate size, in general, seems to have a pronounced effect on secondary metabolite production. The analysis of aggregate size as a process variable seems therefore feasible, and it has already been discussed in a recent study [15]. However, particle size is not a bioprocess variable that can be measured easily. While there are a handful of methods for particle size analysis, only very few are applicable for plant cell aggregates.

So far, plant cell aggregate sizes were often measured by mechanical sieving analysis [119]. While this method is considerably easier, it has the potential to alter the aggregate size distribution [120] and is prone to generating erroneous results. Recently, the Coulter counter principle, based on the electrical resistance pulse seizing, was used as an alternative method for plant cell aggregate characterization [14]. This method requires a dilution and has a measuring range between 50 and 2,000 μm. It is therefore not suitable for measuring single cells within the cultivation broth.

Further methods for plant cell aggregate analysis include image analysis and the *focused beam reflectance measurement* (FBRM). FBRM is a size-determining method previously used for plant cells [6, 121] or fungal systems [122]. This method is an optical technique based on the detection of backscattering laser light. The duration of reflectance of a particle passing a laser corresponds to the chord length of a particle. A photodetector converts the reflected light signal into an electronic signal, and the duration of backscattering, multiplied by the scan speed of the laser, results in a chord length [123]. For nontransparent spherical particles, the chord length is equivalent to the object's diameter. However, for near-transparent objects like plant cell aggregates, it is not as straightforward, as chord length is also a function of particle shape, opaqueness, and surface properties. Here, chord length correlates with particle diameter but is not equal to it [121].

Image analysis techniques allow for the identification of aggregate size and have the advantage of enabling the identification of further morphologic parameters, like circularity, roughness, and elongation, which might provide an overall culture performance. The process of image analysis is mostly divided between sample dilution, image acquisition by microscope, and the more or less automated step of image analysis [22]. While providing high resolution for single cells, estimation of means for the whole culture remains troublesome. Due to the usually very laborious process and exceptionally small throughput, it is hard to obtain statistically valid results by application of this method [14]. To cope with this disadvantage, a minimum of analyzed objects based on a "running average" was suggested by *Pearson* et al. [124] to achieve a statistical robustness.

A new method to analyze the size of biological aggregates is the laser diffraction technique, also known *as low-angle laser light scattering* (LALLS). This method works on the basis of particles of a given refraction index interacting with a laser beam passing through an inverse Fourrier-type lens. The angle of the diffracted laser beam is hereby inversely and the intensity of the diffracted laser beam directly proportional to the particle size. [125–127] have introduced the laser diffraction technique to characterize spore aggregation and pellet size of *Aspergillus niger*. This technique was also used for size determination of *Streptomyces coelicolor* cell clumps and pellets [128]. In association with plant cell systems, laser diffraction has been previously used to study the aggregation of rubber particles of *Hevea brasiliensis*, which are sized between 0.3 and 6.7 μm [129]. Because of the very broad measuring range of 0.02–2,000 μm, an application of this method for whole plant cell aggregates seems feasible.

Wucherpfennig et al. [5] compared the most common microscopic image analytic approach to the laser diffraction technique to characterize aggregate size of *T. chinensis* cells (Fig. 6).

Laser diffraction was found superior to microscopy and image analysis, which had a tendency to underestimate the aggregate size up to 20%. While microscopy and image analysis might indicate that a median *Taxus* aggregate after 6 days of cultivation is made up of 100 cells, laser diffraction measurements in contrast indicated a median cell number of 200 cells per aggregate (Fig. 7) [5]. These differences can be explained by the cubic relationship between the characteristic

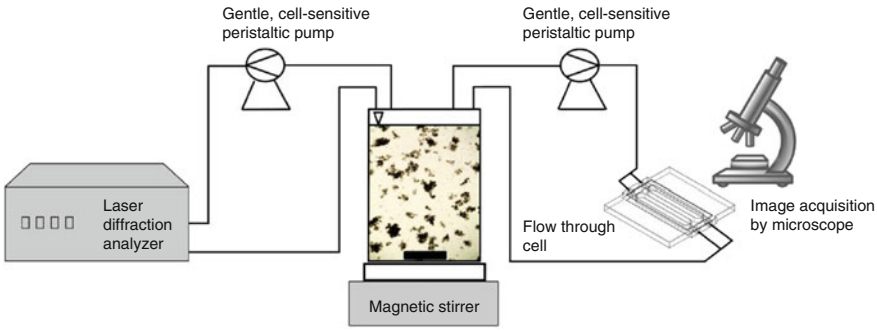

Fig. 6 Setup of equipment for continuous measurement of plant cell aggregate size. The flow through cell for microscopic image acquisition is made of two chambers of 45 × 8 mm with a 2 mm orifice. The cavities are covered by two removable 24 × 60 mm microscopic cover slides [5]

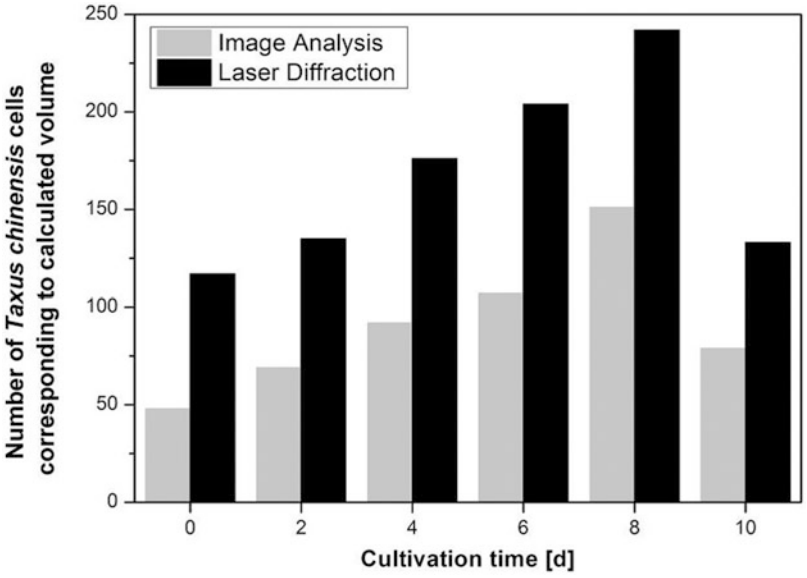

Fig. 7 Number of *T. chinensis* cells corresponding to the median aggregate diameter determined by both laser diffraction and image analysis, assuming a median cell diameter of 80 μm [5]

length and the volume of an aggregate, which should be considered when comparing number distributions (generally by image analysis) and volume-based distributions (generally by laser diffraction). So, while image analysis favors smaller particles and produces lower values, laser diffraction is prone to overestimate larger particles, leading to higher values for mode and median [5].

The advantage of the laser diffraction technique in comparison to image analysis is the quickness of the measurement and, most important of all, the improved

statistical evaluation of measured data. This seems a practicable, rapid, robust, and reproducible way for quality control or production purposes [130].

3.2 Mechanical Stress Sensitivity of Plant Cells

For the commercially established process of paclitaxel production with *Taxus* plant cell cultures, the size of plant cell aggregates was often acknowledged as an intangible parameter, which might be responsible for general variability in plant cell culture processes. In this chapter, hydromechanical stress on plant cells will be reviewed as an important parameter for this industrially relevant phyto-pharmaceutical production process.

The effects of stirrer- and pneumatic-induced hydromechanical stress on various kinds of living cells in suspension were studied intensively [131, 132]. Plant cells were commonly regarded as extraordinarily mechanical stress sensitive because of their relatively large size, rigid cell wall, and large vacuoles [131, 133]. The theory of isotropic turbulence by *Kolmogorov* provides an elucidation of why plant cells are more susceptible to mechanical stress introduced by the hydrodynamic turbulent environment than other cells [134, 135]. In this theory, the turbulent flow in the bioreactor is characterized by a large primary vortex. The eddies roughly correspond in size to the diameter of the stirrer in a stirred tank reactor and/or the diameter of the rising gas bubbles in pneumatic bubble columns or airlift bioreactors. The primary vortices are unstable and tend to fall apart. They transfer energy to smaller eddies [136]. The energy transfer takes place gradually, and the information with respect to the direction of the vortex is lost. Isotropic turbulence represents the state when the movement of eddies and the transfer of kinetic energy in all directions at all points in the flow field of the reactor are equal. Thus, the energy transfer is statistically independent of the direction. The smallest eddies in a turbulent isotropic flow field are called *Kolmogorov* eddies. These micro vortices cannot break down to smaller eddies but dissipate into heat energy [135]. Cells in suspension are strained when the diameter of the vortices is equal to the diameter of the cell. Since plant cells, which often occur in aggregates, are relatively large structures, they can easily interact with these eddies. Therefore, plant cells are very sensitive to hydrodynamic stress [119, 131].

A possible positive effect of hydromechanical stress on a plant cell production system is the stimulation of secondary metabolite production; furthermore, cell metabolism and gene expression can be promoted [131]. However, mechanical stress was mostly regarded as negative for the survival of plant cells. Mechanical stress may have a negative impact on cell growth, metabolism, and cell-cell organization in aggregates. Several symptoms indicate cell damage. The most prominent one is often a change in cell morphology, e.g., by reduction in aggregate size [137–140]. Moreover, hydromechanical stress can also lead to the release of intracellular substances, which can lower the pH of the suspension [141]. Changes in the metabolism and product yields are also common [131, 142]. As a final result, a loss of viability is mentioned, which may lead to cell death. In many cases, however, the

cells are able to repair the damage [131]. With increasing hydrodynamic stress, the ability of regrowth, the cell membrane integrity, and the respiratory activity decrease [119, 134, 141].

Furthermore, plant cells are able to adapt to hydromechanical stress after they have been exposed to shear stress for a certain time [143]. They can develop a kind of robustness against hydrodynamic disorders [131]. A possible explanation for the mechanical stress tolerance is the ability of plant cells to repair cell wall damages through disposal of polysaccharides like callose [137]. Other cells have the ability to compensate for shear damage through increased growth [141].

Different plant cell lines show considerable differences in shear susceptibility [144]. Variations in the shear sensitivity in different cell lines are most likely due to the different morphological characteristics and general stability of the cell wall [134, 145]. The culture age, the cultivation conditions, and also the cultivation history were identified as factors affecting hydromechanical stress sensitivity [137, 141]. In various experiments, it was found that cultures of the late exponential and early stationary growth phase are most susceptible to damage caused by hydromechanical stress [119, 131]. The high stress sensitivity was argued to be related to the increase in aggregate size during this time [119].

Also the color of the callus was previously found to correlate with variability in cell growth. Calli were found to produce more paclitaxel at an old age with brown coloration than at a younger age without the brown stain [146–148]. Generally, *Taxus* aggregates are observed to get larger and to increase in brown pigmentation with time (Fig. 8). Thus, general viability and dark/brown coloration very likely correlate. A large grayish-brown area is distinctive for either inactive or dead cells, since aggregates with low viability have a large dark area, whereas healthy aggregates show only a small or no dark area.

Determination of the healthy to dead cell area ratio by image analysis allows to determine the percentage of dead cells, which well correlated with the *T. chinensis* viability measured by the Alamar Blue assay. Figure 9 shows the relationship between the dark cell area and the percentage of living cells,

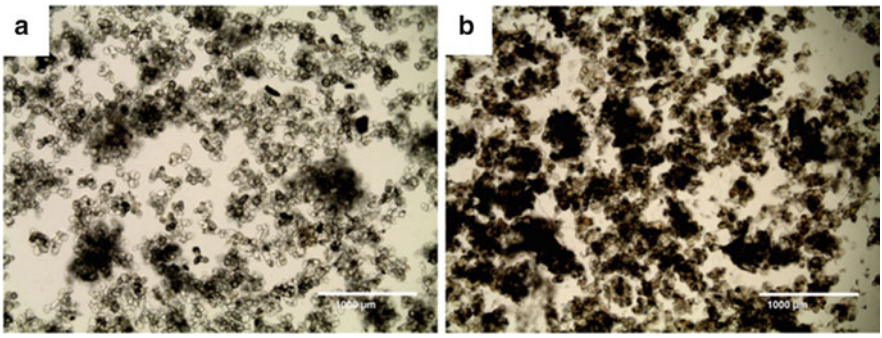

Fig. 8 Micrographs of *Taxus chinensis* cells after 3 h (**a**) and 48 h (**b**) of shear stress exposure. The scale corresponds to 1,000 µm [149]

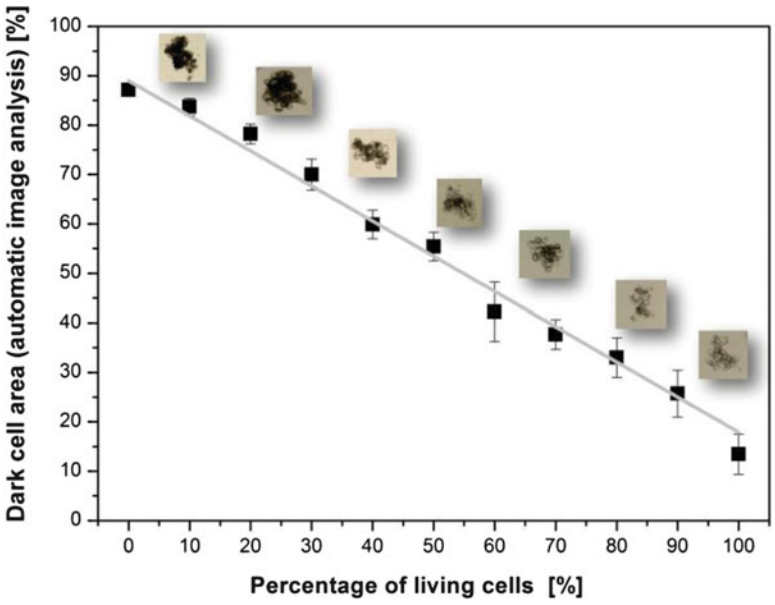

Fig. 9 Relationship (R2 = 0.99) between dark cell coloration determined by automatic image analysis and *T. chinensis* cell viability measured in the AB assay. Values are means of three replicates of 5-day-old shaking flask samples (adapted from [149])

indicating a clear linear relationship. The significant reproducible behavior between cell coloration and viability allows to estimate cell viability solely from morphological appearance [149].

4 Conclusions

Production of metabolites via plant cell suspension cultures is a renewable, environmentally friendly, and economically feasible alternative to extraction from whole plant material. Advancements have been made in understanding metabolite production in plant cell cultures, but there is still a high optimization potential to increase the final product titer and to transform these processes into the industrial scale. This chapter has provided an overview of the challenges in the cultivation of plant cell systems with respect to two applications, *H. perforatum* and *T. chinensis*.

The growing market demand for *H. perforatum* preparations led to a number of global studies focusing on the levels of secondary metabolites, which accumulate in field-grown plants. Ontogenetic, morphogenetic, and environmental factors play an important role in the variability of the valuable biomarkers hypericin and hyperforin, possibly functional at a pre-commercial level in the future, and hence in the chemical diversity of *H. perforatum* extracts. Cryostorage of apical meristems and roots offers the chance of preserving somaclonal variants with unique properties. Improved plant

cell culture technology for selected cell lines provides promising systems for producing metabolites of complex biosynthetic pathways. Combining the scalability with the approach of genetic tailoring offers an efficient biotechnological tool to realize a key step for commercialization.

In the second example, *T. chinensis* plant cell aggregate size was identified as an important parameter for industrially relevant plant cell processes. Aggregate size was influenced by stirring-induced hydrodynamic mechanical forces. Excessive shear forces led to lysis and significantly reduced the viability and productivity of plant cells. From the process engineering point of view, a fundamental impulse into the plant engineering community for the development of new sophisticated low mechanical stress-inducing stirrers and if possible even more important bioreactors is still missing. This gap shall be filled by identifying the key parameters to provide unique relationships between productivity and shear forces, which is also true for other biotechnological processes using filamentous microorganisms, e.g., enzymes of *Trichoderma* sp. as key players in modern biorefineries.

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Mass Production of Artemisinin Using Hairy Root Cultivation of *Artemisia annua* in Bioreactor

12

Nivedita Patra and Ashok K. Srivastava

Contents

Abstract

Malaria is endemic disease of the tropical countries primarily due to their specific climatic conditions. Artemisinin has been widely used for the treatment of patients of cerebral malaria in combination therapy with other antimalarial drugs such as

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quinine and chloroquine. It has been extracted from the leaves of the plant *Artemisia annua* which grows naturally in many countries except for humid tropical countries. However, yield of the drug from dry tissue has been in the range of 0.01–0.5%; with the result, a 1000 kg of dry plant leaves yield only 6 kg of artemisinin after solvent extraction and liquid chromatography-based purification protocols. In order to alleviate these problems, scientists have been exploring alternate in vitro production protocols particularly by plant cell/hairy root cultivation of *A. annua* to supplement the overall artemisinin availability. Hairy root cultivation could be one of the potent in vitro alternative production techniques for artemisinin as it has an inherent advantage of better biochemical stability and less doubling time than plant cell cultivation. The present report attempts to provide a comprehensive overview of mass production of artemisinin particularly in vitro production using various bioreactors and different cultivation modes.

Keywords
Artemisinin · Hairy root culture · Gas-phase bioreactors · Liquid-phase bioreactors · Mathematical modeling

1 Introduction

The drug artemisinin or "qinghaosu" was first reported to treat malaria patients in China in 1979 [1]. It is produced from the plant *Artemisia annua.* Artemisinin and related compounds have unique structure which plays an important role in their mode of action against the deadly parasite *Plasmodium falciparum*. The biochemical mode of action of artemisinin involves bio-activation of the drug by free intracellular iron as well as heme derived from lysed red blood cells. This activation is necessary for the covalent binding of activated artemisinin to macromolecules, such as transporter proteins, iron-sulfur proteins, and translationally regulated tumor protein homolog of the malarial parasite, which finally leads to the destruction of the parasite [2–4]. Artemisinin degrades within a few hours in the human body; therefore, it has to be readministered more number of times daily. Moreover, artemisinin needs to be derivatized to artesunate, artemether, and dihydroartemisinin in order to be more effective during combination therapy of malaria. As a result, the cost of this drug is high and demand is even higher [5]. Large-scale production of the drug artemisinin using plant cell/hairy root cultivation has been indicated to be a promising alternative production protocol to ensure sustained availability of this drug.

1.1 Structure and Properties of Artemisinin and Its Derivatives

Structurally artemisinin can be classified as a sesquiterpene lactone that contains an endoperoxide bridge. Artemisinin and its derivatives have been reported to be effective in combating all the stages of malarial parasite. The key category of

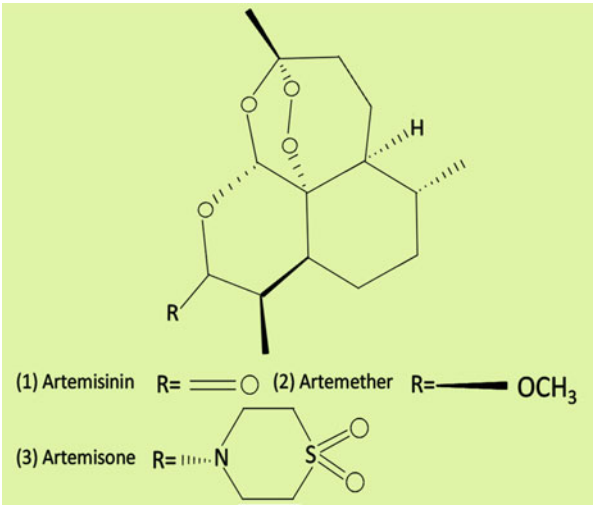

Fig. 1 The molecular structure of artemisinin and its semisynthetic derivatives

antimalarial drugs includes artemisinin and its semisynthetic derivatives [6]. The chemical structure of artemisinin and its derivatives has been depicted in Fig. 1.

Direct precursors of artemisinin such as dihydroartemisinin and other semisynthetic derivatives (artemether and artesunate) have been used as effective antimalarial drugs. Recently, artemisone has been reported as semisynthetic artemisinin which is less toxic and more effective. The water-soluble derivative of artemisinin (artesunate) has been one of the widely used antimalarial drugs. It is primarily due to the endoperoxide ring of artemisinin class of compounds which have significant antimalarial activity [7]. The drug has been chemically synthesized by a series of ten reactions, but the process had been reported to be uneconomical and wasteful [7]. The reduced Fe^{+2} heme complex activates artemisinin to form a highly reactive free radical. The free radical interferes with essential protein metabolism of malarial parasite in the human body [8]. The dosage of the drug that has a curing rate of 99% has been about six tablets (20 mg artemether, 120 mg lumefantrine) in 60 h [9]. The method of administering six tablets of artemether and lumefantrine is a highly effective method to combat *Plasmodium falciparum* malaria [10]. In the present scenario, adequate supply of artemisinin is a major challenge as the only source of artemisinin at the moment are plant parts such as leaves and flowers of *Artemisia* plant species. Besides the content of artemisinin in different varieties of *Artemisia annua* has been reported to be as low as 0.01–1.1% [11], and complete chemical synthesis of artemisinin is not yet established [7, 12].

1.2 Natural Source for Artemisinin Production

The extract of qinghaosu (green herb) or *Artemisia annua* has been the major source of artemisinin so far (Fig. 2). The herb of Chinese origin has been reported

Fig. 2 Plant source of artemisinin *Artemisia annua*, IIT Delhi (India) Garden Nursery

in treating fever since 340 AD. The artemisinin content in the most wild plant varies from 0.01% to 0.4%. However, some high-yielding varieties can even produce up to 1% artemisinin [13]. The critical photoperiod for artemisinin production by *A. annua* is about 13.5 h [14]. The cross-pollinated plant is from Compositae family. Various plant-breeding techniques have been extensively applied to develop high-yielding varieties of *Artemisia annua*. However, the maintenance of high-yielding varieties has been difficult due to cross-pollination by birds and by wind. As a result, the development of alternative in vitro production techniques has become essential. Cultivation of plant cell/callus of *Artemisia annua* under in vitro conditions for several generations has the disadvantage of genetic instability [15], and cultivation of plant cells in bioreactor has an inherent limitation of shearing by moving parts (agitator) of the bioreactor which leads to loss of cell viability. Hairy roots, on the other hand, are genetically stable for several generations, are fast growing, and are able to grow even in hormone-free media. It is therefore desirable to focus on in vitro propagation of hairy roots for artemisinin production. Induction of hairy root is done by genetic transformation of plant parts of *A. annua*, (explants) by infection of *Agrobacterium rhizogenes*. Hairy root tissues are not so fragile, are shear tolerant, and can easily produce secondary metabolites equivalent or even more than the parent explants used for transformation [16]. Several uses of hairy root have been reported. It can be used for heterologous protein production [17]. Hairy root cultures can also serve as a tool to study gene silencing using RNA_i (RNA interference) protocols. By the help of somatic embryogenesis, whole transgenic plants can also be generated from the hairy roots [18]. In general, hairy root cultivation can serve as a useful alternative production protocol for secondary metabolite production in a bioreactor which could be much better than large-scale bioreactor cultivation of *A. annua* for mass production of artemisinin [19].

2 In Vitro Plant Cell/Hairy Root Cultivation for Mass Production of Artemisinin

Plants are known to provide us diverse products of human need. Plant-based natural products have been used since ancient times. It includes the active molecules useful for the treatment, diagnosis, or cure of any malady(ies) and can possibly act as a constituent of medicine [20]. It has been indicated that the crucial Chinese medicinal plant *Artemisia annua* has been used worldwide for the treatment of cerebral malaria caused by the parasites *Plasmodium falciparum* and *P. vivax*. However, the limitation of using *Artemisia annua* as chemical factories is the yield of artemisinin in this plant which has been significantly low; therefore, huge amount of biomass is required to get enough amount of the drug for diagnosis and complete cure. Traditional malaria has been treated using quinine which has to be extracted from the bark of *Cinchona* since ages. Lately, the Chinese weed *Artemisia annua* had been used to extract artemisinin from the leaves and now forms the most reliable medicine for different forms of malaria [21]. As reported by the WHO, more than one million people worldwide die of malaria each year. *Artemisia annua* cannot grow in hot and humid tropical countries, which are even more exposed to malaria due to the lack of required temperature as well as photoperiod [22]. The artemisinin production from cultivated plants has also been highly affected by climatic changes. In a study, it has been reported that highest (0.86% g/g dry weight) artemisinin production was found after 5 months of plant growth after that the artemisinin production reduces significantly [5, 23]. Moreover, the artemisinin yield from dry tissue has been about 0.01–1.0% [24, 25]. In yet another study, it was demonstrated that a ton of dry weight of plant material yields only 6 kilos of artemisinin after rigorous purification steps [26]. In the last couple of decades, researchers have started exploring the alternative in vitro production techniques to supplement the overall supply of artemisinin. The studies have been highly successful, and attempts are being made to establish commercially viable production protocols, and the use of callus/suspension culture, large-scale shoot cultivation, and hairy root cultivation techniques have emerged as possible newer protocols of production of artemisinin [27–29]. The available literature indicates that hairy roots can be maintained on basal growth media (such as MS and B5) and their dilutions without the supplementation of any exogenous phytohormones. Moreover, these differentiated tissues (hairy roots) are genetically and biochemically stable. Artemisinin production by hairy root cultivation has been successfully established in mist and airlift reactors [30, 31]. Even otherwise, mass propagation of hairy roots has been extensively reported for the production of a large variety of phytochemicals [32, 33].

3 Large-Scale Hairy Root Cultivation of *Artemisia annua*

Hairy roots or adventitious roots induced from cut plant parts after infecting with *Agrobacterium rhizogenes* are transformed roots which have a distinct ability to propagate in basal growth medium for several successive cycles of subculture. Hairy

roots of *Artemisia annua* have been subcultured routinely in shake flask cultures for artemisinin production for even longer than 5 years [34–36]. The effect of *Agrobacterium* strain and transformation conditions on artemisinin production indicated that transformation as well as growth kinetics of hairy roots were influenced deeply by these factors [37].

3.1 Technique of Hairy Root Induction

Hairy root induction takes place by the transfer of a DNA segment (T-DNA) from *Agrobacterium rhizogenes* to cut parts of leaves (explants) which eventually gets integrated in plant genome. The various factors that influence this induction process and secondary metabolism in the resulting hairy root culture of *Artemisia annua* are health of explants, infection method, and strain of *Agrobacterium* used [37, 38].

3.2 Advantages of Hairy Root Cultivation of *Artemisia annua*

Hairy roots (adventitious roots) are phenotypically roots of plant; therefore, genotypically they resemble the parent explant used for transformation and can produce secondary metabolites which are seldom produced by untransformed plant roots [37, 39]. This is mainly due to the transformed nature of hairy roots [40]. The genetic stability and biochemical stability enable the reliable scaled-up, stable production of natural products. Unlike normal tap roots of plant, hairy roots grow uniformly in all directions. In particular, hairy roots propagate only geotropically, thus enabling three-dimensional growth in large scaled-up (liquid- as well as gas-phase) bioreactors [31, 41].

4 Mass Production of Artemisinin by Bioreactor Hairy Root Cultivation

In general, bioreactors are the "devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled environmental and operating conditions (e.g. pH, temperature, pressure, nutrient supply and waste removal)" [42]. However, mass hairy root cultivation becomes difficult as only little information is available about the multigene regulated biosynthesis of useful secondary metabolites; with the result, the scale-up of bioreactor cultivation is difficult and still not cost-effective [43].

Design of bioreactor for mass cultivation of hairy roots is a great challenge primarily because hairy roots are propagated from the aerial parts. Upon cultivation of these roots in liquid conditions, their morphology undergoes several changes, and eventually they lose their hairy appearance. This leads to hydrodynamic stress which may lead to reduction in secondary metabolite production as has been observed in

shake flask cultivation cultures of *Artemisia annua* and even in bubble column reactors [44–46].

4.1 Bioreactor Cultivation Modes for Enhanced Artemisinin Production

It has been indicated that mass hairy root cultivation in bioreactor is possible by different modes of cultivation: liquid-phase reactors (wherein hairy roots are soaked in liquid medium) and gas-phase reactors (where nutrient mist is sprinkled on to the growing hairy roots). However, liquid-phase reactors are generally not preferred over gas-phase reactors as they lead to accumulation of liquid layer of nutrients on hairy roots, which becomes yet another barrier for nutrient transport to growing cells, thereby significantly reducing the overall biomass/product accumulation.

In literature, also it has been indicated that for large-scale hairy root cultivation of *Artemisia annua*, gas-phase reactors and a combination of both gas- and liquid-phase cultures are preferable [31, 47, 48]. Aerated liquid-phase bioreactors are more suitable for biomass production of hairy roots and as a result produce lesser artemisinin as compared to gas-phase reactors. However, it has been observed that the kinetic parameters in shake flask cultivation and liquid-phase stirred tank bioreactors are comparable, and therefore design and conduct of fed-batch and continuous mode of bioreactor operation and scale-up are easier in liquid-phase bioreactors. Besides yield enhancement studies in *Artemisia annua* are also more effective and easier only in liquid-phase bioreactors [38]. Also liquid-phase reactors are simpler to design and therefore are significantly easier to operate. Nevertheless, artemisinin production in gas-phase bioreactors is generally higher due to lesser stress and more effective nutrient concentration available to the growing roots due to evaporation of media water [44].

4.1.1 Liquid-Phase Bioreactors for Mass Production of Artemisinin

Stirred Tank Bioreactor

Normally a stirred tank bioreactor used for microbial cultivation consists of flat-blade turbine impellers, multiple baffles, and an air sparger. However, this configuration is not suitable for the shear-sensitive plant cells of *Artemisia annua* as they may experience wear and tear effect arising from the rotation of flat-blade turbine impellers. Plant cells normally get lysed in such a reactor, thereby releasing toxic polyphenolic compounds. During bioreactor cultivation, hairy roots of *Artemisia annua* may also get entangled in the rotating impeller, and as a result the growth of these floating hairy roots may get stunted [49]. The solution to this problem could be operation of the impeller at lower than 100 rev/min or by segregating the growing tissue from the impeller by a mesh or cage [50–52]. The latter technique not only ensures better oxygenation and mixing but also provides higher biomass density. The flat-blade turbine impeller can also be replaced by setric impeller or by centrifugal impeller which are more gentle (thereby leading to less shearing of hairy roots)

and are therefore more suitable for plant cell/hairy root cultivation [53]. Hairy root culture has been reported to be much more fragile as compared to cell suspension cultures (particularly in liquid suspension cultures); with the result, it cannot withstand the beating action of agitator of any conventional stirred tank reactor. To ease out the shearing effect, baffles have to be completely removed from the bioreactor. The use of various kinds of root supports have also been very promising as they can prevent root damage and support better growth due to immobilization. *Daucus carota* hairy roots were segregated from impeller using a stainless steel sieve [54], and it was observed that this strategy led to much better growth due to separation of growing roots from the vigorous mixing zone of the impeller (agitator). However, this kind of design was indicated to be not suitable for higher biomass density which choked the pores of the mesh leading to oxygen and mass transfer limitations in the root mass.

Bubble Column Reactor and Airlift Reactor

Bubble column and airlift reactors are highly specialized reactors which do not have impeller-based agitation. Aeration and agitation in this bioreactor are implemented by sparger alone, which is located at the bottom of the bioreactor vessel [55]. In a normal stirred tank bioreactor, a draft tube is sometimes placed in the center of the reactor vessel to convert it to an airlift reactor to avoid shearing of hairy root tissues by the fast-moving air bubbles. In the airlift reactor, the flow pattern is simpler due to motion of air bubbles in concentric circles through the center of the draft tube. However, effective mixing as well as aeration is not feasible in this bioreactor configuration due to tissue channeling and less nutrient availability. This kind of bioreactor is also unsuitable for hairy root cultivation primarily due to less light penetration in hairy root core mass which might lead to less growth of hairy roots [56]. Suitable modifications in the bioreactor design were done to overcome this, for example, multiple mesh led to better yield of artemisinin in these bioreactor configurations [57].

4.1.2 Gas-Phase Bioreactors for Mass Artemisinin Production

Shear stress and direct impact of agitators have been a major limitation in liquid-phase reactors. This may be minimized by use of gentle mixing. This, however, may lead to poor nutrient availability and slow growth of biomass [58]. This situation can be easily reversed in gas-phase bioreactors like spray and droplet bioreactors.

Spray Bioreactor or Nutrient Mist Reactor

In a nutrient spray reactor, the nutrients are mixed with air and sprayed on to the growing hairy roots of *Artemisia annua*. In this way, the growing hairy roots get nutrients in the form of tiny mist or spray of droplets [59]. Generally the hairy roots are attached to some support or matrices in the form of hooks or mesh, and an intermittent supply of nutrient mist is sprinkled on it. The smaller the size of nutrient droplets, the better biomass growth is obtained. The hairy roots need to be uniformly kept on the porous support in order to reduce water logging particularly in the dense regions of growing hairy root mass.

4.2 Practical Bioreactor Designs for Mass Hairy Root Cultivation of *A. annua*

Non-stirred-type bioreactors (such as airlift bioreactor) and gas-phase bioreactor (such as nutrient mist bioreactor) have been the most popular types of bioreactors used for mass propagation of hairy roots [60, 61]. One of the earliest successful bioreactor-based study on hairy root cultivation of *Artemisia annua* had been done in an airlift bioreactor [19, 45]. It was observed that the addition of a draft tube through the center and a mesh for hairy root support resulted in high biomass and secondary metabolite production in an airlift reactor [57]. However, in liquid-phase bioreactors such as airlift bioreactor, the hairy roots experience hydrodynamic stress and hypoxic stress due to low oxygen solubility. Highly aerated gas-phase reactors such as nutrient mist bioreactor are more suitable for the cultivation of these transformed, hairy, adventitious, aerial roots [59]. Stirred tank reactors are particularly suitable due to proper aeration and agitation; however, its impellers may damage roots significantly if it comes in contact with roots. Modified bubble column and airlift reactors do not have these limitations. In a stirred tank reactor, the shear stress depends on the RPM of agitation, whereas the rheological properties of fluid and superficial gas velocity decide the shear stress in a bubble column reactor [62].

4.3 Bioreactor Hairy Root Propagation for Mass Production of Artemisinin

The use of hairy root cultivations in liquid-phase bioreactors has been reported for some plant species [40]. It has also been reported that liquid-phase bioreactors used for the cultivation of hairy roots had been modified (isolated impeller zone) stirred tank bioreactor, airlift reactor, convection flow-based reactor, turbine blade-fitted reactor, and multistage bubble column reactor [16, 45]. It has been invariably observed that conventional flat-blade turbine stirred tank reactors are unsuitable for growth of hairy roots due to shearing by moving parts of the reactor. This leads to little or no growth of hairy roots. The damage to the hairy roots has been reflected in tissue necrosis and browning of growth media which adversely affects artemisinin production [49]. However, by the help of modifications like wire mesh support for hairy roots, damage to roots can be prevented [52].

Hairy root cultivation has been very successful for the production of secondary metabolites such as artemisinin in gas-phase nutrient mist bioreactors and combination of bubble column and nutrient mist bioreactors [31]. It was observed that the droplet size had significant effect on unnecessary liquid holdup by the hairy root cultures. The biomass concentration obtained in gas-phase bioreactors is slightly reduced as compared to liquid-phase reactors and shake flasks [63]. This has been due to the mutilation of *Artemisia annua* roots in gas-phase reactors over time primarily due to formation of a mucilaginous coating on the growing hairy roots which are not washed of as in the case of perfectly agitated liquid-phase shake flask cultures/liquid-phase bioreactors.

4.4 Artemisinin Concentration/Productivity in *Artemisia annua* Hairy Roots in Different Bioreactor Configurations

The production of artemisinin in different bioreactor configurations has been compared in different literature reports (Table 1). It was invariably observed that artemisinin production was highest only in modified stirred tank bioreactors as compared to cultivations in alternate bioreactor designs [52]. For example, a bubble column reactor with root support and large-scale shake flask cultures were reported to produce negligible artemisinin content of 0.025 mg/l [45] and 0.088 mg/l [46], respectively. However, a nutrient mist bioreactor has been reported to accumulate higher artemisinin of 0.031 mg/l. This was assumed to be partly due to evaporation of liquid media from gas-phase bioreactors (e.g., in nutrient mist reactor) which leads to higher effective concentration of nutrients which in turn inhibits root growth [45]. It has also been indicated that the artemisinin biosynthetic genes are overexpressed during scale-up from shake flask to bioreactor [19]. To summarize, it can be said that bubble column/mist bioreactors are most suitable for biomass growth and artemisinin accumulations, respectively [45].

It was also observed that the changes in the process variables (substrate, biomass, and artemisinin) are much more rapid in a shake flask cultivation as opposed to modified stirred tank bioreactor having immobilized hairy roots; with the result, higher average growth rate (Q_x) and volumetric productivity (Q_p) were observed during shake flask cultivations. However, the growth yield (Y_g) and specific productivity (q_p) values were statistically similar in both shake flask and large-scale liquid-phase bioreactors [52].

Table 1 presents the summary of biomass production, growth yield with respect to substrate, growth rate, artemisinin content, and artemisinin productivity in different gas-/liquid-phase hairy root cultivation studies for *A. annua*.

In general, it was observed that as the scale of operation was increased from shake flask (0.5 L) to bioreactor (BCR (3 L), NMB (3 L), and STR (3 L)), the biomass production decreased significantly due to shear stress (wear and tear) primarily due to uncontrolled motion of free-floating roots in large-scale liquid-phase reactors and insufficient nutrient capture by the unconditioned inoculum in gas-phase reactors. This shear stress was significantly reduced by immobilization of roots in large-scale liquid-phase bioreactor (modified STR (3 L)) and by conditioning of roots (in the liquid-phase growth) in modified nutrient mist bioreactor which led to threefold increase in biomass production as compared to conventional nutrient mist bioreactor [31]. Contrary to the biomass production statistics, the biomass yield per unit limiting substrate consumed remained invariably constant (0.64 ± 0.025) in various scales of operation and in both gas- and liquid-phase bioreactors [31, 52]. A correlation between substrate concentration and biomass yield was also observed which can be utilized for an indirect (in situ and noninvasive) assessment of hairy root biomass in large-scale bioreactors particularly where the medium concentration is non-inhibitory and cultivation conditions remain growth supporting. Although the biomass yield per unit substrate consumed was independent of scale and mode of bioreactor operation, it was found to be dependent on initial substrate concentration in the medium (as high substrate

Table 1 Summary of shake flask/bioreactor cultivations results for artemisinin production using hairy roots

S. no.	Type of cultivation – batch mode	Scale (liters)	Biomass production g/l	Growth yield (g/g sucrose)	Growth rate d^{-1}	Artemisinin content mg/g	Artemisinin productivity mg/(l.d)	Refs.
1.	Shake flask	0.5	12.49 ± 0.94	0.65	0.76	0.27 ± 0.05	0.22	[52]
2.	Modified BCR	3.0	5.68 ± 0.02	0.61	0.17	0.27 ± 0.001	0.06	[31]
3.	NMB	5.0	8.52 ± 0.93	Not reported	0.10	0.22 ± 0.030	0.07	[31]
4.	STR	3.0	6.3 ± 0.45	Not reported	0.21	0.32 ± 0.05	0.08	[49]
5.	Modified STR	3.0	18.52 ± 2.01	0.63	0.62	0.25 ± 0.05	0.17	[52]
6.	Modified NMB	5.0	23.02 ± 2.61	0.67	0.45	1.12 ± 0.140	1.29	[31]

Values are average ± standard deviation
BCR bubble column reactor, *NMB* nutrient mist reactor, *STR* stirred tank bioreactor

concentration was found to be inhibitory for hairy root growth). In a liquid-phase stirred tank bioreactor, the growth yield (Y_g) and specific productivity (q_p) were reported to be 0.85 g/g sucrose consumed in fed-batch cultivation of *Artemisia annua* hairy roots [29]. This observation contradicts the hypothesis of high artemisinin yield in gas-phase nutrient mist bioreactors due to evaporation of liquid media from gas-phase bioreactors (nutrient mist reactor) [45] which results in higher effective concentration of nutrients, because in this cultivation the media evaporation was significantly minimized using medium recycle and yet the artemisinin yield was high. The artemisinin content of hairy roots in mg/g on dry weight basis was also higher for free-floating hairy roots (in shake flask, BCR, and STR). The artemisinin content was increased by more than four-fold in a gas-phase-modified nutrient mist bioreactor wherein the roots were initially conditioned in liquid-phase cultivation for 5 days and later on switched to gas-phase cultivation for the next 15 days. This production protocol effectively reduced shear/hydrodynamic stress which led to increase in effective inoculum volume for mist-phase cultivation and also conditioned the roots for growth in gas-phase nutrient addition [31]. The growth rate of hairy roots per day was maximum for shake flask cultivation (0.76 d^{-1}) followed by modified STR (0.62 d^{-1}) [52]. Scale-up led to decrease in growth rate of hairy roots in liquid-phase as well as gas-phase bioreactors (0.45 d^{-1}). The artemisinin productivity (mg/L.d) was high for shake flask cultivation and lower for scaled-up liquid and gas-phase bioreactors. However, the artemisinin productivity was six-fold higher in the innovative combination of gas-phase and liquid-phase mode of operation in the modified nutrient mist bioreactor [31].

5 Use of Mathematical Modeling for Enhanced Hairy Root Growth in Bioreactors

Mathematical models are useful tools for study of system behavior and development of optimum bioreactor operation strategies for improved biomass/product formation by different microorganisms [64, 65]. It has been indicated that mathematical model can significantly reduce the number of experimental trials to achieve optimized response of system. The use of mathematical model of hairy root growth and artemisinin production for understanding the system behavior and process optimization has been demonstrated [29]. In yet another mathematical model, the growth rate was predicted in terms of nutrient gradient, aerosol deposition, and diffusion of nutrients to root tips by the aerosol model. Aerosol deposition model predictions were used for mist bioreactor to optimize process operation protocol for profuse hairy root growth [63].

6 Scale-Up of *Artemisia annua* Hairy Root Cultures in Bioreactor

Scaled-up cultures of *Artemisia annua* hairy roots face severe mass transfer, oxygen transfer, and heat transfer limitations particularly in large bioreactor vessels [15, 40]. The productivity of artemisinin by hairy root cultures is severely affected by oxygen

limitation in scaled-up cultures [59, 66]. However, some successful reports of hairy root cultivation in large-scale bioreactors are available [45, 52]. A combination of gas-phase and liquid-phase culture has been reported to be better than single-phase mode of operation [31, 45]. The effect of scale-up of hairy root cultures on *Artemisia annua* hairy roots was observed in three different bioreactor configurations on gene expression level. The level of gene expression of artemisinin biosynthesis genes was higher in large-scale cultures. It was also reported that single bioreactor study may lead to misleading conclusions due to the presence of gradient of gene expression pattern in hairy root mass in the bioreactor [19].

7 Future Perspectives

Hairy root cultivation provides a useful method for production of artemisinin in scaled-up bioreactors. Using improved designs of bioreactor, artemisinin productivity can be improved several folds. Screening studies of different root supports such as alginate, polyurethane foam, gelatin, agarose, chemically synthesized polymers, and super-adsorbing materials can help identifying most suitable support system for adequate oxygen availability to the growing hairy roots for improved artemisinin production. Different nutrient limitation/oxygen transfer studies particularly supply of oxygen to the core of hairy root mass can be studied for *A. annua* hairy roots which can form the basis of scale-up of *A. annua* hairy roots as has been previously reported for other plant species [67, 68].

The cultivation cost can be further economized by the use of disposable one-time (single use) large-scale presterilized bioreactors for commercial production of artemisinin [69].

Reports are also available for production of artemisinin precursor by microbial cultures using genetic modification of *Saccharomyces cerevisiae* [70]. By the use of cloning of plant dehydrogenase gene and cytochrome in yeast, a new bioprocess production strategy was developed, which demonstrated production of 25 g/L artemisinic acid (precursor of artemisinin). However, as the process involves a series of genetic manipulation steps as well as final chemical semi-synthesis, the final product obtained may have undesirable side effects or might be less potent as has been in the case of semi-synthetic drug etoposide [71]. However, this innovative breakthrough research provides an opportunity for further improvement in the bioprocess production strategy for artemisinin, which significantly minimizes the number of bioprocessing steps. However, the characteristic properties of semisynthetic artemisinin should be monitored to predict its effectiveness, general acceptability, and absence of side effects arising out of unnecessary contaminants and modifications. The genetic engineering strategy should also be revamped to obtain the final product artemisinin with a higher yield. Efforts, which will be interdisciplinary in nature, can prove significant improvement in the existing new protocol of artemisinin production. The close cooperation of genetic engineering experts as well as bioprocess engineers may significantly improve and optimize the bioprocess for mass production of native artemisinin.

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Plant In Vitro Systems as Sources of Food Ingredients and Additives

13

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Contents

Abstract

In the recent years, people prefer to consume food with natural additives, especially those with plant origin because of the increased reports for carcinogenic and other side effects of some synthetic ones. Plant in vitro cultures have recently received great attention as an effective technology for the production of valuable secondary metabolites used as food ingredients and additives. The advantages of plant cell, tissue, and organ cultures over living plants, in terms of secondary metabolite production, are well understand: in the laboratory, growth conditions and parameters can be controlled and optimized; separation of target compounds is much easier; large-scale growth of plant cells in liquid culture in bioreactors

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can be achieved and ultimately commercialized. This chapter provides an overview and examples of plant in vitro systems producing food colorants, antioxidants, flavors, and sweeteners.

Keywords
Plant in vitro cultures · Food colorants · Food additives · Food supplements

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
2-iP	6-(γ,γ-Dimethylallylamino)purine
B5	Gamborg medium
BAP	6-Benzylaminopurine
CP	Chee and Pool *Vitis* medium
cv.	Cultivar
DW	Dry weight
FW	Fresh weight
HPLC	High-performance liquid chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
JA	Jasmonic acid
L-Phe	L-phenylalanine
LS	Linsmaier and Skoog medium
MJ	Methyl jasmonate
MS	Murashige and Skoog medium
NAA	α-Naphthaleneacetic acid
SA	Salicylic acid
SLSs	Stigma-like structures
TIS	Temporary immersion systems
TLC	Thin-layer chromatography
UV	Ultraviolet light

1 Introduction

Food is any substance from plant or animal origin consumed to provide nutritional support for the human body. Plants have been utilized as a main source of natural food nutrients – pigments, flavors, fragrances, food additives, and food supplements.

Visual characteristics such as color are first assessed by food quality. Fresh food is often highly colored by the plant pigment especially carotenoids, betalains, anthocyanins, and chlorophylls in green vegetables [1]. The antioxidants were other important application substances for prevention of oxidative damage in food systems and oxidative stress-associated diseases in humans, as well used in food industry. The plants produced different chemical types of antioxidants, namely, polyphenols (phenolic acids, flavonoids, lignans, stilbenes), terpenoids, and vitamins [2]. However, for processed foods the pigmentation and antioxidants were often lost during

manufacturing, and the visual appeal of the final product is enhanced using added colorants. During the last years, there is a growing interest of natural antioxidant and pigments compounds, especially those with plant origin because of the increased reports for carcinogenic effect of some synthetic ones, currently applied in foods, cosmetics, and pharmacy [2].

Natural plant-derived food additives and supplements are sourced directly from plant material by extraction from generated through traditional plant breeding, and the target compounds may be available only seasonally in low yields. However, the amount of most plant-derived metabolites, and the structural variation within each type, is affected by seasonal and environmental factors, making availability of defined source material variable. Thus, there is much interest in applying plant biotechnology to production of plant deriver nutrients, additives, and supplements.

Plant in vitro culture has recently received a lot of attention as an effective technology for the production of valuable secondary metabolites. Plant cell cultures produce higher quantities of secondary metabolites, often with different profiles compared to their parent plants. Several strategies have been developed to increase the productivity of these compounds: media optimization, selection of a suitable plant tissue culture, elicitation, and addition of precursors [3–6]. One of the most effective strategies for enhancing the biotechnological production of secondary metabolites is elicitation by different biotic (microorganism-derived elicitors; plant cell wall compounds; peptides, cyclodextrins) and abiotic elicitors (metal salts, UV light) [4, 7, 8].

The advantages of plant tissue and cell cultures over living plants, in terms of secondary metabolite production, are clear. In the laboratory, growth conditions can be controlled; therefore, reproducible yields of end product can be achieved. Growth parameters such as pH, temperature, medium components, and other microenvironmental factors can be optimized to obtain metabolite production, preferably significantly higher than in the living plants. Separation of target compounds is also much easier due to lower complexity of the cultured materials. Lastly, large-scale cultivation of plant cells in liquid culture in bioreactors as a source of secondary products can be achieved and ultimately commercialized. With the increasing awareness concerning the health risks caused by synthetic food colorants, the focus now is to look for pigments from natural resources to substitute the synthetic ones [6].

2 Food Colorants from Plant In Vitro Cultures

In the recent years, humans prefer to consume food contain natural colorants, and there was an increased interest in new sources and improving their performance in food applications. Four plant pigment types are widely used as food colorants: annatto, curcumin, anthocyanins, betalains (beetroot pigment) (the main pigment of turmeric spice), and saffron (Fig. 1). Together with the insect-derived pigment cochineal, they account for over 90% of the market for natural food colorants [9].

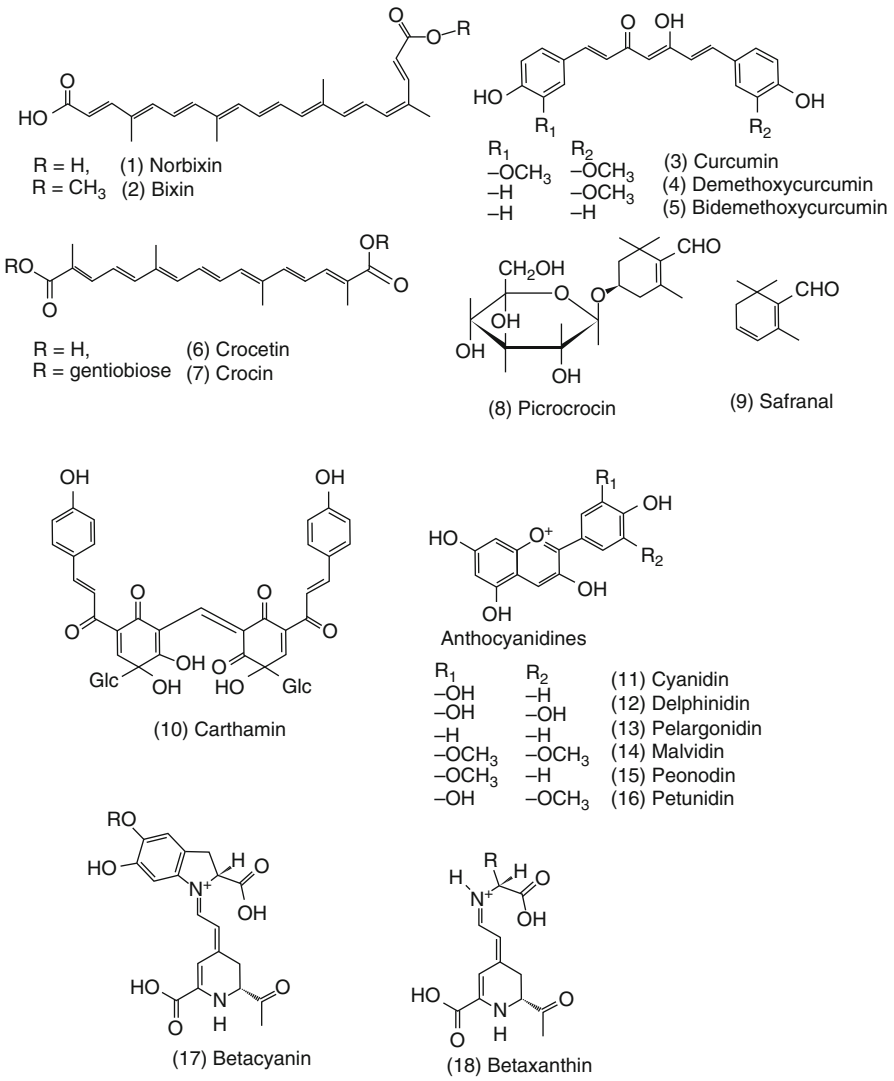


Fig. 1 Chemical structures of some food colorants obtained from plant in vitro cultures

2.1 Fat Solubility Food Colorants

2.1.1 Annatto

Annatto (E160b, CI 75120) is one of the oldest known dyes used for foods, textiles, and cosmetics. It is extracted from the resinous coating on the seeds of the tropical bush *B. orellana*. The species occurs in the wild in tropical North America and was used by Native Americans in pre-Columbian times as a source of pigment. The main apocarotenoid pigments of annatto are cis-bixin (methyl (9-cis)-hydrogen-6,6-diapo-

Ψ,Ψ-carotenedioate) (**2**) and norbixin (**1**) (approximately 80% of the total carotenoids), and these carotenoids are responsible for the yellow to orange-red colors. Annatto is sparingly soluble in oil and is principally used in dairy industry for coloring butter, cheese, chocolate, ice cream, and fat-based foods. Present in smaller quantities in the pigment extract is a water-soluble carotenoid, cis-norbixin, which can also be generated by alkaline treatment of bixin (**2**) [10].

It was developed two methods for in vitro accumulation of carotenoid from plant in vitro cultures of *B. orellana* (normal root cultures and callus cultures) [11–13]. Normal root cultures of *B. orellana* were established under in vitro conditions on MS medium containing α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), and indole-3-acetic acid (IAA) at 0.05–0.2 mg/L. The maximum amount of annatto pigment and maximum root biomass were achieved after 45 and 60 days of growth. (Table 1).

Also it was investigated the biosynthetical potential of callus culture obtained from different explants (seeds, roots, leaf, hypocotyl and stem) of *B. orellana* for carotenoids production [12–14]. Narváez et al. [12] established that type of explants and phenotype of obtained callus culture influence of carotenoid biosynthesis. The highest levels of bixin (**2**) were observed from brown callus from fruit (400 mg bixin /kg). Poornima and Ambika [13] were tested for the growth of callus under different wavelengths of light (red, blue, green, yellow) and established that red light increased the callus growth rate and the callus production of carotenoids. Also, Castello et al. [14] investigated the effect of the carotene precursor geranylgeranyl pyrophosphate, mevalonic acid, gibberellic acid, and manganese (a cofactor for carotenoid) on biosynthesis of bixin and establish that geranylgeranyl pyrophosphate and mevalonic acid did enhance the accumulated bixin content in the first month.

However, the normal root cultures biosynthesized more carotenoids than callus culture. In comparison, yields of carotenoids obtained from plant in vitro systems of *B. orellana* were lower than the bixin derived from natural plants (from 12 to 23 mg bixin/g) [10].

2.1.2 Curcumin

Curcumin (E100, CI 75300, Natural Yellow 3) (**3**) is the principal pigment in the spice turmeric, which is extracted from the rhizomes of , a perennial member of the ginger family (Zingiberaceae) that has been cultivated in Asia for many centuries. It supplies strong yellow color and is generally oil soluble. As a food additive, its E number is E 100. Turmeric has traditionally been used for coloring and flavoring of meals; it is still used in large quantities for this purpose, as well as extensively in a wide range of processed foods. Around 300,000 tons are produced annually in India, mostly for spice with a small amount for preparation of pure curcumin. Polyphenolic curcuminoid compounds including curcumin (**3**), bisdemethoxycurcumin (**5**), and demethoxycurcumin (**4**) as well as volatile oils (tumerone, atlantone, and zingiberene) are the main secondary metabolites in *Curcuma longa* and other *Curcuma* species [15]. Curcumin is best studied and has been shown to exhibit anti-inflammatory, anticarcinogenic, and antioxidant activities [15, 16]. Current researches of the in vitro multiplication and rhizomes induction of *Curcuma* ssp.

Table 1 Food colorants from plant in vitro cultures

Species	Family	In vitro system	Pigment	Content	References
Fat solubility food colorants					
Annatto					
Bixa orellana L.	Bixaceae	Root cultures	Annatto pigment	346 mg/100 g dw	[11]
Bixa orellana L	Bixaceae	Callus cultures	Total bixin	0.008%	[13]
Bixa orellana L	Bixaceae	Callus cultures	Bixin	5%	[14]
Bixa orellana L cv. India	Bixaceae	Callus cultures	Total carotenoids	635 mg/kg	[12]
			Bixin	400 mg/kg	
Saffron					
Crocus sativus	Iridaceae	Callus cultures	Crocin	0.43 g/L	[22]
Crocus sativus	Iridaceae	Stigma-like structures	Crocin	1.08%	[19]
			Picrocrocin	0.17%	
Crocus sativus	Iridaceae	Callus cultures	Crocin	90 mg/L	[23]
				4.4 mg/g	
Curcumin					
Curcuma longa L.	Zingiberaceae	Rhizomes	Curcumin	–	[16]
Curcuma aromatica Salisb.	Zingiberaceae	Microrhizome	Curcumin	107.34 μg/g	[17]
			Demethoxycurcumin	854.60 μg/g	
			Curcuminoids	961.94 μg/g	
Water solubility food colorants					
Safflower pigments					
Carthamus tinctorius	Asteraceae	Cell suspension culture	Total pigments carthamin and safflower yellow A		[26]
Carthamus tinctorius L.	Asteraceae	Root cultures	Total pigments	46.38 mg/g FW	[25]

Anthocyanins					
Aralia cordata	Araliaceae	Cell suspension culture	Total anthocyanins	17.2% DW	[41]
Fragaria ananassa cv. Shikinari	Rosaceae	Cell suspension culture	Total anthocyanins	11 mg/g FW	[33]
Panax ginseng C.A Meyer		Cell suspension culture	Total anthocyanins	2–3% DW	[40]
			Cyanidin		
			cyanidin-3-*O*-β-D-xylcopyranyl-(12)-β-D-glucopyranoside		
Camptotheca acuminata Decne	Nyssaceae	Cell suspension culture	Cyanidin-3-*O*-galactoside		[39]
			Cyanidin-3-*O*-glucoside		
Prunus cerasus cv. Vladimir	Rosaceae	Cell suspension culture	Total anthocyanins		[34]
Melastoma malabothricum	Melastomaceae	Cell suspension culture	Total anthocyanins		[38]
Melastoma malabothricum	Melastomaceae	Cell suspension culture	Total anthocyanins	0.69 CV/g FW	[42]
Prunus cerasus L.	Rosaceae	Callus cultures	Cyanidin 3-glucoside	4.5 mg /100 g FW	[35]
			Cyanidin 3,5-diglucoside	1.4%	
			Cyanidin 3-glucoside	86%	
			Cyanidin 3-rutinoside	6.6%	
			Peonodin 3-glucoside 4.5	4.5%	
			Malvidin 3-glucoside	1.4%	
Vitis vinifera L. cv. Gamay Fre'aux		Cell suspension culture	Total anthocyanins	4.2 mg/g DW	[29]
			delphinidin-3-*O*-glucoside		
			Cyanidin-3-*O*-glucoside		
			Petunidin-3-*O*-glucoside		
			Peonidin-3-*O*-glucoside		
			Delphinidin-3-*O*-p-coumaryl-glucoside		
			Cyanidin-3-*O*-p-coumaryl-glucoside		
			Peonidin-3-*O*-p-coumaryl-glucoside		

(*continued*)

Table 1 (continued)

Species	Family	In vitro system	Pigment	Content	References
Vitis vinifera L.		Cell suspension culture	Total anthocyanins	2 mg/g FW	[28]
Vaccinium macrocarpon Ait.		Callus cultures	Total anthocyanins	140 μg/g FW	[36]
			Cyanidin 3-galactoside		
			Cyanidin 3-glucoside		
			Cyanidin 3-arabinoside		
Ipomoea batata L cv. Kintoki		Callus cultures	Total anthocyanins	7.9 μmoll/g FW	[30]
Daucus carota		Cell suspension culture	Cyanidin 3-*O*-lathyroside, cyanidin 3-*O*-(2″-*O*-beta-D-xylopyranosyl-6″-*O*-beta-D-glucopyranosyl-beta-D-galactopyranoside)		[32]
Daucus carota		Cell suspension culture	Total anthocyanins	15.88% w/w	[31]
Cleome rosea Vahl ex DC.	Capparaceae	Cell suspension cultures			[37]
Betalains					
Amaranthus tricolor L.	Amaranthaceae	Callus cultures	Amaranthin	3.53 mg/100 g FW	[45]
			Methylated arginine-betaxanthin	0.34 mg/100 g FW	
Pereskia aculeata Miller		Callus cultures	Total betalains	0.91 mg/100 mL	[44]
			Betanin	0.34 mg/100 mL	
			Vulgaxantin I	0.80 mg/100 mL	
		Cell suspension cultures	Total betalains	4.10 mg/100 mL	
			Betanin	1.10 mg/100 mL	
			Vulgaxantin I	2.70 mg/100 mL	
B. vulgaris L. cv. Detroit dark red		Hairy root	Betacyanins	16.0 mg/flask	[49]
			Betaxanthins	26.2 mg/flask	
			Total betalains	42.2 mg/flask	

Beta vulgaris L. var. altissima		Transformed cell suspension cultures	Total betanin	53 mg/L	[50]
Phytolacca americana L.		Cell suspension	Betacyanins	–	[46]
Celosia argentea var. plumosa		Cell suspension	Miraxanthin V	1.47 mg/g DW	[47]
			Betanidin	6.27 mg/g DW	
			Decarboxy-betanidin	2.30 mg/g dw	
			Vulgaxanthin I	2.01 mg/g dw	
			Betanin	0.75 mg/g dw	
			Iso-betanin	0.67 mg/g dw	
			Amaranthin	0.48 mg/g dw	
			Iso- amaranthin	0.22 mg/g dw	
Portulaca grandiflora Hook		Cell suspension	Betaxanthins		[51]
Bougainvillea cv. Bhabha		Callus culture	Betacyanins	0.61 mg/g FW	[48]
			Betaxanthins	0.42 mg/g FW	

are summarized in numbers of publications. It was reported the obtained in vitro microrhizomes cultivated in combination of 1.0 mg/ L NAA and 1.0 mg/L kinetin or 2.0 mg/L BAP-produced curcumin [16] (Table 1).

Wu et al. [17] obtained microrhizomes from *C. aromatic* and studied specific light regimes and combinations of sucrose and plant growth regulators on curcuminoid levels. 5% sucrose medium supplemented with 3.0 mg/L of BAP and 0.5 mg/L of NAA, and exposure to red light enhanced the levels of curcumin in microrhizomes of *Curcuma aromatic*. The obtained amount of curcuminoids from in vitro culture were lower than intact rhizomes.

2.1.3 Saffron

Crocus sativus L. (Iridaceae), commonly known as saffron (E 164), is a perennial stemless herb that is widely cultivated in Iran and other countries such as India and Greece. Characteristic components of saffron are carotenoid derivatives crocin (**7**) and crocetin (**6**) (responsible for the color), picrocrocin (**8**) (responsible for the bitter taste), and safranal (**9**) (responsible for odor and aroma) (Fig. 1).

Saffron callus was grown and produces carotenoids in a two-stage culture on B5 medium supplemented with casein hydrolysate at 22 °C in dark. The maximum crocin production (Table 1) was achieved by this two-stage culture method, which was three times higher than that by a one-stage method [22]. Chen et al. [23] investigated influence of different metal ions on production of crocin from *Crocus sativus* callus cultures. It was found that Ce^{3+} and La^{3+} significantly promoted crocin production but had little effect on cell growth. Active compounds from saffron through in vitro multiplication of stigmas have been artificially produced [18]. Himeno and Sano [19] cultured young half ovaries excised from young flower buds on LS and Nitsch and Mitsch media supplemented with different amounts of NAA and kinetin or BAP. At this condition, after 10 weeks, stigma-like structures (SLSs) were formed directly on explant, and crocin (**7**) (Table 1) and picrocrocin (**6**) (Table 1) (Fig. 1) have been detected. The safranal (**9**) has been appeared only after heat treatment at 50 °C for 120 min. Stigma-like structures from the ovary explants of *C. sativus* on MS medium supplemented with NAA and BAP were produced from Sarma et al. [20, 21] for the first time compared by sensory analysis the production of spice from tissue cultures with saffron obtained from intact flowers. The sensory data indicated that the saffron pigments produced in tissue cultures were one tenth of the amount of natural stigmas. Sensory profile test showed that the tissue culture saffron was low in floral, spicy, and fatty characteristics as compared to saffron obtained from flowers [21].

To date, there is no report that shows a technique for in vitro production of saffron on a commercial scale. Hence, there is a great scope for the refinement of the existing techniques of saffron production either through micropropagation of SLSs and/or through the production by callus cultures with major secondary metabolites yields on a larger scale for its use at the commercial level. The content of major secondary metabolites in SLSs was similar to those obtained from intact young stigmas, and they were also morphologically similar [19]. These results define that SLSs

structures cultivated in in vitro condition as appropriate cultures for industrial production of saffron spice and application in food systems.

2.2 Water Solubility Food Colorants

2.2.1 Safflower Pigments

Carthamus tinctorius L. is an annual crop cultured mainly for its seed and flowers. Safflower contains yellow and red pigments with characteristic C-b-D-glucopyranosyl-quinochalcone structure [24]. The petals of safflower flowers contain a red pigment carthamin (**10**) (Fig. 1). This pigment was low solubility in water and was mainly used in colored chocolate. The yellow safflower pigments (safflower yellow A and B) are water soluble. These pigments also have been used as natural colorants of food – juice, jelly, and candy [24, 25].

High yellow pigments productions have been achieved by root in vitro culture grown in static MS liquid medium under light condition [25]. Under these conditions the production of safflower pigment amounted to 13.18 mg/g FW. After treatment of root culture with 18% (v/v) gas oil, the pigment concentration increases to 46.38 mg/g FW [25] (Table 1).

The pigment production from cell suspension cultures of *Carthamus tinctorius* L. (safflower) and effects of sucrose concentration and light have been investigated from Gao et al. [26]. It has developed a two-stage bioreactor system for pigment accumulation using balloon- and column-type bioreactors. Growth phase was realized successfully in a 10-L balloon-type airlift bioreactor that contained one half MS medium. While, the high pigment formation has been achieved in 5-L column-type airlift bioreactor [26].

2.2.2 Anthocyanins

Anthocyanins (Fig. 1) are present in many sources and widely used as food colorants. Commercial anthocyanin extracts are predominantly prepared from *Vitis* species grape skin obtained from the wine industry, which is available, cheap, and in large amounts. However, although around 20 different anthocyanins have been reported for grapes, the common commercial preparations contain principally only relatively simple 3- and 3,5-diglucosides of cyanidin (**11**), delphinidin (**12**) and malvidin (**14**) (Fig. 1). The colors of anthocyanin vary greatly according to the pH of the food, but generally they are used only in acidic foods and provide red to blue colors. Commercial extracts are now available from plant in vitro cultures. Research of anthocyanin production from new sources has been intensified by the last year because the health benefits of anthocyanins were proven [27].

Anthocyanin production by plant in vitro systems has been intensively investigated. It has been reported mainly in cell cultures of edible plants like grapes [28, 29], sweet potato [30], carrot [31, 32], strawberry [33], sour cherry [34, 35], American cranberry (bearberry) [36], as well as *Cleome rosea* [37], *Melastoma malabothricum* [38], *Camptotheca acuminata* Decne [39], *Panax ginseng* CA Meyer [40], and *Aralia cordata* [41]. The yield of cell biomass and anthocyanin

production have influenced the plant regulators, source of nitrogen, light, and elicitors [34, 35, 37, 39, 42].

By using a scale-up technique, Kobayashi et al. [41] established an effective process for the anthocyanin produced from *Aralia cordata* cells suspension in 500-L jar bioreactor. The suspension culture grown and produced anthocyanin without light irradiation. The cell damage was completely prevented cell by the administration of CO_2. In 500-L fermenter, cells were cultivated for 16 days in this condition; cell mass was increased by more than 26 times; and the amount of anthocyanin increased by more 55 times.

From plant in vitro cultures of *Prunus cerasus* L., *Camptotheca acuminate* have been established the cyanidin-3-glucoside [35, 39]. Six anthocyanins were isolated from cell suspension cultures of an Afghan cultivar of *Daucus carota* [32]. The structures of these compounds were elucidated by spectroscopic methods as cyanidin-3-*O*-lathyroside and cyanidin-3-*O*-(2″-*O*-beta-D-xylopyranosyl-6″-*O*-beta-D-glucopyranosyl-beta-D-galactopyranoside) and the latter acylated with 4-coumaric, ferulic, 4-hydroxybenzoic, or sinapic acid [32]. Lazar and Petolescu [43] obtained suspension culture from six grapevines – Burgund Mare, Cabernet Sauvignon, Merlot, Oporto, Negru Tinctorial, and Pinot Noir. The biosynthetic potential of cells cultures was investigated in the laboratory Braun bioreactor. Anthocyanins produced varied between 0.87 mg anthocyanins/g biomass for Cabernet Sauvignon and 10.23 mg anthocyanins/g biomass for Negru Tinctorial variety. Negru Tinctorial variety recorded the higher values which were very significantly for the anthocyanins amount synthesized in cell cultures of all varieties, followed by Burgund Mare (5.42 mg/g), Oporto (4.57 mg/g), Merlot (2.52 mg/g), and Pinot Noir (2.26 mg/g) varieties.

The effects of yeast extract, different polysaccharide (chitosan, pectin, alginate, and gum Arabic), and methyl jasmonate elicitors on secondary metabolite production, mainly of anthocyanin production, phenolic acid, and cell biomass in cell suspension cultures, were investigated [29, 42]. Chitosan, pectin, and alginate enhanced production of anthocyanin. The highest amount of total anthocyanins was produced by pectin-treated cells. In V. *vinifera* cell cultures the major established anthocyanins cyanidin-3-O-glucoside and peonidin-3-O-glucoside, and also, three minor anthocyanin compounds were found: delphinidin 3-O-glucoside, petunidin-3-O-glucoside, and delphinidin 3-O-p-coumaryl-glucoside [28, 29]. Furthermore, chitosan, alginate, and gum Arabic significantly promoted accumulation of phenolic acids, particularly 3-*O*-glucosyl-resveratrol, in *V. vinifera* cultures, as well as in the culture medium. Thaw Saw et al. [28] used salicylic acid and ethephon as elicitors and phenylalanine and shikimic acids as precursors to improve the productivity of anthocyanins from *Vitis vinifera* suspension cultures. When the cells were elicitated with 50 μL/25 mL suspension salicylic acid, the anthocyanin concentration was increased to 0.03 μg/mg in 18 days of cultivation as compared to the unelicitated cells. Both precursor shikimic acid and phenylalanine could promote the synthesis of anthocyanin in the grape cell cultures. After 18 days of the treatment with shikimic acid, the anthocyanin concentration was increased more than the control cultures (Table 1).

2.2.3 Betalains

Betalains are water-soluble unstable to heat and light pigments. They are used in foods with short shelf lives that do not need high heat treatment. Beetroot is the main source of betalains for food colorants. Betalains consist principally two subclasses of red-violet pigments betacyanins (**17**) and yellow-orange pigments betaxanthins (**18**) (Fig. 1). In Europe about 20,000 tons of beetroot are processed for juice and pigment extraction [5].

Pereskia aculeata Mill. is a native cactus found in the Americas. Lage et al. [44] established protocols for new source of betalain production for commercial purposes from callus and cell suspensions of *P. aculeate* Mill. Pigmented friable calluses were induced from leafy cotyledon and hypocotyl segments of *Pereskia aculeata* Mill. cultivated on MS medium supplemented with 4-amino-3,5,6-trichloro-2-pyridine-carboxylic acid (picloram). The highest callus biomass accumulation was achieved in cultures initiated from hypocotyl explants on medium supplemented with 10 μM. In the cell suspension cultures, the highest total betalain accumulation (4.10 ± 0.47 mg/100 mL) was achieved in cultures kept on a 1:4 ratio of NH_4^+/NO_3^-. The main betacyanin betanin was produced in the amount of 1.10 ± 0.11 mg/100 mL and the main betaxanthin vulgaxantin I – 2.70 ± 0.35 mg/100 mL (Table 1).

Callus culture from *Amaranthus tricolor* L. was established, and its ability for production of food colorants was studied [45]. The best betacyanin-producing callus culture was induced on MS medium supplemented with 0.25 mg/L NAA and 2 mg/L BAP using stem segment as explants. Betalain production in callus culture was detected by HPLC and LC-MS. It has been identified as red-violet pigment amaranthin (3.53 mg/100G FW), isoamaranthin, methyl derivative of arginine – a yellow pigment betaxanthin (0.34 mg/100g FW) – and 18 other bioactive phenylpropanoids.

A new betacyanin (**17**) of higher plants – betanidin 5-*O*-[(5″-O-E-feruloyl)-2″-*O*-β-D-apiofuranosyl]-β-D-glucopyranoside and betanidin 5-*O*-(6″-O-E-feruloyl)-β-D-glucopyranoside (lampranthin II) – together with their isoforms have been identified as major compound in *Phytolacca americana* cell cultures [46] (Table 1).

The betaxanthins dopaxanthin and miraxanthin V and the betacyanins betanidin and decarboxy-betanidin have been produced in cell cultures established from hypocotyls of the plant *Celosia argentea* var. *plumosa* [47]. Two stable and different-colored cell lines – yellow and red – were obtained on MS medium supplemented with 6-benzylaminopurine (6.66 μM) and 2,4-dichlorophenoxyacetic acid (6.79 μM). In the yellow line, has been identified the betaxanthins (**18**) – vulgaxanthin I (1.78 mg/g DW) and miraxanthin V (1.64 mg/g d DW). Other detected betaxanthin was dopaxanthin. In addition to the yellow line, the betacyanins amaranthin, betanin, dihydroxylated betanidin, and decarboxy-betanidin were also identified. In the red line, higher content of the betacyanins was determined. In this culture the violet pigment amaranthin (4.92 mg/g DW) and red pigments – decarboxy-betanidin (1.32 mg/g DW), betanin (0.80 mg/g DW), and betanidin (0.74 mg/g DW) – were detected. Betaxanthins were also present in red line – dopamine-derived betaxanthin (3.04 mg/g DW). The

presence of the bioactive betalains showed the ability of cell cultures of *C. argentea* to become a stable source of food yellow or red colorants [47].

The effects of various biotic elicitors (methyl jasmonate (MJ) and β-glucan) and abiotic elicitors (calcium chloride, Fe-EDTA, and copper sulfate) on biosynthesis of betacyanin and betaxanthin pigment production in *Bougainvillea* callus cultures into basal MS medium have been investigated [48]. Treatment with 0.5 μM MJ was found to be most effective in inducing betalain biosynthesis in the callus (betacyanins, 0.65 mg/g FW; betaxanthins, 0.48 mg/g F). Among the abiotic elicitors, calcium chloride at 5 g/l showed maximal yield of betacyanin (0.61 mg/g FW) and betaxanthin content (0.42 mg/g FW).

3 Food Additives with Antioxidant Activities

Antioxidants have an important application against oxidative damages in food systems and oxidative stress-associated diseases in humans, as well. During the last years, there is a growing interest of natural antioxidant compounds, especially those with plant origin because of the increased reports for carcinogenic effect of some synthetic ones, currently applied in foods, cosmetics, and pharmacy [2]. Higher plants and their in vitro cultures are immense and valuable sources of antioxidants employed in food industry. Antioxidants produced by plant in vitro cultures could be differentiated in some main chemical types, namely, polyphenols (phenolic acids, flavonoids, lignans, stilbenes, terpenoids, and vitamins). Worldwide there are many successfully developed biotechnological approaches for production of antioxidants by different type of plant in vitro cultures (Table 2) (Fig. 2).

3.1 Plant Compounds with Antioxidant Activities

Polyphenols are the most abundant secondary metabolites generally involved in plant adaptation to environmental stress conditions such as infection by microbial pathogens, mechanical wounding, and protective agents against excessive ultraviolet or visible light levels [52]. These compounds have a number of beneficial health properties related to their potent antioxidant activity, as well as hepatoprotective, hypoglycemic, anticancer, and antiviral activities [53]. These plant secondary metabolites can be classified into nonsoluble compounds, such as condensed tannins, lignins, cell wall-bound hydroxycinammic acids, and soluble compounds such as phenolic acids, phenyl propanoids, flavonoids (**20**–**24**), and quinones [52].

Rosmarinic acid (**19**) (ester of caffeic acid and 3,4-dihydroxyphenyllactic acid) (Fig. 2) is one of the strongest plant-derived antioxidant compound. Its redox properties play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [54–56]

There are plenty scientific reports describing rosmarinic acid production by plant in vitro systems. *Lavandula vera* MM (*Lamiaceae*) cell suspension culture has been shown to be a good producer of rosmarinic acid after optimization of nutrient

Table 2 Secondary metabolites of plant in vitro systems with potential for application in foods as antioxidants

Plant species	Type of plants in vitro cultures	Compounds	Amount	References
Plant compounds with antioxidant activities				
Lavandula vera (Lamiaceae)	Cell suspension	Rosmarinic acid	3.92 g/L.d	[58, 59]
			0.65 g/L.d	
			3.35 g/L.d	
Nepeta cataria (Lamiaceae)	Hairy roots	Rosmarinic acid	19.2 mg/g DW	[60]
Mentha piperita (Lamiaceae)	Cell suspension	Rosmarinic acid	117.95 mg/g DW (12% DW)	[62]
			110.12 mg/g DW	
Melissa officinalis L. (Lamiaceae)	Callus	Rosmarinic acid	28.12 μg/mg DW	[61]
	Shoots		38.25 μg/mg DW	
Ocimum basilicum L. (Lamiaceae)	Callus	Rosmarinic acid	13.20 μg/mg DW	[61]
	Shoots		25.30 μg/mg DW	
Cyperus rotundus (Cyperaceae)	Callus	Quercetin, kaempferol	1.21 mg/g DW	[69]
			0.75 mg/g DW	
Dionaea muscipula	Callus	Myricetin, Quercetin		[65]
Drosera capensis (Droseracea)				
Echinacea angustifolia (Asteraceae)	Shoots	Caftaric acid	2.5 mg/g DW	[66]
		Chlorogenic acid	11.2 mg/g DW	
		Cichoric acid	30.5 mg/g DW	
Dracocephalum kotschyi (Lamiaceae)	Hairy roots	Apigenin	28.1 μg/g DW,	[70]
		Rosmarinic acid	1438.7 μg/g DW	
H. perforatum (Hypericaceae)	Shoots	Chlorogenic acid	0.16% of DW	[63]
		Hyperosid	0.27% of DW	
		Rutin	0.22% of DW	
		Quercitrine	0.02% of DW	
Momordica charantia (Cucurbitaceae)	Shoots	Luteolin	2.15 μg/g DW	[64]
		Kampherol	0.22 μg/g DW	
		Quercetin	0.57 μg/g DW	
Salvia miltiorrhiza (Lamiaceae)	Hairy roots	Caffeic acid	0.06 mg/g DW	[67]
Salvia tomentosa Mill. (Lamiaceae)	Hairy roots	Gallic acid Protocatechuic acid	39* 188 446	[72]

(*continued*)

Table 2 (continued)

Plant species	Type of plants in vitro cultures	Compounds	Amount	References
		Salicylic acid Chlorogenic acid Vanillic acid Caffeic acid Syringic acid p-Coumaric acid Sinapic acid Ferulic acid Hesperidin Quercetin Luteolin Kaempferol	331 332 131 167 44 521 232 27 47 21 25 11 *Adsorbed on Amberlite XAD-4, μg/RITA apparatus (200 ml medium)	
Ephedra alata Decne (Ephedraceae)	Callus culture	Chlorogenic acid,	0.17 μg/g DW	[52]
		Rutin	21.6 μg/g DW	
		Catechin	2.05 μg/g DW	
		Quercetin	3.18 μg/g DW	
		Coumaric acid	0.65 μg/g DW	
Vitis vinifera L. cv. St. Laurent (Vitaceae)	Cell suspension	Catechin	1.48–2.74 mg/g FW	[71]
		Epicatechin	2.05–1.16 mg/g FW	
		Trans-resveratrol	0.45 mg/L;	
		rutin	1.32–3.10 mg/g fw	
Vitis vinifera L. cv. Cabernet Sauvignon (Vitaceae)	Cell suspension	Trans-resveratrol	3.96 mg/L (extracellular)	[80]
Vitis vinifera L. cv. Öküzgözü (Vitaceae)	Callus culture	Trans-resveratrol	62.23 μg/g callus FW	[81]
Vitis vinifera cv. Barbera (Vitaceae)	Cell suspension	Free and monoglucosylated resveratrol	32.72 μmol/g DW	[82]
V. vinifera cvs Pinot Noir	Cell suspension	*Trans*-resveratrol	0.51 mg/L* 5.80 μg/g **	[83]
V. vinifera cv. Merzling			4.31 mg/L 3.91 μg/g	
V. amurensis			225.22 mg/L 187.35 μg/g	
V. riparia x V. berlandieri (Vitaceae)			911.25 mg/L 622.90 μg/g *in the medium, in the cells	

(*continued*)

Table 2 (continued)

Plant species	Type of plants in vitro cultures	Compounds	Amount	References
Vitis vinifera cv. Feteasca Neagra	Shoots	Trans-resveratrol	41.30 μg/g DW	[77]
Vitis vinifera Cabernet Sauvignon (Vitaceae)			97.94 μg/g DW	
Vitis vinifera L cv. Muscat de Frontignan (Vitaceae)	Cell suspension	3-*O*-glucosyl-resveratrol	150 μM/ g DW	[79]
		4-(3, 5-dihydro-xyphenyl)-phenol	140 μM/g DW	
S. tomentosa (Lamiaceae)	Cell suspension	Oleanolic acid	71.89 μg/ml	[88]
		Ursolic acid	256.279 μg/ml	
S. officinalis (Lamiaceae)	Shoots	arnosic acid	4.37 mg/g DW	[86]
		Carnosol	0.88 mg/g DW	
S. scabiosifolia (Lamiaceae)	Rhizogenic callus	Oleanolic acid	829.14 μg/g DW	[87]
Salvia sahendica (Lamiaceae)	callus	Betulinic acid	17.28 mg/100 g DW	[89]
		Oleanolic acid	126.27 mg/100 g DW;	
		Ursolic acid	121.59 mg/100 g DW	
Elaeagnus angustifolia L (Elaeagnaceae)	Cell suspension	α-Tocopherol	3.2 ppm	[92]
			9.82 ppm	
Helianthus annus L. (Asteraceae)	Cell suspension	α-Tocopherol	11.26 μg/g FW	[91]
Arabidopsis thaliana L (Brassicaceae)	Cell suspension	α-Tocopherol	9.00 μg/g FW	[91]
Helianthus annus L. (Asteraceae)	Cell suspension	α-Tocopherol		[93]
Plant-flavoring compounds				
Vanilla planifolia (Orchidacea)	Callus	Vanillin	0.28 μg/g FW; 5.10 μg/g FW	[97]
	Shoots	Vanillic acid	0.46 μg/g FW; 6.40 μg/g FW	
Vanilla planifolia (Orchidacea)	Suspension	Vanillin		[98]

(*continued*)

Table 2 (continued)

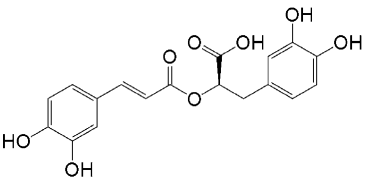
Plant species	Type of plants in vitro cultures	Compounds	Amount	References
Zingiber officinale (Zingiberaceae)	Callus culture	Gingerols	15 mg/g DW	[99]
Zingiber officinale (Zingiberaceae)	Callus culture	Gingerol	191.07 μg per culture bottle	[100]
Capsicum chinense Jacq (Solanaceae)	Immobilized placentas in a calcium alginate	Capsaicinoids	15.6 mg/g DW)	[103]
Allium sativum L. (Alliaceae)	Callus	Alliin		[104]
	Shoots			
Allium sativum L. (Alliaceae)	Shoots	Alliin		[105]
Plant sweeteners				
S. rebaudiana (Asteraceae)	Suspension	Stevioside	381.03 μg/g DW	[113]
S. rebaudiana (Asteraceae)	Shoots	Stevioside	0.451 mg/g DW	[116]
S. rebaudiana (Asteraceae)	Callus	Stevioside	12.62% of FW	[112]

medium composition and culture conditions in laboratory bioreactor [49, 57]. Its elicitation with different biotic elicitors (cell walls and homogenates from *Pseudomonas fluorescens*, *Bacillus subtilis*, and *Penicillium expansum*) has been investigated, and it was established that they did not influence rosmarinic acid biosynthesis. As distinguished, elicitation of *L. vera* MM with methyl jasmonate or vanadyl sulfate increased the amount of rosmarinic acid about 2.4 and 3.3 times, respectively, compared to the non-elicited cells [58, 59], (Table 2).

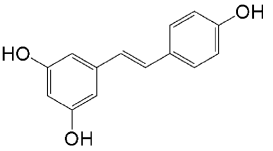
High quantities of rosmarinic acid (**19**) were successfully obtained by hairy root culture of *Nepeta cataria* L, cell suspension culture of *Mentha piperita*, callus and shoot cultures of basil (*Ocimum basilicum* L.) and lemon balm (*Melissa officinalis*) L. [60–62].

In the last few years, there were many successfully developed approaches for production of other valuable phenolic acids (chlorogenic acid, cichoric acid, caffeic acid) and flavonoids (rutin, quercetin (**22**), hyperosid, kaempferol (**20**), luteolin (**23**), apigenin (**24**), myricetin (**21**)) by different type of plant in vitro cultures [63–71] (Table 2) (Fig. 2).

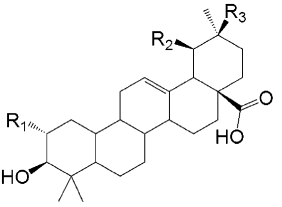
Marchev et al. [72] described an efficient protocol for polyphenols synthesis by hairy roots of *Salvia tomentosa* Mill. obtained by *Agrobacterium rhizogenes* transformation using two-phase temporary immersion cultivation system with Amberlite



(19) Rosmarinic acid

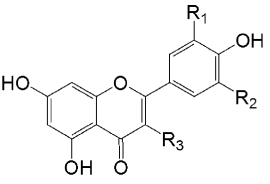


(25) Resveratrol



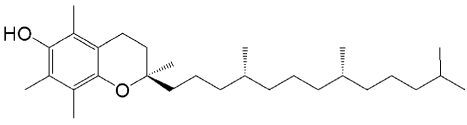
Triterpenes

R_1	R_2	R_3	
-H	$-CH_3$	-H	(25) Ursolic acid
-OH	$-CH_3$	-H	(26) Corosolic acid
-H	-H	$-CH_3$	(27) Oleanolic acid
-OH	-H	$-CH_3$	(28) Maslinic acid

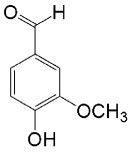


Flavonoids

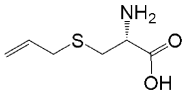
R_1	R_2	R_3	
-H	-H	-OH	(20) Kaempferol
-OH	-OH	-OH	(21) Myricetin
-OH	-H	-OH	(22) Quercetin
-OH	-H	-H	(23) Luteolin
-H	-H	-H	(24) Apigenin



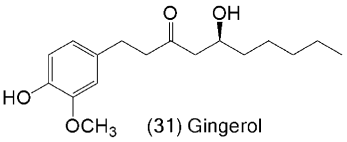
(29) a-Tocopherol



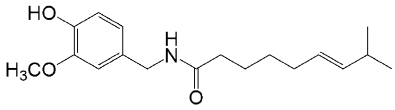
(29) Vanillin



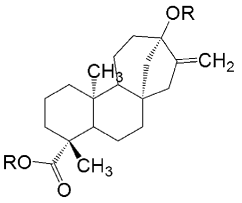
(30) Alliin



(31) Gingerol



(32) Capsaicin



R	
-H	(33) Steviol
-glycoside	(34) Steviol glycosides

Fig. 2 Chemical structures of some secondary metabolites obtained from plant in vitro systems with potential for application in foods

XAD-4 resin. The resin adsorbed more than 93% of the released phenolic acids and 100% of the released flavonoids.

Elicitation of the callus cultures of *Ephedra alata* Decne with casein hydrolysate and feeding with the precursor amino acid L-phenylalanine significantly increased the production of phenolics (chlorogenic acid, rutin, catechin, quercetin, p-coumaric acid) compared to those in the stem of the wild plant [52].

Suspension cultures of grape have been reported to accumulate a wide range of catechins, anthocyanins, proanthocyanins, and stilbenes like *cis*- and *trans*-resveratrol (**25**) with multiple biological properties such as antioxidant, antibacterial, anticancer, and estrogenic and heart-protecting activities [73] (Fig. 2). Reservatrol and its related compounds have been investigated for the biological activity in a wide range of biological assays including breast, lung, and central nervous system cancer cell lines [74, 75]. Production of stilbenes by grape cell suspension culture was enhanced by different biotic (soluble glucan elicitors, prepared from acid hydrolysis of *Botrytis cinerea* cell walls, cyclodextrins) and abiotic elicitors (metal salts, UV light), alone or in combinations [76–79]. Combination of UV light and JA enhanced the production of total intracellular stilbenes production to the 2005.05 μg/g DW and the trans-resveratrol accumulation to 3.96 mg/L by cell suspension culture of *Vitis vinifera* [80]. Belchí-Navarro et al. [81] also reported increased trans-resveratrol production (1447 μmol/g DW) of suspension culture of *Vitis vinifera* by the joint use of cyclodextrins and methyl jasmonate. Significant trans-resveratrol accumulation (62.23 μg/g FW) was observed when 12-day-old callus cultures of *Vitis vinifera* L. cv. Öküzgözü were exposed to UV light (254 nm) for 15 min and incubated for 48 h [82] (Table 2).

Scale-up of cell culture of *Vitis vinifera* cv. Barbera from flasks to stirred tank bioreactor resulted in a 22-fold increase of the endogenous resveratrol production and a tenfold increase of the extracellular resveratrol content. Within 28 days of culture, the total stilbenes accumulated in this cell culture system were around 32.72 μmol/g DW, equivalent to 48 mg/L upon elicitation with 50 mg/L chitosan [83].

Dimethyl-β-cyclodextrin an oligosaccharide consisting of 2,6-methylated cyclic α(1 → 4)-linked glucopyranose moieties was shown to be capable of inducing stilbene biosynthesis in liquid grape cell cultures of the *Vitis vinifera* cultivars Pinot Noir and Merzling, *V. amurensis*, and a cross between *V. riparia* and *V. berlandieri*. The highest content of trans-resveratrol was defined in suspension cultures of the cross between *V. riparia* and *V. berlandieri* [84].

The abiotic elicitor $AlCl_3$ resulted in twofold (41.30 μg/g DW) and sevenfold (97.94 μg/g DW) increase of trans-resveratrol production by *Vitis vinifera* cv. Feteasca Neagra and *Vitis vinifera* cv Cabernet Sauvignon shoot cultures, respectively, compared to the nontreated cultures [77].

Other widespread group of plant secondary metabolites are terpenoids (**25**–**28**), such as betulin, betulinic, carnosic, oleanolic (**27**), and ursolic acid (**25**) (Fig. 2). These pentacyclic di- and triterpenes were mainly found in plants of the genus *Salvia* and exhibit valuable pharmacological activities: antioxidant, anti-inflammatory, hepatoprotective, anticancer, anti-HIV, antimicrobial, antiulcer, and gastroprotective

[85]. A variety of tissue cultures of sage have been established as a source of di- and triterpenes such as *S. officinalis* [86], *S. scabiosifolia* rhizogenic callus [87], *Salvia tomentosa* [88], and *Salvia sahendica* [89] (Table 2). The antioxidant activity of in vitro cultures of common sage, especially in lipophilic systems such as the inhibition of lipid peroxidation, can be attributed to terpenes rather than to phenolics [86].

Tocopherols are widely used in human nutrition as vitamin E and in food conservation as powerful free radical scavengers. α-Tocopherol (**29**) (Fig. 2) is the most active form of vitamin E, while the extraction from plant oils usually yields a mixture of β, γ, δ, and α- tocopherols and tocotrienols. Therefore there is a demand for searching alternative sources of pure α-tocopherol from plant tissues. Various in vitro culture systems of *Helianthus annuus* (sunflower) have been established for this purpose. Increased tocopherol content in plant cell cultures has been achieved by media optimization, elicitation, and precursor feeding [90, 91].

Elicitor-induced α-tocopherol production can be suppressed by both JA and SA elicitors, as the 1-mM concentration of SA was most effective in alpha-tocopherol production (9.82 ppm) in the cell cultures of *Elaeagnus angustifolia* L. [92]. JA increased tocopherol content in *Helianthus annus* L. (sunflower) cell suspension culture with 50% and about 66% in cell suspension culture of *Arabidopsis thaliana* L compared to the both type untreated cell cultures [91]. Probably, the observed enhancement of tocopherol production in sunflower and *Arabidopsis* cell cultures could be due to the ability of the exogenously added jasmonic acid to upregulate the biosynthetic pathway through the induction of gene expression of two genes of the tocopherol biosynthetic pathway, namely, p-hydroxyphenylpyruvate dioxygenase and homogentisate phytyltransferase [90].

The considerable increased α-tocopherol content (max. 230%) was obtained within the photomixotrophic cell suspension culture of *Helianthus annus* compared to a heterotrophic cell culture [93] (Table 2).

4 Plant-Flavoring Compounds

Other widely used group of food additives are the flavors. A variety of plant in vitro systems are now available for studying and production of food flavors [94].

Vanillin (4-hydroxy-3-methoxybenzaldehyde) (**29**) (Fig. 2) is one of the most important flavoring compound used in foods and beverages, as its world consumption was around 32,000 tons annually. Besides it imparts the aroma and taste of foods, this compound has antimicrobial, anticancer, antioxidant, and immunosuppressant activities [95, 96]. The major components of vanilla flavor are vanillin, vanillic acid, vanillyl alcohol, p-hydroxybenzaldehyde, p-hydroxybenzoic acid, and p-hydroxybenzylalcohol. The main commercial source of vanillin is the bean or pod of the tropical *Vanilla planifolia*, belonging to the Orchidaceae family. The conventional cultivation of this plant is laborious, and that one has to manually pollinate the flowers. To overcome this problem, there is a need to develop a viable and simple method for the production of vanilla flavor metabolites using cell culture systems

[95]. There are many successful protocols for in vitro production of this flavoring compound by *Vanilla planifolia* [97, 98] (Table 2).

Different type of plant in vitro cultures of *Zingiber officinale* and *Capsicum chinense* Jacq are sources of pungent flavors, such as gingerols (**31**) and capsaicin. (**32**) (Fig. 2). Anasori and Asghari [99] established that no gingerol and zingiberene were detected in the dedifferentiated callus of *Zingiber officinale* Rosc. grown in light or dark environment and their production is correlated with some sort of differentiation.

Cafino et al. [100] subjected multiple shoot culture of *Zingiber officinale* to different light wavelengths and established that gingerol production under green, blue, indigo, and violet lights were not significantly different with that of the control treatment (58.84 ± 0.06 μg per culture bottle). The highest gingerol content (191.07 ± 1.25 μg per culture bottle) was observed in multiple shoots cultured under red light, which represents about threefold increased gingerol production as compared with that of the control sample.

Since the biosynthesis of capsaicinoids of *Capsicum chinense* is located in the placental tissue, two methods for the in vitro culture of pepper placentas have been used: free and immobilized tissues. Free placentas cannot maintain their integrity as they become swollen and disintegrate after short periods in culture. This effect can be avoided by immobilizing the tissue. Tissue immobilization consists in entrapping sections within an inert support, keeping it alive and functional in a medium with mineral salts and organic compounds under sterile conditions [101, 102].

Aldana-Iuit et al. [103] detected a sharp increase in capsaicinoid level in *Capsicum chinense*-immobilized placentas when available nitrate in MS medium increased to 60 mM (6.4 mg/g DW) or 80 mM (15.6 mg/g DW) for 7 days in contrast when the full MS nitrate content (40 mM) was employed (0.3 mg/g DW).

The flavors of onion and garlic alliin (**30**) were also produced by plant in vitro cultures [104, 105] (Table 2) (Fig. 2).

5 Plant Sweeteners

In the recent years there is need to find non-nutritive and safety sweeteners for various food applications. The most important plant-derived sweetener is stevioside (**33–34**) from *Stevia rebaudiana* [95] (Fig. 2). All of the diterpene glycosides (stevioside and rebaudiosides) biosynthesized by *S. rebaudiana* are nontoxic, nonmutagenic, and 250–300 times sweeter than sucrose. Beyond their sweetness they possessed a number of therapeutic activities, namely, antihyperglycemic [106], antihypertensive [107], antitumor [108], vasodilator [109], antimicrobial [110], and neuroprotective [111]. These compounds are heat and pH stable with a good shelf life and can be added in cooking, baking, or in beverages [112].

The data on the content of steviol glycoside (**34**) in in vitro cultures of *Stevia rebaudiana* differ very much in the literature. According to study carried out by Swanson et al. [113], callus culture was not able to synthesize stevioside that is contrary with the results obtained by Taware et al. [114] who showed high content of

stevioside in the callus cultures (about 12% of DW). Comparative analysis of the stevioside content in the fresh leaves and callus culture revealed approximately equally quantities – 12.19% and 12.62%, respectively. Maximum amount of callusing was observed on the MS medium supplemented with combination of 2.5 mg/L 2, 4-D and 0.5 mg/L BAP after 3 weeks of culture initiation. In this medium, calluses were globular and whitish in color, and average sizes of these calluses were 1.80 cm [112] (Table 2).

Mathur and Shekhawat [115] established an efficient protocol of the stevioside accumulation in *S. rebaudiana* suspension culture as the maximal content of stevioside (about 381.03 μg/g DW) in the cells was observed at the beginning of exponential growth phase, remained unchanged at the end of exponential phase, and decreased significantly at the end of stationary phase. This indicates for a positive correlation between active cell growth and steviol glycoside synthesis in suspension culture. Higher production of stevioside (0.451 mg/g DW) was established in callus-regenerated in vitro shoots of *S. rebaudiana* cultured in MS medium [116].

6 Plant Polysaccharides with Prebiotic Activities

Fructans are storage polysaccharides that accumulate in different vegetal part in approximately 15% of flowering plants [117]. In higher plants can distinguished three different types of fructans – inulin, levan, and graminan. Inulin-type fructan is a fructose polymer that has mostly or exclusively the β (2 → 1) fructosyl-fructose linkage, whereas levan-type fructan consists mostly or exclusively of β (2 → 6) fructosyl-fructose linkages. Inulin-type fructans typically accumulate in the core eudicot families (mainly in Asteraceae), while levan-type fructans with β (2 → 6) linkages and branched, graminan-type fructans with mixed linkages are characteristic for monocot families [118, 119]. Inulin-type fructans and its subgroup fructo-oligosaccharides are the most widely applied prebiotics and immunomodulator isolated mainly from chicory roots and topinambour tubers and added to a variety of food products [119, 120].

The production of fructans from callus presents interesting field of investigation. The isolation, characterization, and potential application of callus fructans will be a challenge for design of new products for food industry. Many reports deal with fructan accumulation from different callus culture from plants that naturally accumulate fructans: comfrey (*Symphytum officinale* L.) [121, 122], *Vernonia herbacea* [123], *Helianthus tuberosus* L. [124], *Viguiera discolor* Baker. [125], and *Gomphrena macrocephala* [126]. *Helianthus tuberosus* and *Vernonia herbacea* are natural source of inulin in higher amount above 60% dry weight [123, 127]. Application of this in vitro technique for callus production from these two plants enables the inulin synthesis as accumulation not only in the whole plant but mainly in plant tissues, organs, or undifferentiated cells [123, 124].

Fructan accumulation in callus derived from comfrey (*Symphytum officinale* L.) depends on various phytohormones (NAA, IBA, 6-BAP, kinetin, and zeatin). The concentration of NAA had no influence on the fructan content. Highest rates of fructan

synthesis occurred at low zeatin concentrations up to 1.5 mg/L. Only zeatin at all concentrations induced the synthesis of polyfructans, whereas appreciable amounts of oligofructans were formed under the influence of all other phytohormones [122].

In vitro propagation of *V. discolor* may allow fructan production under this condition. Analysis showed the presence of inulin-type fructans (3.82 mg/g fresh weight). Incubation of stem nodes in the presence of 2,4-D induced growth of friable callus (callus type 2), in which fructans and their synthesis enzymes sucrose, sucrose 1-fructosyltransferase (SST), and fructan, fructan 1fructosyltransferase (FFT), were found [125].

The production of fructose-containing carbohydrates by leaf and node callus of *Gomphrena macrocephala* St.-Hil. grown in three different auxin to cytokinin ratios was also reported. Higher fructan content was detected in node callus grown in NAA. A homologous series of fructans was detected only in callus grown in 1:2 auxin to cytokinin ratio. These fructans were from inulin series with the mean molecular weight 40 kDa which was 3 times greater than that of fructan from intact plants [126].

But increasing demand for different health-promoting fructan structures requires a more cost-effective production of tailor-made fructans. The expression of fructosyltransferase genes in plants that ordinarily do not accumulate fructans leads to the synthesis of fructans in plants as patato, maize, and sugar beet [128]. Sugar beet is an economically important plant that lacks the enzymes to produce fructans but accumulates high levels of the substrate sucrose. The introduction of a pair of fructosyltransferases from onion, namely, 1-SST and 6G–FFT, resulted in high-level accumulation of neokestose-type fructans in the sugar beet [129]. These neo-series inulin are of interest, as neokestose (1 and 6G–kestotetraose) because its prebiotic and bifidogenic effect [130].

Transformation of a *Bacillus amyloliquefaciens* SacB gene into maize (*Zea mays* L.) callus by particle bombardment was done to introduce fructan biosynthesis. Accumulation of high-molecular-weight fructan in mature seeds was found after tissue-specific expression and targeting of the SacB protein to endosperm vacuoles. Accumulation of fructan in the vacuole had no detectable effect on kernel development or germination. Fructan content was approximately ninefold higher in sh2 mutants compared to wild-type maize kernels [131].

7 Conclusion and Future Perspectives

During the last years, the consumer interests have been direct to consumption of foods mainly from natural sources. The plant in vitro systems are alternative sources for producing of natural colorants, additives, and supplements. It has been several highly productive plant in vitro systems including cell suspensions and hairy root cultures, but has been not developed large scale-up processes, yet. Low-cost bioreactors systems, such as the TIS, plastic-lined reactors, and wave bioreactors, can also be successfully applied to production of various substances from in vitro plant culture application in the food industry. Nonconventional methods, such as elicitation, cell permeabilization, and two-phase cultivations, could significantly increase

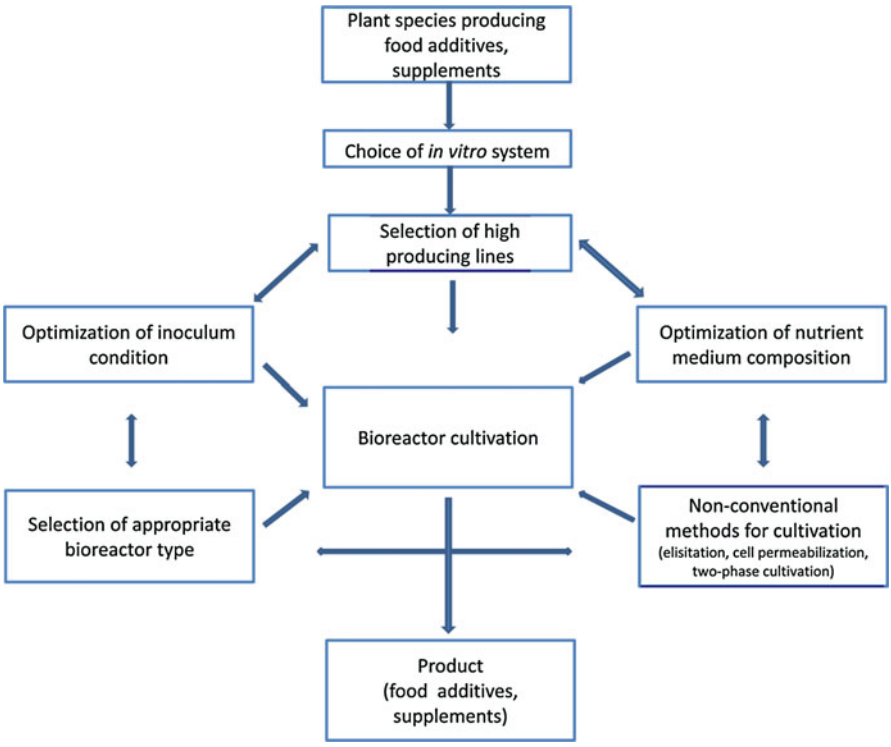


Fig. 3 Integrated approach for improving production of food supplements and additives by plant in vitro systems according to Georgiev et al. [5]

the yields of target compounds. The choice of appropriate type of bioreactors has been found to influence significantly cultivation condition and the yield of target metabolites, as well (Fig. 3). Food and food additives are subjected to a strict control from number of institutions responsible for food safety. The main problem for plant cultures is the synthetically growth regulator 2,4-D. Although it is added in small quantities to culture mediums it has a carcinogenic effect on humans. It is necessary to select productive lines that are independent of 2,4-D or must be replaced with natural ones (IBA, IAA) that do not have negative effects on people and the environment.

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Safety Assessment and Regulations for Food Ingredients Derived from Plant In Vitro Systems

14

Angel Angelov and Velitchka Gotcheva

Contents

Abstract

Plant cell and tissue cultures present numerous advantages for the production of food ingredients. This technology has made a great progress in the past two decades, with a few examples of products reaching the market. Safety assessment of plant in vitro products is a key issue for the development of sustainable commercial processes. Experts have proposed various strategies and methods for assessing the potential risks for the end product arising from the production processes, and throughout the years these have been implemented as guidelines and national regulations. The international approach in safety assessment of single chemically defined plant and cell culture food ingredients is based on the concept of substantial equivalence developed for other categories for novel food products. For products in the form of complex mixtures, conventional metabolism and toxicokinetic studies should be provided for toxicologically relevant constituents with known or demonstrable biological or toxicological activity on a case-by-case basis. The present chapter gives an overview of the current

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international regulatory basis and proposed schemes for the safety assessment of plant cell and tissue culture-derived food ingredient with some suggestions for the future needs in this field.

Keywords
Safety assessment · Regulations · Food ingredients · Plant cell and tissue cultures

Abbreviations

ADME	Absorption distribution metabolism excretion
ANS	Panel on Food Additives and Nutrient Sources Added to Food
CE	Council of Europe
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
FAIM	Food Additive Intake Model
FAO	Food and Agriculture Organization of the United Nations
FDA	U.S. Food and Drug Administration
FEDCA	Federal Food, Drug and Cosmetic Act
FEMA	Panel of the Flavor and Extract Manufacturers Association
GMPs	Good Manufacturing Practices
GRAS	Generally Recognized As Safe
IFBC	International Food Biotechnology Council
LOD	Limit of Detection
LOQ	Limit of Quantification
NDA	Panel on Dietetic Products, Nutrition and Allergies
PCTC	Plant Cell and Tissue Cultures
QSAR	Quantitative structure-activity relationship
SAR	Structure-activity relationship
SE	Substantial equivalence
TTC	Threshold of Toxicological Concern
WHO	World Health Organization

1 Introduction

The use of bioactive compounds derived from plants dates thousands of years back in human history and is continuously increasing. However, nowadays a number of issues must be solved in order to respond to this rising demand. Such issues include climate changes and geographical constraints, decreasing natural habitats and extinction of numerous valuable plant species, availability of farming land and the negative environmental footprint of the production of plant-based products [1, 2, 3].

The alternative of producing plant metabolites by plant cell and tissue cultures (PCTC) has been intensively explored since the introduction of this concept in the beginning of XX century, developing a whole new field of green biotechnology. This approach for deriving biologically active plant ingredients for cosmetic, medicinal,

and nutritional applications offers a number of benefits related to environmental biodiversity, ecological sustainability, product quality, and safety [2, 4, 5].

Application of PCTC reduces the necessity of land and water, as well as the use of pesticides. It allows easier product extraction and, therefore, lowers the use of chemicals. The positive environmental effect is also related to less accumulation of agricultural and industrial waste. The use of plant cell and tissue cultures also allows the production of bioactive compounds from rare or endangered species [1].

Also, unlike traditionally grown plants, cultivation of PCTC in bioreactors guarantees defined controlled process conditions for optimal environment for effective cell growth and production of bioactive substances and allows the application of good manufacturing practices (GMPs). This results in minimum or even no variations in product yield and quality and, therefore, simplified process validation and higher level of product standardization. Further benefits include improved safety of the products based on the absence of environmental pollutants and agrochemicals, which is important for products legal registration as food ingredients [1, 4, 6].

In order to introduce a PCTC-derived product to the market as a food ingredient, it should be recognized as safe by the authorities, and for this purpose data from extensive safety assessment must be provided. Regulatory agencies in many countries have developed policies and guidelines for regulating food ingredients produced by PCTC, and several safety assessment schemes have been developed by various organizations. The present chapter gives an overview of the current international regulatory basis and views on safety assessment of food ingredients from plant cell and tissue cultures.

2 Advances in the Production of Food Ingredients by Plant Cell and Tissue Cultures

The main stages for the development of secondary metabolites production technologies with in vitro plant cultures include:

- Establishment of callus/cell suspension/organ cultures of plant with the content of the required phytochemicals
- Selection of highly productive clones
- Optimization of culture conditions
- Scale up from small to large vessels [7]

Secondary metabolites can be produced by different types of cultures, such as callus, cell suspension cultures, or organ cultures. The potential of PCTC for the production of phytochemicals for food use has been recognized by FAO in 1994. At that time plant cell culture technology is not cost-efficient and only two products are commercially produced (shikonins and ginseng cells), and the main barriers identified in the report are low yield of plant metabolites, unstable producing ability of cultured cells and their slow growth rate [8]. In the recent years, various scientific strategies have been employed in attempts to enhance the production ability of

cultured plant cells and tissues. Secondary metabolite production is a two-stage process, in which the first phase is biomass growth, and in the second phase metabolite biosynthesis occurs. Therefore, production improvement strategies are specific for each stage. The starting point is the selection of plant cells or organ clones with high production ability. Further, conventional optimization of the first phase includes optimization of medium parameters (suitable medium, salt, sugar, nitrogen, phosphate, and plant regulator levels) and physical factors (temperature, illumination, light quality, medium pH, agitation, aeration, environmental gas) [9]. In the second stage, strategies for improving the biosynthesis of metabolites and assisting their accumulation are employed, such as addition of nutrients and precursors for the synthesis of secondary metabolites [6, 10], elicitation [3, 11], and permeabilization and immobilization methods [9].

A huge effort has been put in fundamental research to elucidate the biosynthetic pathways of many useful secondary metabolites in plants and of the regulation mechanisms in their biosynthesis [12, 13]. Genetic manipulation is also employed for altering the metabolite profile to increase production of desired products. Recent molecular biology approaches to affect the productivity of PCTC employ the methods of metabolic engineering, including overexpression of the genes encoding the production of the target metabolite(s) or repression of genes to modify secondary metabolic pathways [14–18].

As a result, a wide range of food ingredients including flavours [19, 20], colorants [21–23], essential oils, sweeteners [24, 25], antioxidants, and nutraceuticals have been produced in plant cell and tissue cultures, and many large-scale cell suspension systems have been developed for the production of various compounds such as vanilla flavor components [26, 27], anthocyanins [28–30], and ginseng products [2, 31, 32].

Different types of bioreactors have been used for the cultivation of plant cells and tissues. However, a number of various problems are yet to be solved before this technology can be adopted on wide scale for the production of useful plant secondary metabolites. In addition to the above listed factors affecting plant cell and tissue cultivation, other factors such as the bioreactor design, gaseous atmosphere, oxygen supply and CO2 exchange, the liquid medium rheology and the cell density, agitation systems, and sterilization conditions also influence the whole process and present a serious challenge in process scale-up and optimization [6, 7, 31, 32].

Since it is generally estimated that production of secondary metabolites is higher in differentiated plant tissues, attempts are made to cultivate whole plant organs (i.e., shoots or roots) in in vitro conditions. Organized tissue cultures offer a number of advantages over the cultivation of undifferentiated cells including higher production stability and similar patterns of secondary metabolites as the control intact plants, the last being very important for the safety assessment of a product for food applications [12, 33].

In this line, the use of plant organ cultures or transformed roots (hairy roots cultures) offers a promising potential [34–37]. A review of Georgiev et al. [21] summarizes the advantages and challenges of hairy root culture systems, viewed as “green factories” for high value molecules. New molecular research is aiming at

genetic transformation of yeast and bacteria with plant genes for the production of plant secondary metabolites. The new approaches look promising so far, but still a lot of effort is needed to develop commercially viable technologies.

Despite their numerous advantages over conventional methods and the promising developments, production of food ingredients from plant cell and tissue cultures still needs a lot of efforts in order to be fully developed on an industrial scale because of the low product yields, high cultivation costs, high level of investment required for industrial scale up, and the long period of time for product development and testing [1].

3 International Guidelines on the Safety Assessment of Food Ingredients from Plant Cell and Tissue Cultures

Besides production obstacles, another reason for limited commercialization of PCTC-based processes for the production of food ingredients is the complex legislation which differs between countries and makes the introduction of a new food ingredient time consuming and costly [4, 37].

Guidelines for the safety assessment of foods derived from modern biotechnology have been elaborated by the World Health and the Food and Agriculture Organisations (WHO and FAO), the Organisation for Economic Co-operation and Development (OECD), the International Food Biotechnology Council (IFBC), the International Life Science Institute (ILSI), the US Food and Drug Administration (FDA), the UK Advisory Committee on Novel Foods and Processes (ACNFP), the Nordic Council, the German Research Community (DFG), and other national bodies. These organizations base their recommendations for the safety assessment of a modified organism on the comparison with the nonmodified counterpart in order to identify equivalencies and differences, which is the so-called concept of substantial equivalence (SE) [38].

The concept has been suggested regarding safety assessment of novel foods only recently, but it has been applied for a long time in many fields of science and technology when new developments were introduced. It is based on the idea that existing food products can serve as a basis for comparison when assessing the safety and the nutritional value of a food or food ingredient that has been modified by modern biotechnological methods or is new. It implies that if a novel food or novel food component is found to be substantially equivalent to an existing food or food component, it can be treated in the same manner with respect to safety, and no additional safety concern would be expected. On the other hand, if a novel food or novel food ingredient has not been found to be substantially equivalent to its conventional counterpart, this does not imply that it is unsafe. In this case, the safety of the novel food should be evaluated on the basis of its unique composition and properties [39].

The SE concept was also referred to in the Regulation (EC) No 258/97 concerning novel foods and novel food ingredients, according to which it is sufficient for an applicant to provide the European Commission scientific evidence that a

novel food is substantially equivalent to existing product(s) with regards to their composition, nutritional value, metabolism, intended use and level of undesirable substances contained, or to notify the Commision of the opinion of a competent state authority which has made the assessment based on the provided evidence [38].

This approach was adopted by the WHO in 1995 for the evaluation of PCTC-derived flavourings and is also applied for other food ingredients obtained from PCTC. It is based on establishing the similarities or the differences between a PCTC product and its traditional counterpart and, when the conventional product has a safe history of use, then the PCTC product is also considered safe to the same extent. The most important factors in establishing the SE of PCTC products are the food ingredient composition and the presence of the key substances for safety considerations (those important with regard to toxicological, antinutritional or allergenic effects) [40]. However, since most PCTC systems are not able to deliver the same product profile as that of the whole plant, and product profile is also affected by the various yield improvement techniques, the substantial equivalence may not be established and the culture-derived product should undergo safety assessment [41].

Another issue which also complicates the determination of SE is the large variation between food ingredients derived from plants of different cultivars in different regions. Therefore, it is very difficult to determine whether the composition variation of a PCTC-derived product exceeds the range of variation found among plant families [41].

Different approaches are applied around the world for safety assessment of food ingredients derived from plant cell and tissue cultures. They differ in various extent from country to country, but in general, they share the same common principles.

In the USA, the Expert Panel of the Flavour and Extract Manufacturers Association (FEMA) has elaborated a safety assessment program for single, chemically defined flavor ingredients, which is recommended as a model for other food ingredients with benign chemical structures which are often naturally present in food and are normally consumed at very low levels. The specific criteria developed by FEMA for the evaluation of flavor ingredients include exposure to the assessed substance in specific foods, total amount in the diet and total poundage; natural occurrence in food; chemical identity and specific chemical structure; metabolic and pharmacokinetic characteristics; and animal toxicity [42].

According to Hallagan et al. [42], the safety focus when assessing a single flavor ingredient produced by using PCTC should be on the end product, and not on the process, through which it is produced. Of course, this does not eliminate the necessity of knowledge on the process with regard to factors which could affect product composition and bring in possible impurities. For the safety of food ingredients from PCTC which are mixtures, the authors recommend the principles described by the International Food Biotechnology Council (IFBC) in 1990, where the concept of SE is also applied.

For food and dietary supplements, the Food and Drug Administration (FDA) applies the Federal Food, Drug and Cosmetic Act (FEDCA), and the product should be declared as “generally recognized as safe” (GRAS) by qualified experts. For products which are not of GRAS status, the regulations of the Dietary Supplement

Health and Education Act should be applied [43, 44]. Beru [44] presents an overview on how food ingredients including those produced through PCTC are regulated at the FDA and how it assesses the safety of these food ingredients. Currently, three types of petitions are submitted for the different groups of food ingredients: a petition for authorization, GRAS affirmation, and color additive petition. For these applications, the common information required for the safety assessment of a proposed ingredient includes chemical identity of the substance, its production process, the intended technical effect, and proposed maximum level of use in food and safety studies.

The latest detailed FDA Guidance for industry and other stakeholders regarding the toxicological principles for the safety assessment of food ingredients (also called Redbook 2000) was published in 2000, and further revised in 2007 [45].

The approach of Health Canada to the regulation of novel foods which include PCTC-derived food ingredients is similar to that of the USA and the general international view [46]. The safety evaluation is also based on substantial equivalence concept and is conducted according to the Guidelines for the Safety Assessment of Novel Foods, published in 2006 [47].

In Japan, the regulation for PCTC-derived products in Japan is considered in three categories: drugs, foods, and food additives [48]. In 2010, the Food Safety Commission of Japan issued the Guideline for assessment of the effect of food on human health regarding food additives. According to the definition of Food Sanitation Act (FSA) in Japan, food additives are "(i) substances used in or on food in the process of manufacturing food, or substances used for the purpose of processing or preserving food. Consequently, "food additive" includes both substances remaining in the final products, such as food colors and preservatives, and substances not remaining in the final products, such as microorganism control agents and filtration aids." [49]. Regardless of whether they are from natural origin, all substances used for the above purposes are categorized as food additives; therefore, the Guidelines cited above would apply for a wide variety of PCTC products.

Strategies for the safety assessment of PCTC-derived food ingredients are reviewed by a number of authors. The schemes are based on the careful consideration of the potential risks for the end products safety at each step of a PCTC technology.

Fu [41] discussed the potential safety concerns starting from (1) cell line development, including different types of cultures and techniques for obtaining high-yield cell lines, and continuing through (2) process scale-up, (3) production (specifically focusing on elicitation and in situ product removal), and (4) purification. The author outlines the issues with applying the SE concept on PCTC food ingredients which are mainly due to the difficulty to determine whether a modification made by a PCTC process exceeds the range of variation among plant families. Since prediction of the effect of a PCTC process on the product profile is not currently possible, monitoring of the level of potential toxic substances present is essential, as well as safety testing.

Safety assessment approaches applied to food and food ingredients are also relevant to novel foods and PCTC-derived products, respectively. An extensive

overview of Blaauboer et al. [50] concluded that any strategy for the safety evaluation of food ingredients should address the following items:

- Chemical identity/structural activity relationships
- Relevant exposure scenarios
- Digestibility/stability
- Absorption and distribution (internal exposure)
- Metabolism
- Genetic toxicity and Carcinogenicity
- Repeated dosing
- Tolerance/allergenicity
- Other systemic endpoints case by case (e.g. reproductive and developmental toxicity)
- Target population specific testing (e.g. term infants and pregnant women)

The authors discussed a number of newly developed in vitro and in silico methods for the assessment of the toxicological effects of PCTC-derived products, such as the use of (stem) cell cultures for studying absorption and systemic toxicity, 3D cell cultures and organotypic tissues for metabolism, organs-on-chips, models for investigating digestion and bioaccessibility, biokinetics (ADME), biotransformation and the application of structure-activity relationship (SAR), and quantitative structure-activity relationship (QSAR) models. An evaluation roadmap for safety assessment of food and food ingredients is suggested, which is also applicable to PCTC-derived products [50].

The safety-related considerations at the various stages of PCTC-based processes for food ingredient production are also described by Murthy et al. [43]. For the stages of biomass production and the production of secondary metabolites, the suggested points of assessment includes (1) selection of suitable plant material, (2) PCTC method for selection of raw material (selection of suitable explants; production of cell or organ lines for the production of biomass; optimized culture conditions/parameters for in vitro cultivation; type of culture vessel (bioreactor) used and the bioreactor conditions during growth and production cycles; elicitation methodology; biomass types such as cells, adventitious roots, hairy roots, embryos, shoots and harvesting of bioactive ingredients from the medium, method of harvesting), and (3) method of processing PCTC-obtained raw materials: drying and processing of biomass, storage conditions.

The group also proposed a range of biosafety evaluation/toxicological tests to be carried out at each of the above-listed process stages:

At stage (1): Chemical and biological analysis of raw material or product; nutritional facts.

At stage (2): Assessment of the in vitro evaluation – in vitro mutagenicity tests in bacteria and in vitro mutagenicity tests in mammalian cells; assessment of in vitro evaluation – animal studies.

At stage (3): Chemical authentication of ingredients; efficacy tests, such as antioxidant assay, antidiabetic, anticancerous, etc.; approval from competent authorities

for commercialization; and specific recommendations for the safety assessment of PCTC raw material or ingredients produced by genetically modified organs [43].

So far, the proposed approaches for the safety evaluation of PCTC-derived products reviewed above apply the same common principles with a different level of details. The same principles have found their sound establishment on the EU regulations further discussed.

4 EU Guidance on the Safety Assessment of Food Ingredients from Plant Cell and Tissue Cultures

Food ingredients derived from plant cell and tissue cultures are one of the food groups falling under the scope of Regulation (EU) 2015/2283 on novel foods ("food consisting of, isolated from or produced from cell culture or tissue culture derived from animals, plants, microorganisms, fungi or algae") [51].

With the adoption of a new Regulation (EU) 2015/2283, following the request of the European Commission, the European Food Safety Authority (EFSA) issued a draft scientific and technical guidance for the preparation and presentation of applications for authorization of novel foods. This guidance sets the minimum requirements on the data needed to carry out the safety assessments of novel foods, which includes description of the novel food, production process, compositional data, specification, proposed uses and use levels, and anticipated intake of the novel food, history of use of the novel food and/or its source, absorption, distribution, metabolism, excretion (ADME), nutritional information, toxicological information, and allergenicity [52]. A brief overview on the main requirements set in this document is provided below.

With regards to food ingredients consisting of, isolated from or produced from plant cell or tissue cultures, the following information on **product description** should be provided:

- Biological source (taxonomic information on family, genus, species, subspecies, variety)
- Organ and tissue or part of the plant sourced
- Laboratory or culture collection sourced
- Information on the identity of cells
- Cell or tissue substrate used as a novel food
- Type of culture

Further, the **production process** of the novel food should be described with sufficient details to allow conclusions regarding the impact of the process on the safety and nutritional value of the novel food. Information on the raw materials, culture medium, other reagents, and solvents used in the process should be provided. In the case of genetic modification, details of the construction used including the incorporation of markers, if any, should be provided. Information about the reactor

conditions during the growth and production cycles should be present, as well as a detailed description about the process by which the raw material is converted into a preparation, for example, extraction and purification procedures, as well as the standardization procedures. A production flow chart with quality control checks should be included in the description, with special accent on potential by-products, impurities or contaminants that could raise safety concerns. Information on the quality and safety management system(s) in place is also required [52].

Some commonly used synthetic growth factors added to the PCTC media have been found to exert toxic effects on humans. Such is the example of 2,4-Dichlorophenoxyacetic acid (2,4-D), the first commercially developed synthetic herbicide, which was later used as the most common synthetic auxin for inducing callus growth and maintaining callus and suspension cells in dedifferentiated states [53, 54]. Data from numerous studies show that 2,4-D generates free radicals causing depletion of ATP, NADPH, and GSH concentrations and lipid peroxidation, which may induce DNA modification and potential carcinogenesis [55]. It also causes mutagenic changes in cellular DNA, A→G mutation chromosome aberrations, sister chromatid exchange and DNA damage, and increase in the frequency of DNA strand breaks. 2,4-D is also related to apoptosis development due to disturbance of mitochondrial membrane [56].

Murthy et al. [43] provide an overview on the safety-related considerations to be made at the various stages of PCTC-based processes for food ingredient production. Techniques used to increase secondary metabolites yield during the production stage of a plant cell or tissue culture include addition of precursors to the cultures, elicitation, and in situ product removal. At this stage, it is important to avoid toxic elicitors, such as heavy metals, detergents, xenobiochemicals, fungicides, herbicides, and other harmful chemicals which could affect the safety of the product. In addition, elicitors of biological origin (methyl jasmonate, salicylic acid) as well as physical elicitors (UV irradiation, differential light sources) can be used for the improvement of product accumulation [43].

In situ product removal techniques used to remove the products from the cultivated plant cells and organ cultures should also be carefully considered since they may result in the accumulation of toxic compounds in the culture [57].

Selection of suitable extraction procedures is also important in view of product safety [58]. The conventional procedure involves solvent extraction of the target compounds, which is mostly affected by the matrix properties of the raw material, solvent temperature, pressure, and duration of treatment [43]. Azmir et al. [59] summarize the techniques for extraction of bioactive compounds from plant materials, which include soxhlet extraction, maceration, hydro-distillation, ultrasound-assisted extraction, pulsed-electric field extraction, enzyme-assisted extraction, microwave assisted extraction, pressurized liquid extraction, and supercritical fluid extraction. Consideration shows that soxhlet extraction is still considered to be one of the reference methods to which the success of newly developed methodologies is compared and in some cases may be superior to nonconventional methods of extraction [43].

Next, qualitative and quantitative data on the **composition** of the novel food should be provided, as well as physico-chemical, biochemical, and microbiological

properties. The specific information required is based on the complexity of the product – whether it is a single substance or a simple mixture (a mixture which components can be fully chemically characterized), or a complex mixture.

For **single substances**, data from identity tests should be provided, physico-chemical properties, solubility data in water and other common solvents, particle size, shape, distribution and the minimum purity value.

For **simple mixtures** of defined substances, information on the identities and the relative ratios of all components should be provided.

In **complex mixtures** (e.g., extracts) where all constituents cannot be fully chemically characterized and/or identified, a qualitative and quantitative characterization of the main constituents should be performed, and based of these data, a mass balance should be set up. The amount of unidentified constituents should also be indicated and should be as low as possible.

Comprehensive qualitative and quantitative data should also be provided for the classes of the PCTC-derived components which characterize the nature of the novel food (e.g., phytosterols, flavonoids, betalains, carotenoids, alcaloids). In addition, qualitative and quantitative data on nutritionally relevant inherent constituents (e.g. micronutrients) should be given.

It is possible that some PCTC produce potential toxic substances in vitro conditions, which may not occur or may occur in very small amounts in the field-grown plant [60]. Also, if the cultures have been genetically modified, they may contain allergenic substances which are not associated with the control plant [40]. Therefore, special attention should be given on toxic, addictive, psychotropic, or other substances of possible concern to human health and the presence of potential allergens. Information on the identities and the quantities of impurities or by-products, residues and contaminants should also be provided together with the type and the spectrum of potential target analytes based on the sources and the production process. Examples of such analytes are undesirable metabolites or residues of extraction solvent.

Compositional data should be obtained by validated methods described with their limit of detection (LOD) and limit of quantification (LOQ).

The physico-chemical, biochemical, and microbiological **stability** of the novel food should be evaluated in order to identify hazards, which might arise during storage under normal conditions, including the effects of packaging, the storage temperature, and the environment factors (light, oxygen, moisture, relative humidity).

Stability of food ingredients should also be investigated with regards to possible effects of processing temperature, pH, and interactions with other ingredients in the processed food.

The key parameters which characterize and substantiate the identity of the novel food should be defined in detailed **specifications** together with other relevant physico-chemical, biochemical, or microbiological parameters. Specifications should include the acceptable limits for impurities, degradation products, and contaminants (microorganisms, mycotoxins, etc.), as well as the methods used for the analysis of all parameters.

To perform risk characterization of PCTC-derived food ingredients, information for the target population, **proposed uses and use levels**, precautions, and restrictions

of use should be provided together with safety relevant data. If potential health hazards have been identified based on the composition, toxicological, or other data, they should be adequately addressed in the proposed conditions of use to ensure that the consumption of the novel food is safe for the target population.

The information on proposed use and use levels of the product should include its form of uses (e.g., food ingredient, food supplement), the food categories for use, whether the novel food is intended to replace another food, the proposed maximum amounts in final product(s), and the proposed average and maximum daily intakes for different age/gender groups.

Also, estimations of anticipated intakes of the novel food are required (per kg body weight and in absolute amounts) for each target population group (and, where relevant, vulnerable groups). The concurrent consumption of all food categories in which a novel food ingredient is proposed to be used should be addressed in the estimations with consideration of different consumption scenarios.

Since exposure assessment of food additives and intake assessment of novel food ingredients share common principles, EFSA Food Additive Intake Model (FAIM) tool for the calculation of chronic exposure to food additives may be used for the intake assessment of novel foods used as ingredients. Data from the EFSA Comprehensive European Food Consumption Database may also be used for intake calculations [61].

In order to assess the total exposure to the novel food, other potential sources of intake should also be taken into account where relevant, such as natural occurrence in food, intake from food fortification and supplements, and intake from other sources.

Exposure to undesirable substances identified in the compositional analysis (e.g., secondary plant metabolites, residues, contaminants, or degradation products) for the relevant population groups should also be estimated by the same approach as for the intake estimate.

When proposing **precautions and restrictions of use**, all available information on safety should be taken into consideration.

Data on toxicokinetics **absorption, distribution, metabolism, and excretion (ADME)** is necessary to assess both the nutritional and toxicological impact of a novel food. It is also critical for the development of appropriate toxicity testing strategy, including the selection of appropriate animal models, and for the interpretation of study results. These data are an important element of the risk assessment to account for differences between experimental animals and humans [62].

For toxicokinetic testing of PCTC-derived food ingredients that are single substances and simple mixtures, the same principles apply as those for food additives. Detailed information is provided in the EFSA ANS Guidance for food additive evaluations [63].

For products in the form of complex mixtures, conventional metabolism and toxicokinetic studies should be provided for toxicologically relevant constituents, which are generally considered to be the major components and those other components with known or demonstrable biological or toxicological activity, and should be determined on a case-by-case basis with a scientific justification and a rationale for their selection. Toxicokinetic data on nutritionally significant

constituents are also important for the evaluation of the nutritional impact of the novel food.

Risk assessment is also based on the **nutritional information** on the novel food. The product should not be nutritionally disadvantageous for consumers at the proposed conditions of use. Nutritional information should include details of its nutrient composition taking into account influences of production, storage and further processing, handling, and cooking. The content and effect of antinutritional factors in the novel food in terms of known and suspected interactions with nutrients (e.g., inhibiting absorption or modifying bioavailability) should also be assessed.

Toxicological studies should be carried out with test material representative of the novel food as intended to be marketed. In order to decide whether and which toxicity studies are necessary, all relevant knowledge on the novel food should be considered. Toxicity testing approach for food additives is considered relevant for PCTC-derived food ingredients. It integrates the core areas of toxicokinetics, genotoxicity, repeated dose toxicity testing (subchronic, chronic toxicity, and carcinogenicity), and reproductive and developmental toxicity [63]. Other specific biological processes may also be studied where relevant, such as immunotoxicity, hypersensitivity and food intolerance, neurotoxicity, endocrine activity, and mechanisms and modes of action.

To assess the risk of low exposure to substances such as impurities, metabolites, and degradation products present in (or derived from) the novel food if toxicity data for those is not available, the Threshold of Toxicological Concern (TTC) approach described in EFSA Guidance on the concept of Threshold of Toxicological Concern (TTC) might be applied [64].

The allergenic potential of the PCTC-derived novel food should also be assessed based on its composition, particularly if it contains protein(s), its source, the production process, and available experimental and human data. Information on appropriate methods to investigate the potential allergenicity of foods is provided by the NDA Opinion on the evaluation of allergenic foods and food ingredients for labeling purposes [65].

By January 2018, the EC will publish the new regulatory documents regarding the content and the presentation of the application for a novel food ingredient, the rules for validity verification of an application, and the type of information to be present in the opinion of EFSA after assessing an application [51].

5 Conclusions

In the last two decades, research on the use of plant cell and tissue cultures, called also “plant cell factories,” for the bioproduction of food ingredients has accumulated huge knowledge and has led to the development of various production technologies. Despite some limiting factors such as low product yields, high cultivation costs, and challenges with industrial scale-up, it is expected that plant in vitro cultures will soon be commercially viable and a large number of food ingredients will be produced by this technology.

A key factor for the market introduction of a food product is its safety for the consumer. Various guidelines and regulations have been elaborated and implemented around the globe to facilitate the safety assessment of plant cell and tissue culture-derived food ingredients, which share similarities on the use of the concept for substantial equivalence and the overall approach as main stages to follow in a safety assessment process.

However, one of the issues with safety assessment is that the available information on characterized chemically defined food ingredients and inherent plant toxicants in traditionally grown plants is still scattered, which creates difficulties with applying the SE concept. In this regard, international databases would significantly aid developers, producers, and assessing bodies. Also, current safety assessment schemes are challenging to apply and require long time to complete; therefore, new methods are necessary to make safety assessment more efficient and less time-consuming process. In the future, modern and emerging "omics" technologies might offer solutions for higher efficacy of the assessment methods used and thus ensure higher confidence regarding the safety of PCTC-based products [43].

In addition, current differences in the regulatory requirements and processes regarding the introduction of PCTC-derived food ingredients to the market also present a significant obstacle to high-investment businesses with global markets. Therefore, international harmonization of legislation and safety assessment schemes would greatly facilitate the processes of product development and admission to the market, which would increase the interest to commercialization of plant cell and tussue-derived food ingredients.

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Part IV

Bioreactor Technology and Monitoring

Bioreactor Technology for Sustainable Production of Plant Cell-Derived Products

15

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Contents

Abstract

The successful cultivation of plant cell and tissue cultures for the production of valuable chemical components requires the selection of an appropriate bioreactor. Selection criteria are determined based on a number of factors that are intrinsic to particular plant cell or tissue cultures and are influenced by the process objectives. Due to the specific properties of plant cell and tissue cultures, bioreactor systems may differ significantly from those used for microorganism or animal cell cultures. Furthermore, the differences from one plant culture to another can be

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immense; it is obvious that the optimal bioreactor system for a plant suspension cell culture is different to one for a plant tissue culture in many ways.

General considerations are presented, and based on these key points, selection criteria are used to establish a "bioreactor chooser" tool. The particular details of the most relevant bioreactor types for plant cell and tissue cultures are listed and described.

To produce valuable products, the process also needs to be scaled up to an economically justifiable size, which is usually done either by scaling up the size of the bioreactor itself or by bioreactor parallelization. Therefore, the most significant influencing factors are also discussed.

Keywords

Plant cell cultures · Plant tissue cultures · Bioreactor selection · Production scale · Scale-up

Abbreviations

2G12	Human monoclonal antibody 2G12
DAF-Fc	Decay-accelerating factor-fragment crystallizable region
DPP4-Fc	Dipeptidyl peptidase-4 fragment crystallizable region
FDA	Federal Drug Administration
GAD65	Glutamate decarboxylase 65
GMP	Good manufacturing practice
HA	Hemagglutinin
HCPS	Hantavirus cardiopulmonary syndrome
hG-CSF	Human granulocyte colony-stimulating factor
hGM-CSF	Human granulocyte-macrophage colony-stimulating factor
ICAM-1-IgA2	Intercellular adhesion molecule 1-Immunoglobulin A2
ICH	International Conference on Harmonization
IL-12	Interleukin 12
MERS	Middle East respiratory syndrome
OUR	Oxygen uptake rate
QbD	Quality by design
RITA	Récipient à immersion temporaire automatique

1 Introduction

Plants are an essential component in human diets as they produce carbohydrates, lipids (fatty acids), proteins (amino acids), and vitamins (e.g., ascorbic acid),as well as storing macro (e.g., magnesium) and trace elements (iron). The relevance of plant cell and tissue cultures has been drawing more and more attention in the biotechnological industry [1–3] over the past decade, with the production of secondary metabolites and recombinant proteins being of particular interest.

Besides plants being fundamental components in animal, mammalian, and human food chains since prehistoric time, mankind has also used plants to cure illnesses and

injuries, to dye cloths and for spiritual purposes. Knowledge has been passed down and enhanced from generation to generation. Most of the positive effects of herbs and other plant material are based on complex chemical compounds, often referred to as secondary metabolites. These secondary metabolites are usually small but complex molecules, which are in many cases impossible or prohibitively expensive to synthesize chemically [4, 5]. Based on their metabolic pathways and their biogenetic precursors, they can be classified into three groups: terpenoids (e.g., paclitaxel, ginsenosides), alkaloids (e.g., morphine), and phenolics (e.g., shikonin, rosmarinic acid) [6]. Today, secondary metabolites are used directly or as precursors for the production of pharmaceuticals, cosmetics, fragrances, flavors, dyes, insecticides, and much more [7–9]. The extraction of secondary metabolites from plants, which were traditionally grown in fields, is still the main production method for these substances [1]. However, there are a number of disadvantages associated with traditional farming, one of which is the excessive variation of environmental conditions over time and region, which leads to unpredictable differences in the quality and quantity of the raw materials.

Many secondary metabolites are produced in plants that are not suited to agricultural production or can be hard to grow outside their local ecosystems [10], and thus the extensive exploitation of these plants could potentially lead to their extinction. As a result, it is not surprising that around one fifth of the 50,000 medical plants that are used today are on the list of threatened species [1]. Therefore, plant cell and tissue cultures grown in bioreactors offer an eco-sustainable alternative. Furthermore, the metabolic pathways of secondary metabolites often contain many branches, and thus the transfer of the genetic information to common biotechnological production organisms often fails to deliver the desired results [4]. As a result, plant cell and tissue cultures are believed to represent an appropriate method that addresses the main drawbacks of traditional farming of herbs and other useful plants and avoids the problems associated with extracting products from protected wild plants.

A famous example for a pharmaceutically used secondary metabolite derived from plant cell cultures is paclitaxel, an anticancer drug. Previously produced by harvesting the bark from *Taxus sp.* trees, today, the large-scale production of paclitaxel is performed in stirred stainless steel bioreactors up to a culture volume of 75 cubic meters using plant suspension cell cultures of *Taxus sp.* by Phyton Biotech from Ahrensburg, Germany [11, 12]. The Swiss cosmetics company Mibelle uses two different types of bioreactors for the production of apple suspension cells for their PhytoCellTec™ product. This involves using a single-use vibrating disk bioreactor at a scale of 50 L and numerous single-use wave-mixed bag bioreactors to produce biomass for the cosmetic industry [11, 13, 14].

Recently, more and more attention has been paid to how plants can be used as hosts for the production of therapeutic proteins [15–17]. Currently, one plant-based therapeutic protein is approved for human treatments: there combinant glucocerebrosidase known as Elelyso®, which is used to treat Gaucher disease and has been produced by the Israeli company Protalix and Pfizer since its registration and approval by the Federal Drug Administration (FDA) in 2011 [18]. It is produced with a carrot-based suspension cell line in single-use pneumatically driven bioreactors at a scale of 400 L

[16, 18, 19]. In order to scale up to production quantities, many bioreactors are used in parallel. A poultry vaccine for the Newcastle disease virus has been approved by US regulatories for veterinary use only in 2006 [15], which is produced with tobacco cells. The cells are lysed and injected subcutaneously into chickens. It is the only veterinary vaccine; however, it was never commercialized [20].

Recently, many more different modern biopharmaceutical compounds [3, 21–23] have been investigated for plant production processes: ZMapp, a vaccine against Ebola [24]; GAD65, a key autoantigen in type 1 diabetes; Norwalk virus-like particles; the monoclonal antibody 2G12 for utilization against HIV [25]; HA vaccine against influenza [26]; personalized medicines like a vaccine against non--Hodgkin's lymphoma for individual patients [27]; CaroRx targeting cavity-causing bacteria *Streptococcus mutans* [28]; and many more. Furthermore, there have also been descriptions of how immunoadhesins, antibody-like, chimeric molecules, which possess the functional domain of a binding protein (a receptor, ligand, or cell-adhesion molecule) with immunoglobulin constant domains [29], like ICAM-1-IgA2 (against human rhinoviruses), DPP4-Fc (against Middle East respiratory syndrome, MERS), and DAF-Fc (against hantavirus cardiopulmonary syndrome, HCPS), have been successfully produced in tobacco plants [30].

Besides their application for pharmaceuticals, different recombinant proteins are available for cosmetic applications. Growth factors (e.g., hGM-CSF, IL-12, hG-CSF) are produced in rice suspension cells for cosmetic application [31].

Producing pharmaceutical compounds require strict compliance with rules laid down by the International Conference on Harmonisation (ICH) and its participating government authorities from the USA, Europe, and Japan [32]. In order to comply with these rules, undefined conditions that exist in agriculturally produced plant cells need to be avoided. Besides the ease of complying with good manufacturing practice (GMP) regulations thanks to the quality by design (QbD) approach when using bioreactor-based production processes with plant cell or tissue cultures, shorter production cycles (days to weeks) can also be expected in comparison to using whole plants that involve a production cycle of months. Furthermore, the complete elimination of environmental variations leads to improved consistency between batches, which is crucial for gaining official acceptance. The avoidance of labor-intensive greenhouse or field production of whole plants reduces costs: not only in upstream processing but also in downstream processing, in particular in the case of products which are secreted into the medium [33]. Obviously, the safety of the process with regard to product contamination with endotoxins and mycotoxins and, of no less importance, with regard to environmental contamination with artificial, genetically modified plants is tremendously enhanced when operating in a closed bioreactor system [23, 34].

2 Plant Cell Culture Demands

The scaling up potential of a bioreactor is one of the main aspects that should be considered very carefully (see Fig. 1). While it is reported that stirred devices can be scaled up to seventy-five cubic meters [12], other bioreactor types available

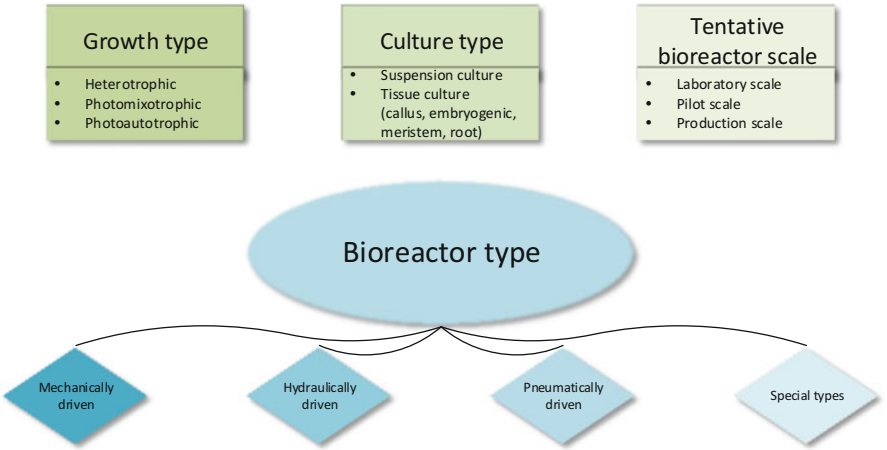

Fig. 1 Factors that influence the bioreactor decision process. The growth type, the culture type, and the final indented bioreactor scale represent key factors of the decision process

on the market are limited to several hundred liters (e.g., wave-mixed bioreactors) [35]. Furthermore, the footprint of a bioreactor plays a crucial role, which is a further disadvantage of wave-mixed and orbitally shaken bioreactors. However, as energy input represents a critical limitation for suspension and tissue cultures in particular, alternative agitated systems are important for these culture types. Gas exchange capacity is another relevant parameter for optimal cultivation results and depends heavily on the energy input method and magnitude. Finally, special cultivation requirements, such as constant illumination for photoauto- and photomixotrophic cultures, further increase complexity, since it is hard to dose light as a non-mixable reactant.

One of the most important functions of a bioreactor is adequately dosing the energy that is input into the culture broth. Sufficient mixing (to prevent nutrient limitations or the accumulation of hazardous by-products), gas exchange (to provide enough oxygen/carbon dioxide for respiration), and dispersion (to prevent sedimentation, especially in suspension cultures) must be ensured. However, excessive energy input may harm the tissue (e.g., the root networks and the "hairy" extensions of hairy roots) [36, 37], decrease the embryonic potential of embryogenic cultures, or damage the cells and thus leads to reduced biomass concentrations and product titers. Finally, it is vital to remember that mechanical stress may influence secondary metabolite production in two ways: either as an increasing elicitor of product formation or as an inhibiting disturbance, which varies depending on the amount of energy, the distribution method, and the plant species.

Mixing is heavily influenced by the rheological properties of the liquid. Plant cell suspensions exhibit water-like fluid characteristics at the inoculum stage. An increase in viscosity and often non-Newtonian rheology is inherent to most plant cell suspensions [38].

Guaranteeing sufficient aeration is another crucial factor in bioreactor design. In heterotrophic plant cultures, the supply of oxygen may limit growth. However, high aeration rates also induce shear stress and lead to increased foaming and evaporation. The average oxygen demand of plant cells is comparably low (compared to microbial processes). Typical values for the oxygen uptake rate (OUR) of plant suspension cells are in the range of 5–10 $mmol_{O2}\ L^{-1}\ h^{-1}$, which is comparable to animal cells with an OUR value of approximately 0.05–10 $mmol_{O2}\ L^{-1}\ h^{-1}$ but much lower than microbial cell values of 10–90 $mmol_{O2}\ L^{-1}\ h^{-1}$ [22]. For slow-growing suspension cells or tissues in particular, the importance of aeration is eclipsed by the potential damage that can result from shear forces. In addition, high aeration provokes increased foaming and thus reduces surface gas exchange and increases the risk of clogged sterile filters.

To deal with the abovementioned issue, the first step is to select the bioreactor type with the most suitable specific oxygen transfer coefficient (k_La) to specific power input (P/V) ratio. Afterward, the bioreactor design and operating method may be adapted, e.g., by employing different spargers or by supplying oxygen-enriched inlet air. The utilization of antifoam solutions must be carefully considered, since they may reduce biomass growth, product quality, and oxygen transfer, and thus lead to even higher aeration rates [39–41].

Photoautotrophic and photomixotrophic cultures with suspension cells or tissue require light as an energy source for their metabolism. In order to provide light with a certain photon density to the plant cells or tissue, it is crucial to keep the layer thickness of the culture as thin as possible. Adequate distribution of illumination is quite difficult, since light is not mixable. Furthermore, higher biomass concentrations drastically increase the absorption properties of the culture broth, leading to reduced illumination as penetration depth rises. As a result, different cells are exposed to different amounts of light, and the specific growth rate may vary in different regions of the bioreactor [42]. However, increased illumination does not increase growth rates in equal measures, and light inhibition may occur [43].

3 Bioreactor Selection Process

The selection of a suitable bioreactor is anything but easy. As a rule of thumb, large-scale suspension cell cultures are most profitable in stirred tanks. Cells with a high sensitivity to mechanical stress may be cultivated in orbitally or wave-mixed bags; however, scalability is limited and parallelization may be required. A simplified selection scheme is depicted in Fig. 2. More detailed information can be found in the following subsections.

3.1 Stirred Tank Reactor

The classical stirred tank bioreactor (Fig. 3a) is widespread in biotechnology and has multiple uses [1]. Agitation and mixing is performed by one or several stirrers [44].

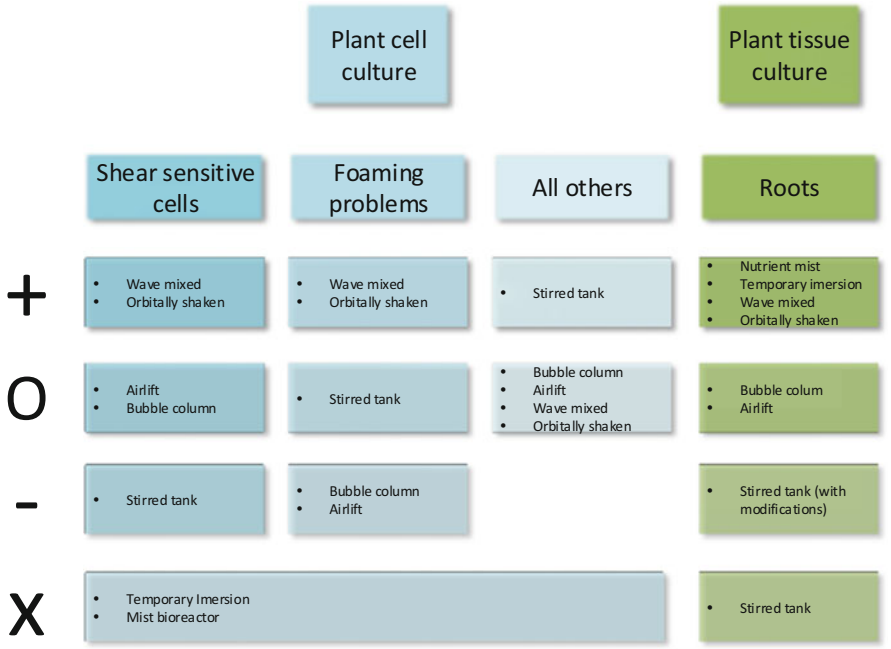

Fig. 2 Bioreactor chooser, based on culture type and limiting factors

Frequently used stirrers for plant cell cultures are marine impellers and pinched-blade turbines, which create axial fluid flow patterns at a comparably low tip speed (up to 2.5 m s^{-1}) [45]. An overview of suitable stirrers is provided by Eibl and Eibl [45] and Doran [4].

Due to variations in vessel geometry and stirrers [46], stirred bioreactors are highly adaptable [47]. Furthermore, the controllability of process parameters like pH, temperature, and oxygen concentration in stirred tanks is superior to all other bioreactors [48], making stirred bioreactors extremely well suited to the cultivation of robust suspension cells. Unfortunately, all these benefits cannot outweigh the fact that the moving stirrer mechanically damages the tissue and thus leads to reduced growth or death. Therefore, there are two reasonable options for growing plant cell tissues: either a stirred tank reactor can be adapted (e.g., with a constructive separation between agitation and growing compartments [49]) to the requirements of plant cell tissue cultures or bioreactors can be used that are agitated in a different way.

3.2 Bubble Column Reactor

A bubble column bioreactor (Fig. 3d) can be simpler than nearly any other bioreactor, consisting of a cylindrical vessel and a bottom-mounted sparger. Mixing and agitation are both performed by the rising air bubbles without further mechanical energy input [23, 50]. The lack of moving parts reduces the risk of contamination.

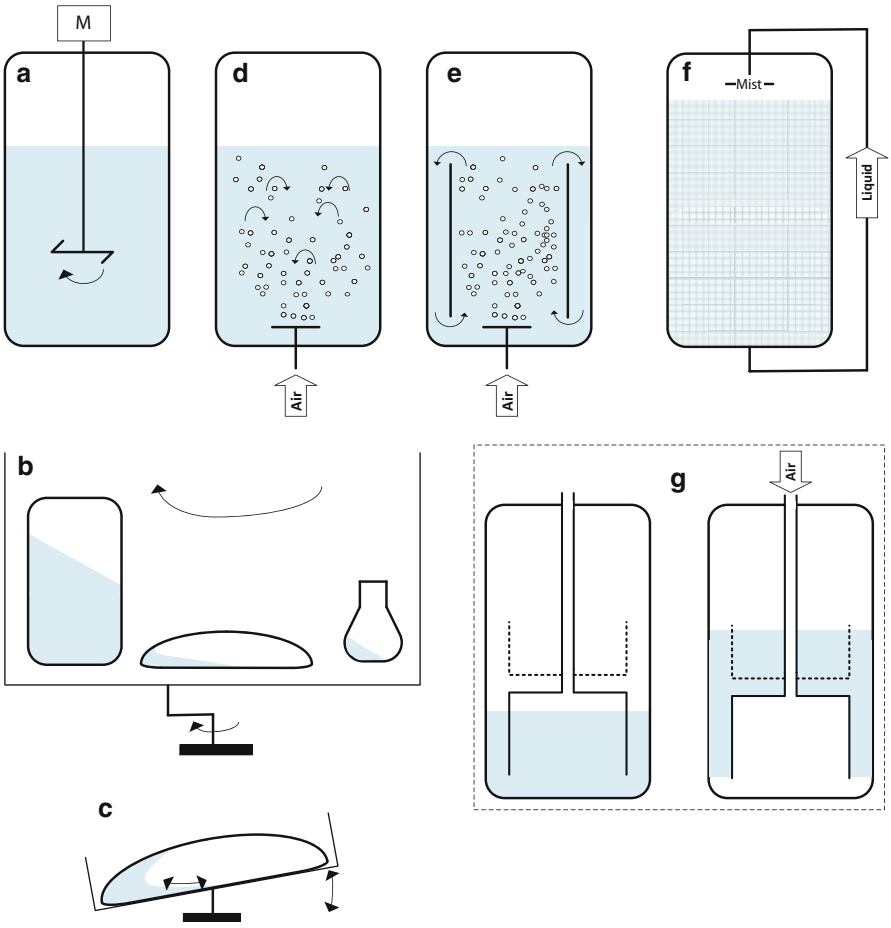

Fig. 3 Mixing principles of the most important bioreactor types (**a**) Stirred bioreactor, (**b**) Orbitally shaken bioreactors with different vessel shapes, (**c**) Wave-mixed bioreactor, (**d**) Bubble column, (**e**) Airlift bioreactor. (**f**) Mist bioreactor, and (**g**) Temporary immersion system)

Due to their homogeneous and low power input, bubble columns are well suited to plant tissue cultures. However, the potential occurrence of shortcuts, mass transfer limitations, foaming, and floatation limits their application [48], especially for high-density suspension cultures [23]. Bubble columns can be further improved by incorporating several stages or static mixers.

3.3 Airlift Reactor

Another pneumatically agitated bioreactor is the airlift reactor (Fig. 3e). It resembles a bubble column but includes the addition of a draft tube (used for the creation of an

internal or external loop) [51]. As a result of this circulation, the oxygen transfer is higher, and the mixing times and shear forces are lower than in a comparable bubble column reactor [1, 45]. Different modifications exist, e.g., a combination with a perfusion system for high cell densities proposed by Wie Wen Su et al. [52] or an illuminated system for photoautotrophic cultivation of plant cell suspension cultures proposed by Fischer and Alfermann [53].

3.4 Orbitally Shaken Bioreactors

The group of orbitally shaken bioreactors (Fig. 3b) consists of several geometrical dissimilar vessels from milliliter to cubic meter scales. Centrifuge tubes and Erlenmeyer, Fernbach, and Thomson Optimum Growth™ shake flasks are used for screening purposes (e.g., media optimization) and inoculum production at the laboratory scale and have been quite well examined [13, 54, 55]. The next step in a scale-up process may include orbitally shaken bag bioreactors (which are similarly shaped to wave-mixed bags). Due to the low shear forces combined with moderate aeration and good mixing, orbitally shaken bag bioreactors seem promising for plant cell and tissue cultures [56]. Large-scale cultivations utilizing orbitally shaken reactors containing *Nicotiana tabacum* suspension cells for manufacturing recombinant antibodies have shown that volumes can be increased up to a small-scale production size of 200 L [57, 58].

Since there are no moving parts inside the reactor, energy is provided via the vessel walls. Aeration may be passive (e.g., for shake flasks) or active (e.g., for bag bioreactors). In the case of bag bioreactors, the aeration also ensures that the bags are fully inflated. The straightforward design and the availability of disposable orbitally shaken vessels make it easier to conform to GMP and reduce contamination risks. Despite their lower aeration and mixing rates and inferior controllability in comparison to a stirred tank, orbitally shaken bioreactors have the great advantage of being able to be used for cell suspension and tissue cultures without needing to be adapted.

An intermediary between orbitally shaken and wave-mixed systems is the traveling wave bioreactor, which is orbitally agitated to produce a traveling wave [59–61].

3.5 Wave-Mixed Bag Bioreactor

As the name implies, energy dissipation and hence mixing and oxygen transfer in the wave-mixed bioreactors (Fig. 3c) are realized by inducing waves. Adjustments may be made by changing the bag itself (e.g., size and length to width to height ratio), the operating conditions (e.g., rocking rate and angle, and aeration rate), and the cultivation conditions (e.g., working volume) [35, 62, 63]. Oxygen mass transfer is bubble-free due to gas exchange on the surface, lowering foaming tendencies. Usually, these types of bioreactors are disposable and are delivered

presterilized. Hence, the cross-contamination risk is relatively low, and it is comparatively easy to perform cultivations that conform to GMP. However, for academic research or for cultivations that do not conform to GMP, reusable options may be promising, e.g., the polycarbonate bioreactor proposed by Scholz and Suppmann [64].

One of the main advantages of the wave-mixed bioreactors is its applicability for plant cell suspensions and tissue cultures. Shear sensitive or foaming suspensions as well as fully and partially submersed root cultures can be cultivated with low rocking rates in the wave-mixed bioreactors. However, not all commercially available bag bioreactors are suitable for plant suspension cultivations, since ports made for animal cells or microbial cultivations are not wide enough, and thus tubes may become blocked. For this reason, plant culture bags have enlarged sampling and inoculation ports. More detailed information about disposable wave-mixed bioreactors can be found elsewhere [35, 65, 66].

3.6 Nutrient Mist and Nutrient Sprinkle Reactors

The nutrient mist and the nutrient sprinkle (also refered to as spray bioreactors) bioreactors (Fig. 3f) are both designed to satisfy the needs of hairy and adventitious root cultures. In both systems, the roots are immobilized on a mesh, a steel matrix, or a porous structure. The medium is distributed as an aerosol (mist reactor) or as small droplets (sprinkle reactor) from the top of the bioreactor [67]. It is then recirculated and may be stored in a reservoir tank. This class of bioreactors is characterized by extremely low mechanical stress and high gas mass transfer rates [68, 69]. However, the designated plant tissues should be adapted to non-submersed growth. Furthermore, the absence of shear forces may lead to reduced growth and product formation rates, as mechanical stress may act as an elicitor of secondary metabolites. A comparison of nutrient mist and sprinkle bioreactors with other systems has been done by Mishra and Ranjan [51] and Nuutila et al. [70].

3.7 Temporary Immersion System

Temporary immersion systems (Fig. 3g) are another type of bioreactor focused on cell tissue cultures. The operating principle is the alternation of submerged and non-submerged periods, equivalent to low and high tide. Adjusting these tidal times is a simple but effective method for controlling metabolite and gas exchange while keeping the mechanical stress low. Some common implementations of the temporary immersion approach are the twin-flask system, the ebb-and-flow system, the RITA® system, and the thermos-photobioreactor [71–73]. A broad review of temporary immersion systems has been provided by Georgiev et al. [74].

4 Engineering and Scale-Up Considerations

4.1 Plant Cell Suspension Rheology

In many cases, the low productivity of secondary metabolites by plant suspension cells requires a scale-up in bioreactor technology often of cubic meters in order to achieve an economical production process. In general, a correlation of fixed costs per kg product to bioreactor volume to the power of minus two thirds is assumed, meaning products become about five times cheaper with a tenfold increase in production volume [75]. Currently, the scale-up of agitated vessels for plant suspension cell cultures generally follows particular rules that date back several decades and are mostly based on a certain level of geometric similarity between the vessels [76]. A lack of geometric similarity often leads to scale-up approaches based on a number of engineering parameters, such as the volumetric oxygen mass transfer coefficient k_La [77, 78], mixing time [79, 80], or power input [81].

One of the most important engineering parameters for dimensioning and scaling up of bioreactors and for the design of process parameters is the Reynolds number, which can be calculated by

$$\mathrm{Re} = \frac{\rho \cdot l \cdot u}{\mu} \tag{1}$$

Here, l represents the characteristic length of the fluid flow, u the characteristic velocity, and ρ and μ the material properties of the fluid. For stirred bioreactors, the length and the velocity is replaced by a geometrical (stirrer diameter d) and a bioreactor operating (stirring speed n) parameter for the bioreactor, which can be written as $l \cdot u = n \cdot d^2$. As a result, the stirrer Reynolds number is given by

$$\mathrm{Re} = \frac{\rho \cdot n \cdot d^2}{\mu} = \frac{n \cdot d^2}{\nu} \tag{2}$$

It can easily be seen that the material properties, namely, the density and the dynamic viscosity (both can be combined to the kinematic viscosity $\nu = \mu/\rho$) are factors that influence the Reynolds number.

During the cultivation of plant cells, the density of the fluid can be considered to be constant. However, the viscosity of the fluid is changed considerably due to growth and the secretion of polysaccharides [82–84]. Therefore, from an engineering point of view, the most important material property of plant suspension cell cultures, unlike other processes, is fluid viscosity. Variations are a result of polysaccharides, which are secreted into the medium and change the rheological behavior and, often more importantly, change the aggregation tendency, shape, and number of large cells. Generally, plant suspension cell fluids are considered to be suspensions which are a more or less water-like fluid loaded with a varying number of heterodisperse particles.

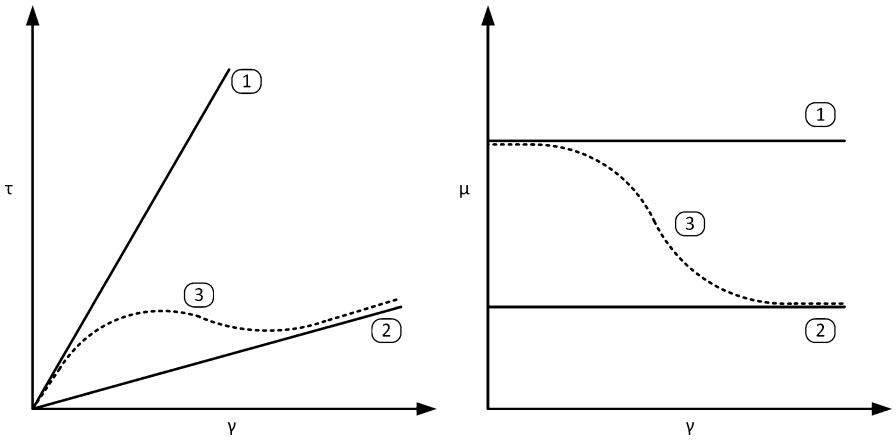

Fig. 4 Typical flow curve (*left*) and viscosity curve (*right*) of Newtonian fluids (*1* and *2*) and non-Newtonian fluids with pseudoplastic flow behavior (*3*), adapted from [85]

Suspended particle fluids often exhibit non-Newtonian flow characteristics with shear-thinning behavior (some well-known examples are blood or sand in water); this means that the viscosity is dependent on the shear rate $\dot{\gamma}$. Typical flow and viscosity curves are presented in Fig. 4. The classical power-law approach (often referred to as the Ostwald-de Waele relationship) is often used to describe rheological behavior and can be written as

$$\mu = k_\gamma \cdot \dot{\gamma}^{(a-1)} \tag{3}$$

Here, k_γ represents the flow consistency index and the factor a is the flow behavior index, with $a = 1$ for Newtonian fluids, $a < 1$ for pseudoplastic or shear-thinning fluids, and $a > 1$ for dilatant or shear-thickening fluids. For plant cell suspensions, the flow behavior index is often in a range between 0.5 and 1, meaning the fluids exhibit a slight shear-thinning behavior [38].

As a result of the shear rate dependency of viscosity, it is obvious that the Reynolds number and the subsequently derived engineering parameters might not be estimated correctly. Therefore, it is necessary to adapt calculations of engineering parameters in order to account for variations in viscosity.

The most commonly used approach dates back to 1957 and was proposed by Metzner and Otto [86]. It describes the effective shear rate $\dot{\gamma}_{MO}$ in relation to the stirring speed n and an empirical determined factor k_{MO} as follows

$$\dot{\gamma}_{MO} = k_{MO} \cdot n \tag{4}$$

Here, the so-called Metzner-Otto constant k_{MO} is only dependent on the type of the stirrer and must be determined experimentally. Typical values found in the literature are as follows: approximately 10 for a marine impeller, 12 for a Rushton

turbine, 11.5 for a pitched-blade impeller, 25 for an anchor impeller, and 30 for a helical ribbon impeller [85, 87].

However, the linear relationship of the effective shear rate to the stirring speed with a correlation factor based only on the geometry of the stirrer is obviously highly simplified and, in the strict sense of its original derivation, only valid for laminar flows [86]. Furthermore, many researchers have proposed a dependency between the Metzner-Otto constant and the flow behavior index [88]. This is particularly important for highly shear-thinning fluids, and thus the Metzner-Otto approach has been used extensively for plant cell suspensions with their minor shear-thinning behavior [38, 89].

Besides the shear rate, cell density also heavily influences apparent viscosity. Depending on the correlation of the apparent viscosity to different cell mass measurements (cell dry weight, cell fresh weight, packed cell volume), masking effects may occur due to variations in individual cell sizes and water contents (e.g., in the vacuole) over the course of the growth cycle.

After calculating the Reynolds number based on the previous description, the power number (often referred to as the Newton number) can be calculated as follows

$$Po = \frac{P}{\rho \cdot n^3 \cdot d^5} \tag{5}$$

The power number is known for a wide variety of stirrers, and therefore it can be used to calculate the power input P for the bioreactor, which can be helpful in estimating mixing and oxygen mass transfer to the bioreactor. Furthermore, the power input P or, even better, its volume-normalized pendant, the specific power input P/V, is related to the shear stress applied to the liquid, which is often an important parameter for shear-sensitive plant cells.

Mixing ensures a sufficient supply of nutrients and prevents the accumulation of toxic metabolites during fluid homogenization and particle suspension. In order to determine the quality of mixing, the mixing time is often used [90, 91]. For small-scale bioreactors, it is generally easy to ensure good mixing and thus low mixing times. However, this task frequently becomes one of the constraints during scale-up of bioreactors, which leads to concentration gradients and poor mass transfer. Improved mixing can be achieved by increasing the agitation rate; however, this may not be the appropriate method for shear-sensitive plant suspension cells. Alternatively changes can be also be made to the stirrers or the overall mixing principle of the bioreactor. For example, mixing can become the limiting factor in larger airlift bioreactors when reaching a cell density of more than 20 gL^{-1} cell dry weight. Increasing the aeration rate is the only operating response that can increase mixing [4]. However, studies have shown that overventilation can reduce cell growth and product formation due to the CO_2 and other growth-related gases like ethylene being stripped [92]. This obviously emphasizes the importance of considering the final bioreactor scale when designing processes and the careful selection of a suitable bioreactor system from the very beginning.

The supply of oxygen is crucial for all heterotrophic cells. However, the oxygen demand of plant cells is, in general, relatively low [93]. Nevertheless, the high cell densities which can be achieved with plant suspension cells, the high viscosity of the culture broth, and strong foaming and cell floating tendencies can be potential issues. The characterization of plant cell cultures and the characterization of the bioreactors to be used are an essential part of process design. How to measure the specific oxygen transfer coefficient has recently been described [90]. However, the measurement is often made using water, and as a result, transferring the coefficient to plant cell culture processes is complicated. In such cases, the use of a model liquid like carboxymethyl cellulose water solution is recommended, which can be adjusted to certain growth stages of plant suspension cells by altering the concentration [56].

The sensitivity of plant suspension cells to shear forces is considered relatively high due to the large size of the cells compared to microorganisms. Furthermore, large vacuoles, which occur during the late growth phase in particular, may even increase sensitivity [94]. In general, shear stress can be reduced by reducing the power input. However, reducing power input can lead to inadequate mixing and can also reduce oxygen and heat transfer rates in high viscosity plant cell culture broths. Another alternative to reducing shear stress to plant cells is to employ special types of bioreactors that are characterized by very homogeneous power inputs. This eliminates high-shear regions, which usually occur when mixing is performed by a stirrer or similar device.

4.2 Tissue Culture Specialities

Plant tissue cultures can be used for various applications, e.g., the production of pharmaceutical active ingredients like secondary metabolites, the vegetative reproduction of highly productive clones, and the growth of genetically modified plants [44]. Several types of biotechnologically applicable plant tissues exist, and their usage depends heavily on the objective of the cultivation: Adventitious and hairy roots are the predominant forms used in research and for the production of secondary metabolites; embryogenic and shoot cultures are primarily used for plant micropropagation. Besides their different uses, the appearance of plant tissues differs greatly and leads to particular cultivation requirements.

Differentiated cells are characterized by their high genetic stability and their ability to grow in hormone-free media. Furthermore, constant secondary metabolite production and high biomass productivity can be observed for hairy roots [95, 96]. The reason for the biotechnological use of tissues for producing secondary metabolites is obvious because they are typically produced in differentiated cells [97, 98].

Due to their dissimilar structure, the requirements for plant tissue cultures depend greatly on their shape. Although high-shear forces do not inevitably reduce the growth rate, they may destroy agglomerations and hence reduce growth potential. Root cultures tend to form large, connected networks which react sensitively to mechanical forces. Furthermore, some root species favor anchoring structures for faster growth and thus reduce the range of potential bioreactors.

In contrast to plant cell suspension cultivations, the viscosity increase in tissue cultures is considerably lower and thus of minor importance in terms of process design. However, major concentration gradients can appear in larger cell tissues (e.g., in dense root networks) and may lead to nutrition deficits and reduced oxygen concentrations in the tissue center [99]. Nevertheless, higher energy input (e.g., due to an increased stirring rate) can be problematic, since it may disrupt cell agglomerates or tissues and thus lead to reduced growth or increased cell death. Therefore, the selection of a suitable reactor system is of major importance.

5 Concluding Remarks

Selecting an optimal bioreactor system for a particular plant cell or tissue culture is a complex task. There are many different bioreactor systems commercially available. Small-scale bioreactors, in particular disposable bag bioreactors, are more or less ready to use and can be used for both cell and tissue cultures. Because this choice is influenced by the culture type (suspension or tissue culture), the growth type (e.g., the need for light), and the targeted production scale, there are only a limited number of bioreactor types remaining that represent appropriate choices. Nevertheless, large production scale bioreactors for plant cell suspension cultures are mainly stirred or pneumatically mixed and are usually tailor made to the specific process.

Mixing, aeration, and – related to both – shear forces can be considered to play a crucial role in many cultivation processes. Thus, these parameters need to be well equilibrated in order to gain an optimal result for cell growth and product formation. An ideal bioreactor provides good and homogenous mixing and thus sufficient gas exchange and nutrient supplementation while keeping shear forces low. Therefore, it is not surprising that it is almost impossible to provide a general. In fact, every single process needs to be optimized and scaled up individually to the desired production size.

The “bioreactor chooser” can be a useful tool during the first steps of the selection of an appropriate bioreactor system. More detailed information for a deliberated selection can be found in Sect. 3.

Various challenges during scaling up the process due to the particular nature of plant cell or tissue cultures have been described. It is particularly important that the drawbacks of increasing viscosity for suspensions cells and nutrient shortage in larger root networks be considered. The pitfalls associated with (large scale) plant cell cultivations may differ from mammalian or microbial cultivations, but they can be avoided as long as the reactor selection is carefully thought through.

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Monitoring of Plant Cells and Tissues in Bioprocesses

16

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Abstract

Plant cell and tissue cultures represent a suitable alternative as production systems for valuable plant secondary metabolites. Unlike traditional extraction from agricultural grown plants, the active ingredient production in biotechnological processes with in vitro cultures takes place in closed bioreactors under controlled conditions. This allows a year-round production with constant quality and quantity. However, the development of biotechnological processes with plant in vitro cultures is often time-consuming and requires parallelized screening systems. Furthermore, the design, optimization, and control of economic processes presuppose knowledge about the physiological state of the biological system and the kinetic parameters of biomass and product formation. To gain access to these data, suitable process-monitoring methods are required which provide information about the physiology of the process, both on a macroscopic and on the single cell level. However, due to the morphology of plant cell and tissue cultures, many methods for bioprocess monitoring that are used for mammalian and microbial cultures are not applicable. This chapter covers methods that are appropriate for monitoring of biotechnological processes with plant cell and tissue cultures: The conductivity of the growth medium is a powerful parameter to estimate the growth of complex plant cell aggregates and tissue structures. The next section describes the application of the RAMOS – a small scale cultivation system – for heterotrophic and phototrophic plant cell and tissue cultures. Flow cytometry is a tool to obtain segregated data of bioprocesses. Further, we describe a novel approach of cell immobilization for physiological studies and the design of bioprocesses, the 3D *Green Bioprinting*.

Keywords

Monitoring · Conductivity · Respiration activity · Oxygen transfer · Shake flask · Evaporation · Flow cytometry · Ploidy · Growth kinetic · *Green Bioprinting* · Immobilization

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BrdU	Bromodeoxyuridine
CAD	Computer-aided design
CAM	Computer-aided manufacturing
c_{bulk}	Concentration of substrates/products in culture medium [g l^{-1}]
CLSM	Confocal laser scanning microscopy
c_{pore}	Concentration of substrates/products in a hydrogel pore [g l^{-1}]
cs	Cell suspension culture
CT	Carbon dioxide transfer [mmol l^{-1}]
CTR	Carbon dioxide transfer rate [mmol l^{-1} h^{-1}]
CTR_{ev}	Evaporation-corrected carbon dioxide transfer rate [mmol l^{-1} h^{-1}]
ctr_{max}	Maximum biomass-specific carbon dioxide transfer rate [mmol g^{-1} h^{-1}]
CTR_{max}	Maximum carbon dioxide transfer rate [mmol l^{-1} h^{-1}]
C-value	DNA content of the holoploid genome with chromosome number n
Cx-value	DNA content of the monoploid genome with chromosome number x
DW	Concentration of biomass dry weight [g l^{-1}]
DW_{max}	Maximum concentration of biomass dry weight [g l^{-1}]
EdU	5-Ethynyl-2′-deoxyuridine
F_{ev}	Rate of evaporation [ml h^{-1}]
FUCCI	Fluorescent ubiquitination-based cell-cycle indicator
G_0/G_1 phase	Cell cycle phase
G_2/M phase	Cell cycle phase
HPLC	High performance liquid chromatography
hr	Hairy root culture(s)
LED	Light emitting diode(s)
LS	Linsmaier and Skoog medium
MS	Murashige and Skoog medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n	Nuclear phase status: n – means the chromosome number of the meiotically reduced genome irrespective of the ploidy 2n – means the chromosome number of the non-reduced genome

N	Number of data points
OLED	Organic light emitting diode(s)
OT	Oxygen transfer [mmol l^{-1}]
OTR	Oxygen transfer rate [mmol l^{-1} h^{-1}]
OTR_{ev}	Evaporation-corrected oxygen transfer rate [mmol l^{-1} h^{-1}]
otr_{max}	Maximum biomass-specific oxygen transfer rate [mmol g^{-1} h^{-1}]
OTR_{max}	Maximum oxygen transfer rate [mmol l^{-1} h^{-1}]
OU	Oxygen uptake [mmol l^{-1}]
OUR	Oxygen uptake rate [mmol l^{-1} h^{-1}]
p	Overall pressure [bar]
PAR	Photosynthetic active radiation
PBR	Photobioreactor(s)
p_{CO_2}	Carbon dioxide partial pressure [bar]
PFD	Photon flux density [μmol m^{-2} s^{-1}]
p_{O_2}	Oxygen partial pressure [bar]
R	Universal gas constant (0.08314 bar l mol^{-1} K^{-1})
RAMOS®	Respiration Activity MOnitoring System®
RQ	Respiration quotient
S	Concentration of substrate [g l^{-1}]
S phase	Cell cycle phase
SEM	Scanning electron microscope
t	Time [d]
V_L	Initial liquid filling volume [ml]
V_t	Total flask volume [ml]
x	Number of chromosomes of the monoploid genome
α	Proportionality constant
κ	Conductivity [mS cm^{-1}]
κ_m	Measured conductivity [mS cm^{-1}]
μ	Specific growth rate
μ_{max}	Maximum specific growth rate

1 Introduction

Plants represent not only an important food source. Since time immemorial, plants and their extracts are also used as remedies, cosmetics, or dyes. They produce more than 30,000 different chemicals used by humans, including pharmaceuticals, pigments, and other fine chemicals. Accordingly, today four times more compounds are recovered from plants, as from microorganisms [1]. Particular importance is attached to the plant secondary metabolites. They occur in different parts of all higher plants in large numbers and structural diversity. So far, more than 100,000 different substances have been identified. It can be assumed that this is only a small part of compounds, which occur in the nature [2–4]. Plant secondary metabolites always occur as mixtures and vary over time, the development status, and in the tissues and organs. Furthermore, their synthesis depends on numerous external stress factors. In their natural

function, they serve the plant defense, for example, against herbivores, pathogenic microorganisms, and competing plants. On the other hand, they act as signaling molecules for pollinating insects or seed spreading animals. They also play a major role as protective factors against UV light as well as in plant development [2, 5, 6]. According to their natural function, plant secondary metabolites are bioactive and interact with nucleic acids or proteins, possess antimicrobial or insecticidal effects, and have numerous pharmacological properties. Therefore, plants are an important source of active pharmaceutical ingredients and fine chemicals. These include, for example, substances for hormone replacement therapy, polyphenols, or triterpenes [7, 8]. Phytochemicals are widely used not only in pharmaceutical products, but also as food additives, natural dyes, pesticides, and in cosmetic products.

So far, herbal active agents are obtained mainly in the traditional way from plants, which are grown on fields or in greenhouses. Here, the continuous decrease of agricultural land in favor of traffic and settling areas and the associated increasing competition with food, feed, and energy crops is a growing problem. Some structures can also be synthesized chemically. However, chemically produced compounds often exhibit a much lower bioactivity compared to the natural agents. In addition, very high processing costs are associated with the chemical production due to the generally high proportion of worthless, partly toxic byproducts [9, 10]. However, the ever-growing world population increasingly demands more effective and less expensive methods for the supply with bioactive plant ingredients. To serve this growing demand, the field of plant biotechnology offers a promising alternative to conventional drug synthesis or extraction from agricultural grown plants [11]. With the help of plant in vitro systems cultivated in bioreactors, a GMP-conform production independent from biotic and abiotic factors is possible. Resulting metabolites are available all year in constant quality and quantity [12]. The yields can be higher than in the plant of origin [13, 14]. Furthermore, there are additional options to enhance product yield (e.g., genetic modification, exclusion of pathogens, or targeted increase of secondary metabolism) with less impact on nature or climate. Additionally, product isolation from in vitro systems is easier than extraction from the complex matrix of an entire plant containing many different cell types due to organ structure [15].

For the studies described here, callus or cell suspension cultures (cs) and hairy roots are the plant in vitro culture types of interest. The totipotent callus acts as wound closure tissue and consists of undifferentiated cells [16–18]. It is naturally developed – controlled by phytohormones – to heal plant injuries and can be induced for biotechnological applications by the use of auxins and/or cytokinins with specific amount and composition [19, 20]. Hairy root cultures (hr) are plant in vitro cultures that arise from manipulation of plant tissue with *Agrobacterium rhizogenes*. Detailed description about the mechanism involved in the genetic transformation of plants by *A. rhizogenes* can be found in literature [17, 21]. Grown for maintenance on solid plant medium, callus, and hairy roots can be transferred in liquid medium and subsequently, used for the production processes in different types of bioreactors. Bioreactors are technical devices that allow the cultivation of plant cell and tissue cultures under defined conditions and therefore, to reproduce and quantify parameters like the productivity of a target metabolite of a specific culture under these conditions.

The conditions that can be controlled are – among others – temperature, pH, aeration, agitation, and supply of substrates. Changes of these operational conditions may evoke changes in the cell biology and metabolism. As the operational conditions can be controlled, their influence on desired process properties like the product formation can be investigated systematically and hence, optimized. In classic development of upstream processes, typically online monitored parameters are of chemical or physical nature like pH or mass transfer, for example, of oxygen to facilitate the respiration of the cells. Biological parameters (e.g., biomass, viability, intracellular metabolites) are usually not measured online due to a lack of suitable sensors that measure these values directly. However, since the biology is responsible for conversions inside the bioreactor, biological parameters are of significant interest for the control and development of bioprocesses. Furthermore, the economic management of biotechnological processes, their design, and optimization increasingly requires knowledge of the kinetics of the fermentation. Here, among others, the determination of the current biomass concentration is of particular interest. Hence, for the development and monitoring of biotechnological processes, the determination of biomass growth and kinetic is indispensable. During the process, the (online) knowledge about biomass formation is a very sensitive parameter to evaluate the progress and to recognize critical points for the process control.

Common methods for the determination of the concentration of biomass are based on optical interactions, as, for example, the optical density. Plant cell and tissue cultures, such as hairy roots, consist of aggregates of heterogeneous size distribution and/or network-like structures and therefore, interfere with such methods. As a consequence, these optical methods for the determination of biomass growth in bioprocesses frequently fail when applied for plant cell and tissue cultures. Thus, in many cases it is necessary to use alternative methods to monitor biotechnological processes applying plant in vitro systems.

In this chapter, we describe the great potential of some innovative methods for monitoring plant cell and tissue cultures. The aim is to get information on the macroscopic level, like by means of the electrical conductivity and the respiration activity on the one hand, and information on the single cell level, for example, via flow cytometry and 3D-bioprinting on the other hand.

2 Conductivity: A Parameter for Growth Monitoring in Plant Cell and Tissue Cultures

A widely used parameter to obtain data about the growth of plant cell and tissue cultures are changes of conductivity in the culture medium [17]. The electrical conductivity κ in mS cm^{-1} of an (electrolytic) solution is a degree of its ability to conduct electrical current in a fluid. The conductivity, therefore, depends on the concentration of ionic components in the solution [22]. Due to cellular uptake of ionic nutrients from the culture medium, mainly NO_3^- and NH_4^+ anions [23], the conductivity will be further reduced within the cultivation process. The conductivity of the culture medium is a frequently used parameter to estimate cell growth,

respectively, for plant cells and tissues [20]. For several applications on plant cell and tissue cultivation, a linear correlation between the increasing biomass and the decrease of conductivity of the culture medium can be observed [17, 24, 25]. The uptake of ionic nutrients can differ between the different growth phases of the cells within the course of cultivation [17, 18]. Therefore, the linear correlation of biomass growth and conductivity decrease needs to be proved. This is often valid before the cells enter the stationary growth phase.

Equation 1 presents the linear proportionality between the change of biomass dry weight ΔDW in g l^{-1} and the corresponding change of the conductivity $\Delta\kappa_m$ in mS cm^{-1} using the proportionality constant α in g cm mS^{-1} l^{-1} [24, 26].

$$\Delta DW = \alpha \cdot \Delta\kappa_m \tag{1}$$

The proportionality constant α is specific for each culture and has to be determined experimentally. Table 1 gives some examples of α showing its specificity for individual plant cells and tissue cultures.

Cultivation experiments using plant cell suspension or tissue cultures like hairy roots are usually performed in standard shake flasks. Since the cultivation period of plant cell and tissue cultures is quiet long compared to microbial processes, it is important to take the evaporation of water into account. This evaporation and its influence is intensified for shake flasks compared to highly instrumented bioreactors with exhaust gas coolers or the possibility of using water-saturated inlet air. If the evaporation remains unconsidered, the conductivity values will be erroneously. The evaporation-corrected κ in mS cm^{-1} can be calculated (Eq. 2) considering the reduction of the initial culture volume V_L by the volume of evaporated water $V_{ev,}$ expressed by the flow rate $F_{ev.}$

$$\kappa(t) = \kappa_m \cdot \frac{(V_L - F_{ev} \cdot t)}{V_L} \tag{2}$$

A correlation of biomass formation and change of conductivity in the culture medium was performed as exemplarily shown in Fig. 1 for a cs of *Helianthus annuus* (*H. annuus,* annual sunflower). As previously described, a relation between the biomass growth and the changes of conductivity during the cultivation in shake flask can be observed.

The cells grew intensively and reached a maximum of biomass concentration of about $DW_{max} = 17$ g l^{-1} at day 10 of cultivation. Comparable to the increase of biomass, the conductivity decreased until the minimum at day 10 according to the

Table 1 Examples of proportionality constants a for various plant cell suspensions (cs) and hairy root cultures (hr)

Species (cell type)	α [g cm^{-1} l^{-1}]	References
Coffea arabica (cs)	3.6	[26]
Catharanthus roseus (cs)	4.1	[26]
Nicotiana tabacum (cs)	2.8	[26]
Withania somnifera (cs)	3.2	[26]
Beta vulgaris (hr)	2.2	[27]

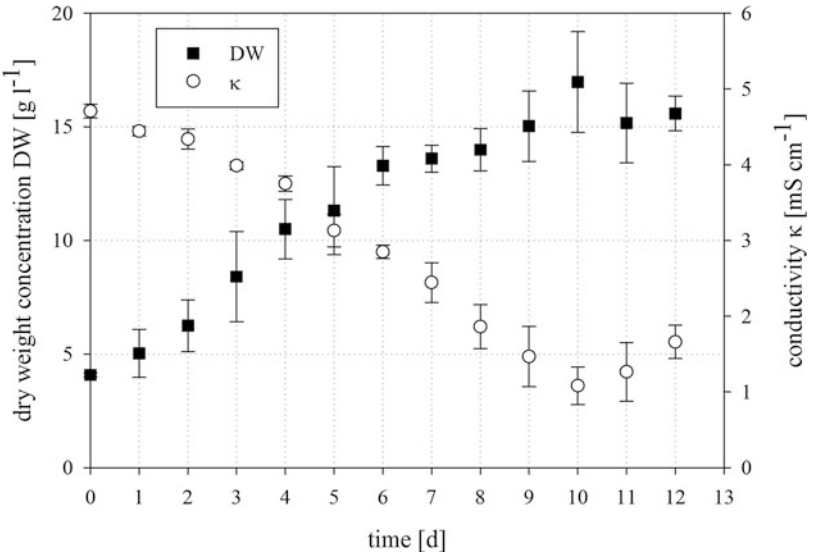

Fig. 1 Biomass dry weight concentration (DW) and electrical conductivity (κ) in the culture medium during the cultivation of a cs of *H. annuus* in the dark (N = 4, LS, 3% sucrose 0.2 mg l^{-1} 2,4-D), 110 rpm, 26 °C, shake flask 250 ml nominal volume, 50 ml V_L, Qcond 2400 Conductivity Meter Set

DW_{max} at the same day. At this time, the culture entered the stationary phase. After reaching its minimum, the conductivity rose, while DW declined slightly. Causative for the conductivity rise is the beginning of cell lysis and, subsequent, of release of ionic cell compounds to the culture medium.

As previously described and depicted in Fig. 1, the change in conductivity indicates a correlation with the biomass formation until day 9 and 10 before the cells entered the stationary phase. Consequently, the relation between biomass growth and change of conductivity can be determined as exemplarily shown for cultivations of three cs and one hr (Fig. 2). For each culture, a linear correlation equivalent to Eq. 1 with culture-specific proportionality constant α was calculated.

The correlation between the conductivity changes and the changes in biomass concentration shows a similar pattern for the considered cs and hr. A linear relationship according to Eq. 1 was observed for each culture before entering the stationary phase of biomass growth. The cultures enter the stationary phase after 18 days for the hr of *Beta vulgaris* (*B. vulgaris,* red beet) and after 10 days in the case of the *H. annuus* and *Salvia fruticosa* (*S. fruticosa,* sage) cs.

The proportionality constant α was 4.0 g cm^{-1} l^{-1} ($r^2 = 0.97$) for the hr of *B. vulgaris,* 4.5 g cm^{-1} l^{-1} ($r^2 = 0.87$) for the cs of *H. annuus*, and 4.8 g cm^{-1} l^{-1} ($r^2 = 0.96$) for the *S. fruticosa* cs. These values exhibit a high statistical significance r^2, indicating a reliable correlation of biomass concentration via conductivity change in the period observed. These values are close to the highest α-value from Table 1 for *Catharanthus roseus* (*C. roseus,* Madagascar periwinkle) (4.1 g cm^{-1} l^{-1}).

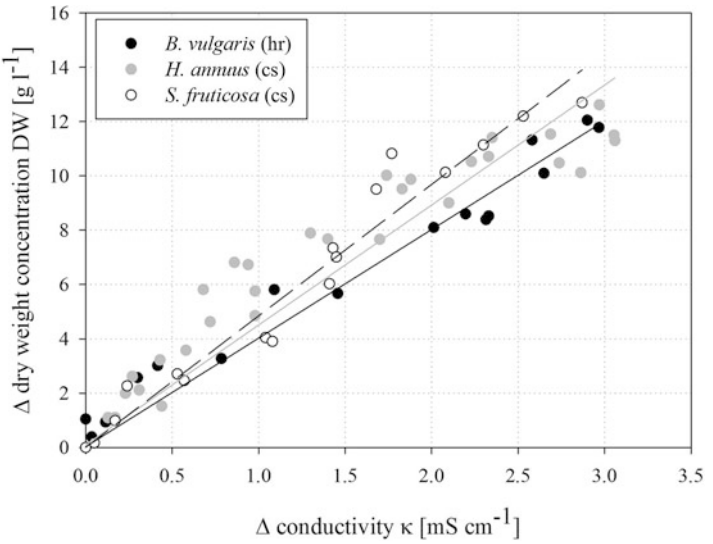

Fig. 2 Correlation between the changes of the biomass dry weight concentration (ΔDW) and electrical conductivity (Δκ) in the medium during the cultivation with 3% sucrose of a hr of *B. vulgaris* (N = 3, MS), cs of *H. annuus* (N = 4, LS, 0.2 mg l^{-1} 2,4-D) and cs of *S. fruticosa* (N = 2, LS, 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} zeatin) in the dark; all in shake flasks, 110 rpm, 26 °C, 250–500 ml flask volume, 0.02 g_{DW} (hr) and 20% (cs) inoculum, linear correlation for *B. vulgaris* (*black line*) $\Delta DW = 4.01\ \Delta\kappa$, $r^2 = 0.97$; *H. annuus* (*gray line*) $\Delta DW = 4.46\ \Delta\kappa$, $r^2 = 0.86$, *S. fruticosa* (*dashed line*) $\Delta DW = 4.87\ \Delta\kappa$, $r^2 = 0.96$

Regarding the experiments in Fig. 2, the values for α are in a similar range, when compared with the range of those values given in Table 1. This might result from the similar culture medium (Murashige and Skoog medium, MS [28] or Linsenmaier and Skoog medium, LS [29]) and the likewise growth characteristic and nutrient uptake. An additional important factor is the temperature of the sample during conductivity measurement, as the electrical conductivity is known to be strongly temperature dependent. A temperature compensation is necessary. Our measurements are always conducted without compensation in media filtrates at the reference temperature of 25 °C. Therefore, beside culture-specific factors, different measuring temperatures could be additional reasons for the different values in Fig. 2 compared to Table 1.

As previously shown, the electrical conductivity is an appropriate parameter for the determination of biomass growth. After providing an evidence of linear correlation between ΔDW and Δκ, the growth behavior of the plant cell and hairy root cultures can be predicted via the change of conductivity. This makes the conductivity a beneficial parameter to estimate the biomass growth of plant cell and tissue cultures, which is nondestructive and independent from scales [23]. To indicate the linear relation, the proportionality constant α has to be predetermined for specific cultures and conditions.

A continuous monitoring of the conductivity over the cultivation period enables to pursue the cell growth and identify characteristic time points of the cultivation such as the beginning of the stationary phase. The linear dependency and correlation

offers the opportunity to track the biomass progress. For bioreactors starting from laboratory scale (>1 l), the application of conductivity probes is well established [30]. But in the case of mass screening systems such as shaking flasks, there is no feasible solution to determine the conductivity online, sterile, and so far continuously without restrictions. This is the reason why the data presented in this publication were collected from offline measurements. To monitor plant biotechnological processes continuously, the Respiration MOnitoring System® (RAMOS®) will be introduced as a proper alternative to the conductivity measurement in the following chapter.

3 Online Monitoring in Shake Flasks with the Respiration Activity MOnitoring System® (RAMOS®)

3.1 Cultivation System, Parameter and Equations

As previously described, an appropriate monitoring of biomass growth and product accumulation is important for the development of biotechnological processes and strategies for enhancing the yields of subsequent phytoproducts. The most important fact for development and monitoring of these processes is knowledge of the growth kinetic of the respective culture.

The RAMOS® device (Respiration Activity MOnitoring System®, HiTec Zang GmbH, Herzogenrath, Germany) joins the benefits of shaken flasks and conventional bioreactors in terms of biotechnological aspects as it provides growth monitoring in small scale cultivation systems [31–33]. This shake flask device consists of eight measuring flasks, each equipped with an exhaust gas detector (refer to Fig. 1c in [20]). The measured values of oxygen partial pressure and carbon dioxide partial pressure in the gas phase can be used to calculate growth-dependent data like oxygen and carbon dioxide transfer (OT, CT), oxygen and carbon dioxide transfer rate (OTR, CTR), as well as the respiration quotient (RQ) at any time of the cultivation period. Furthermore, the RAMOS® provides in parallel to the measuring flasks room for six standard Erlenmeyer flasks as references which are similar in size and geometry to the measuring flasks (refer to Fig. 1 in [34]). In comparison to the manometric measuring method from Wen and Zhong published in 1995 [35], the RAMOS® offers the ability to monitor cell growth noninvasively and, therefore, without changing the hydrodynamic flow conditions in the shaken system [33].

In this chapter, an overview of relevant aspects, equations, data, and helpful information for interpretation of the data for the application of the RAMOS® device with plant in vitro systems, will be given. The flasks used in all experiments had a nominal volume V_t of 250 ml. They were made of glass, without baffles, and the input of energy and the mass transfer of the gas result solely through orbital shaking. By default, all cultivations started with 20% (v/v, 50 ml) initial filling volume V_L. The autoclaved flasks have been always dried prior usage for an increase of reproducibility because “the wetting of the sterile closure … may result in a significant reduction of the gas permeability of the plug” [36].

Moreover, each RAMOS® experiment was realized with permanent series of rinsing and measuring phases. For the realization of an exact measurement, the flasks were closed automatically in advance of each measuring phase for measurement of cell respiration followed by the rinsing phase for flushing the flasks with fresh air. These two phases alternate continuously. In comparison to the monitoring of microbial systems (usually 10 or 20 min and 10 min), both phases were extended for the slow-growing plant in vitro systems (50 and 40 min). According to Anderlei and Büchs [31, 33], the adjusted flow rate of 10 ml air min^{-1} in the rinsing phase equates the indirect aeration for narrow-necked flasks while orbital shaking of standard culture flasks. The rates of the respiratory activity used for the evaluation of cell growth were calculated (Eqs. 3 and 4) according to Büchs and Anderlei [31–33]. Furthermore, the Eqs. 5 and 6 have been used to determine overall oxygen transfer OT and the otr_{max} (CT and ctr_{max} analogous). The maximum biomass-specific oxygen transfer rate otr_{max} was calculated by dividing the determined OTR_{max} by the DW at that time point of the maximum OTR (Eq. 6) and indicates how much O_2 is consumed by a certain cell mass (1 g), whereas the OTR just indicates the overall O_2 consumed by the all cells.

$$OTR_{RAMOS} = \frac{\Delta p_{O_2}}{\Delta t} \cdot \frac{V_t - V_L}{V_L \cdot R \cdot T} \tag{3}$$

$$CTR_{RAMOS} = \frac{\Delta p_{co_2}}{\Delta t} \cdot \frac{V_t - V_L}{V_L \cdot R \cdot T} \tag{4}$$

$$OT = \int_0^t OTR \cdot dt \cong \sum_{i=0}^{n} \left(\frac{OTR_i + OTR_{i+1}}{2} \cdot (t_{i+1} - t_i) \right) \tag{5}$$

$$otr_{max} = \frac{OTR_{max}}{DW} \tag{6}$$

Besides using offline values like DW, also online values like the respiration activity are suitable for the calculation of specific growth-related parameters. Such growth values are the specific growth rate μ(t) in d^{-1} (Eqs. 7 and 11), the maximum specific growth rate μ_{max} in d^{-1} (Eq. 8), and the yields $Y_{DW/S}$ in g_{DW} gs^{-1} (Eq. 9) and Y_{DW/O_2} in g_{DW} ${g_{O_2}}^{-1}$ (Eq. 10). In order to calculate μ by help of the OTR instead of the biomass data, Eq. 7 changes accordingly (Eq. 11). The doubling time t_d in d is determined according to Eq. 12.

$$\mu_{DW}(t) = \frac{\ln(DW_2) - \ln(DW_1)}{t_2 - t_1} \tag{7}$$

$$\mu_{max} = \frac{\ln(DW_{max}) - \ln(DW_1)}{t_{max} - t_1} \tag{8}$$

$$Y_{DW/S} = \frac{DW}{S} \tag{9}$$

$$Y_{DW/O_2} = \frac{DW}{O_2} \tag{10}$$

$$\mu_{OTR}(t) = \frac{\ln(OTR_2) - \ln(OTR_1)}{t_2 - t_1} \tag{11}$$

$$t_d = \frac{\ln 2}{\mu_{max}} \tag{12}$$

For the calculation of μ solely, the values of OTR (online) and DW (offline) within the growth phases were used, to ensure values independent from adapting/lagging or stationary influences.

3.2 Fundamental Aspects of Plant Cell and Hairy Root Cultivations in the RAMOS®

The number of published data concerning the cultivation of microbial cell systems with the RAMOS® has risen continuously since development and product placement in the early twenty-first century. The publications originate mainly from the group of Jochen Büchs, RWTH Aachen University, Germany (e.g., [31, 32, 37–42]), who invented the RAMOS®. Currently, there are known only few publications on animal cell cultures (e.g., [43, 44]). Data related to applications of this device for studies of plant in vitro systems are mainly limited to the characterization of undifferentiated heterotrophic cell cultures (e.g., [45–50]), but increase continuously. Data concerning the cultivation of hr are very marginal published, and Geipel et al. characterized the growth of hairy roots for the first time with the help of the RAMOS® using *H. annuus* [9, 51].

The differences between microorganisms and plant cells (e.g., in size, structure, agglomeration tendency) involve numerous contradictions in terms of ability of in vitro cultivation and, especially, concerning inoculation and sampling [45, 52]. Furthermore, limited data regarding the respiratory activity of plant in vitro systems often lead to difficulties in interpretation of own results and the subsequent scale-up. Therefore, for example, Winkler et al. [9, 20, 53], Haas et al. [48], and Kümmritz et al. [50] carried out extensive studies on the suitability of the RAMOS® for the characterization of plant cell cultures and demonstrated advantages and limitations of this device. These investigations revealed some relevant criteria, which have to be taken into account when dealing with plant in vitro systems and will be discussed in the following.

Plant cells tend to agglomerate and therefore pipette transfer of plant cells is complicated or even impossible. Based on own experiments in shake flasks, the method of preinoculation was determined as best suited for a reproducible cultivation of plant cell suspensions [20]. In contrast, to gain an appropriated hairy root

culture for inoculation, young single tips with a length of about 1 cm were cut from the emers stock culture, therefore, injured, and precultured in liquid medium. After 7 days of preculture, uninjured young hairy root networks were used for inoculation of the subsequent characterization experiments. The differentiation between young and old cells was realized by manual, visual evaluation: old networks are dark-brown, whereas young plant tissues are brighter. Using the two-staged inoculation-method described, (age-related) growth differences due to different ages of the cells are minimized and the death of injured cells due to cutting the tissue in the main experiment reduced.

Compared to traditional microbial systems (e.g., bacteria or yeast cells), plant in vitro systems are characterized by slower growth and, accordingly, longer cultivation times. Consequently, the effect of evaporation out of the liquid phase of the bioreactor system is significantly and has to be considered for the evaluation of measurement data [9, 20]. The calculation of the OTR_{RAMOS} and CTR_{RAMOS} (Eqs. 3 and 4) by means of the RAMOS® is based on the liquid volume V_L in the culture vessel. Due to evaporation, this volume is decreasing incremental over the period of cultivation, but the RAMOS® software uses at any time the constant initial volume V_L as input. Disregarding this volume decrease results in significant errors – especially at long cultivation courses as typical for plant in vitro systems. The degree of evaporation as a function of the flask geometry (wide-necked or narrow-necked), the sterile barrier (cotton plug or paper plug), and the type of ventilation (ambient air, moisture-saturated air) was examined in a study by Geipel et al. [20]. The results are shown exemplarily for suspension cultures of *H. annuus* in Fig. 3 (refer additionally to Table 3 in [20]).

First, the respiration results are not comparable without considering the problem of different evaporation out of different cultivation flasks. Regarding the measuring flasks after a 7-day-cultivation period at standard conditions (26 °C, 110 rpm, 20 % (v/v) filling), an average evaporation volume of 2.3 ml is detected, which corresponds to 4.7% of V_L (filled squares). Using aeration with water-saturated air, this value was reduced to 0.47 ml (empty squares) which is less than 1% of V_L. Thus, the humidification of the inlet air is a suitable strategy to reduce overall evaporation, increase the accuracy of the respiration data (online), and unify concentration-related data like biomass or medium conductivity (offline). However, this method can only be realized easily in the measuring flasks, since the reference flasks are not actively streamed by the inlet airflow. Considering a cultivation period of 7 days, in the reference flasks the evaporation volume was – dependent on the flask neck geometry and sterile barrier used – 6.8 ml for wide-necked flasks with paper plugs and 4.0 ml for narrow-necked flasks with cotton plugs (Fig. 3, refer additionally to Table 3 in [20]). The different degrees of evaporation detected out of measurement, respectively, reference flasks represent a problem in terms of comparability and correlation of online (measurement flask) and offline (reference flask) data.

One way to solve this problem is the frequent filling of the reference flasks with sterile distilled water which, unfavorable, rises the risk of contamination, the effort, and leads to dilution of all medium components. Another alternative is the subsequent correction of the transfer rates after a model approach developed by

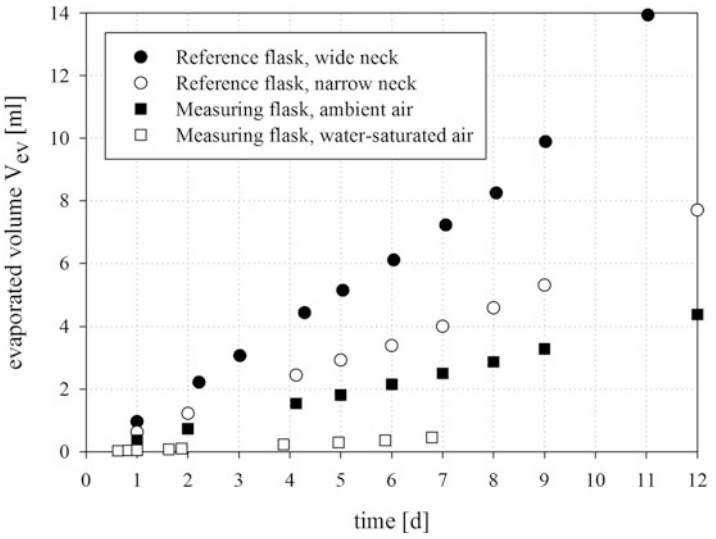

Fig. 3 Level of evaporation during cultivations of cs of *H. annuus*. *Empty squares*: in RAMOS® flasks with water-saturated air (N = 7). *Filled squares*: in RAMOS® flasks with aeration using ambient air (N = 7). *Empty dots*: in reference flasks with narrow necks (N = 6). *Filled dots*: in reference flasks with wide necks (N = 6). Cultivation conditions: RAMOS®, orbital shaker, 50 mm shaking diameter, 110 rpm, 26 °C [54], 250 ml nominal flask volume, 50 ml V_L, LS [29] with 3% sucrose and 0.2 mg l^{-1} 2,4-D, pH 5.7 prior autoclaving; in darkness; aeration with ambient air (10 ml min^{-1}) [20]

Geipel et al. [20]. Accordingly, V_L is mathematically reduced by the predetermined volume of evaporation in the equations for the calculation of OTR values (Eq. 12, CTR, OT and CT analogous). Thus, for the determination of the evaporation-corrected transfer rate OTR_{ev}, the following modified equations are obtained [20].

$$OTR_{ev} = OTR = \frac{\Delta p_{O_2}}{\Delta t} \cdot \frac{V_t - (V_L - F_{ev} \cdot t)}{(V_L - F_{ev} \cdot t) \cdot R \cdot t} \tag{13}$$

Figure 4 shows the progress of OTR and corrected OTR_{ev} of three cultivations of a suspension culture of *H. annuus*. The expected increasing separation of both curves during the cultivation period due to incremental water loss is clearly visible. As the corrected graph OTR_{ev} runs above the noncorrected curve, evaporation results in a lower increase of the respiration rate during cell growth. The deviations between the two curves are slightly (refer to Table 5 in [20]). In this case, it has to be taken into account that the presented OTR trend originates from a measuring flask with an appropriate small shaft diameter (refer to Fig. 1 in [20]). The differences between the respiration activities are expected to be intensified when comparing RAMOS® measuring with reference (wide neck Erlenmeyer) flasks. Possible causes of the major differences in respiration activity and resulting evaporation behavior

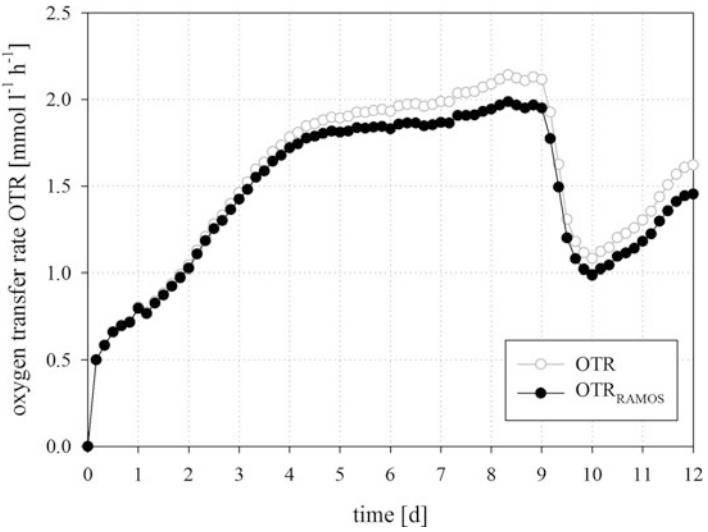

Fig. 4 OTR of the cultivation of a cs of *H. annuus* in the RAMOS® (N = 3). *Filled dots*: OTR_{RAMOS} calculated without regarding evaporation (Eq. 3). *Empty dots*: OTR considering evaporation according to Eq. 13. Cultivation conditions: RAMOS®, orbital shaker, 110 rpm, 50 mm shaking diameter, 26 °C, 250 ml nominal flask volume, 50 ml initial filling volume, LS with 3% sucrose, 0.2 mg l^{-1} 2,4-D, pH 5.7 prior autoclaving; in darkness; aeration with ambient air (10 ml min^{-1}) [20]

(refer to Tables 3 and 4 in [20]) are the different flasks necks and sterile closures used. To confirm this statement, it is necessary to measure the respiration activity in the reference flasks for a comparison with data from the RAMOS®. But such measurements are not easy to execute because the reference flasks do not contain measuring devices. Subsequently, the detection of (different) growth behavior depending on flask type, sterile closure, and/or aeration moisture content as well as possible further evaporation-related parameters is required. In the meantime, the equation for correction of the OTR (Eq. 13) is a useful tool to compare online respiration data with offline data. The volume-related data obtained from the reference flasks (e.g., biomass or product concentration) have to be corrected accordingly.

Since the evaporation in addition to the flask geometry and the sterile barrier used strictly depends, for example, on the shaking frequency, the temperature, and fluid viscosity, the evaporation volumes published by Geipel et al. are only of exemplary importance and need to be redefined for modified cultivation conditions. Additional effects on growth and product formation resulting from the concentration of media components due to water loss remained neglected in this study and have to be examined more closely in future works. As mentioned in Sect. 2, concentration-dependent values like electrical conductivity or substrate concentration have been corrected according evaporation.

3.3 Growth Kinetics of Heterotrophic Plant Cell Cultures

Respiration parameters are correlated to the growth of plant in vitro systems. Figure 5 illustrates OTR, OT (both according to Eq. 13), and biomass data of the heterotrophic cultivation of a cs of *H. annuus* in the RAMOS®. The relative standard deviations (N = 3) for the OTR are on an average of 4% and a maximum of 16%, which confirm the RAMOS® to be an appropriate measuring device for plant cell respiration. Furthermore, those small deviations represent the preinoculation strategy applied for the plant cs (Sect. 3.2) as suitable for reproducible inoculation with plant cells.

For comparison with the cell respiration (online), the concentration of biomass DW (offline) is shown (Fig. 5). Apart from the online device, analysis and growth monitoring of plant in vitro cultures are difficult, for example, due to thick suspension and formation of cell agglomerates. Therefore, as already mentioned (Sect. 2), the electrical conductivity is an excellent (offline) parameter for process monitoring. Additionally, the respiration activity determined perfectly represents the biomass changes during cultivation of plant cs cultured at heterotrophic conditions (Fig. 5). After mathematical correction of evaporation, the maximum OTR achieved by the heterotrophic grown cs of *H. annuus* is $OTR_{max} = 2.4$ mmol l^{-1} h^{-1} at day 8.3 of the cultivation. The DW determined at the same time is 11.8 g l^{-1}, which results in an otr_{max} of 0.2 mmol g^{-1} h^{-1} (Eq. 6). The overall DW_{max} was detected 14.5 g l^{-1} at day 12 of the process.

Regarding the OT in Fig. 5, a classification into growth phases is feasible. After a short adaption phase, an alteration to exponential growth is detectable. Subsequently, due to a limitation of one or more nutrients after 3.5–4 days, a linear growth phase seems to follow up. A limitation of O_2 as reason seems impossible. Experiments with varying V_L (10, 20, 30, 40, and 50 ml) did not expose significant differences in the oxygen transfer rates (refer to Fig. 13 in [9]), which would be expected in case of an O_2 limitation. While the overall rise in biomass is detectable until the end of the experiment, the sudden drop of the OTR curve at day 9 indicates a change to a phase with reduced respiration caused by limitation of an important nutrient, for example, the carbon and energy source [32, 55]. Similar results have been determined investigating a cs of *S. fruticosa* [20]. To compare the OTR values presented with respiration activities of other biotechnologically applied (micro)organisms, please refer to the literature (e.g., [20, 31, 32]).

The maximum specific growth rate μ_{max} of the *H. annuus* cells investigated was calculated to 0.39–0.67 d^{-1} (Eqs. 8 and 11). Resulting doubling times t_d (Eq. 12) calculated in the exponential phase are between 1.0 and 1.8 d. This broad range is due to the use of different types of flasks and sterile closures (refer to Fig. 1 in [20]) during cultivation for obtaining online and offline data [20]. Furthermore, the different resolution of the two methods for calculation of growth-specific parameters (online versus offline) is essential: While the RAMOS® recorded 60 values in each measuring phase (approx. a single value min^{-1}, every 50 min), offline sampling only enabled a single value every 2 days.

Haas et al. determined specific growth rates of different *Salvia* sp. cs, based on the determination of the OTR, ranging from 0.17 to 0.4 d^{-1} [48]. In contrast to published

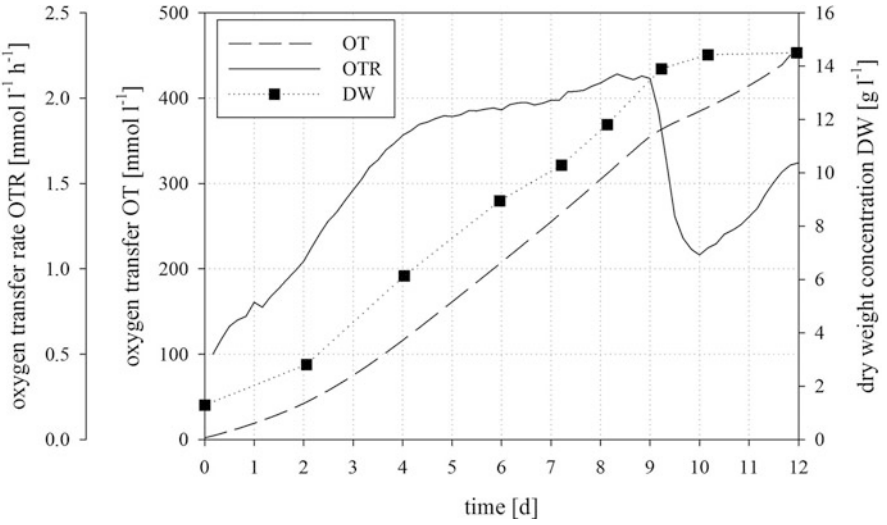

Fig. 5 Respiration (OTR, OT; N = 3) and biomass data (DW) of a cs of *H. annuus*. Cultivation conditions: RAMOS®, orbital shaker, 50 mm shaking diameter, 110 rpm, 26 °C, 250 ml nominal flask volume, 50 ml initial filling volume, LS with 3% sucrose and 0.2 mg l^{-1} 2,4-D, pH 5.7 prior autoclaving; in darkness; aeration with ambient air (10 ml min^{-1}) [20]

data for suspension cultures of *Helianthus* sp. (μ_{max} = 0.21–0.42 d^{-1}, refer to Table 1 in [20]), the μ_{max} calculated using the DW (μ_{max} = 0.39 and 0.53 d^{-1}) are high but authentic. In addition, μ_{max} calculated from the online data (0.66/0.67 d^{-1}) is up to three times higher, suggesting the measured OTR is greater than the O_2 demand caused by pure biomass formation. This higher O_2 demand leads to the conclusion that not the whole volume of O_2 supplied has been metabolized into biomass. For some reason, there have to be other factors influencing the O_2 consumption (e.g., secondary and/or fermentative metabolism, storage of molecular O_2) which, in addition, can be extracted from the values of the respiration quotient (RQ > 1). Moreover, small fluctuations in the gas composition and moisture content of the inlet air due to the experimental setup cannot be excluded.

3.4 Growth Kinetics of Hairy Root Cultures

In contrast to plant cells, hairy roots form filamentous, network-like structures, and therefore, the application of most of the established methods (e.g., determination of optical density) is limited or not applicable to monitor the biomass growth. For the direct quantification of biomass, the sampling of the complete hairy root network is required resulting in offline data like DW. Accordingly, the cultivation process has to be terminated which means further monitoring is not possible. Due to this fact, a continuous online monitoring of hairy root growth is required.

In general, the monitoring of changes in the characteristics of the culture medium (e.g., electrical conductivity, osmolarity, substrate concentration) due to metabolic activity of the culture by, for example, nutrient uptake is reasonable. As shown in Sect. 2, the determination of changes of the electrical conductivity of the culture medium through the uptake of ionic nutrients provides a good correlation to the hairy root formation and is, therefore, suitable as indirect growth parameter. Nevertheless, currently, common applications for the online determination of conductivity are limited to engineered bioreactor systems (e.g., stirred tank or airlift systems). Conventional sensors are not suitable for the process monitoring in shake flasks. Therefore, an alternative continuous online monitoring of growth kinetic, respectively, growth-related values for shake flask cultivations as well as all bioprocesses is required. The respiration activity is known to be related to the metabolic activity of cells through the depletion of carbon and energy source under O_2 consumption and CO_2 production [31] and, therefore, can be used to gain information about the growth-kinetic of hr.

The RAMOS® device offers the possibility to online receive respiration data (Sects. 3.1 and 3.2). Assuming that the storage capacity of aqueous solutions for O_2 is very low, the OTR equals the oxygen uptake rate OUR and the OT equals the oxygen uptake OU [49]. These respiration data represent an indirect parameter for the respiration activity of the hr, which is dependent on the metabolic consumption of carbon sources. Publications regarding the cultivation of hr using the RAMOS® are marginal. Geipel et al. characterized the growth of hr of a model cell culture of *H. annuus* using the RAMOS® [51] the first time. Hairy roots from *B. vulgaris* were further characterized by use of this device (Figs. 6 and 7). The inoculation was carried out with a 7-day-old submerged culture (intact network) with a fresh biomass of approximately 0.2 g (DW $\approx$ 0.02 g) instead of separated, counted, and wounded root tips. The substrate (sugar) concentration (Fig. 7) in the medium was examined via high performance liquid chromatography (HPLC; for method details refer to [53]). Offline values (DW and substrate concentrations) were determined by harvesting and analyzing the whole content of a reference flask at time points as shown. All final values of substrate concentration and respiration activity were calculated considering the reduction of V_L by the evaporation of water from the culture vessel analogous to Eq. 2.

Figure 6 presents the concentration of DW of hairy roots of *B. vulgaris* as well as the evaporation-corrected respiration activity (OTR and OT; Eq. 13). The relative standard deviations (N = 6) for the OTR average at 14% with a maximum of 40%, which is differing from the standard deviations determined for plant cs (Sect. 3.3). Possible reasons for this observation are the inoculation strategy due to the heterogeneous hr network. The OTR increased slowly after inoculation for approximately 2–4 days, followed by an intense increase of metabolic activity to the maximum respiration rate of $OTR_{max} = 2.0\ mmol\ l^{-1}\ h^{-1}$ on day 13–14. Subsequent, the OTR decreased until the end of the cultivation. Considering the OT course, the adaption phase (day 1–2) and the following lag phase are similar to these phases at the OTR curve, leading to an exponential growth phase starting approximately on day 4. Afterwards, a linear growth phase is following until the end of the cultivation. By means of the OT, a clear switch to stationary phase is difficult to detect.

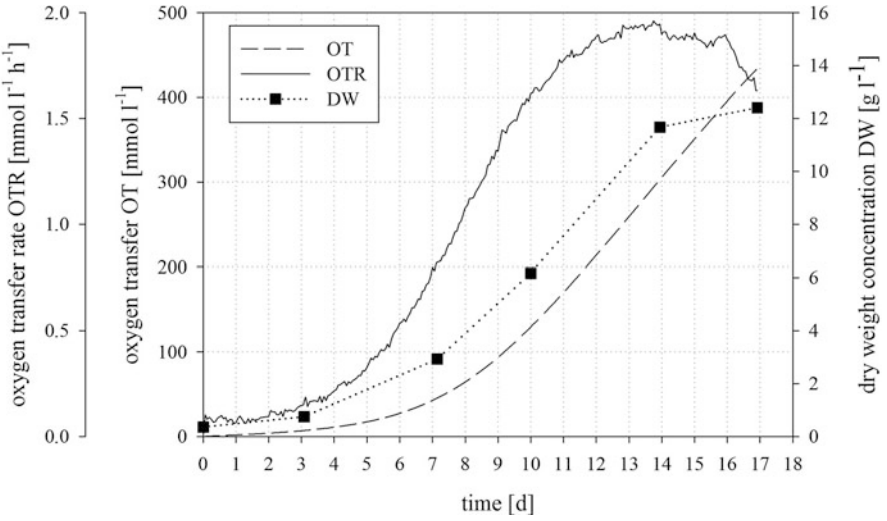

Fig. 6 Respiration (OTR, OT; N = 6) and biomass data (DW; N = 2) of a hr of *B. vulgaris*. Cultivation conditions: RAMOS®, orbital shaker, 50 mm shaking diameter, 110 rpm, 26 °C, 250 ml nominal flask volume, 50 ml initial filling volume, Murashige and Skoog medium (MS) with 3% sucrose, pH 5.7 prior autoclaving; in darkness; aeration rate 26 ml^{-1} min^{-1}, with ambient air, evaporation of water 0.026 ml^{-1} h^{-1}

The DW (Figs. 6 and 7) increased slightly during adaption phase and, subsequently, rose intensively beginning at day 4 to a maximum of $DW_{max} = 12.4$ g l^{-1} on day 17. The curves representing the substrates (Fig. 7) showed for sucrose the expected decrease during the whole cultivation time to a total depletion on day 10 due to the enzymatic cleavage of sucrose by the cells. Simultaneously, the concentration of glucose and fructose (monosaccharides) reached a maximum on day 10 before decreasing due to assimilation.

To sum up, OTR and OT initially increased slowly (Fig. 6) comparable to the slight biomass growth and substrate uptake (Fig. 7). Around the 4th day, the OTR and DW rose associated to higher metabolic activity. The OT increased time-delayed and seemed to skip over in an exponential progress.

In Fig. 7, a rapid decrease of sucrose is detectable starting at day 2, which implies a change from adaption to exponential growth phase. Approximately, on day 10 of the cultivation, a transition into a linear growth phase occurred indicated by a change of the OT value from exponential to linear rising. The detected weakening of the OTR after day 10 happens simultaneously to the sucrose consumption and is possibly due to the limitation of O_2 in the cell network (hr tissue). After 13–14 days the OTR reached its maximum with 2 mmol l^{-1} h^{-1} and is comparable to published values of *H. annuus* and *Salvia* cs (using LS medium instead of MS) that were characterized using the RAMOS® but reached the OTR_{max} of 2 mmol l^{-1} h^{-1} already on day 9, respectively day 11 [20, 48]. In the same time, a strong increase in DW was detected additionally. The following limitation of respiration appeared after

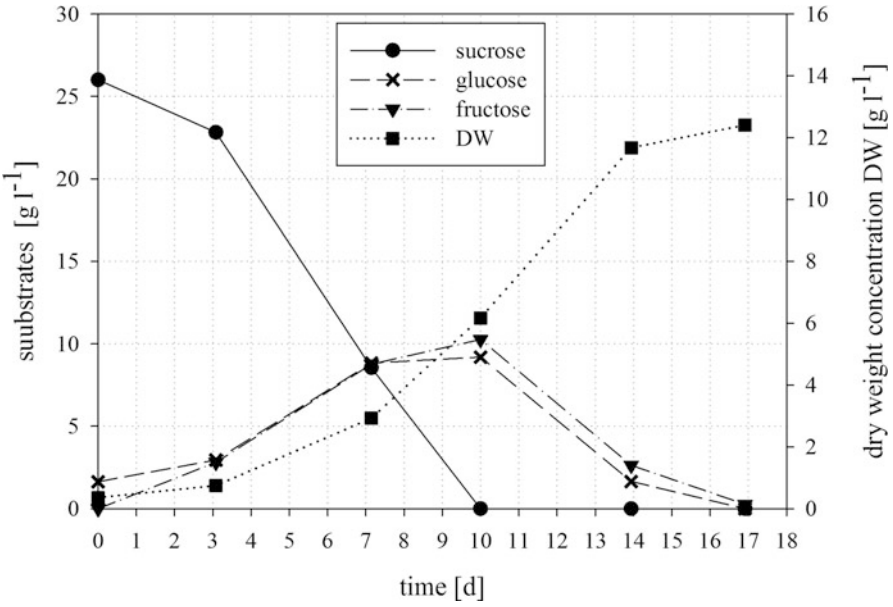

Fig. 7 Concentration of sugars in the culture medium and biomass data (DW; N = 2) of a hr of *B. vulgaris.* Cultivation conditions: RAMOS®, orbital shaker, 50 mm shaking diameter, 110 rpm, 26 °C, 250 ml nominal flask volume, 50 ml initial filling volume, MS with 3% sucrose, pH 5.7 prior autoclaving; in darkness; aeration rate 26 ml^{-1} min^{-1}, with ambient air, evaporation of water 0.026 ml^{-1} h^{-1}

reaching the OTR_{max} on day 14 due to substrate limitation and probably due to O_2 limitation. Hence, the hr formed dense networks in this state of the cultivation. The further decrease of the OTR indicates the culture entering the stationary phase. After reaching $OTR_{max,}$ the OTR decreases which corresponds to the almost final depletion of the carbon sources. After 10 days of cultivation, the sucrose (disaccharide) was completely hydrolyzed to glucose and fructose. These monosaccharides are known to be metabolized by the cells simultaneously [48, 56]. They were depleted at day 17, which indicated the end of the cultivation experiment and coincidences with drop of the OTR.

The online data (OTR, OT) were used to assess the biomass growth of the hr culture compared to the DW (offline data). The otr_{max} for the cultivation of hr of *B. vulgaris* was calculated to 0.17 mmol g^{-1} h^{-1} and is similar to published values. Geipel et al. (2014) reached otr_{max} of 0.22 mmol g^{-1} h^{-1} during the cultivation of hr of *H. annuus* [51], and Haas et al. reached otr_{max} between 0.17 and 0.38 mmol g^{-1} h^{-1} with cultivations of cs of *Salvia* species [48]. As previously described, for the calculation of the specific growth rate μ, only the values in the exponential growth phase of DW (offline, Eq. 7) and OTR (online, Eq. 11) were used. Therefore, the specific growth rates for the hr culture of *B. vulgaris* were calculated to $\mu_{DW} = 0.20$ d^{-1} and $\mu_{OTR} = 0.22$ d^{-1}. Compared to μ of hr of *H. annuus* ($\mu_{DW} = 0.48$ d^{-1} and

$\mu_{OTR} = 0.53\ d^{-1}$) [51], the hr from *B. vulgaris* grew faster. In general, the calculated μ based on the OTR and on the DW are similar to each other. The main advantage of the determination of μ via the respiration activity is the availability of more data points and the continuous online measurement without sampling.

In order to characterize the hr of *B. vulgaris* more detailed, the biomass yields for the consumption of the substrates sucrose (S) and O_2 were calculated. For estimation of the yield for the O_2 consumption Y_{DW/O_2}, the OT, representing the amount of O_2 uptake, was used (Eq. 10). The yields are calculated to $Y_{DW/S} = 0.4\ g\ g^{-1}$ and $Y_{DW/O_2} = 1.2\,g\,g^{-1}$. The yield coefficient for oxygen Y_{DW/O_2} is due the low amount of published data not comparable with values from literature. In further cultivations hr of *B. vulgaris* using the RAMOS® similar yield coefficients were achieved (unpublished data). The yield determined for sucrose consumption is for example, comparable to published values of the cultivation of *B. vulgaris* hr in bubble columns ($Y_{DW/S} = 0.38\ g\ g^{-1}$ [24]).

3.5 Innovative Development for the Monitoring of Photosynthetically Active Plant In Vitro Systems

Several valuable metabolites are exclusively synthesized by phototrophic organisms [57]. Photobiotechnology – biotechnology focused on photosynthetically active systems – is an emerging sector in sustainable biotechnological production. Therefore, photobioreactors (PBR) and their efficiency are permanently improved [58].

Screening and characterization of phototrophic production strains are typically performed in illuminated shake flask systems [59, 60], which are rarely beneficial regarding the light distribution and online monitoring of phototrophic growth [61]. In 2007, Rechmann et al. published a study concerning the characterization of a photosynthetically active *Wolffia australiana* (duckweed) plant cell line (cs) using the RAMOS® device [46]. The photosynthetic active radiation (PAR) within the RAMOS® device was provided by fluorescent lamps which were installed top-side within the shaker. Applying this type of illumination, cells were permanently shaded by the sterile plugs and the RAMOS® sensor rack mounted above the shake flasks. Additionally, the installed fluorescent lamps just provided a low maximum photon flux density (PFD) of 200 $\mu mol\ m^{-2}\ s^{-1}$. Since PAR photons are the sole source of energy for photosynthetically active cells, this low PFD intensity range is not suitable to investigate the phototrophic growth from compensation to inhibition.

To overcome these drawbacks, Socher et al. developed an innovative system which combines an adequate illumination of shake flasks with adjustable PFD intensities. The invention called “CultiLux” includes an illumination unit based on light emitting diodes (LED) and serves as an excellent upgrade for the RAMOS® device [62] as well as other conventional shake incubators. The illumination unit of the CultiLux was designed to provide PAR illumination for each of the 14 shake flasks from below. The LED lighting unit enables a reproducible adjustment of PFD

intensities up to 2,000 μmol m^{-2} s^{-1} and flexible durations of light/dark cycles. The RAMOS®-CultiLux set-up was successfully applied for cultivation and characterization of cs of *H. annuus* [51, 53].

As indicated in Fig. 8, the LED lighting unit was designed to provide the PAR illumination throughout the transparent bottoms of the shake flasks to prevent shading effects and to maximize the illumination homogeneity and reproducibility. To enable the insertion of dimming, diffusing or filtering elements space was left between the mounting of the shake flasks and the LED lighting unit ((3) in Fig. 8). Each LED panel consists of six warm white LED (Fig. 9) circularly mounted onto a stainless steel plate. Because LEDs are punctual light sources, the PFD intensity varies – depending on the position (refer to Fig. 2 and Table 1 in [62]) – between 2,300 μmol m^{-2} s^{-1} directly above the LED (cross in Fig. 9) and 0–90 μmol m^{-2} s^{-1} in poorly illuminated zones between the LED. PFD intensities greater than 2,000 μmol m^{-2} s^{-1} are rarely beneficial for cultivation because they inhibit or even damage the photosystems of most phototrophic cells [63].

Optional filter elements can be used to block or enhance particular wavelengths from the standard spectrum of the LED and, therefore, enlarge the application spectrum to narrow-bandwidth wavelengths, for example, for studying the excitation of specific photosynthetic molecules in the photosystems of the phototrophic cells. Alternatively, the LED panels can be changed completely, if LEDs with different continuous spectra are required.

In conclusion, the innovative CultiLux setup provides a wide range of PFD intensities and light spectra to cultivate and characterize phototrophs with varying cultivation requirements. However, there are also several drawbacks related with the presented LED lighting unit which have to be addressed in future research and development. First, a high thermal energy input was detected at high PFD intensities, which results in an increased evaporation within the flasks. The thermal management could be facilitated using advanced cooling devices with high capacity and fast response time.

An increased cooling effect could be attained by the installation of a fan inside the incubator, cooling elements with larger surfaces or adjusting a larger distance between the light sources and shake flasks (with reduction of light intensity). Indeed, the temperature effect caused by the light input could have a significant impact on the data deviation if light conditions are changing, for example, during light/dark cycles. This issue could be addressed by coupling the measurement and light regimes, that is, LED are switched on at the beginning of each RAMOS® rinsing phase and not during the measurement phase.

Socher et al. inserted diffuser materials, to attain a more homogenized PFD intensity distribution emitted from the punctual LED [62]. For future applications, more effective materials or alternative light sources should be used, for example, organic light emitting diodes (OLED) that provide the PFD in an ideal homogeneous way. However, implementation of OLED light sources resulted in reduced PFD intensity [64] compared to LED technology. Further developments will lead to more efficient OLED devices, regarding the quick development of LED in the past few years [65]. Implementation of light/dark intervals in order to study the effects of light

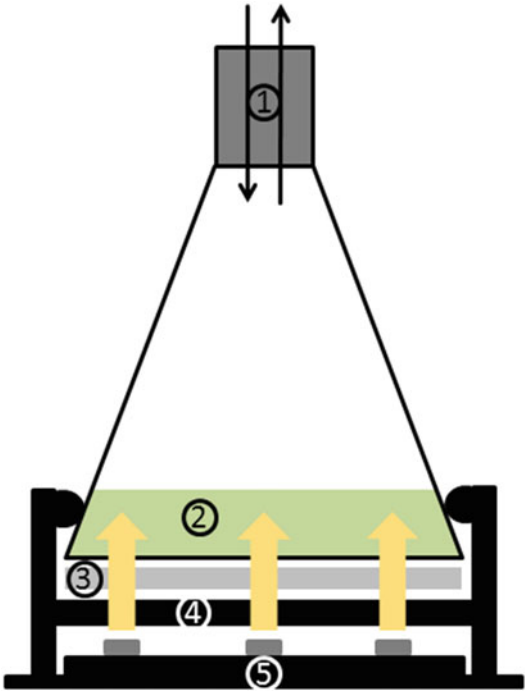

Fig. 8 Design of CultiLux – the lighting unit for incubator shakers like the RAMOS®, sketch: frontal view of arrangement of flask and LED panel with (*1*) O_2 and pressure sensor with gas exchange unit, (*2*) photosynthetically active cs, (*3*) diffusor/dimming/filter insert, (*4*) mounting for flask, and (*5*) LED panel [62]

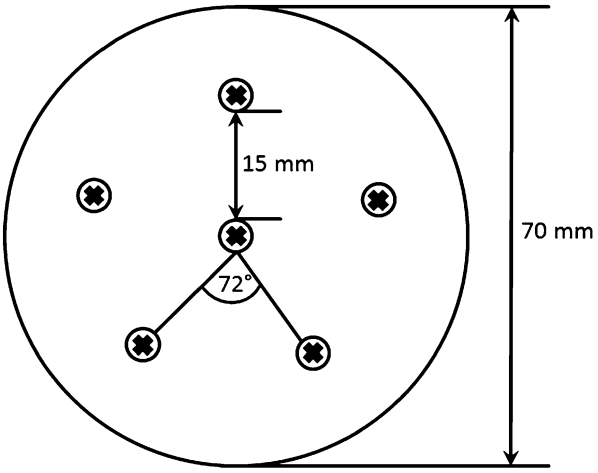

Fig. 9 Design of CultiLux – the lighting unit for incubator shakers like the RAMOS®, sketch: top view of a single LED panel arrangement (positions of LED are cross-marked) [62]

flashes would also be beneficial, because this is a very important step for investigating phototrophic growth and improving the performance of PBR.

The presented RAMOS®-CultiLux setup is an innovative and useful tool to investigate light-dependent parameters like growth rate (including compensation, saturation, and inhibition), and metabolic interrelations like pigment content, to optimize cultivation and practical exploitation of photosynthetically active cell (and tissue) cultures.

4 Flow Cytometry of Plant Cells and Tissues

4.1 Typical DNA-Related Parameters and Particularities in Flow Cytometry with Plant Cell and Tissue Cultures

Flow cytometers are optical analyzers that are capable to measure the properties of several thousands of individual particles suspended in a liquid in 1 s. Measured properties are light scatter, which provides information on size and surface of the particle or fluorescence intensities. The fluorescence usually results from staining of specific properties of the particle, in most applications biological cells. The utilization of flow cytometry is dominated by the analysis of blood cells for diagnostic use. For a long time, the application of flow cytometry for plant cell and tissue cultures was hampered by the fact that single cell suspensions are required to enable the analysis. As already mentioned, plant in vitro systems, however, form clusters of cells and even undifferentiated suspension cultures consist of cell aggregates. Consequently, the cells need to be separated from each other before they can be analyzed. Frequently, this was performed by the generation of protoplasts. This process is time-consuming and evokes changes in the morphology and the physiology of the cells, implicating a change of dynamic metabolic parameters during sample preparation. The breakthrough in the field of flow cytometry of plant cells was the application of a knife. In 1983, Galbraith et al. published a method to release nuclei from plant tissue by chopping it with a razor blade and analyzed the released nuclei using flow cytometry [66]. In that way, also the extraction of nuclei from cs can be realized, but also other methods of mechanical cell disruption like slight grinding [67] are applicable. The most appealing facts of these approaches are that they are simple, inexpensive, and fast. On the other hand only nucleus derived parameters can be measured.

Therefore, the dominant applications of flow cytometry in the field of plant in vitro cultures are related to the analysis of nuclear DNA, mainly the determination of genome size and ploidy. In the following, the most important parameters acquired by flow cytometry are shortly explained and described. According to Greilhuber et al. (2005), the “holoploid genome size” is the “DNA content of the whole complement of chromosomes” and its quantity (in pico gram) is denominated as C-value [68]. The 1C-value is the haplophasic, meiotically reduced genome (nuclear phase status n), whereas the 2C-value (2n) refers to nonreduced, diplophasic genome, for example, the DNA content in somatic cells in the G_1/G_0 phase (Fig. 10).

Somatic tissue normally has at least two sets of chromosomes (diploid), but in plants also polyploidy and polysomaty are common. Polyploidy means that a nucleus contains multiple sets of chromosomes, while the number of chromosomes per set is defined by x. As the C-value does not reflect polyploidy, the Cx-value is used to express the monoploid genome size of all cells of any ploidy (Fig. 10). Considering, for example, a diploid and a hexaploid plant of one species with 10 chromosomes per set: Here, the 2C-value of the diploid variant has the DNA content of 20 chromosomes ($2n = 2x = 20$). While the 2C-value of the hexaploid variant possess 60 chromosomes ($2n = 6x = 60$). The 2Cx-value is the same for both ploidies as it corresponds to the DNA content of a nonreduced monoploid genome (20 chromosomes). Polysomaty describes the fact that tissues are heterogonous concerning ploidy and consist of cells with different ploidy. This is frequently found to be associated to differentiation [69] and correlates among others with taxonomic affiliation, species, and organ type [70]. It emerges from endoreduplication, which means the DNA replication without mitosis. The term endopolyploidy is synonymous to the term polysomaty. To express the degree of endopolyploidy, Barow and Meister [70] defined a so-called cycle value. The detection of the holoploid genome size (C-value) by flow cytometry is performed by quantitatively staining the DNA in isolated nuclei. The simultaneous measurement of nuclei of the unknown sample and nuclei from an internal standard with known genome size allows the calculation of the C-value. Ploidy can be calculated by dividing the measured C-value by the 1Cx value or C-values (of known ploidy) from the literature or databases (http://data.kew.org/cvalues/).

4.2 Typical Applications of Flow Cytometry in Plant Breading and Micropropagation

Plant in vitro cultures like calli, tissue, or organ cultures play an essential role in the propagation of plants and their breeding. Their induction, cultivation and the regeneration to intact plants require growth regulators that are known to cause genetic changes like polyploidy or aneuploidy (additional or lost chromosomes) [71–73]. Plants regenerated from the in vitro status are often desired to have the same ploidy as the mother plant. This is verified by ploidy analysis using flow cytometry. By ploidy analysis, for example, Javadi et al. successfully verified that the ploidy of regenerated pear shoots was identic to the donor shoot [74]. Frequently, calli that kept the ploidy of the explant they were induced from have a higher chance to regenerate to a plant [72]. Therefore, it is of economic interest to screen the calli for this parameter in order to proceed only with material of the desired ploidy. In breeding, a common aim is the production of homozygous plants that can be obtained via haploid plants or cells, respectively, that are diploidized [72]. Here, the ploidy measurement is a convenient method to screen for successfully induced haploids and dihaploids. This is of importance as, for example, the production of haploid plantlets from baby primrose out of anther cultures resulted in only 2% haploids, whereas 65% were diploid and the rest exhibited other levels of ploidy [75].

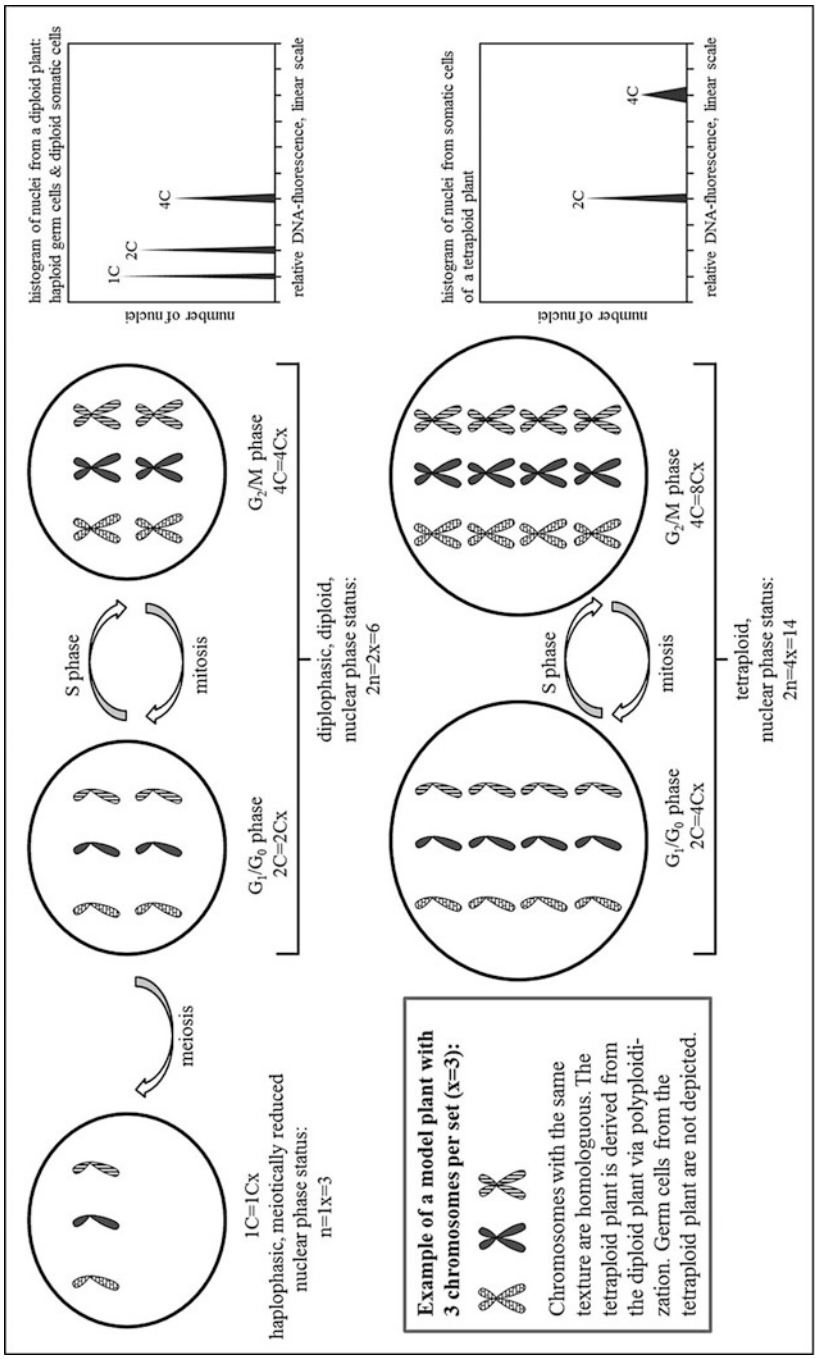

Fig. 10 Scheme of chromosome numbers, DNA contents (C-values), and corresponding histograms of quantitatively stained DNA of nuclei in an imaginary three chromosomes containing model plant. *Upper line*: a haploid germ cell of the diploid model plant and the corresponding diploid somatic cells in the cell cycle phases G_1/G_0 and G_2/M. *Lower line*: somatic cells of the tetraploid model plant in the cell cycle phases G_1/G_0 and G_2/M

Another interesting application for breeding and propagation is the discrimination of the sex of dioecious plants via comparing their genome sizes. Due to different sex chromosomes, these plants exhibit slight differences in the genome size [76].

A supporting method for plant breeding and basic research is the technique of chromosome sorting. It increases the efficiency of next generation sequencing because target chromosomes can be selected by sorting and reducing the amount of sequencing since only the target chromosomes need to be sequenced [77]. This is of special interest in crop plants that often are polyploid and contain high numbers of chromosomes. The discrimination of single chromosomes solely by its DNA content is often impossible but succeeds with other staining techniques described in detail by Doležel et al. and Vrána et al. [77, 78]. Flow cytometric applications with more biological or basic research background are the determination of C-value, ploidy, or base composition (AT to GC ratio) in plants for taxonomical studies [76], for example, the investigation of wild *Beta* species in Portugal [79]. Recently published studies on in vitro cultures apply flow cytometry, for example, for the investigation of the pollen germination process in pollen cultures of petunia [80] or the investigation of cell growth and proliferation activity in cs of *Arabidopsis thaliana* (thale cress) under microgravity conditions [81].

The generation of polyploids is a strategy, which is often helpful when it comes to the breeding of plants. This approach can be adapted to the development of plant cell and tissue cultures for the production of secondary metabolites. Polyploid cells exhibit different physiology and metabolic activity than their diploid counterparts, and polyploid plants can have favorable properties like bigger fruits or higher stress resistance. Many crop species are polyploid as a result of centuries of breeding [82]. Similar to observations in plants, ploidy also influences the physiology and metabolic activity of in vitro cultures. It is, for example, reported that tetraploid *Artemisia annua* (sweet wormwood) hr lines produce more artemisinin [83] or tetraploid hairy roots of *Datura stramonium* (thornapple) exhibited different alkaloid profiles [84] than their diploid counterparts. Two calli with different phenotypes were derived from one callus exhibiting a mixed phenotype from *B. vulgaris* "Detroit dark red". The analysis of nuclear DNA content revealed different levels of endopolyploidy/polysomaty in all three calli (Fig. 11). The authors concluded that "the degree of endoploidy may also influence the spectrum of the metabolite of interest and therefore be a parameter of interest for the choice of the right cell culture for its production" [16].

Often the plant in vitro cultures need to be cultivated in the presence of plant growth regulators. Their presence frequently leads to increased levels of polyploidization. As outlined above, increase in ploidy may have favorable influence on the productivity of the metabolites of interest. Ploidy analyses could support selection of polyploid clones or at least to be used to confirm stability and/or homogeneity of ploidy of a culture. Even cryopreservation actually used to preserve plant in vitro cultures in its current state can evoke genetic changes and to proof a successful conservation ploidy analysis or determination of C-value are necessary [85]. A broad and detailed overview about methods and applications of flow

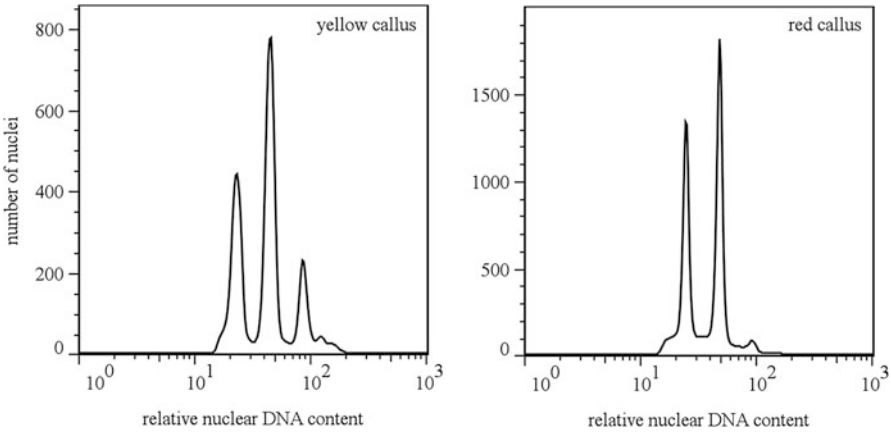

Fig. 11 Histograms of yellow and red calli separated from heterogeneous colored rhizogenic calli of *B. vulgaris* (Adopted from Weber et al. [16])

cytometry with plants and plant in vitro cultures can be found in "Flow Cytometry with Plant Cells: Analysis of Genes, Chromosomes and Genomes" by Dolezel et al. [86].

Besides these rather static parameters, the kinetic of DNA content is an interesting parameter to monitor the cultivation process for the production of target metabolites. The kinetic of DNA content is governed by the cell cycle (Fig. 10). The cell cycle is closely correlated to growth, a parameter of paramount interest in a cultivation process. Usually, the biotechnological process consists of two phases: the first is the proliferation of cells in order to produce biomass that is capable to synthesize the desired metabolites. Second phase is about to create the right conditions for the cells for the synthesis of these metabolites. Here, the conditions typically differ significantly from the conditions in the growth phase. In classic biotechnology, growth is monitored by the measurement of DW throughout the course of the cultivation. This is a bulk parameter reflecting the overall growth of the whole culture. However, it does not reflect the heterogeneities in the growth of the individual cell: Some cells may cycle readily trough the cycle under favorable conditions, while others remain dormant and do not participate in the cell cycle [87]. Therefore, the analysis of the cell cycle by flow cytometry allows receiving segregated data. This means to discriminate cells which contribute to the increase in DW by division from those, which do not divide but grow only in terms of volume.

4.3 Why Apply Flow Cytometry for Monitoring of Plant Cell Suspension Culture Based Processes?

Biomass and its kinetic of accumulation in the bioreactor or – in other words – growth is one of the most important biological parameters to characterize

biotechnological cultivation processes. This is as the biomass is the catalytic agent responsible of the conversion of substrates into products and on the other hand frequently specific data – which means parameters normalized to biomass – play an important role in the development, evaluation, and control of these processes. To gain online information about cells, a common approach is to correlate physical or chemical online parameters with biological ones. As already mentioned, the growth and concentration of biomass in a bioreactor is often estimated by a correlation with the course of the O_2 mass transfer rate – respiratory activity – of the cells [88]. Since such biological parameters are measured indirectly and derived from a correlation with physical parameters, this approach is referred to as the indirect measurement principle [89]. The major drawback is that the measured parameters are bulk measurements in the environment of the cells: These measures give average information of the whole culture in the bioreactor only. However, the culture in the bioreactor consists of millions to billions of individual cells and it is a well-known fact that even in isogenic cultures of bacteria and yeasts individual cells differ considerably [90]. This point is even more critical for plant cell cultures. For example, the production of anthocyanin in different *C. roseus* cs varied more than 30-fold while on single cell level the intracellular content varied only by factor 2 revealing heterogeneous distribution of producing and nonproducing cells [91]. Another example for heterogeneity is the proportion of 48% of cells in a *Solanum aviculare* (*S. aviculare,* kangaroo apple) cs which were not active in cell cycle and further increasing during the cultivation [87]. Plant cells in cs often grow in aggregates where each individual cell has different access to metabolites and O_2, their subjection to shear stress depends on their position in the aggregate, and intercellular communication intensity can vary between close contacted cells comparing to single cells [92]. Beside these factors, other unknown factors are conceivable that cause observed heterogeneity in plant cs. These variations effect the overall performance of a biotechnological production process [93] but cannot be resolved by the sensors that are routinely applied for bioprocess monitoring.

Flow cytometry is a technology that measures biological parameters on single cell level and, hence, allows resolving the physiological differences of the individual cells. Therefore, it provides segregated data and the dynamics of subpopulations inside the bioreactor can be observed.

4.4 Flow Cytometry Can Resolve Heterogeneities in Growth in Plant Cell Suspension Culture Based Cultivation Processes

Growth – the change of biomass concentration over time – is of vital importance for the production of bioactive substances by plant in vitro cultures. These substances are secondary metabolites and their synthesis is usually not growth associated but rather occurs during the stationary phase [48, 94]. Thus, the transition of the growth phase to the stationary phase is important for the productivity of the process. Growth is usually associated with the cells passing through the cell division cycle growing to a critical cell size and then divide again. Before cells divide, their DNA has to be

doubled (S phase) so that after the division each cell has the same number of chromosomes as the mother cell before (Fig. 10). The analysis of the distribution of DNA content (cell cycle analysis) in plant in vitro cells by flow cytometry should therefore be a valid parameter to measure the growth of a culture in a bioreactor. In the course of a batch cultivation, there is an initial excess of substrate allowing fast growth. In a later stage, the substrate becomes limiting causing the growth to slow down until the substrate is depleted and the cells reach a stationary phase characterized by a constant biomass. In a homogenously growing culture, this would be reflected in the cell cycle analysis as follows: In the initial phase of fast growth, an enhanced fraction of cells has doubled their DNA content meaning that they are in the G_2/M phase. When the substrate availability decreases under a certain threshold, the cells do not divide as readily and less cells can be found in the G_2/M phase and more cells have the undouble DNA content that characterizes the G_1/G_0 phase in the DNA histogram acquired by a flow cytometer. When the substrate is about to be exhausted, the fraction of cells of the G_2/M phase decreases until in the stationary phase no G_2/M cells are present since the culture stopped to grow.

Haas et al. analyzed batch cultivations of *H. annuus* cs in shake flasks and a stirred tank reactor [30]. The growth curve in terms of dry biomass over time exhibited a typical pattern of a batch culture (for shake flask compare Fig. 1). After a very short lag phase, the cells grew fast and after 6 days the accumulation of DW slowed down marking the transition into the stationary phase. Though the DW showed a typical pattern for a batch process, a different pattern in the DNA distribution was observed than the one described above: The fractions of cells in both the G_2/M and G_1/G_0 phase remained rather constant over the time of cultivation (Fig. 12). Only on the second day, a significant increase in the G_2/M phase portion and consequently a decrease of the G_1/G_0 phase fraction is observed. After that peak on day 1, the fractions of cells in the individual phases remain rather constant. However, a slight tendency to an increasing fraction of cells in the G_1/G_0 phase and a slight decrease of G_2/M cells can be found.

Though this trend confirms that the cell cycle analysis correlates with the growth what was also described by Georgiev et al. [95], these results raise several questions. Firstly, at the end of the batch, a rather high proportion of cells (approximately 25%) are detected in the G_2/M peak. This may be explained by cells that enter the G_2/M phase and remain there or by a slight tendency of the cells to undergo endoreduplication (genome duplication without mitosis). These polysomatic cells usually do not proliferate by mitosis [71, 96]. If this would be the case, then the cells underwent one round of endoreduplication and have the same DNA amount as the G_2/M cells. Further, the fraction of cells in the G_1/G_0 phase hardly fell below 40% and was comparably constant over the course of the batch cultivation. Similar results, intensive changes of proportions of cells in the different cell cycle phases at an early stage and higher fractions of G_2/M cells at the end of the batch cultivation were found for a single parametric cell cycle analysis of batch cultures of a *Harpagophytum procumbens* (devil's claw) suspension culture [97] and for other cs [98].

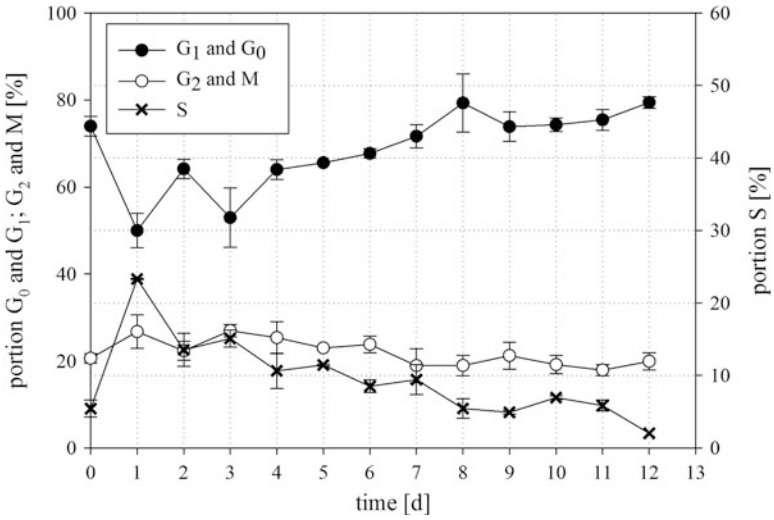

Fig. 12 Proportions of cells in different cell cycle phases in the course of a batch cultivation of a *H. annuus* cs in 250 ml shake flasks, $V_L = 50$ ml, LS medium with 3% sucrose, 0.2 mg l^{-1} 2,4-D, pH 5.7 prior autoclaving, in darkness. DNA of isolated nuclei was stained with propidium iodide (Adopted from Haas et al. [30])

Unfortunately, the method of measuring the DNA content as the single parameter cannot answer these questions: For example, it cannot differentiate between the nonproliferating G_0 cells from active proliferating G_1 cells. Two parametric methods that detect not only the content of DNA but also its synthesis are capable to distinguish these two phases and cycling from noncycling cells. Well-established methods for this are the addition of the thymidine analogs bromodeoxyuridine (BrdU) or 5-ethynyl-2′-deoxyuridine (EdU) which are incorporated into the DNA during its synthesis and can be detected by antibodies or in case of the EdU by click-it chemistry [99, 100]. However, the sample preparation for these analyses is harsh and, for example, requires the denaturation of the DNA for the antibody detection of BrdU and numerous centrifugations steps. Especially in the case of plant in vitro cultures and the measurement of their extracted nuclei, this is a pronounced problem, as the nuclei are without their protecting cell envelope. Yanpaisan et al. [87] successfully analyzed incorporated BrdU via antibodies in a *S. aviculare* cs and identified subfractions of quiescent cells in G_0 phase in the fraction of G_0/G_1 phase cells. The method, however, is harsh and does not allow the analysis of fragile nuclei [67]. An elegant way to detect BrdU incorporation is the so-called Hoechst quenching [100]. This is based on the effect of a reduced fluorescence of the DNA stained with Hoechst dyes when BrdU was incorporated into the DNA. Counterstaining by propidium iodide allows the simultaneous detection of the DNA content. Though Glab et al. and Tréhin et al. showed the feasibility of this method for plant mesophyll cells and cs, these authors went via the step of protoplast formation [101, 102]. Protoplast generation prolongs the sample preparation and

evokes a change of the physiology of the cells. This renders this method unsuitable for the monitoring of the cells status in bioreactor cultivations. Therefore, Haas et al. developed a method that allows to measure the DNA content and the DNA synthesis via BrdU incorporation by Hoechst quenching for directly extracted nuclei [67].

With this method a batch culture of *H procumbens* cs was investigated at shake flask scale. The authors found high cell cycling activities during the first 48 h of the batch and a slower progress thereafter. However, a small but significant fraction of the cells did not show any BrdU incorporation. After 72 h the distribution of the cells among the cell cycle phases remained rather constant. These data are in good agreement with the data from the one-parametric cell cycle analysis of a batch culture and prove the existence of heterogeneities concerning proliferation. This means that most of the cells progress through the cell cycle but a small fraction does not. The constancy of the distribution of the cells among the cell cycle phases at the later stage of the batch is likely to be caused by the presence of BrdU in the culture medium. To measure the influence of BrdU on the growth, the course of both the biomass accumulation and the online measurement of respiration of a BrdU-containing culture and a reference culture without BrdU was monitored. The respiration analysis showed that for the first 60 h the growth of the culture is not affected, while in the later course the activity of the cells was reduced. After 72 h the biomass accumulation is slowed down by the BrdU (5 + 10 μM). Consequently, the developed BrdU method successfully detected heterogeneities in the growth in the early stage of the cultivation, but the data after that period are influenced by the BrdU in the media. Various cultures react very differently to the BrdU as the investigations of Yanpaisan et al. demonstrate. In their study, the use of 10 μM BrdU claim growth to be unaffected on a *S. aviculare* cs [87]. The BrdU-detection via Hoechst quenching is perfectly suited for mechanically labile isolated nuclei. Especially for cultures with a high tolerance to BrdU, the Hoechst quenching is feasible to determine cell cycle heterogeneities in cultivations for the production of valuable metabolites.

This is of importance as processes frequently comprise of a growth stage and a production stage. The detection of heterogeneities allows modifying the operational parameter of the process in a way that biomass accumulation is promoted in the growth phase, while in the second phase the physiology of the cells may be directed into a way that is favorable for production. This is of special importance for the production of secondary metabolites as their synthesis often peaks in phases where the growth is limited.

The combination of flow cytometry with modern molecular biological methods seems promising. A dominant field of application of flow cytometry is the investigation of mammalian cells for medical research. Many protocols are developed for that purpose. One of these examples is the FUCCI technology which was introduced by Sakaue-Sawano et al. [103]. FUCCI is an acronym for “fluorescent ubiquitination-based cell-cycle indicator” [103, 104]. The proteins Cdt1 and Geminin are involved in the cell cycle regulation and their levels alternate throughout the cell cycle. Specific proteolysis is an important mechanism to regulate the levels of these proteins in the nucleus [105]. The FUCCI technology is based on the constitutively expression of these proteins fused to different fluorescent proteins:

mAG-Geminin(1/110) emits green and mKO2-Cdt1(30/120) emits red fluorescence [104]. Depending on the phase of the cell cycle, one of these fusion proteins is degraded by specific proteolysis, while the other is not. This allows the detection of the individual cell cycle phase by the color of the fluorescence. For example, nuclei of cells in the G_1 phase exhibit a red fluorescence as the green fluorescent mAG-Geminin(1/110) is degraded. Only if both are not degraded, as it happens in the early S phase, a yellow fluorescence is detected as result of both red and green fluorescence. This technology was further modified and optimized within the last years and up to now successfully applied to cells from mouse, human, zebrafish, fruitfly, and others [106]. The adaption of this technology to plant cells would be a great step forward in understanding cell cycle progress in plants. The advantage is that the fluorescence appears in the cell nuclei and fast nuclei extraction from cells and tissues allows simple and timely analysis by flow cytometry. Further, the FUCCI technology does not require the addition of potentially growth inhibiting agents like BrdU; however, the genetic modification potentially influences the growth kinetics and the cell cycle behavior. Currently the FUCCI technology is unable to distinguish between quiescent G_0 and active cycling G_1 cells [106]. Unfortunately, the adaption of the FUCCI technology to plant cells is not simple as the cell cycle regulation systems between plant and animal cells differ strongly. Cytrap, the "Cell Cycle Tracking in Plant Cells" based on the idea of FUCCI is an encouraging development into this direction [106, 107].

4.5 Product Formation and Productivity Related Measurements of Plant Cell Suspension Cultures by Flow Cytometry

Beside proliferation and growth, the productivity of the desired metabolites is another important parameter for developing plant cs-based bioprocesses. The possibility to acquire segregated data about the productivities of individual cells in the populations of a bioreactor would be a huge step forward in understanding the culture's behavior. Methods that allow detecting productivities may enable a purposeful development of highly productive processes, for example, by being able to correlate growth and productivity. Wilson et al. [92] recently discussed the problem of heterogeneities in plant cs and how new methods could help to solve these problems on intracellular (pathway), intercellular (aggregation), and extracellular (external environment) level that still hinder broad industrial application of such cultures.

As mentioned above, the product formation of secondary metabolites on single cell level can be very heterogeneous. Concerning the production not only the production rate of the cells is of interest. It turned out that also intracellular product storage capacity and degradation influence overall productivity of the culture [108]. The latter one especially concerns heterologous protein expression in plant in vitro cultures where unspecific protease may cleave the product in the cells [109]. Whether a product is stored in or secreted from the cell is mainly governed by cellular transporters. In some cases, transporter may even transport already

secreted product back into the cell and cause its intracellular accumulation. Recently, experiments to gain data about product transport and storage mechanisms on single cell level were performed with a *Taxus canadensis* suspension culture and the product paclitaxel (Taxol®). The cs consisting of aggregated cells and the protoplasts generated out of the cs were incubated with the fluorescent paclitaxel analog Flutax-2® in the medium [108]. The uptake kinetic of Flutax-2 was analyzed in both aggregated cells and protoplasts. The Flutax-2 in the cells was detected via flow cytometry. Protoplasts could be measured directly, while the detection of the aggregated cell suspension required the previous generation of protoplasts. From these data, it was concluded that Flutax-2 is appropriate to study the transport of paclitaxel and that the transport into cells is partly realized by specific transporters [108]. Deeper understanding of the active paclitaxel transport aids its manipulation in order to reduce the transport back to the cells and, therefore, to reduce feedback inhibition and support product secretion [108].

The possibility to monitor product synthesis on single cell level is described for a transformed *Nicotiana tabacum* cs [110]. In this culture, the heterologous M12 antibody was fused to a fluorescent DsRed protein. Protoplasts were generated from transformed calli, and based on the DsRed fluorescence intensity, highly productive single cells were sorted. The minimum cell concentration necessary for the proliferation of the single cells succeed using untransformed feeder cells. When the sorted single cells proliferated to a density high enough, the callus cells were transferred to selective media to kill untransformed feeder cells. This strategy for selection on single cell level resulted in a homogenously producing suspension culture exhibiting 13-fold higher productivity of monoclonal antibody compared to the original callus [110]. Further, the production proved to be stable over at least 1 year. This example shows how selection on single cell level using flow cytometry and cell sorting can help to optimize heterologous protein production and to decrease heterogeneities with respect to product formation in cs. The selection of high producing single cells in suspension cultures used to synthesize secondary metabolites is hampered by the fact that fluorescent products are rare.

For several years, also the “omics” investigations explore data on single cell level [111]. Different cell types of organs can have different expression and regulation patterns specific to their special role in the development and function of an organ. This may render the analysis of whole organs or tissues not representative as they are heterogeneous concerning cell types and reflect average physiology of all cells. In several studies, specific cell types of organs were labeled with fluorescent markers. After protoplast production, the cell types were sorted and investigated on single cell level for, for example, metabolite profiles [111] and transcription profiles or signal transduction [112]. Most of these investigations are related to basic research on plants but not to plant cell cultures as production platform. Using similar techniques, for example, to correlate the expression of target metabolites with fluorescent markers could support future selection processes as shown by Kirchhoff et al. [110] and have the potential to follow production kinetics during the cultivation process. However, the application of these technologies requires the knowledge of the pathways of the metabolite of interest including involved genes and promoters.

Often this knowledge is missing. Further, the analysis and sorting with flow cytometry again require the generation of single cells or protoplasts. The method of microbead encapsulation of living plant protoplasts [113] could support sorting and handling of fragile protoplasts and further analyses at single cell level, irrespective of whether protoplasts deriving from plants or plant in vitro cultures. Heterogeneity of plant cs still is an outstanding challenge but also bears the advantage that it allows selection.

5 *Green Bioprinting*: Novel Environment for Immobilization of Plant Cells

5.1 Immobilization of Plant Cells: New Perspectives by Using 3D-Bioprinting

Owing the specific properties of plant cells, that is, slow growth rates, cell aggregation, and high shear sensitivity [114, 115]; cell immobilization offers a set of options for physiological studies and the design of bioprocesses. In general, matrix materials for cell immobilization should be supportive of cell culture, nontoxic, and nonreactive to the cells, mechanically stable over the time of application, easy to fabricate and available in sufficient quantities [116]. Immobilization procedures that have already been applied to plant cells are categorized into (1) gel encapsulation, (2) surface immobilization, and (3) entrapment by membrane carriers whereas natural (e.g., alginate, agar, agarose, chitosan, k-carrageenan, or gelatin) or synthetic matrix materials (e.g., polyacrylamide, synthetic foams, polyesters, or fibers) have been used [117].

The interaction between immobilized cells and the surrounding matrix is complex since the matrix acts as a diffusion barrier which directly influences the cellular nutrient supply, promotes the cell aggregation, and can function as a mediator, inducer, or stress factor for the cells, respectively [118]. Besides studying the physiological impact and interaction of cells and matrix, immobilized cells can be further applied for several modes of process operation or bioreactors, for example, two-stage processes, which are separated into a growth and production phase [119].

It is well known that aggregation of plant cells is an important factor influencing the productivity. Therefore, cell immobilization as a promoter of aggregation has been intensively studied related to secondary metabolite production. Several authors proofed an increased secondary metabolite production of immobilized plant cells compared to the corresponding suspension cultures [120, 121].

A major drawback of the immobilization methods described so far is the nonaccessibility of locally and time resolved data on the cellular physiology and productivity simultaneously. Therefore, correlations between these parameters are not completely understood yet. Due to this fact, the authors are developing a novel agglomeration environment based on an Additive Manufacturing technology that is called *Green Bioprinting*. This term defines the computer-aided fabrication of

structured three-dimensional cell-laden immobilization matrices by 3D-bioprinting using cells from the plant ("green") kingdom.

Several Additive Manufacturing technologies are applicable for processing of hydrogel materials; however, extrusion-based technologies such as 3D-bioprinting are currently considered as the most suitable method for producing 3D-cell-laden constructs [122, 123]. The 3D-structure is generated by extruding a cell-laden highly viscous or pasty hydrogel material through a dosing needle. The movement of the dosing system, controlled by CAD/CAM software, results in deposition of material strands which finally build up the 3D-structure in a layer-by-layer fashion (Fig. 13). 3D-bioprinting enhances cell immobilization by creating geometrically defined and complex structures from a wide range of biomaterials. The complexity can be increased by using multichannel systems which allow the combination of either different materials or various cell types within the same structured matrix.

Although 3D-bioprinting has been used for mammalian cells in the field of Tissue Engineering for several years [125, 126] this technology has not been applied for biotechnological approaches yet. Using the *Green Bioprinting* approach, there are two levels which have to be considered with respect to their influence on the cell culture (Fig. 14). The macro level includes the architecture and construction of the whole cell-laden 3D construct – here, the macro pore size (Fig. 15) and geometry, the matrix accuracy according to the CAD model and the distribution of the plant cells play an important role. Metabolic parameters of the whole population like growth rate and productivity of secondary metabolites under different cultivation conditions (e.g., temperature and elicitors) are observed on the macro level.

The micro level includes cell-specific parameters, that is, cell aggregation characteristics, cellular viability, and morphology, respectively. Thanks to the high transparency of most hydrogel biomaterials, the micro level is accessible via fluorescence microscopy in combination with image analysis tools [127]. The high structural organization of the immobilized cells therefore enables the detection of time- and local-dependent information about the cellular physiology (Fig. 16). The cell-specific parameters were directly influenced by the material-specific parameters

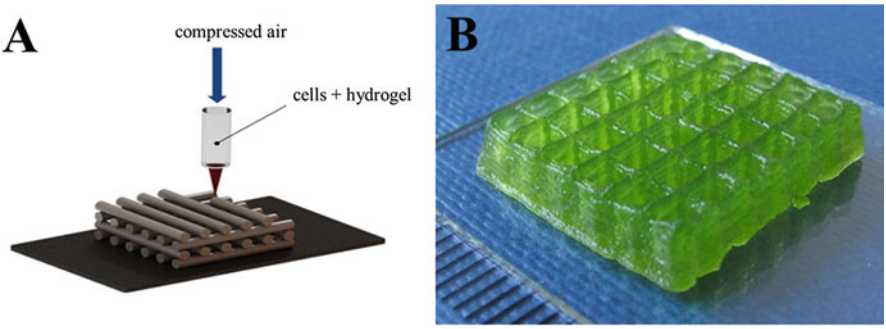

Fig. 13 *Green Bioprinting* – a novel immobilization method for cells from the plant kingdom (**a**): Principle of fabrication of cell-laden 3D constructs by extrusion-based 3D printing; (**b**): printed alginate-based hydrogel matrix, laden with microalgae [124]

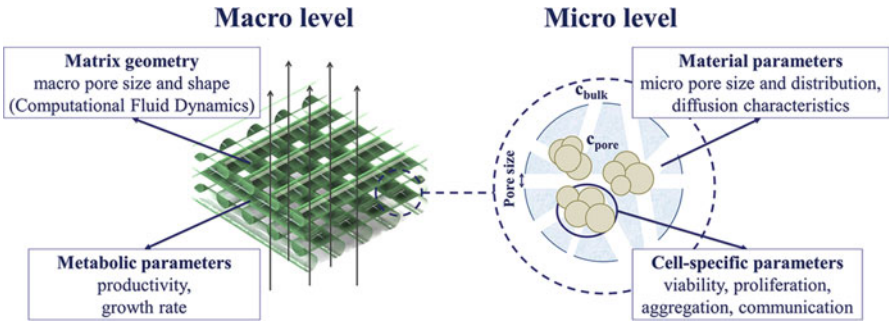

Fig. 14 *Green Bioprinting* – Macro and micro level parameters and their mutual dependencies

of the matrix (e.g., biocompatibility, permeability, micro pore size, and distribution). Mass transfer characteristics of the hydrogel are crucial for viability and productivity of the embedded cells. Diffusion through the hydrogel can be observed using several fluorescence labeled model substances, that is, fluorescein, fluorescein isothiocyanate–dextran of different molecular weight.

5.2 Challenges for Bioprinting with Plant Cells

Applied hydrogel materials should be suitable for both fabrication of 3D-matrices and cultivation of embedded cells. There are three main parameters, which should be considered for the design of a 3D-structured hydrogel environment for plant cells by *Green Bioprinting*: the material properties (e.g., viscosity, microporosity of the hydrogel matrix), the shear forces which the cells are exposed to during the printing process, and the cell-type-dependent biocompatibility of the final hydrogel matrix. Figure 17 gives an overview on the challenges, which are associated with the development of a suitable hydrogel material and a compatible fabrication strategy for the 3D-bioprinting process. Hydrogels like alginate, gellan gum, agarose, and gelatin in low concentrations (0.5–3% by weight) enable growth of embedded plant cells but not the generation of highly accurate 3D-structures according to a predefined CAD model. An increase of the polymer concentration results in facilitated printing characteristics but also has a nonnegligible impact on the mass transport characteristics and cellular physiology. As described by Malda et al. for 3D-bioprinting with mammalian cells, commonly applied immobilization approaches use a compromise solution between the needs of fabrication and those of cellular physiology. New strategies have to be developed avoiding a high polymer concentration during cultivation while maintaining the desired matrix shape [122].

Schütz et al. described such a new method: blending of a low-concentrated alginate with methyl cellulose leads to a temporary increase of the biopolymer concentration during printing, allowing the fabrication of 3D constructs with high accuracy and shape fidelity [128]. After the printing process, the alginate chains are

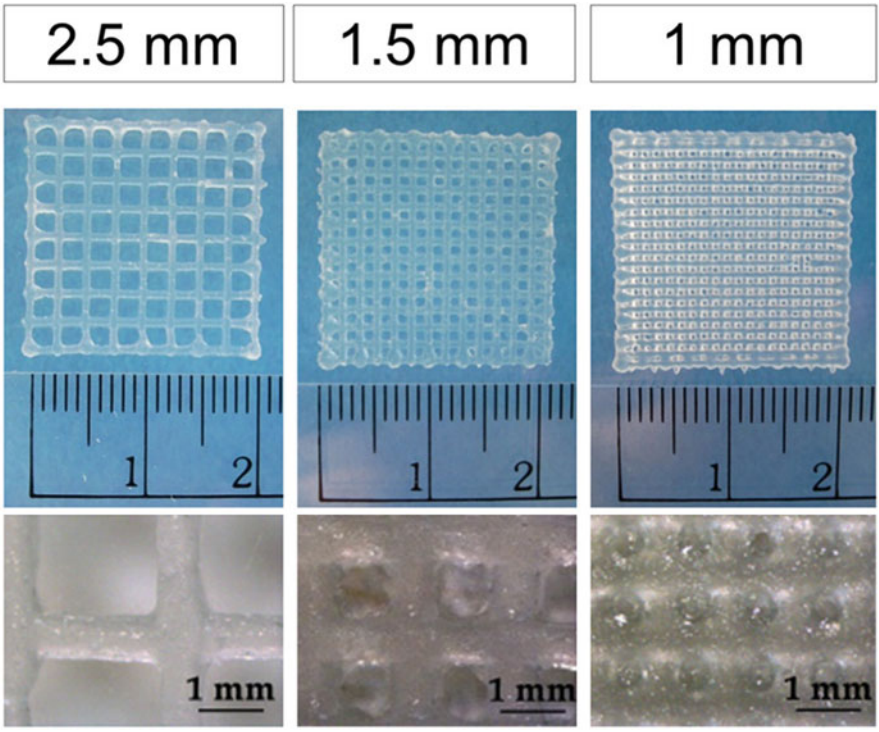

Fig. 15 3D-printed alginate-based hydrogel matrices; variation of the macro pore size by using different strand distances (length from the center line of one strand to those of the neighboring strand; Pictures are kindly provided by K. Schütz)

crosslinked with Ca^{2+} ions, but the methyl cellulose is washed out creating micro pores within the hydrogel matrix (Fig. 18) which enhances the mass transfer into the gel.

As proven by the authors [124] for the microalgae strain *Chlamydomonas reinhardtii* 11–32b, 3D-bioprinting with the alginate/methyl cellulose blend is also applicable for the immobilization of green microalgae (Fig. 16). Monitoring of the cellular viability by fluorescence microscopy and an image analysis indicated that the shear forces during the 3D-printing process had no negative effect on the viability of the cells [127]. The algal cells were homogenously distributed in the hydrogel environment by the printing process. At cultivation conditions promoting the photoautotrophic growth of *C. reinhardtii* 11-32b, the number of cells increased over 12 days of cultivation. Additionally, the immobilized cells formed highly viable and photosynthetic active cell clusters (30–40 μm), which was evidenced by fluorescence microscopy using the autofluorescence of chlorophyll and measuring the increase of oxygen concentration in the surrounding medium, respectively.

First experiments by the authors with an *Ocimum basilicum* (*O. basilicum,* basil) in vitro suspension culture provided the proof-of-concept for plant cell bioprinting. The plant cells were embedded in a Gellan Gum matrix by 3D-bioprinting and cell

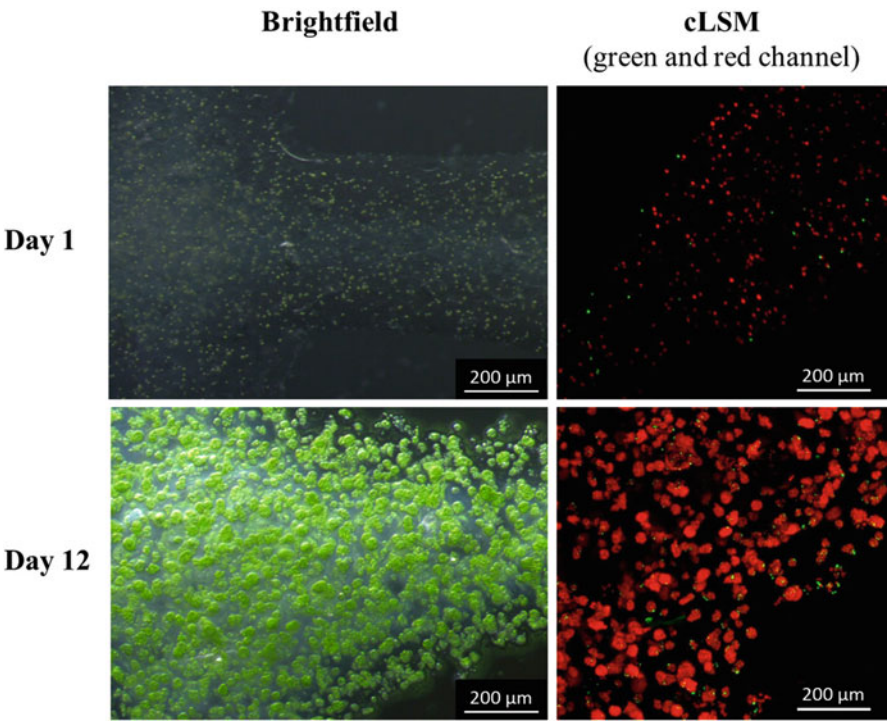

Fig. 16 Investigation of cellular properties at the micro level: viability and proliferation of microalgae *Chlamydomonas reinhardtii* in 3D-printed alginate-based hydrogel; *left*: brightfield images at day 1 and 12 shows the proliferation of the cells; *right*: cLSM (confocal laser scanning microscopy) analysis: live/dead imaging at days 1 and 12 shows viability of the cells: red – life cells (autofluorescence of chlorophyll), green – dead cells (stained by SYTOX® green)

viability was tested over a culture period of 14 days (Fig. 19). Our observations indicate that the cells are able to grow within the matrix. Although the shape fidelity of these matrices were not sufficient for printing of defined and complex 3D-constructs, it has been demonstrated that the plant cells in principle are appropriate candidates for *Green Bioprinting*.

Embedding *O. basilicum* cells in the microporous and well-shaped hydrogel (alginate/methyl cellulose) previously used for microalgae was not successful. Already after 1 day of cultivation no viable cells were detected. To meet the requirements of plant cells for 3D-bioprinting, further investigations and adaptions of the fabrication process, material, and process conditions are necessary. A hydrogel blend with optimal fabrication characteristics and a feasible environment for plant cells has to be designed, which means the development of new strategies for 3D-bioprinting as explained above (see Fig. 17).

To sum it up *Green Bioprinting* as a completely new technology has the potential to lead to better monitored and highly adaptable bioprocesses in order to optimize the productivity of the cell culture.

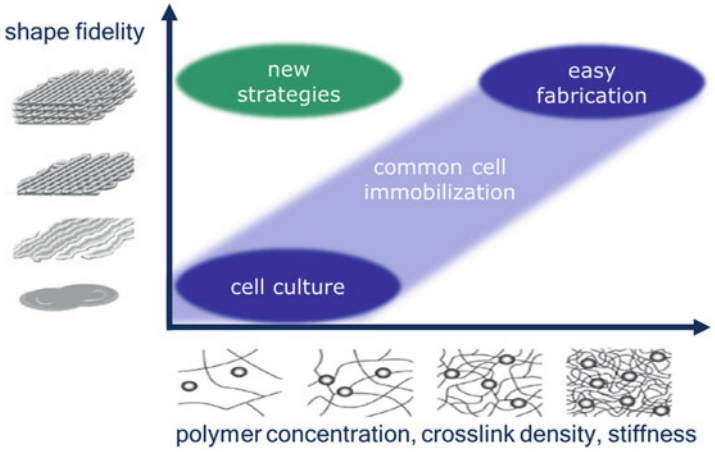

Fig. 17 New strategies should achieve good shape fidelity with a low polymer concentration supporting cell culture within the matrix (Adapted from Malda et al. [122])

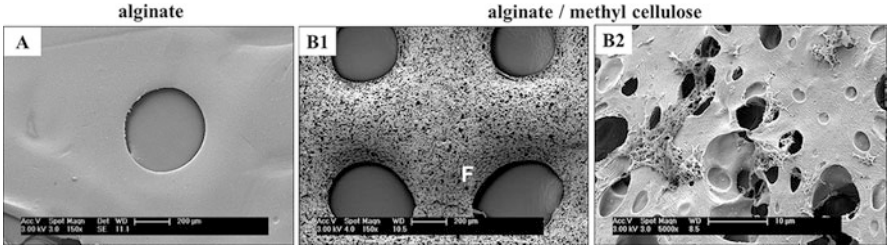

Fig. 18 Scanning electron microscope (SEM) analysis of alginate-based hydrogels: Pictures of the hydrogel surface after crosslinking and 1 day lasted incubation in culture media (**A**) alginate (without methyl cellulose), (**B**) alginate with methyl cellulose (Pictures are kindly provided by K. Schütz)

6 Conclusions

Plants produce a wide range of interesting secondary metabolites, which are used as natural pigments and flavoring agents in foods and cosmetics as well as phytopharmaceutical products. The biotechnological production of plant secondary metabolites allows a year-round production with constant quality and quantity. However, the design, optimization, and control of economic processes presuppose knowledge about the physiological state of the biological system and the kinetic parameters of biomass and product formulation. Most of the common methods for the determination of the biomass concentration are based on optical interactions. Because of their special morphological characteristics, these methods are often not applicable to plant

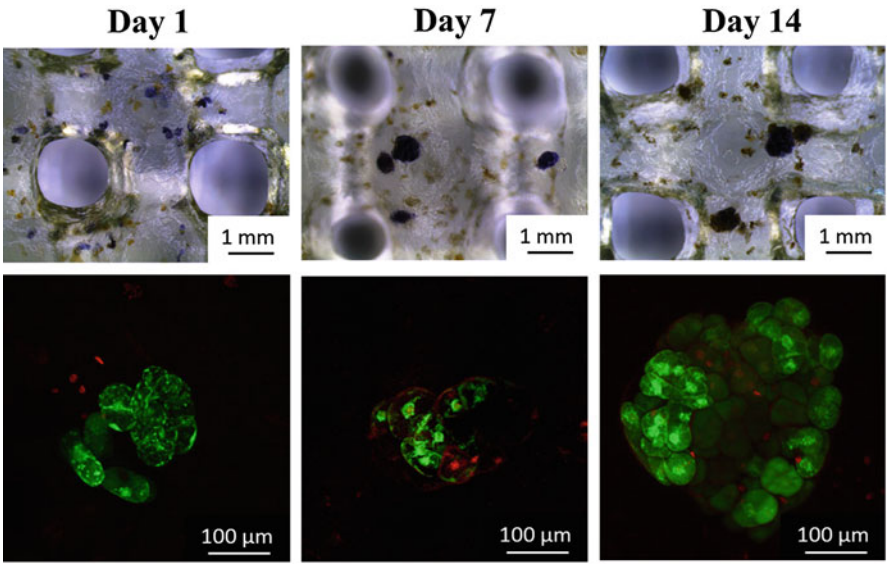

Fig. 19 Viability analysis of an *O. basilicum* cell culture embedded in 3D-printed Gellan Gum; on *top*: assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), purple – living cell agglomerates (MTT reduced to formazan), *below*: live/dead staining of single agglomerates, green – living cells (stained with fluorescein diacetate), red – dead cells (stained with propidium iodide)

in vitro cultures. The electrical conductivity is an appropriate parameter for the monitoring of biomass growth. The uptake of ions from the nutrient medium in the cells results in a decreasing conductivity of the medium. After providing an evidence of linear correlation between changes in the biomass concentration and the conductivity, the growth behavior of the plant cell and tissue cultures can be predicted via the change of conductivity. This makes the conductivity a beneficial parameter to estimate the biomass growth of plant cell and tissue cultures, which is nondestructive and independent from scales. The linear dependency and correlation offers the opportunity to track the biomass progress. For bioreactors starting from laboratory scale, the application of conductivity probes is well established. But in the case of mass screening systems such as shaking flasks, there is no feasible solution to determine the conductivity online, sterile, and continuously without restrictions, yet.

The Respiration Activity MOnitoring System (RAMOS®) represents a suitable alternative. This system enables the simultaneous, not invasive detection of respiration activity of pro- and eukaryotic microorganisms. By coupling with a gas mixing device and/or a feed module, the RAMOS® futhermore offers the possibility to realize different process strategies. Thus, this monitoring system combines the advantages of conventional cultivation in shake flasks (small-scale, parallelized) and instrumented bioreactors. With the presented RAMOS®-CultiLux setup is it also possible to investigate light-dependent parameters like growth rate, and

metabolic interrelations like pigment content, to optimize the cultivation of photosynthetically active cell and tissue cultures. Therefore, the RAMOS proved to be an advanced tool for fast and parallel screening of plant in vitro cultures. Nevertheless, the relationship between measured respiration activity and evaporation was revealed as important factor in the long cultivation period of plant cells and has to be considered in applying the equations to determine the growth-specific parameter.

The major drawback of classical process analysis is that it relies on bulk measurements in the environment of the cells giving average information of the whole culture in the bioreactor only. Not only due to their complex morphology plant cells in cs or hr are heterogeneous, for example, to their access to metabolites and oxygen, their subjection to shear stress, and intercellular communication intensity [99]. Flow cytometry is a technology that measures on single cell level and, hence, allows to resolve these heterogeneities in the culture or in other words it provides segregated data. This technology enables the measurement of DNA-related parameters like genome size, ploidy, as well as polysomaty. These are meaningful data that support the screening and selection process in plant breeding, micropropagation, and establishment of plant in vitro cultures. The aims are different; in plant micropropagation unchanged ploidy is favorable, whereas in breeding and plant cs increased ploidy can be desirable to get improved phenotypes. There are different methods available for flow cytometry to follow the cell cycle activity on single cell level and to determine the fraction of cycling (proliferating) and noncycling (nonproliferating) cells in cultivations of plant cs. Assuming that cycling cells contribute to DW increase, while noncycling cells stronger contribute to formation of secondary metabolites, this knowledge helps to search for parameters which influence the different fractions and to optimize growth and production conditions. To some extend the product formation especially of proteins and the transport of fluorescent products can be monitored in plant cs by flow cytometry as well. Flow cytometry can be applied in many aspects of the process development, for example, the screening and establishment of plant cs and the monitoring of heterogeneities of growth and to determine differences in the contribution of the individual cells to the productivity of the whole culture.

Owing the specific properties of plant cells – that is, slow growth rates, cell aggregation, and high shear sensitivity – cell immobilization offers a set of options for physiological studies and the design of bioprocesses. The matrix acts, for example, as a diffusion barrier which directly influences the cellular nutrient supply, promotes the cell aggregation, and can function as a mediator, inducer, or stress factor for the cells, respectively. Immobilized cells can be further applied for several modes of process operation or bioreactors, for example, two-stage processes, which are separated into a growth and production phase. Furthermore, immobilized plant cells show often an increased secondary metabolite production compared to the corresponding suspension cultures. A major drawback of the immobilization methods described so far is the nonaccessibility of locally and time resolved data on the cellular physiology and productivity simultaneously as well as the reduced mass transport through the immobilization matrix. The innovative manufacturing technology of *Green Bioprinting* defines the computer-aided fabrication of

structured three-dimensional cell-laden immobilization matrices by 3D-printing using plant cells. The immobilization of plant cells in these well-defined 3D-structures provides time and location-resolved information about the physiological state of the individual cells and agglomerates. Additionally, the high porosity of the immobilization matrix produced by bioprinting reduces mass transport limitations compared to common immobilization strategies. Therefore, *Green Bioprinting* has also the potential to lead to better monitored and highly adaptable bioprocesses in order to optimize the productivity of the cell culture.

In last times, great efforts have been made, to develop suitable methods for monitoring of bioprocesses using plant in vitro cultures. The potential of some techniques was described and existing challenges were highlighted in this chapter. The focus of future research should lie, inter alia, on the development of more sensitive online methods for the screening of plant cell and tissue cultures even on a smaller scale. In this way, the long times in process development can be significantly shortened and existing processes can be further optimized.

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Bioreactor Technology for Hairy Roots Cultivation

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Abstract

Bioreactor technology is an integral requisite to the development of scale-up production process of many plant-based high-value products. The proper selection and design of the bioreactor is required to determine the optimal industrial scale bioprocess and the subsequent capital investment. A primary cause of the lack of success in commercial production of secondary compounds using hairy root culture systems is their low yield. To increase the production of hairy root-based bioactive compounds, several strategies, like elicitation, metabolic engineering, and up-scaling, etc., have been adopted. Out of these strategies, the up-scaling in bioreactor deals with the principle of large-scale metabolite production in proportion of high biomass growth. The goal of an effective bioreactor is to control, contain, and positively influence a biological reaction in an incessant way in order to get desired productivity. This chapter provides a descriptive account on up-scaling of hairy root cultures for various purposes including secondary metabolite production. This chapter also discusses the hitherto reports on up-scaling of hairy root cultures of various plant species in terms of modifications in designing of bioreactors for incessant tissue growth concomitantly with metabolite production.

Keywords

Agrobacterium rhizogenes · Hairy roots · Bioreactor designing · Fermentor · Scale-up · Secondary metabolites · Root clump · Mass transfer

Abbreviations

ALR	Air lift reactors
BCR	Bubble column reactors
HRCs	Hairy root cultures
NMR	Nutrient mist reactor
STR	Stirred tank

1 Introduction

Biotechnological intercession has triggered tremendous interest in utilizing in vitro culture systems for the production of a variety of promising phytochemicals [1–4]. Majority of phytochemicals are plant secondary metabolites which are commercially important in the form of pharmaceuticals, nutraceuticals, flavors, essential oils, food additives, feed stocks, and antimicrobials. Till date, several biotechnological strategies have been adopted to produce these bioactive secondary metabolites by establishing in vitro cell and organ cultures [5–8]. However, in general, when compared to the intact plants, the production of these secondary metabolites remains at lower side in usual lab-scale cell and organ cultures. Alternatively, scale-up of an in vitro culture by replicating a lab-scale bioprocess in larger culture vessels as closely as possible to produce larger amounts of product may provide a solution for large-scale

production of desired compounds [4, 9]. In vitro cell and organ cultures growing in liquid medium have a unique blend of physical and chemical culture environment and thus easily extend their use in up-scaling in bioreactors. In the same context, a bioreactor is a mechanical device that can simulate particular physio-chemical environment required for incessant growth and physiological activities of cultured cells and tissues. Besides increased working volumes, the semi or fully automated control system and round the year production efficiency that are independent of seasonal or climatic barriers are the additional advantages of up-scaling of an in vitro culture using bioreactors [10, 11]. However, the growth behavior of a biological entity not only depends upon their morphological and physiological status but also influenced by their physical environment. Therefore, successful operation of these bioreactors involve skillful implementation of engineering principles of designing the culture vessels in a way that can significantly influence the growth of cultured tissue at larger volumes [12]. The prime focus during the designing of bioreactor should be on adequate mixing of culture medium with minimized shear stress and optimized mass transfer with reduced hydrodynamic pressure.

Besides microbial cultures, the use of bioreactors has largely been focused on plant cell suspensions and hairy root cultures (HRCs) for secondary metabolite production [13]. Various reports on large-scale culture of cell suspension and hairy roots in various types of bioreactors are available [14–17]. However, genetic instability of cells and their higher sensitivity toward variability in culture conditions limit the broader use of cell suspension cultures for desired metabolite production. Further, many a times, the metabolite production is restricted to differentiated cell/tissues that too at their certain physiological age, and thus, in most cases, culture aging leads to consequent reduced production of desired metabolites. Therefore, keeping in mind the vast potential of HRCs for secondary metabolite production, the up-scaling of these cultures has shown striking opportunities in commercializing a bioprocess for desired metabolite production [18–20]. In up-scaling of hairy root-based production process, there are various factors which greatly influence the production. These include selection of reactor, optimization of culture conditions, automation and interpretation of synergistic effects of various conditions, etc., which needs judicious scientific attention. The upcoming text provides a descriptive account on bioreactor technology for up-scaling of HRCs for secondary metabolite production. The chapter also discuss the hitherto reports on up-scaling of HRCs of various plant species in terms of modifications in designing of bioreactors for incessant tissue growth concomitantly with metabolite production.

2 Hairy Roots as Potential Secondary Metabolite Production System

In the past three decades, hairy roots and secondary metabolite production became synonymous, and plentiful reports have came into existence which reveals various aspects of growth and production potential of HRCs [3, 20–24]. Hairy roots are disease expression of plants that are infected by soil bacterium *Agrobacterium rhizogenes*. The

etiology behind the disease symptoms describes the stable insertion of bacterial T-DNA from extrachromosomal root-inducing (Ri) plasmid into the host plant genome and a consequent possible interruption in host secondary metabolism [25, 26].

Hairy roots being an easy "establish and explore" low-cost culture obligation have progressively materialized into full-fledged global technology for plant-based secondary metabolite production. Miscellaneous beneficial properties like multi-enzyme biosynthetic paraphernalia and close physiological and biochemical similarity with parent plant contribute to the preferential lead for choosing the genetic and biochemically stable HRCs over other in vitro systems for the production of variety of phytochemicals that is commercially important [21, 27]. In addition to such exclusive properties, hairy roots are also unique as in some cases they produce compounds that are not known in normal roots. The examples of production of glycoside conjugates of flavonoids in HRCs of *Scutellaria baicalensis* Georgi and sarpagine alkaloids from *Rauwolfia serpentina* are relevant to mention here [28]. Normal roots of *S. baicalensis* are known to produce glucose conjugates only. Additionally, sometimes HRCs are also known to produce compounds in higher quantities as compared to those that are present in normal intact roots [28].

A primary cause of the lack of in commercial production of secondary compounds using HR culture systems is their low yield. Several strategies, like elicitation, metabolic engineering, etc., that manipulate inherent property of hairy root tissues to enhance their production potential have been adopted to increase the production of bioactive compounds utilizing HRCs [20, 29–31]. Out of these strategies, the bioreactor up-scaling deals with the principle of large-scale metabolite production in proportion of high biomass growth. Succinctly, higher biomass means higher metabolite production [32]. Thus, keeping in mind high biomass production as the main objective, various types of bioreactors have been designed for the cultivation of hairy roots [32, 33]. A continuous culture in bioreactor can sort out the problems related to the manual handling of cultures and thus rescue the growing tissues from possible microbial infection that occur during regular subculturing. All kinds of bioreactors have heterogenous systems comprising of two or more phases of liquid, solid, and/or gaseous phase. The major confront during the operation is to maintain a homogenous condition which assures optimized mass and heat transfer among the different phases [34]. To deal with the situation, a typical bioreactor consists of various probes and devices to monitor and maintain different physical (temperature, flow rates and turbidity etc.) and chemical (dissolve oxygen, pH) conditions (Fig. 1). The following text discusses the challenges during the maintenance of optimized culture conditions for hairy root growth.

3 Major Challenges in Up-Scaling of Hairy Roots in Bioreactors

Although, HRCs have shown tremendous possibilities in secondary metabolite production at large scale; at the same time, there are certain challenges in using bioreactors for their up-scaling [34, 35]. Normally, a typical scale-up that starts in laboratory includes jars or shake flasks of 50–250 ml which further moves to

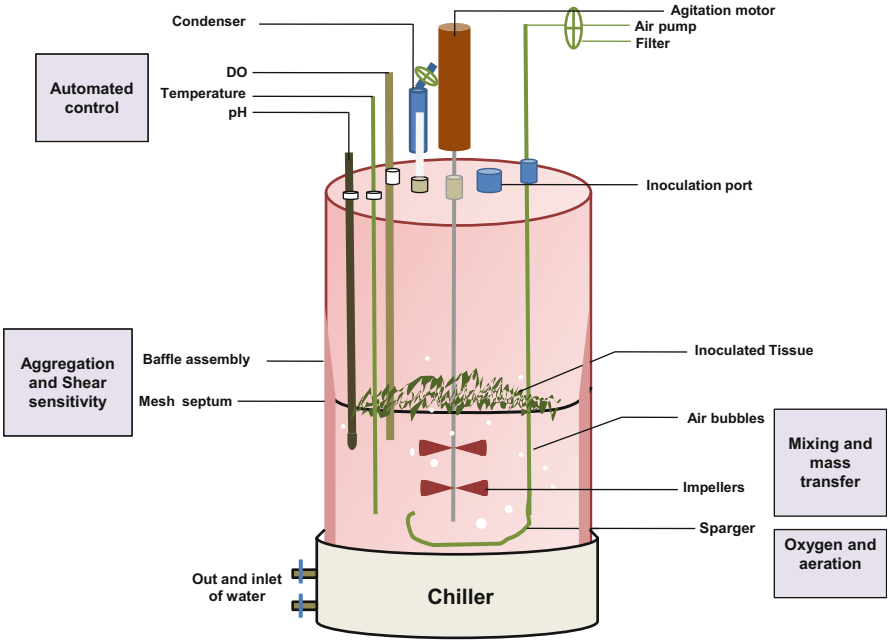

Fig. 1 Schematic presentation of bioreactor configuration

500 ml–10 L small-scale bioreactor. However, for large or industrial scale, stainless steel vessels of varying sizes (>10 L) are required (Fig. 2). It is well known that before going for up-scaling of any culture, optimization of process at bench scale is a prerequisite. However, at this stage, it is relevant to state that the results of bench-scale optimization cannot be directly transferable to higher scales. This is because a large vessel bioreactor may provide totally different culture conditions, and with the increase of vessel size, the homogenous culture ambience remains no longer effective. At this stage various physical factors like gas and liquid flow rates, mass transfer rate, concentration gradients, etc., start affecting the tissue growth simultaneously. These challenges are scientifically handled with the use of proper bioreactor technology that deals with the utilization of engineering principles and mathematical formulations to optimize a bioprocess [20, 36]. At laboratory bench scale, one can only check the practical feasibility of up-scaling of bioprocess in terms of desired productivity and production cost at industrial scale [35]. Various physical and chemical aspects during up-scaling of HRCs that are needed to be judiciously attended are described in upcoming text.

3.1 Aeration, Agitation, and Mixing

The O_2 supplementation in reactor vessel is determine by the air flow supply. The basic objective of continuous O_2 supplementation is to perform all biological

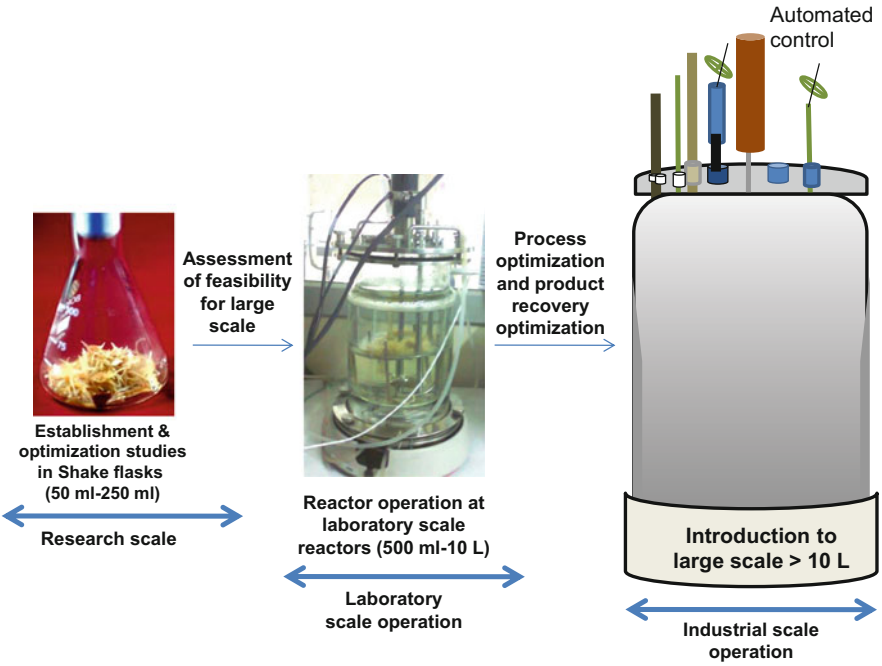

Fig. 2 Gradual process scale-up of culturing hairy roots

activities by growing root tissue, while agitation, on the other hand, assures the homogeneity of liquid medium [37]. The aeration system consists of sparger, while for mass/heat transfer and uniform air distribution, agitator or impellors are required. Keeping in mind the shear sensitivity and bubble tolerance of growing tissue, normally, stainless steel spargers are used to aerate the bioreactor vessel [38, 39]. However, the bubble size and distribution of bubbles within in the liquid significantly influence the gas holdup and unit time for gaseous exchange or mass transfer by the cells in contact. Thus, a judicious attention to the bubble size and distribution improves the understanding of cell behavior under hydrodynamic pressure and mass transfer phenomenon [40]. For this purpose different types of spargers (porous, orifice, etc.) in combination with different types of impellers (turbine or marine blade, propellers, etc.) are used [41]. This designing of reactor vessel depends upon the morphology and growth pattern of hairy roots to be grown. One more point which needs attention during the selection of vessel design is vortex formation. To prevent vortex formation due to continuous mixing, baffles or metal strips attached on radial sides of vessel are used (Fig. 1). In an effort to develop a bioreactor for enhancing the biomass yield of plant organs, the HRCs of *Hyoscyamus muticus* were grown in a 5 L capacity bioreactor of which culture vessel was provided with an elongated baffle assembly [42]. The baffle not only prevented vortex formation but also facilitated rotary transport of radial flow of aqueous medium in the vessel.

Similar reactor was later on successfully used to enhance the biomass yield of HRCs of *Picrorhiza kurroa* [43] HRCs, and multiple shoot cultures of *Glycyrrhiza glabra* [44, 45].

3.2 Root Morphology and Clump Formation

Hairy root cultures of different plant species exhibit differential morphology. Furthermore, the interclonal morphological difference is also significant in hairy root clones of a plant [46]. The randomness of insertion sites of T-DNA into host genome and the number of copies inserted collectively contribute to these interclonal morphological differences. The unique feature of hairy root growth is that they readily form clumps having densely packed rigid central core surrounded by loose highly entangled peripheral root mass [14, 47–49]. The dense mass of root clumps endorses bubble coalescing and channeling, poor liquid mixing, and ultimately the localized liquid stagnation. This hampers the continuous air and nutrient flow to the tissues growing toward the center by the resistance provided by peripheral root mass. This creates a phase difference between liquid (medium) and solid (tissue clump) components of growth matrix. The poor localized intraclump penetration of liquid flow greatly impose nutritional and concentration gradients near and within the clump [50, 51]. Such deficiency may lead to the cell necrosis and death at the clump center. These kinds of limitations can be overcome by applying controlled agitation rate and aeration with appropriate impellers and spargers, respectively [52]. For example, porous polypropylene membrane tubing as a supplementary aeration device was used to directly deliver the O_2 to *Atropa belladonna* hairy root clumps. This was found helpful in overcoming mass transfer resistance related with insufficient intraclump penetration of liquid current and improved the biomass production by 32–65 % higher than sparging only of air [53]. Earlier, in similar context of analyzing the effects of oxygen limitations on *Atropa belladonna* hairy root growth in 250 mL shake flask cultures, oxygen limitations are likely to affect biomass production and kinetic measurements in shake flask cultures of hairy roots [54]. These kinds of optimization and investigation studies at flask level paved the way for acquiring novel strategies for site-directed oxygen delivery into the zones of highest root density in large culture vessels of reactor.

The presence of root hairs also plays an important role in increasing mass flow resistance. Studies reveal that root hairs which improve nutrient uptake under natural conditions are observed unfavorable in liquid medium as they produce resistance to fluid flow and limit the O_2 supply to entangled root masses. In bioreactor cultivation of *H. muticus* transformed root cultures, the root hairs were found as substantial contributor of high fluid flow resistance [55]. To deal with such morphological constraints, some studies proposed the idea of growing hairless roots. In such studies either a root hair mutant line was selected or the root hairs in a normal hairy root clone were chemically removed. In an experimental system, hairy and hairless transformed roots of *H. muticus* were developed to assess the inhibitory role of root hairs in mass transport. Hairless lines of the test plant were initiated using

pyrene butyric acid (PBA) and phosphate. The mixing was significantly enhanced in hairless root cultures grown in a 15 L bubble column reactor. The growth rate of the hairless culture in the bioreactor was as much as 2.4 times greater than growth of the hairy culture under similar conditions. The improved reactor performance was reflected in greater biomass accumulation and respiratory activity. These results show that the root hairs – which facilitate nutrient uptake in a static soil environment – are detrimental to growth in a liquid environment due to their involvement in stagnating fluid flow and limiting oxygen [56, 57].

3.3 Shear Stress and Hydrodynamic Pressure

Effects of hydrodynamic stress on suspended cells and tissues have been the matter of investigation since the beginning of reactor technology. Shear stress is the condition which reflects the extra burden on cell caused by continuous agitation of medium, distribution and fragmentation of gas bubbles due to the stirrer, and bubble rupture at the liquid surface. Hairy roots are also sensitive to hydrodynamic shear. However, no generalized strategy to minimize the shear stress has been concluded as shear stress on hairy roots of a plant species is supposed to be collectively governed by certain factors like tissue morphology, age of culture, aeration and agitation speed, viscosity of liquid medium, etc. Therefore, not only the designing of reactor vessel but also the selection of suitable root line and composition of medium may reduce the shear stress during culture. Although various practical strategies to minimize the shear stress and improve shear tolerance have been discussed for up-scaling of animal cells, for HRCs this aspect has been less explored [58, 59]. Furthermore, mathematical formulations have also been explored to calculate and optimize the shear rate in a continuously operating vessel. Nevertheless, for HRCs and their up-scaling in bioreactors, such studies are sparse and yet to be explored [60].

3.4 Mass Transfer

In multiphase system of bioreactor vessel, mass transfer takes place over phase boundaries. Computational analysis of this mass flux enables to assess the mass distribution in different phases over time and space in the system. The purpose of such computational analysis is to understand the growth behavior of roots and further design as well as control the process [61–63]. The efficient mass transfer between roots and their growth environment may define the growth characteristics of roots. In a case study of *Tagetes patula* hairy roots, methodical testing was performed in order to determine the relative significance of gas-liquid and solid-liquid mass transfer [64]. In shake flask cultures, the biomass growth largely depends upon the volume of medium and shaker speed. Taking an insight, this dependency of root growth indicates the importance of mass transfer in relation to different volumes of culture medium and shaker speed. Such calculation for a specific hairy root line

also makes the basis for the selection of suitable bioreactor for up-scaling of that root line.

The gradient in chemical composition of phase boundaries, which occur due to continuous utilization of O_2 by rapidly growing tissue, is responsible for the driving force for mass transfer. Several studies have been performed to investigate the O_2 requirement and mass transfer phenomenon in HRCs [21, 54, 65]. Taking *A. belladonna* as model hairy root culture, convection was found to be the dominant mechanism for mass transfer in dense areas of root growth [65]. The mass transfer phenomenon was observed through local dissolve O_2 level and rate of O_2 uptake at the vessel areas where roots are loosely and/or densely packed in clumps. Specific growth rate and an exponential increase in root growth in terms of root length and number of growing root tips were observed at oxygen tensions between 70 % and 100 % air saturation. As the O_2 supplementation decreases by 50 %, the root growth got negatively affected specially in the areas where root tissues have formed dense clumps. Due to the dense entangled mass, the air saturation at such points decreased by half or even low when compared to the areas where roots are loosely packed [65]. Such results indicated mass transfer resistances near the gas-liquid and liquid-solid boundary layer which dominantly affect the oxygen delivery to the growing hairy roots.

In the past few years, computational and mathematical simulations have been used to model the mass transfer behavior in bioreactors for hairy root growth [66, 67]. In a study, a computational fluid dynamics (CFD) model was developed to simulate the hydrodynamics and oxygen mass transfer in hairy roots growing in bioreactor. The CFD model predicted increase in mass transfer rates in root clumps that are stimulated by ultrasound. The model predictions were validated through experimental results in which increased O_2 transfer was observed. This increased O_2 was correlated with increased membrane permeability of root tissues by ultrasound stress [67]. Similarly, in another study a mathematical model was developed to investigate O_2 transfer kinetics in *Azadirachta indica* hairy roots [68]. The model simulates and predicted the onset of O_2 transfer limitation in dense intraclonal areas which eventually indicated the need of increased O_2 supply to prevent the subsequent inhibition in growth of the hairy root biomass due to oxygen transfer (diffusional) limitation. Thus, computational/mathematical simulations of mass transfer phenomenon in a hairy root-based bioprocess may help in monitoring and controlling the process.

3.5 Optimization of Process: Medium Components, Temperature, Light, pH, and Inoculation

Chemical composition of nutrient medium directly influences the biomass yield and productivity of metabolites in HRCs. Therefore, studies on the effect of preferred key medium components on tissue growth, as well as product accumulation for incessant productivity, are a primary requisite. Further, this is also crucial to estimate secondary metabolite production as conditions suitable for growth may sometimes adversely affect the product formation and vice versa. Besides the conventional

method of growth medium optimization which includes the alteration in various components individually and observing their effect on tissue growth, nowadays for the optimization of various growth conditions, the computational modeling is observed as a flexible strategy [69, 70]. Recently, the use of artificial neural networks (ANN) alone or in combination with other mathematical concepts was proposed to optimize various physical and chemical culture conditions for desired productivity in HRCs [70]. These studies pave the way for comparisons and simulations of similar bioprocess at larger levels involving bioreactors. With particular reference to the scale-up cultures using large reactor vessels, the computational simulations of growth under varying chemical conditions provide results mostly near to accuracy. Besides, this also helps to overcome the cost and time overflow of the conventional optimization. Spatial variations in culture temperature and medium pH lead to variations in physiological performance of the cultured roots. Continuous effect of light and temperature variation on growth and metabolite production has been observed in hairy roots of *Catharanthus roseus*, *Artemisia annua*, *Echinacea purpurea*, and *Panax ginseng* [71–75]. In *P. ginseng* hairy roots growing in 5 L bioreactor setup, biomass accumulation and ginsenoside production were found highest under red light fluorescence [75]. Therefore, optimization of the physical parameters for best results of hairy root up-scaling in bioreactors is as important as other factors. Though rather much explored in microalgae culture, the present hype in hairy root biotechnology has shown the viewpoint of using photobioreactors for large-scale culture of light-sensitive roots [76]. In case of light-sensitive roots, formation of clump leads to uneven distribution of light. Therefore, the basic principles for developing a reactor for such root cultures must include efficient and quantitative understanding of both mass and light transfer. On the other hand, the temperature of the liquid medium can easily be adjusted by circulating water in a chiller jacket outside the glass vessel.

Another aspect of process optimization that requires proper attention is the inoculation of tissue for scale-up. This not only includes optimization of inoculum density-media volume ratio and the selection of explant material of desired physiological age but also the aseptic procedure through which transfer of tissue from smaller culture container to reactor culture vessel is done. The former is done by subculturing the hairy roots in shake flasks and allowing them to grow till their logarithmic/exponential phase begins. At the onset of exponential phase, the root tissues that are well adapted to fast growth are aseptically transferred to the reactor vessel through the inoculation port of the bioreactor. Expert handling and incessant maintenance of aseptic conditions are the prerequisites of the inoculation procedure which is done under sterile laminar hood. Sometimes, as an extra precautionary measure during inoculation, the inoculation port is optimally flamed with the sterile cotton swabs soaked in alcohol [43]. Further, in an experiment, alginate-encapsulated *R. serpentina* hairy root tips were used as inoculum for the reactor vessel (unpublished results). This made the inoculation procedure a bit easier as simple pouring of small amount of liquid medium containing beads was required rather than transferring the whole root tissue using sterile forceps. However, pragmatic results of a detailed study on growth performance of alginate-encapsulated hairy root tips of

R. serpentina were behind the idea of using encapsulated tips as inoculum of bioreactor [46]. Thus, a prior knowledge of growth performance of alginate hairy root tips of other plants is required before using them as explants for inoculating the bioreactor.

4 Overcoming the Challenges: Designing of Bioreactor for Hairy Root Cultures

By definition, a bioreactor is a culture vessel used for any biological conversion in terms of cellular growth and related productivity. These conversions are either in the form of cells/tissues/organisms cultured in a set of defined conditions or chemical compounds that are converted or transformed through specific metabolic reactions by mediation of a biological entity. The only difference between bioreactors and conventional chemical reactors is that the former is specifically used to culture biological entities. Another term that is used parallel to bioreactor is "fermenter" which is strictly used for anaerobic processes. Bioreactor designing is an engineering practice which inroads to a field that is known only for biology-based phenomenon. Thus, bioreactor designing characterizes an amalgamation of engineering principles of designing and analysis into growth and production processes of biological entities. The key issue in designing and operation of bioreactor is to control a biochemical phenomenon for a defined period in a consistent optimized way to get incessant maximum productivity. This optimization can be done on two scales; first, the biological entity and its products which include physiological basis of cell line/clone selection, metabolite synthesis, and accumulation, etc. The second scale of optimization includes physical parameters of culture vessel like temperature, pH, air supply, medium continuity, product removal, etc. The second scale basically includes designing a reactor vessel in such a way that maximum physical parameters of culture remain optimized and function in equilibrium to result in desired and consistent productivity. Several reports on cultivating hairy roots in large culture vessels endorse the idea of designing to facilitate adequate O_2/nutrient supply during the entire culture duration [32, 33, 77].

On the basis of mode of operation, the bioreactor culture can be batch culture, continuous culture, and semi-continuous culture. Selection of bioreactor operation for hairy root cultivation depends upon various objectives. With particular reference to the HRCs, the bioreactor technology follows the concept of proportionate production of biomass and respective metabolites and, thus, can also be considered as a potential yield enhancement strategy [20]. Initially, Rhodes et al. [47] published a successful report on *Nicotiana rustica* HRCs in a bioreactor for nicotine production. However, during the past two decades, the bioreactor technology for HRCs has significantly uplifted from laboratory bench scale to industrial scale as various companies are now ready to adapt modified versions of technology to cultivate hairy root biomass for metabolite production [16; ROOTec bioactives Ltd, Switzerland; http:// www.rootec.com; CBN Biotech, South Korea]. Nevertheless, the key challenge in commercialization of bioreactor technology for HRCs is the low

productivity of cultures and overall cost of technology [16, 48]. Persistent inflow of ideas can be observed in various reactor configurations that have been advised time to time to overcome limitations of low productivity like shear stress, heterogeneity, mass transfer, and nutrient uptake [32]. A detailed account on various types of bioreactors designed to cultivate HRCs has been given in Table 1 and Fig. 3. Such designing of reactors vessels is made to achieve the major objectives like its suitability to the morphology and physiology of tissue to be grown, adequate optimized culture conditions for maximum productivity, and finally the cost of the procedure. The reactors used to cultivate hairy roots can be divided into liquid phase, gas phase, and hybrid reactors.

4.1 Liquid Phase/Submerged Reactors

In liquid-phase reactors, HRs are allowed to grow throughout in liquid ambience comprised of growth medium. As the roots always grow under submerged conditions, liquid-phase reactors are also known as submerged reactors. Stirred tank (STR) and air lift (ALR) are the simple submerged reactors which are used to grow hairy roots. The STRs consist of impeller to facilitate mass transfer to the growing root tissue. In an earlier study, STR was used to grow HRCs of *Datura stramonium* [78]. However, the major challenge in these reactors is shearing and wounding of root tissues due to continuous rotation of impeller blades. Besides, in compactly grown root culture, poor liquid circulation and chemical gradients also contribute to the insufficiency of these reactors [79]. To deal with such challenges, the use of steel mesh or any porous substrate made up of nonreactive material was proposed [78]. HRCs of *Glycyrrhiza glabra*, *Rauwolfia serpentina*, and *Picrorhiza kurroa* were successfully grown in these modified STRs where a nylon mesh (pore size 200 μ) was provided to avoid submergence of inoculated tissue and its damage from the impeller [43, 44, 46]. Further, the idea of using of reactors without any mechanical agitation came into existence, out of which air lift and bubble column reactors are the most common examples. In air lift reactors, at the bottom of the vessel, aerators are provided that supply compressed air moisten with liquid medium at slow rates. The air lift reactors were used for HRCs of *Lithospermum erythrorhizon*, horse radish, and carrot [48, 81]. Further, *Solanum chrysotrichum* HRCs were also grown separately in a 2L air lift reactor with basic design and a novel modified mesh-draught reactor [82]. The tissue growth and distribution within the vessel was found more desired in later as in continuous operation, the insufficient mixing and nonuniform nutrient supply to the rapidly growing root tissue mass in air lift reactors led to the limiting factors for growth.

The much similar to air lift, bubble column reactors (BCRs) are designed to provide air in the form of bubbles to the liquid medium where the roots are submerged. In these reactors the rate of gaseous supply increased gradually with the tissue growth. Several studies have been performed on growing hairy roots using bubble column reactors with little attachments and modifications. For example, in the case of *Tagetes patula* and *Lithospermum erythrorhizon* HRCs, the division of bubble column into two segments with sparger in each segment was done to increase the mass transfer as a whole [83, 84]. Successful use of bubble column reactor is also

Table 1 Comparative account on various major types of bioreactor used for up-scaling of hairy root cultures

Type of reactor	Bubble column (BCR)	Stirred tank (STR)	Air-lift (ALR)	Convective flow (CFR)
Features and modifications	Vertical cylindrical column in which gas is inserted from the bottom in the form of bubble Can be segmented or non-segmented Mesh septum can be provided for anchorage	Fitted with impellers that facilitates mass transfer Mesh septum can be provided for anchorage and to avoid tissue damage from impeller blades	Glass grid / sparger fitted at the bottom is used to provide compressed air. Different from BCRs as they contain a draft tube which improves circulation and oxygen transfer and equalizes shear forces in the reactor	STRs attached to a tubular culture chamber having support mesh at the lower end Mass transfer occurs by means of convective medium flow around growing tissue Air supply takes place by means of external aeration vessel
Advantages	Low capital costs Simple mechanicalconfiguration Low energy requirements	Ideal for continuous operation Better mass transfer in comparison to BCR	Simple design with no moving parts or agitator for less maintenance Air lifts give more vigorous recirculation for the same air flow	Allows oxygen rich medium to flow simultaneously through external aeration
Disadvantages	Not suitable for viscous liquids Enlargement and Entrapping of bubbles in root clumps, resulting in gas flow channeling around clumps and total localized depletion of oxygen	High possibility of tissue damage More energy consumption Insufficient control of concentration gradients near dense root clumps	Greater air throughput and higher pressures needed. May cause excessive foaming and requires more energy for their generation at porous distributors	May not be a realistic large-scale system due to the pressure required to circulate the culture medium at a velocity high enough to overcome the flow resistance of the root bed
References	[32, 83–85]	[32, 48, 78, 80, 107]	[48, 80–82, 108, 109]	[55]

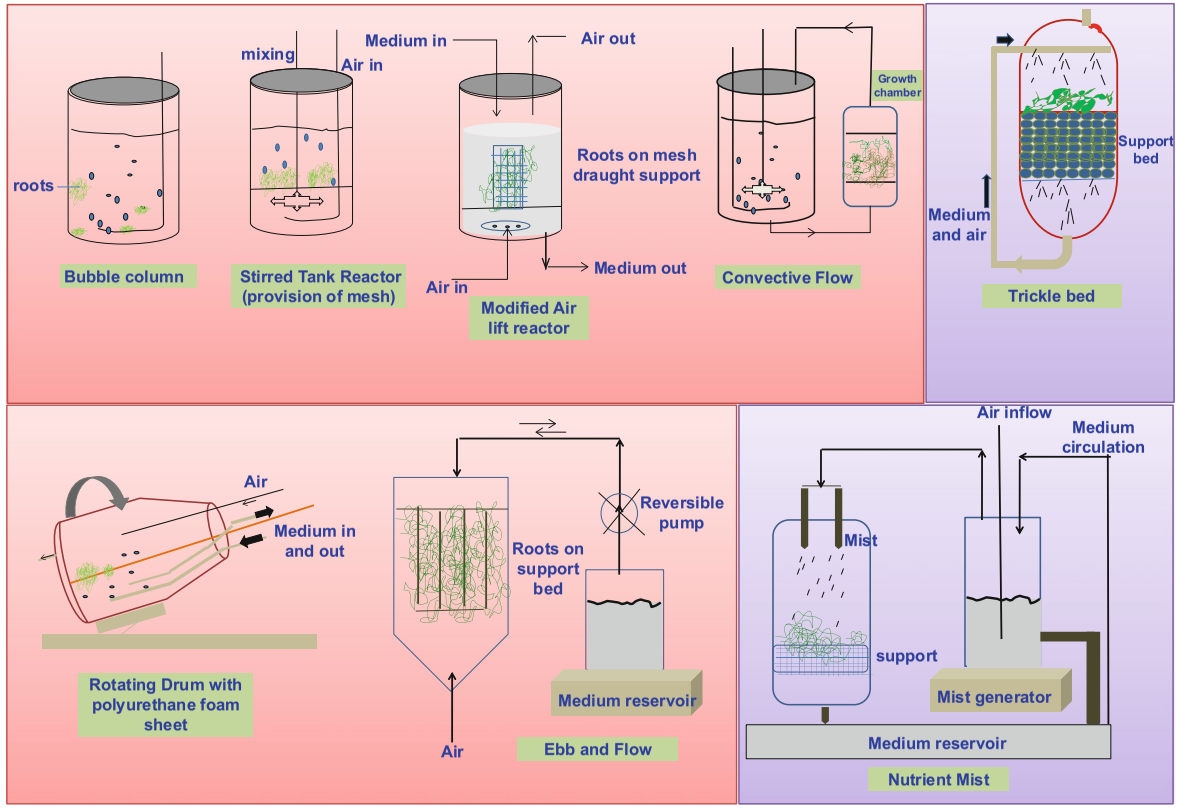

Fig. 3 Schematic presentation of various types of major liquid (red background) and gas phase (blue background) bioreactors used for up-scaling of hairy root cultures

reported for betalain production from HRCs of *Beta vulgaris* challenged with different elicitors [85]. However, the betalain productivity was hampered because of its inhibitory action on biomass. In an extension of this, cetyl trimethyl ammonium bromide (CTAB) was used for pigment release which resulted in optimization of concentration for better efflux of betalains without showing any inhibitory effect on hairy root viability. These studies on product enhancement and online extraction of pigment are useful for developing a bioreactor system for specific hairy root cultures. In particular the use of elicitors and efflux studies provide an insight for integrating unit operations and developing a process for continuous operation and higher production of phytochemicals.

Acquiring the concept of mass transfer based on convective fluid flow, convective flow reactors (CFR) were designed for HRCs of *Hyoscyamus muticus* [55]. The reactor consisted of a stirred tank along with a separate tubular culture chamber for root growth. The medium circulate through the two chambers at a velocity sufficient to overcome the flow resistance of the dense root bed. In a comparison with BCR, the *H. muticus* hairy root growth in CFR exhibited enhanced biomass production [55]. Proportionate to tissue growth, the sufficient medium supplementation at optimized velocity contributed enhanced convective mass transfer subsequently led to the enhanced growth. In other studies, to assess the effect of liquid flow velocity on the growth, oxygen uptake and productivity of hairy roots single column CFR were developed for *Beta vulgaris* [86]. In this study, the optimal range of velocity for good growth was observed 15 mh^{-1} and the increased flow velocity up to 28 mh^{-1} though enhanced the root elongation rate, but the viability of root tips was reduced due to damage from shear stress.

Based on this result, a radial flow reactor was developed to minimize the pressure on root tissue surface created by the fluid flowing in at a specific velocity. A substantial increase in biomass was observed in this reactor configuration where air-saturated medium flow in at a flow velocity 15 mh^{-1} through the ports on the sidewall of the reactor and flow out through the ports at the center of the top and bottom plates. Likewise, *Atropa belladonna* and *Solanum aviculare* [63] HRCs were also grown in packed bed recirculation reactor to analyze the liquid solid hydrodynamic layer and oxygen uptake. In the experiment, the liquid medium was flown in high velocity to minimize the hydrodynamic boundary layers around the tissue surface. However, it was observed that the rate of oxygen uptake and growth by the roots was dependent on the tissue mass, and very thin or the absence of hydrodynamic boundary layer did not affect the growth. It was proposed that under submerged conditions, roots were covered with thick layers of hydrated mucilage which acts as an additional barrier to oxygen transfer.

Keeping such observations in mind, the idea of cultivating hairy roots in alternate fill and drain cycles of liquid and gas phase has came into existence. To materialize this concept, rotating drum (RDR) and ebb and flow reactors (EFBR) were developed [48, 49, 80]. Rotating drum consists of a drum-shaped vessel mounted on rollers for rotation at low speed of 2–6 rpm. The difficulty observed with the rotating drum reactor was that at the beginning of culture, roots did not adhere to the vessel wall. Consequently, as the roots were rotated above the culture medium, they

detached from the wall and were damaged ultimately resulting in low productivity. For the cultivation of carrot hairy roots, Kondo et al. [80] used polyurethane foam to immobilize the roots to overcome this problem. Though such modification had solved the challenge up to much extent, the RDRs were found unsuitable for industrial scale as they require high energy consumption [87]. On the other hand, the EFBR derived its name from the process behavior of liquid medium in reactor which follows repetitive cycles of ebbing and flowing (filling and draining) between the reactor vessel and medium reservoir [51, 88, 89]. Designed for *H. muticus* HRCs, the EFBRs demonstrated successful scale up to 50× of a 50 ml flask. In a comparative study with bubble column reactors, the EFBRs were found superior in terms of productivity, and liquid holds up profiles by dense entangled root mass. The study concluded that it was back and forth convective flow of EFBRs that made it superior to bubble columns which have negligible fluid convection.

Based on the concept of temporary contact between the cultured tissue and liquid medium, temporary immersion systems (TIS) were also used for the cultivation of hairy roots. These systems allow the cultured tissue to be immersed in liquid medium for a defined duration (flooding) followed by standby stage. Basically, TIS are designed for in vitro plant propagation; however certain advantages of these systems, like ease of medium changes, limited shear damage, less chances of hyperhydration of tissue, etc., have attracted researchers to use them for hairy roots [90]. In a study of *Beta vulgaris* HRCs, RITA® (Recipient for Automated Temporary Immersion System) apparatus was used to grow hairy roots for betalains biosysnthesis [91]. The maximum accumulation of betalain pigment was observed with 15 min immersed/60 min standby cycles, whereas optimal biomass in terms of intensively branched and morphologically healthy roots was obtained with 15 min immersed/75 min standby cycles. Thus, this was observed as an advantage of TIS in root growth in comparison to the ALR, STR, and bubble column reactors where clump formation and shear damage followed by nutrient and oxygen limitations in root tissues at central core and callus formation, respectively, are associated with loss of productivity. Forced and repeated air replenishment in the system and lesser hydrodynamic pressure to the growing tissue are supposed to be the reasons behind optimal growth of the root tissues in these systems. However, the success of using temporary immersions depends upon (1) the ratio of inoculum density and medium volume that too in relation with the size of container and (2) the optimization of length and frequency of medium immersions. In TIS, the time and frequency of the immersion are the most decisive parameter for optimized productivity, as they influence nutrient and water uptake. Thus, a prior optimization of such parameters is a must requisite. Another point of limitation that would be in need of attention is that, normally, TIS are smaller in size with small interior space which may prove insufficient for fast-growing root tissues.

4.2 Gas-Phase Reactors

In gas-phase reactors, the roots are immobilized in culture vessels with the help of horizontal sheets or rings made up of inert material and exposed to air or a gas

mixture. The liquid medium is provided in the form of spray or mist of micron-sized droplets. In such reactors liquid is the dispersed phase and gas is the continuous phase in which roots are exposed. Such setup diminishes many of the limitations associated with liquid-phase reactors. Out of various gas-phase rectors, trickle-bed reactors (TBR), nutrient mist rectors (NMR), and droplet phase are the common reactors. Since the medium is supplied in the form of droplet, there is a considerable variation in the size of droplets according to the requirement, and various attachments, like ultrasonic transducers, spray nozzles, etc., are used to create perfect sized droplets ranging from 0.5 to 50 μm [62, 92, 93]. In trickle-bed reactors, the medium trickles over roots from the top of the vessel. The used up medium is drained from the bottom of the vessel to a pool and is recirculated at a specific rate. The flow of liquid depends upon gravity and the distribution of liquid depends upon the mechanism of liquid delivery from the top. The growth of *H. muticus* hairy roots in gas-dispersed reactor exhibited excellent performance and accumulated tissue mass in submerged air-sparged reactors was 31 % of liquid-dispersed controls [49]. A study concluded that the distribution of the roots becomes a key factor in controlling the rate of growth. Noticeable results for hairy root growth, fluid dynamics, and oxygen mass transfer in a trickle-bed reactor were also obtained from the study conducted by Ramakrishnan and Curtis 2004 [94]. These results demonstrate that trickle-bed reactor systems can sustain tissue concentrations, growth rates, and volumetric biomass productivities substantially higher than other reported bioreactor configurations. Mass transfer and fluid dynamics are characterized in trickle-bed root reactors to identify appropriate operating conditions and scale-up criteria. Bioreactor characterization is sufficient to carry out preliminary design calculations that indicate scale-up feasibility to at least 10,000 L. On the other hand, in mist reactors, mists are more water efficient, thus, eliminating the need for extensive recirculation equipment. In comparison to large droplets of spray, provision of mists facilitates nutrient and gaseous exchange by reducing the thickness of the liquid film deposition on the surface of the root tissue [62, 95, 96]. Irrespective of spray or mist, the liquid that forms thin layer on the tissue surface acts as a barrier for efficient mass transfer [96, 97]. On the other hand, roots in a mist reactor are often too sparsely packed to capture mist particles efficiently and cannot, therefore, meet the nutrient demands required to maintain high growth rates. Keeping this in mind, an aerosol model was proposed for *Artemisia annua* hairy roots growing in NMR [98]. Growth rate was increased when mist medium containing high sucrose concentration was used in which sucrose acted as aerosol particles. In another recent study, an ON/OFF strategy was analyzed for optimizing the operating conditions of a mist reactor for the growth of hairy roots [99, 100]. A mathematical model was developed to optimize the ON/OFF mist duty cycle for the specified growth of hairy roots. Considering the availability and rate of transport of nutrients to the roots as vital parameters for growth, the ON/OFF cycles were proposed as the thin liquid film which continuously builds up during the ON cycle over the root surface is a key limiting factor mass transfer. However, the same film can also act as a finite reservoir of nutrients in the absence of any replenishment during the OFF cycle.

Since the continuous phase is gas, the roots are required to be immobilized in the reactor. For the immobilization of roots, various equipments like horizontal or vertical mesh sheets or rings made up of nylon or stainless steel [63, 101–105] were used. Ramakrishnan and Curtis [61] used Intalox metal process packing elements to immobilize roots in gas-phase reactors.

4.3 Hybrid Reactors

Similar to liquid-phase reactors, in gas-phase reactors the major problem includes liquid channeling and holdup within the root bed [61]. The stagnant holdup of liquid may have different nutrient levels than the bulk fluid [106] or make the roots within effectively submerged and depleted of oxygen [107]. To overcome these limitations, hybrid reactors for hairy root cultures are designed which follow the concept of growing roots under submerged conditions initially so that roots get adapted to the process and start growing. After an initial growth when submerged condition no longer remains effective due to dense growth and poor mass transfer rate, the reactor operation switches over to gas phase. Ramakrishnan and Curtis [61] proposed a combination of bubble column and trickle bed for *Hyoscyamus muticus* roots. In the study, bubble column was initially inoculated with roots to allow them to evenly distribute and attach to the anchorage provided in the reactor. Following 2 weeks of growth, when roots started forming clumps, the reactor was switched to a trickle-bed operation for another 4 weeks, thus exposing roots to a gas environment. In another study, *Datura stramonium* hairy roots were grown initially under submerged phase for 21 days followed by droplet phase for 40 days [93]. A hybrid reactor system made up of bubble column and nutrient mist bioreactor was used to study the transient growth characteristics and nutrient utilization rates of *Artemisia annua* hairy roots [108].

5 Conclusion

Hairy root culture technology has offered perspectives for in vitro large-scale production of valuable plant secondary metabolites. Together it also provides many challenges during large-scale cultivation as with the increase in medium volume, vessel size, and culture density, various physical factors, like gas and liquid flow rates, mass transfer rate, concentration gradients, etc., start affecting the tissue growth simultaneously. Besides, the unusual rheological properties and growth patterns of HRCs also contribute to challenges in their up-scaling. This has laid the background of acquiring and investigating novel approaches of bioreactor designing and process optimization. Optimized mass transfer concomitant with low shear stress and hydrodynamic pressure on growing root tissue is the utmost objective of designing a reactor for desired growth and productivity. The selection of right configuration of reactor vessel also depends upon the morphological and physiological properties of hairy roots. Further, a major issue of the commercial

use of a bioreactor setup for hairy root cultivation deals with low capital cost. Keeping this in mind nowadays, bioreactor technology utilizes computational and mathematical simulations for incessant operation. These simulations provide help in meeting the challenges on issues like optimization of physical, biological, and chemical culture conditions, offline and/or online measurement of growth, mass transfer behavior, synergistic effects of various physical and chemical parameters on growth, downstream processing (intracellular/extracellular), product recovery, etc. However, online measurement of biomass growth is a difficult but perennial requirement during bioreactor cultivation. Precise measurement of growth is difficult due to the nonhomogenous nature of cultured tissue. Nevertheless, for offline measurement there are different methods like packed cell volume (PCV), fresh and dry weight estimation, etc. Growth estimation with these methods is relatively time consuming and also require frequent sampling from the running culture at a threat of contamination. Additionally, withdrawing large sample volumes may become problematic while measuring overall productivity against medium volume and inoculum density. To avoid these problems, online measurement is rather observed as an easy and noninvasive method of growth. These include utilization of artificial neural network and response surface methodology-based computational scheming of growth based on variables in culture conditions and predictions of final productivity [109]. Further, measurement of medium conductivity and osmolarity are the other methods that are being used for the growth measurement [110]. Moreover, online fluorescence measurement of metabolic activities of cultured tissues and online monitoring of cell growth by conductometry are the methods that cannot only predict the final biomass at the end of culture but also become helpful in optimizing various parameters during the culture duration [111, 112]. Although such measurement studies have been much explored in cell suspension and microbial cultures, an insight and proper retrieval of information from these studies may provide help in online growth monitoring and up-scaling of hairy root cultures in bioreactor. This may fairly help in filling the gap between capital cost and the benefits of technology at industrial scale.

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Part V

Other Applications

Platforms for Plant-Based Protein Production

18

Jianfeng Xu, Melissa Towler, and Pamela J. Weathers

Contents

Abstract

Plant molecular farming depends on a diversity of plant systems for production of useful recombinant proteins. These proteins include protein biopolymers, industrial proteins and enzymes, and therapeutic proteins. Plant production systems include microalgae, cells, hairy roots, moss, and whole plants with both stable and transient expression. Production processes involve a narrowing diversity of bioreactors for cell, hairy root, microalgae, and moss cultivation. For whole plants, both field and automated greenhouse cultivation methods are used with

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products expressed and produced either in leaves or seeds. Many successful expression systems now exist for a variety of different products with a list of increasingly successful commercialized products. This chapter provides an overview and examples of the current state of plant-based production systems for different types of recombinant proteins.

Keywords
Molecular farming · Recombinant protein expression · Therapeutic proteins · Transient expression · Bioreactor

1 Introduction

Photosynthesis or use of simple growth media provides clear advantages for using plants to produce biomass and protein products at low cost [1–3]. The majority of posttranslational modifications important for many complex eukaryotic proteins can be performed by plants, whose species diversity offers variety in production platforms from in vitro cultures through field crops, all within established regulatory guidelines. Of particular benefit is that plants cannot harbor human and animal pathogens that can plague in vitro mammalian production systems, so plants provide major advantages in product safety, especially for therapeutics [4, 5]. Such safety advantages also reduce purification costs and minimize production shutdowns and facility decontamination, thereby affecting patient/customer demand. Costs to purify plant-produced proteins are comparable to microbial or mammalian cell culture systems, but plants offer key advantages with lower up-front capital costs and potential economies of scale. Direct use of cultivated plant cells, whole or minimally processed plants, or plant parts (e.g., seeds, dried leaves) is in development for industrial/bioenergy applications as well as for therapeutics and vaccines, thereby further reducing costs of recombinant proteins [6–12]. Boosting protein yields is always a challenge for economic feasibility [2, 5] as are regulatory considerations, some of which are unique to plant bioproduction systems [1, 13].

Plant expression platform diversity includes: whole plants, suspension cells, hairy roots, moss, duckweed, and microalgae. There are strengths and weaknesses to each platform with selection often determined by type of recombinant protein, market, scale, cost, and up and downstream processing constraints of the specific protein product. Within each platform is also a diversity of plant species that can host the protein product. Platform diversity provides flexibility in expressing novel recombinant proteins, enables customizing and meeting scale-up needs, and provides opportunity for oral-based delivery of proteins. However, such diversity also may complicate regulatory compliance, which prefers more uniformity. Here we summarize prospects and challenges associated with each type of plant production platform and production strategy, and where appropriate, comments are provided on important regulatory issues and progress toward commercialization.

2 Types of Recombinant Proteins Produced by Plants

Plant-made recombinant proteins are generally categorized into three classes, usually based on function and/or application: biopolymers, industrial proteins/enzymes, and therapeutic proteins. Examples of each of these groups are briefly described in the following sections.

2.1 Biopolymers

Although plants are expert at bulk production of biopolymers such as cellulose and starch, here the focus is on recombinant protein-based biopolymers such as elastin-like polypeptides (ELPs), spider silk proteins, collagens, and plant gums (see review [14]). The ELPs that are repetitive pentapeptide sequences (VGVPG) mainly serve as thermally responsive tags for non-chromatographic purification of recombinant proteins [15]. ELP tags significantly enhanced production yield of different recombinant proteins in plant leaves [16, 17]. Collagen and spider silk proteins (spidroins) serve as two interesting examples.

Collagen is a critical extracellular matrix (ECM) material and connective tissue in animals; commercial supplies are typically extracted from cadavers or animal sources, so a safer source is desirable [18]. Large-scale production of recombinant human collagen type I (rhCOL1) in tobacco is now providing low-cost functional biocompatible ECM that is safer than cadaver or animal sources for use in creating scaffolds for tissue engineering, skin, and wound healing [19, 20].

Spidroins are modular fibrous proteins with highly repetitive amino acid sequences consisting mainly of glycine and alanine [21, 22]. Silk fibers spun from these spidroins have exceptional flexibility, elasticity, and toughness – three times as strong as Kevlar and five times as strong as steel [23]. Plant production is more efficient and cheaper than microbial recombinant spidroins. Transgenic tobacco and potato plants expressing *Nephila clavipes* synthetic genes of dragline spidroin have yielded recombinant silk proteins up to 2% of total soluble protein (TSP) in the ER [22]. *Arabidopsis* dragline spidroins reached 18% of TSP in seeds [24]. Challenges remain in developing manufacturing technology for spinning fibers into more useful products [25].

2.2 Industrial Proteins/Enzymes

Industrial proteins are defined as commodity chemicals used in very large quantities and thus must be produced at very low cost [26]. Transgenic field plants are particularly attractive for producing industrial proteins/enzymes because agricultural production is low cost, protein products stored in specific organs such as seeds are stable, scale-up is easy and relatively fast, and crude plant materials can often be used directly in industrial processes [6, 7]. Industrial proteins of most interest are hydrolases, including glycosidases (e.g., cellulase, α-amylase, and β-glucuronidase

(GUS)) and proteases (e.g., trypsin). The first company to develop and commercialize plant-based recombinant proteins/enzymes, with GUS and avidin being their first two commercialized products, was ProdiGene Inc. (formerly in College Station, TX), which is unfortunately now out of business [3, 7, 27].

Corn seed is considered an ideal platform for industrial protein/enzyme production because this plant has the largest annual grain yield and relatively high seed protein content (10%), offering the highest potential recombinant protein yield per hectare [28]. Regulation is a major hindrance to using plant-made industrial proteins/enzymes because a large acreage of transgenic plants is needed. Use of a food/feed crop for nonfood/feed products may also meet political resistance when, as projected, global food supplies become limited, therefore requiring new solutions.

2.3 Therapeutic Proteins

Biopharmaceutical sales are projected to reach $US 278.2 billion by 2020. Although transgenic plants can produce fully functional mammalian proteins, including blood proteins, vaccine antigens, monoclonal antibodies (mAbs), cytokines, therapeutic enzymes, growth factors, and growth hormones [5, 29–31], bioactivity requires proper folding, disulfide bond formation, subunit assembly, and often proteolytic cleavage and/or glycosylation. Plants can produce lower-cost, safer therapeutic proteins than mammalian cells [30] and may be the only production system available, e.g., for production of secretory antibodies (sIgAs) [2]. These recombinant therapeutic proteins are produced using many different plant-based platforms including cells and field crops; the most common production species is tobacco. Protein yields have reached well over 25% of TSP [32] 247 mg L^{-1} [33].

Plants successfully perform N-linked glycosylation of proteins at the signature recognition motif (N-X-S/T) with subsequent processing in the Golgi complex. As a result, plant glycans differ from those found in mammalian cells. Using plants as hosts to produce therapeutic glycoproteins results in addition of plant-specific xylose and α-1,3-fucose sugars; these may alter bioactivity or immunogenicity in humans [34, 35]. The β-1,4-galactose or sialic acid residues synthesized in mammals are not naturally produced in plants [31, 35–37], so RNAi strategies have been used to engineer more humanlike glycosylation machinery in plants by knocking down fucosyl- and xylosyltransferases in plant transgenic lines expressing a human or chimeric β-1,4-galactosyltransferase [38–40]. Furthermore, tobacco plants infiltrated with *Agrobacterium tumefaciens* produced human antibodies with humanlike N-glycans when co-expressed with a chimeric human β-1,4-galactosyltransferase [41]. The first human-injected therapeutic with greatest clinical experience (Protalix's taliglucerase alfa; discussed further below) did not trigger significant patient antibody production [42–44]. Indeed, plant-specific posttranslational modifications (PTMs) may offer opportunities for producing novel recombinant proteins with enhanced function and efficacy as well as biosimilars [45]. "Second generation" plant-made pharmaceutical proteins are emerging, wherein therapeutic targets are specifically engineered to enhance or produce new therapeutic proteins that integrate

novel motifs or fusion to facilitate protein assembly, delivery, trafficking, protein stability, serum longevity, or protein solubility in either the production host or the target organism (e.g., [31, 46–49]).

3 Platforms for Plant Expression

Advances in plant molecular engineering technologies have expanded the diversity of plant bioproduction platforms, ranging from cell and tissue cultures under sterile and contained conditions to whole plants grown under glass or in the field [2]. These bioproduction platforms can be classified as: (1) in vitro culture systems including cell suspensions, hairy roots, and moss protonema, (2) aquatic plants including duckweed and microalgae, and (3) whole plants using both stable and transient expression. The stable expression of whole plants also includes leaf- and seed-based systems. Characteristics of each platform and their strengths and weaknesses are described here. A comparison of the cost, applicability, time needed for production, scalability, and regulatory compliance of different platforms is shown in Table 1.

3.1 In Vitro Culture Systems

Plant biomass (e.g., suspension cells, hairy roots, and moss) can be propagated in confined bioreactors under sterile conditions for large-scale production of recombinant proteins. In vitro culture allows for precise control over growth and protein production, batch-to-batch product consistency, and a production process aligned with current good manufacturing practices (cGMP) [5]. Due to its relatively high cost of production, in vitro cultures are often used to produce high-value protein therapeutics. Compared with the other plant-based platforms, in vitro cultures are more acceptable to the pharmaceutical industry with fewer regulatory and environmental concerns [50]. Like other bioreactor-based culture systems, scalability of in vitro cultures is limited by bioreactor capacity. However, because recombinant proteins can be secreted into culture media, downstream processing of recovery and purification of the proteins becomes less expensive than from whole plants [51]. Indeed, the first licensed pharmaceutical protein derived from plants for human use – taliglucerase alfa (Elelyso™) – was produced in carrot suspension cells. Examples of representative recombinant proteins produced using in vitro cultures are shown in Table 2.

3.1.1 Plant Cell Suspensions

Undifferentiated clusters of plant callus can be dispersed and propagated in a liquid medium to generate stable cell suspension cultures that retain the same production capacity as whole plants. The production of recombinant proteins in plant cell culture was first demonstrated in 1990 with the expression of a human serum albumin in tobacco cells [78]. Since then, a wide array of biologically active proteins, including antibodies, vaccine antigens, growth hormones and factors,

Table 1 Comparisons of different plant-based bioproduction platforms

Platforms	Viable species	Time for production[a]	Types of proteins applicable	Scalability	Production cost[b]	Regulatory compliance
In vitro culture system						
Plant cell suspensions	Tobacco (*Nicotiana tabacum* cv. BY-2), carrot, rice	7–14 days	Therapeutic proteins	LBC[c], as high as 100,000 L	Medium	Easy
Hairy roots	*N. tabacum*	14–30 days	Therapeutic proteins	LBC, as high as 20,000 L	Medium	Easy
Moss	*Physcomitrella patens*	14–30 days	Therapeutic proteins	LBC, as high as 500 L	Medium	Easy
Aquatic plants (contained system)						
Duckweed	*Lemna* sp., *Spirodela* sp.	20–40 days	Therapeutic proteins; industrial enzymes	LBC, as high as 10,000 L	Low	Moderate
Microalgae	*Chlamydomonas reinhardtii*, *Dunaliella salina*	14–30 days	Therapeutic proteins	LBC, as high as 10,000 L	Low	Easy
Whole plants (leaf-based and seed-based system)						
Stable transgenic plants	Corn, soybean, safflower, rice, tobacco	3–6 months	Therapeutic proteins; biopolymers; industrial enzymes	Unlimited field culture	Very low	Difficult
Transient plants	*N. benthamiana*, lettuce	2–7 days	Therapeutic proteins	Greenhouse limited	Low	Easy

[a]The time required to accumulate maximum amounts of recombinant proteins in a culture system after planting or bioreactor inoculation
[b]Compared with mammalian cell culture platform
[c]*LBC* limited by bioreactor capacity

Table 2 Example of representative recombinant proteins produced via in vitro cultures

Recombinant proteins	Host plant species	Promoter	Protein yields[a]	References
Plant suspension cells				
Anti-HIV antibody 2G12	*N. tabacum* cv BY-2	*CaMV35S*	12 mg L^{-1}	[52]
Human antibody M12	*N. tabacum* cv BY-2	*CaMV35S*	107 mg L^{-1}	[53]
Anti-hepatitis B surface antigen (HBsAg) mAb	*N. tabacum* cv BY-2	*CaMV35S*	15 mg L^{-1}	[54]
Human growth hormone (hGH)	*N. tabacum* cv BY-2	*CaMV35S*	35 mg L^{-1}	[49]
	O. sativa cv Donjin	*RAmy3D*		[55]
Human interferon α2b (hIFNα2)	*N. tabacum* cv BY-2	*CaMV35S*	28 mg L^{-1}	[48]
Human α-1-antitrypsin (rAAT)	*O. sativa*	*RAmy3D*	247 mg L^{-1}	[33]
	D. carota	*CaMV35S*		www.protalix.com
hGM-CSF	*O. sativa*	*RAmy3D*	129 mg L^{-1}	[56]
Glucocerebrosidase	*D. carota*	*CaMV35S*	N/A	[57]
α-Galactosidase-A (PRX-102)	*D. carota*	*CaMV35S*	N/A	[58]
				www.protalix.com
Human interleukin-12 (IL-12)	*O. sativa*	*RAmy3D*	31 mg L^{-1}	[59]
Human serum albumin (hSA)	*O. sativa*	*RAmy3D*	45 mg L^{-1}	[60]
Enhanced GFP	*N. tabacum* cv BY-2	*CaMV35S*	125–131 mg L^{-1}	[61]
Hairy roots				
Human antibody M12	*N. tabacum*	*CaMV35S*	5.9 mg L^{-1}	[62]
Human antibody H10	*N. tabacum*	*CaMV35S*	2–3 mg L^{-1}	[63]
	N. benthamiana (DeltaXTFT)			
Hepatitis B surface antigen (HBsAg)	*Solanum tuberosum (*var. *Kufri bahar)*	*$(Aocs)_3$AmasPmas*[a]	97.1 ng g^{-1}FW	[64]

(*continued*)

Table 2 (continued)

Recombinant proteins	Host plant species	Promoter	Protein yields[a]	References
14D9 murine IgG_1	*N. tabacum*	*CaMV35S*	64 mg L^{-1}	[65]
Human epidermal growth factor (hEGF)	*N. tabacum*	*CaMV35S*	2 ug g^{-1}FW	[66]
Thaumatin	*N. tabacum*	*CaMV35S*	2.6 mg L^{-1}	[67]
GFP	*Brassica rapa rapa* (turnip)	*CaMV35S*	120 mg L^{-1}	[68]
Human tissue plasminogen activator (tPA)	*Cucumis melo* L cv Geumssaragi-euncheon or *N. tabacum*	*CaMV35S*	798 ug g^{-1}FW or 900 ng mg TSP	[69, 70]
Moss				
Vascular endothelial growth factor (VEGF)	*Physcomitrella patens*	*CaMV35S*	656 μg g^{-1}DW	[71, 72]
Erythropoietin (EPO)	*P. patens*	*PpUbq1* [b]	250 μg g^{-1}DW	[73, 74]
Complement factor H	*P. patens*	*Ppact5-P* [c]	23.2 μg g^{-1}DW (secreted); 25.8 μg g^{-1}DW (intracellular)	[75]
Multi-epitope fusion protein (HIV vaccine)	*P. patens*	*Pca200P* [d]	3.7 μg g^{-1}FW	[76]
α-Galactosidase, β-glucocerebrosidase, FGF7/KGF	*P. patens*	*N/A*	N/A	[77]; www.greenovation.com

[a]*FW* fresh weight, *DW* dry weight, *TSP* total soluble protein, *NA* date not available
[b]Chimeric super-promoter consisting of three copies of the octopine synthase activator (*Aocs*) and one copy of the mannopine synthase activator (*Amas*) located upstream of the mannopine synthase promoter (*Pmas*)
[c]*Ppact5-P*: *P. patens* actin5 gene promoter
[d]*PpUbq1*: *Physcomitrella* ubiquitin gene-derived promoter
[e]*Pca200P*: *P. patens* carbonic anhydrase 200 promoter

cytokines, and therapeutic enzymes, have been successfully produced in plant cell culture. See recent reviews [5, 50, 79].

Plant suspension cultures integrate the merits of whole plant production with those of microbial fermentation and mammalian cell culture [80, 81]. Plant cell culture inherits most of the advantages of plant-based production systems, particularly the ability to produce complex proteins with correct posttranslational modifications without risk of contamination by human pathogens. Plant cell cultures also can be rapidly propagated in bioreactors as homogeneous suspensions for large-scale production, growing fast in simple synthetic media with doubling times as short as 16 h [5]. A breakthrough in plant cell culture technology was made in May 2012 by Protalix Biotherapeutics (http://www.protalix.com), an Israel biopharmaceutical company, for a plant cell-produced therapeutic enzyme – taliglucerase alfa – finally approved by the US Food and Drug Administration (FDA) as an orphan drug for Gaucher's disease. Taliglucerase alfa is a hydrolytic lysosomal glucocerebrosidase for intravenous infusion and commercially known as Elelyso™; it is the world's first plant-made human pharmaceutical and made by Protalix using carrot cells.

Plant cell lines most widely used for recombinant protein production are derived from tobacco (*Nicotiana tabacum*), particularly cultivar BY-2 (*N. tabacum* cv. Bright Yellow 2) cells. BY-2 cells are robust and fast growing and can multiply ≥100-fold in a week. They readily undergo *Agrobacterium*-mediated transformation and cell cycle synchronization [5, 81, 82]. Other commonly used cell lines include rice (*Oryza sativa*), alfalfa (*Medicago sativa*), and carrot (*Daucus carota*). In fact, these cell lines derived from common edible crops may be more favorable than tobacco cells in terms of by-product levels and regulatory compliance [81]. Rice cell suspension cultures are used almost as widely as tobacco BY-2 cells due to availability of the sugar-sensitive α-amylase promoter system (*RAmy3D*) [83]. This promoter is induced by sugar starvation and has enabled high-level expression of many pharmaceutical proteins in rice cells, e.g., α1-antitrypsin (rAAT) [33, 83], hGM-CSF [56], interleukin-12 [59], and human serum albumin [60], with highest secreted protein yields reaching 247 mg L^{-1} for rAAT [33]. While these production levels are impressive, growth rates, and general characteristics and stability of rice cell lines are inferior to those of tobacco BY-2 cell lines; rice cell viability is significantly decreased when cultivated in a sucrose-starvation medium [80].

The major bottleneck to full adoption of plant cell culture technology for commercial purposes has been low productivity with protein yields ranging from 0.01 to 10 mg L^{-1} [84]. To substantially improve protein expression for commercial success, strategies at both the molecular and at the process development levels are required to maximize efficiency of all stages of the production pipeline [5, 85]. This is similar to the strategy that has been systematically adopted by the mammalian cell culture industry over the past 25–30 years. Notably, in tobacco BY-2 cells, productivity of M12 mAb was ~8 pg $cell^{-1}$ day^{-1} [86] vs. 20–40 pg $cell^{-1}$ day^{-1} for Chinese hamster ovary (CHO) cells [79]. Besides low productivity, other major challenges remain including non-mammalian glycosylation, genetic instability, and cell culture scale-up in bioreactors [8, 34, 87].

3.1.2 Hairy Roots

Hairy roots are generated by infection of plant tissues with *Agrobacterium rhizogenes* that harbors a root-inducing (*Ri*) plasmid [88]. Similar to suspension cells, hairy roots can be grown in a controlled and sterile environment suitable for cGMP production of pharmaceutical proteins. However, as a more organized organ, hairy roots offer additional benefits, including genotype and phenotype stability and autotrophy for plant hormones [89, 90].

Hairy roots expressing a specific recombinant protein can be readily generated either by infecting stably transformed plants (expressing the target protein) with *A. rhizogenes* or by infecting wild-type plants with genetically modified *A. rhizogenes* harboring binary vectors containing the gene of interest [67]. After the first success producing a full-length murine IgG_1 in tobacco hairy roots [91], more than 20 recombinant proteins, including reporter proteins (e.g., GUS and GFP), enzymes (e.g., human acetylcholinesterase and tPA), antibodies (e.g., human M12 mAb and murine 14D9 IgG_1), antigens (e.g., HBsAg and cholera toxin B surface protective antigen), and cytokines and growth factors (e.g., interleukin-12, hGH, and hEGF), have been expressed in hairy roots [90, 92] with protein yields up to 3.3% TSP of an acetylcholinesterase [93] and >120 mg L^{-1} of GFP [68].

Hairy roots also secrete expressed proteins from cultured tissues, termed *rhizosecretion* [89, 94, 95], offering a simplified, low-cost approach for purification of foreign proteins from inexpensive and well-defined media. Because root tissue is not destroyed for recovery of secreted proteins, a given culture can be used for several cycles of bioproduction. Using an optimized induction protocol for the secretion of M12 mAb from tobacco hairy roots by addition of extra KNO_3, α-naphthaleneacetic acid, and polyvinylpyrrolidone to the standard MS medium, antibody yield was improved by 30-fold, yielding 5.9 mg L^{-1} [62].

The bottleneck to exploiting hairy root technology for commercial purposes has been low protein productivity [90]. Expression systems have been strategically designed to include a strong promoter such as a double-enhanced *CaMV35S* promoter (*2 × 35S*) [93], a chimeric super-promoter (*Aocs*, *$_3$AmasPmas*) [96], and inducible promoters [97]. In addition, the special morphological characteristics of hairy roots including nonhomogeneous growth and highly branched phenotypes present major challenges to culture scale-up in bioreactors [98].

3.1.3 Moss

Moss protonema can be suspension cultured in bioreactors to provide another promising platform for producing recombinant products. While plant cells grow on sugar-based media without the need of light, moss is able to grow using light as a sole source of energy, needing only water and inorganic salts as a medium. This greatly reduces production cost and facilitates product recovery from the medium [51]. Moss also can perform complex posttranslational modifications of expressed proteins [99]. Because it relies on differentiated instead of undifferentiated plant cell cultures, moss cultures are genetically stable over long periods of time [100]. The moss *Physcomitrella patens*, with its genome fully sequenced in 2006 (http://www.cosmoss.org/), is the main species used for bioproduction.

A wide variety of biopharmaceutical human proteins has been produced in moss including tumor-directed mAbs with enhanced antibody-dependent cell-mediated cytotoxicity (ADCC) [101, 102], keratinocyte growth factor (FGF7/KGF) [77], asialo-erythropoietin (asialo-EPO) [73, 74], α-galactosidase and β-glucocerebrosidase, etc. [77] (www.greenovation.com). See a recent review [103]. Some of the recombinant biopharmaceuticals produced from moss are not only similar to those produced in mammalian cells, but even of superior quality ("biobetters"). For example, moss-made α-galactosidase lacks the terminal mannose phosphate and thus is taken up by cells via mannose receptors instead of mannose-6 phosphate receptors, yielding better pharmacokinetics in Fabry mice [103]. In addition, moss N-glycans are free of core α-1,6-fucose, a sugar structure typically present on the N-glycans of mammalian cell-derived proteins. The moss-made IgG lacking this sugar moiety was more efficient in antibody-dependent cell-mediated cytotoxicity (ADCC) than the mammalian cell-made counterparts [101–103].

A unique feature of *P. patens* is that its genome can be readily engineered through gene targeting, a gene replacement strategy based on homologous recombination [51]. The approach has been efficiently used for precision glyco-engineering of moss-produced proteins by knocking out or knocking in certain glycosyltransferase enzymes, allowing for production of humanized glycoproteins [101]. For example, moss mutants were engineered with genes encoding plant typical glycosyltransferases knocked out from the moss genome [72, 74] and further engineered to knock in the gene encoding *β*-1,4-galactosyltransferase into the xylosyltransferase or fucosyltransferase locus, respectively [104]. To avoid unwanted potential *O*-glycosylation at the hydroxyproline residues of human proteins, a gene responsible for prolyl hydroxylation was identified and deleted from the moss genome [99]. See recent reviews [105, 106].

3.1.4 Scaling Up In Vitro Systems

Large-scale cultivation of in vitro systems focuses mainly on cell suspensions and hairy roots. While there are many bioreactors that have been designed for in vitro culture as described in prior reviews that also cite specific examples (e.g., see [107–109]), only a few types of bioreactors seem to be sufficiently scalable for commercial application. These are briefly described below and schematically illustrated in Fig. 1.

While the stirred tank reactor (STR; Fig. 1a) is the industry workhorse for microbial systems, it is not the most ideal option for plant cultures, though as proof of concept some plant cell suspensions have been successfully scaled to 600 L [110, 111]. On the other hand, the bubble column, balloon, wave reactors, and variations thereof seem to be more effective thus far for scaling up in vitro cultures. Plant cell suspensions have been grown in nearly all types of liquid-phase bioreactors. The bubble column reactor (BCR; Fig. 1b) is easy to construct in-house with a variety of design variations. Gas enters at the base of the vertical cylinder through a frit forming small bubbles that rise through the chamber, resulting in aeration and mixing; gas vents through a sterile filter at the top. The BCR

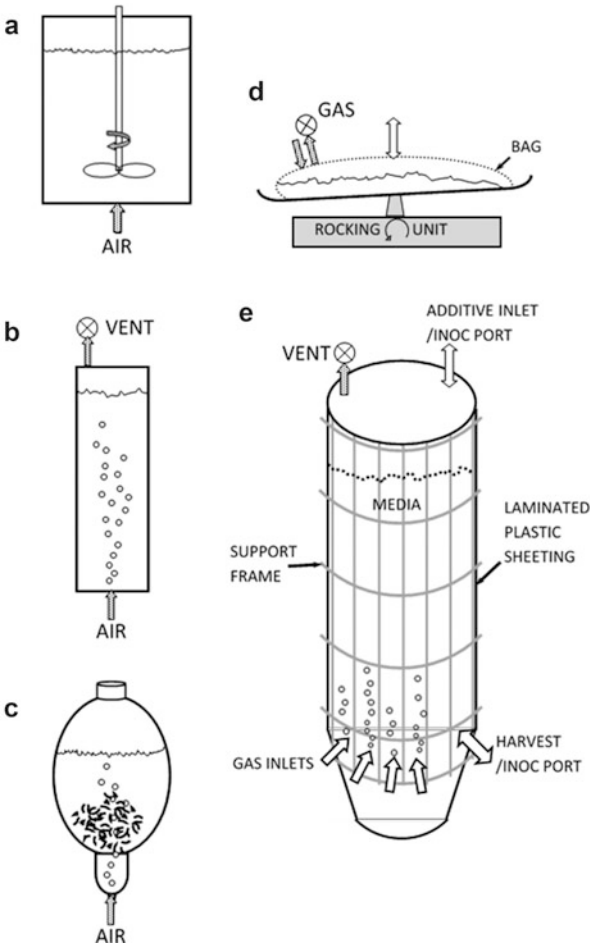

Fig. 1 (**a**–**e**) Schematic of main bioreactors used for in vitro cultures

unfortunately suffers from foaming. This led to development of the balloon-type bubble reactor (BTBR) (Fig. 1c) with a broader liquid surface area.

This design alleviated foaming, providing better gas exchange than the BCR. While the BTBR scales from 4 to 500 L, it is constructed of glass, so at large scale it requires a stainless steel superstructure. This greatly increases capital costs and availability of these reactors is limited. The BTBR has been used to culture a wide variety of cell suspensions and hairy roots. See above cited reviews for more in-depth discussion of different requirements for growing cells vs. hairy roots including many species examples.

The wave bioreactor (Fig. 1d) is a horizontal, transparent plastic bag, seated on a slowly rocking platform that through wave action within the bag provides good agitation. With gas input into the headspace above the large surface area of the liquid in the bag, there is also good gas exchange. Although the wave reactor is being commercially used, it unfortunately scales horizontally with a relatively large

footprint compared to the vertical reactors, for example, that used by Protalix as subsequently discussed.

Other than the glass balloon reactor, plastic single-use disposable culture chambers are also becoming the norm. These can include isolated bags or liners within a supporting superstructure. A vessel with a plastic liner was designed, demonstrated, and patented (US 6,709,862 B2) as versatile and functional some time ago by the Curtis lab [112]. For example, the SB200-X (OrbShake, Kühner AG) is comprised of a large 200 L cylindrical vessel with an inner disposable bag that sits on a platform and is orbitally shaken. This reactor has shown some success in cultivation of recombinant BY-2 tobacco cells for production of human M12 mAb. Yields of 300–387 g FW L^{-1} and ~20 mg L^{-1} M12 were equivalent to yields obtained in shake flasks [113]. Single-use cultivation chambers obviate cross contamination of products or cultures from run to run and are less capital intensive, and because there is no need for sterilize in place (SIP) or clean in place (CIP) plumbing, they also have simpler accommodation requirements [114]. Plastics approved for use by FDA include polypropylene, polystyrene, polyethylene, polytetrafluoroethylene, or ethylene vinyl acetate, facilitating regulatory approval of the overall production process [114].

Moss can also be grown in many of these same bioreactors [103]; however, if light is needed for autotrophic growth, then a photobioreactor is required along with attendant challenges that are further addressed in Sect. 3.2.3. Indeed, successfully grew *P. patens* under GMP-certified conditions in 100 and 500 L wave bioreactors under artificial illumination, so for high-value products smaller volume reactors may be reasonable. If moss could be grown heterotrophically, then the same bioreactors described for cells and hairy roots could be employed. Nevertheless, Greenovation Biotech GmbH (www.greenovation.com/) is using its BryoTechnology™ platform to commercialize recombinant biopharmaceuticals. Several therapeutic enzymes targeted for enzyme replacement therapies, such as α-galactosidase, β-glucocerebrosidase, and complement factor H are under preclinical or Phase I development (www.greenovation.com/).

Modularity is a valuable tool in modern bioprocess design. Implementation of multiple, smaller scale, e.g., $\leq$500 L, reactors is useful in production facilities because it provides the ability to scale in response to demand, minimizes risks of loss due to contamination, and is less capital intensive. An example of a successfully scaled up commercial cell suspension culture is that by Protalix using carrot cells engineered to produce recombinant glucocerebrosidase as a replacement therapy for managing Gaucher's disease, taliglucerase alfa (Elelyso™) manufactured by Pfizer. The large-scale patented [115] cultivation system (ProCellEx™) for growing these carrot cells uses $\geq$400 L vertical conical-shaped plastic culture bags similar in design and operation to a BCR with aeration inlet ports near the bottom of the bag and a gas vent/regulator at the top to help maintain bag inflation (Fig. 1e). Rising gas bubbles from the gas inlet ports provides culture agitation and mixing. The large suspension-filled bags have a grid-like superstructure providing external support (Fig. 1e). The system is modular, thereby allowing cultivation and harvest cycles; a central unit provides aeration and nutrients [116]. Inoculant and culture media are provided to

each reactor, and excess air and waste gases are removed. All units are housed in a clean room. Schematics and details of the reactor technology are in US Patent 2010/0112700 A1.

3.2 Aquatic Plants

Some aquatic plants also are promising bioproduction platforms, including duckweed and microalgae. Many functional industrial enzymes or human therapeutics have been expressed in duckweeds or microalgae at lower cost than in vitro systems [85].

3.2.1 Duckweed

Duckweed, the common name for *Lemnaceae*, is a monocot plant family consisting of four major genera: *Lemna*, *Spirodela*, *Wolffia*, and *Wolffiella*. Duckweed is propagated clonally without the need for pollen or seeds, which simplifies line management, propagation, and the process feed stream [32, 117]. Duckweed is safe, fast growing in simple inorganic media (doubling time $\geq$ 36 h), capable of making complex proteins, and easy to grow and harvest and has a high protein content (up to 45% dry weight) [85, 118]. Cultivation only requires inexpensive upstream facilities and is very scalable. The absence of pollen or seeds also makes duckweed environmentally safer than other transgenic flowering plants. Moreover, duckweed is edible, offering an attractive system for oral delivery [119, 120].

Duckweed can be transformed using either biolistics or *A. tumefaciens.* Efficient nuclear transformation protocols for two species of duckweed, *L. gibba* and *L. minor*, were established in 2001 [121], and >20 recombinant proteins were produced with expression levels up to 25% of TSP [32]. Products include industrial enzymes, e.g., E1 endoglucanase [122], and many therapeutic proteins, e.g., mAb [123], plasminogen [124], interferon α2 [125], vaccine antigen avian influenza including H5N1 hemagglutinin [126, 127], and M2e peptide [117].

In 1997, US-based Biolex, Inc. developed the duckweed (*Lemna*)-based expression (LEX) system and successfully expressed at least 12 therapeutic proteins, including small peptides, mAbs, and large multimeric enzymes [2]. However, in 2012, Biolex's technology was sold to Synthon, a Netherlands-based pharmaceutical company, and interest in the LEX system subsided; research is currently focused on wastewater treatment (phytoremediation) and biofuel production.

3.2.2 Microalgae

Microalgae integrates the merits of microbes, including rapid growth and ease of culture with those of higher plants in performing posttranslational modification and photosynthesis, thereby offering another promising platform for cost-effective production of recombinant proteins [128–130]. Microalgae have a very simple structure, grow in simple media, and produce large amounts of biomass with short life cycles [131]. Downstream purification of proteins from microalgae is similar to yeast and bacterial systems and thus is generally less expensive than from whole plants

[132]. Many species also are generally regarded as safe (GRAS) for human consumption, thus providing a potential platform for oral delivery.

Both nuclear and chloroplast genomes of microalgae can be transformed for expressing heterologous proteins. However, due to the nuclear silencing, nuclear transformants generally accumulate less recombinant protein than chloroplast transformants [129]. Thus, the chloroplast expression system is currently regarded as more feasible for commercial production [128]. The disadvantage of chloroplast transformation is that this organelle lacks posttranslational modification capability, e.g., glycosylation [130]. However, this may provide a benefit for antibodies produced in chloroplasts, because glycan-free antibodies do not activate the immune system in humans [133, 134].

Chlamydomonas reinhardtii is the unicellular green algal species on which the majority of genetic engineering has been performed [128, 135, 136]. This alga grows fast with doubling time of ~10 h, supports easy nuclear and chloroplast transformation, and can be cultivated either photoautotrophically or with acetate as a carbon source [85]. A variety of high-value recombinant proteins, including antibodies, vaccines, growth factors, and industrial enzymes have been produced in microalgae from either nuclear or chloroplast transformation, as documented in several recent reviews [131, 137, 138]. Most of these were produced in the chloroplast of *C. reinhardtii*, but some were produced in other species, such as *Dunaliella salina* and *Phaeodactylum tricornutum*.

Development of economically viable bioproduction is still hampered by a lack of effective and consistent transformation methods for a wider variety of species, as well as low (nuclear expression) or inconsistent (chloroplast expression) recombinant protein yields [129]. PhycoBiologics (www.phycotransgenics.com) is currently attempting to commercialize microalgal production with indoor photobioreactors yielding axenic algae with >20% of TSP in the chloroplast (www.phycotransgenics.com) [132].

3.2.3 Scaling Up Aquatic Systems

Duckweed, moss, and microalgal aquatic species require light for autotrophic growth to achieve high biomass yields, which in turn affects product yields. Autotrophic cultivation requires either sunlight or significant artificial lighting intensity that is in close proximity to the cultured cells. Options include open systems such as ponds and raceways and closed systems such as tubular or plate photobioreactors. While possibly suitable for low-value commodity or industrial products, for products requiring strict GMP compliance, open systems are not recommended because they can be contaminated with a variety of other species and particulates and do not provide production source material consistency. There are also issues regarding environmental release of genetically modified species grown in an open pond. Although tubular or flat plate photobioreactors would seem best, these also have challenges. While the development of more cost-effective LED lighting is now available, these reactors cannot exceed certain culture depths. As culture density increases, light does not penetrate beyond several centimeters, and thus as the culture increases in density, growth decreases significantly. Although cGMP industrial

facilities now exist for moss cultivation, to our knowledge, there is none yet for microalgae [31]. For a more in-depth discussion of photobioreactor designs and limitations, see the review by Xu et al. [139].

3.3 Whole Plants: Stable and Transient Expression Systems

Whole plant expression still dominates plant-based bioproduction platforms and uses either transient expression via viral or nonviral vectors or stable transformation with transgenes targeted to either the nuclear or chloroplast genome. Stable transformation advantages include (1) a heritable transgene, permitting establishment of seed stock for future use, and (2) protein production scalable to field production. However, establishing and characterizing stable transgenic lines can be costly and time consuming. Other challenges include gene silencing, position effects, and GMO environmental concerns [132, 140].

When establishing stable transgenic plant lines, choices are often made regarding the gene integration site (nuclear vs. plastid), the subcellular compartment for recombinant protein accumulation (e.g., cytosol, apoplast, endoplasmic reticulum, and vacuole), and the plant tissue expression target (leaves vs. seeds). Decisions depend on a variety of considerations, including posttranslational modifications needed for protein functionality, stability of the expressed foreign protein in the plant host, desired expression levels of product, and downstream purification costs, as well as size and cost constraints of the product market [85, 95].

3.3.1 Stable Expression: Leaf Based

Leaf tissues of nonfood crops have traditionally been used as a viable expression platform for either research or production purposes. The first plant-produced recombinant protein, an immunoglobulin, was expressed in transgenic tobacco leaves [141]. Tobacco has emerged as the leading plant species for leaf-based protein expression [142] because it produces high biomass yields (~300 tons per acre), is genetically well studied, and is readily amenable to genetic engineering [15]. Importantly, tobacco is a nonfood, nonfeed crop, which minimizes regulatory barriers by eliminating the risk of plant-made recombinant proteins entering the food supply [143]. The first clinical trial of a plant-produced biopharmaceutical was the secretory antibody variant of Guy's 13 produced in field-grown tobacco leaves by Planet Biotechnology Inc. [144]. Besides tobacco, other leafy crops, e.g., lettuce, alfalfa, and clover, have also been exploited as an expression platform. Alfalfa is advantageous because it is a perennial that fixes nitrogen and displays notable homogeneity of N-glycosylated recombinant proteins [85, 145].

Both leaf nuclear and plastid genomes have been targeted for integration of heterologous genes. The choice of gene integration site is generally dictated by the posttranslational requirements of the target proteins. Nuclear integration is necessary for expression of functional glycoproteins, such as EPO and tPA, that need proper processing in the endo-membrane system [35]. Thus far >100 functional proteins have been successfully expressed in leaves with nuclear transformation. These

include mAbs [141, 146–148], vaccines [149], cytokines [150, 151], and industrial enzymes [152, 153]. See reviews [3, 84, 132, 154]. Unfortunately, low copy of gene integration, gene silencing, and proteolytic degradation in the aqueous environment of leaves resulted in <1% TSP [155, 156].

Each plant cell contains an average of 50–100 chloroplasts, and each chloroplast contains ~100 copies of its genome, thus the chloroplast enables thousands of copies of a given transgene to be expressed [157–159]. With chloroplast transformation, accumulation of recombinant protein up to 70% of the total leaf protein has been achieved [160]. See the special issue on Chloroplast Biotechnology in *Plant Biotechnology Journal* (June 2011). A wide range of proteins, ranging from very small antimicrobial peptides or hormones to very large viral or human proteins, have been successfully expressed in plant chloroplasts [161]. Except for exceptionally high-level expression, therapeutic proteins accumulated in the chloroplasts of lyophilized plant cells can be stored for several months or years without a decrease in their functionality, thereby eliminating costs of cold storage and transportation [162]. However, because the chloroplast does not support many important posttranslational modifications desired for expressing complex proteins, this technology is limited to production of proteins whose functions are independent of glycosylation. Another disadvantage of chloroplast transformation is the difficulty of transforming many important crop plants using current methods [163].

Chloroplast transformation also provides a unique bioproduction system for oral medicines [164]. When a pharmaceutical protein is accumulated in plant leaves, the plant cell wall can protect the protein drug from acids and enzymes in the stomach via bioencapsulation after oral delivery. However, microbes residing in the human gut have evolved to break down the plant cell walls to release the target protein [164]. If a transmucosal carrier (receptor-binding protein) such as cholera toxin subunit B (CTB) and heat-labile toxin subunit B (LTB) that binds to GM1 receptors is fused to the protein drug, it will efficiently cross the intestinal epithelium and be delivered to the circulatory or immune system [161]. Several drugs have advanced to the clinic, including glucocerebrosidase for treating Gaucher's disease [12], clotting factor IX for treating hemophilia B [165], myelin basic protein for treating Alzheimer's disease [160], acid α-glucosidase for treating Pompe disease [166], and, most importantly, a variety of oral vaccines for treating infectious diseases [158, 167]. See recent reviews [164, 168, 169].

Limitations of leaf-based platforms compared to seeds are the short shelf life of leafy tissue and the high variability of the production system. Plant leaves degrade faster upon harvest and must be processed immediately to ensure product stability and quality. Product yields in field-grown materials can be highly variable due to environmental changes (both biotic and abiotic). Finally, large-scale, regulatory-compliant disposal of transgenic biomass waste may have volume and cost implications [85].

3.3.2 Stable Expression: Seed Based

Recombinant protein expression targeted to plant seeds can overcome the major limitations associated with leaf tissue: protein stability and storage [170, 171]. Plant

seeds are naturally suited to produce and store proteins. High protein content (7–10%), low protease activity, and low water content make seeds an attractive alternative bioproduction platform [155, 156]. Antibodies or vaccines expressed in cereal seeds remained stable at ambient temperatures for years [85, 172]. In addition, edible seeds such as maize and rice have GRAS, status making them particularly suitable for developing oral vaccines that can be administered as flakes or flour with minimal purification [173]. In the past decade, significant advances have occurred using seeds as bioreactors including the commercialization of the first plant-derived recombinant protein, avidin, produced in maize seeds [170].

Cereal seeds, e.g., rice, wheat, barley, soybean, and maize, are commonly used as expression hosts [174]. Other typical production platform seed crops include legumes (pea, cowpea, and soybean), oil crops (canola and safflower), tobacco, and *Arabidopsis*. Stable expression yields up to 10% of total seed proteins have been achieved [170, 175]. Products include therapeutic proteins such as antibodies [176], vaccines [177], and cytokines [178]; industrial enzymes such as trypsin [179], phytase [180], and cellulase [181]; and biopolymers such as spider silk protein [182]. See recent review [173].

Maize seeds are the most widely used host for commercial therapeutic proteins and industrial enzymes [173]. Compared to other cereals, maize has a larger grain size, a higher proportion of endosperm ($\leq$82% of the seed), and a higher biomass yield per hectare at lower production costs [173, 183, 184]. Many industrial enzymes, including GUS, cellulase, laccase, and trypsin, have been successfully produced in maize seeds and marketed [27, 184]. Therapeutic proteins such as the HIV neutralizing antibody 2G12 [185], influenza virus H3N2 nucleoprotein [186], and α-galactosidase [187] in maize seeds were produced cost-effectively with simple downstream purification processes. Other seed-based platforms in commercial development include rice seed-produced human transferrin [188] by US-based Ventria Biosciences (www.ventria.com/) and barley grain-produced **e**ndotoxin-free growth factors and cytokines (Orfeus™ expression system) [189] by Iceland-based ORF Genetics Ltd. (www.orfgenetics.com/).

Seeds from some oil crops such as safflower and rapeseed have been used to develop a novel "oleosin-fusion" bioproduction platform, in which recombinant proteins are expressed as a fusion with oleosin, an endogenous protein that localizes within oil bodies of rapeseed and safflower [190, 191]. Fusion proteins accumulated in the oil bodies are easily separated in the lipid fraction from the bulk seed homogenate. SemBioSys Genetics, a Canadian biotech company, was developing and commercializing this technology for the low-cost "biosimilar" insulin [85], but operations ceased in 2012 due to financial problems.

Although a seed-based platform has many positive attributes, major hurdles still exist. Compared to the leaf-based platform, seeds have a relatively lower biomass and high possibility of gene leakage into the environment via the seed or from pollen dispersal [15, 192]. There is strong reluctance among scientists, regulators, and the general public to use seeds of major crops (e.g., maize, rice, and wheat) for recombinant protein production because of the possibility of contaminating the food chain [192]. Considerable time is also required to create high-expressing

transgenic plant lines [155]. However, the stability of foreign proteins, post-harvest processing, and overall cost makes the seed-based platform suitable for many recombinant proteins produced on a large scale [193].

3.3.3 Transient Expression

Transient expression is achieved either by epichromosomal expression of *A. tumefaciens* directly infiltrated into plant tissues (agroinfiltration) or by viral-based expression vectors [194, 195]. During transient expression, foreign genes are typically introduced into leaves of plants by vacuum infiltration of *Agrobacterium* containing genes of interest in an expression vector [85]. Recombinant protein production within the plant tissues (usually leaves) can be initiated quickly, within 24 h, continuing for several days depending on vector and target protein. Because a high copy number of foreign genes is introduced into each plant cell and free of "position effect" on gene transcription, the protein yields are usually higher in transient than in stably expressed plants [196]. Since there is no transgenic plant created, this also addresses regulatory issues and public concerns for GMO plants [197]. As a result, transient expression has been increasingly used for production of biopharmaceuticals, in particular, antigen vaccines and antibodies [195, 197].

N. benthamiana is the most common host plant for transient expression because it is amenable to genetic transformation and rapidly yields large amounts of biomass from seeds for scale-up production [198]. Potato, green pea, *Arabidopsis*, lettuce, and other *Nicotiana* species (e.g., *N. debneyi*, *N. excelsior*, and *N. simulans*) also serve as alternative hosts for transient expression [85]. There are two basic strategies for introducing transgenes into host plant cells for bioproduction: nonviral vector based or viral mediated [199, 200]. Nonviral vector-based expression using common plant expression vectors enables rapid accumulation of recombinant proteins in plant leaves, typically 2–4 days post-infiltration, with protein yields of 0.1–200 μg g^{-1} FW [85]. The viral-mediated expression process takes ~2 weeks for protein expression, but generally produces higher levels of recombinant protein up to 5.0 mg g^{-1} FW GFP [201]. Some effective viral vector-based expression platforms include Geneware® (Kentucky BioProcessing LLC) and magnICON® (Icon Genetics) based on a tobacco mosaic virus (TMV) RNA replicon and the geminiviral expression system based on a bean yellow dwarf virus (BeYDV) DNA replicon (Arizona State University) [202–204].

Transient systems also provide the ability to simultaneously co-express several genes to produce complex proteins, such as antibodies, viruslike particles (VLP), and other multichain proteins [195]. Numerous therapeutic proteins, especially those addressing sudden viral epidemics (e.g., an outbreak of Ebola, severe acute respiratory syndrome, or influenza pandemic) have been successfully produced by a transient expression system [132, 205, 206]. For example, Mapp Biopharmaceutical Inc. (San Diego, CA) used the geminivirus technology to transiently express the humanized antibodies MB-003 (Mapp) and ZMab in tobacco leaves (http://mappbio.com/z-mapp/). An optimized cocktail combining the best components of the MB-003 and ZMab was ZMapp™, which cured 100% of Ebola-infected rhesus macaques primates [207, 208]. In another example, plant-produced influenza

vaccine candidates, such as VLP antigens against avian flu (H5N1) virus and swine flu (H1N1) virus, were developed by the Canadian biotech company Medicago Inc. (Quebec, Canada) and have undergone Phase I and II clinical trials with positive results (http://medicago.com/pandemic-flu/). Plant-produced influenza vaccines are regarded as quicker to develop and potentially cheaper than egg-produced vaccines. Many other therapeutic proteins reportedly produced by the transient expression platform include IgG and IgA antibodies [209, 210], vaccine antigens against malaria, influenza and HIV [211–215], and therapeutic enzymes treating lysosomal storage diseases [216]; see recent reviews [140, 195, 217, 218].

Transient expression is easily applied to industrial scale for mass production of recombinant proteins. Milligram to gram quantities of target proteins can be rapidly produced in weeks to allow animal and clinical testing. Several plant biotech companies, including Kentucky BioProcessing LLC (Owensboro, KY), Medicago Inc. (Quebec, Canada), Fraunhofer CMB (Newark, DE), Mapp Biopharmaceutical Inc. (San Diego, CA), and iBio Biotherapeutics (Bryan, TX), have developed cGMP manufacturing facilities for biopharmaceuticals using vacuum agroinfiltration of *N. benthamiana* [219]. A major disadvantage of the transient system is the necessity of harvesting at a particular time. If using the product in humans or animals, an additional purification step to remove endotoxins derived from the infiltrated *Agrobacterium* is required [85]. Transient expression systems have been continuously optimized in the past decade for rapid, high-yield, and large-scale production of recombinant proteins, which expedites the acceptance of this production platform for the commercial production of a broad range of biopharmaceuticals.

3.3.4 Scaling Up Whole Plant Systems

While field production of plants provides a distinct advantage given its apparent low cost, considerations of weather, pests, and seasonal effects on productivity will increase costs. Similarly there are concerns regarding the risk of unintended environmental contamination of native gene pools by genetically modified field crops. An alternative is the use of controlled environment agriculture (CEA), where the transgenic crop is grown in glasshouses. Of course this increases production cost substantially, but may be worthwhile if there is high product value, e.g., for a therapeutic. CEA is not subject to seasonal variation, providing greater control over cultivation conditions and thus the quality of the crop and its contents. A number of companies have developed more or less automated cultivation systems under glass where product quality is maintained and aseptically handled in its final form. Medicago has developed such a CEA system whereby they hope to produce a number of different products via transient expression in tobacco. The production system begins after there is a synthesized gene of interest (GOI) from, for example, a virus. Then young *N. benthamiana* tobacco plants grown in pots in contained greenhouses are handled robotically including the vacuum infiltration step. Large pallets of plants are inverted into a solution containing the GOI and vacuum infiltrated. Afterward the inverted plants are drained and rotated back into vertical position and then moved into another more stringent culture facility for a 4–6-day

incubation for gene expression and protein synthesis. Plants are subsequently moved to a harvesting area where leaves are stripped by hand and then extracted. Using methods applicable to any other clinical grade in vitro or microbial product, the target protein is purified. The process is documented in this video: https://www.youtube.com/watch?v=lAk_HkFi9-s.

There are different GMP concerns for products produced from whole plants that extend beyond those for products produced from in vitro cultures (Fischer et al. 2012). Guidelines drafted in the USA by USDA and FDA exist to cover all platforms described herein (FDA-USDA 2002 http://www.gmp-compliance.org/guidemgr/files/BIOPLANT.PDF). These include selection of a crop species platform suitable for industrial production; in industry a diversity of platforms is problematic because of the need for consistent and stringent regulatory compliance, especially for therapeutics. Subsequently there should be an early stage determination of field vs. CEA cultivation. Similar to in vitro or microbial production, master seed or tissue banks must be established and maintained to provide batch to batch consistency. Isolation of GM plant material, controlled seed stock, APHIS/BRS permits for field-grown plants, appropriate confinement for transport of source plant material from field/greenhouse to production facility, control of harvested material and its transfer to the processing facility, waste biomass control, storage control, are all needed for production operations that can be validated.

Whole plant production can be further streamlined through second generation propagation via shoot regrowth from a retained axillary bud on the rootstock after harvesting the initial shoot Kim [220]. Functionality and yields of protein in harvested leaves from three successive harvests remained relatively consistent. Time required for seed production and germination and second and third generation shoots required >50% less time to harvest vs. initial planting. Another obvious alternative strategy would be rooting of clonal cuttings of transgenic plants to rapidly scale-up whole plants. Such a strategy should also minimize time to harvest as there is no need to generate seed or use in vitro micropropagules.

4 Commercialization Status and Outlook

Since conceptualization in 1989, the plant molecular farming industry has grown rapidly. While some plant-made industrial proteins/enzymes (e.g., avidin, GUS, and trypsin) and one plant cell-made pharmaceutical for human use (Elelyso™) have been commercialized, many are in various developmental stages. Examples of some successful plant-produced products, either on the market or in commercial development, are listed on the Molecular Farming website last updated in March 2014 (http://www.molecularfarming.com/PMPs-and-PMIPs.html), or in some recent reviews [50, 132, 221, 222]. The major biotech companies involved in research and development of plant-produced protein products that are still in business are listed in Table 3. These companies include the representatives of the very first commercial ventures (Large Scale Biology Corp., Planet Biotechnology, Ventria

Table 3 Companies involved in molecular farming over the past 25 years

Company name and country	Plant species/ expression platform	Main products	Status of products	Status of companies
Biolex Therapeutics, USA	Duckweed/stable expression – LEX platform	Anti-CD20 antibody, controlled release interferon α2b for the treatment of HCV and HBV, etc.	Preclinical and Phase I, II, and III clinical trials	LEX platform sold to Synthon in 2012
Epicyte Pharmaceutical, USA	Maize, tobacco/seed-based and leaf-based expression	Antibodies for infectious and inflammatory disease	Preclinical development	Acquired by Biolex in 2004
Fraunhofer IME, USA, Germany	Tobacco/leaf based	HIV antibody	Phase I clinical trials	Active
Fraunhofer CMB, Germany	*N. benthamiana*/ transient expression	Influenza vaccine	Phase I clinical trials	Active
		Malaria vaccine		
Greenovation Biotech GmbH, Germany	Moss (*P. patens*)/ stable expression – BryoTechnology™ platform	α-Galactosidase, β-glucocerebrosidase, and factor H	Preclinical and Phase I clinical trials	Active
Kentucky BioProcessing Inc., USA (formerly Large Scale Biology Corp.)	*N. benthamiana*/ transient expression – Geneware® platform	Aprotinin and pharma (antibody ZMapp™, vaccines, and therapeutic enzymes)	Aprotinin marketed by Sigma-Aldrich. Others under Phase I and II clinical trials	KBP (active now) offers contract biomanufacturing services; LSBC out of business in 2006
iBio Biotherapeutics, USA (formerly Caliber Biotherapeutics)	*N. benthamiana*/ transient expression	iBio-CFB03 for anti-fibrosis	iBio-CFB03 granted orphan drug designation in 2016	Active
Icon Genetics, Germany	Tobacco/transient expression – magnICON platform	Vaccine for non-Hodgkin's lymphoma	Phase I clinical trial	Purchased by Nomad Bioscience in 2012
Mapp Biopharmaceutical, USA	Tobacco/transient expression – magnICON platform	Antibody (ZMapp™)	Phase I and II clinical trials	Active

Medicago, Canada	Alfalfa, *N. benthamiana*/ transient expression – Proficia™ platform	Pharma (vaccines for influenza, rabies, and rotavirus)	Phase I and II clinical trials	Acquired by Mitsubishi Tanabe Pharma in 2013
Nomad Bioscience, Germany	Tobacco/transient expression – magnICON platform	Cellulase enzymes and antimicrobial proteins (colicins, lysins, and bacteriocins)	Development	Active
ORF Genetics Ltd., Iceland	Barley/seed-based expression	Growth factors and cytokines	Marketed for diagnostics, research, and cosmetics	Active
PhycoBiologics Inc., USA	*Chlamydomonas*/ algal-based expression	Vaccine growth factor and enzymes	Under development	Active
Planet Biotechnology, USA	Tobacco/leaf-based expression	CaroRx™ for dental caries; PBI-220	CaroRx™ under Phase II clinical trial; PBI-220 Phase I clinical trial	Active
		antibody for anthrax; DPP4-Fc for		
		MERS coronavirus infection		
Protalix Biotherapeutics, Inc., Israel	Tobacco or carrot/cell suspension culture – ProCellEx® platform	Pharma (β-glucocerebrosidase, α-galactosidase, DNase I, anti-TNFα)	One commercialized; others under clinical trials (Phases I/II/III)	Active
Synthon, Netherlands	Duckweed/stable expression – LEX platform	Anti-CD20 antibody for non-Hodgkin's	Preclinical development	Active
		lymphoma and thrombolytic drug		
Ventria Bioscience, USA	Rice/seed-based expression – ExpressTec platform	Lactoferrin and lysozyme; VEN120 for inflammatory bowel disease; VEN150 for HIV-associated chronic inflammation; nontherapeutics for basic research	Therapeutics under Phase II clinical trial or clinical development; nontherapeutic products marketed	Active

Bioscience, SemBioSys Genetics, and Protalix), companies established in the mid-1990s (Epicyte, Medicago, Biolex, Icon Genetics, Greenovation, and ORF Genetics), and companies that emerged from the earlier pioneers (Mapp Biopharmaceutical, Kentucky BioProcessing, and Nomad Bioscience) [219]. In 1999, Large Scale Biology Corporation (Owensboro, KY), now Kentucky BioProcessing, designed and opened the first cGMP manufacturing facility for production of recombinant therapeutics by using the plant-virus transient expression system Geneware®. For plant transient expression systems (vacuum infiltration of *N. benthamiana*) to rapidly manufacture vaccines in response to epidemics and outbreaks, the US Defense Advanced Research Projects Agency (DARPA) sponsored four pilot-scale cGMP manufacturing facilities in the USA, including Fraunhofer CMB (Newark, DE), Kentucky BioProcessing (Owensboro, KY), Medicago (Durham, NC), and Caliber Biotherapeutics (now iBio Biotherapeutics, Bryan, TX) [206]. So far, iBio Biotherapeutics runs the world's largest plant-based vaccine production facility with the capacity to process over 3500 kg of plant biomass per week (http://www.ibioinc.com/). With the advance in both technologies and manufacturing facilities, plant molecular farming is now reaching the stage at which it may challenge established microbial and mammalian bioproduction systems. Commercialization status of the three classes of plant-made recombinant products is briefly described as follows.

4.1 Plant-Produced Industrial Proteins/Enzymes

Due to ProdiGene's pioneering work on the development and commercialization of several hydrolases including avidin, GUS, lysozyme, trypsin (TrypZean™), and aprotonin (AproliZean™) in the late 1990s [3, 7, 223, 224], plant-made industrial proteins spearheaded commercialization over the other two classes of proteins [85]. However, ProdiGene went bankrupt in 2002 due to mishandling US field tests of maize genetically modified to make pharmaceutical products and received more than a $250,000 fine from USDA. Since then, efforts on commercializing plant-made industrial proteins have declined. Most of the research is now focused on production of biomass-degrading enzymes for the biofuel industry, e.g., cellulase, hemicellulase, xylanase, ligninase, α-amylase, and laccase [181, 225–227]. For example, an Arkansas-based start-up company, *Infinite Enzymes* (http://www.infiniteenzymes.com/), has been developing a cost-effective maize seed production system for cellulase enzymes for cellulosic biofuel production. It is noteworthy that in 2011, a transgenic variety of maize expressing thermostable α-amylase was approved by the USDA for commercial use as an improved biofuel feedstock. This transgenic maize line, marketed as Enogen™, was developed by Syngenta (http://www2.syngenta.com/en/index.html) to produce – within the kernel – an enzyme needed to break down starch for biofuel production. However, it sparked a controversy not only from anti-GMO organizations but also from some biotech supporters because of environmental and human health issues [228].

4.2 Plant-Produced Therapeutic Proteins

This class of protein products has attracted the most interest in research and development in molecular farming. More than 20 plant-made pharmaceuticals have been placed in preclinical or clinical trials to date [132], and specific product examples are further discussed.

4.2.1 Antibodies

Production of therapeutic antibodies (plantibodies) is of great interest for the plant molecular farming industry [209]. Although none to date has been approved by pharmaceutical regulators, several plant-made antibodies have made it to human clinical trials. For example, CaroRx™, a secretory IgA (SIgA) plantibody produced by Planet Biotechnology Inc., was the world's first clinically tested antibody for preventing adhesion of decay-causing bacteria to the tooth surface. Since 1999 CaroRx™ was subjected to Phase II clinical trials (topical) for dental caries in the USA under an FDA-approved Investigational New Drug Application [229], but was discontinued in early 2016. ZMapp™, a cocktail of three mAbs produced in tobacco leaves by Mapp Biopharmaceutical Inc. to combat the 2014 Ebola virus outbreak in Africa, underwent clinical Phase I and II trials in 2015 in the USA, Liberia, Sierra Leone, and Guinea, sponsored by the National Institute of Allergy and Infectious Diseases (NIAID) [132]. In September 2015, ZMapp™ was granted a fast-track status by the US FDA (http://mappbio.com). Although it has not yet received final approval by FDA, to date, ZMapp™ is the only drug that has been effectively used to treat patients infected with the Ebola virus [206]. In addition, a tobacco-derived HIV neutralizing antibody 2G12, produced by an EU funded project, Pharma Planta, recently completed a Phase I clinical trial [147].

4.2.2 Vaccine

Although several plant vaccines, for either animals or human, are now in clinical trials with encouraging results [229, 230], none thus far has been commercialized. Despite Dow AgroSciences receiving the world's first regulatory approval by USDA in 2006 for a tobacco cell-produced vaccine against Newcastle disease virus in chickens, it only remained a proof of concept; Dow AgroSciences never intended to market the product [231]. However, since the scale of animal vaccination is so large, plant-based systems may represent the only cost-effective production platform on a scale for which other (non-plant-based production) methods are not competitive. Furthermore, plant-made veterinary vaccines, such as those made in seeds, fruits, and leaves, can be orally delivered as part of the animal feed, thus offering great convenience and economy in immunizing large populations of animals on farms [232]. In terms of commercialization potential, regulatory approval for the plant-made veterinary vaccines can be significantly less onerous than that for human vaccines [233]. Therefore, the most likely near-term possibilities for commercialization of plant-derived vaccines will be the veterinary products. Key examples of plant-produced vaccines tested in target animal species, including those against avian influenza, foot-and-mouth disease, Newcastle disease, diarrheal disease caused

by enterotoxigenic *E. coli*, porcine reproductive and respiratory syndrome, and swine transmissible gastroenteritis, are listed in recent reviews [231, 234]. These antigens were expressed in potato tubers, rice seeds, corn seeds, and *N. benthamiana* leaves. With continuing efforts to optimize the bioproduction platform, successful commercialization of plant-made veterinary vaccines is imminent.

The recent outbreak of avian and swine flu spurred development of seasonal and pandemic influenza vaccines. Medicago developed the Proficia™ technology, a transient plant expression platform for rapid and high-yield production of vaccines and antibodies, in particular, the viruslike particle (VLP)-based antigen (VLP*Express*™ platform). Their products are at various stages of development. For example, the vaccine candidates against various influenza strains (e.g., H5N1 and H1N1) in Phase I and II human clinical trials were found safe and well tolerated, and potency was among the most effective of the industry (www.medicago.com). Mitsubishi Tanabe Pharma (Osaka, Japan) acquired Medicago in 2013 and announced that its tobacco-based flu vaccine could hit the market by 2018 or 2019. Fraunhofer CMB (Newark, DE) developed another type of plant-made VLP vaccine, Pfs25-VLP, for blocking malaria transmission [236]. The Phase I trial of Pfs25-VLP was completed in 2016 (https://clinicaltrials.gov/ct2/show/NCT01867463). One of the unique features of plant-made vaccines is that plants not only serve as the production "bioreactor" but can serve as the delivery vehicle for oral vaccines [236, 237]. Professor Charles J. Arntzen, a pioneer in plant-made oral vaccines at Arizona State University, has been developing plant-based oral vaccines including HBsAg (Phase I in lettuce and Phase II in potato), *Vibrio cholerae* vaccine, heat-labile toxin B subunit of *E. coli*, and the capsid protein of Norwalk virus (all are Phase I in potato) [1, 2, 85].

4.2.3 Therapeutic Enzymes

Therapeutics targeted for enzyme replacement therapies have been mainly developed by the Israel biopharmaceutical company Protalix Biotherapeutics. They developed and commercialized several recombinant therapeutic proteins through their ProCellEx® plant cell-based expression system (www.protalix.com). In 2012 Protalix commercialized Elelyso™ (taliglucerase alfa for injection), the very first plant-made therapeutic ever approved by the FDA for human use and has been marketed in the USA, Canada, and many Latin American countries (Uplyso™ in Latin America). Elelyso™ has comparable enzymatic activity and uptake in macrophages and is structurally homologous to Cerezyme® (imiglucerase), manufactured in CHO cells by Genzyme [57, 238]. Elelyso™, however, is ~25% less expensive than Cerezyme® because the plant cell-synthesized enzyme does not require further modification of its N-glycans for clinical use after bioproduction (terminal mannose residues are already exposed) [57]. Protalix's development pipeline for therapeutic enzymes includes PRX-102, a pegylated version of a recombinant human α-galactosidase enzyme for the treatment of Fabry disease; PRX-110, a chemically modified DNase I for the treatment of cystic fibrosis; and PRX-112, an orally delivered glucocerebrosidase enzyme for treatment of Gaucher's disease. To date,

Protalix has successfully completed a Phase I clinical trial of PRX-112 in Gaucher's patients. In June 2016, the company initiated its Phase III clinical trial of PRX-102.

There are also three moss-derived enzyme products targeted for enzyme replacement therapies: α-galactosidase for Fabry disease, β-glucocerebrosidase for Gaucher's disease, and factor H for atypical hemolytic uremic syndrome are under Phase I and/or preclinical development by the German biotech company, Greenovation (www.greenovation.com/). With the cGMP-compliant manufacturing process well established in Greenovation, the first moss-expressed biopharmaceutical is expected to come to market in the near future.

4.2.4 Other Therapeutics

Other plant-made therapeutics such as human growth factor and cytokines are in clinical trials or are already on the market. For example, using barley grain as a production host (Orfeus™ platform), ORF Genetics produced high-grade, animal-free, and endotoxin-free human growth factors and cytokines (ISOkine™ growth factors and cytokines) targeted for stem cell research (www.orfgenetics.com/). About twenty ISOkine™ products, such as IL-3, GM-CSF, EGF, TNF, SCF, etc., are also on the market (http://orfgenetics.com/ISOkine/ProductList/). Commercialization of several moss-made human growth factors, including FGF7/KGF (keratinocyte growth factor), EGF, and HGF (hepatocyte growth factor) intended for mammalian cell culture, has also been achieved by Greenovation [77]. FGF7/KGF is the first commercially available moss-made human protein. In addition, PRX-106, an oral formulation of anti-TNFα for treatment of immune and inflammatory-mediated disorders, is through Phase I clinical trial by Protalix, showing that the drug was safe and well tolerated and had gut biological activity and induction of regulatory T cells (www.protalix.com).

4.3 Plant-Produced Biopolymers

Compared to industrial enzymes and therapeutic proteins, commercial development of plant-made protein biopolymers lags. The major biopolymer products expressed in transgenic plants include collagens [239], spider silk proteins [22, 182], elastin-like polypeptides [15], and plant gum [240], which are promising biomaterials for regenerative medicine and tissue engineering as well as for the food industry [241].

5 Conclusions

Large-scale production and commercialization, especially of therapeutic proteins using plant production platforms, has passed proof of concept. While there are always challenges for each product and downstream process efficiency can certainly be improved, the technology for producing recombinant proteins in plants is past its infancy. More work is still needed to realize the full potential for plant-made industrial proteins and enzymes. Although not all industrial sectors have equally

progressed, the list of successfully commercialized plant-produced recombinant proteins is rapidly growing, suggesting a bright future for the biotechnology industry.

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Hairy Roots and Phytoremediation

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Abstract

Contamination of the environment arises either from natural geological processes or due to human activities and has created an alarming situation worldwide. Biological strategies for cleaning up contaminated biosphere have gained much importance in recent years and are preferred over other conventional physical and chemical methods because these are environmentally friendly and cost-effective. Phytoremediation is an ecologically compatible approach using plants to remediate polluted environment. Currently hairy roots have emerged as a notably competent research tool for phytoremediation among the various biological systems investigated for this purpose. Infection of certain plants caused by *Agrobacterium rhizogenes* is expressed in the form of hairy root disease. The disease is characterized by adventitious roots with copious root hairs developing elaborately from or next to the infection site. The plant genome receives a set of genes from a segment of the large root inducing (Ri) plasmid of *A. rhizogenes.* Under the effect of these genes, the inherent hormonal balance of the plant is altered resulting in the development of hairy roots. In nature, plant roots are the primary organs having contact with the environmental contaminants. Thus, hairy roots have been used in phytoremediation research as physiologically they resemble the normal roots of the mother plants. Several studies demonstrate the potentiality of hairy roots in removing a vast array of both organic and inorganic pollutants from the environment. In addition, microorganisms colonizing the rhizosphere of hairy roots have also proved to improve the efficacy of hairy roots in eliminating contaminants. The purpose of this review is to summarize the applications of hairy roots in different phytoremediation strategies and provide examples and prospects of the use of hairy roots in the removal of organic and inorganic contaminants from the environment.

Keywords

Agrobacterium rhizogenes · Hairy roots · Inorganic pollutants · Organic pollutants · Phytoremediation

Abbreviations

2,4-DCP	2,4-dichlorophenol
AMF	Arbuscular mycorrhizal fungus
Cd	Cadmium
cv	Cultivar
DDT	1,1,1-trichloro-2,2-bis-(4′-chlorophenyl)ethylene
DNA	Deoxyribonucleic acid
FTIR	Fourier transform infrared spectroscopy
GC–MS	Gas chromatography–mass spectrometry
h	Hour
HPLC	High-performance liquid chromatography
kb	Kilobase
min	Minute

NADH–DCIP reductase	Nicotinamide adenine dinucleotide reduced–dichlorophenolindophenol reductase
Ni	Nickel
OBZ	Oxybenzone
PCB	Polychlorinated biphenyl
Px	Peroxidase
TCE	Trichloroethylene
T-DNA	Transferred DNA
TNT	2,4,6-trinitrotoluene
U	Uranium
UV	Ultraviolet

1 Introduction

Contamination of the biosphere is a crisis severely threatening the welfare of all living organisms. Contaminants polluting the environment may be classified as organic and inorganic and are released either naturally through geological processes (viz., erosion, saline seeps) or from anthropogenic activities and extensive industrialization (viz., agriculture, construction, wastewater treatment, mining, melting, military activities, chemical works, electroplating, energy and fuel production, sludge dumping, pharmaceuticals, paper mills, tanneries, textile plants, etc.) [1–5]. These pollutants are ecotoxic substances which are either non-biodegradable or their degradation is very slow, leading to their accumulation in the biosphere, ultimately upsetting the harmony of the ecosystem. Development of remediation technologies to prevent this disruption is of utmost importance. Various techniques (viz., incineration, irradiation, soil washing, pump and treat, surfactant flushing, activated carbon adsorption, or extraction) are available for cleaning up the contaminated environment, but in most cases the methods are expensive and less efficient, require application of huge labor, and cause disorders in the soil or produce by-products which further enhance environmental toxicity [4, 6–8]. Thus, these methods have got limited public acceptance [4]. Consequently research efforts were diverted toward the development of other efficient and reliable technologies which would make the environment cleaner and healthier.

Biological methods score higher over conventional physical and chemical methods in being cost-effective and eco-friendly. Bioremediation of contaminated sites by indigenous microbial flora, a process commonly called natural attenuation, presents certain drawbacks such as prolonged time requirement, production of hazardous by-products, and difficulty in restoration to normal environment [9]. **Phytoremediation** is a technology where plants are being used to clean up environmental contaminants and is ecologically harmless for restitution and remediation. It is less expensive, does not cause any invasion, is safer than conventional strategies [3, 10], and is thus attracting attention worldwide. Other advantages and limitations of phytoremediation have been discussed in our previous review [11].

The principal mechanism of phytoremediation is based on the solar energy-driven uptake of chemicals from polluted air, water, and soil by plants [10]. The two most vital aspects of phytoremediation are the eradication of pollutants from contaminated sites, also known as **phytodecontamination** and pollutant stabilization, thereby preventing its transport and toxic effects [12].

Although the method was originally developed for removing heavy metals from the soil [13], phytoremediation now relies on the ability of certain plant species to uptake, tolerate, assimilate, detoxify, and store a diverse range of pollutants present in the environment and render them harmless [8]. The recent era of "-omics" has made metabolism of diverse pollutants by plants more effective, and practical technologies are being developed for improving the applicability of phytoremediation [8, 14].

Based on the type of pollutant present and the decontamination strategy applied by the plants, phytoremediation treatments can be of various types, viz., phytoextraction, phytodegradation, phytostabilization or phytosequestration, phytovolatilization, rhizoremediation, rhizofiltration, or phytofiltration [10, 15, 16]. The process by which contaminants are absorbed from the soil and translocated to aerial plant parts is called **phytoextraction** and has been successfully applied for the removal of edaphic contaminants [10, 17]. "Hyperaccumulating" plants, i.e., unusual plants capable of absorbing, storing, and tolerating huge amounts of heavy metals in the foliage, play a crucial role in phytoextraction. Compared to normal plant species growing under identical conditions, these hyperaccumulator species have the capability to accumulate about 100-folds more concentration of heavy metals [17, 18], without affecting the plants in an adverse manner. About $<0.2\%$ of angiosperms [19, 20] can hyperaccumulate heavy metal ions, and the phenomenon is reported from members of 45 angiosperm families [4, 21]. **Phytomining**, on the other hand, involves commercial recovery of accumulated metals from plants by ashing, smelting, or liquid extraction [22]. In **phytodegradation,** also known as **phytotransformation** [23], certain organic pollutants such as chlorinated hydrocarbons, polycyclic aromatic hydrocarbons, herbicides, trichloroethylene, and explosives are degraded either through endogenous plant enzymes or enzymes which are secreted [10, 24]. The three phases of metabolism include transformation, conjugation, and compartmentation, leading to the detoxification and breakdown, ultimately accumulating the contaminants [23, 25]. The tropical leguminous tree *Leucaena leucocephala* and *Populus* hybrids [26–28] have been frequently used for phytodegradation. **Phytostabilization** or **phytosequestration** is a strategy, where instead of removing the contaminant from the soil, the polluted soil is stabilized by plants to check the movement of the contaminant to the surrounding neighborhoods [12, 17]. Weathering of contaminated soil by natural elements is reduced by planting of vegetation at the polluted site. Another approach of phytostabilization is to prevent leaching by the addition of various chemicals and organic matter, which prevent solubilization of the metals [17]. After uptake by the roots, contaminants can be transported to the aerial parts from where they can be volatized through **phytovolatilization** [15]. **Phytoimmobilization** denotes the immobilization of pollutants taken up by plants in a soil containing mineral or a geomat (mineral-

containing mat) [3]. Certain pollutants cannot be completely degraded by plants only [29]. In such cases, their alliance with bacteria, colonizing the rhizosphere, has been proposed to enhance phytoremediation potential, a process known as **rhizoremediation** [29, 30]. Plant root exudates provide the microbial population with nutrients and energy; the microbes in turn degrade contaminants, assisting plants in pollutant remediation. **Phytofiltration** or **rhizofiltration** is the plant-aided elimination of pollutants from aquatic bodies [3]. Usually, a variety of these processes utilizing plants are associated with remediation. However, in-depth analysis of plant metabolic pathways, the enzymes involved therein, and understanding the mechanism of tolerance are needed for greater applicability of phytoremediation strategies.

Currently hairy roots have emerged as an important tool for phytoremediation research among the different biological systems investigated for this purpose.

2 Hairy Roots

The history of hairy roots and their causative agent dates back to the early 1900s. The bacterium causing the hairy root syndrome was initially named *Phytomonas rhizogenes* [31]. *P. rhizogenes* later came to be known as *Agrobacterium rhizogenes*, which is a Gram-negative bacterium present in the soil. It induces hairy root syndrome in higher plants, which can be distinctly identified by adventitious roots with copious root hairs developing elaborately at or next to the infection site [32, 33]. The Ri (root inducing) megaplasmid (>200 kb) [32, 34, 35] determines the infectivity of *A. rhizogenes*. Distinct segments of DNA, the "transferred DNA" or "T-DNA," carried by the plasmid are transferred to the plant genome [32]. Hairy roots originate from plant wound sites following infection with *A. rhizogenes* resulting in transfer of T-DNA genes from bacteria to the plant followed by their stable integration and expression in the plant genome. Pacurar et al. [36] and Chandra [37] recently studied in details the molecular mechanism lying behind the genetic transformation of plants by different strains of *Agrobacterium*. A number of genes of the Ri plasmid, viz., the *vir* genes (located in the virulence region), which are not delivered into the plant and a group of genes residing on the bacterial chromosome (*chv*) [14, 38] are essential for T-DNA transfer from the bacteria to the plant cell. Another set of genes, the *rol* genes, located in the T-DNA, affect the development and phenotype typical to the hairy root disease. Ri-transformed root cultures have been reported for more than 500 plant species, most of them being dicotyledons [23]. However, with improvements in strategies, new species of plant or species which are recalcitrant to transformation are being utilized to produce hairy roots.

Biotechnological research has advanced greatly based on the immense potential of hairy roots for commercial exploitation. Highly branched, plagiotropic hairy roots grow fast, indefinitely on medium without phytohormones under aseptic conditions and are characterized by genetic and biochemical stability over extended periods [14, 32]. They are often called "phytochemical factories" as these roots can biosynthesize

compounds naturally produced in the native plant roots, and the amounts are often analogous to or more than that of the roots and shoots of mother plants [14, 39, 40]. The laboratory maintenance of hairy root cultures is a low-cost method and requires simple tissue culture procedures. Furthermore, for proper functioning, these roots need not be associated with aerial plant organs and remain free of microbes. Currently, hairy roots are being used for the production of valuable 'secondary metabolites' of plant origin, for expression of genes for the production of foreign proteins to be used for therapeutic purpose (viz. antibodies, vaccines, cytokines), enzyme production, molecular farming, elucidation of biosynthetic pathways, biotransformation of exogenous substrates and environmental decontamination by phytoremediation [41–49]. Great advances have also been made in culturing hairy roots in bioreactors and optimizing conditions for large-scale production [49, 50].

The present review deals with the instances of the use of hairy roots for phytoremediation; relevant studies demonstrating the tolerance, metabolization, and storage of a vast array of organic and inorganic contaminants by plant cells using hairy roots of different plant species are discussed.

2.1 Hairy Roots in Phytoremediation: A Rational Approach?

Among the different experimental systems currently utilized for research on phytoremediation, hairy root cultures of various plant species have proved to be promising experimental tools and suitable isolated model systems to understand not only the mechanisms associated with the elimination or decomposition of contaminants but the activity of pivotal enzymes involved in detoxification processes as well. Hairy roots can be transformed genetically [14] and because of their genetic stability, foreign proteins can be produced by them for a long term [51]. The resultant functional proteins might be involved in metabolization of environmental pollutants. Moreover, introduction of foreign genes from plants, animals, or microbes along with *A. rhizogene*-mediated transformation of suitable plants results in the production of proteins by the hairy roots which are capable of metabolizing chemical compounds. These genes can also be overexpressed via genetic transformation of hairy roots to improve these metabolic traits further. Hairy root cultures serve as model experimental system for standardization of variables prior to large-scale field application of a particular remediation technique. Keeping this in mind, Flocco and Giulietti [52] designed protocols concerned with *Armoracia lapathifolia* hairy root production and utilization of the same in detoxification of phenol, a model organic compound.

The initial reactions against the environmental pollutants take place in the roots which are the primary organs having contact with the contaminants [14]. In terms of physiology, hairy roots are more similar to normal roots than undifferentiated cell cultures. Another advantage of hairy roots is that they use common metabolic pathways to metabolize harmful compounds [53]. They can be propagated indefinitely and their prolific growth rates shorten their subculture period, thus yielding stable and large amounts of biomass over the whole year, independent of the season.

This is a necessary prerequisite for phytoremediation research. This is also helpful as large surface area of the hairy root mass comes in contact with the contaminants. Moreover, hairy roots grow in microbe-free environment, and thus it is possible to discriminate the exact role of plant root cells against those of microorganisms residing in the rhizosphere in removal of soil contaminants. Hairy roots are organs without shoots, a characteristic aiding in elucidating the mechanisms of root remediation only, bypassing the event of translocation. Easy regeneration of whole plants from hairy roots is another attribute which can be exploited for clonal selection of plants with appropriate phytoremediation potentialities. Their organized nature makes them suitable candidates for cultivation in bioreactors for studying remediation processes on a large scale and can be reused consecutively in several cycles [54–56]. They also allow medium to be manipulated easily and make the availability of end products easier, thus reducing the procedures of purification [14]. Hairy roots are also advantageous in the sense that they can produce root exudates which cause detoxification and sequestration of harmful pollutants by the action of secretory enzymes or heterologous enzymes which influence the secretory pathway. However, these areas of research are still not completely elucidated. Hairy roots have also contributed to our understanding regarding the compartmentalization and nature of a few of the end products of detoxification processes [55] and the effects of contaminants and their metabolism on certain physiological and biochemical processes, viz., antioxidative stress responses and lipid peroxidation [57]. In addition, hairy root cultures act as indicators of the plants capable of phytoremediation as these roots carry the genetic capacity of its parent plant to transform a particular compound [8].

However, certain disadvantages are also associated with the use of hairy root cultures for phytoremediation research, viz., application on a large scale might become complex in some cases, aseptic culture conditions are required, sugars are required in the culture medium, and maintenance of such cultures could be a bit expensive for some species [14].

Nevertheless, the use of hairy root in phytoremediation research is well documented (as will be described in the following sections) and reviewed widely [5, 11, 14, 58, 59]. Figure 1 demonstrates the application of hairy roots in phytoremediation research.

3 Remediation of Organic Pollutants by Hairy Roots

Several reports demonstrate the phytoremediation of different organic pollutants by hairy roots of different plant species as follows:

3.1 Phenols and Chlorophenols

Aromatic compounds are present in industrial effluents resulting from coal processing, coke ovens, petroleum refineries, as well as manufacture of phenolic resins, fiberglass, herbicides, pesticides, disinfectants, and other activities including paper,

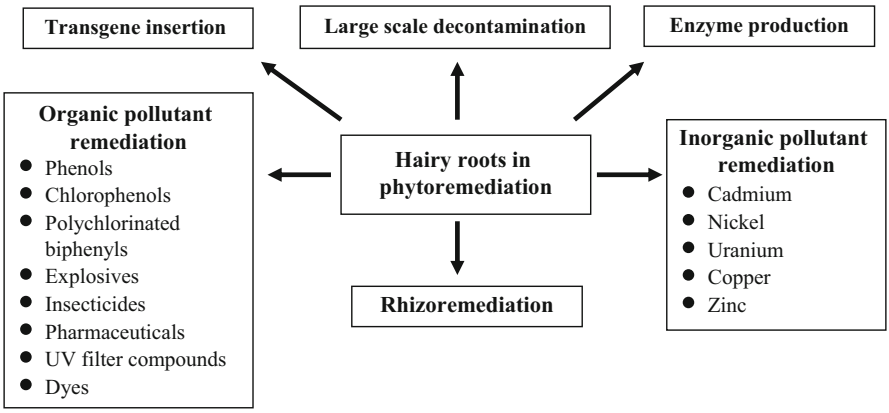

Fig. 1 Schematic representation of the application of hairy roots in phytoremediation research

wood, metal, and plastic industries [60–64]. The fractional degradation of certain aromatic organic pollutants (viz., polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and some surfactants) is also responsible for the release of phenolic compounds [14, 65]. Phenols are categorized as one of the major classes of hazardous pollutants as they are toxic and recalcitrant to degradation and have low volatility [5, 66]. Phenol exposure may cause liver damage, hemolytic anemia, blindness, and nervous disorder and is also alleged to cause paralysis [62, 67]. Decontamination processes for eradication of phenol by conventional and commonly used techniques applied to contaminated areas are less efficient and expensive, and the metabolic by-products often have higher toxicity than phenols [6, 54, 61, 62]. In recent years, hairy roots derived from different plant species have not only been successfully applied for the removal of phenol from aqueous solutions but also to test the tolerance capacity of plants to high concentrations of this pollutant. The effect of 2-week-old hairy roots of different species obtained through transformation using *A. rhizogenes* LBA 9402, on the detoxification of phenol from the culture medium, was studied by Singh et al. [61] demonstrating that the remediation of phenol by hairy roots was species dependent. They observed that *Brassica juncea* was the most prospective species for phenol removal, removing 97% phenol, followed by *Beta vulgaris*, *Raphanus sativus*, and *Azadirachta indica* in the absence of H_2O_2 supplementation to the medium. Plant peroxidases (Px), mainly those restricted to the cell walls, are crucial for the removal of toxic compounds. Chemical assays related to contaminant removal potential and kinetics of root extracts as well as purified Px suggested the formation of "inextricably bound residues" resulting due to covalent bond formation between plant cell wall and hydroxylated pollutants and metabolites [68]. They are categorized as the chief enzymes behind the removal of phenolic compounds [14]. In their study, Singh et al. [61] also demonstrated that exposure to phenol increased Px activity in the roots. H_2O_2 was synthesized in situ and its level was enhanced in the presence of phenol thereby eliminating the necessity of addition of H_2O_2 to the culture medium.

The susceptibility of hairy roots to phenols and chlorophenols was investigated by Araujo et al. [62]. They demonstrated that hairy roots of *Solanum aviculare* were most efficient for phenol remediation, removing 98.6% from the medium within 72 h followed by *Ipomoea batatas* and *Daucus carota*. *D. carota*-transformed roots were most effective in removing 2,6-dichlorophenol (2,6-DCP). However, inherent Px activity was highest in *I. batatas*, in the presence of phenol, demonstrating that transforming efficiencies and Px activity in the root cultures were not directly correlated.

Lycopersicon esculentum cv Pera hairy roots derived by *A. rhizogenes* LBA 9402-mediated transformation of sterile leaf explants were also found to be suitable for removing phenol from water [63]. Conditions (pH, temperature, etc.) for efficient removal of phenol with minimum inactivation of the enzymes possibly involved in the removal process were optimized. Doubly transgenic tomato hairy roots engineered for overexpression of *tpx1* (basic tomato peroxidase 1, p*I* 9.6) have also been tested for enhanced removal of phenol from aqueous solutions; Px activity was higher in the transgenic hairy roots overexpressing *tpx1* and *Nicotiana tabacum* hairy roots which were double transgenic for *tpx1* and *tpx2* (tomato peroxidase genes) as compared to the hairy roots developed following infection with wild-type strain [57, 68].

In another study, Coniglio et al. [64] observed that up to 500 mg l^{-1} phenol could be removed by hairy roots of *Brassica napus* developed following transformation with wild-type *A. rhizogenes* LBA 9402 in the presence of exogenously added H_2O_2. The authors reported that removal efficiency was maximum within 1 h of treatment and the hairy roots when reused for consecutive cycles, the efficiency for removing phenol gradually decreased, together with a decrease of Px activity. They suggested that this decline in Px activity could be due to its inactivation caused by H_2O_2 or irreversible bonding between Px and phenyl or phenoxy radicals formed during oxidation of phenolic compounds. Competitive inhibition by the end product for the Px active site or the inaccessibility of root biomass due to adsorption of the polymer might be the other causes of reduction in Px activity. But in contrast to studies by González et al. [63], the authors demonstrated that acidic Px might play the crucial role in phenol removal, rather than the basic and neutral Px.

Hairy roots of another plant species *Helianthus annuus* could effectively metabolize phenol at concentrations ranging from 100 to 400 mg l^{-1} [69]; however, a decline in phenol removal efficiency was noted with increasing phenol concentrations. When *l*-proline was added to the reaction mixture, more than 90% phenol (100 mg l^{-1}) was removed after 24 h. Px activity was also induced in the roots in the presence of phenol, and catechol was detected as a major metabolite in the process of biodegradation. Treatment with hairy roots also reduced toxicity of phenol solutions in comparison to untreated solutions.

Mazaheri and Piri [70] reported the phenol-metabolizing potential of *Atropa belladonna* hairy roots up to a concentration of 500 mg l^{-1} from wastewater in the presence of H_2O_2 within pH ranging from 4.0 to 9.0. Reuse of the hairy roots in the fifth cycle showed decrease of phenol removal efficiency from 98% to 62%. Toxicity tests using *Lactuca sativa* seeds revealed that the treated solution had less toxic effect than the initial solution.

Chlorophenols are another group of hazardous compounds which are recalcitrant to degradation, extremely toxic, and widely distributed in the biosphere [71]. Several chemicals utilized in agriculture and industries such as herbicides (viz., 2,4-dichlorophenoxyacetic acid), pesticides, germicides, resins, and antiseptics are manufactured using 2,4-dichlorophenol (2,4-DCP), a substituted phenol [54], which is released to the environment through industrial effluents. Exposure to this toxic compound for extensive periods is harmful to both aquatic organisms and human beings [5, 72]. Thus, efforts have been made toward the remediation of 2,4-DCP using eco-friendly phytotechniques. Agostini et al. [54] demonstrated that hairy root cultures developed by *A. rhizogenes* LBA 9402 inoculation of *B. napus* leaves could remove 2,4-DCP from solutions in concentrations ranging from 100 to 1,000 mg l^{-1} in the presence of external H_2O_2 (5–10 mM), within an incubation span of 15 min to 1 h, probably due to the effect of Px. Reuse of the roots for six successive removal cycles (with a high efficiency of ~90% after six cycles) distinguished the results from those of Singh et al. [61] and Coniglio et al. [64] and is thus suitable for continuous decontamination purposes in a large scale. 2,4-DCP was also efficiently (98%, 88%, and 83%) removed by tobacco hairy root cultures in a short time for solutions containing 250, 500, and 1,000 mg l^{-1}, respectively [55]. When 10 mM H_2O_2 was used, 500 mg l^{-1} 2,4-DCP was removed in 60 min. The hairy roots could also be reused for almost three consecutive cycles. The role of Px in 2,4-DCP dehalogenation was suggested.

Isolation of pure enzymes is expensive and the enzymes are more readily subjected to inactivation during reaction. The studies discussed so far indicate that enzyme isolation might not be an obligatory prerequisite for decontamination processes, and hairy root biomass or extracts from the roots might be used as economical enzymatic systems for eradication of phenol from contaminated waters. Tissues of the roots might act as protective and stabilizing agents, thereby avoiding enzyme inactivation.

The removal of 2,4-DCP by hairy roots in the presence of H_2O_2 was applied by Angelini et al. [56] in a novel study related to eradication of the contaminant in a large scale using a discontinuous stirred tank reactor. The authors achieved 98% removal of 2,4-DCP using *B. napus* hairy roots in 30 min with reduction to 86% after six consecutive cycles.

3.2 Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) add to the list of recalcitrant chlorinated aromatic pollutants frequently encountered in the ecosystem. These are carcinogenic, mutagenic, teratogenic, and immunotoxic [73]. PCBs are used in the production of electrofluids, hydraulic lubricants, gas turbines, paints, plasticwares, pesticide extenders, adhesives, dedusting agents, cutting oils, flame retardants, heat transfer fluids, etc. PCBs affect human beings after entering the food chain and accumulating in the adipose tissue [74–77]. Thus, removal of PCBs from the environment is important, and considerable scope of PCB remediation has been shown by

phytoremediation. The ability of hairy roots of different species to degrade PCB has been studied. A patent had been obtained by Morita et al. [78] who described that *A. belladonna* hairy roots could absorb and decompose significantly large amounts of PCBs and dioxins in comparison to the natural roots of the plant, thereby providing a cheaper treatment. Kučerová et al. [79] and Rezek et al. [73] demonstrated that hairy root cultures of black nightshade (*Solanum nigrum*) could successfully metabolize an array of PCB congeners. Rezek et al. [77] also demonstrated that *S. nigrum* hairy root-mediated transformation of PCBs produced known hydroxy-PCBs as well as novel metabolites such as methoxy-PCBs and hydroxymethoxy-PCBs [80]. Lack of knowledge on the effect of these compounds has opened up interesting challenges in the field of metabolism of these compounds by plants.

3.3 Pharmaceuticals

Bioremediation by hairy roots has also proved to be an inexpensive and green technology that has been used for the removal of other organic pollutants as well. Aquatic bodies and drinking water often contain pharmaceuticals and the metabolites produced from them. Of these compounds, some [viz., antibiotics and *N*-acetyl-4-aminophenol/acetaminophen (paracetamol), a broadly used analgesic, antipyretic, and anti-inflammatory agent] are detrimental to aquatic organisms. Hairy root cultures of *H. annuus* developed by *A. rhizogenes* 15834 were effective in removing tetracycline and oxytetracycline from liquid media. The cell-free exudates of the hairy roots also showed similar activity. The rate of modification was found to decrease with the increase in oxytetracycline concentration and increase with the increase in age of culture [81]. The authors also suggested that reactive oxygen species (ROS) and not enzymatic catalysis was responsible for the modification of antibiotics. Huber et al. [82] demonstrated that *Armoracia rusticana* (horseradish) hairy roots could absorb and detoxify the xenobiotic compound paracetamol. It was suggested that paracetamol-glucoside, a predominant metabolite, is stable and nondegradable as glucoside serves as a precursor to cell wall lignin-associated insoluble residues. Such studies paved way for remediation of pharmaceuticals from wastewater using plants.

3.4 Explosives

Another area of considerable concern is the phytoremediation of explosives, which are hazardous. The most prevalent and persistent among the explosives is 2,4,6-trinitrotoluene (TNT) and its metabolization is extremely difficult. Research has been largely directed to analyze the ability of plants to alter TNT. Uptake and transformation of TNT have been reported by periwinkle (*Catharanthus roseus*) hairy root cultures; products of transformation and their chemical characteristics were identified and the transformation events leading to removal processes were studied [83, 84]. Using *A. rusticana* hairy roots, Nepovím et al. [53] analyzed the effect of explosives, viz., 2,4-dinitrotoluene (DNT), TNT, aminodinitrotoluenes

(ADNTs), and diaminonitrotoluenes (DANTs), on the activity of certain enzymes, viz., glutathione S-transferase (GST) and Px, involved in the metabolism of pollutants upon exposure to the pollutants for different time intervals.

3.5 Insecticides

Hairy root cultures have emerged as a vital tool in research related to the remediation of insecticides. One of the most commonly used pesticides, DDT [1,1,1-trichloro-2,2-bis-(4′-chlorophenyl)ethylene], has gained popularity over decades owing to its broad-spectrum activity, easy and cheap formulation, and high residual biological activity. However, inclusion in the food chain has resulted in carcinogenesis and endocrinal abnormalities in human beings [85]. *Cichorium intybus* and *B. juncea* hairy root cultures (obtained by inoculation of germinated seedlings with *A. rhizogenes* 15834) were used for the uptake and breakdown of DDT [86]. Their work also suggested that endogenous enzymes from the roots assisted in the degradation of this persistent insecticide.

3.6 Dyes

Dyes are another class of xenobiotic chemicals which are produced industrially. The largest group of dyes, azo dyes, is recalcitrant to biodegradation [87]. Various industries (viz., textiles, leather, plastics, cosmetics, food processing) are presently using a huge number of dyes including approximately 2,000 types of azo dyes, as coloring agents [14, 87]. Certain azo, xanthene, and anthraquinone dyes are toxic and mutagenic agents which may lead to severe health issues and affect aquatic life when discharged into water bodies [88–90]. Although categorized as harmful compounds, till date there are very few reports on remediation of these pollutants utilizing hairy roots. Hairy root cultures of marigold (*Tagetes patula*) could remove reactive red 198 dye up to concentrations of 110 mg l^{-1} and could be consecutively used for five successive cycles of decolorization. GC–MS analysis revealed that the dye was transformed into nonhazardous metabolites [91]. These roots could also decolorize other dyes, viz., golden yellow HER, methyl orange, orange M2RL, navy blue HE2R, and reactive red M5B after 10 days. However, in another study, 92% decolorization of methyl orange was achieved within 4 days using hairy root cultures of *B. juncea*, suggesting that these hairy roots are extremely potential for degrading textile dyes [92].

Studies demonstrate the association of certain intracellular enzymes, viz., laccase, lignin Px, tyrosinase, NADH–DCIP reductase, azoreductase, and riboflavin reductase with the decolorization of textile dyes by plants or hairy roots [91, 93–95]. These observations led to the purification and characterization of an intracellular laccase from *B. juncea* hairy roots which was applied for removing textile dyes [92]. Among the various redox mediators studied, 2, 2′-azinobis, 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) appeared to be the most appropriate in step-up of the decolorization rate of laccase, suggesting that the combination of

laccase and ABTS could be efficiently used for treating textile dyes. These outcomes demonstrate the feasibility of using enzymes and/or other compounds from hairy root exudates for dye detoxification/sequestration purposes.

Recently, hairy root cultures of the facultative halophyte *Sesuvium portulacastrum* were induced by inoculating pre-cultured leaf and stem explants with *A. rhizogenes* NCIM 5140 [96]. The roots could degrade a wide variety of textile dyes (viz., reactive orange 14, reactive pink MB, reactive red 2M5B, reactive green 19A-HE4BD, Remazol yellow 3GL, Remazol navy blue RGB, Remazol blue RGB, Remazol yellow RGB, and red brown H4R) after 5 days of incubation. Maximum decolorization (98%) was noted in the case of reactive green 19A-HE4BD used at concentration of 30 mg l^{-1}. However, decolorization efficiency was reduced when higher concentration (150 mg l^{-1}) of the dye was used. Degraded nature of the dye was confirmed by HPLC and FTIR analyses. Also, nontoxic nature of the products of degradation was demonstrated by germination assay using seeds of *Phaseolus mungo*.

3.7 Trichloroethylene

Industrial effluent and a xenobiotic compound, trichloroethylene (TCE), is a key pollutant. P450 2E1, an enzyme from mammalian liver, metabolizes TCE. Hairy roots of *A. belladonna* expressing a P450 2E1 enzyme from rabbit were able to metabolize TCE [51].

3.8 UV Filter Compounds

In recent times, another compound which is gaining much attention as a contaminant is oxybenzone (OBZ) or benzophenone-3, frequently used in cosmetics which serve as UV filter in sun tans and skin protectants. It is detected in water from swimming pools and surface water samples, and treatment of wastewaters is not adequate to get rid of this contaminant completely. Aquatic organisms tend to accumulate OBZ and related metabolites which induce abnormalities of the endocrine and reproductive system [97–100]. Richardson's water analysis [101] has categorized OBZ as an emerging pollutant since 2005. A very recent study by Chen et al. [102] demonstrates that hairy root cultures of *A. rusticana* could remove more than 20% of the initial amount of OBZ from the medium after 3 h of exposure, with oxybenzone-glucoside and oxybenzone-(6-*O*-malonyl)-glucoside being identified as novel metabolites. These findings paved the way for future applications of hairy roots in remediation of UV filter compounds.

4 Remediation of Inorganic Pollutants by Hairy Roots

Anthropogenic activities and extensive industrialization have resulted in the release of various types of inorganic pollutants, viz., heavy metals, metalloids, and radionuclides into the environment. These accumulate largely in agricultural and

industrial areas, affecting crop yields, soil biomass, and fertility, finally entering into the food chain [14, 103, 104]. A few of the heavy metals, viz., manganese (Mn), iron (Fe), copper (Cu), zinc (Zn), and nickel (Ni), are essential micronutrients for the normal development of plants and animals, playing key roles in the induction and reaction of enzymes, membrane function, activity of isozymes, etc. [105]; these metals might become toxic at higher concentrations. Heavy metals cannot be destroyed by any chemical or biological means [5] and thus significantly contribute to environmental pollution. Currently, phytoremediation techniques are gaining much commercial importance for the elimination of hazardous inorganics from the environment, and hairy roots, in particular, have proven to be competent tools for investigating the underlying mechanisms involved in metal uptake, accumulation, and tolerance. Extraction and sequestration of metals by plants as well as the physiological and biochemical processes lying behind metal accumulation have also been elucidated utilizing hairy roots as model systems. Remediation of different inorganic pollutants is being mentioned in the following sections.

4.1 Cadmium

Cadmium (Cd) is a heavy metal contaminant derived from the use of fertilizers, sewage sludges, compost, and from metallurgical industries [5]. Nedelkoska and Doran [105] developed transformed roots of Cd hyperaccumulator species *Thlaspi caerulescens* with *A. rhizogenes* 15834 and compared with transformed roots of non-hyperaccumulator species *N. tabacum* and *A. belladonna*. They reported that Cd accumulation was 1.5–1.7-fold greater in roots of *T. caerulescens* than in the hairy roots of *N. tabacum* and *A. belladonna. T. caerulescens* hairy roots continued to grow till 100 ppm Cd, whereas roots of *N. tabacum* turned brown under similar treatment.

A comparative study on Cd tolerance was carried out by Wu et al. [106] using hairy roots *Adenophora lobophylla*, an endangered species, and *A. potaninii*, spread broadly in the same habitat. They demonstrated that although closely related, the plants might use different metabolic strategies for detoxification of Cd. *A. lobophylla* synthesized a high amount of phytochelatin, whereas *A. potaninii* harbored a Cd removal system and maintained GSH (reduced glutathione) level in the cell via homeostasis, together with phytochelatin synthesis.

Boominathan and Doran [107] also used hairy roots of the Cd hyperaccumulator *T. caerulescens* to study the function of antioxidative metabolism in tolerance of heavy metal. Their work demonstrated that although growth is unaltered by heavy metals, oxidative stress induced by metals occurs in hyperaccumulator tissues. Their results also suggested that enhancement of the antioxidative defenses of plants like *N. tabacum* (which have high biomass and are non-hyperaccumulating) is required in order to genetically improve the metal hyperaccumulating characteristics of these plants. Overexpression of certain enzymes like catalase and/or superoxide dismutase in addition to other molecular approaches could be considered.

A huge literature indicates the involvement of organic acids in heavy metal tolerance, transport, and storage in plants [88, 108–115]. Although high concentrations of citric, malic, and malonic acids are a constitutive property of *T. caerulescens* and *Alyssum bertolonii* hairy roots hyperaccumulating Cd and Ni, respectively, after exposure to 20 ppm Cd and 25 ppm Ni, 13% Cd and 28% Ni taken up by the respective roots were associated with the organic acids [116]. Cd was mostly found in *T. caerulescens* cell walls, similar to the results of Nedelkoska and Doran [105], whereas Ni was located in the symplasm of *A. bertolonii* hairy roots. Treatment with diethylstilbestrol, a plasma membrane H^+-ATPase inhibitor, resulted in retention of viability of *T. caerulescens* roots, while concentration of Cd in the symplasm was increased by about six times. However, similar treatment caused reduction in Ni transport across the plasma membrane and root viability in *A. bertolonii*. Hence, plasma membrane depolarization does not affect hyperaccumulation of Cd in *T. caerulescens* hairy roots. However, Ni hyperaccumulation is negatively affected in *A. bertolonii* hairy roots under such conditions.

4.2 Nickel

Ni hyperaccumulation by hairy roots of *Alyssum tenium*, *A. bertolonii*, and *A. troodii* was reported by Nedelkoska and Doran [117]. Using long-term hairy root cultures, the authors demonstrated that Ni tolerance and hyperaccumulation are not essentially shoot dependent or dependent on transport from root to shoot. Hairy roots and whole plants also varied noticeably in their capacity to uptake Ni; hairy root-regenerated plants of *A. tenium* were much more tolerant to Ni, accumulating greater amounts compared to the hairy roots of the same species. Shoots of *A. murale* were inoculated with *A. rhizogenes* A4M70GUS [118] producing hairy roots, and the shoots regenerated from the roots could accumulate up to 24,700 μg g^{-1} dry weight Ni.

4.3 Uranium

Brassica and *Chenopodium* can uptake heavy metals from aqueous solutions [119]. To exploit such potentiality, hairy root cultures of *B. juncea* and *C. amaranticolor* were induced by *A. rhizogenes* A4 and these were utilized for the removal of uranium (U) from solutions [119]. Up to a concentration of 5,000 μM U, the efficiency of *B. juncea* hairy roots was found to be two- to fourfolds greater than that of *C. amaranticolor* and thus appeared to be more suitable for U removal from contaminated solutions. Also, 97% U uptake was noted in *B. juncea* roots in medium lacking phosphate compared to only 40% uptake in medium containing phosphate. Reduction in the levels of free uranyl cations and uranyl hydroxides due to formation of uranium–phosphate complex in the presence of phosphate was suggested as the cause of greater U uptake in *B. juncea* hairy roots in the absence of phosphate. In contrast to these results, Soudek et al. [120] demonstrated that the presence of phosphates could stimulate accumulation of U

by hairy roots of *A. rusticana*. Straczek et al. [121] studied U toxicity on *D. carota* hairy roots. Under the experimental conditions, U accumulation was recorded to be 4–563 mg kg^{-1} fresh weight in the presence of 2.5 and 20 mg l^{-1} U in 34 days with progressive decrease in the threshold of U toxicity for root length over a period of time. Such accumulation level would be suitable in terms of contaminated soil.

4.4 Copper

Uptake and tolerance of Cu were analyzed using *Hyptis capitata*, *Polycarpaea longiflora*, and *Euphorbia hirta* hairy root cultures [122]. While similar levels of Cu uptake were noted in *H. capitata* and *P. longiflora* hairy roots, *E. hirta* hairy roots accumulated lower amounts of Cu. The authors also demonstrated the biphasic uptake of Cu by *H. capitata* hairy roots, with faster accumulation during the initial phase as compared to the second phase.

4.5 Zinc

Zn is another dominant heavy metal contaminant commonly encountered in water bodies. Subroto et al. [123] reported that up to 98% Zn was taken up from the culture medium and accumulated by *S. nigrum* hairy root cultures, and thus these roots can be used as an efficient means for the remediation of areas polluted with Zn.

5 Phytoremediation by Hairy Root–Microorganism Association

An emerging area of interest in the field of phytoremediation is **rhizoremediation** or phytoremediation assisted by bacteria, which refers to the confluence of phytoremediation and microbial bioremediation [30, 124]. This approach employs microorganisms residing on or near plant roots, called rhizospheric microorganisms. Apart from the removal of contaminants, plants are also benefitted by the presence of these rhizospheric microbial communities as they can provide plants with important nutrients, can protect plants by reducing plant stress hormone levels, or can protect them from plant pathogens [125]. Natural substances exuded by the plants in turn create a nutrient-rich environment for the microorganisms, which boost up their biological activities and help in the degradation of pollutants by induction of enzymes in the microbial populations. This synergism between plants and the rhizospheric microbes can result in either enhanced elimination or breakdown of harmful compounds. As mentioned, hairy roots of different plant species have proved to be ideal model systems to study different aspects of phytoremediation. But to our knowledge, there are very few reports demonstrating association of hairy roots with microbes which would provide useful basis for enhancing the process of phytoremediation. *Burkholderia* and *Agrobacterium* are two genera of microorganisms

which have been shown to degrade phenolic compounds [126, 127]. *Burkholderia* species are a very diverse group of organisms, thriving on rhizosphere surrounding crop plants such as coffee, maize, legumes, tomato, etc. [128]. With the aim to improve phenol phytoremediation techniques, *B. napus* hairy root cultures growing on phenol-supplemented media were inoculated with *B. kururiensis* KP 23 or *A. rhizogenes* LBA 9402 [29]. A promotive effect on phenol eradication from the medium was noted by the existence of both the microorganisms; phenol removal was improved by 34% by co-inoculation of *B. kururiensis*, whereas a 40% improvement was noted in the presence of *A. rhizogenes* compared to roots without the rhizobacteria. The results were encouraging enough to test the rhizoremediation potential of whole plants using these strains.

Another Gram-negative bacterial strain, *Pantoea* sp. FC 1, resistant to phenol and chromium [Cr (VI)] was used in association with *B. napus* hairy roots to test the potentiality of this system in rhizoremediation of phenol and chromium [Cr (VI)] [30]. Significant enhancement in phenol and Cr (VI) removal efficiencies were noted by the hairy roots inoculated with the bacterium than in non-inoculated hairy roots, indicating a positive effect of the co-inoculation process. Their results indicated that *B. napus* hairy roots–*Pantoea* sp. FC 1 association would be an attractive alternative for the rhizoremediation purposes.

The effect of symbiotic association between arbuscular mycorrhizal fungus (AMF) and hairy roots on phytoremediation has also been studied. Ibáñez et al. [129] observed that colonization of transgenic hairy roots of *N. tabacum* with *Glomus intraradices* resulted in higher activity of antioxidative enzymes, viz., Px, superoxide dismutase, and ascorbate peroxidase in the presence of phenol. Furthermore, these roots showed lower oxidative damage when exposed to phenol in comparison to wild-type hairy roots colonized by AMF.

6 Conclusions

The importance of phytoremediation lies in its being an effective and optimal model system for the elimination of harmful contaminants from the biosphere. In this aspect, the application of hairy roots has proved to be beneficial. The success of the technique is based on the ability of hairy roots to predict the response of a particular plant species to the pollutant, and thus these roots are important means for screening the plants for phytoremediation. The hairy roots also help in better understanding of plant–soil microbe interaction for rhizoremediation. The challenge lies in applying the knowledge obtained from experimental systems using hairy roots to polluted field sites. Furthermore, the efficiency of phytoremediation ability of a particular species might be enhanced by introduction and expression of genes producing degradation enzymes, proteins capable of binding heavy metals, etc., in hairy roots developed by *A. rhizogenes*-mediated genetic transformation. However, in-depth elucidation of xenobiotic detoxification pathways, enzymes involved, and rate-limiting steps are necessary for optimization of experimental setups using hairy roots. As hairy roots originating from a single plant can be propagated extensively, difficulties arising due to variability among individual specimens can be overcome.

Since hairy roots do not possess identical characteristics to whole plants, they may serve as supporting systems to provide information prior to whole-plant field trials. Rapid rate of hazardous contaminant accumulation in the environment due to either geological or man-made reasons necessitates the utilization of hairy root cultures in phytoremediation, keeping in mind their stable nature, fast growth rate, and easy maintenance.

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