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Víctor M. Loyola-Vargas
Neftalí Ochoa-Alejo *Editors*

Plant Cell Culture Protocols

Third Edition

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Edited by

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Preface

Cell culture methodologies have become standard procedures in most plant laboratories. Today, facilities for in vitro cell cultures are found in practically each plant biology laboratory, serving different purposes since tissue culture has turned into a basic asset for modern biotechnology, from the fundamental biochemical aspects to the massive propagation of selected individuals.

The subject of this book is taught around the world as part of undergraduate and graduate courses in agronomical and biological sciences. However, the apparent simplicity of cell culture technology should not lead to mistakingly consider it as trivial. It would be very difficult to understand modern plant biotechnology without in vitro cell cultures and even today, the application of recombinant DNA technology to important crops is hampered by the poor embryogenic or morphogenetic response of in vitro cultured tissues.

This third edition of Plant Cell Culture Protocols follows a similar plot as its predecessors. It also pursues similar goals, i.e., to provide an updated step-to-step guide to the most common and applicable techniques and methods for plant tissue and cell culture. A total of 29 chapters, divided into five major sections, have been included. Topics selected cover from general methodologies, such as culture induction, growth and viability evaluation, statistical analysis and contamination control, to highly specialized techniques, such as clonal propagation, haploid production, somatic embryogenesis, organelle transformation, passing through the laborious process to measure the epigenetic changes in tissue cultures. The protocols are currently used in the research programs of the authors or represent important parts of business projects aimed to generate improved plant materials. Two appendices have also been included; the first of them discusses common principles for the formulation of culture media and also lists the composition of the eight most commonly used media formulations. The second appendix compiles a list of useful Internet sites for cell culture scientists. A total of more than 100 sites have been selected, based on the quality of the information offered in them, as well as on their users' friendliness.

We hope that readers will find this version of Plant Cell Culture Protocols, which belongs to the Methods in Molecular Biology series, as a helpful source of information in their research projects, since this has been its real purpose.

We would like to thank the authors of each chapter for responding to our constant requests for the materials, despite their reckless work schedules. They made us possible to carry this job to its completion.

Finally, we should express our profound gratitude to Professor John Walker for his trust in our experience to complete this project. We have certainly enjoyed the opportunity to interact with colleagues from all over the world.

Mérida, Yucatán, México
Irapuato, Guanajuato, México

Víctor M. Loyola-Vargas
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Chapter 1

An Introduction to Plant Cell Culture: The Future Ahead

Víctor M. Loyola-Vargas and Neftalí Ochoa-Alejo

Abstract

Plant cell, tissue, and organ culture (PTC) techniques were developed and established as an experimental necessity for solving important fundamental questions in plant biology, but they currently represent very useful biotechnological tools for a series of important applications such as commercial micropropagation of different plant species, generation of disease-free plant materials, production of haploid and doublehaploid plants, induction of epigenetic or genetic variation for the isolation of variant plants, obtention of novel hybrid plants through the rescue of hybrid embryos or somatic cell fusion from intra- or intergeneric sources, conservation of valuable plant germplasm, and is the keystone for genetic engineering of plants to produce disease and pest resistant varieties, to engineer metabolic pathways with the aim of producing specific secondary metabolites or as an alternative for biopharming. Some other miscellaneous applications involve the utilization of in vitro cultures to test toxic compounds and the possibilities of removing them (bioremediation), interaction of root cultures with nematodes or mycorrhiza, or the use of shoot cultures to maintain plant viruses. With the increased worldwide demand for biofuels, it seems that PTC will certainly be fundamental for engineering different plants species in order to increase the diversity of biofuel options, lower the price marketing, and enhance the production efficiency. Several aspects and applications of PTC such as those mentioned above are the focus of this edition.

Key words: Aseptic culture, Genetic modified organisms, Large-scale propagation, Metabolic engineering, Plant cell culture, Techniques

1. Introduction

Plant cell, tissue, and organ culture (PTC) techniques were developed and established as an experimental necessity for solving important fundamental questions in plant biology, but they currently represent very useful biotechnological tools for a series of important applications such as commercial micropropagation of different plant species, generation of disease-free plant materials, production of haploid and double-haploid plants, induction of epigenetic or genetic variation for the isolation of variant plants, obtention of novel hybrid plants through the rescue of hybrid

embryos or somatic cell fusion from intra- or intergeneric sources, conservation of valuable plant germplasm, and is the keystone for genetic engineering of plants to produce disease and pest resistant varieties, to engineer metabolic pathways with the aim of producing specific secondary metabolites or as an alternative for biopharming. Some other miscellaneous applications involve the utilization of in vitro cultures to test toxic compounds and the possibilities of removing them (bioremediation), interaction of root cultures with nematodes or mycorrhiza, or the use of shoot cultures to maintain plant viruses. With the increased worldwide demand for biofuels, it seems that PTC will certainly be fundamental for engineering different plants species in order to increase the diversity of biofuel options, lower the price marketing, and enhance the production efficiency. Several aspects and applications of PTC such as those mentioned above are the focus of this edition.

PTC technology also explores conditions that promote cell division and genetic reprogramming in in vitro conditions and it is considered an important tool in both basic and applied studies, as well as in commercial application (1).

The theoretical basis for plant tissue culture was proposed by Gottlieb Haberlandt in 1902. He predicted that eventually a complete and functional plant could be regenerated from a single cell. Although all multicellular organisms share almost the same life cycle fate, plant cells in contrast with animal cells have the quality to be totipotent. This means that a single cell can become a complete plant and backwards, and all this is governed by a precise and regulatory mechanism during cell division. This regulation is what makes one organism different from another and roots from leaves. The knowledge of the events that govern the transformation of that single cell into a complete and functional individual lies at the core of the understanding of life itself.

Recently, some of the regulatory steps that govern the totipotentiality and cell differentiation have been uncovered. PTC propitiates the best condition to induce or repress some key genes for the morphogenesis process, allowing the formation of new structures in vitro that otherwise cannot be formed under natural conditions.

PTC is a set of techniques for the aseptic culture of cells, tissues, organs, and their components under defined physical and chemical conditions in in vitro aseptic and controlled environment (Fig. 1). Throughout the years, the techniques developed from the early 1960s to the mid-1980s (see Chapter 2) are found today in practically each plant biology laboratory and have turned into a basic asset to modern biotechnology. These tools are used for different purposes, from massive propagation of selected individuals to fundamental biochemical aspects.

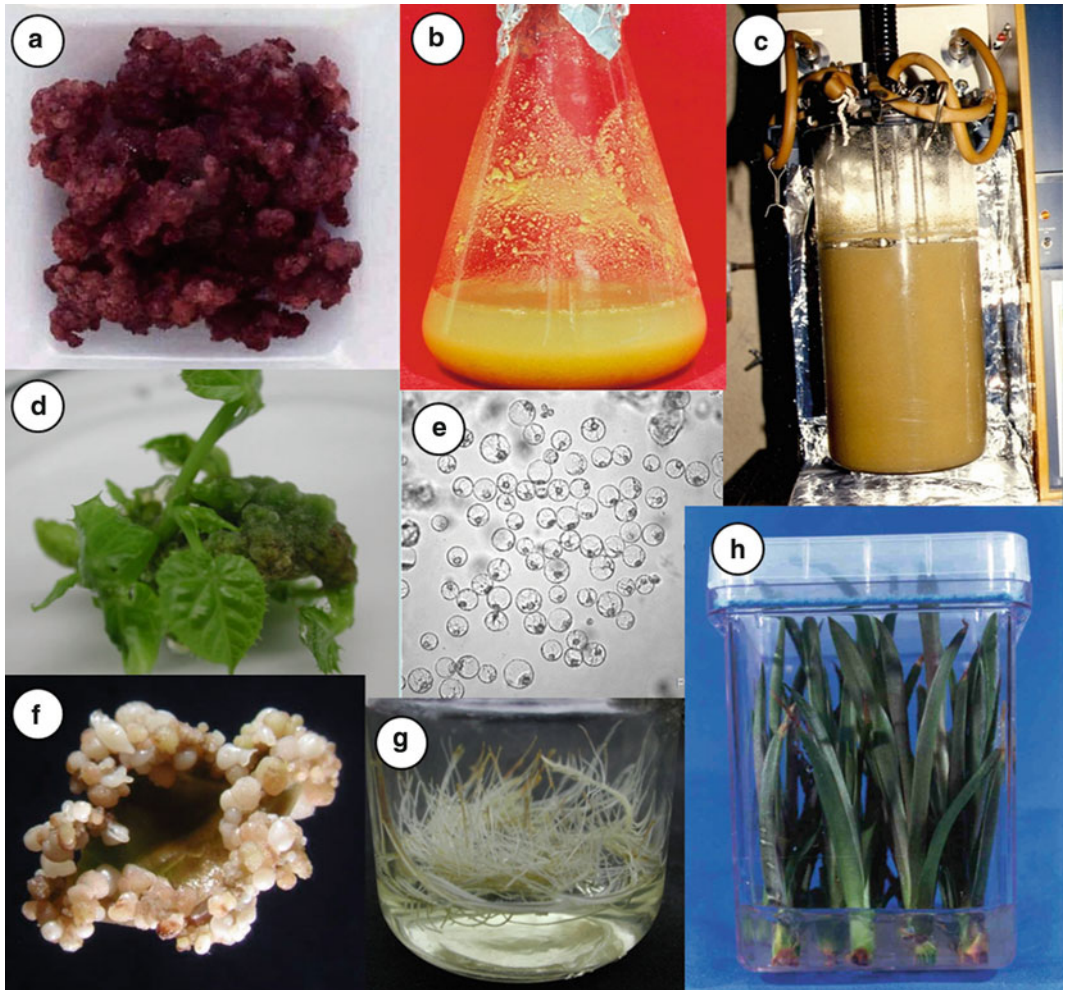


Fig. 1. (a) Callus from *Coryphantha* spp. (b) Suspension culture from *Canavalia ensiformis*. (c) Scale-up *C. roseus* suspension cultures. (d) Regeneration of *Jatropha curcas* plants from callus. (e) Protoplasts from *Coffea arabica*. (f) Somatic embryogenesis in *Coffea canephora*. (g) Root culture from *Daucus carota*. (h) Micropropagation of *Agave fourcroydes*. Pictures a, b, c, d, f, and g are from the authors' laboratories. Pictures e and h are a gift from the laboratories of Dr. Teresa Hernández-Sotomayor and Dr. Manuel Robert, respectively, from Centro de Investigación Científica de Yucatán.

2. General Aspects of Cell, Tissue, and Organ Culture

It is of primary importance for those who are using plant tissue culture techniques for the first time to know about the history of those researchers whose contributions led to the development of these experimental systems and biotechnological tools. Professor Thorpe (Chapter 2) had written a wonderful article about the main historical facts in PTC advances.

The basic principles of in vitro plant tissue culture involve the selection of the adequate explant from a plant source, the subsequent surface sterilization to eliminate microbial contaminants,

inoculation in a proper culture medium to allow growth and differentiation of the tissue, incubation under controlled conditions, and finally the adaptation of in vitro regenerated plants to greenhouse conditions. Different culture systems are usually used such as cell cultures (cell suspensions and protoplasts), tissue cultures (callus and differentiated tissues), and organ cultures (anthers, embryos, meristems, shoots, and roots). These systems are applied for several basic and biotechnological goals, as mentioned before. Some aspects of the general procedures, basic principles, and applications of PTC have been included in this edition (see Chapters 3–6).

3. Plant Micropropagation

After the demonstration of totipotency in plants, the potential of PTC for vegetative or clonal propagation was first recognized. Clonal propagation implies that the same genetic background of donor plants is maintained through the next plant generations.

Although micropropagation techniques are theoretically applied to all plant species, they are recommended for those species that are usually asexually propagated or in cases of seed-recalcitrant species. Undoubtedly, micropropagation has been the most important commercial application of in vitro cultures, and many companies worldwide are currently producing millions of clonal plants from different species. Micropropagation is one of the most used applications of PTC with commercial proposes, mainly in ornamentals (2–7) and medicinal plants (8), although it has also been used in some important crops such as potato, banana (see Chapter 11), herbaceous plants (see Chapter 12), or some forest tree species (*Pinus*, *Eucalyptus*, *Cedrela*, etc., see Chapter 10). There are three ways by which micropropagation can be achieved; these are enhancing axillary bud breaking, production of adventitious buds directly or indirectly via callus, and somatic embryogenesis directly or indirectly on explants (9, 10). Culture conditions, mainly nitrogen source, light regime, temperature, and the container's atmosphere can play critical roles in favoring bud development in in vitro plants (11–15).

Although the propagation protocols for a number of plant species were developed in the 1960s, transition of in vitro to ex vitro conditions even now frequently represents a bottleneck step. Therefore, successful commercial propagation protocols also require procedures for the establishment of plants to field conditions in addition to the efficient multiplication of plantlets (see Chapters 10 and 11). The massive production of embryos, and their later development into entire plants, can also provide a methodology for the propagation of selected materials (see Chapter 14).

4. Genetic Improvement

Genetic improvement in crops is mainly used to produce resistant plants or plants with high phenotypic or organoleptic qualities. Microspore and anther culture (see Chapters 13 and 17) is one of the PTC applications that allows for the regeneration of haploid plants exhibiting unique alleles in just one set of chromosomes. When these haploid plants are treated with the alkaloid colchicine, double-haploid plants with homozygous sets of chromosomes are produced. This phenomenon is used for the generation of isogenic or homozygous lines in one generation in comparison with 5–10 cycles of autogametic reproduction that usually take using the traditional breeding techniques. Furthermore, it is possible to fix hybrid characteristics from parental crosses faster than through the backcrosses usually employed by conventional methods.

Hybrid plants from partially sexual compatible parental species (interspecific crosses) that do not occur naturally can be produced by rescuing and culturing *in vitro* the hybrid embryos before seed abortion (see Chapter 18). Alternatively, it is possible to generate interspecific or even intergeneric hybrids between sexually incompatible plants through somatic fusion using two parental protoplast sources. However, very often these somatic hybrids are unfertile, which prevent their further utilization in breeding programs.

5. Genetic Engineering

PTC in combination with recombinant molecular biology techniques have been exploited to introduce and integrate foreign genes from any source (microorganisms, plants, or animals) into the plant genome with the aim of conferring a new characteristic to the transformed or transgenic plant. This process called genetic transformation is carried out by biological or physical methods, the most common being through the infection with *Agrobacterium tumefaciens* and by microparticle bombardment (biolistic) (see Chapters 27 and 28). By using this technology, different important crops such as corn, cotton, and soybean, among others, have been manipulated to improve their resistance/tolerance to pests, herbicides, or diseases caused by virus, and more recently, production of transgenic corn resistant to drought has been achieved, opening new perspectives for increasing the food production worldwide. Millions of hectares of genetically modified crops are currently under culture in the USA, Argentina, Brazil, Canada, and China, and to a lesser extent in countries such as Paraguay, Mexico, and India, among others.

There is a great concern regarding the possibility of genetic contamination of wild species from transgenic crops. Since the ecological impact of this type of genetic contamination is still unpredictable, some approaches have been developed to reduce or to eliminate this risk; that is the case of organelle genetic transformation (see Chapter 29).

Undoubtedly, plant genetic transformation has been also a key tool for basic studies in Plant Biology. Regulation of gene expression analysis in plant tissues and organs, and the study of gene function are some of examples of applications of genetic transformation.

6. Preservation and Conservation of Plant Germplasm

Plant genetic resources are of fundamental importance, since they are the primary sources of genetic variability for breeding and crop improvement programs. In general, germplasm from sexually propagated species are conserved in the form of seeds maintained in dry environments and at low temperatures for long time. However, germplasm from species that are usually propagated through vegetative methods or that produce recalcitrant seeds implies that tubers, rhizomes or some vegetative organs must be conserved. This is not an easy task, since very often the volume of vegetative material that should be handled represents a problem involving more space and higher expenses in comparison with conservation of seeds. Furthermore, vegetative organs must be preserved under specific conditions for each plant species. PTC, under minimum growth or through cryopreservation, has been used for plant germplasm conservation (see Chapters 7 and 16). Institutions such as the International Potato Center in Peru or the IPK Gatersleben, Genebank Department, Foundation Leibniz, Institute of Plant Genetics and Crop Plant Research (IPK) in Germany are currently applying these systems as an alternative for germplasm conservation. Since plant tissue cultures are under aseptic conditions, this can allow the exchange of germplasm around the world without quarantine.

7. Epigenetics in the Process of Development and In Vitro Culture

In vitro cultures represent an advantageous system for the study of different processes in the cell. Conditions can be strictly controlled allowing monitoring the effects of a single factor on a given process. Elicitation of secondary metabolism in cell cultures has been used for many years to turn on the genes and biosynthetic enzymes

involved in the biosynthesis of secondary compounds (see Chapter 21). Cell mechanisms for removing metals (see Chapter 20), salinity, or drought among others can be analyzed without having the interference of tissue organization. Furthermore, in vitro cultures submitted to morphogenetic conditions provide an optimum system for the study of the biochemical and molecular aspects associated with plant differentiation (see Chapters 10 and 14).

In general, specific foreign genes encoding specific characteristics have been manipulated and transferred by genetic transformation to plant species; however, new strategies involving genetic transformation with microRNAs have opened new possibilities for crop improvement (see Chapter 23).

Sometimes, plants produced in in vitro induce variation in their morphology, growth index, productivity, etc. There are some indications that variation in DNA methylation patterns seems to be much more frequent and in some cases it has been directly implicated in phenotypic variation (16). To understand the variation in PTC, as well as different phenotypes produced by cultured cells, the analysis of epigenetic status of the cultures is crucial for the understanding of this phenomenon. An update on the detection of epigenetic variation in plant cell cultures is provided (Chapters 22 and 25), as well the description of a powerful technique to determine the histone methylation is described (Chapter 24).

8. Future Perspectives

Plant cell cultures have turned into an invaluable tool to plant scientists, and today, in vitro culture techniques are standard procedures in most laboratories around the world. This technology goes beyond academic laboratories. Companies around the world are using plant tissue culture techniques for the massive propagation of plants. The development of genomics, proteomics, metabolomics, and more recently epigenetics has allowed the advances in different techniques relates to PTC and in the understanding of basic biological process. These approaches, with no doubt, will accelerate the discovery, isolation, and characterization of genes conferring new agronomic traits to crops. Between the most important challenges ahead are the increments in yields in commercial important crops, the production of better raw material for biofuel production, the increment in vitamins and nutritional proteins in food crops, as well as the generation of plants capable of to absorb contaminants from the environment. In order to achieve this goal, we need to produce resistant crops against the major diseases pathogens agents, as well as to abiotic stress. Since many of these traits are multigenic characters, the introduction of several genes in each transformation event will be important. The metabolic engineering techniques and

the uses of PTC must provide new raw materials for the production of bioethanol and biodiesel. The development of the techniques will keep cell culture scientists busy for many years.

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Chapter 2

History of Plant Tissue Culture

Trevor Thorpe

Abstract

Plant tissue culture, or the aseptic culture of cells, tissues, organs, and their components under defined physical and chemical conditions *in vitro*, is an important tool in both basic and applied studies as well as in commercial application. It owes its origin to the ideas of the German scientist, Haberlandt, at the beginning of the twentieth century. The early studies led to root cultures, embryo cultures, and the first true callus/tissue cultures. The period between the 1940s and the 1960s was marked by the development of new techniques and the improvement of those that were already in use. It was the availability of these techniques that 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 (mainly secondary metabolite) formation, starting in the mid-1960s. The 1990s saw 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 areas of plant biology and biochemistry and have assumed major significance in studies in molecular biology and agricultural biotechnology in the twenty-first century. The historical development of these *in vitro* technologies and their applications is the focus of this chapter.

Key words: Cell behavior, Cell suspensions, Clonal propagation, Organogenesis, Plantlet regeneration, Plant transformation, Protoplasts' somatic embryogenesis, Vector-dependent/independent gene transfer

1. Introduction

Plant tissue culture, also referred to as cell, *in vitro*, axenic, or sterile culture, is an important tool in both basic and applied studies, as well as in commercial application (1). Plant tissue culture is the aseptic culture of cells, tissues, organs, and their components under defined physical and chemical conditions *in vitro*. The theoretical basis for plant tissue culture was proposed by Gottlieb Haberlandt in his address to the German Academy of Science in 1902 on his experiments on the culture of single cells (2). He opined that, to his knowledge, no systematically organized attempts to culture-isolated

vegetative cells from higher plants have been made. Yet the results of such culture experiments should give some interesting insight to the properties and potentialities that the cell, as an elementary organism, possesses. Moreover, it would provide information about the interrelationships and complementary influences to which cells within a multicellular whole organism are exposed (from the English translation (3)). He experimented with isolated photosynthetic leaf cells and other functionally differenced cells and was unsuccessful, but nevertheless he predicted that one could successfully cultivate artificial embryos from vegetative cells. He, thus, clearly established the concept of totipotency, and further indicated that the technique of cultivating isolated plant cells in nutrient solution permits the investigation of important problems from a new experimental approach. On the basis of that 1902 address and his pioneering experimentation before and later, Haberlandt is justifiably recognized as the father of plant tissue culture. Other studies led to the culture of isolated root tips (4, 5). This approach of using explants with meristematic cells produced the successful and indefinite culture of tomato root tips (6). Further work allowed for root culture on a completely defined medium. Such root cultures were used initially for viral studies and later as a major tool for physiological studies (7). Success was also achieved with bud cultures (8, 9).

Embryo culture also had its beginning early in the first decade of the last century with barley embryos (10). This was followed by the successful rescue of embryos from nonviable seeds of a cross between *Linum perenne* $\leftrightarrow$ *Linum austriacum* (11), and for full embryo development in some early ripening species of fruit trees (12), thus providing one of the earliest applications of in vitro culture. The phenomenon of precocious germination was also encountered (13).

The first true plant tissue cultures were obtained by Gautheret (14, 15) from cambial tissue of *Acer pseudoplatanus*. He also obtained success with similar explants of *Ulmus campestris*, *Robinia pseudoacacia*, and *Salix capraea* using agar-solidified medium of Knop's solution, glucose, and cysteine hydrochloride. Later, the availability of indole acetic acid and the addition of B vitamins allowed for the more or less simultaneous demonstrations with carrot root tissues (16, 17), and with tumor tissue of a *Nicotiana glauca* *Nicotiana langsdorffii* hybrid (18), which did not require auxin, that tissues could be continuously grown in culture and even made to differentiate roots and shoots (19, 20). However, all of the initial explants used by these pioneers included meristematic tissue. Nevertheless, these findings set the stage for the dramatic increase in the use of in vitro cultures in the subsequent decades. Greater detail on the early pioneering events in plant tissue culture could be found in White (21), Bhojwani and Razdan (22), and Gautheret (23). This current article is based on an earlier review by the author (24) (used with permission from Elsevier).

2. The Development and Improvement of Techniques

The 1940s, 1950s, and 1960s proved an exciting time for the development of new techniques and the improvement of those already available. The application of coconut water (often incorrectly referred to as coconut milk) allowed for the culture of young embryos (25) and other recalcitrant tissues, including monocots. Callus cultures of numerous species, including a variety of woody and herbaceous dicots and gymnosperms, as well as crown-gall tissues, were established as well (23). It was recognized at that time that cells in culture underwent a variety of changes, including loss of sensitivity to applied auxin or habituation (26, 27), as well as variability of meristems formed from callus (27, 28). Nevertheless, it was during that period that most of the *in vitro* techniques used today were largely developed.

Studies by Skoog and Tsui (29) showed that the addition of adenine and high levels of phosphate allowed nonmeristematic pith tissues to be cultured and produced shoots and roots, but only in the presence of vascular tissue. Further studies using nucleic acids led to the discovery of the first cytokinin (kinetin), as the breakdown product of herring sperm DNA (30). The availability of kinetin further increased the number of species that could be cultured indefinitely, but, perhaps most importantly, led to the recognition that the exogenous balance of auxin and kinetin in the medium influenced the morphogenic fate of tobacco callus (31). A relative high level of auxin to kinetin favored rooting, and the reverse led to shoot formation and intermediate levels to the proliferation of callus or wound parenchyma tissue. This morphogenic model has been shown to operate in numerous species (32). Native cytokinins were subsequently discovered in several tissues, including coconut water (33). The formation of bipolar somatic embryos (carrot) was first reported independently by Reinert (34, 35) and Steward (36) in addition to the formation of unipolar shoot buds and roots.

The culture of single cells (and small cell clumps) was achieved by shaking callus cultures of *Tagetes erecta* and tobacco, and subsequently placing them on filter paper resting on well-established callus, giving rise to the so-called nurse culture (37, 38). Later, single cells could be grown in the medium in which tissues had already been grown (i.e., conditioned medium) (39). As well, single cells incorporated in a 1-mm layer of solidified medium formed some cell colonies (40). This technique is widely used for cloning cells and in protoplast culture (22). Finally, in 1959, success was achieved in the culture of mechanically isolated mature differentiated mesophyll cells of *Macleaya cordata* (41), and later in the induction of somatic embryos from the callus (42). The first large-scale culture of plant cells was obtained from cell suspensions of

Ginkgo, holly, *Lolium*, and rose in simple sparged 20-L carboys (43). The utilization of coconut water as an additive to fresh medium, instead of using conditioned medium, finally led to realization of Haberlandt's dream of producing a whole plant (tobacco) from a single cell by Vasil and Hildebrandt (44), thus demonstrating the totipotency of plant cells.

The earliest nutrient media used for growing plant tissues *in vitro* were based on the nutrient formulations for whole plants, for which there were many (21); but Knop's solution and that of Uspenski and Uspenska were used the most, and provided less than 200 mg/L of total salts. Based on studies with carrot and Virginia creeper tissues, the concentration of salts was increased twofold (45), and was further increased ca. 4 g/L, based on the work with Jerusalem artichoke (46). However, these changes did not provide optimum growth for tissues, and complex addenda, such as yeast extract, protein hydrolysates, and coconut water, were frequently required. In a different approach, based on an examination of the ash of tobacco callus, Murashige and Skoog (MS) (47) developed a new medium. The concentration of some salts was 25 times that of Knop's solution. In particular, the levels of NO_3^- and NH_4^+ were very high and the arrays of micronutrients were increased. MS formulation allowed for a further increase in the number of plant species that could be cultured, many of them using only a defined medium consisting of macro- and micronutrients, a carbon source, reduced N, B vitamins, and growth regulators (48). The MS salt formulation is now the most widely used nutrient medium in plant tissue culture.

Plantlets were successfully produced by culturing shoot tips with a couple of primordia of *Lupinus* and *Tropaeolum* (9), but the importance of this finding was not recognized until later when this approach to obtain virus-free orchids, demonstrated its potential for clonal propagation (49). The potential was rapidly exploited, particularly with ornamentals (50). Early studies had shown that cultured root tips were free of viruses (51). It was later observed that the virus titer in the shoot meristem was very low (52). This was confirmed when virus-free *Dahlia* plants were obtained from infected plants by culturing their shoot tips (53). Virus elimination was possible because vascular tissues, within which the viruses move, do not extend into the root or shoot apex. The method was further refined (54), and is now routinely used.

Techniques for *in vitro* culture of floral and seed parts were developed during this period (55). The first attempts at ovary culture yielded limited growth of the ovaries accompanied by rooting of pedicels in several species (56). Compared to studies with embryos, successful ovule culture is very limited. Studies with both ovaries and ovules have been geared mainly to an understanding of factors regulating embryo and fruit development (56). The first continuously growing tissue cultures from an endosperm were

from immature maize (57). Plantlet regeneration via organogenesis was later achieved in *Exocarpus cupressiformis* (58).

In vitro pollination and fertilization was pioneered using *Papaver somniferum* (59). The approach involves culturing excised ovules and pollen grains together in the same medium and has been used to produce interspecific and intergeneric hybrids (60). Earlier, cell colonies were obtained from *Ginkgo* pollen grains in culture (61), and haploid callus was obtained from whole anthers of *Tradescantia reflexa* (62). However, it was the finding of Guha and Maheshwari (63, 64) that haploid plants could be obtained from cultured anthers of *Datura innoxia* that opened the new area of androgenesis. Haploid plants of tobacco were also obtained (65), thus confirming the totipotency of pollen grains.

Plant protoplasts or cells without cell walls were first mechanically isolated from plasmolysed tissues well over 100 years ago, and the first fusion was achieved in 1909 (23). Nevertheless, this remained an unexplored technology until the use of a fungal cellulase by Cocking (66) ushered in a new era. The commercial availability of cell wall-degrading enzymes led to their wide use and the development of protoplast technology in the 1970s. The first demonstration of the totipotency of protoplasts was by Takebe et al. (67), who obtained tobacco plants from mesophyll protoplasts. This was followed by the regeneration of the first interspecific hybrid plants (*N. glauca* ↔ *N. langsdorffii*) (68).

Braun (69) showed that in sunflower *Agrobacterium tumefaciens* could induce tumors not only at the inoculated sites, but also at distant points. These secondary tumors were free of bacteria and their cells could be cultured without auxin (70). Further experiments showed that crown gall tissues, free of bacteria, contained a tumor-inducing principle (TIP), which was probably a macromolecule (71). The nature of the TIP was worked out in the 1970s (72), but Braun's work served as the foundation for *Agrobacterium*-based transformation. It should also be noted that the finding by Ledoux (73) that plant cells could take up and integrate DNA remained controversial for more than a decade.

3. The Recent Past

Based on the availability of the various in vitro techniques discussed in Subheading 2, it is not surprising that, starting in the mid-1960s, there was a dramatic increase in their application to various problems in basic biology, agriculture, horticulture, and forestry through the 1970s and 1980s. These applications can be divided conveniently into five broad areas, namely: (1) cell behavior, (2) plant modification and improvement, (3) pathogen-free plants and germplasm storage, (4) clonal propagation, and (5) product formation (1).

Detailed information on the approaches used can be gleaned from Bhojwani and Razdan (22), Vasil (74), and Vasil and Thorpe (75), among several sources.

3.1. Cell Behavior

Included under this heading are studies dealing with cytology, nutrition, primary, and secondary metabolism, as well as morphogenesis and pathology of cultured tissues (1). Studies on the structure and physiology of quiescent cells in explants, changes in cell structure associated with the induction of division in these explants and the characteristics of developing callus, and cultured cells and protoplasts have been carried out using light and electron microscopy (76–79). Nuclear cytology studies have shown that endoreduplication, endomitosis, and nuclear fragmentation are common features of cultured cells (80, 81).

Nutrition was the earliest aspect of plant tissue culture investigated, as indicated earlier. Progress has been made in the culture of photoautotrophic cells (82, 83). In vitro cultures, particularly cell suspensions, have become very useful in the study of both primary and secondary metabolism (84). In addition to providing protoplasts from which intact and viable organelles were obtained for study (e.g., vacuoles) (85), cell suspensions have been used to study the regulation of inorganic nitrogen and sulfur assimilation (86), carbohydrate metabolism (87), and photosynthetic carbon metabolism (88, 89), thus clearly showing the usefulness of cell cultures for elucidating pathway activity. Most of the work on secondary metabolism was related to the potential of cultured cells to form commercial products, but has also yielded basic biochemical information (90, 91).

Morphogenesis or the origin of form is an area of research with which tissue culture has long been associated, and one to which tissue culture has made significant contributions both in terms of fundamental knowledge and application (1). Xylogenesis or tracheary element formation has been used to study cytodifferentiation (92–94). In particular, the optimization of the *Zinnia* mesophyll single-cell system has dramatically improved our knowledge of this process. The classical findings of Skoog and Miller (31) on the hormonal balance for organogenesis have continued to influence research on this topic: a concept supported more recently by transformation of cells with appropriately modified *Agrobacterium* T-DNA (95, 96). However, it is clear from the literature that several additional factors, including other growth active substances, interact with auxin and cytokinin to bring about de novo organogenesis (97). In addition to bulky explants, such as cotyledons, hypocotyls, and callus (97), thin (superficial) cell layers (98, 99) have been used in traditional morphogenic studies, as well as to produce de novo organs and plantlets in hundreds of plant species (50, 100). As well, physiological and biochemical studies on organogenesis have been carried out (97, 101, 102).

The third area of morphogenesis, somatic embryogenesis, also developed in this period with over 130 species reported to form the bipolar structures by the early 1980s (103, 104). Successful culture was achieved with cereals, grasses, legumes, and conifers, previously considered to be recalcitrant groups. The development of a single cell to embryo system in carrot (105) has allowed for an in depth study of the process.

Cell cultures have continued to play an important role in the study of plant–microbe interaction not only in tumorigenesis (106), but also in the biochemistry of virus multiplication (107), phytotoxin action (108), and disease resistance, particularly as affected by phytoalexins (109). Without doubt, the most important studies in this area dealt with *Agrobacterium* and, although aimed mainly at plant improvement (see Subheading 3.2), provided good fundamental information (96).

3.2. Plant Modification and Improvement

During this period, in vitro methods were increasingly used as an adjunct to traditional breeding methods for the modification and improvement of plants. The technique of controlled in vitro pollination on the stigma, placenta, or ovule has been used for the production of interspecific and intergeneric hybrids, overcoming sexual self-incompatibility, and the induction of haploid plants (109). Embryo, ovary, and ovule cultures have been used in overcoming embryo inviability, monoploid production in barley, and seed dormancy and related problems (110–112). In particular, embryo rescue has played a most important role in producing interspecific and intergeneric hybrids (113).

By the early 1980s, androgenesis had been reported in some 171 species, many of which were important crop plants (114). Gynogenesis was reported in some 15 species, in some of which androgenesis was not successful (115). The value of these haploids was that they could be used to detect mutations and for the recovery of unique recombinants because there is no masking of recessive alleles. As well, the production of double haploids allowed for hybrid production and their integration into breeding programs.

Cell cultures have also played an important role in plant modification and improvement, as they offer advantages for isolation of variants (116). Although tissue culture produced variants that have been known since the 1940s (e.g., habituation), it was only in the 1970s that attempts were made to utilize them for plant improvement. This somaclonal variation is dependent on the natural variation in a population of cells, either preexisting or culture induced, and is usually observed in regenerated plantlets (117). The variation may be genetic or epigenetic and is not simple in origin (118, 119). The changes in the regenerated plantlets have potential agricultural and horticultural significance, but this potential has not yet been realized. It has also been possible to produce a wide spectrum of mutant cells in culture (120). These include cells showing

biochemical differences, antibiotic, herbicide, and stress resistance. In addition, auxotrophs, autotrophs, and those with altered developmental systems have been selected in culture; usually, the application of the selective agent in the presence of a mutagen is required. However, in only a few cases has it been possible to regenerate plants with the desired traits (e.g., herbicide-resistant tobacco) (121) and methyl tryptophan-resistant *Datura innoxia* (122).

By 1985, nearly 100 species of angiosperms could be regenerated from protoplasts (123). The ability to fuse plant protoplasts by chemical (e.g., with polyethylene glycol (PEG)) and physical means (e.g., electrofusion) allowed for the production of somatic hybrid plants, the major problem being the ability to regenerate plants from the hybrid cells (124, 125). Protoplast fusion has been used to produce unique nuclear-cytoplasmic combinations. In one such example, *Brassica campestris* chloroplasts coding for atrazine resistance (obtained from protoplasts) were transferred into *B. napus* protoplasts with *Raphanus sativus* cytoplasm (which confers cytoplasmic male sterility from its mitochondria). The selected plants that contained *B. napus* nuclei, chloroplasts from *B. campestris*, and mitochondria from *R. sativus* had the desired traits in a *B. napus* phenotype, and could be used for hybrid seed production (126). Unfortunately, only a few such examples exist to date.

Genetic modification of plants has been achieved by direct DNA transfer via vector-independent and vector-dependent means since the early 1980s. Vector-independent methods with protoplasts include electroporation (127), liposome fusion (128), and microinjection (129), as well as high-velocity microprojectile bombardment (biolistics) (130). This latter method can be executed with cells, tissues, and organs. The use of *Agrobacterium* in vector-mediated transfer has progressed very rapidly since the first reports of stable transformation (131, 132). Although the early transformations utilized protoplasts, regenerable organs, such as leaves, stems, and roots, have been subsequently used (133, 134). Much of the research activity utilizing these tools has focused on engineering important agricultural traits for the control of insects, weeds, and plant diseases.

3.3. Pathogen-Free Plants and Germplasm Storage

Although these two uses of in vitro technology may appear unrelated, a major use of pathogen-free plants is for germplasm storage and the movement of living material across international borders (1). The ability to rid plants of viruses, bacteria, and fungi by culturing meristem tips has been widely used since the 1960s. The approach is particularly needed for virus-infected material because bactericidal and fungicidal agents cannot be used successfully in ridding plants of bacteria and fungi (22). Meristem-tip culture is often coupled with thermotherapy or chemotherapy for virus eradication (135).

Traditionally, germplasm has been maintained as seed, but the ability to regenerate whole plants from somatic and gametic cells and shoot apices has led to their use for storage (22, 135). Three in vitro approaches have been developed, namely, use of growth-retarding compounds (e.g., maleic hydrazide, B995, and abscisic acid [ABA]) (136), low-nonnfreezing temperatures (1–9°C) (22), and cryopreservation (135). In this last approach, cell suspensions, shoot apices, asexual embryos, and young plantlets, after treatment with a cryoprotectant, are frozen and stored at the temperature of liquid nitrogen (ca. –196°C) (135, 137).

3.4. Clonal Propagation

The use of tissue culture technology for the vegetative propagation of plants is the most widely used application of the technology. It has been used with all classes of plants (138, 139), although some problems still need to be resolved (e.g., hyperhydricity, aberrant plants). There are three ways by which micropropagation can be achieved. These are enhancing axillary bud breaking, production of adventitious buds directly or indirectly via callus, and somatic embryogenesis directly or indirectly on explants (50, 138). Axillary bud breaking produces the smallest number of plantlets, but they are generally genetically true to type, whereas somatic embryogenesis has the potential to produce the greatest number of plantlets, but is induced in the lowest number of plant species. Commercially, numerous ornamentals are produced, mainly via axillary bud breaking (140). As well, there are many lab-scale protocols for other classes of plants, including field and vegetable crops, fruit, plantation, and forest trees, but cost of production is often a limiting factor in their use commercially (141).

3.5. Product Formation

Higher plants produce a large number of diverse organic chemicals, which are of pharmaceutical and industrial interest. The first attempt at the large-scale culture of plant cells for the production of pharmaceuticals took place in the 1950s at the Charles Pfizer Co. The failure of this effort limited research in this area in the USA, but work elsewhere in Germany and Japan in particular, led to the development; hence, by 1978, the industrial application of cell cultures was considered feasible (142). Furthermore, by 1987, there were 30 cell culture systems that were better producers of secondary metabolites than the respective plants (143). Unfortunately, many of the economically important plant products are either not formed in sufficiently large quantities or not at all by plant cell cultures. Different approaches have been taken to enhance yields of secondary metabolites. These include cell cloning and the repeated selection of high-yielding strains from heterogeneous cell populations (142, 144) and by using enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay techniques (145). Another approach involves selection of mutant cell lines that overproduce the desired product (146). As well, both abiotic

factors—such as ultraviolet (UV) irradiation and exposure to heat or cold and salts of heavy metals—and biotic elicitors of plant and microbial origin have been shown to enhance secondary product formation (147, 148). Lastly, the use of immobilized cell technology has also been examined (149, 150).

Central to the success of producing biologically active substances commercially is the capacity to grow cells on a large scale. This is being achieved using stirred tank reactor systems and a range of air-driven reactors (141). For many systems, a two-stage (or two-phase) culture process has been tried (151, 152). In the first stage, rapid cell growth and biomass accumulation are emphasized, whereas the second stage concentrates on product synthesis with minimal cell division or growth. However, by 1987, the naphthoquinone, shikonin, was the only commercially produced secondary metabolite by cell cultures (153).

4. The Present

During the 1990s, continued expansion in the application of in vitro technologies to an increasing number of plant species was observed. Tissue culture techniques are being used with all types of plants, including cereals and grasses (154), legumes (155), vegetable crops (156), potato (157), other root and tuber crops (158), oilseeds (159), temperate (160) and tropical (161) fruits, plantation crops (162), forest trees (163), and, of course, ornamentals (164). As can be seen from these articles, the application of in vitro cell technology went well beyond micropropagation, and embraced all the in vitro approaches that were relevant or possible for the particular species, and the problem(s) being addressed. However, only limited success has been achieved in exploiting somaclonal variation (165) or in the regeneration of useful plantlets from mutant cells (166); also, the early promise of protoplast technology has remained largely unfulfilled (167). Substantial progress has been made in extending cryopreservation technology for germplasm storage (168) and in artificial seed technology (169). Some novel approaches for culturing cells, such as on rafts, membranes, and glass rods, as well as manipulation of the culture environment by use of nonionic surfactants have been successfully developed (170).

Cell cultures have remained an important tool in the study of plant biology. Thus, progress is being made in cell biology, for example, in studies of the cytoskeleton (171), on chromosomal changes in cultured cells (172), and in cell-cycle studies (173, 174). Better physiological and biochemical tools have allowed for a reexamination of neoplastic growth in cell cultures during habituation and hyperhydricity, and to relate it to possible cancerous growth in plants (175). Cell cultures have remained an extremely important

tool in the study of primary metabolism, for example the use of cell suspensions to develop *in vitro* transcription systems (176) or the regulation of carbohydrate metabolism in transgenics (177). The development of medicinal plant cell-culture techniques has led to the identification of more than 80 enzymes of alkaloid biosynthesis (reviewed in ref. 178). Similar information arising from the use of cell cultures for molecular and biochemical studies on other areas of secondary metabolism is generating research activity on metabolic engineering of plant secondary metabolite production (179).

Cell cultures remain an important tool in the study of morphogenesis, even though the present use of developmental mutants, particularly of *Arabidopsis*, is adding valuable information on plant development (see ref. 180). Molecular, physiological, and biochemical studies have allowed for an in depth understanding of cytodifferentiation, mainly tracheary element formation (181), organogenesis (182, 183), and somatic embryogenesis (184–186).

Advances in molecular biology are allowing for the genetic engineering of plants through the precise insertion of foreign genes from diverse biological systems. Three major breakthroughs have played major roles in the development of this transformation technology (187). These are the development of shuttle vectors for harnessing the natural gene transfer capability of *Agrobacterium* (188), the methods to use these vectors for the direct transformation of regenerable explants obtained from plant organs (189), and the development of selectable markers (190). For species not amenable to *Agrobacterium*-mediated transformation, physical, chemical, and mechanical means are used to get the DNA into the cells. With these latter approaches, particularly biolistics (191), it has become possible to transform virtually any plant species and genotype.

The initial wave of research in plant biotechnology has been driven mainly by the seed and agrochemical industries, and has concentrated on the agronomic traits of direct relevance to these industries, namely, the control of insects, weeds, and plant diseases (192). At present, over 100 species of plants have been genetically engineered, including nearly all the major dicotyledonous crops and an increasing number of monocotyledonous ones, as well as some woody plants. Current research is leading to routine gene transfer systems for all-important crops, for example the production of golden rice (193). In addition, technical improvements are further increasing transformation efficiency, extending transformation to elite commercial germplasm, and lowering transgenic plant production costs. The next wave in agricultural biotechnology is already in progress with biotechnological applications of interest to the food processing, specially chemical, and pharmaceutical industries.

The current emphasis and importance of plant biotechnology can be gleamed from the last three *International Congresses on*

Plant Tissue and Cell Culture and Biotechnology held in Israel in June 1998, in the USA in June 2002, and in China in August 2006. The theme of the Israeli Congress was *Plant Biotechnology and In Vitro Biology in the 21st Century*; the theme of the 2002 Congress was *Plant Biotechnology 2002 and Beyond*, while the theme of the 2006 Congress was *Biotechnology and Sustainable Agriculture 2006 and Beyond*. The proceedings for these three congresses (194–196) were developed through scientific programs that focused on the most important developments, both basic and applied, in the areas of plant tissue culture and molecular biology and their impact on plant improvement and biotechnology. They clearly show where tissue culture is today and where it is heading (i.e., as an equal partner with molecular biology) as a tool in basic plant biology and in various areas of application. In fact, progress in applied plant biotechnology is fully matching and is without doubt stimulating fundamental scientific progress, which remains the best hope for achieving sustainable and environmentally stable agriculture (197). Indeed, the advancements made in the last 100 years with in vitro technology have gone well beyond what Haberlandt and other pioneers could have imagined.

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Chapter 3

Callus, Suspension Culture, and Hairy Roots. Induction, Maintenance and Characterization

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Abstract

The growth is a characteristic of each culture and it is determinate by the origin of the species, culture conditions, and type of culture. In this chapter, we make a comparison of the different growth parameters among three different species and three different types of cultures.

Key words: Callus, Conductivity, Growth, Hairy roots, Suspension cultures

1. Introduction

Callus induction is necessary, as the first step, in many tissue culture experiments. Callus is produced when the initial response of the tissues to a wound is followed by the external addition of growth regulators in an aseptic medium in order to maintain the rapid cell division response and sustain it indefinitely (1). Callus formation from an explant involves the development of progressively more random planes of cell division, less frequent specialization of cells, and loss of organized structures (2, 3).

Calli can be obtained from almost any part of the plant. However, it must be taken into account that different factors can influence the initiation of the calli. These factors include the physiological or ontogenic age of the organ or tissue that serves as the explant source; the environmental conditions in which the plant is cultivated before the explant is taken; the organization of the cells; and the size and location of the explant in the plant. Another consideration to keep in mind is the ultimate goal of the cell culture (4), and the fact that not all the cells in the explant produce callus.

The source of the primary explant can be an aseptically germinated seedling or surface-sterilized roots, stems, leaves, or reproductive structures. The level and kind of plant growth regulators as well as the culture medium are the major factors that control callus formation. Culture conditions are also important in callus formation and development as well as the level of gases inside the flask or container.

For the subculture of callus, small portions of friable callus, normally 0.2–0.5 g, must be transferred to the new medium in order to have fresh callus. The calli can be used as a source of material for biochemical and molecular studies, and to produce suspension cultures, protoplasts, organogenesis, somatic embryos, and secondary metabolites (1, 4). Calli cultures are normally maintained in growth chambers under controlled light conditions, which need to be established for each species and genotypes. Also, for most species, the callus cultures need to be maintained at a temperature of 25°C. Callus cultures are subcultured under these conditions every 2–6 weeks depending on the growth rate (5). Cell suspension culture consists of a population of single cells and clumps suspended from a single callus, in flasks with liquid nutrient medium (6). The flasks are then placed on a shaker with constant agitation to provide aeration to the cells (7–9). As new cells are formed, they are dispersed into the liquid medium and become clusters and aggregates (9). In general, cells in suspension exhibit much higher rates of growth than cells in callus culture. The suspension cultures are used when rapid cell division or a more uniform treatment application is required, such as during cell selection procedures. Cell suspension can be maintained by transferring a small portion of the culture into fresh medium.

1.1. Growth Curves

The callus and cell suspension cultures' growth rate parallels in many ways to a sigmoid curve seen in populations of single-celled organisms (10). There are several stages of growth (10, 11) independently whether the culture is callus, cell suspension, or hairy root culture. A typical growth curve for fresh weight and dry weight of different cultures is shown in Figs. 1–4. There is an initial lag phase (I) followed by a period of rapid growth which may approach exponential (II), then lineal (III), a period of declining growth after the lineal phase begins (IV) followed by a stationary phase (V), and eventually a decline in the biomass as the cells in the culture begin to senesce.

The behavior of the cells is different during each growth stage. There are several factors influencing the growth of the cells, such as the culture medium, origin of the explant, growth regulators' amount, environmental culture conditions, etc. The cultures must be transferred to fresh medium before they reach the stationary phase. The deceleration of growth is due to several factors; among them are the depletion of nutrients, cell density, and production of toxic by-products.

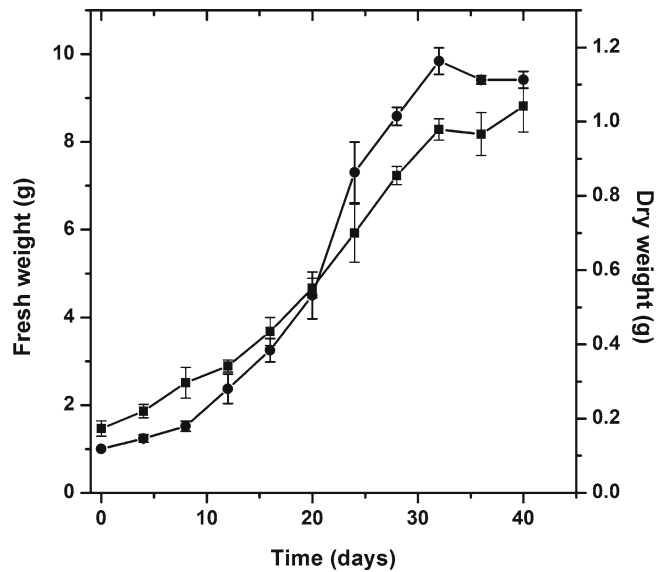


Fig. 1. Growth cycle for *Jatropha curcas*. Fresh weight (filled circle) and dry weight (filled square). A gram of callus is inoculated in 25 mL of MS medium supplemented with 2.69 μM naphthalene acetic acid and 4.64 μM kinetin contained in baby food jars. The flasks are incubated under dark conditions at $25 \pm 2^\circ\text{C}$. Triplicate samples are taken every 4 days, beginning with day 0, until day 40.

The first two phases of growth of the culture are the exponential and linear. The culture must be transferred to fresh medium at the end of the linear phase (phase number III).

Cells' growth can be monitored measuring different parameters depending on the cultures, such as fresh and dry weight, cell number, medium conductivity, percentage of the cell packet, image analysis, light absorbance, osmolarity, focused beam reflectance, image processing, enzymatic activity, and turbidimetric measurement (12–21).

The behavior of the tissue culture is different during each stage of growth. The medium and the incubation conditions can affect how long the callus remains at a particular stage (4). For many experimental procedures, it is necessary to use cultures at a specific developmental point. This is the reason why it is very important to characterize the growth cycle before to start with any experiment.

During the whole growth cycle, the viability must be checked up in order to make sure that the culture is growing properly. The viability can be followed using different methods; among them, dye exclusion (22, 23), vital dying (24), and electron transport activity (25) are the most used. Viability can also be determined by noninvasive methods (26) or using intracellular esterase activity (27). In some cases, the reduction of tetrazolium salts can be a better indicator of metabolically active cells, such as in the study of reactive oxygen species-mediated responses during the critical early hours of fungal elicitation (28).

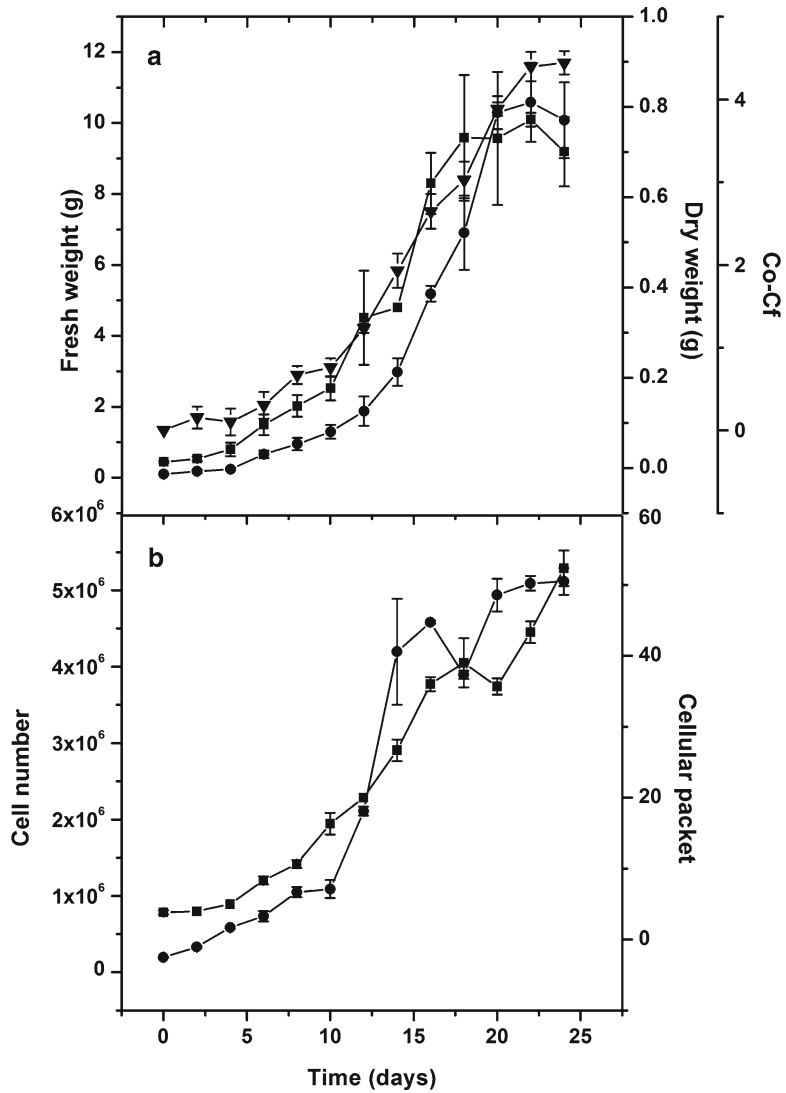


Fig. 2. Growth of *Coffea arabica* suspension culture line L1. (a) Fresh weight (filled circle); dry weight (filled square); conductivity (filled inverted triangle). (b) Cell number (filled circle); cellular packet (filled square). One hundred milligrams of cells are transferred into 25 mL of MS medium supplemented with 13.6 μM 2,4-D and 4.4 μM BA contained in baby food jars. The flasks are incubated under photoperiod (16-h light to 8-h dark at $25 \pm 2^\circ\text{C}$). Triplicate samples are taken every 2 days, beginning with day 0, until day 24.

2. Materials

2.1. Biological Material

- 2.1.1. *Jatropha curcas* L. plants in in vitro culture.
- 2.1.2. *Coffea arabica* L. plants in in vitro culture.
- 2.1.3. *Catharanthus roseus* plants in in vitro culture.
- 2.1.4. *Agrobacterium rhizogenes*.

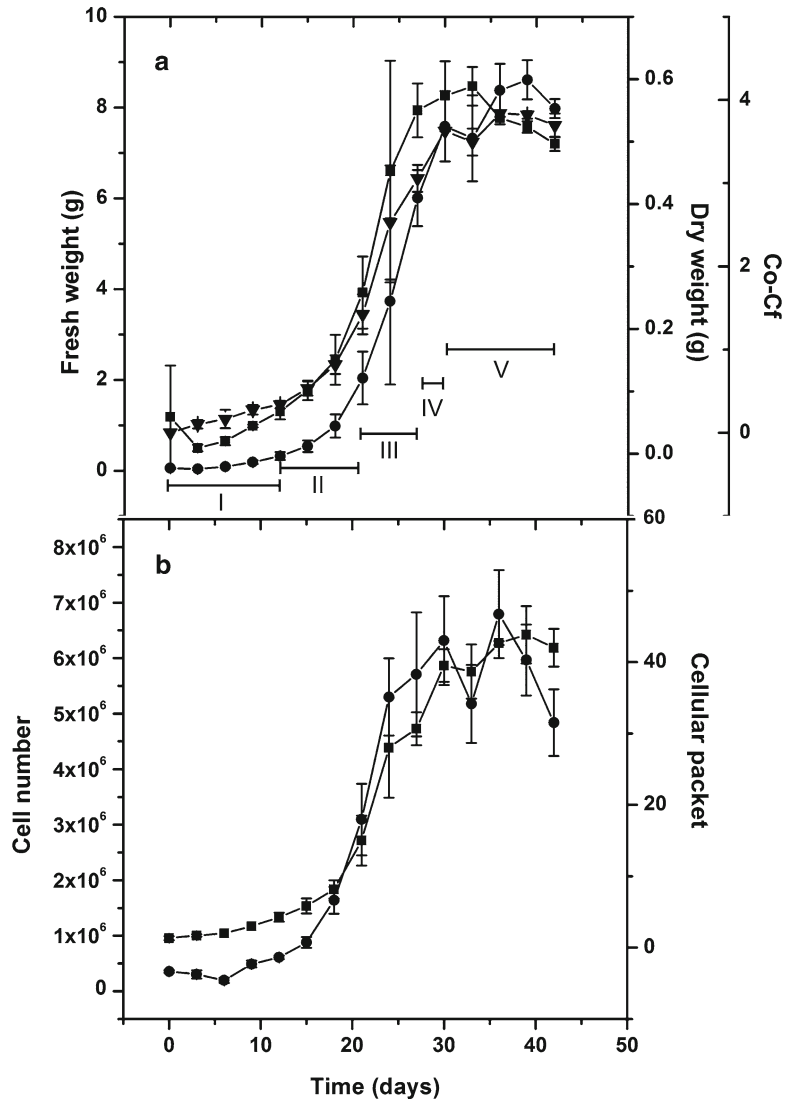


Fig. 3. Growth of *Coffea arabica* suspension culture line L2. (a) Fresh weight (filled circle); dry weight (filled square); conductivity (filled inverted triangle). (b) Cell number (filled circle); cellular packet (filled square). One hundred milligrams of cells are transferred into 25 mL of MS medium supplemented with $13.6 \mu\text{M}$ 2,4-D and $4.4 \mu\text{M}$ BA contained in baby food jars. The flasks are incubated under photoperiod (16-h light to 8-h dark at $25 \pm 2^\circ\text{C}$). Triplicate samples are taken every 3 days, beginning with day 0, until day 42.

2.2. Glassware

1. Erlenmeyer flasks (250 mL).
2. Glass bottles (250 mL).
3. Petri dishes (100×15 mm).
4. Beakers (500–1,000 mL).
5. Baby food jars.

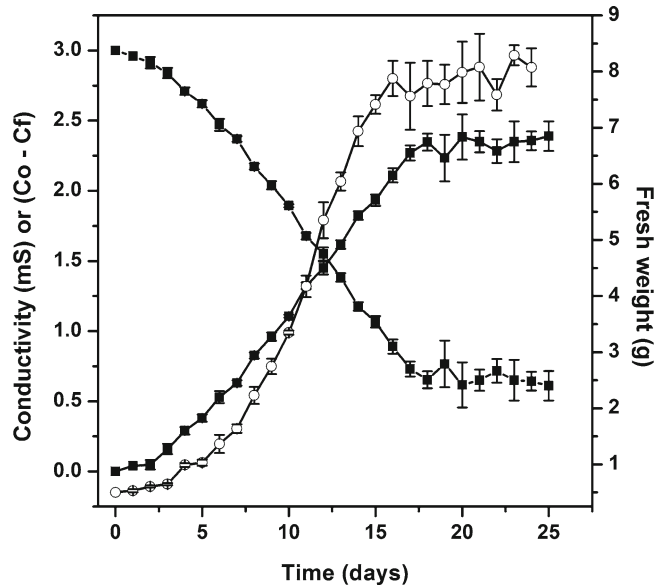


Fig. 4. Growth of *Catharanthus roseus* hairy roots. Fresh weight (filled circle). Conductivity or (Co – Cf) (filled square). Half of a gram of the hairy roots is transferred into 50 mL of B₅ half strength without growth regulators in 250-mL Erlenmeyer flasks. The flasks are incubated under dark conditions at 25 ± 2°C. Triplicate samples are taken every day.

2.3. Instrumentation

- 1. Analytical balance.
- 2. Hot/stirrer plate.
- 3. pH meter.
- 4. Autoclave.
- 5. Conductimeter.
- 6. Centrifuge.
- 7. Hemocytometer.
- 8. Laminar flow cabinet.
- 9. Bunsen burner.
- 10. Micropipets.
- 11. Microscope.
- 12. Sterile Petri dishes.

2.4. Chemicals

- 1. Plant growth regulators: 2,4-dichlorophenoxy acetic acid (2,4-D); naphthalene acetic acid (NAA); kinetin (K); benzyl adenine (BA).
- 2. Murashige and Skoog (MS) culture medium (29).
- 3. Gambor (B₅) medium (7).
- 4. Commercial bleach solution (Cloralex®).
- 5. Tween 20.
- 6. Sterile distilled water.

7. Agar.
8. Ethyl alcohol.
9. Trypane blue.
10. Fluorescein triacetate.
11. Chromium trioxide.

3. Methods

3.1. Induction

3.1.1. Establishment of *Jatropha curcas* Callus Cultures

Two-month-old *J. curcas* plantlets cultivated in vitro are used as a source for the explants. Leaves explants, eliminating midvein and edges, are put in baby food jars with 25 mL of solid (agar) MS medium with 2.69 μM NAA and 4.64 μM K under dark conditions at $25 \pm 2^\circ\text{C}$. The calli appear around the wounding after 1 week. After 4 weeks, the calli obtained are separated from the original leave explant and transferred into a fresh medium. The callus cultures are first selected for their ability to accumulate biomass. Once established, the calli are subcultured every 30 days.

3.1.2. Establishment of *Coffea arabica* Callus and Suspension Cultures

Three-month-old *C. arabica* plantlets cultivated in vitro are used as a source for the explants. Leaves explants, eliminating midvein and edges, are put in baby food jars with 25 mL of solid MS medium (agar) supplemented with 13.52 μM 2,4-D and 4.43 μM BA under dark conditions at $25 \pm 2^\circ\text{C}$. The calli appear around the wounding after 3 weeks and they are transferred into a fresh medium. The callus cultures are first selected for their ability to accumulate biomass. Once established, the calli are subcultured every 30 days.

Two grams of *C. arabica* calli in the linear phase of growth are transferred to a 250-mL Erlenmeyer flask with 50 mL of MS medium supplemented with 13.52 μM 2,4-D and 4.43 μM BA. The callus is disaggregating with a sterilized forceps inside of a laminar flow cabinet. The flasks are incubated in a shaker at 100 rpm under dark conditions at $25 \pm 2^\circ\text{C}$. After 4 weeks, the culture is filtered through a 60- μm mesh in order to eliminate the big pieces of callus. The cells and clumps are precipitated by centrifugation ($1892 \times g$) and the old medium is eliminated and replaced with new medium. After two or three transferences, the growth of the cells is good enough to dilute and start the suspension culture.

Five milliliters of the stock solution of *C. arabica* suspension culture are transferred into 45 mL of fresh MS medium supplemented with 4.5 μM 2,4-D and 9.2 μM BA in 250-mL Erlenmeyer flasks. The cultures are subcultured every 14 days.

3.1.3. Establishment of Transformed Root Cultures

The *A. rhizogenes* strain 1855 pBI 121.1 is cultured in solid yeast extract-mannitol broth (YMB) medium (pH 8) at 25°C for 2 weeks before it is transferred into 50-mL Erlenmeyer flasks containing

10 mL of liquid yeast extract-tryptone (YT) medium. The flasks are incubated at 25°C in the dark in a gyratory shaker (100–120 rpm) for 48 h. An aliquot (1.5 mL) is taken and centrifuged for 3 min at $14,000\times g$. The supernatant is eliminated and the pellet resuspended in 300 μL of YT medium to a final concentration of 1.7×10^5 bacteria per microliter. This suspension is used for the infection of explants.

Two-month-old *C. roseus* plantlets are dissected in a sterile Petri dish. Leaves and stems are put in baby food jars with 25 mL of solid B₅ medium (7) at half ionic strength for both major and minor components and vitamins, without growth regulators and supplemented with 1 mg/mL of antibiotic (carbenicillin or cephotoxime, depending on the *A. rhizogenes* strain used). The leaves must be placed in such a way that the upper surface touches the medium and the extreme end of the petiole remains exposed. In the case of stems, the apical part is exposed. Four stems or two leaves must be placed in each jar. An aliquot of 5 μL of the bacteria solution is applied to the wounded area of each of the explants. For each bacterial strain, 40 leaves and 20 stem cuts are infected. The infected explants are incubated at $25\pm 2^\circ\text{C}$ in the dark in a growth chamber.

The roots obtained from the explants infected by the bacteria are harvested when they are approximately 1 cm long. The roots are placed in a solid B₅ medium, at half ionic strength, with 1 mg/mL of antibiotic. After several transfers to eliminate the bacteria, the axenic root cultures are put in liquid B₅ medium at half ionic strength. The root cultures are first selected for their ability to accumulate biomass and scaled up to 250-mL flasks with 100 mL of medium. Once established, the hairy roots are subcultured every 18–25 days (30).

3.2. Measurement of Growth

3.2.1. *Jatropha curcas* Calli

In order to characterize the growth of the *J. curcas* calli, a growth curve must be established. A gram of callus is inoculated in 25 mL of MS medium supplemented with 2.69 μM NAA and 4.64 μM kinetin contained in baby food jars. The flasks are incubated under dark conditions at $25\pm 2^\circ\text{C}$. Triplicate samples are taken every 4 days, beginning with day 0, until day 40. Every sample is weighted immediately in order to measure the fresh weight. This is a nondestructive method. After the samples are weighted, they are freezed with liquid nitrogen and stored at -80°C until the last sample is finished and freeze-dried. The freeze-dried samples are weighted in order to get the dry weight. Dry weight is a more reliable criterion to follow growth than fresh weight, but this method requires sacrifice of the samples.

The results of fresh and dry weight are shown in Fig. 1a, b. After the growth cycle curve is built, the data are analyzed by using the doubling time software package (Roth V. 2006 <http://www.doubling-time.com/compute.php>) (Table 1).

Table 1
Growth parameters of the tested cultures

Species	Culture	Technique	Doubling time (days)	Growth rate	Growth index
<i>Jatropha curcas</i>	Calli	FW ^a	7.95	0.0871	1.96
		DW	12.95	0.0535	0.86
<i>Coffea arabica</i> L1	Suspension culture	FW	3.62	0.1916	3.51
		DW	3.69	0.1877	5.63
		NC	2.95	0.2346	4.72
		C	3.35	0.2068	5.36
		CP	4.82	0.1437	3.37
<i>C. arabica</i> L2	Suspension culture	FW	3.55	0.1954	10.12
		DW	4.86	0.1425	4.41
		NC	4.84	0.1433	5.48
		C	4.76	0.1456	4.73
		CP	4.86	0.1427	4.25
<i>Catharanthus</i> <i>roseus</i>	Hairy roots	FW	3.53	0.2289	3.04
		C	3.14	0.2207	2.37

^aFresh weight (FW); dry weight (DW); number of cells (NC); conductivity (C)

3.2.2. *Coffea arabica* Suspension Culture

In order to characterize the growth of the *C. arabica* suspension culture, a growth curve must be established. One hundred milligrams of cells are transferred into 25 mL of MS medium supplemented with 13.6 μM 2,4-D and 4.4 μM BA contained in baby food jars. The flasks are incubated under photoperiod (16-h light to 8-h dark at $25 \pm 2^\circ\text{C}$). Triplicate samples are taken every 2 days (L1), beginning with day 0, until day 24, or every 3 days (L2), beginning with day 0, until day 42.

Growth of suspension cultures is measured by filtering the cell suspension through a wet Whatman No. 1 filter paper with a funnel attached to a vacuum line. The vacuum is maintained until the excess medium is removed as judged by the appearance of the cell mass. The cells are then carefully scraped off from the surface of the paper and transferred into a preweighed vial. The vials are kept at -80°C and freeze-dried later in order to get the dry weight. The results are shown in Figs. 2 and 3.

Another method to measure the growth of the suspension cultures is counting the number of cells. In order to determine the number of cells (density) in each flask, an aliquot of the suspension culture is allocated into a hemocytometer and the cells are counted under a microscope. The results are reported as cells per milliliter (Figs. 2b and 3b).

An additional method to measure the suspension culture growth is by the packet cell volume. Ten milliliters of the suspension culture are put in a conical graduate tube and centrifuged at $1892 \times g$ in a centrifuge for 15 min at room temperature. The packet formed by the cells is recorded, and the volume of the cells

expressed as a percentage of the volume of cell suspension is used to follow the growth of the culture (Figs. 2b and 3b).

3.2.3. *Catharanthus roseus* Hairy Roots

In order to characterize the growth of the *C. roseus* hairy roots culture, a growth curve must be established. Half of a gram of the hairy roots is transferred into 50 mL of B₅ half strength without growth regulators in 250-mL Erlenmeyer flasks. The flasks are incubated under dark conditions at 25 ± 2°C. Triplicate samples are taken every day. The medium is used to measure the conductivity and the roots are used for fresh and dry weight determination as already mentioned above (Fig. 4).

After a growth cycle curve is built, the data are analyzed by using the following formula. To calculate the speed of growth, the following formula can be used:

$$\mu = \frac{\ln X_f - \ln X_0}{t_f - t_0},$$

where μ = growth speed; X_f = final weight; X_0 = initial weight; t_f = final time; and t_0 = initial time.

In order to calculate the duplication time of the culture, the following formula can be used:

$$Td = \frac{\ln 2}{\mu},$$

where Td = duplication time and μ = the speed of growth.

To calculate the growth index, the following formula can be used:

$$I = \frac{X_f - X_0}{X_0},$$

where I is the growth index, X_f is the final weight, and X_0 is the initial weight.

The growth of the three species described in this chapter shows a similar growth curve with an initial lag phase, an exponential phase, and a stationary phase (Figs. 1–4). The major difference is that the growth cycle is completed more rapidly in suspension and hairy root cultures than in callus cultures (Table 1).

The duplication time of *C. roseus* hairy roots is 3.53 days using the fresh weight as a parameter to follow the growth of the roots. When the conductivity is used instead of the fresh weight, the duplication time is 3.14 days. Similar results were found for the other three cultures. For the *C. arabica* L1, the duplication time oscillated between 2.95 and 4.82 days when we used the number of cells and the cellular packet to calculate its growth, respectively. However, the calculation of the duplication time using fresh and dry weight as well as cell number gives a very close result around 3.6 days.

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Chapter 4

Growth Measurements: Estimation of Cell Division and Cell Expansion

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Abstract

The main parameters for the estimation of growth within in vitro cultures are reviewed. Procedures to measure these parameters are described, emphasizing in each case their convenience of use, depending on the features of the culture evaluated.

Key words: Cell counting, Duplication time, Dry weight, Fresh weight, Growth index, Packed cell volume

1. Introduction

The accurate, fast, and reliable determination of cell growth is of critical importance in plant cell and tissue culture. The precise assessment of growth kinetics is essential for the efficient design of bioprocess engineering. However, the measurement of growth parameters in the different types of cultures and concomitantly the use of various containers along with the heterogeneity in cell morphology introduce diverse problems that must be addressed by using a specific methodology for each case (1). Since callus and cell suspension cultures represent two of the most common in vitro systems, this chapter concentrates on growth measurements in such systems.

1.1. Callus Cultures

Plant cell cultures are initiated through the formation of a mass of nondifferentiated cells called “callus.” A callus culture is obtained by excising a piece of tissue, the explant, from the parent plant and placing it onto a nutrient base, solidified with agar. This nutrient base contains macronutrients (nitrogen, potassium, phosphorus,

etc.), micronutrients (cobalt, copper, iron, etc.), a carbon source (usually sucrose), and various plant growth regulators. Callus tissue is not an ideal system to work with, due to their slow growth rate and high biochemical variability. Nevertheless, since they are an almost obligatory step to initiate an *in vitro* culture, the proper assessment of the effect of different media composition or growth regulators on their growth rate is an important parameter to define the optimal media to use. The most common growth parameters used for callus cultures include fresh weight, dry weight, and growth index.

1.2. Suspension Cultures

The success in the establishment of a cell suspension culture depends, in a great extent, on the availability of “friable” callus tissue, i.e., a tissue that, when stirred in liquid medium, rapidly disaggregates into single cells and small clusters. Such a system is much more amenable for biochemical studies and process development than calli, since they generally grow in a faster rate and allow cells to be in direct contact with the medium nutrients. Suspension or liquid cultures of plant cells are usually grown as microbial cells. However, plant cell dimensions are larger than those of bacteria or fungi, ranging from 20 to 40 μm in diameter and from 100 to 200 μm in length (2, 3). The central vacuole occupies a large portion of the mature cell volume. Plant cell cultures tend to contain clumps, formed by a variable number of cells. These clumps arise as a result of the failure of new cells to separate after division or from the adherence of free cells among themselves. In some cases, such clumps (also known as aggregates) may contain up to 200 cells, and reach up to 2 mm in diameter. Although cell stickiness can be overcome by modifying the culture medium, cells in culture become “sticky” in late lag phase of growth as a rule.

Methods to obtain suspensions composed largely of free cells (fine cell suspensions) include the use of cell wall degrading enzymes and sieving. Unfortunately, once established, a fine cell suspension has a tendency with time to revert to a clumped condition (4).

1.3. Methods for Evaluating Growth in *In Vitro* Cultures

There are several methods for evaluating growth kinetics in plant cell cultures. Selected examples include: fresh cell weight, dry cell weight, settled cell volume, packed cell volume, cell counting, culture optical density, residual electrical conductivity, and pH measurements, among others (1).

In cultures originated from different plant species, settled volume, packed cell volume, as well as fresh weight, all show a very good linear correlation with dry weight data. Thus, any of these estimations can be used for measuring cell growth. However, during the stationary phase of the culture, there are important deviations in this correlation. At this phase, plant cell cultures show a high degree of aggregation, cell lysis, and a marked heterogeneity in cell morphology.

The measurement of cell concentration by cell counting and turbidity (optical density) has also shown a reasonably good correlation with the dry cell weight parameter. When cultures reached the stationary phase, the same problems stated above render unsatisfactory results regarding to the correlation of these parameters to dry weight estimation. On the other hand, protein, RNA and DNA contents fail to show the same good correlation with the dry weight, which may be ascribed to changes in cell physiology along the growth cycle, which frequently results in wide variations.

Although complicated and time consuming, cell counting represent the best way to assess culture growth in suspension cultures. Nevertheless, it often shows a good correlation with other parameters, such as electric conductivity. Cell density is obtained by direct counting of cells under the microscope, using a cell counting chamber, such as the Sedgewick rafter cell (Graticulates Limited, Tonbridge England) or the Newbauer chamber (Sigma-Aldrich, San Luis, MO). Such devices hold a fixed volume of the suspension over a defined area. The base of the chamber is divided in squares, frequently containing a 1 mm^3 ($1\text{ }\mu\text{L}$) volume. By observing the suspension with a low magnification objective, cells contained in such a volume are identified and counted. The use of digital methods, consisting in the capture of a microphotography and then processing it with one the several software packages, such as MATLAB, MathWorks Co. (5), may significantly reduce the time invested in this kind of analysis. Recently, Millipore has developed a hand-held cell counter, based on the Coulter principle, or flow cytometry (the ScepterTM), which might provide a fast and accurate cell estimation at an affordable cost. However, it has been designed for animal cells, which are smaller than plant cells (10–30 vs. 30–100 μm in diameter, respectively). Therefore, its application for plant cell cultures will be limited until this size restriction is overcome.

Electric conductivity of culture medium decreases inversely to biomass gain. This is a consequence of ion uptake by cells. The monitoring of this decrease to assess cell growth offers several advantages over other methods, such as the following: (1) it allows continuous and in situ or on-line monitoring of cell growth; (2) no sampling or no wet chemical analysis is required; (3) it is economical and efficient; (4) it provides an accurate, reliable, and reproducible measurement of plant cell growth rate; and (5) it is independent of cell aggregation, growth morphology, and apparent viscosity (1).

2. Materials

2.1. Biological Material

1. Callus and cell suspension cultures of any species.

2.2. Glassware

1. Glass desiccators, or any other hermetic container, with silica gel.
2. 50-mL sterile, graduated centrifuge tubes.
3. Counting cell chamber (Sedgewick rafter cell).

2.3. Instrumentation

1. Analytical balance (resolution to 0.01 mg).
2. Top loading balance (resolution 0.1 g).
3. Convection oven (resolution 0.1°C).
4. Conductivity meter (range 0–200 μ S and 0–200 mS; resolution 0.1 units).
5. Universal centrifuge (with a speed range including 4,000 $\times g$).
6. Light microscope with 10 $\times$ and 40 $\times$ objective lenses.

2.4. Chemicals

1. 8% chromium trioxide solution: Dissolve 8 g of CrO₃ in 100 mL of water. Keep in the dark at room temperature.
2. 10% cellulase solution: Dissolve 0.5 g of cellulase from *Aspergillus* sp. (Sigma Chemical Co, St. Louis, MO) in 5 mL 100 mM KH₂PO₄, pH 6.0. Keep at 4°C. Use solution within a week.
3. 5% macerozyme R-10 solution: dissolve 0.25 g of Macerozyme R-10 (Pectinase from *Rhizopus*) (Sigma Chemical Co, St. Louis, MO), in 5 mL 100 mM KH₂PO₄, pH 6.0. Keep at 4°C. Use solution within a week.

3. Methods**3.1. Measuring Growth in Callus Cultures****3.1.1. Fresh-Weight Determination**

1. To determine the total fresh weight, harvest all the tissue on the media surface with flat-end tweezers and weigh on a balance. To avoid tissue desiccation, open the culture jar only when you are ready to process it.

3.1.2. Dry-Weight Determination

1. Dry weight can be estimated by drying the tissue at 60°C in a convection oven, until constant weight (usually 16 h). Take a sample of the fresh tissue, weigh it on a preweighed square of aluminum foil, and evaporate the water contained in the tissue in the preheated oven at 60°C for 12 h.
2. Allow samples to cool down in a desiccator containing silica gel for 15–20 min and then register the sample's weight.
3. Put the tissue sample back into the oven and take a new weight register after 2 h. If no variations are detected, samples have reached constant weight. If variations higher than 10% regarding the previous register are observed, return samples to the oven for another 2 h period.

4. Alternatively, dry weight can be obtained from lyophilized tissues. Once harvested, fresh tissues should be weighed, deposited in lyophilizer flasks and frozen at -20°C for at least 12 h. Flasks with frozen tissues are then connected to the vacuum line for 24 h and weight is registered.

3.2. Measuring Growth in Cells Cultures

Growth of suspension cultures is commonly evaluated as the settled cell volume (SCV), the packed cell volume (PCV), fresh cell weight (FCW), dry cell weight (DCW). Indirect evaluations include pH measurements and medium residual conductivity (mmhos). Finally, parameters describing growth efficiency, such as specific growth rate (μ), doubling time (dt), and growth index, can be determined.

3.2.1. Settled Cell Volume and Packed Cell Volume

Both parameters allow for the quick estimation of culture growth while maintaining sterile conditions. These measurements are useful for monitoring growth in the same flasks along a culture cycle, since suspensions may be returned to prior culture conditions. Care must be taken to maintain sterile conditions. However, volume estimation may not be an accurate way of monitoring growth, given its dependence on cell morphology (cell and clump size, cell density, etc.).

SCV is determined by allowing a cell suspension to sediment in graduated tubes. It is reported as the percentage of the total volume of suspension occupied by the cell mass. The PCV is determined in a similar way, after it has been compacted by centrifugation (see Note 1).

Settled Cell Volume

1. Pour the cell suspension in a graduated cylinder of adequate volume.
2. Allow the suspension to settle for 30 min and record the cell volume.
3. Take a second read 30 min later. If the variation between readings is higher than 5%, record a third measurement after another 30 min wait period.
4. The volume fraction of the suspension occupied by the cells is determined as the SCV.

Packed Cell Volume

1. PCV can be determined by centrifuging 10 mL of the culture in a 15-mL graduated conical centrifuge tube at $200\times g$ for 5 min.

3.2.2. Fresh Cell Weight and Dry Cell Weight

Fresh and dry cell weight represent more precise measurements of cell growth than the sole culture volume. However, both require the manipulation of samples in nonsterile conditions. Fresh weight estimation involves less time than that required for dry weight, but it may not reflect a real measurement of biomass gain, particularly

at the end of the culture period, when most of the culture growth is due to water uptake (6).

1. Collect the cell mass by filtration, using a Büchner funnel under vacuum.
2. Wash the cell package with about 3 mL distilled water and retain under vacuum for a fixed time periods (e.g. 30 s). Weigh immediately to reduce variations due to water evaporation.
3. Fresh and dry weights are determined as described earlier for callus tissue.

3.2.3. Culture Cell Density

In order to obtain a reliable value of the number of cells in a suspension culture, clusters should be first disaggregated. This can be accomplished by incubating the suspension with an 8% chromium trioxide solution, or with hydrolytic enzymes, such as cellulase and pectinase. The chromium trioxide method is quicker and less complicated than the use of enzymes; however, it hinders the estimation of cell viability in the same sample. Since a careful use of enzymes maintains cells viable, the assessment of the number of living cells by the exclusion of vital stains can be performed in the same sample.

Cell Cluster Disaggregation by Chromium Trioxide

1. Take 1 mL of the cell suspension and add 2 mL of 8% CrO_3 .
2. Incubate the mixture for 15 min at 70°C.
3. Vortex the mixture vigorously for 15 min.

Cell Cluster Disaggregation Hydrolytic Enzymes

1. Take 1 mL of the cell suspension and mix it with 0.5 mL of 10% cellulase and 0.5 mL 5% pectinase.
2. Incubate 30 min at 25°C with rotatory agitation (100 rpm) (see Note 2).

Cell Counting

1. Fill the counting cell chamber with the mixture, position carefully the cover glass on top of the chamber, to avoid the formation of bubbles.
2. Observe under the microscope with the 10× objective to locate the squared field.
3. Count all the cells contained in ten squares. Add the values of the ten squares (do not obtain the average). This number represents the number of cells in 10 μL , so multiply by 100 to determine the cell number per mL (7) (see Note 3).
4. Depending on the culture's cell density, further dilution may be required, which should be considered in the calculation.

3.3. Parameters of Growth Efficiency

3.3.1. Growth Index

Both, fresh and dry weight, are measurements of tissue's absolute biomass at a given sampling time. No reference to the actual growth capacity is taken in consideration. Growth index is a relative

estimation of such capacity as it correlates the biomass data at the sampling time to that of the initial condition. It is calculated as the ratio of the accumulated and the initial biomass. The accumulated biomass corresponds to the difference between the final and the initial masses.

$$GI = \frac{W_F - W_0}{W_0}$$

where GI represents growth index, and W_F and W_0 , represent the final and initial masses, respectively (either as fresh or dry weight).

3.3.2. Specific Growth Rate

It is generally accepted that growth of a cell culture with respect to time is best described by the sigmoid curve theory. At the beginning, the cell population grows relatively slow (lag phase). As the population size approaches one half of the carrying capacity (defined by the nutrient status of the culture medium), the culture's growth per time unit increases. The rate of growth is measured by the steepness of the curve, and it is the steepest when the population density reaches one-half of the carrying capacity (in the middle of the sigmoid). After this point, the steepness of the curve decreases, keeping this tendency as the population increases, until it reaches the carrying capacity (stationary phase).

The specific growth rate (μ) refers to the steepness of such a curve, and it is defined as the rate of increase of biomass of a cell population per unit of biomass concentration. It can be calculated in batch cultures, since during a defined period of time, the rate of increase in biomass per unit of biomass concentration is constant and measurable. This period of time occurs between the lag and stationary phases. During this period, the increase in the cell population fits a straight line equation (7):

$$\ln x = \mu t + \ln x_0$$

$$\mu = \frac{\ln x - \ln x_0}{t}$$

where x_0 is the initial biomass (or cell density), x is the biomass (or cell density) after time t , and μ is the specific growth rate.

3.3.3. Doubling Time

Doubling time (dt) is the time required for the concentration of biomass of a population of cells to double. One of the greatest contrasts between the growth of cultured plant cells and microorganisms refers to their respective growth rates. While the pattern of growth may be the same, plant cells have doubling times or division rates measured in days, while this parameter in many microorganisms is in the order of minutes to hours. One of the fastest (and quite exceptional) doubling time recorded for a plant cell culture is

15 h for tobacco cells (2). The doubling time (dt) can be calculated according to the following equation (7):

$$dt = \frac{\ln 2}{\mu}$$

where μ represents the specific growth rate.

4. Notes

1. Volume of the cell package should be taken immediately after centrifugation, since sedimentation could alternate readings.
2. The conductivity of the culture medium can be determined using a digital conductivity meter or equivalent.
3. A very good linear correlation between the decrease in conductivity and the increase in dry weight can be found.

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Measurement of Cell Viability

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Abstract

An overview of the methods for assessing cell viability is presented. Different protocols of the most commonly used assays are described in detail so that the readers may be able to determine which assay is suitable for their own projects in plant biotechnology.

Key words: Cell viability, Evans blue, Fluorescein diacetate, MTT, TTC, Tetrazolium salts, Pollen viability, Microspore viability

1. Introduction

Plant cell cultures have been widely used as model systems for biochemical and physiological studies, as well as in some biotechnological processes, such as cell permeabilization, cell immobilization, or cell cultivation in bioreactors (1).

A cell suspension can be defined by the number of single cells or aggregates that are dispersed in the culture medium; hence, its growth can be estimated by measuring either their biomass concentration in terms of fresh or dry matter per volume unit, or by cell density. Cell viability is a fundamental factor to consider since it is connected not only to the well-being of the culture but also to its productivity. Therefore, the accurate assessment of the number of viable cells in a population is very important to prevent the inclusion of low viable or dead cells in the calculations of results per cell or on a fresh weight basis or to indicate the maximal attainable cell density in production processes (2).

A cell is considered viable if it has the ability to grow and develop (3, 4). Reagent-aided methods to assay viability are based on either the physical properties of viable cells, such as membrane integrity or cytoplasmic streaming, or on their metabolic activity,

such as reduction of tetrazolium salts or hydrolysis of fluorogenic substrates. To assess cell membrane integrity, dyes such as Evans blue (EB, (5, 6)), methylene blue (7), Trypan blue (8), neutral red (9), and phenosafranine (10) have been used. These compounds leak through the ruptured membranes and stain the contents of dead cells and then are accounted for via microscopic observation or spectrometric estimation.

Other methods rely on the measurement of the activity of enzymes, such as reductases and esterases. In the case of reductases, both, MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) and TTC (2,3,5-triphenyl tetrazolium chloride), accept electrons from the electron transport chain of the mitochondria; as a result, these molecules are converted to insoluble formazan within viable cells with fully active mitochondria (11). On the other hand, intracellular esterases hydrolyze a fluorogenic substrate (fluorescein diacetate) that can pass through the cell membrane, whereupon they cleave off the diacetate group producing the highly fluorescent product fluorescein. Fluorescein will accumulate in cells, which possess intact membranes, so the green fluorescence can be used as a marker of cell viability (12). An alternative method to measure the activity of esterases is the use of two-dimensional fluorescence spectroscopy, which eliminates the time-consuming, and often difficult, counting of viable and nonviable cells under the microscope (1). Nevertheless, images can now be easily obtained from the observed microscopic fields and then, they can be assessed with a number of computer programs that use functions or algorithms to discern the cells' status, e.g., MATLAB, MathWorks Co. (13).

Apart from the mentioned reagent-aided methods, there are also approaches for assessing cell growth and viability, which include typical on-line instruments based on capacitance (14), in situ dark field microscopy probe (15), infrared sensing (16), and turbidity (17), among others.

In here, we present three different cases of study, in which the determination of viability was cumbersome to future efforts.

1. Cell death is an important feature to monitor during plant-pathogen interactions, so it must be measured accurately for the proper appraisal of the cellular responses. There have been contradictory reports of the measured cell viability in elicited cell suspension cultures (18–20). Hence, we have analyzed the elicitor induced cell viability changes in a tomato cell suspension using different methods. The use of the reduction of tetrazolium salts is proposed as a better indicator of metabolically active cells during the early hours of fungal elicitation (21); although, during the characterization of the cell suspension's culture cycle, viability was monitored employing the Evans Blue assay.
2. Tissue immobilization in alginate is a suitable alternative in plant protocols for biochemical studies where the conservation

of a complete differentiated state is pivotal. The use of alginate as the matrix of choice is based on its non toxicity to the tissues and its permeability to let nutrients in, and in turn allows the removal of debris or excretion compounds. We have developed a methodology for the establishment of an immobilized placental tissue culture from *Capsicum chinense* Jacq. with the aim of studying placental primary and secondary metabolisms. The use of a modification of Evans Blue assay (22) was proposed to assess the physiological state of this tissue.

3. Anther/pollen culture offers invaluable opportunities for the success of breeding programs, primarily through the production of homozygous lines. In order to efficiently exploit its potential, it is critical for pollen quality and their response frequencies to be as high as possible since further efforts are costly and labor intensive. Thus, reliable methods for testing pollen viability are essential, first in order to identify male sterility factors and subsequently to study environmental factors that affect its development in culture. Pollen viability of two species from the *Capsicum* genus: *C. chinense* Jacq. and *C. annuum* L. were evaluated to assess pollen grains' ability to germinate in vitro (23). Additionally, the embryogenic response of uninucleated and binucleated microspores in media differing in the concentration of maltose and sucrose (24) was assessed using tetrazolium salts. Both MTT (25) and TTC offered a consistent approach to monitor the early response of multinucleated pre-embryo development in culture.

2. Materials

2.1. Cell Suspension Cultures

Calli were induced from young leaves of *Solanum lycopersicum* L. Var. Rio Grande on media with Murashige and Skoog (MS) salts (26) and supplemented with B₅ vitamins (27), 3% (w/v) sucrose, 2.24 µM 2,4-dichlorophenoxyacetic acid, 0.049 µM kinetin, and 0.22% gelrite, pH 5.8. They were incubated in the dark at 25°C and subcultured every 3 weeks. Friable calli were transferred into 50 mL liquid medium (as previous, without gelrite) with orbital shaking (100 rpm). The cell suspensions were subcultured every 7 d by 1:5 dilution with fresh media. In order to observe an adequate response to the elicitor treatments, the number of cells in the suspension cultures was adjusted to 2.5×10^6 cells per mL (see Note 1).

2.2. MTT/TTC Assay

1. MTT stock solution: dissolve 50 mg in 10 mL sterile 50 mM phosphate buffer, pH 7.5 and store in the dark at 4°C.
2. TTC stock solution: dissolve 0.2 g in 10 mL sterile 50 mM phosphate buffer, pH 7.5 and store in the dark at 4°C.

3. Sterile 50 mM phosphate buffer, pH 7.5.
4. 50% (v/v) methanol, containing 1% (w/v) sodium dodecyl sulfate (SDS).

2.3. Evans Blue Assay

1. EB stock solution 0.1%: dissolve 100 mg in 10 mL sterile water (10×) and store in the dark at 4°C. From this solution take 1 mL and dilute in 10 mL sterile water.
2. 50% (v/v) methanol, containing 1% (w/v) SDS.

2.4. Fluorescein Diacetate Assay

1. Fluorescein diacetate (FDA) stock solution: dissolve 25 mg of fluorescein diacetate in acetone. Preserve the solution in the dark at -20°C.
2. 50 mM phosphate buffer, pH 7.5.

2.5. Pollen Germination and Viability Assay

1. Germination media: 2 mM H_3BO_3 , 2 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mM KNO_3 , and 3% sucrose in distilled water.
2. Pollen viability stain [3% (w/v) aniline blue and 2% (w/v) basic fuchsin]: dissolve 150 mg of aniline blue and 100 mg of basic fuchsin in 5 mL of 25% (v/v) ethanol.

2.6. In Vitro Microspore Viability Assay Using MTT/TTC

1. MTT stock solution: dissolve 0.1 g in 10 mL of 3% (w/v) sucrose in sterile water and store in the dark at 4°C.
2. TTC stock solution: dissolve 0.2 g in 10 mL of 3% (w/v) sucrose in sterile water and store in the dark at 4°C.

3. Methods

3.1. MTT/TTC Assay (for Cell Suspension Cultures)

1. Wash aseptically tomato cell suspension samples (1 mL) with 50 mM phosphate buffer, pH 7.5. Repeat twice (see Note 1).
2. Resuspend the cells in 1 mL of the same buffer.
3. Add MTT or TTC to a final concentration of 1.25 or 2.5 mM, respectively.
4. Incubate samples for 8 h in the dark at 25°C.
5. Solubilize formazan salts with 1.5 mL 50% methanol, containing 1% SDS, at 60°C for a period of 30 min.
6. Centrifuge at $1,875 \times g$ for 5 min and recover the supernatant.
7. Repeat steps 5 and 6. Pool the supernatants.
8. Quantify absorbance at 570 nm for MTT and 485 nm for TTC (see Note 2).

**3.2. Evans Blue Assay
(for Cell Suspension
Cultures)**

1. Add EB stock solution to cell suspension samples (1 mL) to a final concentration of 0.025% (v/v).
2. Incubate for 15 min at room temperature.
3. Wash extensively with distilled water to remove excess and unbound dye.
4. Solubilize dye bound to dead cells in 50% (v/v) methanol with 1% (w/v) SDS at 60°C for 30 min. Repeat twice and pool the supernatants.
5. Centrifuge at $1,875 \times g$ for 15 min.
6. Dilute the supernatant to a final volume of 7 mL.
7. Quantify absorbance at 600 nm (see Note 2).

**3.3. Evans Blue Assay
(for Immobilized
Tissue Culture)**

1. Add EB stock solution to immobilized placental tissue (1 g) to a final concentration of 0.042% (v/v).
2. Incubate for 45 min at room temperature.
3. Wash extensively with distilled water to remove excess and unbound dye.
4. Solubilize dye bound to dead cells in 50% (v/v) methanol with 1% (w/v) SDS at 60°C for 30 min. Repeat twice and pool the supernatants.
5. Dilute the supernatant to a final volume of 20 mL.
6. Quantify absorbance at 600 nm (see Note 3).

3.4. FDA Assay

1. Mix cell samples (1 mL) with 10 μ L of FDA stock solution.
2. Incubate for 15 min at room temperature in the dark.
3. Adjust the volume to 4 mL with distilled water.
4. Centrifuge at $1,875 \times g$ for 5 min.
5. Resuspend the pellet in 1 mL 50 mM phosphate buffer, pH 7.5.
6. Freeze quickly in liquid nitrogen.
7. Thaw and dilute samples to 3 mL with phosphate buffer.
8. Homogenize with a Brinkman polytron at high speed for 10 s.
9. Centrifuge at $1,875 \times g$ for 20 min.
10. Dilute a 100 μ L sample of the supernatant to a final volume of 2 mL.
11. Determine fluorescence at 516 nm, using a 492 nm excitation beam (see Note 2).

**3.5. Microscopic
Assay**

1. Stain cell samples with FDA by mixing the samples (1 mL) with 10 μ L of the stock solution.
2. Incubate for 15 min at room temperature in the dark.

3. Wash with 50 mM phosphate buffer, pH 7.5.
4. Centrifuge at $1,875 \times g$ for 5 min.
5. Resuspend in phosphate buffer (1 mL).
6. Counterstain with EB, following steps 1–5 but using 10 μL of the EB stock solution.
7. Determine the number of blue dead cells under a bright field and yellow-green fluorescent viable cells under ultraviolet light (excitation: BP 450–490 nm and emission: LP 520 nm) in an Axioplan microscope in ten randomized fields in a Sedgewick chamber.

3.6. Pollen Germination Assay

1. Collect anthers from newly opened pepper flowers between 8:00 and 11:30 am.
2. Dust the pollen of one dehiscent anther onto each 1.5-mL microcentrifuge tube.
3. Add 100 μL of germination media to each tube and gently invert them twice to obtain a homogeneous pollen suspension.
4. Transfer 25 μL of the pollen suspension to a clean cover glass so it spreads into a uniform film.
5. Invert carefully the cover glass on a clean glass slide without trapping air bubbles, and place the slides horizontally on a black moist humidity chamber at $25 \pm 2^\circ\text{C}$ for 24 h.
6. Monitor pollen germination with a stereoscope under dark field every 3–6 h for 24 h and record data for both germinated and nongerminated pollen.
7. Evaluate pollen viability for each time point using a dye solution containing 3% (w/v) aniline blue and 2% (w/v) basic fuchsin in 25% (v/v) ethanol.
8. Determine the percentage of germination at 3, 6, 12 and 24 h, using the following equation:

$$\% \text{ germination} = \frac{\text{total germinated pollen grains}}{\text{total pollen count}} \times 100.$$

3.7. In Vitro Microspore Viability Assay Using MTT/TTC

1. In all cases, aseptically collect anthers from in vitro cultures after 6, 12, 24, and 48 h, and 15 and 30 days of initiated the experiments, and put them in sterile 1.5-mL microcentrifuge tubes.
2. Liberate microspores in uni/binucleated and multicellular stage from the swollen anthers by mixing them with 1 mL of either 1% (w/v) MTT or 2% (w/v) TTC in 3% aqueous sucrose solution and incubate at 37°C for 24 h in the dark.
3. Transfer the immature pollen solution to a Sedgewick chamber and count 25 randomized fields using a light microscope (100 $\times$ magnification) (see Note 4).

4. Notes

1. Cell densities were determined by the chromium trioxide method. Briefly, 1-mL samples were taken at the indicated time and mixed thoroughly with 2 mL of 8% (w/v) chromium trioxide. Then, they were incubated for 15 min at 80°C. One-milliliter samples were loaded on a Sedgewick Rafter counting cell chamber (London), which holds 100 mm³ and 10 randomized fields were counted twice per slide under bright light microscopy (10×). The mean and standard deviation of cell density values were calculated.
2. There was a robust correlation between enzyme activities and the number of viable cells employed in each assay (MTT, $r^2=0.995$; TTC, $r^2=0.989$; EB, $r^2=0.979$; FDA, $r^2=0.996$) and an internal control of heat-killed cells (75°C for 30 min) was used to estimate the percentages of relative viability ($r^2=0.998$).
3. In the case of immobilized placental tissue, an internal control of heat-killed tissue (100°C for 1.5 h) was used to estimate the percentage of relative viability.
4. Usually, five replicates from each sample were analyzed. Immature pollen was considered viable if it turned deep pink (MTT) or red (TTC), or it presented no color, but showed colored lines over its surface.

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Chapter 6

Pathogen and Biological Contamination Management in Plant Tissue Culture: Phytopathogens, Vitro Pathogens, and Vitro Pests

Alan C. Cassells

Abstract

The ability to establish and grow plant cell, organ, and tissue cultures has been widely exploited for basic and applied research, and for the commercial production of plants (micro-propagation). Regardless of whether the application is for research or commerce, it is essential that the cultures be established in vitro free of biological contamination and be maintained as aseptic cultures during manipulation, growth, and storage. The risks from microbial contamination are spurious experimental results due to the effects of latent contaminants or losses of valuable experimental or commercial cultures. Much of the emphasis in culture contamination management historically focussed on the elimination of phytopathogens and the maintenance of cultures free from laboratory contamination by environmental bacteria, fungi (collectively referred to as “vitro pathogens”, i.e. pathogens or environmental micro-organisms which cause culture losses), and micro-arthropods (“vitro pests”). Microbial contamination of plant tissue cultures is due to the high nutrient availability in the almost universally used Murashige and Skoog (Physiol Plant 15:473–497, 1962) basal medium or variants of it. In recent years, it has been shown that many plants, especially perennials, are at least locally endophytically colonized intercellularly by bacteria. The latter, and intracellular pathogenic bacteria and viruses/viroids, may pass latently into culture and be spread horizontally and vertically in cultures. Growth of some potentially cultivable endophytes may be suppressed by the high salt and sugar content of the Murashige and Skoog basal medium and suboptimal temperatures for their growth in plant tissue growth rooms. The management of contamination in tissue culture involves three stages: disease screening (syn. disease indexing) of the stock plants with disease and endophyte elimination where detected; establishment and pathogen and contaminant screening of established initial cultures; observation, random sampling, and culture screening for micro-organism in multiplication and stored cultures. The increasing accessibility of both broad-spectrum and specific molecular diagnostics has resulted in advances in multiple pathogen and latent contaminant detection. The hazard analysis critical control point management strategy for tissue culture laboratories is underpinned by staff training in aseptic technique and good laboratory practice.

Key words: Culture contamination, Endophytes, HACCP, In vitro contamination, Micro-propagation, Phytopathogens, Vitro pathogens

1. Introduction

Plant cells, tissues, and organs are widely used in research, for example in the commercial production of plant metabolites, biotransformation of pharmaceuticals, production of proteins including antibiotics, plant genetic manipulation, and propagation of plants (1–4). In all applications, it is essential that the cultures and progeny plants be free of microbial contamination. Microbial contaminants, whether pathogens of the plant or not, may influence the behaviour of the tissues *in vitro* by releasing metabolites and proteins which affect the plant tissues and altering the composition and or pH of the culture medium. Furthermore, microbial contaminants may overrun the cultures resulting in the loss of valuable research material or cause production losses in micro-propagation. If released in progeny plants, contaminants may spread disease locally and across international quarantine zones, resulting in crop loss and creation of potential reservoirs of inoculums for the pathogen. Progeny plants contaminated endophytically with non-pathogenic bacteria may also show adverse growth effects (5).

Plant diseases have been described in classical literature, but it was not until the nineteenth century that plant pathology (syn. phytopathology) became an established discipline (6). An infrastructure for phytopathology has been established under the United Nations Food and Agriculture Organization (UN FAO; <http://www.fao.org>) with regional, inter-regional, national, and state agencies, professional bodies, local institutes, university and college departments, and commercial companies involved in disease research and development of diagnostics and diagnostic protocols (see for service providers: <http://wdcm.nig.ac.jp/hpcc.html>; <http://www.rapidmicrobiology.com>). This infrastructure is available to those working with plant cell, tissue, and organ cultures. Phytopathology services are available to plant tissue culturists for pathogen screening of stock plants, but tissue culturists also have to deal with some unique problems, namely, random endophytic contamination of stock plant tissues by environmental micro-organisms and contamination of cultures by environmental micro-organisms and micro-arthropods (7–11).

It is now widely accepted that most plants established from seed start life free of microbial contamination, that is, few diseases are vertically transmitted from generation to generation (12). Seed-derived plants may, however, be colonized epiphytically (externally) and perhaps endophytically by one or more environmental micro-organisms at/after planting. In contrast with seed-derived plants, perennial crops and vegetatively propagated crops should, arguably, always be considered to be locally, if not systemically, endophytically contaminated (13, 14). These endophytes, in contrast with pathogens, are non-pathogenic strains of

locally abundant micro-organisms (15), mainly bacteria, which enter through natural openings on the plant surface, or through wounds, or at sites of pathogen or pest damage. Many grow on plant tissue culture establishment media, but some may be suppressed by the high osmotic pressure of the media, specific media components, and pH of the media or not grow rapidly enough to be visible at the temperature of the culture and to be transmitted clonally in vitro and possibly overrun the tissues when the medium composition is changed, e.g. when the tissues are transferred to reduce strength medium for rooting, etc. (16).

A further source of microbial contamination which is usually expressed rapidly is contamination by common environmental micro-organisms (bacterial and fungal) in the culture laboratory and growth room and by micro-arthropods in the latter (17). Inoculum of environmental micro-organisms is abundant universally but in some climates may show seasonal trends (18). While it is generally difficult, if not impossible, to recover heavily contaminated cultures, it may be possible to use antibiotics to treat slow-growing contaminants (19). There is anecdotal evidence that some laboratories may be routinely using antibiotics to suppress microbial contaminants.

In summary, the elements of pathogen, endophyte, laboratory, and growth-room contamination management depend on applying the principles of plant pathology, clinical microbiology, and Hazard Analysis Critical Control Point (HACCP) management (11, 20). Here, the application of these principles at each stage in the tissue culture (21) is discussed, namely, at Stage 0 (selection and preparation of stock plants); at Stage 1 (establishment of aseptic cultures in vitro); at Stage 2 (multiplication of cultures in vitro); and at Stage 3 (in vitro preparation of plants for return to the environment). Stage 4 (establishment of micro-plants extra vitrum) is not discussed here (see refs. 3, 16, 22).

2. Background: Pathogen and Microbial Plant Distribution in and on Plants

It is necessary to have an understanding of plant anatomy and the distribution of pathogens and other micro-organisms on and in plant tissues and organs to appreciate the background to the selection of plant parts for disease screening and the selection of tissues for culture (6). Plants propagated from certified seed (i.e. seed chemically treated with pesticides) or certified stock plants are free of specified pathogens but especially in the case of vegetative propagules may be contaminated with pathogens which have not been screened for, and endophytes. When the seed or vegetative propagule, e.g. aseptic micro-plant, is planted and germinates, it is colonized

epiphytically by environmental micro-organisms. Microbial growth is abundant in the rhizosphere due to the high leakage of nutrients from plant roots (23). In root growth, the emergence of lateral roots creates openings in the root epidermis and cortex through which soil micro-organisms may enter, colonizing the intercellular spaces. Most such endophytic colonizers are excluded from the root core by the suberization of the exodermis (outer layer of cells around the vascular system) (24). Exceptions are soil-borne viruses which may become systemic (12). Beneficial micro-organisms which colonize root cells, such as mycorrhizal fungi and rhizobia, are not considered here as they are not a contamination issue (23). Some specialized pathogens (fungal vascular pathogens) have the ability also to enter the vascular system in the roots and systemically colonize the plant (6). Micro-organisms, including non-specialist root pathogens, that colonize the intercellular spaces of the root may be restricted at the root crown from moving up into the stem and colonizing the haulm (above ground) tissues (6, 25).

The aerial parts of the plant are colonized by environmental micro-organisms and airborne pathogens, but the nutrient supply on the leaf surface is limited and other factors, such as water availability and UV exposure, may be highly variable (26). Foliar fungal and bacterial pathogens use pressure mechanism and/or enzymes to break down the plant structural barriers to colonization; usually, pathogen damage is easily seen by eye or magnifying hand lens and the affected tissues can be discarded for culture (6, 25). Insect-transmitted viruses/viroids and xylem- and phloem-restricted bacteria are injected into the vascular system and spread systemically therein. Some viruses are introduced into the parenchyma cells of the leaf and spread intracellularly *via* the plasmodesmata between the neighbouring cells and enter the vascular system from which they spread systemically (12). As plant tissues develop, intercellular spaces and cavities may develop which can be colonized by environmental bacteria entering through wounds and natural openings, such as leaf scars (24). These endophytes may increase cell leakage, providing plant nutrients for their colonization (6, 25). In general, where no organic manure is applied to the plant, the colonists are more likely to be from common plant surface-dwelling genera, such as *Pseudomonas* (14).

In summary, plants can be colonized epiphytically and endophytically intracellularly and intercellularly by micro-organisms. Critically intracellular pathogens and presumably endophytes also, are usually restricted to the limit of development of the vascular system which develops back from the apex, and excluded from the solid tissues in the apex (12, 24): hence the basis of meristem culture for the elimination of pathogens and endophytes (16).

3. Contamination Management at the Stage 0

3.1. Selection and Screening of Stock Plants

It is important to observe the industrial maxim of “rubbish in, rubbish out” (referring to raw materials used in manufacturing) when approaching the establishment of tissue cultures. Every effort should be made to ensure that the stock plant(s) used is appropriately screened for pathogens of the crop and for endophytic contamination. Phytopathology databases, crop disease compendia (see <http://www.apsnet.org> for publications of the American Phytopathological Society, APS), should be consulted for information on disease of the crop and official protocols for the selection of mother plants and the production of pathogen-free stock (see <http://fao.org>; <http://www.eppo.org>; <http://www.nappo.org>). Note: Only officially approved screening methods are rigorous enough to satisfy official crop disease-certification schemes (27). Where the crop is not cultivated on a large scale, and this applies to many of the high-value crops which are micro-propagated, there may be no official methods published, but the general principles of guidelines for related species/genera and for the type of plant, e.g. woody ornamental, tuber crop, etc., should be observed (28).

Selection of stock plants for tissue culture begins with the selection of vigorous, symptomless plants and their transfer to an insect-proofed greenhouse, where they are observed for disease symptoms and managed in a way which minimizes the risk of pathogen infection (21). Since expression of pathogen symptoms, especially virus symptoms, may be seasonal and/or nutritionally dependent, it is important to be familiar with the relevant plant pathology literature (6, 12); see also disease compendia of the APS at <http://www.apsnet.org>. It is also important to be aware that some viruses, viroids, and phloem/xylem-restricted bacteria may be symptomless but affect plant vigour (12). Indeed, some subliminal infections of plants, especially ornamentals, may be “beneficial”. Examples of beneficial infections are some flower breaks, notably the “Rembrandt” tulips, foliar variegations, and vein clearings (29). Also more than one virus may be present in a plant (12). Where more than one virus is present, the viruses may interact positively (giving severe symptom), negatively (reducing symptom severity), or not interact (12). It is important in multiple infections to confirm that all viruses have been eliminated.

It is essential to reduce the risk of contamination spread between plants by sterilizing equipment before use on the next plant in the screening. Plants may be grown in Sunbags™, namely, plastic bags with bacterial filters which allow gaseous exchange with the ambient environment. Samples of the plants are taken for screening by grafting, enzyme-linked immunosorbent assay (ELISA), or nucleic acid (NA)-based diagnostics as prescribed in the official protocol or diagnostics producer’s instructions where

applicable. Test results may be available in hours for ELISA to months in the case of grafting. Details of screening methods are given in Table 1. Should this official certification approach be impractical for a researcher, it may be possible to follow an alternative strategy based on the use of ELISA and NA-based diagnostics on the stock plant along with culture screening of the initial cultures (see Subheading 4).

Testing for non-pathogenic endophytes is complicated by the random nature of contamination (13–15). As an aside, while the association between pathogens and their host plants is well studied, colonization by non-pathogenic endophytes is promiscuous and arguably influenced by random events, such as tissue damage and local inoculum pressure. There is insufficient evidence to date to support the hypothesis that some non-pathogenic endophytes are host specific. Methods used to screen for endophytes include expressing sap from the plant and culturing the extract on bacteriological media or surface sterilization of tissue pieces (explants) and their culture on bacteriological media (30, 31). The explants culture method may be more reliable as tannins and other plant materials released during sap preparation may inhibit growth of the endophyte (12, 16) in the preparation of sap samples for testing.

3.2. Methods for Pathogen and Endophyte Elimination from Stock Plants

Methods have been developed to eliminate specific viruses/viroids and intracellular bacteria from mother stock plants for the production of certified planting material for the grower (12, 32, 33). Heat therapy is widely used to eliminate plant viruses, and cold treatment has been used to eliminate some phytoplasmas (12, 16, 27). Thermotherapy is based on the destabilizing effects of heat on the pathogen. Plants are treated in “hot boxes” at temperatures in the range 32–40°C for 4–30 weeks. For species that are not heat tolerant, shorter periods at high temperature are alternated with ambient temperatures, e.g. 4–8 h at 40°C followed by 16–24 h at 20°C (16). Virus elimination, as opposed to suppression, must be confirmed according to the FAO/regional organization’s protocols (see Subheading 2).

The most widely used method to eliminate all classes of pathogens is “meristem culture” (4, 16). This depends on the hypothesis that micro-organisms affecting the plant do not penetrate the apical tissues (12) and are dependent on culturing the smallest apical tip that will survive in vitro on culture medium or when grafted to an in vitro micro-plant or aseptic seedling (16). This hypothesis generally holds up for most viruses and bacterial pathogens and it is not unreasonable to assume that it will also apply to (non-pathogenic) bacterial endophytes. The effective use of meristem culture depends on sterilization of the excised buds to eliminate surface contamination. Commonly, this is achieved by immersion in hypochlorite solution containing a surface-wetting agent. Hypochlorite is unstable and the active concentration should

Table 1
The main methods used to characterize pathogenic and non-pathogenic micro-organisms associated with plants

Micro-organism	Symptom expression	Inoculation of indicator plants	Culture on microbiological medium		Biochemical tests	Serological diagnostics	DNA diagnostics
			Microscopy				
Viruses	Widely used to identify pathogens	Widely used to confirm pathogenicity and identify pathogen strains	Electron microscopy used in research	Non-cultivable	Not applicable	ELISA is widely used to identify pathogens	Widely used to identify viral pathogens
Viroids	Widely used to identify pathogens	Widely used to confirm pathogenicity and identify pathogen strains	Not commonly used	Non-cultivable	Not applicable	Not applicable	Standard method used to identify viroid pathogens
Bacteria	Widely used to identify pathogens	Widely used to confirm pathogenicity and identify pathogen strains	Light microscopy used to investigate morphology	Widely used to identify pathogens and environmental bacteria	Not commonly used to identify plant pathogens; used to identify environmental and human pathogenic bacteria	Not commonly used	Used to identify pathogens and environmental bacteria
Fungi	Widely used to identify pathogens	Widely used to confirm pathogenicity and identify pathogen strains	Light microscopy used to investigate morphology	Not commonly used	Commonly used for identification of environmental yeasts	Not commonly used	Used to identify pathogens and environmental fungi

be determined experimentally or commercial products used by their expiry date (34). George (16) recommends alternative surface sterilants for more intractable contamination.

In meristem excision, the apical bud is surface sterilized and then dissected removing the outer leaflets/leaf primordia until the apical dome or apical dome plus first pair of leaf primordial is reached (4). This explant is then transferred to an appropriate meristem culture medium (16, 22). Menard et al. (35) recommend that the latter includes polyvinylpyrrolidone (PVP) to counteract the production of tannins which may block nutrient availability to the explants and suppress bacterial contamination of the explants and components of bacterial media to encourage the growth of bacterial contaminants. Early expression of bacterial contamination is critical to the prevention of the entry of contaminants into culture with consequential risk of contamination of established clean cultures during multiplication of cultures.

4. Stage 1: Screening of Initial Cultures

4.1. Screening of Initial Cultures of Large-Scale Production Crops

Assuming that pathogen-free stock is available, then the focus is on screening for the presence of latent bacterial endophytic contamination or environmental contamination introduced during the tissue culture process (Fig. 1). In most cases, plants are introduced into culture via meristem culture; however, in some cases, other explants are used, namely, nodes, internodes, leaf and stem explants, and rarely root tissues (16, 22). In contrast to meristem culture, establishment of tissue cultures via other explants posed the high risk of transmission by inter- and intracellular micro-organisms present in those explants. The risk of introduction of contamination with root explants is so great that the latter explants are rarely used to establish cultures (16, 22). Commonly, cultures are only visually inspected for contamination assuming that any bacterial contamination will be expressed, that is, the bacteria will grow on the plant tissue culture medium. This is not an unreasonable assumption but is not universally applicable as some endophytic bacteria may be inhibited by the high salt, pH, and/or plant growth regulators in the culture medium or may not grow rapidly at the culture temperature (35). For temperate crops, it is likely that bacterial endophytes and environmental microbial contaminants will grow at the growth-room temperature of 20–25°C, but bacterial endophytic contaminants of tropical crops may have higher temperature optima. Since most laboratory contaminants and endophytes may be common environmental micro-organisms, they are likely not to be fastidious with respect to the components of their culture and may grow on common bacteriological media. This also applies to yeast endophytes and the media used in screening should

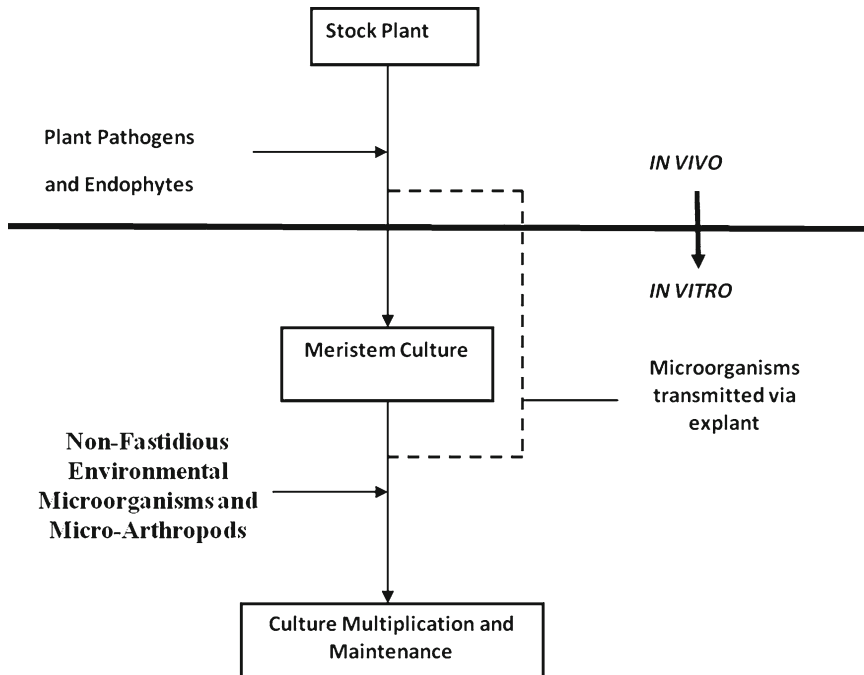


Fig. 1. A scheme illustrating a two-phase strategy for managing contamination in tissue culture, where the focus is on pathogen screening and pathogen and contaminant elimination before or at the establishment of the cultures, and good laboratory working practice is used to exclude environmental organisms during the *in vitro* stages. It is hypothesized that most pathogens and endophytes are eliminated if cultures are established from meristem explants, but this must be confirmed by screening of the initial cultures.

reflect possible contamination with the latter. The minimum screening conditions should be based on plating the excised base of the established explants on broad-spectrum (“universal”) bacterial and yeast diagnostic media (see <http://www.sigma.com> for media formulations) and followed by incubation at 20, 25, and 30°C for approx. 10 days. Cultures may be contaminated also by airborne fungal spores of, e.g., *Penicillium* and *Aspergillus* spp. These rapidly grow on plant tissue culture media and can be identified using a light microscope (36) (Fig. 2).

If pathogen-free stock is unavailable and meristem culture is being used to eliminate pathogens and subliminal endophytes, then the complexity of screening is increased, especially where the cultures will be used to multiply pathogen-free stock for certification as free of specific pathogens. This is because the official certification schemes generally involve screening based on greenhouse/field-grown plants (27), that is, screening is not accepted for *in vitro* cultures and progeny plants must be established and grown *in vivo* before testing can be carried out. In the case of fruit trees, the classic screening for some pathogens involves grafting to root-stock of indicator species (27). In some areas, micro-propagators

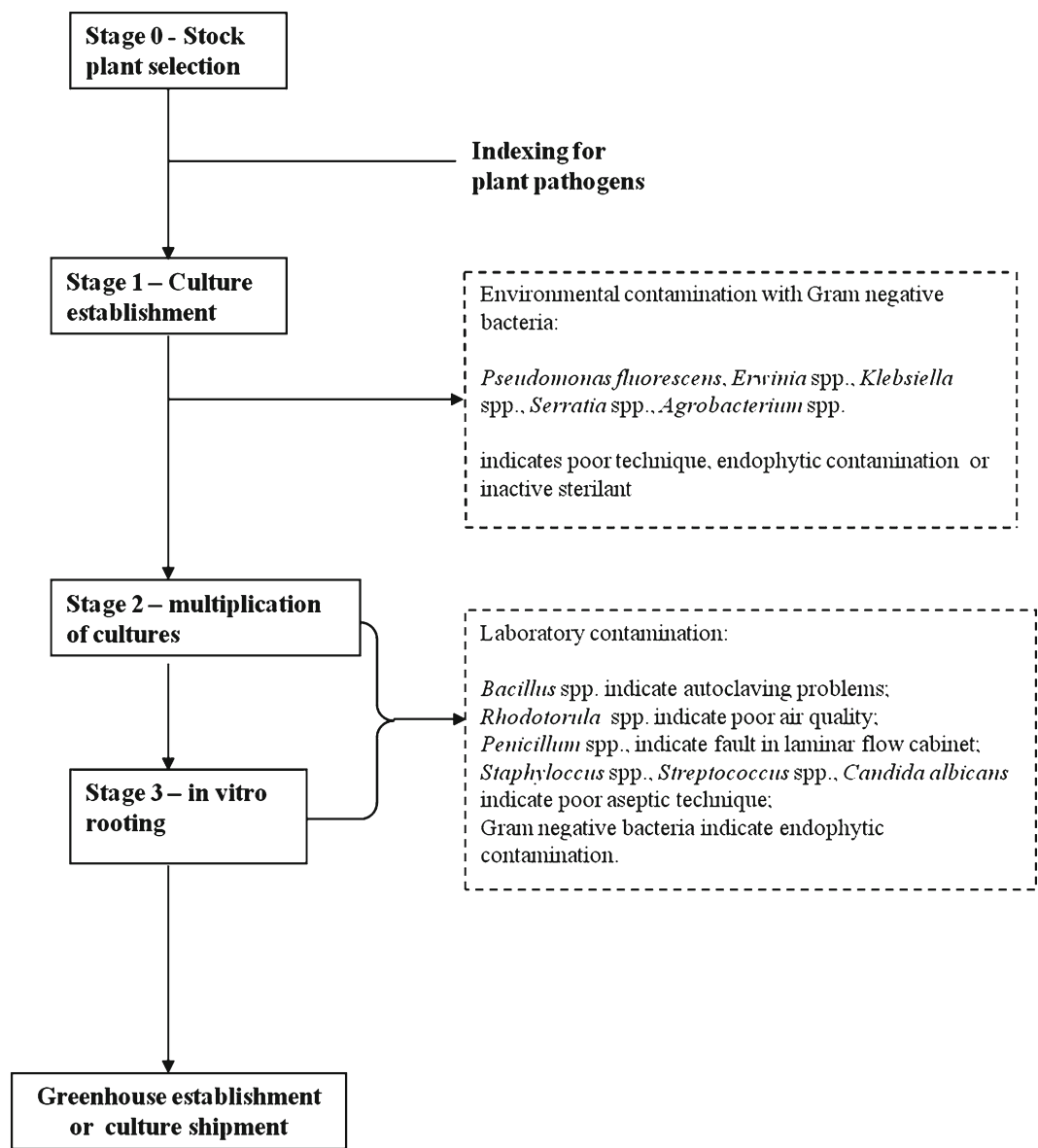


Fig. 2. A flow diagram showing the stages in micro-propagation. Representative micro-organisms, which indicate failure of aseptic technique and equipment at the respective stages, are given.

can obtain cultures established from health-certified mother plants provided by the quarantine authorities. The latter “certified cultures” may, however, only be subcultured for a specified number of generations or have a time limit before their certified status expires and they must be replaced by fresh cultures (9).

4.2. Screening of Minor Crops and Species of Research Interest

Commonly, micro-propagators and researchers are supplied by clients or select themselves plants for research, species for which no official disease-screening protocols exist, implying that disease risks may be unknown or poorly characterized, albeit the disease risk for wild or weakly domesticated plants is less than that for highly bred agricultural and horticultural crop varieties (37).

The micro-propagation client may request that the tissue culture progenies are free of a specified pathogen. However, for researchers, there is the risk that latent contaminants may influence experimental results (38), whereas for micro-propagators there is the risk of significant commercial losses in production and even a “non-pathogenic” endophyte when present in micro-plants from establishment may cause losses in the field (5). If a pathogen is specified by the micro-propagation client, the normal approach is to use a commercially available ELISA or NA-based test to screen the mother plant(s) in vivo or the established cultures in vitro. The cultures should also be screened for endophyte contamination. Screening of cultures poses special problems (see Subheading 2).

4.3. Contamination in Externally Sourced Mother Cultures

It is important to note that cultures obtained from official sources, such as state laboratories and international germbanks (<http://www.biodiversityinternational.org>; <http://www.cgiar.org>), may be certified free of specified pathogens but may not be free of endophytes which can overrun the cultures when subcultured to a different medium. In some cases, mother cultures bought in from external sources may have been grown on antibiotics to suppress endophytes. If antibiotics are present in the medium, the externally sourced cultures may express microbial contamination when transferred to antibiotic-free media in the laboratory. It is important to test a sample of externally sourced cultures as a precaution. Explants should be incubated on antibiotic-free media as for screening of cultures for endophytes above (Subheading 3.2).

There are anecdotal claims of the beneficial growth effects of some microbial contaminants of tissue cultures (39). This may be explained by the effects of these micro-organisms on the pH of the medium, by their enhancement of nutrient availability to the cultures, or by their production of plant growth regulators (40, 41). Any such positive growth effects must be offset against the potential risks of culture losses when the medium is altered or damage to the progeny plants at and after establishment occurs.

4.4. Action to Be Taken When Contamination Is Detected in Stage 1 Cultures

If contamination of the initial explants by fast-growing cultivable contaminants is detected, there are two possibilities: firstly, the stock plants may be treated with appropriate antibiotics to suppress the contaminant in the stock plant and cultures may be established from new growth in the stock plants (42); secondly, if the contaminant is fastidious, or slow growing, an attempt to eliminate the contaminant from the cultures may be made by inclusion of

antibiotics in the culture medium (43). It is important to recognize that antibiotics may be bacteriostatic rather than bacteriocidal and may be toxic to the plant cultures (44, 45), and consequently only new growth should be excised and subcultured. Antibiotics and antibiotic cocktails have been used empirically by many plant tissue culturists (19). Regardless of which antibiotic strategy is used, that is, antibiotic treatment of the stock plant or incorporation of antibiotic into the culture medium, the cultures must be re-screened to confirm elimination of the contaminant before subculture.

Correct bacterial identification procedure for antibiotic screening/identification involves isolation in pure culture (by streak plating), establishment of single-colony cultures (30), and screening for antibiotic sensitivity on diagnostic sensitivity testing (DST) medium (43). Antibiotics are incorporated into the plant tissue culture medium at two to four times the minimum inhibitory concentration and testing should be carried out to confirm that their action is not inhibited by components of the plant culture medium (43). Antibiotics which are potential mutagens should not be used (46). Also only early-generation antibiotics should be used to avoid the risk of selection for antibiotic resistance in the culture.

If virus or viroid contamination is present in the cultures, the antiviral chemical ribavirin (syn. Virazole; 1- β -D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide) may be incorporated into the culture medium (12, 47, 48). Ribavirin is virustatic and as with antibiotics, only new tissue should be excised for subculture and screening (47). It has been reported that ribavirin interacts with cytokinins and it may be the best to either reduce or eliminate cytokinin in the elimination medium (49). Confirmation of virus elimination should be confirmed by inoculation of the appropriate indicator plant (see Subheading 5.3).

5. Screening Methods

5.1. Infrastructure for Plant Pathology

As referred to in Subheading 1, there is a comprehensive infrastructure in plant pathology. The international phytosanitary regulatory authority is the Food and Agriculture Organization of the United Nations (<http://www.fao.org>). This is supported by regional organizations—the North American Plant Protection Organization (<http://www.nappo.org>), the European and Mediterranean Plant Protection Organization (<http://www.eppo.org>), with other regional organizations (see <http://www.fao.org> for further information). The APS (<http://www.apsnet.org>) has an international supporting role in publishing crop disease compendia and details of new diagnostic methods. Working groups of the International Society for Horticultural Science (<http://www.ishs.org>) and of other plant pathology societies contribute also to the

development of new methods of disease diagnosis. There are also governmental organizations and private companies which supply mother cultures, plant disease diagnostics, and disease diagnosis.

5.2. Classical Methods for Pathogen Screening of Plants

The general focus in screening plants is on the detection of viruses, viroids, and fastidious or non-cultivable bacterial pathogens. Historically, viruses and viroids were detected by sap, vector, dodder, or graft transmission to indicator plants (12, 50) while intracellular, generally fastidious, or non-cultivable bacterial pathogens were detected by graft or dodder transmission to indicator plants (32). Indicator plants are species/genotypes which show a specific reaction to inoculation with a specific pathogen or pathogen strain (51). These transmission-based tests were slow and required the maintenance of an array of indicator plants at the appropriate growth stage for the test. For details of the classical methods, see Noordam ((51); Table 1).

5.3. Molecular Plant Diagnostics

Classical transmission tests have been largely superseded by antibody-based methods, principally ELISA (52) or nucleic acid-based molecular diagnostics for virus, viroid, and mollicute (fastidious/non-cultivable xylem- and phloem-restricted bacteria) detection (33, 53, 54). Classical detection/colony identification of cultivable bacterial plant pathogens is based on growth response/characteristics on differential or selective media (30, 31) and to a limited extent on fatty acid profiling (55).

ELISA and NA-based diagnostics have two common features: a specific binding function and a signal amplification function. These functions provide the specificity and sensitivity of the test. In virus antibody tests, the antibody is raised against viral coat protein determinants, where there may be one or many determinants per coat protein. The uniqueness of the determinant(s) to the specific viral coat protein determines the specificity of the ELISA (or other antibody-based tests). ELISA may be based on “monoclonal” or “polyclonal” antibodies to achieve different levels of specificity (56). The sensitivity of ELISA is based on the enzyme–antibody-linked activity on the chromogenic substrate used in the test (52). An important consideration in the interpretation of ELISA results, which are statistical positives/negatives, is to have an appropriate positive/negative threshold (57). The highly conserved proteins involved in virus replication are in low relative abundance, difficult to harvest free of host protein contamination, and, hence, not used commercially in ELISA kits. ELISA is of limited use in identifying bacterial plant pathogens as the available determinants, e.g. membrane and flagellar proteins, may not discriminate between pathogenic and non-pathogenic isolates. In general, while a positive ELISA test, taken in conjunction with disease symptom expression, is taken as a positive pathogen diagnosis, it is advisable that appropriate test (indicator) plants are inoculated with the isolated

micro-organism to confirm pathogenicity. It is important to confirm Koch's Postulates for cultivable micro-organisms (6).

Unlike ELISA, antibodies for which cannot be raised against non-transcribed regions and highly conserved regions of the viral genome, NA probes can be synthesized to detect all regions of the genome (58). Conserved and hypervariable regions of plant viral genomes have been identified and probes can be designed to give narrow- or broad-spectrum detection/identification (12). NA probes are routinely used for the detection of viroids which lack protein coats. NA probes are also used to detect the presence of bacteria; commonly, probes against conserved regions of the ribosomal 16S ribosomal DNA may be used to detect the presence of any/all bacteria, whereas probes against hypervariable regions of 16S rDNA gene can be used to discriminate bacteria to the species and subspecies levels (58). There are various methods of enhancing and detecting NA-probe signals. In the case of plant viruses (most have RNA genomes) where RNA probes are used, the sequence is converted to a DNA copy by reverse transcriptase and then amplified by DNA transcriptase. In the case of bacterial detection, DNA probes are amplified by DNA transcriptase (59). The amplified sequence may be detected by separation on gels and visualized by silver staining, or the probe may be fluorochrome labelled and viewed in liquid (60). DNA micro-arrays may be used to screen for multiple organisms in a sap or tissue extract (58, 61, 62).

5.4. Screening of Plants for Bacterial Endophytes

Miniature biochemical test kits (<http://www.biomerieux.fr>; <http://www.oxoid.com>) and growth on differential or selective media are used widely to identify environmental and human pathogenic bacteria and can be used to detect/identify the latter associated with plants as endophytes ((63, 64); Table 1). For suppliers of media, etc., see <http://www.sigmaldrich.com> and <http://www.oxoid.com>. Moreover, NA probes offer the possibility of detecting any contamination by bacteria of a tissue which is critically what is required in screening for promiscuous/random bacterial contamination. For this purpose, probes against the conserved regions of the 16S rDNA gene are used to screen for bacterial presence, as opposed to probes against the variable regions of the 16S rDNA gene used to develop more specific probes (58, 59).

6. Stages 1, 2, and 3: Laboratory and Growth-Room Contamination Management

As discussed above, when axenic, perhaps more precisely "aseptic", cultures have been achieved, the challenge is to prevent re-contamination in the laboratory (Figs. 1 and 2). While there may be overlap in the species which constitute "pathogens", "endophytes", and "environmental micro-organism" (see above), laboratory

contaminants are generally non-fastidious airborne fungi and bacteria that are fast growing and rapidly overrun cultures and are incurable with antibiotics. Control is based on excluding them from the laboratory environment and cultures by good HACCP management.

6.1. Personnel Training

It is important that all personnel working in or sharing common facilities with the tissue culturists are trained in aseptic technique and have a basic understanding of microbiology. The most important aspects are control of sources of contamination and prevention of contamination of the cultures in the culture laboratory and growth rooms. Training can be provided by digital media (<http://www.sigmaaldrich.com/life-science/cell-culture/learning-center/cell-culture-videos.html>) and can be re-enforced by visits to hospital laboratories and food processing companies which share common contamination management principles. It is important that personnel are familiar with the need to avoid contamination from the external environment which involves removing outdoor shoes before entering the work area or putting on disposable overshoes; wearing of laboratory coats which should be regularly laundered; removal of items of jewellery; washing of hands with aseptic washes; and wearing of disposable headgear.

Tissue culture personnel should also be trained in aseptic laboratory technique from correct autoclaving procedure to cleaning and maintenance of sterile transfer cabinets and use of dissection equipment and laminar flow cabinets. Important aspects of operations in the laminar flow cabinet are that nothing should be placed between the operative and the laminar air source; dissection should be carried out on a sterile tile or similar, which focuses attention on the need to maintain a sterile working surface and avoids cutting the surface of the cabinet; and instruments should be sterilized between each distinct culture event. The use of equipment is discussed further below.

It is important that all personnel be on the look out for sources of contamination at all times. Contaminated cultures, whether detected in the growth room or at subculture, should be autoclaved unopened and subsequently disposed of. Cultures before subculture should be examined carefully for signs of contamination. Contaminated cultures should be autoclaved unopened prior to disposal. If testing for latent endophytes in initial material and brought-in cultures has been carried out, the main contamination risk is from environmental micro-organisms which are generally expressed on plant culture media, and the occasional risk of human-associated micro-organisms (bacteria and yeasts) also probably expressed on plant culture media (14, 64, 65). Personnel with infectious bacterial or yeast diseases (67) should desist from working in the tissue culture laboratory until they have recovered. An important feature of laboratory contamination is the change over

time in culture, predominantly from contamination with plant-associated bacteria to mixed contamination with human-associated bacteria and yeasts (68).

6.2. Maintenance of Laboratory Equipment

6.2.1. Laboratory and Growth-Room Design

Many of the problems associated with laboratory and growth-room contamination can be resolved by good laboratory design or modification, primarily aimed at preventing the entry of environmental micro-organisms. Key aspects are entry airlocks, provision of areas for the personnel to wash and change into laboratory clothing, and filtering of inputs such as water and air into the laboratory. While growth-room shelving may be solid to allow water circulation for bottom cooling of the cultures, it is generally of open mesh to allow air circulation (16). Open-mesh shelving can allow micro-arthropods to drop down from infected cultures on upper shelves to contaminate healthy lower cultures. Where micro-arthropod contamination is detected, plastic or other sheeting should be, at least temporarily, placed on the shelves to prevent this occurrence. For more information on micro-propagation laboratory design, see ref. 69.

6.2.2. Water Supply

In some regions, potable and generally non-potable water may carry heavy microbial contamination and may have a high salt content. It may be desirable to use a UV-water sterilizer or water filtration unit to purify the water entering the laboratory. If used, online water filtration equipment must be maintained in line with the manufacturers' recommendations and under local conditions. Only filtered, single-distilled or double-distilled water should be used for preparing media (16).

6.2.3. Culture Vessels

The problem of microbial contamination of cultures in the growth room can be influenced by the type of culture vessel lid or closure used (16). Cultures need gaseous exchange with the ambient atmosphere to allow oxygen and carbon dioxide exchange and escape of ethylene and other volatiles (70, 71). To achieve gaseous exchange, culture vessels with loose lids are commonly used (Petri dishes, food jars, and customized plant tissue culture vessels) in plant tissue culture (16). The problem with most types of vessel closures (lids) is that while allowing gaseous exchange they also allow spores and micro-arthropods access to the cultures. The medium may also dry out, i.e. the osmotic pressure increases which can suppress the growth of some microbial contaminants and affect the tissue growth. Also, spores and micro-arthropods may be sucked into the vessels at the end of the light period when the atmosphere in the vessels cools and creates a negative pressure in the vessel. It has been shown that plastic packaging materials allow gaseous exchange while preventing microbial/micro-arthropod contamination (72). It is now a common practice in micro-propagation laboratories to wrap conventional culture vessels in "cling film"-type materials (the gaseous permeability of the material

should be checked or advice obtained from the manufacturer) to control growth-room contamination.

Culture vessels are commonly adapted glass food jars and are recycled. Medium is dispensed into the jar which is sterilized by autoclaving. Alternatively, food plastic containers or dedicated plastic tissue culture containers are used (<http://www.sigmaaldrich.com>). The former may not withstand autoclaving but may be sterile as a consequence of the thermal-moulding process used in their manufacture or may be sterilized by gamma irradiation. Pre-sterilized medium is dispensed into these vessels in the laminar flow cabinet.

6.2.4. Autoclaves

Media/equipment/instruments that are autoclaved should always be accompanied by (autoclave) tape which provides an indicator when the appropriate conditions (121°C and 1.05 kg/cm³) have been reached inside the autoclave. Small volumes of media dispensed into culture vessels require 15 min under standard conditions for sterilization, but bulk sterilization of media requires a pre-heating period to allow the medium to reach 121°C (see ref. 16 for information of sterilization times for large volumes of liquid). Vessels should not be tightly lidded during autoclaving to satisfy the conditions for sterilization. Heat-labile materials should not be autoclaved but instead sterilized by ultrafiltration (16).

It is a good practice to rest autoclaved medium for a few days before use to allow any bacterial contamination which passed through autoclaving to be expressed. In general, contamination in solid media is seen as fungal mycelium “ball” development and/or bacterial colony development in, as opposed to on, the surface of the medium (see below). Instruments should be sterilized individually or in sets as opposed to batches to avoid re-contamination when instruments are selected for use.

6.2.5. Automated Medium Dispensers

Equipment for the automated dispensing of medium into Petri dishes is widely used in clinical laboratories. The medium sterilization and peristaltic pump functions of this equipment are used in micro-propagation laboratories, where large volumes of media are used. As above, media should be poured a few days in advance of use and monitored for signs of bacterial/fungal contamination. Contamination on the surface of the poured medium indicates faulty dispensing equipment or procedure. As with other laboratory equipment, the item should be routinely serviced.

6.2.6. Laminar Flow Cabinets

The laminar flow cabinet is designed to provide a positive clean air pressure space for aseptic operations. This is achieved by passing the air through a coarse pre-filter followed by an HEPA filter which removes microbial spores (16). The pre-filter should be removed and washed regularly. Similarly, the HEPA filter should be replaced as recommended by the manufacturers. It should not be assumed that the HEPA filter is functioning. Correct functioning can be

tested by using an airflow meter, and by placing open media plates in the cabinet workspace and by sealing these and incubating them for 3–7 days at 25°C to observe any microbial growth.

It is important that the workspace be cleaned before using a laminar flow cabinet. This can be done by incorporation of a germicidal UV lamp in the cabinet (making sure that the sides of the chamber are not damaged by UV—Perspex sides should be replaced by toughened glass), by covering when not in use, the opening of the chamber with a blind, and/or by swabbing all the interior space with commercial antimicrobial solution. The cabinet should be turned on for a “warm-up” period before use. For a training video, see <http://www.sigmaaldrich.com/life-science/cell-culture/learning-center/cell-culture-videos.html>; see also ref. 16.

6.2.7. Instrument

Sterilization in the Laminar Flow Cabinet

There are several methods used to sterile dissecting instruments during use in a tissue culture session. Traditionally, flame sterilization in ethanol was used, but this method can result in accidental fires and injury to personnel. Alternative methods are the use of hot air guns and hot bead sterilizers (<http://www.sigmaaldrich.com>).

6.2.8. Laboratory and

Growth-Room Atmosphere

The laboratory atmosphere can become contaminated, sometimes seasonally, by atmospheric spores of yeasts, fungi, and bacteria (18). The main inoculum entry points are the access doors and open windows. The problem can be addressed by providing an entry air lock to the laboratory and creating a positive pressure in the laboratory. Advice should be sought from appropriate hospital laboratory designers/constructors. It is also advisable to sample the laboratory/growth-room atmosphere and inspect and screen the cultures in the growth room for contamination regularly, especially slow-growth (long-term storage) cultures which may become reservoirs of infection. Chemical sterilization of the laboratory/growth rooms can be carried out by experts, but this may shut down activities for a period of time.

7. Management of Micro-Propagation Facilities

Depending on the scale of operation, it may be necessary to allocate the contamination management function to one or more of the personnel. Management should be formally structured and based on HACCP analysis as used in the food industry (11, 20). The focus in HACCP is on staff training and equipment servicing, health/contamination status of the mother plants, screening of the initial cultures, sampling and screening of samples of subcultures, and monitoring of the growth rooms for micro-arthropod, bacterial, and fungal contamination. Biomarkers of contamination associated with the stages in tissue culture are shown in Fig. 2. The characteristics of these biomarkers are given in Table 2.

Table 2
Characteristics of HCCP biomarkers of plant tissue cultures

Indicator organism	Type	Characteristics	Quick test
<i>Pseudomonas fluorescens</i>	Eubacteriaceae	Gram-negative, motile rods that oxidize glucose	(31)
<i>Erwinia</i> spp.	Eubacteriaceae	Gram-negative, motile more than 4 peritrichous flagellae, grows anaerobically	(31)
<i>Klebsiella</i> spp.	Eubacteriaceae		(31)
<i>Serratia</i> spp.	Eubacteriaceae	Gram-negative, motile rods, highly mucoid colonies, produces red pigment, facultative anaerobes, oxidase negative, nitrate positive	api 20E ^a
<i>Agrobacterium</i> spp.	Eubacteriaceae	Gram-negative, motile, non-spore-forming rods, grows on D-1 medium	(31)
<i>Bacillus</i> spp.	Eubacteriaceae	Gram-positive, motile rods; produces large, grey-white colonies with irregular margins on blood agar; produces endospores when stressed; motile/non-motile; catalase positive	(31)
<i>Rhodotorula</i> spp.	Basidiomycetes	Unicellular (blasto-)conidia globose to elongate which may be encapsulated; pseudohyphae are absent or rudimentary; colonies cream to pink, coral red, orange, or yellow in colour	api 20C AUX ^a
<i>Penicillium</i> spp.	Deuteromycetes	Conidiophores arising from the mycelium singly or less commonly in groups or fused, branched near the apex, ending in spore-producing cells; the spores are produced sequentially and form chains	(36)
<i>Staphylococcus</i> spp.	Eubacteriaceae	Gram positive, non-motile, spherical in clusters, anaerobic fermentation of glucose with acid production, catalase, nitrate and coagulase positive	api STAPH ^a
<i>Streptococcus</i> spp.	Eubacteriaceae	Gram positive, non-motile, spherical in chains, aerobic action on glucose, catalase negative	api STREP ^a
<i>Candida albicans</i>	Saccharomycetales	Produces blastospores, pseudohyphae, and chlamydospores on cornmeal Tween 80 agar at 25°C after 72 h	api ID32C ^a

For a complete list of commercial diagnostic suppliers for bacterial and yeast biochemical detection kits and media see <http://www.rapidmicrobiology.com>, <http://www.sigmaldrich.com>, and [*http://www.biomerieux.com](http://www.biomerieux.com)

8. Conclusions

In recent years, several developments have influenced how disease and pathogen contamination is approached and managed. Firstly, there has been the recognition that endophytic contamination of perennial/vegetatively propagated plants is possibly widespread (13, 14). These contaminants are non-pathogenic to the stock plant, are intercellular, may be locally distributed, and are potentially cultivable on common microbial media. Some endophytes may be potentially pathogenic to the stock plants depending on the development of a quorum to initiate pathogenesis (73). They may enter initial cultures (Stage 1) in explants subliminally, and if suppressed by components of the plant tissue culture medium or culture environment, they may spread at subculture to emerge when the medium concentration is altered for culture multiplication (Stage 2) or root induction *in vitro* (Stage 3), overrunning the cultures and causing extensive economic losses, especially in micro-propagation and large-scale industrial plant cell culture (74). Bacterial intercellular endophytes are sometimes regarded as potentially beneficial micro-organisms (39). Such contaminants, however, while posing a direct threat of *in vitro* pathogenicity may also cause disease in the progeny plants (5). This difference in behaviour may be that in the stock plants the distribution is localized, whereas in the progeny plants the contaminant may become systemic in the “softer” tissues (21).

Secondly, developments in molecular diagnostics, especially in NA-based diagnostic, have made available the possibility of probes which are of broad spectrum and can detect generically bacteria, fungi, fungal-like micro-organisms, virus families, and virus-like micro-organisms (53, 58). While ELISA has the potential to detect virus strains and some plant viral families, developments in NA diagnostics have greater potential and, when used in micro-arrays, have the potential to screen for all the pathogens of a crop in a single test (58, 61, 62). It takes time to adapt experimental protocols for commercial application, and availability then is dependent on the market demand. Nevertheless, one can be optimistic that methodological advances will continue to become available to plant tissue culturists directly or via their service providers. Plant disease diagnosticians are seeking rapid sensitive tests that will eliminate the need to inoculate indicator plants to confirm pathogenicity, whereas the plant tissue culturists are also seeking a universal test for the detection of latent bacterial endophyte contamination.

Thirdly, there have been a few reports of the transmission of human pathogenic bacteria via tissue cultures to infect personnel during subculture (66). It is unclear if plants infected with human pathogens or plant biocontrol bacteria related to human pathogenic

bacteria (75) pose a threat to purchasers, e.g. when introduced into hospitals and other location housing immune-compromised patients.

Fourthly, the commercial development of plant diagnostics and provision of diagnostic services enable outsourcing of pathogen identification and detection of endophyte contamination, where the expertise is not available within the laboratory. There are many companies and state laboratories operating internationally, offering services to research and commercial laboratories. It is also easier now for micro-propagation companies and researchers to identify laboratories working on specific diseases or diagnostics using Google Scholar (<http://scholar.google.com>), JSTOT (<http://www.jstor.org>), or, if access is available, the Web of Science (<http://www.isiwebknowledge.com>) or journal publishers Web sites such as Science Direct (<http://www.sciencedirect.com>). Thus, the prohibitive cost of developing and validating diagnostics may be defrayed.

Fifthly, international germplasm collections (germbanks) (<http://www.bioversityinternational.org>; <http://www.cgiar.org>), botanic gardens (International Union of Biological Sciences, IUBS; <http://www.iubs.org>), and commercial sources may be able to supply mother cultures from their collections or from a client's material. But a caution: Unless certified, the cultures may not be free of pathogens and/or endophytes and/or they may have incorporated an antibiotic in the medium to suppress bacterial contamination. Externally sourced culture must be rigorously indexed for pathogens and endophytes unless the supplier will undertake to indemnify the client against consequential losses.

In summary, the anticipated further development of broad-spectrum NA-based diagnostics implies that it will be possible in the future to be confident that initial cultures are not only aseptic but also axenic and can be certified (76). However, during subculture, there will remain the risk of microbial contamination. To avoid the latter, the principles of HACCP-based management as outlined above should be followed.

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Chapter 7

Cryopreservation of Embryogenic Cell Suspensions by Encapsulation–Vitrification and Encapsulation–Dehydration

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Abstract

Encapsulation–vitrification and encapsulation–dehydration are two newly developed techniques for cryopreservation of embryogenic cell suspensions. Here, we describe the two protocols using grapevine (*Vitis*) as a model plant. Cell suspensions at the exponential growth stage cultured in a cell suspension maintenance medium are encapsulated to form beads, each being about 4 mm in diameter and containing 25% cells. In the encapsulation–vitrification procedure, the beads are stepwise precultured in increasing concentrations of sucrose medium up to 0.75 M, with 1 day for each concentration. The precultured beads are treated with a loading solution for 60 min and then dehydrated with plant vitrification solution 2 at 0°C for 270 min before a direct immersion in liquid nitrogen. Following cryostorage, the beads are rapidly rewarmed at 40°C for 3 min and then unloaded with 1 M sucrose solution for 30 min. In the encapsulation–dehydration procedure, the beads are precultured in increasing concentrations of sucrose medium up to 1 M, with 1 day for each concentration, and then maintained on 1 M sucrose medium for 3 days. The precultured beads are dehydrated for 6 h under a sterile air flow, prior to rapid freezing in liquid nitrogen. The freezing and rewarming procedures are the same as used in the encapsulation–vitrification technique. The unloaded beads from encapsulation–vitrification and rewarmed beads from encapsulation–dehydration are postcultured on a recovery medium for 3 days at 25°C in the dark for survival. Surviving cells are transferred to a regrowth medium to induce cell proliferation. Embryogenic cell suspensions are reestablished by suspending the cells in a cell suspension maintenance medium maintained on a gyratory shaker at 25°C in the dark. For plant regeneration, surviving cells are transferred from the recovery medium to an embryo maturation medium and maintained at 25°C under light conditions. Embryos at the torpedo stage are cultured on a rooting medium until whole plantlet regenerates.

Key words: Cell suspensions, Cryopreservation, Dehydration, Encapsulation, Somatic embryogenesis, Vitrification

1. Introduction

Embryogenic cell suspensions have widely been used as an efficient system for plant clonal micropropagation and as target tissues for genetic manipulation in many transformation systems (1). However, establishment of embryogenic cell cultures is difficult (1–3).

Once established, cell cultures have to be maintained in vitro by repeated subculture. In vitro maintenance may create risks of culture loss due to contamination or human error during culture (2, 3). Moreover, the morphogenetic potential of embryogenic cultures declines or gets totally lost and genetic alterations may occur as subculture times increase (2–4).

Cryopreservation has been considered as an ideal means for long-term storage of plant genetic resources, which can avoid loss of embryogenic potential and prevent occurrence of somaclonal variation, since all cell divisions and other metabolic processes of the plant materials stored in this way are arrested (5). Theoretically, plant materials can thus be stored without any changes for an indefinite period of time (5). Encapsulation–vitrification and encapsulation–dehydration are among the newly developed cryogenic methods (6–8), which allow samples to be directly immersed into liquid nitrogen, thus eliminating the usage of sophisticated and expensive programmable-controlled freezer that is required in two-step freezing method (5, 6). These two methods are receiving increasing interests and being widely used for cryopreservation of cell suspensions (2, 7). Some examples of successful cryopreservation of cell suspensions by encapsulation–vitrification and encapsulation–dehydration in recent years are documented in Table 1.

Table 1
Some examples of successful cryopreservation of cell suspensions by encapsulation–vitrification and encapsulation–dehydration techniques

Plant species	Method	No. of cultivars	Survival (%)	Ref.
<i>Arabidopsis</i> spp.	En–dehy	1	87	(9)
<i>A. thaliana</i>	En–dehy	1	34	(10)
<i>Catharanthus roseus</i>	En–dehy	1	50	(11)
<i>Chaetoceros muelleri</i>	En–vitri	1	48	(12)
<i>Gentiana cruciata</i>	En–dehy	1	83	(13)
<i>G. tibetica</i>	En–dehy	1	68	(13)
<i>Medicago sativa</i>	En–dehy	1	25	(14)
<i>Nitzschia closterium</i>	En–vitri	1	74	(12)
<i>Polygonum aviculare</i>	En–dehy	1	Not specified	(15)
<i>Vaccinium pahalae</i>	En–dehy	1	30	(16)
<i>Vitis vinifera</i>	En–dehy	1	78	(17)
	En–vitri	4	46–82	(18)
<i>V. berlandieri</i> × <i>V. rupestris</i>	En–vitri	1	76	(18)
<i>V. vinifera</i> × <i>V. berlandieri</i>	En–vitri	1	42	(18)

En–dehy encapsulation–dehydration, En–vitri encapsulation–vitrification

1.1. Encapsulation–Vitrification

Encapsulation–vitrification, which is a combination of encapsulation–dehydration and vitrification procedures, is one of several newly developed techniques for cryopreservation of plant germplasm. Vitrification refers to the physical process by which a highly concentrated cryoprotective solution is supercooled at very low temperatures and eventually solidifies into a metastable glass without undergoing crystallization at a glass-transition temperature (19). Thus, vitrified cells are able to evade the danger of intracellular freezing and to survive freezing in liquid nitrogen. Encapsulation of cell suspensions, normally in 2–3% calcium-alginate beads, can protect the specimen and reduce the chemical toxicity or osmotic stress of the vitrification solution. In addition, encapsulation–vitrification is much easy to handle and can be used to simultaneously treat a large number of samples, compared with vitrification procedure alone.

Encapsulation–vitrification was originally developed by Tannoury et al. (20) for cryopreservation of carnation apices (*Dianthus caryophyllus* L.). In this protocol, encapsulated apices were precultured for 16 h in progressively more concentrated sucrose medium, then incubated for 6 h in a vitrification solution containing ethylene glycol and sucrose, and finally frozen in liquid nitrogen.

In comparison with encapsulation–dehydration, encapsulation–vitrification produced high recoveries in cryopreserved shoot tips, such as wasabi (*Wasabia japonica*) (21) and strawberry (*Fragaria × Ananassa* Duch.) (22). With embryogenic cell suspensions, Wang et al. (18) also demonstrated that encapsulation–vitrification was much more applicable to different grapevine (*Vitis*) species and cultivars than encapsulation–dehydration. Therefore, encapsulation–vitrification would have a great potential for much broader applications to cell suspensions (2, 3, 8).

1.2. Encapsulation–Dehydration

Encapsulation–dehydration is based on the technology developed for producing synthetic seeds, i.e., the encapsulation of explants in calcium alginate (7, 23). Encapsulated explants are then precultured with a high sucrose concentration and partially desiccated before a direct exposure to liquid nitrogen (7). Desiccation is usually performed in a laminar airflow cabinet, but more precise and reproducible dehydration conditions can be achieved by using a flow of sterile compressed air or silica gel (24). In general, water content of the beads that ensures highest regrowth following cryostorage is about 20% on a fresh weight basis, which corresponds to the amount of unfreezable water in the samples (3, 7, 24). Encapsulation of the cells allows the application of subsequent preculture with high sucrose concentrations and drastic dehydration to low moisture contents prior to freezing in liquid nitrogen, which would otherwise be highly damaging or lethal to nonencapsulated samples (7, 23, 25).

Encapsulation–dehydration was originally developed by Dereuddre and Fabre for cryopreservation of *Solanum* shoot tips (26). In this protocol, encapsulated apices were precultured in progressively more concentrated sucrose medium, then dehydrated under a sterile airflow for 4 h, and finally frozen either rapidly or slowly in liquid nitrogen.

Encapsulation–dehydration is a very efficient cryopreservation technique which is simple to implement and easy to manipulate throughout the freezing protocol. Encapsulation–dehydration procedure has been successfully implemented with a large number of species, including agricultural (3, 27) and horticultural (6) crops.

2. Materials

2.1. General Requirements

1. Standard tissue culture facilities.
2. Cryogenic vial (2 mL).
3. Erlenmeyer flask (250 mL).
4. Gyrotory shaker.
5. Pipette (1 mL).
6. Sterile disposable pipette (25 mL).
7. Taylor-Wharton Dewar flask (beaker style wide-mouth, 20–35 L).
8. Magenta GA7 vessels.
9. Whatman paper (9 cm in diameter).

2.2. Chemicals, Plant Growth Regulators, and Media

1. Activated charcoal (AC, Sigma, C9157).
2. Calcium chloride (Sigma, C4901).
3. Casein enzymatic hydrolysate (Sigma, C7290).
4. Dimethylsulfoxide (DMSO, Sigma, D8418).
5. Ethylene glycol (Sigma, E9129).
6. Gelrite (Sigma, G1910).
7. Glycerol (Sigma, G5516).
8. Maltose (Sigma, M5885).
9. Naphthalene acetic acid (NAA, Sigma, N0640).
10. 2-naphthoxyacetic acid (NOA, Sigma, N3019).
11. Nitsch and Nitsch medium (NN (28), Duchefa, N0224).
12. Sodium alginate (Sigma, A2158).
13. Sucrose (Sigma, S7903).
14. Woody plant medium (WPM (29), Duchefa, M0220).

2.3. Media and Solutions

1. 0.1 M calcium chloride solution: Liquid NN medium containing 0.1 M calcium chloride, 2 M glycerol, and 0.4 M sucrose, pH 5.8.
2. Cell suspension maintenance medium: Liquid NN medium supplemented with 18 g/L maltose, 1 g/L casein enzymatic hydrolysate, 4.6 g/L glycerol, and 1 mg/L NOA, pH 5.8.
3. Embryo maturation medium: Solid NN medium supplemented with 18 g/L maltose, 1 g/L casein enzymatic hydrolysate, 4.6 g/L glycerol, and 2.6 g/L gelrite, pH 5.8.
4. Loading solution: Liquid NN containing 2 M glycerol and 0.4 M sucrose, pH 5.8.
5. Plant vitrification solution 2 (PVS2) (30): 0.4 M sucrose, 30% (w/v) glycerol, 15% (w/v) DMSO, and 15% (w/v) ethylene glycol made up in liquid NN medium, pH 5.8.
6. Plant regeneration medium: Solid NN medium supplemented with 18 g/L maltose, 1 g/L casein enzymatic hydrolysate, 4.6 g/L glycerol, and 2.6 g/L gelrite, pH 5.8.
7. Preculture medium: Cell suspension maintenance medium supplemented with 0.25, 0.5, 0.75, and 1.0 M sucrose, respectively, pH 5.8.
8. Recovery medium: Cell suspension maintenance medium containing 2.5 g/L AC and solidified with 2.6 g/L gelrite, pH 5.8.
9. Regrowth medium: Cell suspension maintenance medium solidified with 2.6 g/L gelrite, pH 5.8.
10. Rooting medium: Solid WPM containing 30 g/L sucrose, 1 mg/L NAA, 2.5 g/L AC, and 2.6 g/L gelrite, pH 5.8.
11. Sodium alginate solution: 0.4 M sucrose, 2 M glycerol, and 2.5% (w/v) sodium alginate made up in liquid NN medium, pH 5.8.
12. Unloading medium: Liquid NN medium containing 1 M sucrose, pH 5.8.

3. Methods

As with any method, optimal treatment parameters at each step during the whole procedure may vary greatly with different plant species and even cultivars. In this section, we make every effort to illustrate two protocols: encapsulation–vitrification and encapsulation–dehydration using grapevine (*Vitis*) as an example. The encapsulation–vitrification protocol consists of (a) maintenance of embryogenic cell suspensions; (b) encapsulation–vitrification; (c) freezing and rewarming; (d) unloading; and (e) survival, regrowth, and plant regeneration, while the main steps involved in encapsulation–dehydration procedure include (a) maintenance

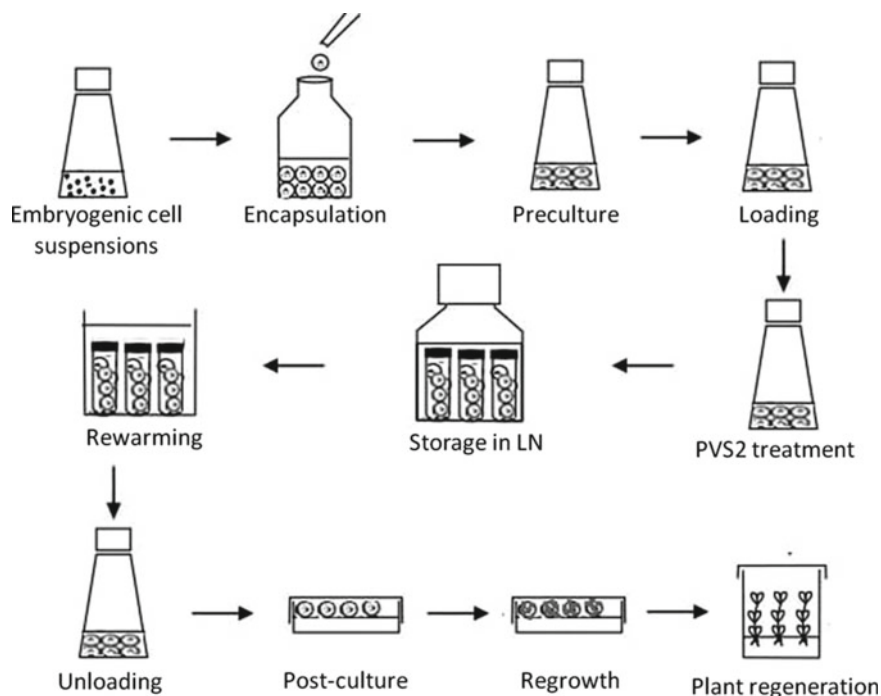


Fig. 1. A flowchart of cryopreservation of embryogenic cell suspensions by encapsulation–vitrification.

of embryogenic cell suspensions; (b) encapsulation–dehydration; (c) freezing and rewarming; and (d) survival, regrowth, and plant regeneration. Flowcharts of the two protocols are illustrated in Figs. 1 and 2, respectively.

To maintain sterility of embryogenic cell suspensions, all appropriate manipulations should be carried out in a laminar airflow cabinet using aseptic techniques and sterile materials.

3.1. Encapsulation–Vitrification

3.1.1. Maintenance of Embryogenic Cell Suspensions

1. Calli (1 g) are transferred to a 250-mL Erlenmeyer flask containing 50 mL of cell suspension maintenance medium to establish cell suspensions.
2. The cell suspensions are incubated at 25°C in darkness on a gyratory shaker at 90 rpm.
3. Subculture is weekly carried out by transfer of cell suspensions to fresh cell suspension maintenance medium.

3.1.2. Encapsulation–Vitrification

1. Three days after subculture (see Note 1), embryogenic cell suspensions are collected by using a sterile disposable 25-mL pipette to remove the entire liquid medium from the 250-mL Erlenmeyer flask. In order to avoid loss of cells while the liquid medium is emptied, the mouth of the pipette is pressed against the bottom of the Erlenmeyer so that the cells do not penetrate into the pipette.

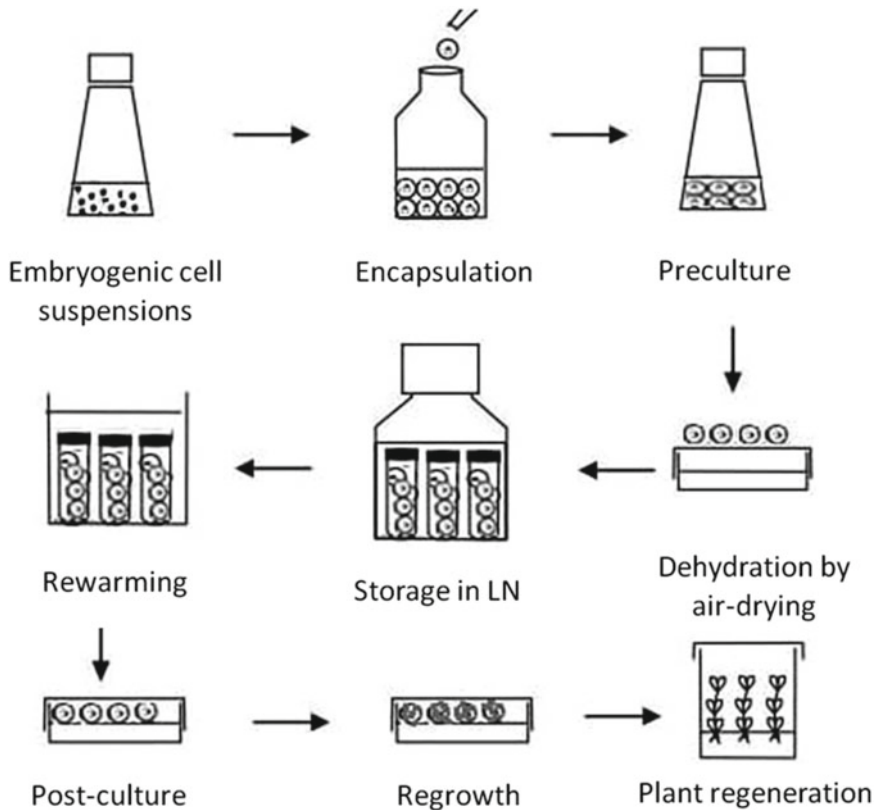


Fig. 2. A flowchart of cryopreservation of embryogenic cell suspensions by encapsulation-dehydration.

2. The cells are transferred to sodium alginate solution at a ratio of 1 g cells to 3 mL solution (see Note 2) and mixed well.
3. The mixture is dripped with a sterile pipette (1 mL) into 0.1 M calcium chloride solution and left for 20 min to form beads, each being about 4 mm in diameter and containing 25% cells (see Note 2).
4. The beads are precultured stepwise on the preculture medium enriched with increasing sucrose concentrations of 0.25, 0.5, and 0.75 M for 3 days, with 1 day for each step (see Note 3). The preculture conditions are the same as for the maintenance of embryogenic cell suspensions.
5. The precultured beads are rapidly surface dried by blotting onto sterile Whatman paper for a few seconds, then transferred to the loading solution, and incubated for 60 min in a laminar airflow cabinet (see Note 4).
6. The beads are rapidly surface dried by blotting onto sterile Whatman paper for a few seconds, then transferred to PVS2 cooled on ice, and incubated at 0°C for 270 min (see Note 5).

3.1.3. Freezing and Rewarming

1. The vitrified beads are rapidly surface dried by blotting on sterile Whatman paper for a few seconds. Next, ten beads are transferred into a 2-mL cryogenic vial and directly immersed in a Taylor-Wharton Dewar flask containing liquid nitrogen for 1 h.
2. Cryogenic vials containing frozen cells are rapidly removed from liquid nitrogen and immediately rewarmed at 40°C in a water bath for 3 min.

3.1.4. Unloading

1. Rewarmed beads are placed in the unloading medium for 30 min (see Note 6).

3.1.5. Survival, Regrowth, and Plant Regeneration

1. The beads are postcultured at 25°C in the dark for 3 days in 9-cm Petri dishes (ten beads/dish) containing 30 mL recovery medium for survival (see Note 7).
2. For regrowth, the beads are transferred from the recovery medium to 9-cm Petri dishes (ten beads/dish) containing 30 mL regrowth medium and placed at 25°C in the dark for 4 weeks (see Note 8). Embryogenic cell suspensions are reestablished by suspending the beads in a 250-mL Erlenmeyer flask containing 50 mL of cell suspension maintenance medium. The cultures are placed on a gyratory shaker (90 rpm) at 25°C in the dark. Subculture is carried out every week.
3. For plant regeneration, the beads are transferred from the recovery medium to 9-cm Petri dish (ten beads/dish) containing 30 mL plant regeneration medium. The cultures are maintained at 25°C under a 16 h photoperiod with a light intensity of 45 $\mu\text{mol}/\text{s}^1/\text{m}^2$ provided by cool white fluorescent tubes. Four weeks later, single embryos at the torpedo stage are transferred to Magenta GA7 vessels containing 50 mL rooting medium. The culture conditions are the same as for embryo maturation. Subculture is done every 4 weeks until a whole plantlet with a profuse root system is developed.

3.2. Encapsulation– Dehydration

This step is exactly the same as described above in the encapsulation–vitrification procedure (18).

3.2.1. Maintenance of Embryogenic Cell Suspensions

3.2.2. Encapsulation– Dehydration

1. Collection of embryogenic cell suspensions and encapsulation are the same as described above in encapsulation–vitrification (18).
2. Following encapsulation, the beads are stepwise precultured on the preculture medium enriched with increasing sucrose concentrations of 0.25, 0.5, 0.75, and 1.0 M for 4 days, with 1 day for each step, and then maintained on 1 M sucrose for 3 days (see Note 3). The preculture conditions are the same as for the maintenance of embryogenic cell suspensions.

3. The precultured beads are rapidly surface dried by blotting with cellulose tissue for a few seconds, placed on sterilized filter paper in 9-cm Petri dish, and dehydrated by air in a laminar flow chamber at room temperature for about 6 h, at which water content in beads is reduced to about 20.6% on a fresh weight basis (see Note 9).

3.2.3. Freezing and Rewarming

1. Following dehydration, ten precultured beads are transferred into a 10-mL cryogenic vials and immersed directly in a Taylor-Wharton Dewar flask containing liquid nitrogen for 1 h.
2. Cryogenic vials containing frozen cells are rapidly removed from liquid nitrogen and immediately rewarmed at 40°C in a water bath for 3 min.

3.2.4. Survival, Regrowth, and Plant Regeneration

Steps involved in survival, regrowth, and plant regeneration are exactly the same as described above in the encapsulation–vitrification procedure (18).

4. Notes

1. Growth phase at which cells are harvested at the onset of the procedure is an important factor for cryopreservation (31). Cells at the exponential growth stage are more tolerant to freezing than those either at the lag or stationary stage because they have small volume and vacuoles, and contain relatively little water. The growth pattern of cell suspensions differs with different plant species. For several *Vitis* species and cultivars, the exponential growth stage is reached 3 days after each subculture (17, 18).
2. Cell density in the beads influences significantly the regrowth and embryo formation of cryopreserved cell suspensions. In general, 25–50% cell density was used in encapsulated beads (11, 16–18). Regrowth of cryopreserved cells of *Catharanthus* was found faster in the beads containing 50% than 25% cells (11), while efficiency of embryo formation in *Vitis* was much higher in beads containing 25% cells (17, 18).
3. Preculture is required to induce resistance of cell suspensions to dehydration and subsequent freezing in liquid nitrogen. Sucrose is the most often used sugar for induction of such tolerance at this step (5), although other sugars, such as glucose (32), glycerol (33), maltose (34), manitol (35), and sorbitol (34, 36), were used. Sugar concentration used in the preculture medium ranges from 0.3 to 1.0 M for periods varying between 1 h (34) and 9 days (11). Progressive increase in sucrose concentration enables to avoid the deleterious effects

of direct exposure to high sucrose concentrations (37). Preculture with high sugar concentrations (up to 0.75 or 1.0 M) has been shown to increase the viability of cryopreserved cell suspensions of *Catharanthus roseus* (11), *Papaver somniferum* (34), *Vaccinium pabhalae* (16), *Medicago sativa* (14), *Asparagus officinalis* (38), and *Vitis* (17, 18).

4. In vitrification-based cryopreservation procedures, cells have to be dehydrated by exposure to a highly concentrated solution, such as PVS2, prior to immersion in liquid nitrogen. However, direct exposure of cells to PVS2 without osmoprotection causes harmful effects on cells because of osmotic stress or chemical toxicity (39), which has been described as a major limitation in determining the success of cryopreservation of cells by vitrification (40). This major limitation can be overcome by the loading treatment (39, 40). In this treatment, cells are placed at room temperature in a solution containing cryoprotective substances, such as sucrose, glycerol, and ethylene glycol, for a short period varying between 10 (40, 41) and 60 min (18), depending on plant species. The most often used loading solution is composed of a basic medium containing 2 M glycerol and 0.4 M sucrose (18, 40, 41).
5. Two plant vitrification solutions are most frequently employed: one described by Langis et al. (42), which comprises 40% (w/v) ethylene glycol, 15% sorbitol, and 6% bovine serum albumin, and the other (PVS2) described by Sakai et al. (30), which consists of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) DMSO. We applied both vitrification solutions to *Vitis* cell suspensions with much better results obtained with PVS2. Overexposure to the vitrification solution can cause chemical toxicity and excess osmotic stress (43), eventually leading to reduced survival of cryopreserved cells. Dehydration can be performed either at 0°C or room temperature. The optimal time of exposure to the vitrification solution varies with plant species, and depending on the temperature during exposure, it ranges from 20 (40) to 270 min (18). Dehydration at 0°C takes longer time, but usually yields a higher survival of cryopreserved cells (18, 40), mainly through elimination or reduction in the chemical toxicity or osmotic stress of vitrification solutions to plant cells (44).
6. Unloading is designed to dilute the vitrification solutions and remove cryoprotectants from cryopreserved cell suspensions (45). Because of the extremely high concentrations of the vitrification solutions, unloading is usually performed either by a one-step dilution in a high sugar concentration solution up to 1.2 M (40) or by a stepwise dilution in decreasing sugar concentrations (14) in order to reduce the osmotic shock. With *Vitis* cell suspensions, the best results were achieved using 1 M sucrose solution for 30 min (18).

7. In many cases, recovery of cryopreserved cell suspensions was largely improved when postcultured for a few days on a solid medium before being transferred to liquid medium (18, 46). Addition of activated charcoal to the recovery medium was also found beneficial to the survival of cryopreserved cell suspensions (5, 17, 18). The beneficial effect of activated charcoal on survival was probably achieved through reduced necrogenesis (46) and adsorption of toxic substances produced by frost-damaged cells (47).
8. Regrowth of cryopreserved cell suspensions generally starts after a lag period that usually lasts from 3 (40) to 10 days (48) and reaches the same growth pattern as that of noncryopreserved cell suspensions after two to three times of subculture (17, 18).
9. Two desiccation methods can be employed: dehydration under the air current of a laminar flow cabinet or dehydration in sealed containers with dry silica gel. In general, the bead water content that ensures the highest regrowth following cryopreservation is around 20% on a fresh weight basis (3, 7, 24), which corresponds to the amount of unfreezable water in the samples. At such water contents, only glass transitions are recorded by differential scanning calorimetry when samples are plunged in LN (49). This value may vary depending on the species and the type of samples.

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Chapter 8

The Study of In Vitro Development in Plants: General Approaches and Photography

Edward C. Yeung

Abstract

Careful examination of how explants react to experimental conditions is the first step of a successful research program. Photographing the specimen is an extremely important method that can be used to document changes of an explant throughout an experiment. This article serves to draw attention to the utility of macroscopic and microscopic examinations in the study of in vitro development, and details some useful methods in the study of the cultured explants.

Key words: Digital camera, Freehand sections, Photography, Photomicrography, Staining

1. Introduction

Developmental processes are complex, and are subject to many internal and external factors and influences which complicate investigation. The use of in vitro culture systems enables researchers to isolate the explants and to reduce the complexity of the experimental system to study the problem of interest. The results often provide new insights and augment in vivo studies. This approach is powerful and is well documented in the literature (1, 2). Besides theoretical interest, the study of in vitro development is also essential to plant biotechnology; the success of shoot and root regeneration and in vitro embryogenesis are the key steps towards the development of protocols in plant transformation and genetic modification.

In the course of studying a developmental process, careful evaluation of responses by the explants to experimental manipulation is essential. Thorough observation and documentation of

results is the first step towards the development of a successful protocol. Some practical comments concerning the use of histology in the study of plant tissue culture systems have been presented earlier (3, 4). The purpose of this article is to focus on simple methods and approaches used to extract information from one's system by careful macroscopic examination of specimens. The information presented in this chapter endeavors to improve the quality of research and presentation.

1.1. How to Examine a Specimen and Evaluate the Results

1.1.1. Approaches and What Needs to Be Documented

Every experimental system is different, with both obvious and subtle features. How are these observed? The best answer is to use an investigative approach—ask questions as you look at a specimen. While you look, be sure to consider physiology, cell biology, and biochemical changes. “Looking and thinking” provides a dynamic interpretation of changes observed.

The majority of experiments should initially investigate how their explant materials respond to an *in vitro* culture environment, and test the effectiveness of various additives such as plant growth regulators in achieving a desired outcome, such as root and shoot regeneration. At present, numerous protocols are present in the literature that can serve as guides in the design and the initial selection of culture media and growth conditions.

If there is a positive response to the initial test trial, the next step is optimization. This requires systematic testing of various parameters. In order to be convincing as a useful protocol, the following information should be presented. A careful morphological description of the starting plant material used in the experiment must be clearly stated. The information such as the age of the explants, where and how the materials were excised from the starting material, and the final size of the explants used for *in vitro* culture should be described carefully. This information is useful for readers to assess the developmental potential of the explants used in the experiment. The culture conditions, e.g., media composition, additives, light intensity, photoperiod, etc. should be detailed. A clear indication of what has been tested such as range of growth regulator concentrations should be clearly indicated in the text, tables, and figures. This information is necessary to justify and prove that optimization of the protocol had been performed. The response of the explants to various combinations of treatments must be documented in a quantitative manner whenever possible. The data such as percentage of explants responding to a treatment, the number of regenerated structures per explant, and the normality of regenerated structures should be documented. Normality can be illustrated with photos and conversion percentage. Conversion is the best approach to describe normality of a regenerated structure. For example, the fact that a somatic embryo can

convert into a normal seedling within a few days of transferring to a germination medium indicates the presence of functional shoot and root meristems. Not all regenerated structures have functional meristems. Simply showing a form without further testing of normal development is unacceptable as a protocol. Another indication of the utility of a protocol is the percentage of plants that can flower and set seeds. Companion photographic documentation of changes will greatly clarify the course of development over time. Proper statistical treatment of data will also add credence to the data presented (5, 6). If all the above information can be presented together with positive outcomes, the protocol is definitely a useful method for others to follow.

1.1.2. Know Your Initial Explants

It is imperative to have a good understanding of the structure and the developmental potential of explants at the time of culture. The basic morphology and anatomy must be clearly understood. In order to appreciate the changes occurring in, for example, the nodal explant system which is a common approach used in the micropropagation of a species (7), it is important to know whether existing axillary buds are present. The newly formed shoots that one sees may originate from existing axillary and accessory buds, and are therefore not newly regenerated structures. A good understanding of the explant is thus needed in order to avoid wrong inferences.

1.1.3. Asking Questions as You Look Is the Key to a Successful Observation Process

Once the cultures are set up, visual examination should be carried out regularly. The best approach to studying changes during the course of an experiment is to ask questions. This approach provides a better focus as one examines the explant; without questions in mind, we are “blind.” Using shoot regeneration as an example, one might ask where the shoot is coming from. If it is formed near explant surface in the absence of a callus, is it derived from the epidermal or subepidermal layers? If so, meristemoids may be present (8). If the shoots come from a cut surface, vascular tissues may be responsible for their formation (9). Careful observation of swellings near or at existing vascular strands can provide clues to their origin. Are the newly formed structures somatic embryos or adventitious shoot primordia? In the case of the former, one should be able to see a narrowing of the root pole suggesting the presence of a functional root tip. Also, if the structures are loosely attached to the explants, they are likely to be somatic embryos (10). Many structures can take on a “somatic embryo-like” structure although functional meristems are absent (11, 12). In a shoot regeneration system, shoot-like structures can be present and yet they may not develop into a functional shoot. In fact, shoot-like structures can simply be leaves; hence, careful observation and further studies are required to discern the true nature of the structures observed.

Once questions begin to be formulated, the examination will be more focused. At the beginning, simply ask one question and look for the answer one at a time. Repeat the process until all potential questions have been exhausted. Remember that the answer is in front of you. Careful observation allows for new discoveries to be made.

1.2. How to Capture the Information?

Information recording is key to any experimentation. Besides written comments, photography is a powerful way to document changes that took place. Morphological changes can be subtle and sometimes difficult to describe. Photographic records are extremely useful for enhancing written descriptions. As a general rule, one should take photos as one examines a specimen. As indicated earlier, it is also important to ask questions. Try to look for the answer and capture it with a photo to complement written notes. If a different question arises, take another set of photos, even though they can be very similar. It is important to remember that we are focusing on “living” specimens, the same exact structure may be difficult to see in a repeat experiment. Photographic records are useful for future comparisons. Be sure to examine your cultures frequently in order not to miss rapid changes that might have occurred. Certain changes occur rapidly such as color changes of cells and tissues; new root initiation can occur within 12–24 h from a regenerated shoot.

Developmental processes are complex. To fully understand a process, one has to be equipped with knowledge from all areas of biology. Morphological and anatomical approaches provide appreciation for some visual changes in the explant during the course of an experiment. This is an observational approach in the study of a problem. It is important to remember that describing the changes using biochemical, molecular and cellular approaches such as presenting gene sequences, gels, etc. is just a different type of “picture,” a different way of show and tell. These approaches are not better or worse than structural presentations. Instead, all these approaches complement one another to answer the problem of interest.

The power of one’s observation is the key to the success of a project. This fuels your imagination and is the basis of new discoveries. This article simply serves as a reminder of how to look at the specimens. Younger investigators tend not to pay too much attention to their culture system and fail to recognize the power of simple observation. A careful, observational, investigative approach can yield valuable results and create additional hypotheses for further testing of ideas.

This chapter details protocols that are useful in the study of macroscopic changes of the explants during the course of in vitro culture.

2. Materials

2.1. Equipment for Stereomicroscopic Observation

1. A good quality stereomicroscope preferable with a trinocular head so that a camera can be attached (see Note 1).
2. Lighting attachments and accessories are needed to provide the necessary light intensity for image capture (see Note 2). Ring light that attaches directly to the lens of the stereomicroscope and gooseneck fiber optics that directs light to specimens are commonly available. The new “mini-Light” from Electron Microscopy Sciences offers high light intensity with a small footprint that fits into most locations.

2.2. Dissecting Tools (See Note 3)

1. Fine forceps.
2. Dissecting knives.
3. Dissecting needles.

2.3. Freehand Sections

1. Double edge razor blades.
2. Supplies: small paint brush, test tubes, slides and cover glasses, Petri dishes, absorbing papers, disposable pipettes.

2.4. Staining and Mounting Solutions

1. Toluidine blue O (TBO) stain: Dissolve 0.1 g of TBO in 100 mL of 0.1 M benzoate buffer, pH 4.4 (benzoic acid 0.25 g, sodium benzoate 0.29 g, water 200 mL). This buffer is recommended for histochemical purposes. If benzoate buffer is not available, for general use, laboratory water can be used as the solvent for TBO.
2. Phloroglucinol-HCl stain: There are various recipes to make up the staining solution but commonly it is prepared as a saturated aqueous solution of phloroglucinol in 20% hydrochloric acid. The hydrochloric acid used is about 2 N. *Be sure to handle the solution with care. Wear gloves. Prepare this solution in the fume hood.* First dissolve phloroglucinol (about 2.0 g) in 80 mL of water and then add 20 mL of concentrated HCl (12 N) to it.
3. IKI stain: The solution is prepared by first dissolving 2 g of KI in 100 mL of water, and adding 0.2 g of iodine into the KI solution. Prepare this solution ahead of time, as iodine takes some time to dissolve. Store the solution in a dark glass bottle and cap tightly. Exposure to light and air degrades the solution's usefulness. Iodine sublimates at room temperature. It is preferable to prepare the solution in a fume hood. The stain, once prepared, can be kept for several months or longer, as long as the bottle is tightly capped.
4. Mounting solutions: water; a 30% glycerol solution.

2.5. Camera and Accessories

1. Digital cameras. Specialized digital cameras for photomicrography are available from major microscope vendors. Selection of a model is based on one's own need and budget (see Note 4).
2. Black agar plates. Charcoal powder about 4% is added to a hot 1.5–2% agar solution before solidification (see Note 5).
3. Other accessories: small camera tripods, hair dryer and ruler, color cardboards for background contrast.

3. Methods

3.1. General Photography of Large Explants

1. Large explants such as plantlets from in vitro cultures can be photographed using standard digital cameras. At present, quality digital cameras are commercially available, and their price has been reduced in recent years. The macro-function enables the photography of large explants.
2. Arrange the objects such as culture boxes, Petri plates, plantlets, etc. against an even background. Cardboard and cloths can be used.
3. Label each object and be sure that the writing is legible.
4. A scale such as a ruler should be photographed together with the object.
5. Arrange the lighting equipment to avoid obvious shadow formation (see Note 6).
6. After photographing the object, be sure to look at the digital files to ensure the images are sharp showing the desired features. Otherwise retake the photos at once (see Note 7).

3.2. Photography Through a Stereomicroscope

For a detailed examination of the object, careful study using a stereomicroscope is preferred. The “zoom” feature easily allows for the change of magnification. The image can be captured using a digital camera directly attached to the stereomicroscope. Before use, one should be familiar with one's own stereomicroscope in order to understand its capability and limitations. For a more detailed theoretical and practical treatment of this topic, see references such as ref. (13).

1. Explants can be examined without removal from the culture vessels. One can follow the developmental changes of the same explants through the course of the experiment. Photos of the specimens can be taken directly through the Petri plates or culture vessel. Condensation often occurs on the lid of a Petri plate impeding observation. It is possible to remove the condensation by using a hair dryer to evaporate the condensate from the surface of the Petri dish or culture vessel.

2. For better close up observation, especially for smaller specimens, a representative explant can be removed from the culture vessel and placed on a black agar plate. The specimen can be examined from different angles by rotating the specimen on top of the agar plate with the help of a fine forceps. Detail surface features can be studied by using the zoom function of the stereomicroscope. The black agar plate provides a better contrast for the specimen (see Note 8).
3. A small scale bar should be placed close to the specimen to provide information about the size of the specimen. A scale bar can be created by simply photocopy of a ruler, cut off a centimeter portion of the image and place next to the specimen.
4. Proper lighting is important as it provides the proper light intensity as well as the shadowing effects highlighting the structures of interest. Ring light provides an even illumination over the entire specimen when examining the specimen using a stereomicroscope. The positioning of the gooseneck arms and or any other lights can create the shadow and provides better contrast of the specimen. Try different positions by moving the arm or other light source to obtain the best desirable image that show case the feature of interest (see Note 9).

3.3. Photomicrography of Liquid Suspension Cells and Freehand Sections

For more detailed studies of cell, small cell aggregates, and hand sections of explants, a compound light microscope is preferred, as it has a better resolving power than a stereomicroscope.

1. Dispense a small amount of liquid suspension cells or freehand sections on a slide. The cells and sections can be stained previously to improve the contrast (see Subheading 3.5).
2. Quickly place a cover glass over it with just enough liquid surrounding the cells or sections. It is imperative that no liquid should be overflowing the slide. The extra liquid can corrode the microscope stage and condenser.
3. Unstained specimens can be examined using phase contrast or interference contrast optics, if such components are present.
4. Capture the image with existing digital camera equipment.

3.4. Simple Histological and Histochemical Staining of Specimens

Certain explants such as calluses may have little color; hence, little contrast to show surface features. One way to increase the contrast of a specimen is through staining. Staining, in addition to enhancing the contrast may also provide some additional information concerning the wall features and cellular details. Thus, one can gain additional insights into the experimental system. A number of staining protocols are available in the literature that can be used to stain explants and suspension culture cells directly without the need of elaborate embedding and sectioning. For large explants that can be handled by “hand,” freehand sections (see Subheading 3.5) can be carried out prior to staining and examination. The toluidine

blue O, phloroglucinol-HCl and IKI procedures are detailed in this section. For additional staining recipes, consult additional references (14, 15).

*3.4.1. Toluidine Blue O
Staining for General
Histology and
Histochemistry (16, 17)*

TBO has the advantage of being a polychromatic dye, i.e., it reacts with different chemical components of cells differently and results in a multicolored specimen (16). The colors generated can provide information on the nature of the cell and its walls. TBO is a cationic dye that binds to negatively charged groups. An aqueous solution of this dye is blue, but different colors are generated when the dye binds with different anionic groups in the cell. For example, a pinkish purple color will appear when the dye reacts with carboxylated polysaccharides such as pectic acid; green, greenish blue or bright blue with polyphenolic substances such as lignin and tannins; and purplish or greenish blue with nucleic acids (for details, see ref. 16). General expected results are as follows: pectin, red or reddish purple; lignin, blue; other phenolic compounds, green to blue-green; thin-walled parenchyma, reddish purple; lignified elements such as tracheary elements and sclerenchyma, green to blue-green; sieve tubes and companion cells, purple; middle lamella, red to reddish purple; callose and starch, unstained (16).

1. Transfer the suspension culture to a small container such as a small tube. Remove excess culture medium using a disposable pipette with a fine tip. Apply the TBO stain to the cells and stains for 1 min. Gently remove the stain and wash the cells two times with water. Transfer the cells directly onto a clean slide, cover with a cover glass and examined with a light microscope.
2. For freehand sections, apply the TBO stain directly to the sections for 1 min. Gently remove the stain by using a piece of filter paper. Wash the sections by flooding them with water followed by its removal. Repeat until there is no excess stain around the sections. Add a drop of clean water over the material and apply a cover glass. The slide is ready for examination (see Note 10).
3. The images can be captured using a digital camera attached to the microscope.

*3.4.2. Phloroglucinol-HCl
Test for Lignin (18, 19)*

Lignin is a common constituent in the secondary wall of plant cells, e.g., the walls of xylem elements and sclerenchyma tissue. The cinnamaldehyde end groups of lignin appear to react with phloroglucinol-HCl to give a red-violet color (18, 19). Although the reaction is not very sensitive, because of the ease of staining, this procedure is still often used as one of the tests for the presence of lignin in plant cell wall. General expected results: Lignified walls become red.

1. Since the phloroglucinol stain has a high concentration of hydrochloric acid, suspension cells, cell clusters, and hand

sections are placed in a small Petri dish and stained with the phloroglucinol–HCl stain for 2 or more minutes. If lignified elements are present, the specimen will turn red in a few minutes.

2. Use a wet brush to transfer the cells or sections onto a clean slide, add a drop of water or a drop of 30% glycerol solution to the section. Be sure to place a cover glass over the cells or tissues before examination. Examine the specimen at once. The color fades gradually.
3. Be careful when handling the stain. Be sure that the stain does not get in contact with any part of the objective and microscope stage as the stain is highly corrosive. Wash the brush with running tap water to remove the acid.

3.4.3. Starch: Iodine–Potassium Iodide Test (18, 19)

The iodine–potassium iodide (IKI) stain is specific for starch. Apparently, the basis of the reaction is the accumulation of iodine in the center of the helical starch molecule. The length of the starch molecule determines the color of the reaction—the shorter the molecule, the more red the color; the longer the molecule, the more blue the color. General expected results: Starches will give a blue-black color in a few minutes. Newly formed starch may appear red-purple.

1. Transfer materials onto a slide as described in Subheading 3.4.1.
2. Place a drop of IKI solution directly on the specimen. Wait for a few minutes and apply a cover glass and examine the specimen with a microscope. The specimen can be examined without the removal of excess IKI solution from the sample.

3.5. Freehand Sections

Most plant parts are too thick to be mounted intact and viewed with a microscope. In order to study the structural organization of the plant material, sections have to be made so that enough light can be transmitted through the specimen to resolve cell structures under the microscope. A freehand section is the simplest method of preparing specimens for microscopic viewing. Chemical fixation of materials is generally not required for temporary preparations. This method allows one to examine the specimen in a few minutes. For in vitro cultures, large explants can be sectioned by hand. The sectioning of smaller explants is possible but requires some practice. Many additional freehand sectioning methods are available in the literature; please consult references (14, 15, 17, 20). A detailed pictorial presentation of hand sectioning procedure can be found in (17). Figure 1a represents a soybean cotyledon explant with nodules developing from the cut surface. Corresponding hand section of the cotyledon explant shows the organization of the nodule with dense cytoplasmic cells (Fig. 1b). Histochemical staining

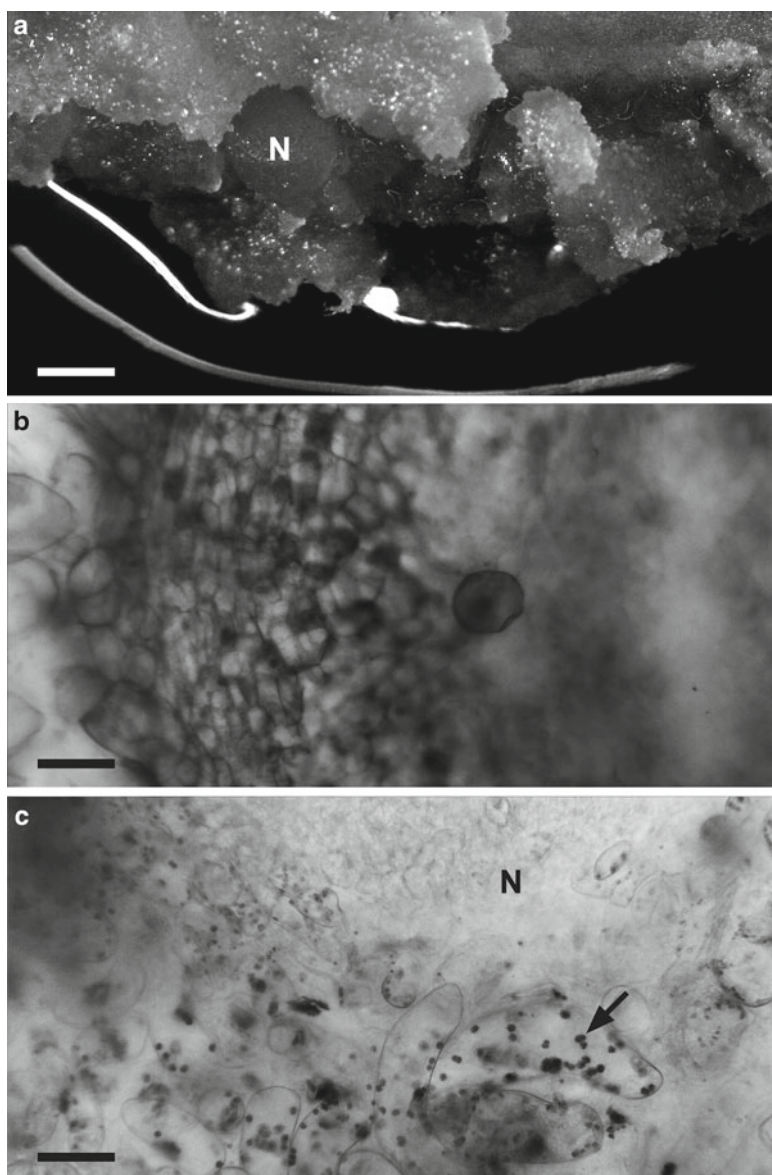


Fig. 1. (a) Soybean cotyledon explants showing nodule (N) formation near the cut surface. Scale bar = 1 mm. (b) A hand section through a developing nodule showing the presence of dense cytoplasmic cells within the nodule. Scale bar = 25 μ m. (c) Histochemical staining for starch using the IKI solution reveals the presence of starch granules (arrow) in cells surrounding the nodules (N). Scale bar = 25 μ m.

with the IKI solution reveals the presence of starch grains in cells surrounding the nodular structure (Fig. 1c).

1. Obtain a new double edge razor blade. To minimize the risk of cutting oneself, cover one edge of the razor blade with masking tape. Rinse the blade with warm tap water to remove traces of grease from the surface of the blade if necessary (see Note 10).

2. *Hold the plant material firmly. The material should be held against the side of the first finger of the left hand (or right hand) by means of the thumb. The first finger should be kept as straight as possible, while the thumb is kept well below the surface of the material out of the way of the razor edge (17, 20). Relax! It is not that easy to cut your own finger.*
3. Flood the razor with water. This will reduce the friction during cutting as sections can float onto the surface of the blade. Take the razor blade in the right hand (or left hand) and place it on the first finger of the left hand (or right hand), more or less at a right angle to the specimen (17, 20).
4. Draw the razor across the top of the material in such a way as to give the material a drawing cut (about 45° in the horizontal direction). This results in less friction as the razor blade passes through the specimen. Cut several sections at a time. The sections will certainly vary in thickness. However, there will be usable ones among the “thick” sections!
5. Transfer the sections to water, always using a brush, not a forceps or needle.
6. Select and transfer the thinnest sections (the more transparent ones) onto a glass slide and stain (see Subheading 3.2).

3.6. Digital Camera and Photomicrography

The process of photography changes dramatically with the development of digital cameras. For better quality camera, the CCD (charge coupled device) type cameras are preferred as they provide better quality images. The improvement of the CMOS (complementary metal oxide semiconductor) type cameras in recent years also makes them suitable for general photomicrography; especially, this type of camera is slightly less expensive.

1. Set up the digital camera according to manufacturer specification.
2. Be sure to understand the various features of one's own stereomicroscope or the compound microscope in order to take full advantage of the equipment at hand.
3. Depending on how the camera is attached to the microscope, different “mounts” alter the magnification ratio of the specimen. The exact magnification can only be determined by taking a set of images of a ruler that correspond to the setting of the stereomicroscope or a fine calibrated scale for the compound microscope. These magnification calibrations are not interchangeable between scopes.
4. Capture images with specific questions in mind.
5. Examine the images and make sure they are of good/excellent quality; if not, retake the photos at once.
6. Copy images to CD or DVD format for safe keeping.

4. Notes

1. For small explants and for a closer look at surface features, a stereomicroscope set up is preferred. Simple photomacrography requires a stereomicroscope, a light source and a digital camera. The images captured need to be transferred to a computer, and can be further manipulated with improved contrast and sharpness using appropriate software such as the Adobe Photoshop. A good quality printer is needed for printing the final composition. Depending on one's budget, instruments with different price ranges can be found. There are many manufacturers of these instruments. Additional information can be acquired through local suppliers or the World Wide Web. The magnification power and the quality of the objective lens determine the price of a stereomicroscope. The presence of a transmitted light base is a useful feature, but will add to its cost.
2. Proper illumination of the object is essential to quality photography. A full range of devices can be found on the Electron Microscopy Sciences Web site (<http://www.emsdiasum.com>) for reference.
3. Dissecting tools. In order to study the specimen thoroughly, careful dissection is required. It is imperative that good quality dissecting tools are used such as fine forceps, microdissection knives and pins (see companies such as Fine Science Tools, <http://www.finescience.ca>). This avoids unnecessary physical damage to the explants during manipulation.
4. Digital cameras are now standard equipment and can be placed in the same light path as the microscope, using a trinocular head piece or by attaching the camera to one of the eye pieces. Again, there is a wide range of prices. The Web site from the Martin Microscope Company (<http://www.martinmicroscope.com>) provides useful information showing the different types of cameras available. If possible, test the camera with your stereomicroscope system before purchasing it to ensure compatibility. Software is usually purchased or included with the camera. It is important that the software be compatible with the computer system.
5. Keep plates in a refrigerator. Excess water condensation can be removed using absorbent paper or a paper towel before use.
6. Be sure not to allow the specimen to dry up during examination. The chances of this are augmented by the fact that the light source can generate quite a bit of heat.
7. The photographed specimens can be stored temporary in a refrigerator for short time (about 24 h) if rephotographing is contemplated.

8. One way to minimize sample desiccation is to examine the explants on an agar plate. Agar is moist, so the specimen will not easily dry out during the course of photography. Agar plate can also serve as background support for the specimen during photography. Large specimens are difficult to focus at high magnifications because the depth of field of a stereomicroscope is limited. Because agar is soft, one can orientate the specimen without damaging it and easily position the feature of interest on the surface of the agar and submerge the remaining portion of the specimen. This way, the feature of attention is in focus.
9. If more light is required, reflectors can be created using aluminum foil near the specimen. Excess moisture around the specimen can cause bright reflections. It can be easily removed by small filter paper wedges.
10. Since water can evaporate from the slide over time, a 30% glycerol solution can be used instead of water. The sections will not dry out as fast as those in water.

Add only a small drop of mounting medium. Excess mounting medium around the cover glass should be removed by gently touching the edge of the cover glass with a filter paper. *Be sure there is no mounting fluid on the surface of the cover glass. Be sure to place a cover glass over your preparation.* Use only one cover glass!

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Chapter 9

Use of Statistics in Plant Biotechnology

Michael E. Compton

Abstract

Statistics and experimental design are important tools for the plant biotechnologist and should be used when planning and conducting experiments as well as during the analysis and interpretation of results. This chapter provides some basic concepts important to the statistical analysis of data obtained from plant tissue culture or biotechnology experiments, and illustrates the application of common statistical procedures to analyze binomial, count, and continuous data for experiments with different treatment factors as well as identifying trends of dosage treatment factors.

Key words: Analysis of variance, Binomial data, Concentration treatment factors, Continuous data, Count data, Data analysis, Logistic regression, Mean separation tests, Plant tissue culture, Poisson regression, Regression analysis

1. Introduction

Most research projects are initiated to solve a problem. In response, we plan and design experiments in the hope that some treatment will be found that solves the problem. Researchers use a logical and stepwise approach for problem solving. We examine the published literature to gain knowledge from prior experiences and apply what we have learned to formulate a hypothesis. Experimental objectives are developed that can be tested objectively and data gathered from observations made during experimentation. Finally, data are analyzed and evaluated to ascertain the effectiveness of each treatment at correcting the problem.

We can easily make measurements of plant cells and tissues, and mathematically calculate and rank the averages of the tested treatment variables. However, simply calculating averages does not consider the variation present among specimens receiving the treatments and does not accurately examine treatment effectiveness.

There are many statistical procedures that can be used to measure variation within and between treatments and provide us a better estimate of treatment effectiveness. For this reason statistics should be employed when evaluating data.

It is important that we avoid personal bias during all phases of experimentation. Personal bias, either occurring intentionally or by accident, will produce unreliable results that will inhibit the researchers' attempts to solve problems. Various experimental designs such as the completely randomized (CR), randomized complete block (RCB), incomplete block (IB), and split plot (SP) have been developed to help researchers reduce personal bias during experimentation and data collection (1–3). For more information on how these designs are used in plant biotechnology the readers should consult prior publications (4–6).

Use of the most appropriate and efficient statistical procedures can aid researchers in planning, conducting, and interpreting experimental results. This chapter is intended to serve as an introduction to the use of statistics in plant biotechnology research. After reading this chapter the reader should be able to select the appropriate statistical methods to analyze, interpret, and present plant biotechnology data.

2. Materials

2.1. Computer Software Used to Perform Statistical Analyses

In this day and age few plant biotechnologists calculate statistical analyses by hand or use a calculator. Instead, most scientists choose to use a statistical software package that is compatible with their personal computer (PC). Several windows-compatible software packages are available for statistical analysis procedures using a PC and include those available from SAS (7), SPSS (8), SYSTAT (9), MINITAB (10), and STATISTX (11). All software programs can be used to efficiently perform data analysis procedures used for plant cell and tissue culture and plant molecular biology data.

To illustrate the use of statistical procedures commonly used in plant biotechnology, two hypothetical experiments are used. The first evaluates the effectiveness of six cytokinins [zeatin, zeatin riboside, N⁶-(δ^2 -isopentenyl)-adenine (2iP), benzyladenine (BA), kinetin, or thidiazuron (TDZ)] on stimulating adventitious shoots from petunia leaf explants while the second was designed to identify the optimum BA dose required for shoot organogenesis in petunia. The hypothetical data collected included the number and percentage of explants that produce shoots, the number of shoots per responding explant, and the length of adventitious shoots.

2.2. Plant Materials

Plants of *Petunia* $\times$ *hybrida* “Blue Picotee” were grown from seed in the greenhouse for 12 weeks before excising leaves for explant preparation. Only the youngest, fully expanded leaves were harvested.

Plants were grown at 20°C and natural light intensities and photoperiod at Platteville, WI, from September to March.

The freshest quiescent seeds should be obtained. Seeds can be stored at 4°C for about 5 years and remain regenerable.

2.3. Shoot Regeneration Medium

Petunia Shoot Regeneration Medium: MS salts and vitamins with (per liter) 0.1 g myo-inositol, 30 g sucrose, and 5 g agar-gel (Phytotechnology Laboratories, LLC, Overland Park, KS) at pH 5.8 (12).

The plant growth regulators tested included zeatin, zeatin riboside, 2iP, benzyladenine, kinetin, or TDZ. All growth regulators were obtained from Phytotechnology Laboratories, LLC, Overland Park, KS, and supplied at 0, 2, 4, 6, 8, or 10 μM concentrations, respectively.

3. Methods

3.1. Explant Preparation and Culture Conditions

The methods used for explant preparation were outlined previously by Preece (12).

The youngest, fully expanded leaves were removed from plants actively growing in the greenhouse. Leaves were washed with soapy water and immersed in a 10% bleach solution (0.6% NaOCl with 1 ml/l antibacterial soap) for 15 min before three rinses with sterile reverse osmosis water.

To prepare the explants, the margins were trimmed from disinfested leaves and the lamina cut into 5×5-mm explants, each containing a portion of the midrib.

Explants were placed abaxial side down in test tubes containing 20 ml of shoot induction medium supplemented with test concentrations of the selected cytokinins. One leaf explant was cultured per vessel.

Test tubes containing explants were incubated in a growth chamber providing a 16-h photoperiod (30–50 $\mu\text{mol}/\text{m}^2 \text{ s}$) and 25°C. Test tubes containing explants were selected at random and placed in racks that held 32 vessels. All racks were placed on the same shelf in the growth room and arranged in a completely randomized design (see Note 1).

Explants were transferred to vessels containing 20 ml of fresh shoot induction medium every 4 weeks.

3.2. Statistical Analysis Procedures

Statistix for Windows© (see Note 2) was used to analyze the experimental data. Like other windows-based versions of statistical software, Statistix for Windows© uses drop-down dialog boxes to guide the user through data analysis (11).

The following paragraphs are divided into sections demonstrating the use of statistical analysis software to analyze binomial,

count, and continuous data for experiments with different treatment factors as well as identifying trends of dosage (concentration) treatment factors.

Explants were placed in treatment media described above. Data for all experiments were recorded at 12 weeks and included the number and percentage of explants with shoots, the number of shoots per responding explant, and the length of each shoot recorded. Data analysis procedures were performed based on a completely randomized design with one replicate per vessel, making each explant a replicate (see Note 3). Vessels were randomly placed into racks positioned on the same shelf in the growth chamber. Therefore, the model statements used for each analysis were constructed to reflect a completely randomized design.

3.3. Analysis of Binomial Data

Logistic regression is the statistical procedure suggested for analyzing response data (13). This is because, unlike ANOVA, logistic regression does not produce separate estimates of experimental error. Because of this feature, logistic regression produces more accurate results than ANOVA when analyzing response data. The logistic regression procedure can be found in Statistix for Windows® in the linear models selection of the statistics drop-down menu. The following steps should be followed when using this software to conduct logistic regression analysis.

While working in the appropriate data file, select the “Statistics” drop-down menu in the tool bar and click on “Linear Models.” This action creates a new box listing several linear model-based procedures, one of which is logistic regression.

Clicking on “Logistic Regression” produces a new window containing boxes in which the dependent and independent variables must be entered. In this example, the dependent variable is “response” and the independent variable is cytokinin. Click OK after entering names of the requested variables.

Clicking the OK button initiates the analysis. Test results appear in a new window that can be printed or saved.

When reading the analysis output, significance of the independent variable, cytokinin type in this case, is indicated by the *P* value (see Note 4).

3.4. Analysis of Count Data

Data, such as the number of shoots produced per regenerating explant or number of somatic embryos per explant, are considered counts (13). Count data are not normally distributed because the variance of each treatment is equal to the average response of the treatment (14). Because of their distribution, Poisson regression (also known as discrete regression) is suggested for analyzing count data (13).

The advantage of Poisson regression is that the procedure uses a logarithmic value of the mean counts, which normalizes the data during analysis (13). The following steps should be followed when using Statistix to conduct Poisson regression analysis.

While working in the appropriate data file, select the “Statistics” drop-down menu and click on “Linear Models.” This creates a new box listing several linear model-based procedures, one of which is Poisson regression.

Clicking on Poisson regression produces a new window in which the dependent and independent variables must be entered. In this example, the dependent variable is the number of shoots (NOSHOTS) and the independent variable is CYTOKININ. Click on the OK button after entering the information.

Clicking the OK button initiates the analysis. Test results appear in a new window that can be printed or saved.

When reading the analysis output, significance of the independent variable, cytokinin type in this case, is indicated by the *P* value (see Note 5).

3.5. Analyzing Continuous Data

The shoot length variable is considered continuous since the value of data observations is unrestricted. Because of this fact, continuous data tend to be normally distributed with treatments having similar variances (13). ANOVA is well suited for analyzing continuous data with a normal distribution (4, 5, 13).

During ANOVA, a model statement is created that identifies the treatment variables and the observations recorded (dependent variables) as well as treatment interactions (independent variable), if present. The model statement must be written based on the experimental design used (4). The procedure generates a random error value by subtracting the value of each datum from the overall mean (13). The influence of independent variables on the dependent variables is tested according to the model statement, generating a summary table that provides the results of the model tested (2). Information in the summary table includes the degrees of freedom (DF), sources of variation (SS), mean square error (MSE) *F*-statistic (*F*), and estimates the probability (*P*) value that determines the level of significance of the *F*-statistic (2). The following steps should be followed when conducting a general ANOVA using Statistix.

While working in the appropriate data file, move the cursor to the “Statistics” drop-down menu. Clicking on “Linear Models” creates a new box listing several linear model-based procedures, one of which is the general ANOVA.

Click on “GENERAL ANOVA” to display a new window in which the dependent variable must be entered. In this example, the dependent variable is shoot length (SHOOTSLTH).

The model statement should be entered in the box identified as “AOV Model Statement.” In this case, the model statement is “CYTOKININ.” No other variables are entered into the model statement in this situation because cytokinin is the only treatment factor. If there were multiple treatment factors, each would be written in the model statement with the interactions of interest (see ref. 4 for instructions on writing model statements for experimental designs).

Clicking the OK button initiates the analysis and the results appear in a new window that can be printed or saved.

In ANOVA, the sources of variation are listed with their DF, SS, MS, F , and P values. In this example, there are two main sources of variation identified as the treatment factor (cytokinin type) and the residual (experimental error). When reading the analysis output, significance of the treatment factor (cytokinin type) is indicated by the P value (see Note 6).

3.6. Analyzing Treatment Means

Treatment means can be analyzed to determine treatment differences once a significant value is obtained in the preliminary statistical test (ANOVA, Poisson regression, or Logistic regression). A separate mean comparison evaluation test is not necessary when there are only two treatments because the general test (ANOVA, Poisson regression, or logistic regression) alone determines the statistical difference (4). However, most researchers evaluate multiple treatments simultaneously. The easiest way to compare treatment means is to rank them in ascending or descending order and pick the best one. However, this method does not measure variation within the treatments and may not, on its own, accurately represent how the explants responded to the treatments (13, 15). For this reason, it is suggested that a mathematical procedure that calculates within-treatment variation be used to compare treatment means.

There are many mean separation procedures that account for within-treatment variation. The procedures most commonly used in plant biotechnology research to compare the means of unrelated or related treatments are multiple comparison and multiple range tests, standard error of the mean (SEM), and orthogonal contrasts.

3.6.1. Multiple Comparison and Multiple Range Tests

Multiple comparison and multiple range tests are statistical procedures that use the population variance to calculate a numerical value for comparing treatment means (4, 5, 15). Means are ranked in ascending or descending order and the difference between adjacent means calculated and compared to a value computed by the statistical test (13). The two treatment means are considered different if their difference exceeds the computed statistical value. However, if the difference between treatment means is equal to or less than the computed statistical value, the treatments are considered similar (13).

Multiple comparison tests calculate one statistical value and use it to compare adjacent and nonadjacent means. Examples of multiple comparison tests are Bonferoni, Fisher's least significant difference (LSD), Scheffe's, Tukey's Honestly Significant Difference Test (Tukey's HSD), and Waller-Duncan K-ratio T test (Waller-Duncan). One problem with these procedures is that they may overestimate treatment differences among treatments ranked far apart. This occurs because treatment variances tend to increase with increasing data values (13). One should use caution if using

one of the above procedures in experiments with a large number of treatments.

Multiple range tests differ from multiple comparison tests because the former employs different critical values to compare adjacent and nonadjacent means (4). This modification improves the accuracy of the test, making it less likely that errors will be made when comparing distantly ranked means. Examples of multiple range tests include Duncan's New Multiple Range Test (DNMRT), Ryan-Einot-Gabriel-Welsh Multiple F-test (REGWF), Ryan-Einot-Gabriel-Welsh Multiple Range Test (REGWQ), and Student Newman-Keuls (SNK). The Ryan-Einot-Gabriel-Welsh Multiple Range Test is recommended because of its moderate level of conservancy. Unfortunately, multiple range tests are not always available in PC statistical software packages.

Mean comparisons are conducted as part of an ANOVA. The following steps demonstrate how to conduct the Tukey's HSD test using Statistix.

While working in the appropriate data file, select the "Statistics" drop-down menu and click on "Linear Models." This creates a new box listing several linear model-based procedures, one of which is the general ANOVA.

Clicking on "GENERAL ANOVA" produces a new window in which the dependent variable must be entered. In this example, the dependent variable is shoot length (SHOOTSLTH). CYTOKININ is written in the model statement box.

Clicking the OK button initiates the test. The results are displayed in the general ANOVA coefficient table window.

Clicking "Results" in the toolbar display a drop-down box listing procedures that can be used to evaluate treatment means or variances.

Clicking on "Comparison of Means" displays a window listing various mean comparison tests. Enter the desired main effect, or interaction, to be tested (CYTOKININ in this case) and click on one of the available tests. In this case, the desired test is Tukey's HSD.

Click OK to initiate the test. An output is generated in a new window that lists the treatments and their means, the homogeneous groups and the number of significant groups, as well as the critical Q value and the critical value for comparisons.

The means with their homogeneous group identification may then be displayed in a table or a graph (see Note 7).

3.6.2. Orthogonal Contrasts

Orthogonal contrasts are used to make comparisons between treatments with similar characteristics (4, 13). In plant tissue culture and biotechnology studies, these may be plant growth regulators (PGRs) with similar activity (e.g., natural vs. synthetic cytokinins) or DNA constructs with the same promoter. Orthogonal contrasts differ from mean comparison tests in that more than two treatments can be compared in one test. However, the number of

comparisons made must be restricted to the number of degrees of freedom for the treatment variable (2).

Orthogonal contrasts are performed as part of the ANOVA procedure (4). A contrast statement is written that specifies the treatments or group of treatments to be compared and the ANOVA procedure calculates the DF, SS, and MS for the comparison. An F statistic is calculated for the contrasts and the level of significance (P value) determined. One DF is used for each comparison. The following steps demonstrate how to perform orthogonal contrasts using Statistix.

While working in the appropriate data file, select the “Statistics” drop-down menu. Click on “Linear Models” to create a new box listing several linear model-based procedures, one of which is the general ANOVA. Click on general ANOVA.

In the ANOVA window, indicate the dependent variable and type in the model statement. Click OK to initiate the test.

Click on “Results” located in the toolbar of the general ANOVA coefficient window. This generates a list of treatment evaluation procedures.

Clicking on “General Contrasts” produces a new window in which the contrast main effect, or interaction, and appropriate contrast coefficients for the desired treatment, or treatment group, comparisons are entered (see Note 8).

Clicking OK initiates the test and displays the results in a new window.

The results are interpreted by identifying the level of significance (P value) for each contrast (see Note 9).

3.6.3. Standard Error of the Mean

The standard error (SE) is frequently used for mean comparison purposes in plant tissue culture and biotechnology research. SEs are obtained by dividing the sample standard deviation by the square root of the number of observations for that treatment (14). Many researchers use SE values like a mean comparison test, generating an individual value for each treatment and comparing the difference between the means of paired treatments with their calculated SE. The researcher often declares two treatments similar or different if the collective values (treatment mean $\pm$ its SE) for the paired treatments do not overlap. This use of SE can be used to compare means ranked adjacently. However, problems occur when using SE to compare means that are ranked far apart. When remembering how SE values are calculated, one would realize that SE values increase with the numerical value of the data, causing the researcher to overestimate treatment differences and violate the assumption of ANOVA that treatment variances are equal (13). Therefore, the use of SE to compare means ranked far apart fails to produce useful results and does not accurately reflect population variance.

The following steps can be used to generate SE values for the accurate comparison of treatment means. This test is most valuable for treatments that are unrelated (5, 13).

While working in the appropriate data file, select the “Statistics” drop-down menu and click on “Linear Models.” This creates a new box listing several linear model-based procedures, one of which is the general ANOVA. Click on general ANOVA.

In the ANOVA window, indicate the dependent variable and type in the model statement. Click OK to initiate the test.

Click on “Results” located in the toolbar of the general ANOVA coefficient window. This generates a list of treatment evaluation procedures. Select “Means and Standard Errors.” Enter the main effect, or interaction, and click OK to initiate the test and generate the results.

The results are displayed in a window. The identity of treatment with its means and SS is listed as well as the number of observations per cell, the standard error of an average, and the standard error of the difference of two averages (see Note 10).

3.7. Dosage, or Concentration Treatments

Researchers that design experiments with treatments consisting of various doses or concentrations of a single treatment factor are usually interested in identifying a single dose, or concentration, that produces an optimal explant response. In other words, the researcher is interested in identifying a trend present among the levels of the treatment factors. The best statistical procedure for these types of experiments is usually trend analysis (5, 13). In trend analysis, models identifying specific trends (linear, quadratic, cubic) are tested in a stepwise fashion from simplest (linear) to most complex (cubic in most cases) until a nonsignificant trend is identified (16). The last significant trend is considered to best describe the response to the treatments. Trend analysis uses SS, T , and R^2 values to indicate significant trends (4). Trends may be tested through regression analysis or polynomial contrast statements in ANOVA. The most effective method of conducting trend analysis depends on the experimental objectives and the statistical software package used.

To illustrate trend analysis, the treatments for the petunia example can be changed from six cytokinin types to six concentrations of BA [0 (control), 2, 4, 6, 8, and 10 μM]. The objective of this experiment would be to identify a trend in explant response to BA that would identify an optimum concentration of the growth regulator for adventitious shoot organogenesis. Data recorded included the percentage of explants that produced shoots, number of shoots per explant, and length of regenerated shoots. The following steps can be used to conduct trend analysis using Statistix.

For simple linear regression:

While working in the appropriate data file, select the “Statistics” drop-down menu. Clicking on “Linear Models” creates a new box listing several linear model-based procedures, one of which is linear regression.

Clicking on linear regression produces a window in which the dependent and independent variables must be entered in their respective boxes.

Click OK after entering these variables.

Clicking OK initiates the procedure and produces a window displaying the linear regression coefficient table (see Note 11).

For trend analysis using polynomial contrasts:

While working in the appropriate data file, select the “Statistics” drop-down menu and click on “Linear Models.” This creates a new box listing several linear model-based procedures, one of which is the general ANOVA.

Clicking on general ANOVA window displays a box in which the dependent variable and type in the model statement must be entered. Enter the appropriate statements.

Clicking on OK initiates the test and produces the ANOVA results with a new toolbar.

Clicking on “Results” located in the toolbar of the general ANOVA coefficient window generates a list of treatment evaluation procedures.

Selecting “Polynomial Contrasts” produces a new window in which the level of polynomial contrasts and the contrast main effect, or interaction, must be entered (see Note 12). Click on OK.

Clicking on OK produces a window displaying the results of the polynomial contrasts for the desired dependent and independent variables indicated in the ANOVA.

The last polynomial degree with a significant *P* value is considered to be the most significant trend (see Note 13).

4. Notes

1. Because one explant was cultured in a vessel and the vessels randomly placed in the test-tube rack, the experimental design was a completely randomized design (2). The CR design is commonly used for plant biotechnology experiments because it is easy to use and allows researchers to maximize the number of replicates examined as well as utilize either equal or unequal replication for the treatments (1). The CR design is also amenable to statistical procedures for binomial, count, continuous, and concentration data (5). These advantages are important as maximizing replicate numbers can lead to a more powerful test in determining treatment differences. Flexibility in the number of replicates for each treatment is important because unequal replication often occurs due to contamination or death of explants.

Table 1
Results of analysis of variance of petunia shoot length data analyzed using a completely randomized design

Source	DF	SS	MS	F	P
Cytokinin	5	1,803.35	360.67	12.46	<0.0001
Experimental error	54	1,563.50	28.954		
Total	59	3,366.85			

Explants were prepared from leaves of petunia plants grown in the greenhouse. The influence of six cytokinins (zeatin, zeatin riboside, 2iP, BA, kinetin, and TDZ) on the length of regenerated shoots was examined. Ten replicate test tubes were cultured per treatment. Data are hypothetical

In a CR design, the sources of variation are the applied treatment(s), in this case cytokinin type, and experimental error, the latter representing variation not associated with the applied treatments. Interpretation of computer outputs from the CR design is considered simple and straightforward (Table 1).

- The use of Statistix for Windows© in this chapter should not be viewed as an endorsement for the product. Similar data analyses can be performed with other statistical software packages.
- While the petunia example uses a CR design with one explant per vessel, the randomization scheme can be modified to include multiple explants per vessel. Such a randomization scheme is referred to as subsampling (17) and is often used in plant biotechnology to conserve resources, space, and time. Subsampling can also be used to minimize the variation associated with explants by “averaging out” the response among culture vessels (6). When using subsampling, it is important that the researcher writes the analysis statements in a manner that the variation present among explants within each culture vessel is calculated; otherwise, the experimental error would be overestimated leading to erroneous results.

Other experimental designs, such as the RCB, incomplete block, and split plot, can be used in situations in which isolation of specific nontreatment or treatment factors is desired. The use of experimental designs that employ blocking has been discussed thoroughly by others (4–6, 18).

- A *P* value of 0.7969 was obtained for cytokinin when analyzing the petunia data using logistic regression (Table 2). This indicates that the explants responded similarly to the cytokinin treatments.

Following logistic regression, a mean comparison test can be used to determine which cytokinin elicited the optimum explant response. When performing a mean comparison test, it is usually best to perform an appropriate data transformation

Table 2
Results of logistic regression analysis of the percentage of leaf explants that produced adventitious shoots

Predictor variables	Coefficients (COEF)	Standard error (SE)	COEF/SE	<i>P</i>
CONSTANT	1.296	0.743	1.74	0.0813
CYTOKININ	0.057	0.196	0.29	0.7696
DEVIANCE	57.08			
P-VALUE	0.509			
DF	58			

Table 3
Results of Poisson regression analysis of the number of adventitious shoots produced per responding leaf explants

Predictor variables	Coefficients (COEF)	Standard error (SE)	COEF/SE	<i>P</i>
CONSTANT	1.120	0.1504	7.45	<0.0001
CYTOKININ	0.101	0.0362	2.79	0.0052
DEVIANCE	205.94			
P-VALUE	<0.0001			
DF	58			

before comparing the means of response data if an ANOVA-based mean comparison or multiple range test is used (4).

Before analyzing response data, it is important to determine if there are treatments in which the response did not vary. This usually occurs when all or none of the explants responded. These values should either be changed or deleted before analyzing data (13). If a decision is made to change the values, zeros should be changed to a slightly higher value (0.000001) and 100% values reduced slightly (0.999999). It is important to decide during planning if values are going to be altered or deleted. You should also indicate if treatments were dropped or values altered when writing reports and manuscripts.

5. In the petunia example, Poisson regression revealed that cytokinin type influenced the number of shoots per responding explant as determined by reading the *P* value of the CYTOKININ variable (Table 3). In this case, a *P* value of 0.0052 was obtained indicating that there was a significant regression associated with cytokinin type.

It is important to delete observations from nonresponding explants before analyzing the data, as retention of zeros will

skew the data set and lead to inaccurate results and interpretation of treatment effectiveness (13). As always, it is important to indicate that values from nonresponding explants were deleted before analysis in all reports and manuscripts.

6. In the petunia example, the results of ANOVA indicated that shoot length was influenced by the cytokinin used (Table 1). An F value of 12.46, which was highly significant ($P < 0.0001$), was obtained. The cytokinin MS reflects the amount of variation caused by the cytokinin treatments, whereas the MSE measures variation due to culture vessels and other nontreatment sources.
7. In the petunia example, Tukey's HSD was used to compare the effect of different cytokinins on elongation of regenerated shoots. The test divided the treatment means into four homogeneous groups as represented by the letters next to each treatment mean. When presenting treatment means in a table or graph, the results of multiple comparison and multiple range tests are placed with the treatment means in a table or a graph. A letter is assigned to each treatment mean and treatment means assigned different letters are considered statistically different. Means assigned the same letter are considered statistically similar (Fig. 1).

Based on these results, shoot length was the best among explants cultured in medium containing zeatin compared to zeatin riboside, kinetin, and TDZ. However, there was no

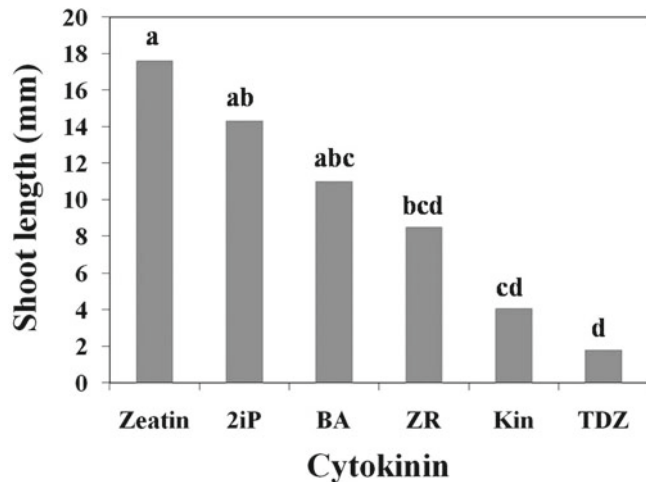


Fig. 1. Bar graph demonstrating the use of Tukey's HSD mean comparison test. The hypothetical experiment tested the influence of cytokinin type on the elongation of adventitious shoots regenerated from leaf explants of *Petunia × hybrida* incubated in MS medium containing 4 μ M of zeatin, 2-isopentenyl adenine (2iP), benzyladenine (BA), zeatin riboside (ZR), kinetin (Kin), or thidiazuron (TDZ). Bars with the different letters are significantly different according to Tukey's HSD. From Compton and Mize (6) with permission.

difference in the length of shoots regenerated from explants cultured in medium supplemented with 2iP or BA.

As you can see, results of mean comparison tests can be complicated and confusing. For this reason, researchers should seriously examine their options for evaluating treatment observations when planning experiments and consider using procedures that produce the most meaningful results. Often, procedures, such as orthogonal contrasts, can produce the most meaningful comparisons (4, 5, 13).

- 8. In the petunia example, five contrasts of interest were planned before the experiment [1—natural cytokinins (zeatin, zeatin riboside, and 2iP) vs. synthetic types (BA, kinetin, and TDZ); 2—cytokinins containing zeatin (zeatin and zeatin riboside) vs. non-zeatin containing cytokinins; 3—BA (as a control) vs. all other cytokinins; 4—BA vs. TDZ; and 5—TDZ vs. all other cytokinins] and contrasts conducted for all dependent variables as part of ANOVA. The software instruction manual explains how orthogonal coefficients are written.
- 9. Results of orthogonal contrasts are usually presented in a table containing ANOVA computations (Table 4). Means of each contrast may be placed in a table or presented in the text with

Table 4
Use of orthogonal contrasts to demonstrate the influence of cytokinin type on the elongation of adventitious shoots regenerated from petunia leaf explants

Cytokinin type	Number of explants	Shoot length (mm)
Zeatin (Z)	9	17.6
2iP	9	14.3
Benzyladenine (BA)	10	11
Zeatin riboside (ZR)	5	8.5
Kinetin (K)	9	4.1
Thidiazuron (TDZ)	7	1.8

Orthogonal contrasts	F-Value	P-Value
Z and ZR vs. 2iP, BA, K, and TDZ	2.54	0.039
Z, ZR, and 2iP vs. BA, K, and TDZ	6.36	0.0001
BA vs. Z, ZR, 2iP, K, and TDZ	0.17	0.9711
BA vs. TDZ	2.92	0.0209
TDZ vs. Z, ZR, 2iP, BA, and K	4.98	0.0008

the means and P value given for each contrast. Examining results for shoot length, shoot elongation was promoted when PGRs containing zeatin (Z and ZR) were used ($P=0.039$). Mean shoot length was 13 mm when explants were cultured in medium containing a zeatin-based cytokinin, whereas non-zeatin-based cytokinins inhibited shoot length (7.8 mm). Likewise, natural cytokinins (Z, ZR, and 2iP) promoted shoot length more than synthetic formulations of BA, kinetin, and TDZ ($P=0.0001$; 13.5 mm vs. 5.3 mm, respectively). Shoot elongation was inhibited ($P=0.0008$) when TDZ (1.8 mm) was used compared to any of the other cytokinin formulations (11.1 mm).

Orthogonal contrasts can be used to analyze binomial or count data given that the data are transformed before analysis. Information gained from orthogonal contrasts is often much more useful than the outcomes of multiple comparison or multiple range tests. Orthogonal contrasts are underutilized in plant biotechnology studies possibly because researchers have not been exposed to the concept or because their usefulness is not completely understood. See Little and Hills (1), Lentner and Bishop (2), Compton (4, 5), Mize et al. (13), or Zar (16) for more information regarding the use of orthogonal contrasts.

10. When using SE values to compare the means of several treatments, one SE value should be calculated from the ANOVA MSE or SE values obtained from Poisson or logistic regression (13). This use of SE is more likely to yield realistic results and reveal true treatment differences compared to the calculation of individual SE's each treatment, which violates the equal variance assumption of ANOVA (13). The correct use of SE is demonstrated in Fig 2.
11. In the petunia example, trend analysis was used to examine the effect of cytokinin concentration on shoot length. Because no shoots were produced by explants cultured in medium without BA, observations recorded for 0 BA were deleted before analysis. In addition, zero values from nonresponding explants were deleted because only shoot length values from responding explants were of interest.

A regression coefficient table is generated by trend analysis. The table identifies the predictor variables with their coefficients, standard errors, student's T , and P values along with the R^2 , adjusted R^2 , MSE, and standard deviation (Table 5). Based on the information from responding explants, shoot length was dependent on BA concentration with the linear model ($X=22.954-2.1014\times\text{BA}$) best describing the effect of BA on shoot length ($P<0.00001$; $R^2=0.86$).

Means of dosage treatments should be presented in graphs displaying the regression equation and fitted line, R^2 , individual

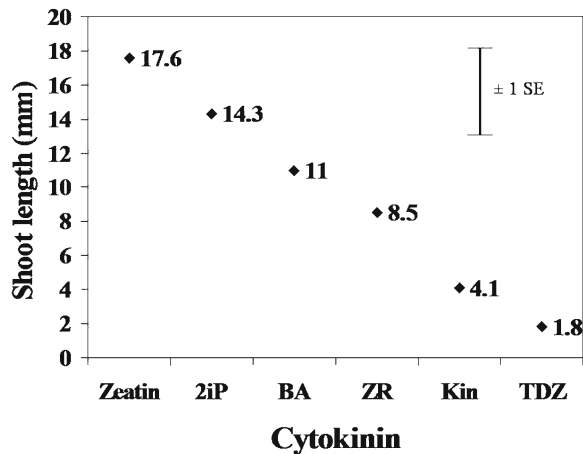


Fig. 2. Graph demonstrating the use of standard error for comparing treatment means. The hypothetical experiment tested the influence of cytokinin type on the elongation of adventitious shoots regenerated from leaf explants of *Petunia × hybrida* incubated in MS medium containing 4 μM of zeatin, 2-isopentenyl adenine (2iP), benzyladenine (BA), zeatin riboside (ZR), kinetin (Kin), or thidiazuron (TDZ).

Table 5
Results of regression analysis of evaluating the influence on benzyladenine concentration on the elongation of adventitious shoots regenerated from petunia leaf explants

Predictor variables	Coefficients (COEF)	Standard error (SE)	COEF/SE	P
CONSTANT	22.9535	0.9164	25.05	<0.0001
BA concentration	−2.1014	0.1366	−15.38	<0.0001
R ²	0.8616	EMS	4.8968	
Adjusted R ²	0.858	SD	2.2129	

Regression summary table

Source	DF	MS	F	P
Regression	1	1,158.70	236.62	<0.0001
Residual	38	4.8968		
Total	39			

Explants were prepared from leaves of petunia plants grown in the greenhouse. The influence of various concentrations of benzyladenine (0, 2, 4, 6, 8, and 10 μM) on the length of regenerated shoots was examined. Data from 0 μM BA were deleted before analysis because no explants produced shoots. Likewise, data from nonresponding explants incubated in media containing 2–10 μM were deleted before analysis. Data are hypothetical

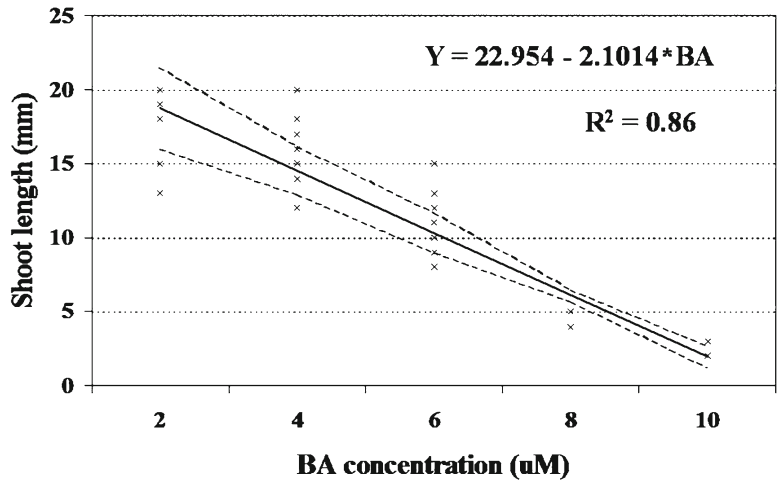


Fig. 3. Graphical presentation obtained from regression analysis testing the influence of benzyladenine (BA) concentration on elongation of adventitious shoots regenerated from leaf explants of *Petunia × hybrida* incubated in MS medium containing 0, 2, 4, 6, 8, or 10 μM BA. Graph from Compton and Mize (6) with permission.

data values and confidence intervals (Fig. 3). Similar to mean comparison tests, confidence intervals of treatments that overlap are considered similar. In the petunia example, confidence intervals for 2 and 4 μM overlap. However, confidence intervals for 6, 8, and 10 μM do not overlap with 2 and 4 μM , and are considered significantly different from those for 2 and 4 μM . The conclusion would be that shoot elongation was inhibited with increasing concentration of BA above 4 μM .

Trend analysis and polynomial contrasts may be used for evaluating optimum treatment levels, even if those levels were not directly tested but lie within treatment boundaries (4). Predicted values can be obtained using information generated from the analyses. These values allow researchers to estimate the effects of treatments that were not evaluated as long as they are within the tested boundaries (16).

Trend analysis can be used to evaluate binomial and count data provided that the data are transformed prior to analysis. As with mean comparison procedures, data are converted back to the original scale for presentation.

12. For the petunia described above, the level of polynomial contrasts is 3 and the main effect is BA. These values are entered in the appropriate boxes and the computer substitutes the correct contrast coefficients.
13. Polynomial contrasts often provide results similar to simple regression. As with regression analysis, the last significant contrast is considered to provide the best description of the trend. Polynomial contrasts can be used when treatments are

Table 6
Use of polynomial contrasts to demonstrate the influence of benzyladenine concentration on the elongation of adventitious shoots regenerated from petunia leaf explants

Benzyladenine concentration (μM)	Number of explants	Shoot length (mm)
0	–	–
2	5	17.0
4	9	15.9
6	10	11.0
8	9	4.56
10	7	2.6

Polynomial contrasts	SS	F-Value	P-Value
Linear	1,615.3	452.91	<0.0001
Quadratic	7.7861	2.18	0.1485

Explants were prepared from leaves of petunia plants grown in the greenhouse. The influence of various concentrations of benzyladenine (0, 2, 4, 6, 8, and 10 μM) on the length of regenerated shoots was examined. Data from 0 μM BA were deleted before analysis because no explants produced shoots. Likewise, data from nonresponding explants incubated in media containing 2–10 μM were deleted before analysis. Data are hypothetical

unequally replicated or spaced as long as specific contrast coefficients are calculated (4, 19).

In the petunia example, results of polynomial contrasts indicated that the linear regression best describes the trend in shoot length (Table 6). This is evident by the fact that the linear contrast was the last significant trend.

Results of polynomial contrasts are presented in a table with ranked means (Table 6). For instructions on how to generate a regression equation from polynomial contrasts, see Carmer and Seif (19), Kleinbaum et al. (16), or Zar (14).

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Chapter 10

Tissue Culture Methods for the Clonal Propagation and Genetic Improvement of Spanish Red Cedar (*Cedrela odorata*)

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Abstract

The choice of a method to culture red cedar tissues depends on the final objectives pursued. If homogeneous clonal material is required for experimental purposes, the easiest way is to generate the lines through adventitious shoot induction from seedlings germinated from seeds. If the objective is to generate high yielding material for plantation purposes, the choice will be the same method but starting from mature vegetative tissues from selected elite plants. Most of the process are the same, but the initial steps are less efficient and much more elaborate. If the purpose is to generate lines with new genetic characteristics through somaclonal variation, mutagenesis, or genetic transformation, somatic embryogenesis will be required. No single method in its present form is suitable for all purposes. Eventually, the efficient production of somatic embryos from rejuvenated shoots collected from mature selected plants is the ideal way to culture this species, but for the time being we have to choose one or the other. In this chapter, we present a grafting procedure to rejuvenate and maintain mother plants in the greenhouse and the in vitro culture systems we have developed for the production of *Cedrela odorata* propagules using explants from both young seedlings and mature tissues from selected old trees. Using a modified TY17 medium and the BioMINT® temporary immersion system, we obtained high multiplication and ex vitro transplantation rates for efficient large-scale propagation of this species.

Key words: Adventitious shoots, Red cedar trees, Clonal lines, Somatic embryogenesis, Rejuvenation, Temporary immersion bioreactor

1. Introduction

Spanish red cedar (*Cedrela odorata* Linnaeus [Meliaceae]) is a tropical timber tree distributed from southern Mexico to northern Argentina (3). Highly prized on the world market, it can reach up

to five times the value of coniferous hardwood (4). However, large-scale demand has led to the overexploitation of natural populations by selective logging which has severely reduced the availability of elite trees for seed production and genetic improvement (5). The natural populations have also declined due to the destruction of their habitat (6). The International Union for Conservation of Nature (7) lists *C. odorata* as a species that faces a high risk of extinction in the wild in the medium-term future.

The pressure on natural populations could be alleviated by commercial plantations, but their establishment has been hampered by the Meliaceae borer *Hypsipyla grandella* Zeller (Insecta: Pyralidae), a moth whose larvae feed on the apical meristem of young trees, causing lateral branching that deforms the trunks and makes them unsuitable for the timber industry (8). Natural populations of *C. odorata* are genetically diverse, and it is possible to find individuals with a certain degree of resistance (9); however, new varieties with improved traits are difficult to produce by means of traditional breeding due to the long life span of this species.

Cloning of selected elite trees through in vitro propagation is an alternative to classic silviculture techniques that has demonstrated its potential to improve the quality and yield of wild perennial species (10). Moreover, the micropropagation of tree species, particularly in *Pinus* and *Populus*, has proved to be a rapid strategy for the production of homogeneous clonal planting stock useful for commercial plantations, forest restoration (11), and conservation of elite or rare germplasm (10).

The main problem limiting the broad use of this technique is the difficulty to select elite individuals in young populations. It is, therefore, necessary to use mature tissues from proven adult plants that are not the most adequate tissues for the establishment of in vitro lines.

The rejuvenation of tissues from mature *C. odorata* plants is essential to culture and propagate elite individuals for the establishment of plantations with elite characteristics.

2. Materials

2.1. Reagents

1. Growth regulators:
 - Benzyladenine (BA).
 - Abscisic acid (ABA).
 - Dicamba.
 - Giberellic acid (GA_3).

2.2. Culture Media and Standard Mixtures

1. Plant Preservative Media (PPM®).
2. Mixture of commercial fungicides: 1% (w/w) of each: Benomyl® (DuPont; Wilmington, DE), Agrimycin® (Pfizer; New York, NY), and Captan® (Bayer; Leverkusen, Germany).
3. Sterilizing solution: 20% (v/v) commercial sodium hypochlorite and 100 µL/L of poly-oxyethylene sorbitan monolaurate (Tween 20®).
4. Sterilizing solution for shoots derived from grafted materials: Mixture of commercial fungicides plus 1 g/L Agrimy (streptomycin sulfate, oxytetracycline chlorhydrate, copper oxychloride).
5. Rooting medium: Half-strength semisolid MS medium, supplemented with 80 mM sucrose, 9 µM GA₃, and 0.25% (w/v) Gelrite®.
6. Embryogenic induction medium (TY 17) (1): 1× WPM basal medium (12), 0.5× MS vitamins (13), 13.57 µM dicamba, 0.1% (w/v) activated charcoal, 2% (v/v) coconut water (CW), 26.02 µM citric acid, 28.38 µM ascorbic acid, 80 mM sucrose, and 0.25% (w/v) Gelrite® adjusted to pH 5.7 prior to autoclaving (14).
7. Maturation medium: Half-strength MS medium without Gelrite® supplemented with 20 g sucrose and 3 g/L GA₃.

2.3. Culture Dishes

1. Magenta culture boxes GA7.
2. Baby food jars.
3. Petri dishes.
4. Temporary immersion bioreactors (2).

2.4. Basic Facilities

1. Airflow cabinets.
2. Autoclave.
3. Growth incubation room.
4. Greenhouse facilities.

3. Methods

3.1. Selection of Elite Plant Material

1. Elite 15–30-year-old *C. odorata* trees from natural populations, selected for their phenotypic features, must have, as recommended by Longman (15), very straight trunks (at least 10 m long and 50 cm in diameter), few lateral branches (see Note 1), and must show no signs of *H. grandella* attack (8) (see Note 2).

3.2. Sources of Explants

As mentioned before, three different sources of explants can be used depending on the objective of the work.

1. For micropropagation of elite individuals, twigs (30 cm long) must be cut from the newest possible branches of the selected trees (see Notes 3 and 4) and maintained in a polystyrene box with ice and transferred to the greenhouse as soon as possible to prevent desiccation or any other sort of stress.
2. For experimental clonal lines, ripe fruits, collected from the selected trees, are incubated at 25°C and 10% relative humidity until spontaneous opening occurs and mature seeds are released.
3. For somatic embryogenesis, unripe fruits 9 week post anthesis (about 25 mm long) from the same trees are collected between January and March and stored with a mixture of commercial fungicides until the seeds are used as a source of immature zygotic embryos (see Note 5).

3.3. Disinfestation and Establishment in Culture

1. The collected twigs are disinfested treating them with the mixture of fungicides before they are established in a warm greenhouse ($\pm 30^\circ\text{C}$) to induce the development of the axillary buds (16). They can be established, either directly in water or in soil beds, to induce rooting or they can be grafted (as soon as possible) into juvenile trees (15) (see Note 6). Both, the directly planted (rooted) twigs and the grafted ones, are kept under greenhouse conditions at 30–35°C and 80% humidity.
2. The seeds from mature fruits are mixed with the fungicide mixture and kept at room temperature in a black plastic bag (for a maximum period of 4 months) before they are germinated. The disinfested seeds are germinated aseptically in 2-L sterile Erlenmeyer flasks (100 seeds/flask) filled with cotton soaked with distilled water. Seed germination and all of the subsequent culture steps are carried out in a culture room at 25°C with a photoperiod of 16-h light (40 $\mu\text{mol}/\text{m}^2 \text{ s}$; Sylvania T12 Gro-Lux[®]; Waltham, MA) and 8-h dark. After 2 weeks, the emerging seedlings are used as explants for micropropagation (see Note 7) or planted in soil to be used as rootstocks for grafting (17).
3. The shoots excised from twigs and the seeds are disinfested by immersing them in a 15% (v/v) commercial sodium hypochlorite solution supplemented with 200 $\mu\text{L}/\text{L}$ of polyoxyethylene sorbitan monolaurate (Tween 20[®]) for 20 min and rinsed three times with sterile distilled water, each for 5 min.
4. The unripe fruits are exhaustively washed with soap and tap water for about 5 min and then transferred to a 1-L Erlenmeyer flask containing 500 mL of a 30% v/v commercial sodium hypochlorite solution. Fruits are maintained in this solution in

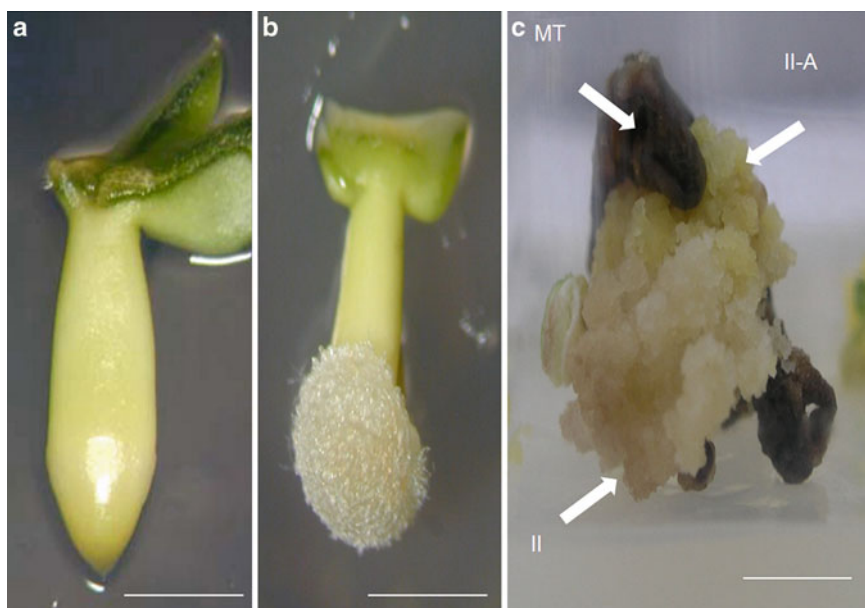


Fig. 1. Induction of somatic embryogenesis: (a) Zygotic embryo isolated from an immature fruit. (b) Initiation of callus formation (bar = 2 mm). (c) Embryogenic callus (bar = 10 mm). MT; mother tissue. II; embryogenic callus type II. II-A; embryogenic callus type II-A.

an orbital shaker at 50 rpm for 30 min and then rinsed three times with sterile distilled water for 5 min each time.

5. The fruits are then dissected with sterile forceps and scalpel under aseptic conditions in an airflow hood to extract the immature zygotic embryos (Fig. 1).
6. The embryos are maintained immersed in a 0.1% (v/v) Plant Preservative Media (PPM®) solution for 24 h at 14°C in the dark before they are transferred to in vitro culture.

3.4. Grafting

Grafting and micrografting (see below) have the double purpose of maintaining the collected branches in order to generate more explants for the propagation of a specific individual and, more important, to try to rejuvenate the explant tissues for a more efficient in vitro propagation.

1. The grafting process is carried out by applying side wedge cuttings on young (6 months old) trees derived from germinated seedlings to be used as rootstocks and “V” cuttings on the twigs used as scions (Fig. 2c).
2. The grafted twigs are kept under greenhouse conditions at 30–35°C and 80% humidity.
3. One week after grafting, the rootstocks are decapitated to induce shoot formation by cutting some 10 cm below the apex and removing all the leaves. The plants are kept under the same

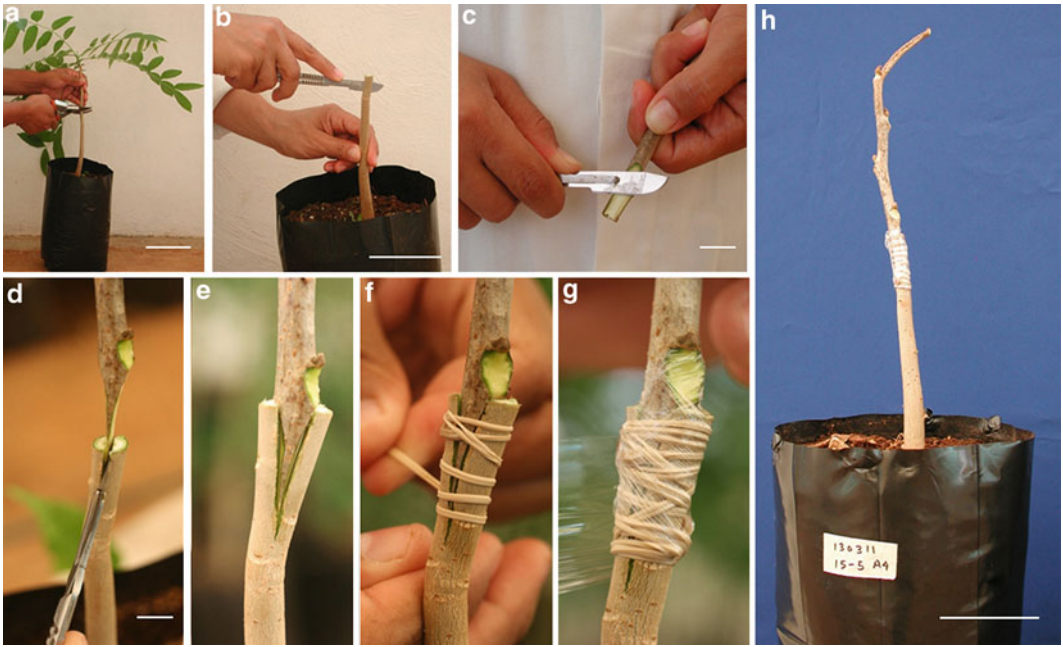


Fig. 2. Grafting process: (a) Top removed 15 cm from the base of a 6-month-old *Cedrela odorata* plant from seed. (b) Radial cut. (c) Preparation of the scion. (d, e) Grafting the scion into the rootstock. (f–g) Tiding and wrapping for protection. (h) Growing in a green house. Scale bars: (a) 10 cm, (b) 5 cm, (c) 1 cm, (d–g) 1 cm, (h) 10 cm.

greenhouse conditions for three additional weeks after which the responding axillary buds from the rootstocks are excised.

4. After 4–6 weeks, the formation of new shoots from the scion is observed and those that are at least 10 cm long are harvested to be used as explants for tissue culture (see Note 8; Fig. 3).

3.5. Micrografting In Vitro

1. New shoots, at least 10 cm long, formed from the grafted twigs are placed in the antifungal solution supplemented with 1 g/l Agrimyl for 1 h, rinsed three times with sterile distilled water, and then incubated in 10% calcium hypochlorite for 20 min (see Note 9) and rinsed again thoroughly with sterile distilled water.
2. The apical sections (3 cm from the tip) to be used as scions are prepared by removing the leaves and making a V cut (1 cm) at the base.
3. The rootstocks are plantlets derived from in vitro germinated seeds that have been maintained in culture for 5 months to allow them to develop a sufficiently thick stem (0.5 cm diameter) to receive the scion.
4. The plantlets are prepared by cutting the apex and removing the leaves. The roots are also trimmed (Fig. 4).
5. A longitudinal cut of 0.5 cm is made at the top, where the scion is inserted.

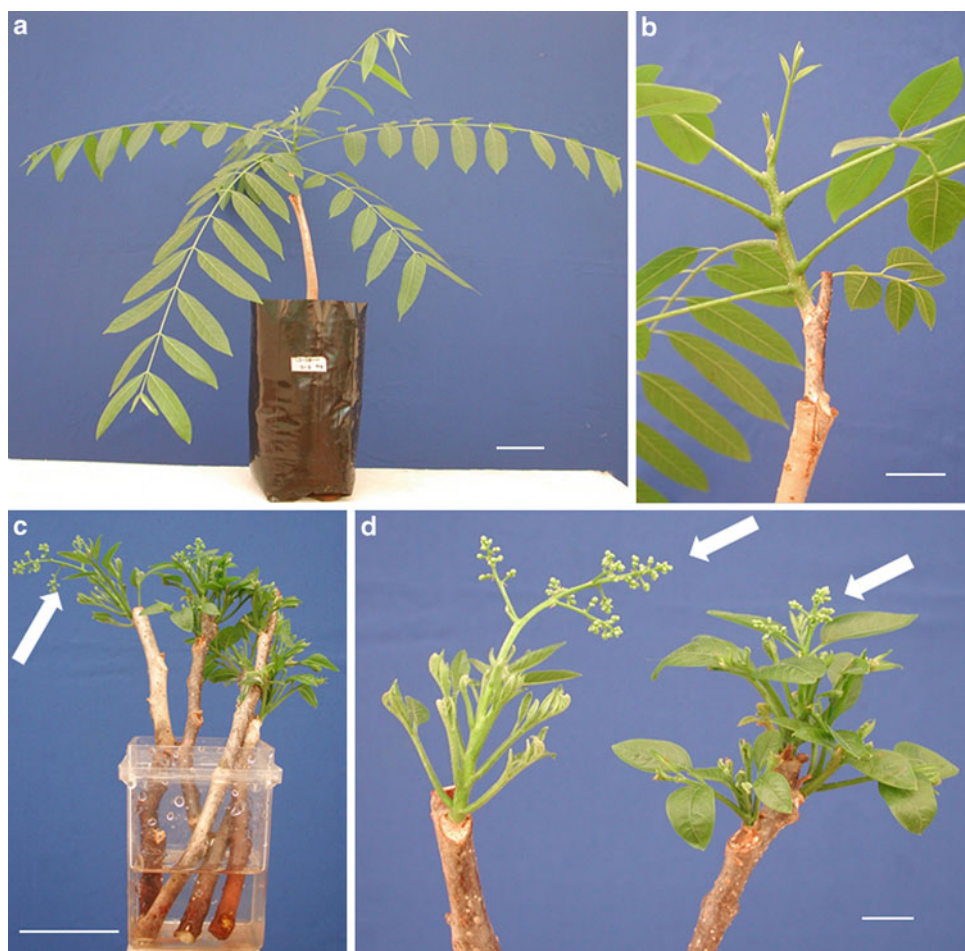


Fig. 3. Grafts and twigs after 5 weeks of culture: (a) A general view of a graft, (b) shoots of about 10 cm, (c) twigs in only water, (d) shoots with inflorescences from twigs in water (the white arrows indicate the position of the inflorescences). Scale bars: (a) 10 cm, (b) 3 cm, (c) 5 cm, (d) 2 cm.

6. The graft zone is protected by wrapping it with sterile aluminum fold (1×3 cm).
7. The micrografts are cultured in test tubes with MS medium supplemented with sucrose (30 g/L) and gelrite (3 g/L) (Fig. 5).

3.6. Micropropagation Through Organogenic Induction of Adventitious Shoots from Mature Tissues

1. To micropropagate *C. odorata*, from mature tissues of selected mother plants, the axenified shoots from mature twigs are tissue cultured individually in baby food jars containing 25 mL of semisolid TY17 medium at a density of ten explants per container. Plants were incubated as described above.
2. After 8 weeks, 9-mm-long hypocotyl segments are excised from the in vitro culture-established shoots.

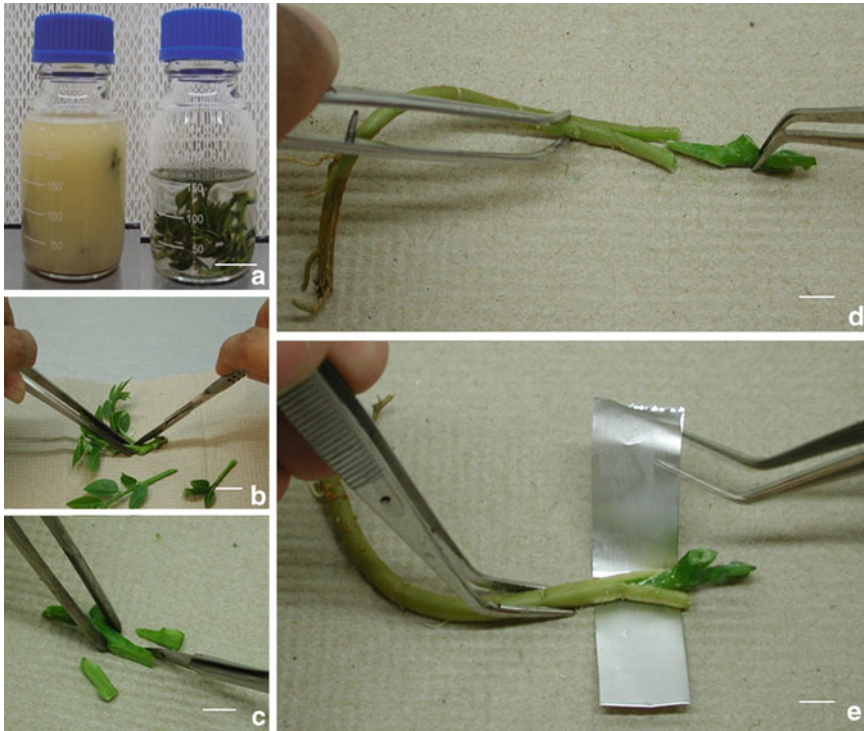


Fig. 4. Micrografting process. (a) Disinfestation of shoots, (b) cleaning, (c) wedge formation by cutting a “V”, (d) union of the graft with the rootstock, (e) the grafting area is secured with a rubber band. Scale bars: (a) 3 cm, (b) 10 mm, (c–e) 5 mm.

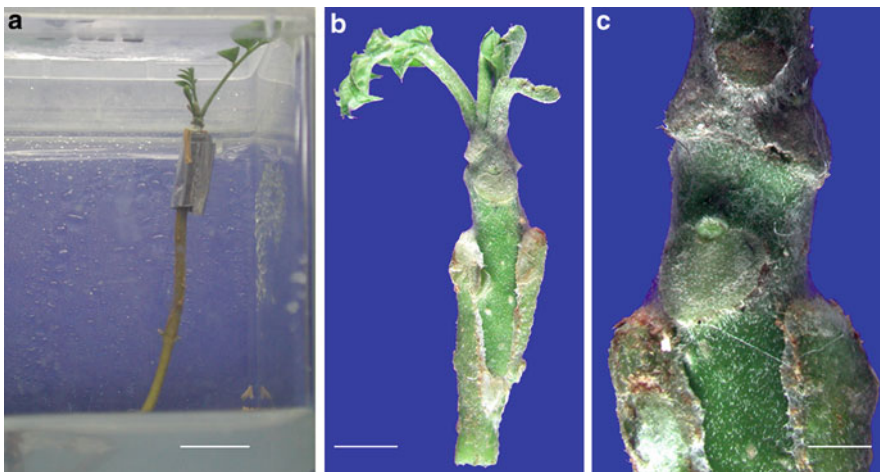


Fig. 5. Micrograft at 5 weeks. (a) Note the formation of new leaves at the apex; (b) buds in the process of growing. Scale bars: (a) 10 mm (b) 5 mm, (c) 3 mm.

3. The cuttings are placed horizontally in baby food jars containing 25 mL of semisolid TY17 medium unless a specific modification is indicated.
4. The jars are placed in a culture room under the conditions described earlier and 3 weeks later the formation of new shoot will be observed.
5. For successive micropropagation rounds, the shoots formed in vitro are allowed to grow for 6 weeks in TY17 medium supplemented with 20% (v/v) CW and 9 μM GA₃ before cutting them again into 9-mm internode segments to be used as fresh explants for a new round of adventitious shoot induction under the same culture conditions (see Note 10).
6. The shoots from the last multiplication round are elongated and rooted in Magenta boxes containing 50 mL of rooting medium.
7. After 9 weeks in the culture room, the plants are taken out of the culture container, washed to remove the agar, and established in soil.
8. Each round of replication produces between 15 and 20 new plantlets.

3.7. Induction of Somatic Embryogenesis

1. The immature zygotic embryos isolated from fruits are placed in a Petri dish containing 25 mL of TY17 semisolid medium and incubated in a culture room under the conditions previously described.
2. All cultures are transferred to fresh medium every 3 weeks.
3. After 9 weeks, various types of calluses are formed classified according to their general appearance, color, and texture (Fig. 1c). Only type II calli are truly proembryogenic and are used to induce embryo formation.
4. Proembryogenic type II calli are aseptically chopped into sections of approximately 60 mg fresh weight, incubated under photoperiodic conditions at 28°C, and transferred to fresh MS-based induction medium every 3 weeks for 12 weeks.
5. After 12 weeks in culture, approximately 60% of the type II calli produce a new type of callus clearly distinguishable from its predecessor by the complete absence of mother tissue and because they are more friable with a more intense yellow color.
6. These new calli give rise to all developmental embryo stages: globular, heart, torpedo, and early cotyledonary (≤ 5 mm long; pale or light green) and late cotyledonary (≥ 5.1 mm long; deep green) (Fig. 6a). All of them in the same explant.

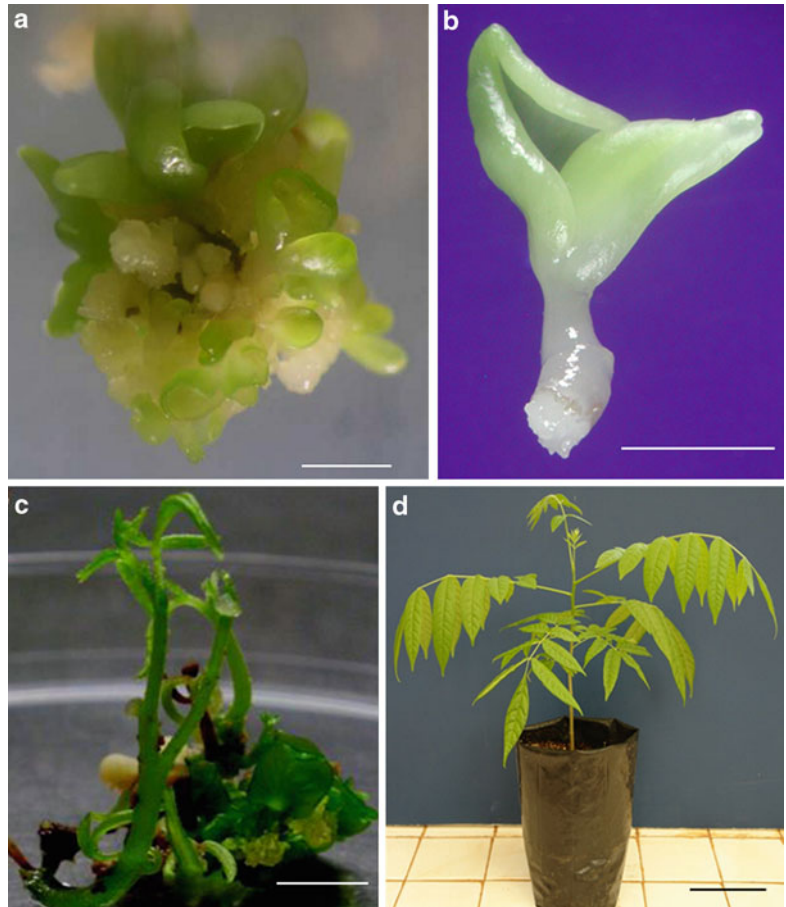


Fig. 6. Development of somatic embryogenesis. (a) Somatic embryos of type II-A, (b) cotyledonary embryo stage after 9 weeks of culture, (c) germinated embryos. (d) plant established in soil under greenhouse conditions. Scale bars: (a) 10 mm, (b) 15 mm, (c) 10 mm, (d) 10 cm.

7. For embryo conversion and germination, type II embryogenic masses are placed in semisolid medium containing 3.8 μ M ABA.
8. Embryo conversion begins with the formation of bipolar structures (Fig. 6b) followed by the appearance of roots (including secondary root formation), shoots with true leaves, and stem elongation (Fig. 6c). About 12% of the embryos germinate successfully after 9 weeks. From this stage on, plants are successfully maintained with virtually no losses prior to soil acclimatization.
9. Plantlets derived from germinated embryos are transferred individually to magenta boxes containing 40 mL of full-strength WPM basal salt medium added with 1 $\times$ MS vitamins,

40 mM sucrose, and 0.13% (w/v) Gelrite®. Plants were kept under photoperiod in an environmental chamber for 2 months before transfer to soil.

3.8. Acclimatization and Transfer to Soil

1. After 6 weeks in culture, rooted plants with stems about 100 mm long are washed *ex vitro* with running tap water to eliminate the agar from the roots, planted on germination trays containing wet Germination Mix® substrate (SunGro Horticulture; British Columbia, Canada), and kept in a greenhouse for 1 month. The survival rate is around 95% and all plants show a normal development similar to plantlets derived from seeds.
2. The plants are then transplanted to plastic bags containing a wet mixture of 70% peat moss sphagnum (SunGro Horticulture, British Columbia, Canada) and 30% perlite (Perlita de La Laguna S.A. de C.V. Saltillo, México) (w/w) with an initial pH of 5.7.
3. The trees are maintained under greenhouse conditions for 3 months and, when they reach a minimum size of 50 cm, can be transplanted to the field. The survival rate of the established plants after 6 months is around 75%.

3.9. Shoot Elongation and Rooting Using Temporary Immersion Systems

An alternative, more efficient way to grow and root micropropagated plants is the use of temporary immersion systems (TISs) (see Note 11).

1. Approximately 20-mm-long adventitious shoots, derived from the last micropropagation round, are transferred to temporary immersion bioreactors for maturation and rooting (see Note 12).
2. Ten plantlets are incubated in 200 ml of maturation medium. The bioreactors are set at a frequency of a 5-min immersion every 8 h and incubated at 28°C for a period of 4 weeks as described by Robert et al. (18).
3. For rooting, fresh maturation medium containing 1.48 µM IBA is added. After 6 weeks, the plantlets reach an average size of 10 cm and develop large root systems (see Note 13).

4. Notes

1. Seedlings germinated from seeds are the most abundant and easy to handle sources of explants to tissue culture large perennial species. They are ideal starting material for the establishment of tissue culture methods and for basic research. However, if the objective of the work is to produce high-quality clonal

material for commercial plantations, the explants must originate from vegetative material collected from mother plants that possess the desired characteristics. The plants derived from germinated seeds will all be genetically different and there is no doubt that some high-yielding lines can be derived from them, but this will not be known until the plants have grown in the field for 6 or 7 years.

2. This does not guarantee that the plants will be tolerant.
3. This is the most difficult part since the distal parts of the highest branches present the newest tissues and can only be reached by climbing the trees.
4. The origin of the twigs must be carefully marked in order to keep track of all lines derived from a single individual.
5. The immature zygotic embryos must be extracted before 72 h to ensure their capacity to form embryogenic calli and their viability.
6. The grafting into juvenile materials permits to maintain the sources of explants for individual lines for longer periods.
7. The seedlings from seeds are cut and cultured in exactly the same manner that is described for the shoots derived from the twigs of mature tissues.
8. The fact that, sometimes, the new shoots from nongrafted tissues flower while none of the grafted ones do suggests that there is a rejuvenating effect in the later ones.
9. The control of bacteria and fungi is the main limitation of the whole process since most explants get contaminated when placed in the *in vitro* culture medium. However, neither sodium hypochlorite nor higher calcium chloride concentrations or incubation times are recommended since the tissues get badly damaged. Only 5% of the grafted or micrografted explants have been established *in vitro* for micropropagation purposes. Some claims of efficient disinfestation methods do not give any information about how it was achieved. Even in specialized courses on the topic, it is maintained as a secret. This is surprising given the enormous contribution that an efficient disinfestation procedure would represent for the advancement of the field.
10. This process can be repeated at least three (maybe many more) times producing at least 50 new plants per clone. For mature tissues, the efficiency is still low.
11. There are many TISs, all with their own advantages and disadvantages. The one we use is the Modular system called BioMINT[®] developed by Robert et al. (18) because it is simple to use, has a large capacity, and is cheaper than other systems.

12. A full description of the system is beyond the scope of this paper but can be found in Robert (18).
13. For comparative purposes, plants rooted in semisolid medium containing 0.25% (w/v) Gelrite®, 80 mM sucrose, and GA₃ (8.66 µM) developed much slower.

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Chapter 11

Micropropagation of Banana

Yıldız Aka Kaçar and Ben Faber

Abstract

Banana (*Musa* spp. AAA) is propagated vegetatively and can be rapidly and efficiently propagated by micropropagation. Conventional micropropagation techniques, however, may be too costly for commercial purposes. Our laboratory has found that depending on the combination of culture vessel and gelling agent more economic methods can be chosen for successfully micropropagating banana.

Key words: Micropropagation, *Musa*, Banana, In vitro, Multiplication, Acclimatization

1. Introduction

Banana (*Musa* spp. AAA) is an important fruit crop of the Musaceae family, widely grown in developing countries, and is the second largest fruit crop in the world after citrus (1). Bananas are vegetatively propagated using corms, suckers, and sword suckers as materials. These materials, however, can harbor pests, such as fungi, nematodes, viruses, and insects. Since diseases are often spread through vegetative propagation, there has been a great deal of effort to create disease-free planting material on a large scale through tissue culture, which also has the advantages of speed and optimized use of plant materials. Similar trends in tissue culture can be seen during this period in fruit propagation of other plant species, especially in tissue culture-propagated rootstocks of apple, pear, and cherry (2). Successful results have been obtained in all these crops; however, the cost of culturing may not be justified in all these species.

Bananas can be micropropagated with high multiplication rates, and the production of high-quality plants is feasible. Many studies have reported on the clonal propagation of *Musa* spp. (3–7). Micropropagation has become a routine procedure, but the high

costs involved have prevented laboratories with limited resources from benefiting from tissue culture technology (7).

As an example protocol for banana tissue culture, an efficient system was developed for in vitro mass propagation of the commercial cultivar *Musa acuminata* cv. Dwarf Cavendish by the cultivation of shoot apices (8). Shoot apices with two to three pairs of leaf primordia were induced from sliced rhizome explants on Murashige and Skoog (MS) (9) medium supplemented with 6.0 mg/L BA, 150 mg/L adenine sulfate (Ads), and 3% (w/v) sucrose. Multiple shoots were induced from meristematic domes on the same medium. Addition of 1.0–1.5 mg/L IAA to the culture medium and incubation in continuous light increased the yield of multiple shoots. The high multiplication coefficient was stably maintained up to the fifth subculture; thereafter, it declined. Rooting was readily achieved upon transferring the shoots onto half-strength MS medium supplemented with 500 mg/L activated charcoal and 2% (w/v) sucrose. Micropropagated plantlets were hardened in a polyethylene house and successfully established in soil. There was no morphological variation among the micropropagated plants (9). Gübbük and Pekmezci (10) published a protocol refined for Turkish Dwarf Cavendish clones: supplementations with 2 μ M TDZ and 1 μ M IAA or 20 μ M BAP and 1 μ M IAA on MS medium, followed by 5 g/L charcoal at the rooting stage, were the best combinations for the in vitro propagation of banana types.

Agar is the most commonly used gelling agent in media for plant tissue culture. Since its introduction more than 100 years ago, it has remained the most frequently used gelling agent in culture media, employed for microbes as well as plants. The properties of agar that make it the gelling agent of choice are stability, high clarity, and resistance to metabolism during culture (11). Agar can be the most expensive component of plant tissue culture media. Because of the high price of tissue culture-grade agar, attempts have been made to identify suitable alternatives.

There are many commercial grades of gelling agents. The importance of using pure-grade gelling agents in experimental tissue culture work is widely acknowledged; however, their use in commercial micropropagation is not necessary, even though lower grades may contain various ions, sulfonated polysaccharides, and long fatty acids (12).

The lid of the culture vessel protects the culture medium from microbial infections and prevents excessive evaporation of water from the culture medium. The type of vessel and lid affects the gaseous composition inside the vessel, as well as light penetration. Therefore, the growth of tissues in culture (shoot elongation, proliferation, fresh weight increase, and possibly the hyperhydric degradation processes) is also affected (13). Different culture vessels, such as disposable containers, may reduce labor costs in commercial tissue culture laboratories.

We tested the effect of different culture vessels (industrial glass jars, Magenta boxes, and disposable containers) and gelling agents (agar-agar, Agargel, Phytigel, and plant agar) on the micropropagation of Dwarf Cavendish banana to identify the combination that produces a high number of good-quality banana seedlings. When shoot number (which is the most important variable in multiplication) was considered, commercial glass jar/Phytigel and jar/plant agar combination yielded significantly higher numbers of shoots than did any other combination. The quality of the seedlings produced by these combinations, indicated by shoot weight and length in our study, was comparable to that of other combinations for shoot weight, but lower than that of many other combinations for shoot length (14).

In a similar study, Neštáková et al. (15) compared the effect of two gelling agents, agar and Phytigel, on in vitro growth and shoot development of miniature rose and gladiolus. They collected data for number of shoots, shoot length, number of leaves per shoot, and fresh weight. For both of the species studied, significantly higher fresh weight was recorded on Phytigel. Shoot multiplication was significantly higher on Phytigel for miniature rose culture, although for gladiolus, agar showed higher numbers. Conversely, the number of leaves per shoot was significantly higher on agar medium for miniature rose, and the length of the main shoot of gladiolus was higher on Phytigel. When agar and Phytigel were compared pairwise in our results, the comparisons were either not significant or Phytigel had higher numbers than with agar. The differences in the two sets of results can be explained by the response of different species.

The cost of the disposable containers is higher than that of glass jars and Magenta vessels, since they are used only once, which may appear to increase the long-term cost of producing banana seedlings by micropropagation. However, glass jars and Magenta vessels must be cleaned, which can add considerable cost. Therefore, depending on the labor cost necessary to clean the vessels, the overall cost of disposables may not be significantly different than that of others. Plant agar is a less expensive alternative to conventional agar, which also helps to reduce overall costs. However, because we found differential patterns among vessel types and media as well as for their interactions, we suggest that selection can be made based on economic factors in banana micropropagation.

2. Materials

2.1. Sterilization of Plant Material

In surface sterilization of banana plants, a distinction must be made between greenhouse-grown and field-grown materials. Field-grown materials are dirtier and more contaminated at the beginning.

The surface sterilization procedure can be made effective by increasing the time in bleach and ethanol or by increasing the concentration of bleach.

1. Sterile beakers and flasks.
2. 70% ETOH.
3. Sterile ddH₂O.
4. 10% Bleach and Tween 20 (few drops).
5. 0.05% Mercuric chloride (HgCl₂ is a toxic chemical, be careful when using, and follow waste disposal regulations when disposing the waste material).

2.2. Initiation Stage and Multiplication Stage

Most plant cell culture media are based on the original formula of Murashige and Skoog salt formulation. MS salt mixture supplemented with Morel vitamins (16) is used for initiation and multiplication stages of banana micropropagation (see Fig. 1).

2.2.1. Initiation Medium and Multiplication Medium

1. 900 mL ddH₂O.
2. 4.3 g MS salts (see Note 1 for MS macro- and micronutrients).
3. 1 mL of Morel vitamins (see Note 2 for Morel vitamins).
4. 2 mg/L BA.
5. 4% sucrose (w/v).
6. Adjust volume to 1 L.
7. Adjust to pH 5.7 with 0.1 N NaOH or HCl.
8. Add 8% agar (w/v).
9. Heat and stir to dissolve.
10. After boiling the media, pour into culture vessels.
11. Autoclave at 1.05 kg/cm² and 121°C for 15 min.

2.3. Rooting Stage

2.3.1. Rooting Media

1. 900 mL ddH₂O.
2. 4.3 g MS salts (see Note 1 for MS macro- and micronutrients).
3. 1 mL of Morel vitamins (see Note 1 for Morel vitamins).
4. 1 mg/L IBA.
5. 4% sucrose (w/v).
6. Adjust volume to 1 L.
7. Adjust to pH 5.7 with 0.1 N NaOH or HCl.
8. Add 8% Agar (w/v).
9. Heat and stir to dissolve.
10. After boiling the media, pour into culture vessels.
11. Autoclave at 1.05 kg/cm² and 121°C for 15 min.



Fig. 1. (a) Culturing the shoots on initiation media (IM). (b) Shoot tips in IM. (c) New shoots form in the multiplication stage on MM. (d) Separation of multiplied shoots to transfer MM or RM. (e) Plantlets on rooting media. (f–h) Rooted plantlets after washing off the agar.

3. Methods

3.1. Sterilization of Plant Material

1. Select the suckers from healthy plant material.
2. Remove outer layers of leaves and corm tissue of the suckers.
3. The plant material should be about 10 cm long and 6–8 cm in diameter.
4. Wash the plant material thoroughly for 30 min under running tap water.
5. Excise the basal part of the plant including 2.5 cm of shoot tip.
6. Prepare a solution containing 0.05% mercuric chloride (see Note 3) with a few drops of Tween 20 to reduce surface tension and allow better surface contact.
7. Soak shoot tips in this solution, stir gently for 10 min, and pour off solution.
8. Rinse explants three times with sterile, tissue culture-grade water.
9. Remove the leaves' length or width 0.5 cm.
10. Rinse the explants with 70% ETOH for 3 min.
11. Prepare a solution containing 10% commercial bleach and a few drops of Tween 20.
12. Soak the explants in this solution for 10 min.
13. Remove the bleach solution.
14. Rinse the explants three times with sterile, tissue culture-grade water to remove all traces of surface-sterilizing agents under a laminar airflow chamber.

3.2. Initiation Stage

1. After surface sterilization, isolate shoot tips (4–5 mm), cut longitudinally into two sections, and then culture on initiation medium (IM) in aseptic conditions.
2. Incubate cultures in the dark at $25 \pm 2^\circ\text{C}$. Depending upon the cultivar, shoots may appear in the first 4–5 weeks.
3. If shoots are not present, subculture the explants for another 4 weeks in the same media until well-formed shoots are visible.
4. Transfer the shoots to the multiplication medium (MM).

3.3. Multiplication Stage

1. When the shoot culture increases in size and multiplies, cultures should be subcultured on a fresh medium (MM) by separating the plantlets.
2. Incubate cultures in the dark at $25 \pm 2^\circ\text{C}$.
3. Transfer the new plantlets to a fresh multiplication medium every 4 weeks.
4. After having targeted the number of plantlets, transfer the plantlets to the rooting media (RM).

3.4. Rooting Stage

1. Place well-formed shoots on to RM medium to induce root formation in a 16/8-h light/dark photoperiod using cool-white fluorescent lights ($50 \mu\text{mol}/\text{m}^2 \text{ s}$) at $25 \pm 2^\circ\text{C}$.
2. Transfer healthy plants in soil to a greenhouse or growth chamber after 6 weeks.
3. Add water to the plants and allow sitting for an hour.
4. This helps loosen the roots from agar.
5. Carefully scoop the plant (roots and agar together) out of the container.
6. Hold the plant upside down under water.
7. Gently wash with tap water to remove residues of agar from the roots.
8. Fill the 10-cm square pot half full of sterile substrate mixture.
9. Hold the plant upright in the pot, and pour more substrate around the roots until the pot is full of soil and the plant is upright.
10. Water the plant and substrate.
11. Plants that have been brought from the aseptic culture require a hardening-off period to acclimate them to the lower humidity of growth chamber.
12. In the greenhouse, and especially in the field, irradiance is much higher and air humidity much lower than those in the vessels. Even if the water potential of the substrate is higher than the water potential of the rooting media, the plantlets may quickly wilt, as water loss of their leaves is not restricted.
13. In addition, water supply can be limiting because of low hydraulic conductivity of roots and root-stem connections. Many plantlets die during this period. Therefore, after *ex vitro* transplantation, plantlets usually need some weeks of acclimatization with a gradual lowering of air humidity (17).

4. Notes

1. Macro- and micronutrients of MS:

Macronutrients	Concentration (mg/L)
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370
KH_2PO_4	170
KNO_3	1,900
NH_4NO_3	1,650
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440

Micronutrients	Concentration (mg/L)
H ₃ BO ₃	6.2
MnSO ₄ ·H ₂ O	15.6
ZnSO ₄ ·7H ₂ O	8.6
Na ₂ MoO ₄ ·2H ₂ O	0.25
CuSO ₄ ·5H ₂ O	0.025
CoCl ₂ ·6H ₂ O	0.025
KI	0.83
FeSO ₄ ·7H ₂ O	27.8
Na ₂ EDTA	37.3

2. Morel vitamins:
The biotin is soluble in ethanol: Weight biotin, dissolve in ethanol 95°C, agitate, add 1 mL of this solution in the graduated flask containing the other components dissolved in water, and calibrate.

Vitamin	Concentration (mg/L)
Biotin	0.010
Ca pantothenate	1.00
Thiamine HCl	1.00
Pyridoxine HCL	1.00
Nicotinic acid	1.00
Myo-inositol	100

3. Mercuric chloride is a very effective sterilizing agent, but it is toxic to the explant tissues. Therefore, the concentration of the solution and duration of the treatment should be optimum to minimize tissue mortality of the explants due to oversterilization.

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Chapter 12

Liquid In Vitro Culture for the Propagation of *Arundo donax*

Miguel Ángel Herrera-Alamillo and Manuel L. Robert

Abstract

We describe a simple and inexpensive plant micropropagation system for giant reed (*Arundo donax* L.) that uses axillary buds from the lateral stems of elite plants selected from field- or nursery-grown plants. The buds, attached to the stems are cultured in stationary liquid MS culture medium, supplemented with indole 3-acetic acid and kinetin. This formulation is the only one required for all the stages. Contrary to what happens in semisolid medium where roots are not formed, the plants cultured in liquid medium are whole plants with shoots and roots that develop at the same time. The survival rate of these plants when transferred to soil is close to 100% during acclimatization. A clonal line of 900 plants from a single mother plant can be produced in 4 months.

Key words: *Arundo donax*, Biofuels, Liquid culture, Direct organogenesis, Giant reed

1. Introduction

Increasing the speed of biomass production is important if biofuels are to make a larger contribution to satisfying our growing energy needs without competing with food production for land, water, and markets, as currently occurs with maize and sugar. Although no single method or species is likely to achieve this, a number of promising plants are being considered as alternatives, on the basis of their highly efficient biomass production. Together with agricultural wastes, sugarcane and maize have already received considerable attention (1–6). For decades, sugarcane (*Saccharum officinarum*) has been in the forefront of initiatives for the production of bioethanol from the fermentation of the sugars accumulated in the stem. Starches accumulated in the grain corn (*Zea mays*) are being used for the same purpose, while other crops produce oils and cellulosic biomass for the production of biodiesel.

Among the various alternative species that are being considered and tested, *Arundo donax* appears to be particularly promising due

to the high biomass yield that it generates (6–10). *A. donax* produces large amounts of cellulosic biomass from the rapid growth of its stems, which is converted into a biofuel. This is achieved by means of an industrial process called fast pyrolysis that consists in the rapid burning of the plants at very high temperatures that will convert them into gases (11–13). These gases are then condensed to produce a mixture of hydrocarbon compounds known as biocrude because of its similarity to crude oil. Just as crude oil, biocrude needs to be refined but, in contrast, it is a rapidly generated renewable resource.

There are several publications on the cultivation, productivity, and chemistry of this grass and the way it can be used for the generation of biofuel (10–17). *A. donax* has potential as a bioenergy feedstock for several conversion processes. Dried *Arundo* has an estimated direct combustion High Heating Value (HHV) of approximately 8,000 BTUs/lb. *Arundo* is also being considered as a candidate for gasification and cellulosic ethanol conversion (18).

The costs associated with the cultivation of *A. donax* should be lower than those of sugarcane mainly due to lower water requirements. The need for pesticides and fertilizers could also be considerably lower (18).

Giant reed (*A. donax* L.) is a perennial rhizomatous grass that produces considerable amounts of lignocellulosic biomass, traditionally used to obtain paper pulp, building materials, and training sticks for orchards and horticultural crops (19). Giant reed is an ideal plant for dealing with extreme situations of soil conditions and water availability: it can grow in all types of soils, ranging from heavy clay to loose sands and gravelly soil, and it also tolerates high salinity; it prefers abundant water supply but it can withstand extended periods of severe drought (20). Therefore, giant reed is well suited to exploit marginal lands (e.g., wetlands, riparian slopes) poorly or totally unsuited for grain crops' cultivation.

A. donax can reproduce sexually through seeds but it also propagates vegetatively by means of rhizomes. Although seeds are not ideal as planting material due to the variability that they generate, at present there are no viable alternatives for establishing plantations. Rhizomes, on the other hand, are advantageous for efficient multiplication of the trees (they may even pose an environmental problem because of their aggressive growth which can be difficult to control).

Once a field is planted, it can be harvested repeatedly since the rhizomes in the ground will generate new plants all the time. However, if originally planted from seeds, the plantation will continue to show a high degree of variability in the quality of its trees. This is where the use of selected cloned material from tissue culture becomes useful.

The energetic advantages and environmental disadvantages that might be associated with this crop and processes are beyond

the scope of this paper but need to be addressed before plantations are established.

Here, we describe a simple and efficient method to rapidly propagate *A. donax* in continuous liquid culture medium from the axillary buds present in the knots of the stems of plants selected for their size and speed of growth.

2. Materials

All solutions are prepared with distilled water and analytical grade reagents are recommended in all cases.

2.1. Disinfesting Solutions

1. Extrán® (EX1007-4, MERCK) diluted to 10% (v/v) with distilled water.
2. Tween® 20 (P2287 Sigma-Aldrich) at 5%.
3. Sodium hypochlorite (commercial bleach) 10% (v/v).

2.2. Culture Media

1. MS® (21), culture medium (M5524, Sigma-Aldrich) 4.3 g/L, supplemented with:
2. Gamborg B5® (22) vitamins (G1019, Sigma-Aldrich), 1 mL/L.
3. Sucrose (21700, BD Bioxon), 30 g/L.
4. 5 mL/L PPM® (071806-1, Plant Cell technology).
5. Indole acetic acid (I2886 Sigma-Aldrich), 3 mL/L.
6. Kinetin (6-furfurylaminopurine), 3 mL/L (K0753, Sigma-Aldrich).
7. pH: 5.8.

2.3. Accessories

Beaker, 1 L.
Erlenmeyer flask, 250 mL.
Volumetric flask, 250 mL and 1,000 mL.
Magnetic stirrer.
Graduated test tube, 100 mL, 1,000 mL.
Aluminum paper.
Scalpel.
Rubber bands.
Kleen pack.
Stainless steel Tweezers of 18 and 25 cm.

2.4. Basic Equipment

Autoclave SU-MI® (Corporation SU-MI®).
Airflow cabinet NOVATECH® (CFLH-120).
Potentiometer Beckman Coulter® (pHi510).
Hot stirred plate IKA® (CMAG HS7).

Micropipet FINNPIPETE® of 250, 1,000 mL.

Analytical balance OHAUS® (PA114).

Sterilizer Steri 250 (Keller®).

3. Methods

3.1. Selection of Elite Plant Material

Plants collected from the wild or other gene banks are established in a nursery, where their performance can be assessed and compared before they are selected for *in vitro* culture. Plants are selected for their vigor and rapid growth. Only plants that have reached a minimum height of 6 m have stems 4–5 cm in diameter and, at least, ten lateral branches are used as mother plants for the establishment of clones (see Note 1). The plants must not show any symptoms of fungal or bacterial infections (Fig. 1a–d).

3.2. Disinfestation of Plant Material

1. The lateral stems of selected plants are cut into 10–12-cm-long segments containing four to five knots (Fig. 1g) with one axillary bud each, and are placed in a 10% Extran solution for 10 min. (see Note 2), followed by six washes with distilled water (see Note 3).
2. The stems are then placed in a solution of 5% Tween 20® for 15 min (see Note 4) and washed again six times with distilled water to remove all the detergent from the tissues.
3. The stems are moved to a sterile environment in an airflow cabinet, treated with 10% sodium hypochlorite for 10 min, and then rinsed three times with sterile distilled water (see Note 5).
4. The stems are kept in sterile distilled water until they are used as a source of explants for tissue culture (Fig. 1h).
5. This disinfestation method eliminates all microbial contaminants in the tissues.

3.3. Isolation of Explants

1. The knots from the stems are individualized by cutting them into 1-cm sections with the knot in the middle. The cuttings are made with a scalpel under axenic conditions in an airflow cabinet.
2. All tissues damaged during the disinfestation procedures are removed as are the remnants of cortex and leaves covering the buds in the knots (see Note 6).
3. Ten axillary buds with stems are aseptically cultured in Erlenmeyer flasks on media containing 50 mL of MS culture medium (see Note 7).

3.4. In Vitro Multiplication and Growth

1. The Erlenmeyer flasks are incubated in a growth room at a temperature of $25 \pm 2^\circ\text{C}$, and a light intensity of $40 \mu\text{mol}/\text{m}^2 \text{ s}$ under a 16-h photoperiod.



Fig. 1. (a) shows the plant selected for cloning; (b) the main stem with several lateral branches; in (c), the apical region of the lateral branches is cut off from the main stem of the mother plant; (d)–(i) show the processing of the lateral stems in the laboratory: (d) shows full lateral stems, (e) the removal of the leaves, (f) the exposed lateral buds; (g) cutting the knots which are disinfested and kept in sterile water (h). (j)–(m) show the development and multiplication of new stems from lateral buds.

2. After 4 weeks, the axillary buds in the knots sprouted forming a whole plant with shoots and roots and at least two new adventitious shoots, as illustrated in Fig. 11 (see Note 8).
3. The plantlets, still attached to the explant stem, are transferred to fresh medium for another 4 weeks in order to allow them to grow before transplanting them to soil.
4. After two transfers, equivalent to 8 weeks in culture, the vitro plants reach a minimum height of 7 cm and develop a good rooting system (Fig. 1m).
5. The plants are now separated from the explant stem and individualized by cutting them with a scalpel and transferring them to polystyrene trays, as indicated below.

3.5. Further Multiplication

1. The *in vitro*-produced plants can be used as a source of new explants to increase the biomass.
2. For this purpose, the roots are removed and the shoots incubated again in the same culture medium.

3.6. Acclimatization Under Greenhouse Conditions

1. Plants that reach a minimum of 7 cm height and develop a good rooting system are transplanted to polystyrene trays filled with a mixture of agrolite and soil 1:1, and incubated in a greenhouse for a period of 4 weeks after which they can be transplanted to soil.
2. The plants are maintained in a fairly high relative humidity during the first 14 days and the trays are covered with a plastic top.
3. Figure 1 illustrates the whole protocol of induction and multiplication.

3.7. Efficiency of the Propagation System

1. A single mother plant with ten lateral branches yields 100 knots. On average, each knot will generate three whole plants after 8 weeks of *in vitro* culture.
2. Considering up until the transfer to soil, survival is close to 100% which implies that a clonal line of 300 plants, from a single mother plant, can be produced in 3 months.
3. If the new plants are used as explants for a new round of replication, this figure will be increased to 900 clonal plants.

4. Notes

1. The selected elite plants must have a minimum of ten 20–25-cm-long lateral branches. This is not only an indicator of vigor, but also the source of the knots that will be used as explants for the production of clonal lines.

2. Use a soft brush to remove as much soil and tissue as possible from the stems before chemical disinfection.
3. Several washes with distilled water are recommended (six times) since it is very important to remove all the detergent from the explants.
4. The volume of Tween 20[®] depends on the number of stems to be treated. We recommend at least 1 L of a 5% solution for every 50 explants. Maintain under continuous agitation for 15 min and then wash thoroughly as before with distilled water (see Note 3).
5. Repeat the hypochlorite wash at least three times and afterwards keep the stems immersed in sterile distilled water until use.
6. After disinfection, it is important to remove all the visibly damaged tissue since its presence reduces the development of new shoots and increases the possibility of microbial contamination.
7. The MS[®] medium is supplemented with 1 mL/L of Gamborg[®] vitamins, 30 g/L sucrose, 3 mL/L AIA (1 mg/mL), and 3 mL/L Kinetin (1 mg/mL). The pH is adjusted to 5.8 before it is sterilized in an autoclave at 120°C for 15 min.
8. The optimum response expected is the development of all the buds present in the knots (one per explant) developing to a height of 5 cm and the presence of at least four roots of 1–2 cm long that develop at the base 4 weeks after induction.

The present protocol makes it possible to produce 900 clonal plantlets from a single mother plant in a period of 4 months. The plantlets are whole plants with shoots and a well-developed root system which have a very high survival rate when transferred to soil.

5. Conclusion

This protocol has advantages over other reported protocols (23). With this protocol, it is possible to produce three sprouts per explant after 4 weeks. These sprouts are converted in plants with a very good radical system.

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Chapter 13

Production of Haploids and Doubled Haploids in Maize

Vanessa Prigge and Albrecht E. Melchinger

Abstract

The in vivo haploid induction approach offers several advantages compared to the in vitro induction approach and recurrent self-pollination. It is currently the method of choice for inbred line development in many commercial maize breeding programs. Here, we describe the in vivo approach for generation of maternal doubled haploids (DHs). It involves four steps: (1) induction of haploidy by pollinating source germplasm with pollen of a haploid inducer; (2) identification of putative haploid seeds (seeds with a haploid embryo) using a seed coloration marker system; (3) doubling of chromosomes of putative haploids by treating seedlings with a mitotic inhibitor; and (4) verification of putative doubled haploids with a stalk color marker and self-pollination of true doubled haploid plants to multiply their seed.

Key words: Doubled haploid, Chromosome doubling, Colchicine, In vivo haploid induction, Haploid, Maize

1. Introduction

Maize (*Zea mays* L.) is a cross-pollinated crop of major relevance for global food production. It currently ranks first in terms of grain production and third, after rice and wheat, in terms of the cultivated area (1). In the past 50 years, hybrid varieties have greatly helped boosting maize production around the world. Hybrid varieties are developed by crossing homozygous inbred lines as parents. Inbred lines are commonly derived from elite source germplasm, such as crosses among two or multiple elite lines. Traditionally, inbred lines have been developed by recurrent self-pollination of source germplasm for six to eight generations.

In recent years, the doubled haploid (DH) technology was established and has been adopted by many maize breeding programs. It enables the development of completely homozygous lines in less than half the time required traditionally. The technology involves

the induction of haploidy and subsequent chromosome doubling of haploids. The induction of haploidy can be achieved *in vitro* (2) or *in vivo* (3). A major advantage of the *in vivo* over the *in vitro* approach is that it is more widely applicable because it does not require the source germplasm to be responsive to tissue culture (for review, see 4). Moreover, the involved processes are simpler to carry out and require only low-cost laboratory facilities.

In both methods, heterozygous source germplasm, such as plants from crosses between two or multiple elite inbred parents within heterotic groups, forms the basis for developing new DH lines. The following four steps are specific to the *in vivo* haploid induction approach (Fig. 1). First, maternal haploidy is induced by pollinating the source germplasm with pollen from a haploid inducer genotype (3). For production of paternal haploids specific inducers are used as female parent (5). Second, a suitable haploid identification system is employed for distinguishing putative haploid seeds (seeds with haploid embryo) from those with regular

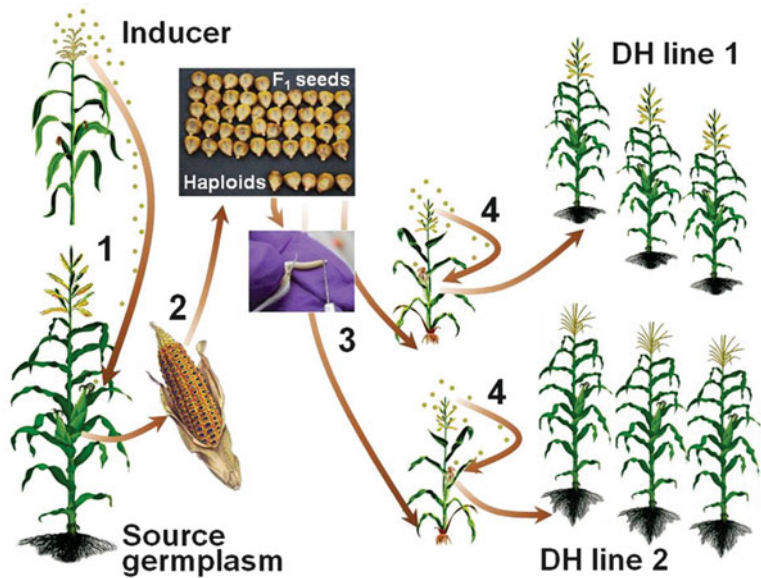


Fig. 1. Schematic description of doubled haploid (DH) line development with the *in vivo* haploid induction approach. (1) Haploidy is induced by pollinating the source germplasm with pollen from a haploid inducer genotype. (2) The pollinated ears of the source germplasm are harvested and a seed marker system is employed for identification of the putative haploid seeds. (3) The haploid seeds are germinated and, after cutting 2 mm off the tip of the coleoptile with a razor blade (see Fig. 3), are treated with mitotic inhibitors. Subsequently, the seedlings are transplanted to the field to produce DH plants. (4) DH plants are self-pollinated to produce seeds for maintenance and multiplication of the DH line.

diploid embryo. Third, these haploid seeds are treated with mitotic inhibitors to artificially double their chromosomes and produce doubled haploid plants. Spontaneous pollen shedding and/or female fertility has been observed in haploids of maize (appearance of partially fertile tassel sectors and/or silks), but was reported to occur at low frequencies (see, e.g., 9–11). Hence, chromosome doubling has to be done artificially for large-scale production of DH lines. Fourth, putative DH plants are confirmed using a stalk color marker and true DH plants are self-pollinated to produce DH line seed for further use in breeding and maintenance. In short, the key to successful DH line production comprises the availability of (1) a haploid inducer genotype, (2) a haploid identification system, (3) an artificial chromosome doubling procedure, and (4) suitable facilities to raise treated plants for maintenance and seed multiplication. The steps for producing maternal haploids and DH lines using the *in vivo* haploid induction approach are described in detail in the following sections. An accompanying video is available free of charge online (6).

2. Materials

2.1. Induction of Haploidy

- (a) Haploid inducer genotype (see Note 1).
- (b) Shoot bags (glassine bags, approx. 10 × 20 cm).
- (c) Pollination bags (paper bags, Lawson No. 502).
- (d) Field or greenhouse space.
- (e) Stapler.

2.2. Identification of Seeds with Haploid Embryo

This step requires containers (paper envelopes, cotton sacks, or metal containers) to separate haploids from diploids, as well as labels to identify the respective source germplasm until the initiation of the next step.

2.3. Artificial Chromosome Doubling

1. Germination trays with filter paper inlay; germination chamber.
2. 0.06% colchicine solution: Colchicine (100% purity, HPLC), DMSO, deionized water (see Note 2).
3. Container for conducting the seedling treatment (see Note 3).
4. Containers for storage of residual waste.
5. Personal protective equipment (see Note 4).
6. Mesh bags (see Note 5) and labels (see Note 6).
7. Scalpel or razor blades.
8. Pots with sterilized soil (see Note 7).
9. Greenhouse and field space.

2.4. Self-Pollination of Treated Haploids for Production of DH Lines

1. Shoot bags (as under Subheading 2.1).
2. Custom-made glassine bags (see Note 8) and common pollination bags (paper bags, Lawson No. 501).
3. Greenhouse or field space.

3. Methods

3.1. Induction of Haploidy

Induction of haploidy is carried out in the field or greenhouse. Plants of the haploid inducer and of the source germplasm are grown under conditions that allow plant growth, pollination, and maturity of seeds. The planting dates of inducer and source germplasm should be adjusted to synchronize pollen shedding of the inducer with silking of the source germplasm (see Note 9). Tassels of all source germplasm plants should be removed to avoid pollen contamination during pollinations with the inducer.

1. Before silk emergence, cover ear shoots of source germplasm with shoot bags to avoid uncontrolled pollinations.
2. During anthesis, cover tassels of inducer plants in time (e.g., previous evening) with pollination bags to avoid contamination of their pollen with random pollen and use that same bag to collect pollen (see Notes 10 and 11).
3. Pollinate plants of the source germplasm (see Note 12).
4. Cover pollinated ears with pollination bags and fasten these tightly around the pollinated ear and stalk, e.g., with a stapler.
5. Harvest cross-pollinated ears of source germplasm after they have reached physiological maturity, subject them to postharvest insecticide treatment, dry them down to storage moisture content, and shell them (see Note 13).

For large-scale in vivo haploid induction, grow inducers and source germplasm in alternate rows in an isolated block (the number of rows of inducer and source germplasm depends on the inducer's pollen shedding ability) and detassel all plants of the source germplasm so that these are pollinated by wind with pollen of the inducer. This eliminates manual pollinations.

3.2. Identification of Seeds with Haploid Embryo

In this step, seeds with a haploid embryo are visually separated from those with diploid embryo using the *RI-nj* marker system which is carried by the inducers. The haploid seeds have unpigmented (haploid) embryo and purple-colored (triploid) endosperm (Fig. 2a), whereas normal F_1 seeds have a purple-colored (diploid) embryo and a purple-colored (triploid) endosperm (Fig. 2b). Further, completely unpigmented seeds (Fig. 2c) may be present at very low frequency due to reasons discussed below.

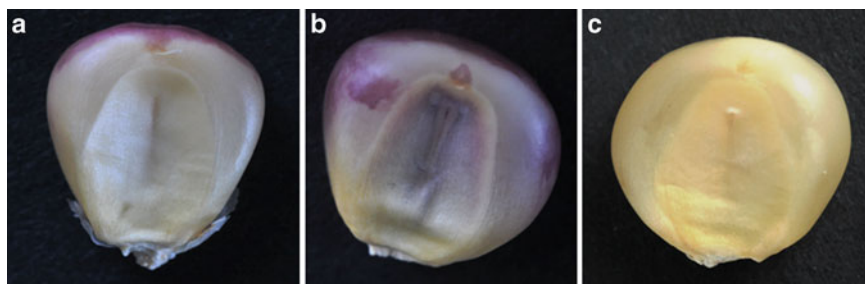


Fig. 2. Seed categories distinguished by the *R1-nj* color marker system. (a) Haploid seed resulting from haploid induction with a purple aleurone (regular triploid endosperm) and a colorless scutellum (haploid maternal embryo). (b) Regular F1 seed displaying pigmentation of both aleurone and scutellum. (c) Seed resulting from color inhibition or (unintended) out-crossing with other colorless source germplasm showing coloration of neither the aleurone nor the scutellum. Photos by X. Fonseca (CIMMYT).

1. Visually score shelled seeds for purple coloration to identify seeds with haploid embryos: a purple-colored endosperm (see Note 14) and a colorless embryo indicate a putative haploid seed (see Note 15). If no purple coloration is visible on the seeds, color inhibition may have occurred (see Note 16). If coloration is present neither on the endosperms nor on the embryos, no haploids can be identified from this source germplasm with the *R1-nj* color marker (see Note 17).
2. Select seeds with haploid embryo and discard the rest.

Haploids have now been generated and the following two steps are only required if the aim is to produce doubled haploids.

3.3. Artificial Chromosome Doubling

Haploid seedlings are weak and very sensitive to stress imposed by colchicine treatment and transplanting. Therefore, they must be handled very carefully.

1. Germinate haploid seeds in labeled (see Note 6) germination trays with wet filter-paper inlay (see Notes 18 and 19) under controlled conditions in darkness (see Note 20) with adequate moisture supply (see Note 21).
2. When coleoptiles of the seedlings are about 2 cm long, prepare seedlings for colchicine treatment as follows (7, 8): take each seedling individually from the tray, cut off a few millimeters of the tip of its coleoptile with a scalpel or razor blade (Fig. 3; see Note 22), and put seedling into a mesh bag (see Note 5) labeled with the corresponding identification number. Avoid drying of seedlings during this step (see Note 23).
3. Place mesh bags into colchicine treatment container.
4. Fill the colchicine solution into the treatment container until all seedlings are well covered. It is compulsory to use personal protective equipment (see Note 4) for all steps involving colchicine! See Note 24 if the addition of chemical is automated.



Fig. 3. Approximately 2 mm from the tip of the coleoptile of each germinated haploid seedling is cut off before colchicine treatment.

5. Keep seedlings submerged in colchicine solutions for 8 h at room temperature (approx. 20°C).
6. After the treatment, drain the colchicine solution from the treatment container and collect it in specially designated residual waste containers. Rinse treated seedlings with tap water three times to remove residual colchicine. For this, fill the treatment container with tap water until all seedlings are fully submerged, then drain, and collect the wastewater (see Note 25).
7. Fill pots with sterilized soil and transfer treated seedlings into pots (see Notes 7 and 26). Protective gloves should be worn when handling colchicine-treated seedlings.
8. Place the potted seedlings in the greenhouse for approximately 10 days so that they recover from the colchicine treatment and grow to the three- or four-leaf stage (see Note 27). During this period, maintain conditions favorable for seedling growth (see Note 28).

Repeat Steps 2–8 for three consecutive days if germination of the haploid seeds is not uniform. Thus, seedlings with delayed germination attain the appropriate growth stage for treatment.

3.4. Self-Pollination of Treated Haploids for Production of DH Lines

In this final step, the putative doubled haploid plants are transplanted and self-pollinated to maintain and multiply seeds of the new DH lines. Usually, colchicine treatment does not lead to uniform or complete doubling of the chromosomes of all cells of a seedling. The effect may be variable, especially on the tassel: some plants may have tassels producing abundant pollen, while, in most instances, tassels will have limited pollen-producing anthers or none at all. Consequently, self-pollination might be extremely difficult and, therefore, well-trained staff is required to avoid losing such genotypes due to unsuccessful self-pollination.

1. Transplant seedlings to the field or into bigger pots in which plants can reach maturity (see Note 29).

2. Follow standard crop cultivation practices, including reliable irrigation, effective weed and disease control, and appropriate fertilization, to provide favorable growing conditions to putative DH plants (see Note 30).
3. Monitor coloration of the stalk of putative DH plants before flowering time and eliminate plants which show purple stalk coloration. These had been incorrectly classified, i.e. they originate from regular F_1 seeds.
4. Cover ear shoots (female flowers) before silk emergence.
5. Monitor silk and anther emergence, cover tassels with pollination bags in time before the intended pollination, and try to self-pollinate each plant (see Note 31). Cover pollinated ears with pollination bags for protection and fasten them tightly with stapler.
6. Harvest self-pollinated ears after they have reached physiological maturity (see Notes 32 and 33).

4. Notes

1. Commonly used genotypes for in vivo induction of maternal haploids in maize are the inbreds RWS, UH400, RWK-76, and UH402, all developed at the University of Hohenheim, Germany, and their single crosses (3, 4, 12). The inducers are adapted to the temperate climate of Central Europe but have also successfully been employed under tropical conditions to induce haploidy in tropical source germplasm (3, 12). The inducers have an average haploid induction rate of 8–12% (3, 12). They carry the dominant marker gene *R1-nj* whose phenotypic expression is a purple coloration of the scutellum and the aleurone of seeds (13, 14), which can be used as embryo and endosperm markers, respectively, to identify putative haploid seeds. Additionally, both inducers carry a dominant purple stalk marker which, in the late-seedling stage, enables the detection of “false positives” among putative haploid plants.
2. For 1 L of 0.06% colchicine solution, dilute 0.6 g of colchicine powder in 995 mL of deionized water and add 5 mL of DMSO. Mix solution for about 15 min on a thermolyne mixer. Follow standard safety procedures as outlined in the material safety data sheet provided by the supplier of the colchicine powder. Prepare the required amount of solution, store it under cool (4°C) and dark conditions, and use it within a week.
3. Common laboratory glass flasks or 10–20 L covered plastic buckets can be used for colchicine treatment on a smaller scale.

A custom-made stainless steel tank equipped with electric pump and timer is suitable for a large-scale application. When such a tank is used, staffs hardly have any contact with the chemical because the filling of the tank starts and stops automatically, e.g., during the night.

4. Persons involved in colchicine treatment must wear respiratory masks, laboratory safety glasses, gloves, and laboratory coats.
5. Mesh bags are cheap and their flexible material allows adjustments to the space required for different quantities of seedlings. Alternatively, perforated stainless steel containers may be used with the disadvantage that they cannot be adjusted to the volume needed.
6. Adhesive tapes or plastic labels may be used for labeling. The former are pasted onto germination trays and the latter are placed into the germination trays. Plastic labels have the advantage that they can also be used for the next step.
7. Various types of pots can be used: (1) typical plastic pots (approx. $5 \times 5 \times 8$ cm) commonly used for greenhouse experiments and horticulture that are made of durable plastic and can be reused; (2) styrofoam cups, more commonly used for coffee or tea, which are very cheap but less durable; or (3) pots made of biodegradable material that decays in the soil allowing transplanting of seedlings along with pots and thereby enabling the use of a planting machine. All pots must have perforated bottoms to allow for drainage of excess water.
8. Noncoated, transparent glassine bags (size approx. 6×20 cm) are most suitable to collect pollen from putative doubled haploid plants for self-pollination. As pollen production is often limited in these plants, the transparent bags allow visual assessment of the quantity of pollen collected. If necessary, the pollination can be repeated with fresh pollen the next day. After successful pollination, common pollination bags can be used to cover and protect the pollinated ear.
9. Staggered planting may be necessary if anthesis of inducers and silking of source germplasm do not synchronize.
10. Follow agronomic practices that help maximizing pollen production of inducers, such as timely sowing and optimal and timely application of input such as irrigation, fertilizer, herbicides, and pesticides. Shading with nets can be very helpful during anthesis, particularly when temperature is abnormally high.
11. Using a single cross between two inducer inbreds not only increases the amount of pollen produced, but also increases the time span of anthesis.
12. To maximize seed production on the ears of source germplasm, pollinate at appropriate time, i.e., when the majority of the silks have emerged.

13. If several source germplasm are induced simultaneously, as is usually the case in maize breeding programs, make sure that ears of different source germplasm are shelled and kept separately and clearly labeled for future handling.
14. Leftover silk attachments on the pericarp of seeds, visible as tiny colored spots at the point where the silks were attached to the developing seed, are often misleadingly perceived as purple coloration, especially by untrained staff.
15. Some seeds will not have developed embryos and, therefore, appear colorless in the embryo region. Screen seeds carefully to make sure that all selected putative haploids actually contain an embryo.
16. The expression of purple coloration governed by the *RI-nj* marker gene is completely inhibited in the presence of dominant antocyanine inhibitor gene(s) in the source germplasm (for review, see 4). Hence, no haploid identification is possible with the *RI-nj* color marker in such source germplasm. Further, unsuccessful pollination of the source germplasm with inducer pollen due to the use of nonviable pollen (caused by delayed pollen collection or pollen desiccation due to high temperature or other factors) and contamination due to pollination with pollen from other nonpigmented genotypes may result in colorless seeds. In this case, repeat the cross and ensure optimal pollination conditions.
17. Other haploid identification systems include purple root coloration (3) or embryo oil content (15). In both cases, the haploid inducer must display the trait to successfully identify seeds with haploid embryo.
18. Other germination procedures may also be used depending on the facilities available in a specific laboratory. Fan-folded filter paper as germination tray inlay is very useful because (1) seeds can be placed in an organized manner along the filter paper furrows and this facilitates easy separation of germinated seedlings from the tray later on and (2) it absorbs water and remains moist for a long time while excess water within the furrows can be avoided easily.
19. Standard fungicide treatment of seeds is recommended to increase the rate of germination.
20. If germination chambers are available, a day and night temperature of 26°C in complete darkness is optimal. Under these conditions, seeds need approximately 72 h to grow and attain a suitable coleoptile length. If such facilities are not available or temperature within a chamber is regulated by light bulbs, good germination success can be obtained by covering germination trays with slightly perforated aluminum foil to simulate darkness. Furthermore, large amplitudes between day and night

temperatures can be tolerated by maize seeds for germination (which is important when several lab groups share a germination chamber and have different temperature requirements), but seedlings may need more time to reach the suitable coleoptile length than under optimal germination conditions.

21. Check germination trays daily for adequate moisture content. The filter paper should be wet but the application of excess water must be avoided. Air humidity seems to play a minor role during germination when trays are covered with perforated aluminum foil.
22. Take utmost care while handling the seedlings. The coleoptile is a very vulnerable tissue. Therefore, handle seedlings by holding the kernel and do not touch the root or coleoptile to avoid damage. Damage to the tissue during preparation of seedlings for treatment and/or during the subsequent transplanting of treated seedlings often leads to necrotic shoot tissue and subsequent seedling death.
23. When preparing large numbers of seedlings from one or more source germplasm simultaneously, keep bags of already prepared seedlings in a moist place (e.g., a covered plastic container with wet paper inlay).
24. To minimize personal contact with colchicine and/or conduct treatment during non-office hours, an electric pump and timer may be used for automatic filling of the colchicine treatment container. In this case, it is crucial to beforehand estimate the amount of colchicine solution required. This can be done by filling the container with tap water until all seedlings are well submerged, draining the water from the container into a measuring device, and determining the quantity. Then, prepare the same quantity of colchicine solution, fill it into its designated container, and place this container next to the treatment container. Finally, install the pump connecting the colchicine and the treatment containers and set the timer.
25. All colchicine residuals must be collected in specially designated and clearly labeled container(s) and disposed of by an authorized company/agency (follow the regulations for disposal of dangerous chemical wastes applicable in your specific country and/or your institution!). Under no circumstances should these toxic residuals be disposed of through the common sink!
26. For planting seedlings into pots, first fill each pot about half with soil. Then, carefully place seedling (holding the attached seed rather than the shoot or root) onto the soil and hold it while filling more soil around it until the pot is well filled and only the tip of the coleoptile is visible. Gently push to make soil compact and prevent soil runoff during watering.

27. Sometimes, deformed seedlings appear whose shoots are not able to emerge fully from clasping cotyledons. In spite of this, shoots and leaves continue to grow and, thus, the shoot is strongly bent downwards and its development is inhibited. Use fine scissors to cut the shoot free from the clasping cotyledon to facilitate normal development of the seedling.
28. Keep soil moist but avoid excess water. Apply the required dose of fertilizer in a soluble form with the irrigation water.
29. Field transplanting can be done manually or with a transplanter (e.g., a common planting machine for vegetable plantlets), especially for large-scale applications.
30. Drip irrigation is particularly suitable. Plastic foils covering the ground suppress the growth of weeds. Some transplanters are equipped with specific devices that automatically lay out foil and then transplant seedlings directly into foil-covered soil. Use shading nets to reduce temperature and radiation stress to the seedlings.
31. Well-trained field staff is crucial for successful pollination. Treated haploid plants are weak, often have only a few pollen-shedding anthers, and may only shed pollen for a day. Hence, the staff must be trained to spot plants shedding pollen (among the many plants which do not have fertile tassels), immediately collect pollen, and perform the self-pollination. It should be noted that the DH genotype is lost if self-pollination is not taken seriously and, consequently, no seed is obtained, even though all previous steps may have been performed perfectly.
32. Often, only few seeds are produced by a DH plant. Therefore, utmost care is needed to avoid seed loss in the field. The ears should be harvested carefully and kept in their cover bags during transport to the warehouse for dehusking and drying. They represent the newly developed, completely homozygous DH line which must be planted again for seed multiplication to be able to use the DH line in further research and breeding activities. Seed production on DH plants is expected to improve in subsequent cycles of DH production because (1) selection is expected to occur in source germplasm for genes imparting favorable response to haploid induction and artificial chromosome doubling and (2) the persons involved gain experience. Hence, the more frequently source germplasm passes through the haploid induction and artificial chromosome doubling processes, the higher will be the success rate in further breeding cycles.
33. If there are some ears bearing purple-colored seeds, discard these because they do not originate from haploid seeds.

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Maize Somatic Embryogenesis: Recent Features to Improve Plant Regeneration

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Abstract

Plant regeneration capacity is maintained through the life of a plant by the stem cell niche present in the meristems. Stem cells are capable of differentiating into any plant organ, allowing propagation of new plants by different techniques. Among them, somatic embryogenesis is a widely used technique characterized by a complex process that involves coordinated expression of genes, mediated by the influence of specific hormones, nutrients, stress, and/or environmental signals. This tool is particularly relevant in the propagation of genetically improved crops. The intrinsic embryogenic potential of the explant used as starting material for plant in vitro cultures varies depending on the genotype of each plant species. Particularly in maize, the regeneration capacity is lost during the course of tissue maturation, since embryogenic callus (E) is almost exclusively obtained from immature zygotic embryos. In this chapter, the latest advances in the literature for maize somatic embryogenesis process are reviewed. Further, a detailed procedure for maize plant regeneration from E callus is described. The callus obtained from immature zygotic embryos is capable to generate somatic embryos that germinate and develop into fertile normal plants.

Key words: Auxins, Culture medium, Embryogenic callus, Plant regeneration, Somatic embryos

1. Introduction

Maize is a major crop spread worldwide required for human and animal feed. Recently, it has also been used to obtain biocombustibles such as ethanol and biodiesel (1). Maize's importance also resides in its great genotype variety, since this crop has been adapted to many different environments in the world. Indeed, Mesoamerica and particularly Mexico are the center of origin of this crop, where populations of Teocintle, the ancestor of maize, are still found in several locations (2). Further, most cultivated genotypes actually are originated from germoplasm generated and cultivated for centuries in México. Traditional breeding techniques have been

widely used to generate high yielded crops, and recently, plant transformation technologies have become the strategy to increase crop production particularly in maize. This crop has been one of the most targeted materials used to generate transgenic cultivars directed for basic as well as applied purposes. Indeed, plant somatic embryogenesis is a useful tool to propagate transgenic organisms and actually almost any attempt to obtain genetically modified plants requires a somatic embryogenesis protocol. Since somatic embryogenesis implies the establishment of a specific genetic program for plant regeneration from cell cultures, it constitutes as well a potential model tool for basic studies on the early regulatory and morphogenetic events during the embryogenic process (3–5). In this regenerating model, somatic embryos are initiated from non-gametic somatic cells in tissue culture, which later resemble their zygotic counterpart in morphology and to a substantial degree in gene expression. The embryos have a single cell origin, although usually they develop from multicellular proembryogenic cell masses (6), allowing to obtain plants that conserve the genotype within the transformed organisms. On the other hand, even though somatic embryogenesis has many advantages, it also presents with some limitations, since every plant species that has been propagated through this technique has shown a variable degree of competence, restricted by the tissue type and developmental phase of the explant and most importantly by the plant genotype (7–10). Maize is a typical example of this behavior where the type of explant as well as the species genetic background plays a determinant role in successfully establishing embryogenic cultures. Indeed, immature embryos, harvested within a period between 12 and 18 days after pollination, are the explants traditionally used to establish maize embryogenic cultures, since the calli obtained from these tissues show high embryo regeneration efficiency (7, 8, 11–14). Nevertheless, recent reports on maize propagation through organogenic and embryogenic systems have selected other plant tissues ranging from pollen (15) or pollinated ovaries (16) to obtain haploid plants for breeding purposes, to nodal regions (17) and shoot meristems (18, 19). Maize embryogenic cultures characteristically show two forms of calli: calli type I and type II that differ mainly in their regeneration efficiency (for a detailed description of each calli see ref. 7). Type II callus is the most used to generate somatic embryos, since it maintains the embryo regeneration capacity on in vitro culture beyond 2 years. In regard to plant genotype, the vast majority of maize embryogenesis systems used to improve regeneration have been from the line denominated Hi II, obtained from the cross of inbred lines A188 × B73 (20) due to its high embryo regeneration frequency of around 80% (21). The Hi II line is a cultivar from temperate climate conditions.

Recently, other genotypes, mainly of Mexican germoplasm background from tropical and subtropical climate varieties, have been subjected to regeneration through somatic embryogenesis as well, with efficient regeneration capacity (12, 14, 20, Garrocho-Villegas et al. unpublished data). This material offers the possibility of propagating maize landraces and inbred lines adapted to particular environmental conditions in countries with complex and varied climate such as Mexico, or those from Asia and Africa continents.

Since the first report of maize regeneration by somatic embryogenesis (22), several improvements to the original method have been established. These improvements are directed to recover or increase plant regeneration capacity, through the addition of polyethyleneglycol to culture medium (21), or by a short callus desiccation period (48 h) (23). Also, in regard to the culture medium formulation, the vitamins from the N6 medium (24) have been substituted by those from modified B5 medium (25) or a combination of nitrogen source and vitamins, named cocktail 20, has also shown to increase embryo regeneration efficiency (26). Further basic research has been developed, directed to study the molecular mechanisms involved in embryo development (25), as well as to unravel gene expression in embryogenesis and germination processes, that ranges from global perspective through microarrays technology (27, Garrocho-Villegas et al. unpublished data), to the role of individual genes (28), or to characterize metabolic profiles of embryogenic callus (29, 30). All this research shares the prospect for identifying key genes that may limit the regeneration process in more recalcitrant lines. Finally, a great number of recent research works have been reported using somatic embryogenesis as support technology for propagation of transgenic plants, obtained through either bioballistic methods (31–34) or *Agrobacterium* (17, 35) or whiskers transformation (36).

In the present chapter, a detail protocol that has been used for several years in our lab is described. It has proved to be efficient in plant regeneration through somatic embryogenesis using maize immature embryos as explants. The protocol is mainly based on the method described by Armstrong and Green (7) with some modifications that are described in detail. This protocol has been used as a tool to obtain embryogenic callus for basic (Garrocho-Villegas et al. unpublished data) as well as applied research endorsed to obtain plants with improved crop yield. The source of plant material used in this example is the Mexican Tuxpeño germplasm, relevant for being one of the three maize landraces from which many commercial hybrids with worldwide distributions have originated. Moreover, it has been proved to have a remarkable adaptation capacity, since seeds from this germoplasm have been successfully introduced to countries all over different continents.

2. Materials

All solutions are prepared using deionized water and analytical grade reagents at room temperature. Store the stocks at 4°C.

2.1. Medium Composition

All media are prepared right before use or might be stored at 4°C for at most 3 weeks.

2.1.1. N6I (Initiation Medium)

N6 salts (24) and vitamins of cocktail 20 (26), 2 mg/L 2,4-D, adenine 10 mg/L, 2.76 g/L proline, 200 mg/L casein hydrolysate, 30 g/L sucrose, and 3.3 g/L gelrite (Sigma-Aldrich); adjust pH to 5.7 before adding gelrite; pour the medium into a flask, cover with an aluminum foil cap, and autoclave at 120°C for 18 min. After autoclaving, the medium is left to cool until the heat of the flask is tolerated by the back of the hand, then it is pour into large petri plates (100×15 mm) within aseptic conditions in a laminar flow bench. The medium is allowed to solidify and stored at 4°C until use.

2.1.2. N6P (Proliferation Medium)

N6 salts (24) and vitamins of cocktail 20 (26), 2 mg/L 2,4-D, 0.1 mg/L kinetin, adenine 10 mg/L, 2.76 g/L proline, 200 mg/L casein hydrolysate, 30 g/L sucrose, adjust pH to 5.7; add 3.3 g/L gelrite, apply low heat until the gelrite has dissolved and then pour approximately 30 mL in culture vessels; cover each vessel with a polypropylene cap and autoclave at 120°C for 18 min. Allow the medium to cool until it reaches room temperature. The medium is ready for use or could be stored for up to 2 weeks at 4°C.

2.1.3. N6R (Maturation and Germination Medium)

N6 salts (24) and vitamins of cocktail 20 (26), 0.05 mg/L kinetin, adenine 10 mg/L, 2.76 g/L proline, 200 mg/L casein hydrolysate, 30 g/L sucrose, 3.3 g/L gelrite, pH 5.7. The medium is prepared and stored as described above.

2.1.4. MSG (Rooting Medium)

MS salts and vitamins (37) 30 g/L sucrose, 3.3 g/L gelrite, pH 5.7. The medium is prepared and stored as describe above.

2.2. Disinfection Solutions

1. Seventy percentage ethanol: Add 700 mL of 100% ethanol to 300 mL of deionized sterilized water (see Note 1).
2. Fifty percentage bleach solution: mix 450 mL of commercial bleach (5.25% hypochlorite) with 450 mL of deionized sterilized water, add two drops of the surfactant Tween-20 (Sigma-Aldrich) and two drops of Triton-X 100 (Sigma-Aldrich), and mix well. Use ~3,000 mL for five ears (see Note 1).
3. Wash solution: Sterile deionized water. Fill two thirds of a flask with deionized water, cover the mouth of the flask with aluminum foil, and autoclave for 30 min at 120°C.

2.3. Plant Material

Ears of inbred Mexican maize (*Zea mays* L.) landrace Tuxpeño. Collect ears from field grown plants of 15–18 days after pollination (see Note 2).

2.3.1. Ears Disinfection

Perform all procedures on a clean flow bench that has been turned on for at least 15 min.

1. Dehusk the ears and divide each into three parts, cutting off the top and bottom. Use only the middle part, since embryos in this part have a similar developmental stage.
2. Place five ears in a 4,000-mL beaker along with a stir bar. Add 70% ethanol solution until all the ears are submerged, cover the beaker with aluminum foil, and place it on a stir plate. Stir at medium speed for 1 min. Decant the ethanol into a liquid-waste container.
3. Rinse the ears by covering with deionized sterilized water and stir for 5 min, and then decant water.
4. Cover the ears with 50% bleach solution, stir for 20 min at medium speed, and then decant the bleach into the liquid-waste container.
5. Rinse the ears three times with deionized sterile water as described before, each wash for a period of 4 min.
6. After the last rinse, drain off water and keep the ears in the beaker covered with aluminum foil; the ears are now ready for embryo dissection to obtain immature embryos.

**2.3.2. Embryo Dissection
for Callus Initiation**

Ears and embryo manipulations should be performed on a sterilized petri plate placed between the flames of two alcohol lamps in the flow bench. Use scalpel blade and clamps previously sterilized in autoclave for 30 min at 120°C (see Note 3).

1. Excise the embryos by inserting the narrow end of a scalpel blade between the endosperm and pericarp, popping the endosperm out of the seed coat to expose the embryo (see Note 4).
2. Place the embryos with the embryo-axis side down (scutellum side up) onto the N6I media, 30 embryos per petri plate (Fig. 1a).
3. Wrap tightly the plate edges with plastic wrap and keep at $25 \pm 2^\circ\text{C}$ in a culture chamber in the dark for 3 weeks (see Note 5).

**2.4. Subculture
in Proliferation
N6P Medium**

1. After 3 weeks, friable, rapidly growing white to yellow embryogenic callus (E) can be seen growing out from the scutellar tissue of the immature zygotic embryo explants (Fig. 1b). Small pieces of E callus are selected and placed on N6P medium very close to each other in the center of the culture vessel.

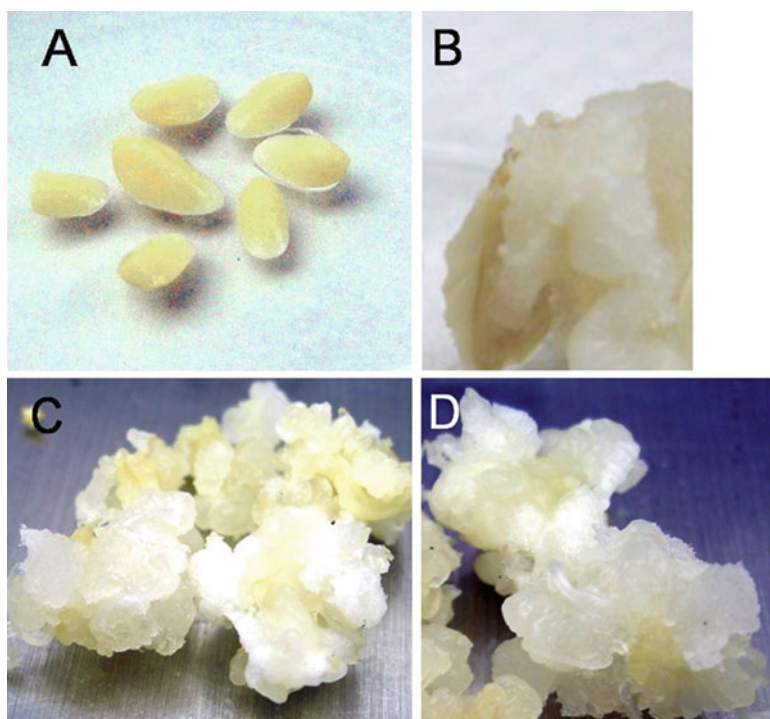


Fig. 1. Embryogenic callus induction and propagation. (a) Immature embryos before callus induction. (b) Friable callus after 3 weeks of culture on induction medium; notice that the callus has originated from the scutellum. (c) Callus cultivated for 3 weeks on propagation medium. (d) Callus morphology after 9 weeks of culture on propagation medium in dark conditions.

Be careful to wrap the polypropylene cap edges with plastic wrap to prevent contamination, and keep the calli under the culture conditions described above. Transfer the calli to fresh N6P medium every 3 weeks (Fig. 1c, d) (see Notes 6 and 7).

2.5. Somatic Embryo Maturation and Germination

1. Transfer small E callus pieces (approximately 5 mm) to medium N6P. The auxin dose in the medium should be lowered to 1 mg/L the first week and 0.5 the second week; in the third week of culture, transfer callus to N6R medium (see Note 8). Cultures are incubated under white fluorescent light photoperiod of 16:8 light/dark and $25 \pm 2^\circ\text{C}$ (see Note 9).
2. After 4 weeks, green callus and germinated embryos are transferred to MSG rooting medium and kept under the same culture conditions (Fig. 2a–d).
3. Transfer plantlets to fresh medium every 3 weeks (Fig. 3a).
4. After 2 months, complete plants are transferred to soil and cultivated in greenhouse (Fig. 3b, c).

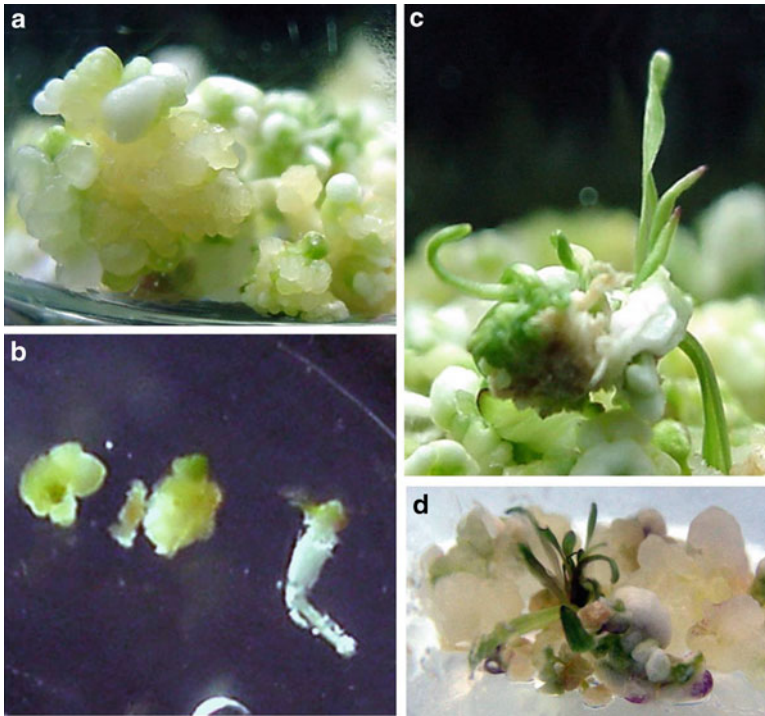


Fig. 2. Maturation and germination of somatic embryos. (a) Globular and scutellar embryos on the surface of the callus after 3 weeks of culture in light/dark photoperiod on N6R medium. (b) Somatic embryos separated from callus mass, representing globular, scutellar, and coleoptilar phases from *left to right*; the last embryo has germinated and root and shoot primordia are visible. (c) Callus after 4 weeks of culture on N6R medium; somatic embryos have germinated and shoots and leaves are visible. (d) Plantlet originated from a somatic embryo growing on the callus.

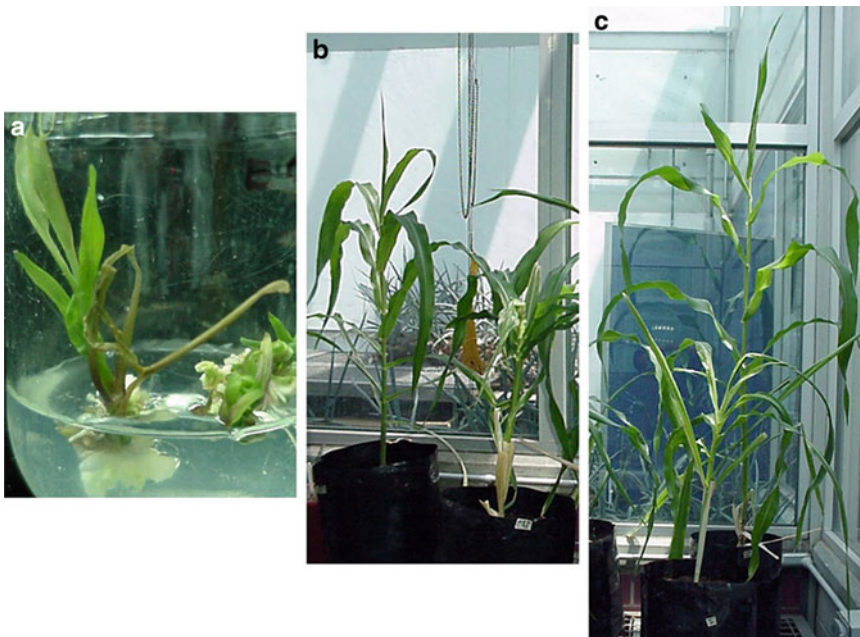


Fig. 3. Growing plantlets and plants. (a) Maize plantlet after 6 weeks of culture on medium MSG; leaves and roots are visible. (b, c) Greenhouse maize plants of 3 and 6 months of culture on soil, respectively.

3. Notes

1. Prepare before use; mix components under aseptic conditions in a flow bench.
2. Immature embryos from 12 to 16 days have been used for several researchers (7–10, 38). However, in our experience, embryos of 15–18 days after pollination produce highly regenerating embryogenic callus. Ears within the husk might be stored in the fridge for up to 2 days.
3. Use 90% ethanol solution to flame utensils (scalpel and clamps) for resterilizing throughout the protocol.
4. Embryos should be placed on a sterile petri plate containing an antibiotic solution of 1 mg/mL of Cefotaxime (Sigma-Aldrich), to prevent contamination by manipulation.
5. Cultures should be observed every 3 or 4 days; if any contamination appears, uncontaminated embryos are rescued and placed on fresh N6I medium.
6. In every subculture, be sure to discard any piece of brown callus and select only yellow to white healthy calli.
7. E callus is maintained with regular subculture on N6P medium for up to 3 years without losing the regeneration capacity.
8. For embryo maturation, absence or a reduced auxin dose and a low cytokinin dose is required to further promote callus differentiation.
9. Green spots should be visible over the callus within the first week of light exposure.

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Improved Shoot Regeneration from Root Explants Using an Absciscic Acid-Containing Medium

Subramanian Paulraj and Edward C. Yeung

Abstract

Arabidopsis thaliana is an excellent experimental system to study various aspects of plant biology. An efficient regeneration protocol of the plant is important to produce in vitro fertile plants, as well as a fundamental step to study the cellular, physiological, and molecular mechanisms of plant development. Here, we describe an efficient and fast regeneration system from root explants of *A. thaliana* ecotypes Columbia-0 and Landsberg *erecta*. Culture conditions and media compositions were optimized for the development of higher number of plantlets. We demonstrate that the combination of the plant regulators abscisic acid and cytokinin in the shoot induction medium increases the formation of shoots as well as reduces the proliferation of abnormal tissues.

Key words: Abscisic acid, *Arabidopsis thaliana*, Auxin, Callus, Cytokinin, Organogenesis, Regeneration, Shoot

1. Introduction

Arabidopsis thaliana is a small flowering plant. It is used as a model system to study different aspects of plant development as it has many desirable features, such as a small genome, a short life cycle, a large number of seeds produced per individual, ease to grow and manipulate, and excellent resources with a large number of mutant lines and genomic resources available from stock centers (1, 2). The genus *Arabidopsis* belongs to the *Brassicaceae* family which includes many important agricultural crop species, such as canola. Hence, besides theoretical interests, the knowledge gained from the study of *Arabidopsis* will have important agricultural implications.

Efficient regeneration protocol of plants through organogenesis is an important prerequisite for crop improvement. Organogenesis is an in vitro process which produces unipolar structure of either shoot or root primordium (3). The success of organized development

through organogenesis is highly dependent on the manipulation of plant hormones, mainly auxin and cytokinin (4). The most commonly used *Arabidopsis* regeneration method is based on Valvekens et al. (5) in which the root explants have been used to produce shoots. There are two important steps in this protocol. First, the root explants are grown in a high auxin/cytokinin ratio medium which produce undifferentiated tissues called callus. In the second step, the calluses produced are differentiated into shoot buds upon transfer to a medium containing low auxin/cytokinin ratio. This simple and efficient method is adapted to study basic developmental processes, such as shoot apical meristem initiation and the role of auxin and cytokinin on meristem gene expression during different developmental stages (6–9). Thus, the *Arabidopsis* root explant system is very useful in the study of plant regeneration processes (10). Though the protocol detailed by Valvekens et al. (5) is highly reproducible and efficient, the problem of abnormal plant structure development is often visible, such as the formation of “finger-like” structures, and the number of plantlets produced is low. Hence, we further optimized the protocol which produces normal functional shoots with few abnormal structures as well as increasing the number of shoots formed. The main modification to the protocol of Valvekens et al. (5) is that the plant growth regulator, abscisic acid (ABA) is added to the shoot induction medium. The inclusion of ABA greatly improves the number of shoots produced when compared to the control treatment in the absence of ABA.

In tissue cultures, ABA has been shown to be involved in many different functions. Mainly, ABA is required for somatic embryo maturation (11). It improves the shoot apical meristem organization and improves the conversion frequencies of somatic and microspore-derived embryos (12–15). Histological sections of ABA-treated samples reveal the presence of dense cytoplasmic cells in the shoot apex and a better organized shoot apical meristem (13, 15).

The current protocol is efficient and applicable to both *Arabidopsis* ecotypes Columbia-0 and Landsberg *erecta*; however, the regeneration frequency is slightly different between these ecotypes. The following organogenesis protocol has four main parts: (a) dedifferentiation of root cells into callus, (b) formation of shoot meristems, (c) conversion of shoot meristems into functional shoots, and (d) rooting of developed shoots.

2. Materials

2.1. Seed Materials

Arabidopsis seeds can be obtained from the Arabidopsis Biological Resource Centre (ABRC) or The Arabidopsis Information Resource (TAIR).

2.2. Seed-Sterilizing Solution

For sterilizing *Arabidopsis* seed, a 30% commercial Javex bleach solution is prepared by mixing with 70% water of required volume.

2.3. Culture Media (See Note 1)

1. Seed germination medium: It consists of 50% strength MS medium (contains both MS salts and vitamins, see ref. 16) with 1% sucrose and 50 mg/L myo-inositol. The pH is adjusted to 5.7. The gelling agent 3 g/L Gelzan™ is added after pH adjustment.
2. Growth regulators and tissue culture chemicals used: 6- γ (dimethyl allyl-amino)-purine (2iP), kinetin, 2,4-dichlorophenoxy-acetic acid (2,4-D), indole acetic acid (IAA), ABA (all plant growth regulators can be obtained from Sigma), Dextrose (VWRinternational, USA), sucrose (EMD, USA), myo-inositol (Sigma), MES buffer (Sigma), Gelzan™ (Sigma).
3. Growth regulator stock solutions: All stock solutions have a concentration of 1 mg/ml of growth regulators. 2,4-D: Dissolve a desired amount in a small volume of 1 N NaOH; once dissolved, make up the solution to the required volume using sterile water. The liquid can be stored at 4°C for up to 2 months. IAA: Dissolve a desired amount in 100% ethanol. Prepare fresh just before use. Filter sterilization is not needed as long as a sterile tube is used. Pipette an appropriate volume directly into the medium before pouring into the plates. Kinetin and 2iP: Dissolve a desired amount in a small volume of 1 N NaOH; once dissolved, make up the solution to the required volume using sterile water. The liquid can be stored at -20°C for 1 month (see Note 2). ABA: Dissolve a desired amount in a small volume of 1 N NaOH. Once dissolved, adjust the pH to 5.7; make up the solution to the required volume using sterile water. Filter sterilize the solution by using a syringe fitted with a 0.20- μ m filter. This procedure should be carried out in a laminar flow hood in order to avoid contamination. Add a desired volume of the ABA solution to the medium before pouring into the plates (see Note 3).
4. The callus induction medium (CIM): It consists of the B5 medium (17) containing 20 g/L dextrose, 0.5 g/L MES buffer, and the plant growth regulators 2.2 μ M 2,4-D and 0.2 μ M kinetin. These growth regulators can be autoclaved; hence, they are added before pH is adjusted to 5.7. The gelling agent 3 g/L Gelzan™ is added after pH adjustment.
5. The shoot induction medium (SIM): The basal medium for shoot induction consists of the B5 medium (17) containing 20 g/L dextrose, 0.5 g/L MES buffer, and the plant growth regulators 5 μ M 2iP and 0.9 μ M IAA. The pH is adjusted to 5.7. The gelling agent 3 g/L Gelzan™ is added after pH adjustment. The IAA solution should be freshly prepared, filter

sterilized using a 0.20- μ m filter unit, and added to the autoclaved medium before pouring it into Petri dishes. For the ABA treatment, the same SIM medium is used with the addition of 10 μ M ABA. Like IAA, ABA should be freshly prepared, filter sterilized, and added to the medium before pouring into the plates.

6. The shoot development medium: It is simply a half-strength MS medium (16). The preparation of this medium is similar to the seed germination medium.

2.4. Equipment and Laboratory Supplies

A laminar flow hood, a microfuge, sterile scalpel with no. 11 blades, forceps and scissors, Parafilm, sterile culture tubes, 0.2- μ m sterilizing units, syringes, disposable sterile pipettes, general-purpose growing medium, such as the Prom-mix from Premier Horticultural, Inc. Canada.

3. Methods

3.1. *Arabidopsis* Seed Sterilization

1. Place about 50 μ l of seeds into a sterile Eppendorf tube.
2. Add 1 ml of a 30% commercial Javex bleach solution.
3. Keep seeds in the bleach solution for 10 min and shake well.
4. Centrifuge the seeds using the pulse function of a microfuge for 10–20 s; it helps to collect the seeds in the bottom of the tube.
5. Remove the bleach solution with a pipette in a sterile environment.
6. Wash seeds three times for 5 min with sterile water (centrifuge between washes) in a sterile environment.
7. Pipette seeds on a sterile filter paper in a Petri dish, and let seeds and filter paper dry inside the laminar flow hood (see Note 4).
8. Once the water has evaporated, wrap the Petri dish with Parafilm and keep in 4°C for a week to break the dormancy. The seeds are ready to use.

3.2. Seed Germination and Explant Preparation

1. Place 15–20 seeds per Petri dish containing the seed germination medium and place in a culture room at $22 \pm 2^\circ\text{C}$ under a 16-h photoperiod with a light intensity of 102 $\mu\text{mol}/\text{m}^2$ s.
2. Root explants are excised from 7-day-old sterile seedlings (see Note 5). Remove seedlings from the germination media, keep in a sterile Petri dish, quickly excise a 4–5-mm portion from the tip of a root using a sterile scalpel and forceps, and transfer it quickly to the CIM (see Note 6).

3.3. Callus Induction

1. Keep 12–15 root explants per Petri dish containing the CIM. Keep the plates under 16-h light and 8-h dark conditions for 6 days.
2. At the end of the CIM treatment, the cut end and the root meristem tip will be slightly swollen.

3.4. Shoot Induction

There are two steps in the shoot induction phase. Incubate the root explants in the SIM for 4 days and then transfer to the same medium which contains ABA as an additional plant growth regulator and keep it for 6 more days.

1. Transfer the root explants from CIM to SIM and keep the plates under a 16-h photoperiod for 4 days.
2. After 4 days in SIM, the explants are transferred to the same medium which contains additional 10 μM ABA. At the time of transfer, small calluses begin to form at both ends, albeit, larger at the cut end.
3. Keep 10–12 explants per Petri dish and incubate the plates under a 16-h photoperiod for 6 additional days.
4. Near the end of the ABA treatment, one or two prominent shoot buds can be seen. These buds can also be seen in SIM without the ABA treatment. In the ABA-treated explants, the callus at the cut end tends to be more compact and takes on a slightly darker color. Besides the few prominent shoot buds, additional shoots will continue to develop upon transferring to the shoot development medium.

3.5. Shoot Development

1. Transfer explants from SIM into the shoot development medium. Keep ten explants per Petri dish and incubate the plates under a 16-h photoperiod for 10 days. Besides the few shoots formed during the shoot induction treatment, additional shoots continue to develop from the compact callus from the ABA-treated explants. Small shoot buds become visible 4 days after transfer. Small clusters of shoot buds appear (Fig. 1) and subsequently develop into shoots. In the absence of the ABA treatment, this population of small buds is absent (Fig. 1).
2. Ten days' half-MS treatment allows for additional shoot development and multiplication. In the same medium, roots form automatically and extra manipulation is not needed to produce roots.
3. To collect the seeds from the regenerated plantlets, the plantlets can be transferred into fine vermiculite or a suitable mix for further growth.

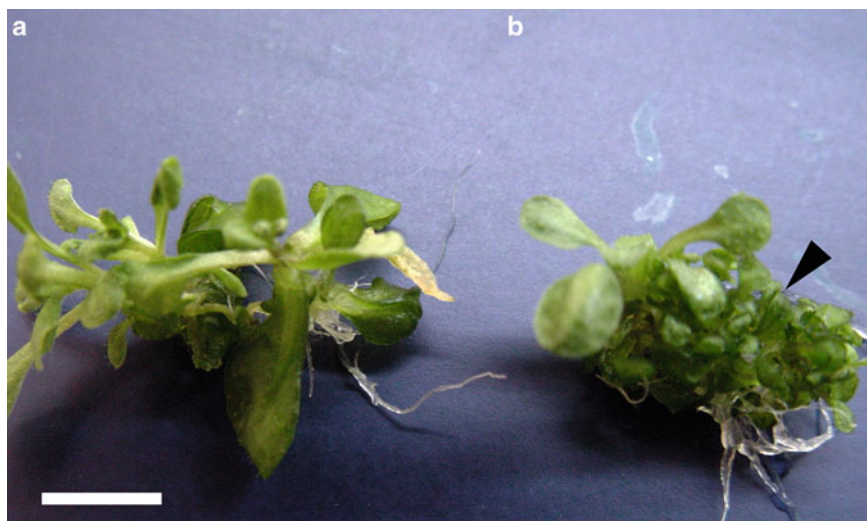


Fig. 1. A photomicrograph showing (a) a control root explant in the absence of ABA having a few prominent shoots and (b) an ABA-treated root explant; in addition to a few prominent shoots, clusters of smaller shoots are also present (arrowhead). Scale bar = 0.1 mm.

4. Notes

1. All the tissue culture plates should be prepared 1 day prior to use. The plates should initially be kept in dark condition at room temperature for 24 h. Unused plates are stored in the refrigerator and should be used within a week; otherwise, discard.
2. When using frozen stock solutions, it is important to make sure that there is no precipitation or crystallization in the stock solution before adding to the media. If precipitates are present, it is advisable to prepare new stock solutions.
3. The plant hormones IAA and ABA are light sensitive and not as stable when compared to 2,4-D and a majority of cytokinins. It is imperative that freshly prepared solutions are used the same day as the media are prepared. This is to ensure reproducibility of results. It is also advisable not to prepare a large number of ABA-containing SIM plates. ABA is expensive; therefore, prepare only a sufficient volume for each batch of medium.
4. For seed sterilization, remove excess sterile water from the Eppendorf tube before transferring the sterile seeds onto a filter paper. This reduces the drying time of the seeds.

5. The roots are small and easily desiccated. It is better to take one seedling from the germination medium, excise a root segment, and transfer immediately to the CIM.
6. The root excision can be done by scissors instead of scalpel. In this case, carefully cut the root tip using a scissors while the seedlings are still on the germination medium. After excision, the excised root tips can be transferred to CIM, one at a time.

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Chapter 16

Cryopreservation of Shoot Tips and Meristems: An Overview of Contemporary Methodologies

Erica E. Benson and Keith Harding

Abstract

Cryopreservation is the storage of viable bioresources at ultra-low temperatures in liquid nitrogen (LN). This chapter provides an overview of those protocols most commonly used to cryopreserve in vitro derived shoot tips and meristems; they are described generically, as sequential technical steps, including preparative and cryogenic treatments and the morphogenetic assessment of recovery. The importance of translating research-generated methods into formal Standard Operating Procedures (SOPs) is considered.

Key words: Cryoprotection, Cryostorage, In vitro gene bank, Standard operating procedures

1. Introduction

The rationale for cryopreserving shoot tips and meristems is motivated by the need to conserve the germplasm of (1) vegetatively proliferating species, (2) clonally maintained elite genetic stocks, (3) species that produce recalcitrant seeds, and (4) micropropagated plants of economic and conservation significance. Cryopreservation of field-derived dormant buds is also used for the long-term storage of perennial plants (1); however, this chapter focuses on in vitro-derived genetic resources. Facilitated by international networking (2–7), progress in the development of shoot tip and meristem cryopreservation has been substantial (8–10). Despite these advances, some species remain persistently difficult to cryopreserve and variable recovery responses are problematic for certain crops and genotypes (11). Cryopreservation is preceded by preparative manipulations which enhance recovery, and protocols are underpinned by two main regimes: (1) colligative cryoprotection and controlled rate cooling or (2) vitrification and ultra-rapid cooling.

**1.1. Best Practices,
Standard Operating
Procedures,
and Quality
Management**

Technical progress has resulted in the routine implementation of cryopreservation in gene banks, particularly those conserving clonal crops (9, 10, 12–14). Genetic resource stakeholders, custodians, and end users (13, 15–17) are developing Best Practices (BPs) and working towards standardized operations (<http://crop-genebank.sgrp.cgiar.org/>).

Transforming empirical cryopreservation methods into robust BPs and Standard Operating Procedures (SOPs) paves the way for developing QMS (see Notes 1 and 2) in plant biorepositories (13, 15). BPs are defined as protocols that are the most effective in producing a particular outcome, as compared to any other approach in a specific facility. They are checked, tested and validated to confirm a desired outcome without complication. By definition, BPs are optimally effective with respect to time, resources, facilities, efficiency, and costs. They should be capable of being repeated by others to produce the “best possible” result with respect to their intended purpose. BPs underpin the formulation of SOPs, which are defined by the International Society for Biological and Environmental Repositories (16) as precisely documented and managed step-by-step instructions that comprise the following components:

Essential components of standard operating procedures (SOPs)

Title	A unique name explicit for the protocol described
Number	A unique number that facilitates easy tracking and reference
Dates	Dates of introduction and most recent SOP version
Version reference	Tracking system, version number, date to ensure use of the most recently authorized version
Affiliations	Department, Division, Facility, and Laboratory
Personnel	Individuals authorized and responsible for compiling, administering, and using the SOP
Purpose	Concise description of procedures and protocols
Protective kit	Description of the Personal Protective Clothing (PPC) that should be worn by staff when undertaking SOPs and a list of required safety equipment
Equipment	Equipment list required to perform SOPs; this can include essential information (name, model, purchase date, serial number, inventory tracking number, manufacturer) and key dates for maintenance, servicing, and safety checks
Materials	List of all the chemicals, reagents, cryoprotectants, media, materials, and supplies needed to perform the procedure. The SOP also includes a checklist of suppliers, catalogue numbers, lot numbers, and expiration dates for materials used

(continued)

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Step-by-step instructions	Procedures written in sufficient and specific detail to ensure that they can be repeated and consistently reproduced by authorized personnel. Instructions include the sequential order of steps that should be followed, where appropriate, these include the time allowed for each step and critical information (e.g. temperature at which steps are performed, environmental control parameters, asepsis, and containment regimes)
Safety	Detailed description of safety steps associated with the procedures including cross-reference to all relevant SOPs, safety documents and pertinent information involving safety, risk assessment and mitigation, and incident reporting

SOPs are precise and detailed descriptions of protocols and the different tasks undertaken within them; they are formally compiled by experienced, trained personnel and administered in a document management system. This is essential to ensure their reliable tracking, updating, and approval. SOPs are collated in a “*Quality and Standard Operating Procedure Manual*” which contains the following: (1) a clear quality statement, (2) a description of the roles and responsibilities of personnel required to work in compliance with legal, regulatory, health, and safety obligations. The SOPs would normally include all the protocols and procedures used by gene bank and biorepository personnel (16, 17). To maintain appropriate standards of Quality Assurance (QA) and Quality Control (see Notes 1 and 2), SOPs are verified by more than one person and a supervisor. As required, each step is either double or triple checked against records to ensure that all steps have been undertaken correctly. Failure to conform to or deviations from SOPs involves formally administered consideration and revision as required.

1.2. Risk Management

Risk management concerns, identifying, managing, and mitigating risks to personnel, samples, the facility, recipient institutions involved in germplasm transfers, and the end users of cryopreserved materials and plants regenerated from them (15). Training should be provided in cryogenic safety and alarm systems; PPC as required is used throughout all liquid nitrogen (LN) handling procedures. Compliance with manufacturers’ special safety instructions should be observed for cryovials and cryogenic equipment. Cryovials specifically manufactured to withstand LN temperatures should be used; internally threaded vials fitted with a silicone “O” ring provide additional containment and security. It is critical to maintain temperatures in cryobanks and dry shippers by ensuring that LN levels are monitored and replenished (15).

1.3. Choice of Plant Germplasm

Shoot germplasm can be derived from proliferating in vitro (micropropagated) tissue cultures (18), nodal cuttings of in vitro plantlets (19), germinated seedlings (20), shoots excised in vitro from mature zygotic embryos (21), in vitro cold hardened shoot tips (22), and the dormant buds of perennial species (1). Non-cryogenic factors play a major role in the recovery of cryopreserved shoot tips and meristems and it is important to select donor plants and shoots in an optimal physiological state (11, 23). For temperate species this can involve inducing a natural or simulated state of cold hardiness and acclimation. Tropical and warm temperate species present special challenges with respect to procuring and conditioning shoot material more vulnerable to cryogenic stress (20, 24, 25). Donor plant and shoot tip physiology are important and high quality in vitro plants are necessary to ensure successful post-storage recovery (11, 23, 26). Keller et al. (12, 13) report that plant vigour, position of shoot tip (i.e. apical or nodal or position from apex), latent (undetected) infections, subculture regime, and explant size critically affect cryopreservation success. Duration of in vitro propagation before cryopreservation influences survival in some species, and meristems from older cultures can be more sensitive to freezing stress (27). Shoot ranking below the apical shoot can be a significant factor with survival decreasing in meristems from nodes more distal from the apex (28).

1.4. Phytosanitary Considerations

Shoot cultures as appropriate, should be pathogen-screened, tested, and disease-indexed and thoroughly checked for viral/bacterial/fungal contamination before cryopreservation. Tissues can harbour covert, systemic, and latent infections that only become apparent in cultures, otherwise presumed contaminant-free after cryogenic treatments. Latent and systemic infections increase the risk of loss of surviving shoot tips/meristems during recovery (12, 13, 15). Cultures should be axenic, with the exception where the co-conservation of an associated organism (e.g. symbiont) is required. Equipment and materials should be sterile and manipulations undertaken in the filtered air of a well-maintained, regularly serviced laminar airflow bench. Evidence is accumulating to suggest that contamination can be perpetrated via cryogenic means (15, 29); therefore, it is prudent to source LN from a reputable supplier, dispense the cryogen under clean, dust-free conditions, and observe cryovial containment.

2. Materials

Unless otherwise stated all materials, media and equipment are presumed sterile and manipulations are conducted using aseptic technique in a laminar airflow cabinet. General requirements

include both minimal and (optionally) specialist cryogenic equipment the procurement of which is dependent upon the choice of protocol.

2.1. General Equipment

1. Aluminium foil.
2. Autoclave, autoclave tape, and performance-testing indicators.
3. Automatic pipettes (e.g. 50, 100, 200, 1,000, and 5,000 μL).
4. Binocular (dissecting) microscope.
5. Clingfilm[®] or Parafilm[®] or similar sealant.
6. Desiccator(s) and activated silica gel.
7. Disinfection and sterilization solutions (e.g. alcohol, Hibitane[®]).
8. Dissecting equipment (e.g. forceps, scissors, scalpels).
9. Environmentally controlled plant growth room or chamber.
10. General laboratory glassware (e.g. selection of 25, 50, 100 mL measuring cylinders, beakers, conical flasks, glass 50- and 90-mm Petri dishes with lids).
11. Glass pipettes (1, 2, 5, 10, and 20 mL).
12. Pasteur pipettes (glass and plastic).
13. Heated magnetic stirrer and magnetic beads/rods.
14. Hypodermic syringes and needles of various sizes/gauges.
15. Laminar airflow bench and instrument sterilizing equipment.
16. Micro and top pan balances and weighing boats.
17. Microfilters for filter sterilization (e.g. Millipore[®]).
18. Oven (for gravimetric weight determinations and dry-heat sterilization).
19. pH meter, buffers, and alkali/acid solutions.
20. Reciprocal (orbital) shaker.
21. Refrigerator (0–4°C) and freezers –20 and –80°C.
22. Sieves (250–500 μm mesh).
23. Standard plant tissue culture reagents, pre-made commercial media formulations; culture media (species specific).
24. Sterile filter papers or blotting paper.
25. Sterile plastics (90- and 50-mm Petri dishes).
26. Stop watch.
27. Water bath (usually set at 45°C).

2.2. Cryopreservation Equipment

1. Cryovials, canes, sleeves, boxes, labels, LN-resistant marker pens, and/or labels or electronic bar-code tracker system for inventory automation.

2. Cryoprotectant chemicals and special additives of which the most commonly used are: bovine serum albumin, calcium chloride (CaCl_2), dimethyl sulphoxide (DMSO), ethylene glycol (EG), glucose, glycerol, mannitol, polyethylene glycol (PEG), sorbitol, sucrose, and sodium alginate.
3. Ice, and ice bucket or ice tray.
4. Long-term storage Dewar equipped with manual or electronic inventory (tracking) system.
5. Low-level cryogen alarms (for cryostores) and automatic LN fill system (see Note 3).
6. Programmable freezer.
7. Reliable source of clean LN.
8. Safety equipment (PPC, low O_2 air quality alarms, low LN alarms).
9. Selection of bench-top (1–2 L), LN supply tank (25–50 L), long-term cryostores.
10. Solutions/media for pre-culture, pretreatment, cryoprotection, loading, unloading, and recovery.

3. Preparative Protocols

These are treatments and manipulations that prepare donor plants and shoot materials prior to cryopreservation; their purpose is to enhance and improve recovery (see Note 4). It is important to optimize micropropagation and any associated in vitro culture regime (e.g. growth regulator composition) before undertaking cryopreservation. Pretreatment additives are usually made in solutions of the appropriate species-specific culture medium in either liquid or solid form, dependent upon application (see Note 5).

3.1. Cold Acclimation

Cold acclimation (sometimes termed preconditioning) is a natural adaptation in which exposure to low chilling and/or high freezing temperatures increases tolerance to much lower temperatures (see Note 6); it is exploited to enhance resistance to cryogenic treatments. The following protocol is based on a method validated for acclimated *Ribes* shoots (30–32).

3.1.1. Materials

1. See Subheading 2.
2. Cold-acclimated in vitro shoots or plants (see Note 7).

3.1.2. Method

1. Grow in vitro cultures to an appropriate physiological stage.
2. Transfer cultures to a controlled environment chamber (e.g. diurnal regime, 8 h day at 22°C and 16 h night at -1°C) for 1 week.

3. Excise meristems (see Subheading 3.3) and proceed with cryopreservation (see Note 8).

Permutations of cold acclimation regimes include extended acclimation over several weeks or months (26, 33, 34) and continuous exposure (i.e. without a diurnal cycle) to refrigerated conditions in the dark (35). Following excision, meristems from cold-hardened in vitro shoots can be further acclimated for 24–48 h in the presence of 5% (v/v) DMSO prepared in culture medium (30). This may facilitate recovery from dissection damage (see Subheading 3.3) whilst maintaining acclimation status. Similarly, applying alternating cold temperatures during pre-culture and just prior to meristem excision enhances survival in potato (36). Cold acclimation protocols usually need to be optimized (see Note 6).

3.2. Pretreatment, Pre-culture, and Pre-growth

The terminology used for these preparative regimes is varied (see Note 4). In this review, pretreatment, pre-culture, and pre-growth are used collectively to mean preparative manipulations that provide additional tolerance (but do not usually confer total protection) when applied prior to cryopreservation. Pretreatment with osmotica, the most common of which are mannitol, sorbitol, or sucrose, dehydrate tissues prior to cryopreservation; they can also simulate some cold acclimation responses (37–39). Osmotic pretreatments are particularly useful for chilling sensitive tropical species incapable or intolerant of cold acclimation (24, 25). The following method developed for *Ribes* spp. enhanced antioxidant protection and changed DNA methylation status in cryotolerant genotypes (11, 18, 39).

3.2.1. Materials

1. See Subheading 2.
2. Shoot cultures selected at an appropriate subculture stage (e.g. 3–4 weeks).
3. Culture medium containing 0.75 M sucrose.

3.2.2. Method

1. Remove nodal stem segments from in vitro shoots or plantlets, place on the sterile surface of a 9-mm filter paper, detach all expanded leaves using a scalpel and forceps and dissect the stem segment to an optimal size (e.g. within the range of 5–10 mm) and comprising one axillary (nodal) meristem.
2. Culture the stem segments on 0.75 M sucrose (prepared in culture medium) for 7 days (see Note 9).
3. Remove stem segments from sucrose medium and excise nodal shoot meristems (see Subheading 3.3).

Pretreatments can be administered to different donor tissues and cultures; in some protocols (e.g. encapsulation-dehydration) osmotica are also applied to meristems after excision. Osmotica are usually applied in the 0.25–0.75 M range, either at the final molarity

or stepwise by slowly increasing the dosage (40). The duration of pretreatment of excised shoot tips is usually 24–72 h; this may be extended to 1 or more weeks for donor cultures and nodal cuttings and careful optimization of osmoticum choice, concentration and time of exposure is required. Pretreatment with “special” additives such as proline, an amino acid associated with enhancing stress tolerance, and abscisic acid (ABA) can improve shoot tip and meristem survival (35, 41). Additives are usually incorporated into solid media; alternatively, they can be delivered on filter papers moistened with osmoprotectants dispensed in liquid media (8, 37).

3.3. Shoot Tip and Meristem Excision

Shoot tips prepared for cryopreservation are normally composed of the meristematic apex (apical dome) and its associated structures (apical or lateral meristem, subjacent tissue and several larger leaf primordia) the composition and size (0.5–5 mm) of which is species dependent. Meristems are smaller (0.1–0.5 mm) and usually comprise the apical dome, with or without the youngest, unexpanded leaf primordia. The size and composition of shoot tips and meristems are critical factors for successful cryopreservation. Newly dissected tissues are frequently susceptible to mechanical wounding (often observed as browning or blackening) exacerbated by cryogenic treatments. As this can compromise survival, a method adapted from potato and *Ribes* shoot tip protocols (11, 27) has been devised for tissues sensitive to excision wounding.

3.3.1. Materials

1. See Subheading 2.
2. Shoot tips from in vitro cultures or in vitro germinated seedlings.
3. Liquid culture medium.
4. Choice of 1–5% (v/v) DMSO solution made up in culture medium.

3.3.2. Method

All excision dissections should be undertaken in a sterile laminar airflow bench.

1. To prevent excessive and uncontrolled dehydration during excision, dissection should be undertaken as quickly as possible. This step is facilitated by using a sterile filter paper moistened with an appropriate (e.g. pre-growth) liquid medium (see Note 10) as a platform for manipulations that are carried out in Petri dishes.
2. Place the Petri dish/filter paper under a microscope viewing platform, select an appropriately sized shoot tip or nodal stem cutting; with the aid of the binocular microscope (20–40×) and two hypodermic needles (or micro scalpel) cut away larger expanded leaves, retaining several unexpanded or partly expanded leaf primordia and some subjacent tissue.

3. Trim the shoot tip or meristem to an appropriate size.
4. Immediately after dissection transfer shoot tips or meristems to filter paper bridges (in a 50 mm Petri dish) moistened with a suitable liquid medium (see Note 10).
5. Proceed to cryopreservation, alternatively follow one or both of the following options:
 - (a) Transfer dissected shoot tips or meristems to moistened filter paper bridges or standard solid or liquid medium and recover for 24–48 h under normal culture regimes or in the dark for tissues susceptible to photooxidation (bleaching).
 - (b) For particularly sensitive dissected shoot tips or meristems, transfer to 1–5% (v/v) DMSO dispensed in either liquid or solid medium to help ameliorate the effects of wounding, proceed as in option (a) in step 5 and recover as described in steps above.

Dissection skills are a critical point factor (42); it is advisable that excision technique is well practised and survival confirmed before proceeding to cryopreservation.

3.4. Cryoprotectants

Cryoprotection involves the application of colligative (penetrating) and osmotic (non-penetrating) cryoprotective additives (CPAs). It is generally advisable to use quality grade chemicals and take care in preparing highly concentrated solutions that are difficult to solubilize. In the case of multicomponent mixtures and percentage (%) formulations, the technical preparations used to make the final volume of a solution should be stated (see Note 11) on the basis of either weight per volume (w/v) or volume per volume (v/v) and the composition of the solvent (i.e. the diluent medium) clarified. This is particularly important to achieve consistency in the preparation of CPA mixtures used in validation exercises and technology transfers between different laboratories. For example, PVS2 consists of liquid chemical components, each having a different density (see Note 11), specifically glycerol 1.258 g/mL, ethylene glycol 1.113 g/mL, and dimethyl sulfoxide 1.100 g/mL (Sigma-Aldrich); these may vary dependent upon chemical purity. Generally, the preparation of PVS2 comprises 30% (w/v) glycerol (Molecular weight=92.09; 300 g/L=3.26 M), 15% (w/v) ethylene glycol (Molecular weight=62.07; 150 g/L=2.42 M), 15% (w/v) DMSO (Molecular weight=78.13; 150 g/L=1.92 M), and 0.4 M sucrose (Molecular weight=342.3; 136.92 g/L) all of which are made up in basal liquid culture medium (i.e. comprising all macro/micro elements and vitamins without sucrose and gelling agent). Thus, the final concentration of sucrose is 0.4 M in PVS2 (see Note 11).

Media pH should be checked and adjusted following the incorporation of CPAs (see Note 12). Preferably, cryoprotectant mixtures should be filter-sterilized with the exception of high viscosity solutions for which autoclaving may be the only practical option. In that case, care is required to avoid unnecessary holding times at high temperatures as these can be detrimental to heat-labile components and cause caramelization (browning) of sugars. Cryoprotectant solutions should be freshly prepared, with the exception of solutions known to remain stable and contaminant free in refrigerated storage.

4. Specific Cryopreservation Protocols

Increasingly diverse protocols are used for cryopreserving shoot tips and meristems; recent advances (8, 9, 43) include “hybrid protocols” which combine different cryoprotective regimes (e.g. encapsulation-vitrification, droplet-vitrification, droplet freezing), some of the most effective are vitrification-based (23, 44–46). These have the advantage of offsetting the purchase and maintenance of programmable freezing equipment; however, the disadvantages are that certain types of germplasm may be sensitive to vitrification and careful stabilization of the glassy state on cooling and re-warming is critical. It is therefore insightful to underpin technical “know how” with cryopreservation theory (15, 47–49). The following protocols are generic descriptions of the most frequently used protocols:

4.1. Controlled Rate Cooling

This method is based on a validated protocol applied to acclimated *Ribes* shoot meristems (31, 42); it describes the basic technical steps of osmotic and colligative cryoprotection used in combination with controlled rate cooling.

4.1.1. Materials

1. See Subheading 2.
2. Appropriately prepared, excised meristems (see Subheadings 3.1–3.3).
3. A solution of (w/v) each of 10% polyethylene glycol (Molecular weight = 8000 or 10,000), glucose, and DMSO, made up in liquid culture medium (see Subheading 3.4 and Note 11) and pH adjusted (see Note 12).

4.1.2. Method

1. Transfer 10–15 excised shoot meristems to a 1.2- or 2.0-mL cryovial (or similar volume) containing (ca. 0.5–1.0 mL) CPA mixture and cryoprotect by incubating at an appropriate temperature (e.g. ambient, on ice, 4°C, 25°C) for 1 h (see Notes 13–16).

2. Transfer cryovials to the chamber of a controlled rate freezer, programmed with an optimized cooling cycle (see Note 17). Parameters are usually set within the following ranges:
 - (a) Cooling rate (0.25–5°C/min).
 - (b) Terminal transfer temperature (to a minimum of -40 to -45°C).
 - (c) Holding time at temperature of transfer (30–45 min).
 - (d) An optional “seeding” (ice nucleation) stage incorporated at ca. -5 to -15°C.
 - (e) A hold at the seeding temperature of 5–15 min to allow for extracellular ice nucleation, achieved by:
 - Manually touching the cryovials with a cold instrument or
 - Automatic seeding by the controlled rate freezer (see Note 18).
3. On completing the programme rapidly transfer cryovials to a 1 L LN-filled Dewar located by the side of the freezer chamber (see Note 19).
4. Thereafter, transfer the cryovials to a long-term cryostore.
5. For thawing, transfer the cryovials to a 40–45°C water bath until all the ice has melted.
6. Transfer to an airflow bench; carefully wipe the outside of the cryovial with a tissue soaked in a sterilant to remove excess and potentially contaminating water before opening.
7. Expel cryovial contents onto filter papers in a Petri dish to remove CPAs by capillary action. Optionally:
 - (a) Wash shoot tips or meristems with liquid culture medium to remove excess CPAs (see Note 20) or,
 - (b) Wash the shoot tips or meristems in a solution of suitable osmotica.
8. Transfer shoot tips or meristems to an appropriate recovery medium (see Note 21).

Controlled rate cooling has declined in favour of vitrification-based methodologies; however, for those species that are amenable to this method, it compares favourably with other protocols (50). Controlled rate cooling has the advantage of efficiency, as it is possible to concomitantly process large numbers of cryovials.

4.2. Vitrification Protocols (PVS2 and PSV3)

Plant Vitrification Solution (PVS) protocols (51) comprise variations of the following manipulations:

1. Pretreatment with an osmoticum or special additive (see Subheading 3.2).
2. Pre-exposure to a loading solution (LS).

3. Cryoprotection with PVS2 or PVS3, optimized in a toxicity trial.
4. Rapid cooling by direct plunging of cryovials in (liquid phase) LN.
5. Rapid re-warming at 40–45°C in a water bath.
6. Removal of CPAs using an unloading solution (ULS), e.g. 1.2 M sucrose.
7. Recovery of shoot tips and meristems on filter papers over layered on culture medium.
8. Daily transfer to fresh filters for the first 1–5 days following re-warming.

The objective of vitrification is to increase cell viscosity to a point, defined by the glass transition temperature (T_g) at which water forms an amorphous glassy state, that under this condition damaging ice crystallization is obviated. Cell viscosity is increased using two main approaches: (a) loading tissues and cells with penetrating (e.g. colligative) CPAs and (b) water removal by osmotic dehydration and/or evaporative desiccation (see Note 22).

4.2.1. Loading Solutions

Loading solutions (LS) are applied prior to vitrification; their purpose is to provide osmoprotection supplementary to or substituting for pretreatments (see Subheading 3.2). The most common LS comprise 2 M glycerol and 0.4 M sucrose (51). Shoot tips and meristems are exposed to the LS for 20–60 min at ca. 25°C; further optimization is often required. The following protocol is based on a method developed for *Hypericum perforatum* shoot tips (52).

Materials

1. See Subheading 2.
2. Prepared and pretreated shoot tips and meristems (see Subheadings 3.1–3.3).
3. LS, 2 M glycerol and 0.4 M sucrose prepared in culture (see Notes 11, 12 and 23).

Method

1. Transfer batches (e.g. 10–25) of excised shoot tips or meristems to LS contained optionally in: (a) 10–15 mL of LS dispensed in tubes or conical flasks agitated on an Orbital (rotatory) shaker or (b) 0.5–1.0 mL LS dispensed in 1.2 mL cryovials.
2. Incubate in LS at ambient temperatures (20–25°C) for 20 min (optimize as required).
3. Using a Pasteur pipette remove the LS by aspiration, whilst retaining the shoot tips or meristems and replace with PVS2 (see Subheading 4.2.2) or PVS3 (see Subheading 4.2.3).

The application of LS prior to exposing shoot tips and meristems to vitrification solutions is a critical factor for some species and genotypes (41), optimization can be undertaken as described for PVS-toxicity trials (see methods in Subheading 4.2.2).

4.2.2. PVS2

The following generic protocol is based on a method developed for cold-acclimated *Ribes* shoot tips for which glass stabilization was confirmed using thermal analysis (30).

Materials

1. See Subheading 2.
2. Prepared, excised (see Subheadings 3.1–3.3), and pre-loaded (if required) shoot tips or meristems (see Subheading 4.2.1).
3. 100 mL of 100% PVS2 (see Notes 11 and 12).
4. 100 mL of ULS comprising 1.2 M sucrose made up in liquid culture medium.

Method

These steps include the preparation of the various solutions and a toxicity test to determine the PVS2 application.

1. Prepare and apply a LS (see Subheading 4.2.1); this may not be necessary for certain species.
2. Prepare PVS2 using basal liquid medium (i.e. comprising all components with the exception of sucrose) as the diluent (see Note 11) and to which is added:
 - (a) 30% (w/v) glycerol.
 - (b) 15% (w/v) ethylene glycol.
 - (c) 15% (w/v) DMSO.
 - (d) 0.4 M sucrose to a *final* concentration (see Subheading 3.4, Notes 11 and 12).
3. Prepare a dilution series (e.g. 20, 30, 40, 50, 60, 70, 80% v/v) of 100% PVS2 solution using basal liquid culture medium as the diluent (see Note 11).
4. Prepare 1.2 M sucrose ULS in basal liquid culture medium (see Subheading 3.4).

PVS2 Toxicity Trial

The PVS2 toxicity trial is best undertaken using cryovials (see Notes 13–16). Prepare shoot tips or meristems (see Subheadings 3.1–3.3) and construct a toxicity test (see Notes 20 and 21) to ascertain the most effective duration and temperature of exposure to PVS2 as follows:

1. Conduct manipulations in parallel at two different temperatures (see Notes 15 and 24).
 - (a) On ice, e.g. contained in an ice bucket or ice trays.
 - (b) At 20–25°C (or ambient temperature).

2. Transfer 10–25 shoot tips or meristems in appropriately replicated batches to cryovials.
3. Using a sterile automatic or graduated Pasteur pipette, dispense (chilled or non-chilled) 0.5–1 mL aliquots of diluted PVS2 (within the range of 20–80%, v/v) into the cryovials, and accurately time each exposure e.g. 5–60 min or longer as required (see Note 25).
 - (a) For comparison, include a treatment involving direct exposure to 100% PVS2.
4. For each dilution, remove by aspiration almost all the CPAs (see Note 20) and replace sequentially with an increased strength of PVS2.
5. Solutions can be dispensed in a single aliquot or by drop-wise addition over a timed period to ameliorate osmotic stress (see Note 16).
6. Continue to add and replace the intermediate strengths of PVS2 until the shoot tips or meristems are bathed in 100% solution (see Note 14).
7. Accurately time the duration of exposure (e.g. 5–60 min) to 100% PVS2.
8. Remove the 100% PVS2 using a Pasteur pipette and replace with ULS containing 1.2 M sucrose prepared in basal liquid culture medium (see Note 20).
9. Wash two to three times in fresh ULS and maintain in the final wash for 10–30 min.
10. Expel cryovial contents onto a filter paper to remove CPA excess by capillary action (see Note 20).
11. Transfer the shoot tips or meristems to recovery medium (see Note 21).
12. Select the PVS2 treatment that permits maximum regrowth and cryoprotection.

PVS2 Cryopreservation

1. Following optimization of PVS2 application, transfer the tissues in cryovials directly to LN.
2. Re-warm cryovials in a water bath at 40–45°C for 1 min.
3. Transfer to an airflow bench and carefully wipe the outside of the cryovial with a tissue soaked in a sterilant to remove excess, potentially contaminating water before opening.
4. Remove 100% PVS2, replace with 1.2 M sucrose ULS (see Note 26).
5. Perform two to three washes in fresh ULS, equilibrating the shoot tips or meristems in the final wash for 10–30 min (this step will require optimization).

6. Expel cryovial contents on a filter paper to remove liquid excess by capillary action (see Note 20).
7. Transfer the shoots tips or meristems to recovery medium (see Note 21).

Critical factors in PVS2 protocols are the temperature of application (41, 53, 54), sequential loading, and duration of applying intermediate and full strength PVS2 solutions (34, 41, 55–57). The efficacy of PVS2 is affected by size; smaller meristems (0.5 mm) are generally more sensitive, whereas larger ones (3 mm) require longer exposure to CPAs (51).

4.2.3. PVS3 Vitrification

This method (51, 58) is preferentially used for species sensitive to PVS2, although some plants are more sensitive to PVS3 (38). Successful recovery using PVS3 usually requires pretreatment (see Subheading 3.2) and the application of LS (see Subheading 4.2.1). Optimization of exposure to PVS3 may be required as described in the PVS2 toxicity trial (see methods in Subheading 4.2.2).

Materials

1. See Subheading 2.
2. Prepared, excised (see Subheadings 3.1–3.3), and pre-loaded (LS) shoot tips or meristems (see Section 4.2.1).
3. PVS3 comprising 50% (w/v) glycerol and 50% (w/v) sucrose (see Notes 11, 12 and 27).
4. 100 mL of 1.2 M sucrose ULS.

Method

1. To optimize exposure to PVS3, conduct parallel steps at two different temperatures: (a) on ice (see Note 15) or (b) at 20–25°C (or at ambient temperatures).
2. Using a sterile automatic or graduated Pasteur pipette dispense 0.5–1 mL aliquots of PVS3 into a 1.2-mL cryovial (see Note 15).
3. Transfer 10–25 batches of shoot tips or meristems to cryovials.
4. Accurately time the duration of exposure of the tissues to PVS3 (e.g. 20–90 min); once optimized, proceed to the cryopreservation step.
5. Transfer cryovials directly to LN.
6. Re-warm cryovials in a water bath at 40–45°C for 1 min.
7. Transfer to an airflow bench and carefully wipe the outside of the cryovial with a tissue soaked in a sterilant to remove excess and potentially contaminating water before opening.
8. Remove PVS3 and replace with ULS containing 1.2 M sucrose.

9. Perform two to three washes in fresh ULS; equilibrate in the final wash for 10–30 min.
10. Expel cryovial contents onto filter papers to remove liquid excess by capillary action (see Note 20).
11. Transfer shoots to recovery medium (see Note 21).

Various adaptations of the basic PVS3 protocol have been developed (26, 35, 52, 54, 58, 59); however, because PVS-based cryopreservation comprises several stages, laborious transfers, and stringently timed exposures, technical rigour is required for the optimization of each step. For some species, this is critical, whereas for others, variations in parameters are not as influential (60). Overall, PVS protocols have been widely used in gene banks (26, 43, 51, 61, 62); they support high to acceptable levels of recovery. Ellis et al. (63) report accessions of garlic as being successfully cryopreserved using either PVS2 or PVS3, when 40% shoot regrowth is achieved.

4.3. Encapsulation Protocols

Fabre and Dereuddre (64) developed the original encapsulation-dehydration methodology for *Solanum phureja*; the protocol is based on artificial seed technology adapted for cryogenic purposes and has the advantage of combining two applications. Encapsulation-dehydration has been successfully applied to a diverse range of plant species (43, 65, 66). The basic protocol involves the encapsulation of shoot tips or meristems in calcium alginate beads followed by osmotic dehydration and evaporative air desiccation, either in sterile laminar airflow or over silica gel (40, 67, 68). Cryoprotection requires the formation of a stable glassy state in the beads and encapsulated tissues (30, 69). Encapsulation is also used in combination with PVS2 cryoprotection (see Subheading 4.2) in which case it is termed encapsulation-vitrification (see Subheading 4.3.2).

4.3.1. Encapsulation-Dehydration

The following generic protocol is based on a method adapted for potato and *Ribes* spp. (18, 39, 70); it incorporates a sucrose pretreatment that simulates cold acclimation (71, 72); thermal analysis (30, 67, 69) was used to confirm vitrification. The success of the protocol is dependent upon achieving a critical moisture content that ensures stable glass formation during cooling and that ice nucleation is circumvented during re-warming. This is usually within the residual moisture range of 20–25% on a fresh weight basis that approximates to 0.4 gH₂O/g dry weight.

Materials

1. See Subheading 2.
2. Prepared, pretreated and excised shoot tips or meristems (see Subheadings 3.1–3.3).

3. A 5 mL Gilson “Pipetteman” or similar automatic pipette, alternatively graduated 3-mL plastic pastettes can be used (see Note 28).
4. Liquid culture medium containing 0.75 M sucrose (see Notes 5 and 9); for convenience this can be aliquoted (10–20 mL) into 50–100 mL conical flasks.
5. Calcium-free, liquid culture medium containing 3% (w/v) sodium alginate (SIGMA® 2% low viscosity, sodium salt derived from sea Kelp) dispensed into small bottles or glass vials as 15–20 mL aliquots (see Notes 11, 29 and 30).
6. Liquid culture medium containing 100 mM CaCl_2 dispensed as 30 mL aliquots into conical flasks or beakers (see Note 29).
7. Choice of a pre-growth media containing 0.5–1 M of an osmoticum, e.g. sucrose, mannitol, or sorbitol (see also Notes 4, 5 and 9).
8. An airtight vessel (e.g. a Parafilm® sealed 9.0 cm glass Petri dish) containing a known amount (30–50 g) of sterile heat-activated (blue) silica gel.
9. An open sterile Petri dish located in sterile, horizontal laminar airflow.

Method

1. Transfer shoot tips or meristems to 3% (w/v) alginate solution (see Notes 28–30) swirling gently to assist tissue immersion and taking care not to form air bubbles as these will produce non-uniform beads.
2. Fill a 3 mL plastic pastette (or automatic pipette tip) with 1–2 mL of alginate solution containing ca. 5 meristems; care is required to prevent air bubble formation.
3. Hold the pastette vertical to ensure that spherical drops are formed by surface tension, deliver droplets at rate of ca. 1/s into 100 mM CaCl_2 made up in liquid medium.
4. On exposure to calcium ions, uniform spherical beads form (45–50 μL in size).
5. Allow the beads to polymerize in the CaCl_2 solution for ca. 15–30 min.
6. Pour off the excess CaCl_2 solution and transfer beads to a 90 mm-sterile Petri dish; retrieve beads containing the shoot tips or meristems. Reserve empty beads for parallel dehydration and moisture content assessments (see Notes 31–34).
7. Transfer the encapsulated shoots tips or meristems to a 100 mL conical flask containing ca. 20–25 mL 0.75 M sucrose made up in basal liquid medium. Optimization of the concentration and stepwise addition (usually in the range 0.25–1.0 M) and duration of exposure (ca. 16–72 h) to osmotica may be required.

8. Place flasks on an orbital shaker (e.g. Gertomat M, 125 rpm) for 16–22 h under standard culture conditions.
9. Pour the beads from the flask into a 90 mm-sterile Petri dish or through a sieve and blot dry on sterile filter papers to remove excess surface moisture.
10. Transfer beads to the base of a sterile empty 90 mm-sterile Petri dish. Ensure the beads do not touch each other and that they are evenly distributed across the base of the dish.
11. Place the dish to the back of a horizontal air stream of a laminar airflow bench for 4 h. The duration of evaporative desiccation may need to be optimized (see Notes 31–33). Alternatively (see Note 35), the beads can be evaporatively desiccated over activated silica gel (68).
12. Relative humidity (RH) and ambient temperature can critically influence the bead desiccation rate and final moisture content. To ensure quality control (see Note 35) between different experiments, record the temperature and RH of the airflow bench at the onset and end point of the period of evaporative desiccation.
13. Place desiccated beads (see Note 34) in a cryovial and immerse in LN.
14. Re-warm at ambient temperature (see Note 36) in the laminar airflow bench for 10–30 min.
15. Wipe the exterior of the cryovials with a sterilant solution before removing the beads.
16. Rehydrate beads in liquid medium (see Note 37).
17. Transfer to recovery medium (see Note 38).

Modifications of the encapsulation-dehydration protocol include the incorporation of sucrose as an osmoticum (e.g. within the range 0.4–0.75 M) in the calcium alginate solutions (56, 73) and the formation of beads in the presence of 2 M glycerol and 0.4 M sucrose (73). Critical factors are duration of exposure and concentration of pretreatment, sequential dehydration in osmotica and duration of evaporative desiccation in the laminar airflow, or over activated silica gel (21, 43, 65–67, 74). Encapsulation-dehydration has also been combined with controlled rate cooling (40). Alginate beads can be supplemented with activated charcoal (21) to aid recovery in some species. Bonnart and Volk (73) have improved protocol efficiency by encapsulating multiple numbers of shoot tips in a single bead.

4.3.2. Encapsulation-Vitrification

This involves shoot tip or meristem encapsulation (64) followed by treatment of the beads with PVS2 (75, 76); the combined approach was developed for desiccation intolerant germplasm. The version of the protocol described was used for shoot tips of *Parkia speciosa*,

a recalcitrant seed producing tropical tree for which thermal analysis was used to confirm stable glass formation (20).

Materials

1. See Subheading 2, materials in Subheadings 4.2.1 and 4.3.1.
2. Prepared, pretreated, and excised shoot tips or meristems (see Subheadings 3.1–3.3).
3. PVS2 solution (see methods in Subheading 4.2.2).
4. Calcium-free, liquid culture medium containing 3% (w/v) sodium alginate (SIGMA® 2% low viscosity, sodium salt derived from sea Kelp) and incorporating 0.4 M sucrose (final concentration) dispensed into small bottles as 20 mL aliquots (see Note 39).
5. Liquid culture medium containing 100 mM CaCl_2 dispensed as 30 mL aliquots into conical flasks or beakers.

Method

It may be necessary to first undertake a vitrification solution toxicity trial (see Subheadings 4.2 and 4.3.1).

1. Suspend pretreated shoot tips or meristems in 3% (w/v) Na-alginate loaded with a final concentration of 0.4 M sucrose or alternatively a mixture of 2 M glycerol and 0.4 M sucrose may be used (see Subheading 4.2.1).
2. Dispense droplets of alginate into 100 mM CaCl_2 solution made up in basal liquid culture medium containing a final concentration of 0.4 M sucrose or a mixture of 2 M glycerol and 0.4 M sucrose (see Subheading 4.2.1).
3. Incubate in this solution for up to 1 h at 25°C. Optimization of exposure to osmotica and bead polymerization time may be required.
4. Remove the encapsulated shoot tips or meristems and place in a 50–100 mL flask containing 100% PVS2 solution.
5. Place flask on a reciprocal shaker e.g. 60 rpm up to 1 h; optimization may be required.
6. Remove beads and place in a 2 mL cryovial (e.g. 5–15 beads/vial) and add 1–1.5 mL of fresh PVS2 solution.
7. Directly immerse the beads (contained in cryovials) in LN.
8. Re-warm the cryovials in a water bath at 35–45°C for ca. 1 min.
9. Wipe the exterior of the cryovials with a sterilant solution before removing the beads.
10. Drain the PVS2 solution away and replace with 1.2 M sucrose ULS for 10–20 min.
11. Blot the beads dry of excess moisture and place on recovery medium.

Using the above protocol (20) pretreated shoot tips were also exposed to trehalose within the range 2.5, 5 and 10% (w/v). Various other adaptations of the encapsulation-vitrification protocol have been devised (43, 51, 75).

4.4. Droplet-Based Protocols

These involve the cryoprotection and ultra rapid cooling and re-warming of plant tissues in microdroplets. When DMSO cryoprotection is used the procedure is termed droplet freezing (77–80) and for PVS2, droplet-vitrification (45). The success of droplet protocols is critically dependent upon achieving ultra rapid rates of cooling and re-warming; this requires direct exposure to LN making containment one of its main drawbacks. Consequently, these protocols require stringent risk management and phytosanitary precautions to respectively offset the hazards of operators directly handling LN and non-containment (15).

4.4.1. Droplet Freezing

This generic method is based on the protocol developed for potato (12, 79, 80).

Materials

1. See Subheading 2.
2. Prepared, pretreated, and excised shoot tips or meristems (see Subheadings 3.1–3.3).
3. Sterile pieces of aluminium foil (dimensions: 20–25 × 5 × 0.003–0.005 mm).
4. Sterile liquid culture medium.
5. 1% (w/v) agarose made up in culture medium with 30 g/L sucrose (optional step).

Method

1. Prepare 10% (v/v) DMSO in liquid medium (see Subheading 3.4).
2. Dispense 1–3 mL aliquots of 10% (v/v) DMSO over sterile filter papers contained in 50 or 90 mm sterile Petri dishes.
3. Transfer the shoot tips or meristems onto the surface of the filter papers and time the duration of exposure to the cryoprotectant at ambient temperatures (20–25°C), usually within the range 30–60 min.
4. Dispense, using an Analytical 2.5–20 µL Gilson® Pipetteman (or equivalent) 2.5–5.0 µL droplets (dependent upon critical meristem droplet size) of cryoprotectant (5–10 droplets/strip dependent upon strip loading and shoot tip or meristem size) onto the surface of sterile aluminium foil strips.
5. Transfer one shoot tip/meristem from the 10% (v/v) DMSO-treated sterile filter papers to each droplet and continue timing the duration of exposure to the cryoprotectant administered in the droplet.

- (a) Alternatively the shoot tips or meristems can be transferred directly to the DMSO droplets and cryoprotected in situ.
6. Using LN-tolerant, sterile forceps immerse the foils directly into LN contained in a small (5–10 mL) Dewar (taking care not to release the foils) or, alternatively, transfer foils to LN-filled cryovials (see Note 40). These operations are undertaken in a sterile laminar airflow bench.
7. Once the LN has finished boiling, rapidly transfer the foils to cryovials (up to 2 foils per cryovial), or replenish the cryovials with LN (see Note 40).
8. Seal the foils in the cryovials and plunge directly into LN (see Note 40).
9. For re-warming, remove foils from cryovials (see Note 41) and rapidly transfer to liquid medium at ambient room temperatures (ca. 25°C); the shoot tips or meristems will dislodge on re-warming.
10. Transfer the shoot tips or meristems to recovery culture medium.
11. Alternatively, recover the shoot tips or meristems in cooled and solidified agarose droplets (2.5–5.0 μ L) dispensed on the surface of 50 or 90 mm Petri dishes that are bathed in liquid culture medium (1–3.0 mL).
12. Transfer to standard culture conditions following initial shoot regrowth.

There is limited information concerning the cryoprotective basis of droplet freezing; under optimal biophysical conditions, it is possible that the microlitre droplets of DMSO become vitrified. Should ice crystals form they are likely to be sufficiently small not to be lethal; the protective principle might also be attributed in part, to several biophysical factors. Ice crystallization may be prevented by the impeded alignment of H₂O molecules in microlitre droplets caused by their insufficient numbers, surface tension-cohesion effects limiting H-bonding and cooling rates of millions of degrees per second (15, 67, 81).

4.4.2. Droplet Vitrification

Droplet vitrification was originally developed by Panis et al. (45) for *Musa* spp., subsequently it has proved successful for the cryoconservation of an increasing number of plant species and is rapidly becoming a popular method of choice for shoot tips and meristems (33, 43, 44, 46, 51, 82–85). The following protocol is based on the method developed for *Musa* germplasm (14, 45).

Materials

1. See Subheadings 2, 4.2 and 4.4.1.
2. Prepared, pretreated, and excised shoot tips or meristems (see Subheadings 3.1–3.3).

Method

The PVS2 component of the protocol is adapted from the standard vitrification method (see Subheading 4.2) and the cryogenic part from the droplet freezing protocol (see Subheading 4.4.1).

1. Prepare LS, PVS2 and ULS in liquid medium (see Subheadings 4.2.1 and methods in 4.2.2).
2. Transfer shoot tips or meristems to LS contained in either cryovials or an appropriate vessel (see Note 13).
3. Osmoprotect in LS at ambient temperatures (20–25°C) for 20–60 min; this stage will need to be optimized; a minimal exposure of 20 min is usually necessary.
4. Remove the LS and substitute for ice-chilled PVS2.
5. Incubate the shoot tips or meristems in the cryoprotectant (usually on ice) for 20–60 min noting that the temperature and duration of exposure may need to be stringently optimized (see Notes 15 and 16).
6. Dispense, using an Analytical 2.5–20 μL Gilson® Pipetteman (or equivalent) 5–15 μL droplets of PVS2 (5–10 droplets/strip dependent upon strip loading and shoot tip/meristem size) onto the surface of sterile aluminium foil strips contained in a 90 mm Petri dish placed on ice.
7. Transfer 1 shoot tip/meristem to each droplet and continue timing the duration of exposure to the cryoprotectant administered in the droplet. Alternatively, the shoot tips or meristems can be transferred directly to the PVS2 droplets and cryoprotected in situ.
8. Following PVS2 cryoprotection, using LN-tolerant, sterile forceps immerse the foils directly into LN contained in a small (5–10 mL) Dewar (taking care not to release the foils) or in LN-filled cryovials (see Note 40). These operations are undertaken in a laminar airflow bench.
9. Once the LN has finished “boiling”, rapidly transfer the foils to cryovials (up to 2 foils per cryovial), as required replenish the cryovials with LN (see Note 40).
10. Seal the cryovials and directly plunge them into LN (see Note 40).
11. For re-warming, remove the foils from the cryovials (see Note 41) and transfer to ULS comprising 1.2 M sucrose.
12. Perform two to three washes in fresh ULS and equilibrate the shoot tips or meristems in the final wash for 10–30 min.
13. Expel cryovial contents on a filter paper to remove liquid excess by capillary action (see Note 20).
14. Transfer the shoots tips or meristems to recovery medium (see Note 21).

15. Transfer to standard culture conditions following initial shoot regrowth.

Practically, the success of this method is critically dependent upon achieving very high rates of cooling (45, 84). Changes to the original (45) droplet-vitrification include modification of the LS substituting 1.9 M glycerol and 0.5 M sucrose and vitrification solutions (82, 83). Halmagyi et al. (28) report that apex size, sucrose concentration during pretreatment, pre-culture, and cold hardening affect recovery of potato shoot tips following droplet-vitrification. Similarly, Yoon et al. (23) comment that donor plant and shoot tip subculture influence survival. Marco-Medina et al. (57) found that optimizing benzyl adenine (BA) concentration in the recovery medium improved regrowth of *Thymus moroderi* shoot tips following droplet-vitrification.

5. Assessment of Morphogenetic Recovery

Environmental conditions and media formulations for post-cryostorage survival, regrowth, and regeneration of shoot tips and meristems are largely species dependent (9). Critical factors are removal of CPAs, pre-culture and unloading of osmoprotectants, plant growth regulator composition of recovery media (57, 86, 87), reducing photooxidation (bleaching) by recovering initially in the dark and oxidative stress mitigation (8, 15, 52). It is essential to establish definitive criteria for assessing recovery of shoot tips and meristems (see Note 21), which can be monitored in three stages:

1. Survival (viability) manifest as new growth (e.g. leaf expansion), tissue expansion (swelling), and greening. However, all initial assessments should be interpreted with caution (see Notes 21 and 42).
2. Confirmed and sustained shoot regrowth or regeneration.
3. Plant development, i.e. shoot cultures are capable of rooting and producing entire new plants that are “true-to-type”.

Only steps 2 and 3 are definitive measures of cryopreservation success. The following protocol can be applied to all types of shoot tips and meristems.

5.1. Materials

1. See Subheadings 2–4.
2. Thawed/re-warmed shoot tips or meristems on an appropriate recovery media.

5.2. Method

Develop robust performance indicators that can be used to assess sequential and sustained recovery; commonly, these include %total survival, %shoot regrowth (other morphogenetic evaluation),

%plant development, and fresh weight gain (see Note 21). It is important to be aware that removing survivors from culture vessels to assess recovery (e.g. gain on fresh weight basis) may impede recovery.

1. Within 7 days of thawing/re-warming examine tissues in situ on recovery medium under a binocular microscope (see Note 43).
2. Record the number of shoot tips/meristems that demonstrate early survival indicators, e.g. greening, expanded leaf primordia, and swelling (see Notes 21 and 42).
3. Continue to undertake weekly assessments for a minimum of 4–8 weeks (dependent upon genotype-specific rates of recovery and species) to (a) determine losses from delayed onset decline in survivors and (b) confirm sustained recovery, growth, and shoot development in survivors (see Notes 42–45).
4. Evaluate recovery as:
 - (a) Normal events: shoot/plantlet formation (i.e. in the absence of callogenesis and adventitious shooting).
 - (b) Abnormal events: callus, adventitious shoots, hyperhydricity, blackening.
 - (c) Sustained shoot regrowth regeneration and plant development (see Notes 21, 42–45).
5. Construct a time course of different recovery events (e.g. leaf expansion, callus formation, sustained shoot regrowth and plantlet regeneration); this will help to devise conditions (see Notes 44 and 45) that support normal regrowth and sustained development of survivors and pinpoint when corrective measures are required (e.g. transfer to a different recovery medium).

Stringent evaluation of abnormal and normal morphogenetic pathways of development following cryostorage is important for storage stability (88). Understanding the dynamics of both short and long-term regrowth and morphogenetic development and competence in plants recovered from cryopreserved shoot tips and meristems has practical implications for the utilization of plants supplied by in vitro cryobanks (11, 70, 89, 90).

6. Choosing a Cryopreservation Protocol

The increasing number of approaches now being used for the cryopreservation of in vitro derived shoot tips and meristems has greatly facilitated the ex situ conservation of plant genetic resources (9, 15).

However, making the appropriate protocol choice is important; this can be facilitated by studies that have compared the efficacy of different cryopreservation methods (15, 31, 34, 42, 50, 86). Whilst choice of protocol is crucially determined by maximum survival, sustained shoot and plant development, other factors are also important. These include, cost–benefit analyses, feasibility of scale-up (13, 45, 60) and confidence in long-term cryostorage security and stability (56, 88, 91, 92). Choice is also influenced by risk mitigation, access to specialist cryobiological expertise and equipment and the infrastructural capability of the facility (e.g. a secure LN supply). These parameters also need to be addressed in the context of protocol validation, risk and quality management (15–17, 93, 94). Securing the long-term economic viability of cryobanks holding shoot-derived germplasm is also enabled by improving the technical efficiency of different protocols (73). This is particularly important for large-scale biorepositories conserving a diverse range of plant genetic resources (13–15, 63).

7. Notes

1. Quality Management Systems (QMS) also termed Quality Management Programmes or QA based on ISBER's BP (16) can be defined as an integrated system of activities that include management, planning, implementation, documentation, and assessment (including measures for improvement) designed to ensure that a procedure, protocol or operation is of the type and quality needed to achieve a desired, consistent and safe outcome.
2. Quality Control (QC) based on ISBER's definitions (16) is the system of technical activities that measures the attributes and performance of a process, assay, protocol, procedure, product or output against defined standards in order to verify that the stated requirements and expectations are met and satisfy performance criteria.
3. Consider the advantages and disadvantages of using manual or automatic replenishment of LN and that the choice will ensure the level of coolant is maintained (15, 81). Automated low level alarm systems can offset the risks of potentially damaging temperature fluctuations; this is particularly important for samples stored in vapour phase of LN or at temperatures critically close to the T_g.
4. As these terms are used interchangeably, pre-growth and pre-culture are defined herein to mean protective treatments applied to growing shoots and meristems, whereas pretreatment is more generally used to describe either: (1) the application of

additives and short treatments (e.g. exposure to high concentrations of osmotica) that confer additional protection, but may retard growth, or (2) the combined application of pre-growth and pre-culture treatments. The term preconditioning has been used to describe cold acclimation. In the context of developing SOPs and BPs, it is important to ensure that terminology is clarified.

5. It is important to make clear the final composition and concentration of osmotica added for cryoprotective purposes to media that already contain sugar additives as energy sources (e.g. sucrose and glucose).
6. It may be necessary to moderate low temperature (e.g. within the range -5 to 10°C), treatment duration (days to months) and temperature cycling (diurnal alternation of growth and low temperature treatments). For cold acclimation *in vitro*, it will be necessary to select regimes on a species, and sometimes genotype basis, this can be facilitated by LT_{50} testing shoot cultures and their meristems using a programmable freezer (22).
7. In the absence of an environmentally controlled cold chamber a standard refrigerator can be used (set at the minimal temperature ca. $0-4^{\circ}\text{C}$) and manual transfers of the cultures undertaken between the growth room (day) and refrigeration (night) conditions; thus creating a simulated diurnal acclimation cycle.
8. To avoid loss of acclimation status in shoots prior to cryogenic treatment, meristems excised from cold hardened cultures should not be exposed to lengthy periods of time at an inappropriate stage of their diurnal cycle before cryopreservation. Dissections may be best synchronized and scheduled for the light/higher temperature stage of the cycle.
9. Pre-growth (pretreatments) require optimization for different species and even genotypes within species. This can be facilitated by constructing a dose response experiment comprising a series of different osmotica (e.g. within the range $0.25-0.75\text{ M}$) selected from mannitol, sorbitol or sucrose (or other pre-growth additives such as proline, trehalose) to which the donor tissues, nodal cuttings or shoot tips/meristems are exposed for a time course (e.g. 1–7 days). After pretreatment, tissues are transferred to standard medium to assess which regime is optimal in achieving maximum effect with minimal loss of shoot regrowth. For some species and tissues pretreatments cause symptoms (bleaching, browning, necrosis at points of dissection injury, hyperhydricity) indicative of stress; these may not necessarily impair development or become lethal. The selected treatment is then tested for its effectiveness in supporting recovery.
10. Under circumstances where shoot tips dehydrate during and following dissection, they should be transferred to a “holding”

substrate or medium. It is important to ensure that stabilization of the meristem during excision does not expose tissues to inadvertent osmotic changes that would invalidate preparative regimes. This stage should maintain the appropriate osmotic equilibrium relative to pretreatment.

11. When dispensing CPAs, appropriate adjustments may be required to account for different specific gravities and densities of viscous liquids. The definition of specific gravity (SG) is generally the ratio of the density of a substance relative to the density of water, which is used as a reference standard at a specified standard temperature and pressure. The SI unit of kilogram per cubic metre (kg/m^3) or gram per cubic centimetre (g/cm^3) is a frequently used unit for density; one cubic centimetre corresponds to a volume of 1/1,000 of a litre or a millilitre ($1 \text{ cm}^3 = 1 \text{ mL}$). The density of a substance is also defined as its mass per unit volume. The density of water is ($1,000 \text{ kg}/\text{m}^3$ or $1 \text{ g}/\text{cm}^3$) equivalent to 1 kilogram per litre ($1 \text{ kg}/\text{L}$) or 1 gram per millilitre ($1 \text{ g}/\text{mL}$).

Preparing multicomponent % formulations on a w/v basis needs some additional care. For example PVS2 (30% glycerol; 15% ethylene glycol; 15% DMSO and 0.4 M sucrose) may be achieved (w/v) gravimetrically (e.g. using a balance) by weighing 30 g glycerol; 15 g ethylene glycol; 15 g DMSO and 13.7 g sucrose and making to a volume of 100 mL using basal liquid culture medium as the diluent.

Alternatively, as the liquid components (glycerol 1.258 g/mL, ethylene glycol 1.113 g/mL and dimethyl sulfoxide 1.100 g/mL) each have a different density > water, the preparation of PVS2 may be adjusted on a w/v basis and dispensed volumetrically as follows: 30% glycerol ($30 \text{ g}/1.258$) = 23.85 mL; 15% ethylene glycol ($15 \text{ g}/1.113$) = 13.48 mL; 15% DMSO ($15 \text{ g}/1.100$) = 13.64 mL and 13.7 g sucrose; these components are made up to 100 mL with basal liquid culture medium. It is cautionary to note, that with highly viscous CPAs a residual volume may be retained within the dispensing pipette or measuring equipment. It is important to ensure that the total amount of each component in PVS2 is fully dispensed. For CPA mixtures, it is convention that all components within the same formulation are prepared on the same basis e.g. either w/v or v/v.

When making CPA mixtures with pre-constituted (i.e. commercially formulated) media, it is good practice to describe how each cryogenic component is added, particularly when creating BPs and SOPs for technology transfer and validation exercises. For complex mixtures prepared in liquid culture medium, this involves making explicit the final concentration of each additive (as g/L or molarity) particularly sucrose which is used as both a CPA and carbon source. Tissue culture media

usually contain 3% (w/v) sucrose (30 g/L), which is equivalent to a concentration of 0.09 M sucrose solution. Preparing PVS2 on a w/v basis in basal liquid culture medium (with sucrose) would effectively increase the final concentration ($0.4 + 0.09$) to 0.49 M sucrose. Therefore, it is preferred to use basal medium, i.e. comprising all components (without sucrose) as the diluent. If the liquid CPAs of PVS2 are prepared on a w/v basis and made up using liquid culture medium as the diluent that already contains 0.4 M sucrose, the addition of the liquid CPAs would effectively reduce the final concentration of sucrose to ca. 0.2 M.

12. Correcting the pH of viscous cryoprotectant solutions should be undertaken with care to ensure sufficient mixing and correct pH adjustment.
13. The choice and size of cryovial is operator dependent. Cryoprotection and CPA toxicity trials are usually undertaken in cryovials to ensure compliance with stringently timed transfers of tissues and cryoprotective manipulations. The CPAs should be added in excess (e.g. 25–50% of capacity). Overloading vials is cautioned as this can cause them to rupture or explode on re-warming.
14. Ideally, CPA mixtures are added in one stage, e.g. as a 100% solution in cryovials to which the shoot tips or meristems are already transferred. In the case of genotypes susceptible to osmotic stress, CPAs may need to be added gradually. This can be achieved in several ways, most usually by the stepwise addition of a double-strength CPA mixture to an equal volume of meristems pre-bathed in liquid culture or if appropriate pre-growth medium. It is important to ensure with accuracy the final concentration of the CPA and that this is reproducibly achieved in validation exercises, across different samples and between different cryopreservation runs. Replacing the final, stepwise-added CPA with an aliquot of full strength CPA solution will make certain that tissues are optimally exposed at the maximum concentration.
15. The efficacy, toxicity, and permeability of CPAs can be affected by the temperature and duration of application; these factors will need to be considered on a case-by-case basis.
16. Standardizing the application of CPAs and testing the effects of non-compliance concerning technical variables is critically important, particularly when scaling up the number of cryovials handled by large gene banks. For some species, reasonable variation in CPA application can be tolerated, whereas for others they may be critical. Tolerance to variability and technical non-compliance should be ascertained and points of caution incorporated into SOPs.

17. Programmable freezers consist of a freezing chamber cooled by LN, sample and chamber thermometers, an electronic computer programming and output device (chart recorder/PC). Rates of cooling and terminal transfer temperature (including hold times) are critical factors to successful controlled rate cooling, and they should be developed on a case-by-case basis.
18. The temperature of “seeding” or ice nucleation manifest as the latent heat of crystallization of extracellular ice is a critical event because it marks the onset of intracellular dehydration. This is an important factor in developing controlled cooling methods for shoot tips and meristems. Once formed, extracellular ice causes a vapour pressure deficit that is compensated by the movement of intracellular water to the outside of the cell. The effect is “cryoprotective” because it reduces the amount of intracellular water available to form ice. Without intervention, extracellular ice nucleation occurs randomly leading to variable freezing responses; therefore, the control of nucleation can be a critical factor in slow freezing. Some programmable freezers are fitted with a device that automatically initiates nucleation by mechanically agitating the cryogenic samples or exposing the chamber to a transient reduction in temperature. Touching the outside of the cryovial with a LN-chilled instrument can manually induce nucleation. The point of homogeneous ice nucleation (ca. -40°C) can also be ascertained with reasonable accuracy using a programmable freezer.
19. Cryovials should be plunged immediately into LN once they are removed from the programmable freezer, as inadvertent re-warming of samples is detrimental to the tissues. For convenience, a small Dewar (e.g. of 1 L capacity) can be located near the programmable freezer and used to transport vials to the cryostore.
20. Washing thawed and re-warmed shoot tips or meristems to remove CPAs may be injurious; to reduce osmotic shock, the tissues are transferred to an appropriate diluent. Removal of CPAs by capillary action using filter papers overlaid on medium and daily transfer to fresh medium can facilitate early recovery stages.
21. Performance indicators for successful shoot tip/meristem cryopreservation are shoot regrowth and plant development arising from the original meristem. Survival can be indicated by morphological changes that do not support further development, e.g. leaf expansion, callus and root formation, whilst these indicate viability, sustained regrowth must be confirmed. This should proceed via the original meristem (primary or axillary) and not (unless desired) through an indirect route involving adventitious shooting and organogenesis from dedifferentiated callus. This assessment criterion is important to ensure the

genetic stability of plants regenerated from cryopreserved tissues (88). Quantitatively acceptable levels of recovery are important. Regrowth of shoots and plants approaching 40–50% of the total number of shoot tips cryopreserved may be considered encouraging and acceptable for certain gene banks.

22. It is commonly assumed that the protective modality of PVS2 is (osmotic) dehydration. However, evidence is accumulating (53) to suggest that it also has colligative properties and changes the behaviour and reduces the mobility of water.
23. LS composition requires careful optimization to avoid excessive osmotic changes.
24. Adding chilled solutions on ice may reduce the toxic effects of vitrification cocktails. It may be possible to expose tissues to higher concentrations of PVS2 if applied at 0°C.
25. The shoot tips and meristems of some genotypes do not survive direct exposure to 100% PVS2; in these cases, a toxicity test should be performed, this involves the step wise addition of PVS2 solutions of increasing strength. Stringently timed periods of exposure at a stable temperature (e.g. on ice, to offset ambient fluctuations) are required in order to identify the optimal dosage and duration.
26. Treatment with an appropriate ULS to offset the risks of osmotic shock is essential.
27. The solvent used to prepare PVS2 and PVS3 can be variable, ranging from water to liquid culture medium (53, 54, 58, 59).
28. Achieving a uniform bead size is critical for protocol reproducibility and quality control; this is particularly important for inter-laboratory technology transfers and validation exercises. Size differences in beads lead to non-uniform desiccation profiles during air and silica gel drying. It is important to be consistent by using the same or similar brand of Pasteur pipette or automatic pipette tip (e.g. with respect to dispensing aperture size). The authors' use Fisherbrand 3-mL plastic pastettes. Any similar product will suffice, so long as bead size uniformity is achieved; adjusting the pipette tip diameter by cutting with a scalpel can create beads that accommodate larger shoots.
29. This involves the preparation of calcium-free liquid medium (Ca^{2+} salts of vitamins accepted). The presence in media of Ca^{2+} derived from nutrients causes alginate to polymerize at an undesirable stage of the process. It is important to use the appropriate alginate product: 3% (w/v) sodium alginate (SIGMA® 2% low viscosity kelp alginate, as the sodium salt).
30. Preparing alginate solutions can be problematic because it is difficult to solubilize; this is best achieved by (1) either adding the alginate to liquid medium on a magnetic stirrer and heating

to boiling whilst agitating vigorously and adding step-by-step, small amounts of alginate (2) or adding all the alginate to the solution which is vigorously agitated by shaking and then autoclaved. Discard any flocculated alginate solutions.

31. Determination of water loss is important for the development of this protocol. This is achieved using a drying oven set at an appropriate oven temperature (e.g. 105°C) and drying to the level of minimally consistent water loss. Lower temperatures and insufficient drying times will underestimate bead water content. Moisture content status should be accurately determined on a fresh and dry weight basis as appropriate. In humid environments, transfers to and from the drying ovens should be undertaken over activated silica gel desiccators.
32. Desiccation can be determined by assessing changes in residual moisture (RM) of a batch of empty (without meristems) beads before and after desiccation. This can be most accurately determined for individual beads on the basis of fresh and dry weights for each desiccation time (e.g. $t=0, 1, 2, 3, 4$ h) as follows:

$$\begin{aligned} &\text{Residual moisture (\% fresh weight)} \\ &= \frac{\text{fresh weight (g)} - \text{dry weight (g)}}{\text{fresh weight (g)}} \times 100. \end{aligned}$$

33. For quality control purposes, desiccation profiles should be determined when working in different laminar airflow benches and/or laboratories.
34. Alginate beads become extremely hard when desiccated making them difficult to manipulate.
35. Failure to maintain environmental quality control parameters (RH and temperature) will result in non-uniform bead desiccation. An alternative option in high RH environments is to desiccate beads over activated silica gel.
36. Encapsulated shoot tips and meristems may be re-warmed at ambient temperatures without the need for a water bath.
37. Following re-warming, beads can be rehydrated by placing in liquid culture medium for ca. 30 min before transfer to recovery medium.
38. Regenerating shoots usually emerge from the bead however, for some plants careful dissection from the alginate may be necessary to allow regrowth to proceed.
39. Reduced levels of alginate e.g. 2% (w/v) may be used in the beads.
40. Droplet protocols involve direct immersion of the foils and droplets into LN-filled cryovials and maintaining the LN level in the vials on closure. This has the advantage of achieving ultra rapid cooling rates and the temperature of LN throughout

manipulations. However, this procedure may contravene Health and Safety guidelines for some countries and their institutions. For example, regarding risk mitigation and following cryovials manufactures' guidelines not to fill cryovials with LN at the risk of operator exposure to LN and the breach or explosion of LN-filled cryovials.

41. LN-filled cryovials risk explosion on re-warming.
42. Green tissues do not necessarily confirm viability, as some oxidation breakdown products of chlorophyll pigments can still remain green (95) even during senescence and following lethal injury.
43. To avoid false positives, due to the delayed demise of lethally damaged meristems, viability assessment is best undertaken at a minimum of 7 days following retrieval from LN.
44. Swelling, greening, and leaf expansion are not necessarily reliable predictors of continued and sustained shoot regrowth after cryopreservation.
45. Delayed onset degeneration (11) and death of recovering shoot tips and meristems can occur particularly in sensitive genotypes. It is prudent to continue weekly assessments for a minimum of 4–8 weeks to confirm sustained recovery.

Disclaimer

Information concerning risk management is incorporated solely as general information; it is ultimately the responsibility of readers to ensure that safety and regulatory issues have been addressed in accordance with their institutional, national, and international obligations. Mention of any trade names or commercial products is for the sole purpose of providing scientific information and does not imply any recommendation or endorsement by the authors.

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Chapter 17

Anther Culture of Chili Pepper (*Capsicum* spp.)

Neftalí Ochoa-Alejo

Abstract

Chili pepper (*Capsicum* spp.) is a very important horticultural crop around the world and is especially important for Mexicans because of its impact in the culture and the cuisine. Biotechnological tools such as tissue culture techniques and specifically anther culture may be applied successfully for plant breeding and genetic improvement in order to generate isogenic lines (100% homozygous) in a shorter time in comparison with the classic breeding methods. In this chapter, a protocol for efficient recovery of chili pepper haploid plants from in vitro cultured anthers is described.

Key words: Anther culture, Androgenesis, Capsicum, Chili pepper, Haploids, Plant regeneration

1. Introduction

Haploid plants of chili pepper have been reported to occur spontaneously as a result of parthenogenetic development of an egg or a haploid accessory cell of the gametophyte, or from sperm cells (1, 2), and some doubled haploid plants have also been recovered (2, 3). This spontaneous haploidization phenomenon has been observed in twin chili pepper seedlings at low frequency (1, 4). The use of haploidy in the breeding of chili pepper is bound with the possibility of getting haploid plants at high rates in materials of high agronomic value (4). A biotechnological approach to accomplish this goal more efficiently is to apply in vitro anther culture. The advantages of using this biotechnological tool for chili pepper breeding and improvement have been previously described (5).

Anther culture is an important tool for plant breeding and crop improvement, since it allows the generation of homozygous lines in shorter time, by doubling of chromosome number of haploids, than the classic self-fertilization procedure (6), or than the

traditional back-crosses used to fix or to stabilize the desired traits in hybrid plants derived from parental crosses (7). This in vitro system also allows for the rescue of plant recessive mutants that otherwise should be very difficult or almost impossible to be obtained by traditional methods (8–11).

Chili pepper (*Capsicum* spp.) is a very important horticultural plant to which anther culture techniques have been applied in order to generate haploid or doubled haploid plants for crop improvement, breeding programs (12), or genetic mapping (13). Wang et al. (14), and George and Narayanaswamy (15), independently, were the first to report on chili pepper anther culture and plant regeneration. In the first case, anthers of *Capsicum annuum* var. Yeo Hsien Small Redpepper showing pollen at the uninucleate stage were cultured on a medium containing combinations of 1 mg/L kinetin + 40 mg/L RNA-nucleotide mixture prepared by enzyme treatment, and 1 mg/L 2,4-D or NAA. After incubation for 2 weeks, both callus and embryos were observed on the cultured anthers with an efficiency of 28.6% and 4.8%, when the medium was supplemented with NAA, and 23.5% and 2.6%, respectively, in the presence of 2,4-D. The regenerated plants showed an $n=12$ chromosome number. George and Narayanaswamy (15) were able to regenerate haploid embryos from *C. annuum* L. var. *groszum* from young immature anthers containing uninucleate pollen and cultured on LS basal medium (16) supplemented with kinetin (0.3 and 3.0 mg/L) combined with IAA (1.0 and 3.0 mg/L); further development of haploid embryos to render haploid plants was attained after frequent transfer on LS medium containing 0.5 mg/L IAA + 400 mg/casein hydrolysate + 100 mg/L inositol. Haploid plant regeneration was a rare event under these conditions. After these reports, a series of protocols have been established by different authors, among them, those described by a French group (17–19) have been the most frequently used; however, further improvements have been reported by other authors (12, 20–25). Additionally, conditions for chili pepper plant regeneration from microspore culture have also been reported (26, 27).

A protocol for chili pepper plant regeneration from anther culture that has shown to be effective for haploid plant recovery is described in this chapter.

2. Materials

2.1. Biological Material

1. Chili pepper seeds from different cultivars (*Capsicum* spp.).

2.2. Glassware

1. Erlenmeyer flasks (500–1,000 mL).
2. Glass bottles (140 mL).

3. Petri dishes (100×15 mm).
4. Beakers (500–1,000 mL).

2.3. Instrumentation

1. Analytical balance.
2. Hot plate stirrer.
3. pH-meter.
4. Autoclave.
5. Stereomicroscope.
6. Forceps.
7. Scalpel.
8. Laminar flow cabinet.
9. Bunsen burner.
10. Micropipettes.

2.4. Chemicals

1. Plant growth regulators (2,4-D and IAA).
2. MS culture medium components (mineral salts and organic additives).
3. Cysteine.
4. Cyanocobalamin.
5. Casein hydrolysate.
6. Commercial bleach solution (Cloralex®).
7. Tween 20.
8. Captan.
9. Foliar fertilizer.

3. Methods

3.1. Seed Germination and Plant Growth

Chili pepper seeds are germinated on wetted filter paper in Petri dishes (100×15 mm), in the presence of a fungicide (Captan). Petri dishes are sealed with Vitafilm® and are incubated in a growth room at 25°C and under a photoperiod of 16 h light (daylight fluorescent lamps; 70 $\mu\text{mol}/\text{m}^2 \text{ s}$)/8 h darkness. After seed germination, the seedlings are transferred to plastic trays containing 5 cm³ of peat moss (Sunshine, Canada) in each compartment, and the seedlings are maintained under the same conditions until they reach 15 cm in length. Plantlets are then individually transferred to plastic pots (4–5 L) with peat moss and grown in a greenhouse until flowering. Chili pepper plants are fertilized twice a week using a foliar fertilizer (FerviaFol 20-30-10 NPK with micronutrients (0.2% Fe, Zn, and Mg; 0.05% Cu and Mn; 0.1% B; 0.01% Mo; and

0.001 Co); 8 g/20 L; 200 mL per plant). Floral buds (3–4 mm in length) are collected when the sepals and petals have the same length or when the sepals (2 mm) are slightly bigger than the petals. At this stage, the floral buds have anthers containing uni-nucleate microspores, which are more responsive to undergo androgenesis in vitro.

3.2. Anther Culture

Floral buds are disinfected with a 50% (v/v) bleach solution (NaOCl, 6% active Cl₂) with 0.1% Tween 20 for 30 min in agitation, and then they are rinsed three times with sterile distilled water under aseptic conditions. Floral buds are dissected with forceps and scalpel using a stereomicroscope in order to carefully get the anthers avoiding wounding them. Anthers (20 per 100 × 15 mm Petri dish) are placed on MS (28) sterile semisolid medium supplemented with 0.01 mg/L 2,4-D, 0.03 mg/L cyanocobalamin (vitamin B₁₂), and 1 mM L-cysteine, adjusted at pH 5.6 (see Note 1). Petri dishes are sealed with Vitafilm® and the cultures are preincubated in dark conditions at 4°C for 2 days, then at 35°C for 5 days, and at 25°C for 8 days. Finally, the cultures are incubated in a growth room under a 16:8 h light–dark photoperiod (daylight fluorescent lamps; 70 μmol/m² s) at 25°C until embryo regeneration occurs (1–3 months). The frequency of embryo formation using this protocol is approximately 2% of the total cultured anthers, but this value may be higher (4–7%) or lower (1%) depending on the chili pepper material used as a source of anthers. After regeneration, the emerging embryos from the anthers are carefully taken up with forceps, avoiding wounding, and are cultured on MS medium supplemented with 0.1 mg/L IAA and 50 mg/L casein hydrolysate in glass bottles (20 mL culture medium in 140-mL baby food jars) to allow further development into plantlets. Finally, the regenerated plantlets are transferred to pots with sterilized peat moss in order to be adapted to ex situ conditions. Pots are covered with plastic bags, and the plantlets are gradually exposed to a lesser relative humidity until they become well adapted in the greenhouse.

This protocol has also been very efficient for in vitro embryo and plant regeneration from anther cultures of the solanaceous Mexican husk tomato (*Physalis ixocarpa* Brot.) (29).

4. Note

1. All culture media were adjusted to pH 5.6 and sterilized in autoclave at 1.1 kg/cm² and 121°C for 20 min.

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Chapter 18

Production of Interspecific Hybrids in Ornamental Plants

Juntaro Kato and Masahiro Mii

Abstract

In breeding of ornamental plants, interspecific hybridization and polyploidization have successfully been used to produce novel cultivars with blended traits of both parents and to introgress useful traits of one species to another. Embryo rescue techniques and molecular cytogenetic methods have successfully been used to produce and characterize interspecific hybrids in various genera. In this paper, recent advances in interspecific hybridization are described based on the results obtained in *Primula*, *Cosmos*, and *Kalanchoe* with special references to the use of embryo culture techniques for rescuing the abortive hybrid embryos. The methods for production and characterization of interspecific hybrids are categorized into three steps, i.e., (1) pollination, (2) rescue culture of immature embryo, and (3) confirmation of hybridity and ploidy level of the plants obtained. For interspecific crosses, emasculation step is usually needed to avoid self-pollination even in the genera with self-incompatibility system, such as *Primula* and *Cosmos*, since self-incompatibility is not always complete. Since interspecific crosses are usually hindered by various cross-incompatibility mechanisms, successful production of interspecific hybrids could be achieved only from limited crosses among those using many cultivars/strains of both parents, suggesting the importance of the selection of the compatible genotypes. Unilateral cross incompatibility is commonly observed in interspecific cross combinations, so reciprocal crosses should be conducted as an indispensable step. At the rescue culture step, addition of plant hormones, e.g., auxin cytokinin and gibberellin, to the culture medium at the appropriate concentrations is proved to be effective and necessary. The hybridity of the plants is efficiently confirmed at the seedling stage by DNA analysis in addition to the observation of morphological characters. The analysis of relative DNA contents by flow cytometry is an easy and rapid means to confirm hybridity and to estimate ploidy level and genomic combination.

Key words: *Cosmos*, Embryo rescue, Flow cytometry, Interspecific hybrid, *Kalanchoe*, PCR-RFLP of ITS, Plant hormone, *Primula*

1. Introduction

Production of interspecific hybrids is useful not only for producing novel cultivars with intermediate characters between the parental species, but also to induce introgression of the useful genes possessed by one species to another species through alien chromosome addition line. After successful production of interspecific

hybrids, several methods have been used for the breeding of novel cultivars. In the case where interspecific F_1 hybrids are fertile, progenies of F_2 and further generations are produced with shuffling of whole genes from both parents through repetition of meiosis and fertilization. For example, *Primula x polyantha*, which is one of the most popular primulas, might be originated from fertile interspecific hybrids between *P. vulgaris* and *P. veris* (1), and numerous seed propagation cultivars have been bred in this hybrid species without any problem. Therefore, genomic difference between these two parental species is considered to be small or none. In contrast, when interspecific F_1 hybrids are completely or incompletely sterile, it is considered that genomic composition of the two parental species is more or less different. In such case, the sterile F_1 progenies with desirable characters are needed to propagate vegetatively if they are worth to be novel cultivars. Sterile interspecific hybrids are also utilized through chromosome doubling, which can yield amphidiploids with restored fertility. Although progenies derived from self-pollination of the amphidiploid (allotetraploid) are uniform when genome differentiation between the parental species is complete, progenies with various characters are frequently obtained because genome differentiation between the parents may mostly be incomplete in the case where interspecific hybridization is possible.

It has sometimes been reported that interspecific hybrids with unexpected ploidy level, e.g., triploid, were produced presumably by the fertilization of unreduced gametes (2, 3). The triploid hybrids consisting of two different genomes are useful as parental plants for producing alien chromosome addition lines by recurrent backcrossing.

Plant materials originated from interspecific hybridization may be utilized to obtain useful information for the molecular genetics analysis. In the case where interspecific hybrid has fertility, differences in various DNA markers between both parents, which may be segregated in the next generation, can be used for making linkage map of the markers at F_2 generation. On the other hand, if interspecific hybrid is sterile, it can be utilized to produce chromosome addition lines, which is useful to identify the chromosome of located objective character (4), through production of its amphidiploid by chromosome doubling treatment followed by recurrent backcrossing with either parent. Molecular phylogeny sometimes gives us effective information to produce interspecific hybrid. In *Cosmos*, comparison of PS-IDs sequence (5) showed close relationships between parents of the interspecific hybrid (6).

For the production of interspecific hybrids, however, there exist various reproduction barriers, which are classified into two categories, pre- and postfertilization barriers. The prefertilization barriers include failure of pollen germination on stigma, and arrest of pollen tube growth on stigma, in style, or in ovary. The postfertilization

barriers include defectiveness or abnormal growth of endosperm, and abnormal growth or abortion of hybrid embryo by genetic unbalance. Moreover, there exist other types of barriers, such as failure of seed germination and lethality of seedlings caused by abnormal development of shoots or roots at the early stage of germination. In the cross combinations with postfertilization barriers, embryo rescue culture techniques including in vitro seedling have been effectively used to obtain interspecific hybrid plants. For example, in interspecific reciprocal crossing between *P. veris* × *P. elatior*, unilateral incompatibility is known for the cross using *P. elatior* as a mother plant (7), in which lethality of hybrid embryo is caused by unbalance between rapid embryonic development and slow endosperm formation. When hybrid seeds obtained in interspecific crossing between *P. sieboldii* and *P. kisoana* were sown on soil, most individuals died after germination without emerging true leaves (Mii unpublished data). These results suggest that embryo rescue culture is important for most interspecific hybridization in wide crosses of ornamental plants. In this paper, we describe the detailed embryo rescue methods used for successful hybridizations in each cross combination tried in three genera, *Primula*, *Cosmos*, and *Kalanchoe*, and at the end, rescue methods of abnormal seedling, which resulted in the successful production of normal and useful hybrid between *Primula filchnerae* and *P. sinensis*.

2. Materials

1. Paraffin paper bag or sewing thread.
2. Scissors.
3. Fine wire for flower arrangement.
4. Pollen parent plant.
5. Ovary parent plant.
6. Laminar flow cabinet.
7. 110 mm filter paper No. 2 (Advantec Co.; JAPAN).
8. 70% EtOH.
9. Forceps (250 mm).
10. Autoclaved sterile distilled water.
11. Sodium hypochlorite solution (1% available chlorine concentration) with two drops of Tween 20 (Sigma Co.) per 100 mL.
12. 30-mL glass vial for tissue sterilization.
13. Parafilm.
14. 120-mm glass dish containing filter papers, which is sterilized with dry heat at 150°C for 1 h.

15. Chemicals and equipment for preparing Murashige and Skoog (MS) medium (8).
16. Gibberellin A₃, which is stored at 4°C in the dark.
17. 100 mg/L 1-naphthaleneacetic acid (NAA) dissolved by 1 mL ethanol, which is diluted with distilled water and stored at 4°C.
18. 100 mg/L 6-benzylaminopurine (BA) dissolved by 1 mL ethanol, which is diluted with distilled water and stored at 4°C.
19. 100 mg/L zeatin dissolved by 1 mL ethanol, which is diluted with distilled water and stored at 20°C.
20. Sucrose.
21. Gellan gum.
22. Beaker for waste fluid.
23. DNA extraction equipment.
24. Polymerase chain reaction (PCR) equipment.
25. DNA polymerase, e.g., ExTaq polymerase (TAKARA Co.; Japan), KOD-plus DNA polymerase and KOD-FX DNA polymerase (Toyobo Co.; JAPAN).
26. Several kinds of restriction endonuclease.
27. Agarose gel electrophoresis equipment and/or DNA PAGE equipment.
28. DNA detection equipment.
29. Flow cytometric (FCM) analysis equipment, e.g., Type PA (Partec Co.; Germany).

3. Methods

The methods are described in the following orders: (1) artificial crossing, (2) embryo and immature seed rescue culture, (3) confirmation of hybridity by PCR-RFLP of ITS region, (4) confirmation of hybridity and ploidy level by FCM, and (5) rescue of abnormal seedling.

3.1. Artificial Crossing

For artificial crossing, two-step procedure, (i.e., emasculation and pollination) is required. Because most species of *Primula* have dimorphic heterostylous self-incompatibility system (2, 9), legitimate crosses are commonly used for interspecific hybridization. However, even in such cross combination, emasculation is needed because seed parent-type progenies are sometimes obtained by wide cross combination.

In the case of *Cosmos*, which includes self-incompatibility species mostly, pollination is performed without emasculation

because each head flower has numerous small anthers, which makes perfect emasculation difficult. Consequently, hybrids should be selected from germinated seedling population.

3.1.1. Emasculation

Emasculation is performed to exclude the possibilities of self-pollination. For emasculation, flower buds 2–3 days before anthesis are incised, and the immature anthers are carefully removed by forceps. Then, each emasculated flower bud is tied up at the tip of corolla by sewing thread or it is covered with a paraffin paper bag and the base of the bag is tied with a flower arrangement wire. If seed parent plant shows complete pollen sterility, this procedure is not necessary. In self-incompatibility plants, self-incompatibility system sometimes fails to function, which is called as mentor effect usually performed in wide cross combinations. Therefore, they should also be emasculated before pollination (see Note 1). In interspecific hybridization of *Kalanchoe*, because maturation of stigma occurs later than that of pollen, emasculation can be conducted after blooming (10). After emasculation, however, the seed parent plant must be isolated alone to avoid unexpected natural crossing with pollen of other individuals.

3.1.2. Crossing

Two or three days after emasculation, the stigma of the seed parent is pollinated with the pollen of partner parent by holding the stamen with forceps. In interspecific hybridization of *K. laxiflora*, the stigma requires 2–3 weeks to become acceptable of pollen at the flowering stage (11). After pollination, the flower is protected from unexpected pollination by tying up corolla or bagging by a flower arrangement wire again. In interspecific hybridization of *Cosmos*, the step of emasculation might be skipped and pollination is conducted by rubbing flowers of pollen parent against those of seed parent (6).

Ovules or immature seeds in enlarged ovary are used for rescue culture (see Notes 2 and 3). The timing for the culture of target ovules is different depending on the cross combination, i.e., 3–4 weeks in the case of *Primula filchnerae* × *P. sinensis* (12), 10 days after pollination in *Cosmos* (6), and 3 weeks in the crosses using *Kalanchoe blossfeldiana* as the seed parent (10). In the cross between *K. spathulata* and *K. laxiflora*, mature seeds can be obtained. However, the time required for maturation is different depending on the seed parent, i.e., 40 days after pollination in *K. spathulata* and 60 days in *K. laxiflora* used as seed parent (11).

3.2. Rescue Culture of Embryo or Immature Seed and In Vitro Sowing

3.2.1. Preparation of Medium

In interspecific hybridization of *Primula*, appropriate amounts of gibberellin A₃ and the stock solutions of NAA and BA are added to half-strength MS medium (1/2 MS) containing 5% (w/v) sucrose to give the final concentrations of 50 mg/L, 0.1 mg/L, and 0.1 mg/L, respectively (2, 3). After pH of culture medium is adjusted to 5.6–5.8, gellan gum is added to the culture medium at

Table 1
Composition of culture media for *Primula*, *Cosmos*, and *Kalanchoe*

	<i>Primula</i>		<i>Cosmos</i>	<i>Kalanchoe</i>
	Intrasectional	Intersectional		
Basal medium	1/2MS	1/2MS	MS	1/2MS
Solidifying material	0.25–0.3% gellan gum	0.25–0.3% gellan gum	0.2% gellan gum	0.8% agar
Sucrose	3%	5%	2%	1.5%
Gibberellin A ₃	50 mg/L	50 mg/L	–	–
NAA	–	0.1 mg/L	0.1 mg/L	–
BA	–	0.1 mg/L	1.0 mg/L	–

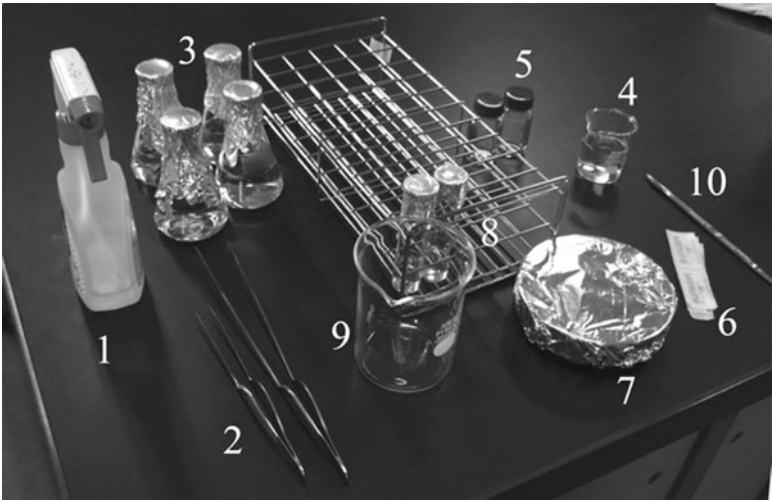


Fig. 1. The materials needed for sterile culture: (1) 70% EtOH, (2) forceps, (3) autoclaved sterile distilled water, (4) sodium hypochlorite solution, (5) 30-mL glass vial, (6) parafilm, (7) 120-mm glass dish containing filter papers, (8) medium, and (9) beaker for waste fluid.

0.2% (w/v) final concentration. After dissolving gellan gum completely in boiling water bath, medium is dispensed to each culture tube and autoclaved. In interspecific hybridization of *Cosmos*, MS medium supplemented with 0.1 mg/L NAA, 1.0 mg/L BA, and 20 g/L sucrose and solidified with 8 g/L agar (pH 5.8) is used for embryo rescue culture (6). For ovule culture and in vitro sewing in interspecific hybridization of *Kalanchoe*, 0.8% agar-solidified 1/2 MS medium supplemented with 1.5% sucrose is used (10, 11). Table 1 shows differences in medium components among *Primula*, *Cosmos*, and *Kalanchoe*.

*3.2.2. Embryo and Immature
Seed Rescue Culture*

The materials needed for sterile culture are shown in Fig. 1. Immature ovaries attaching ovules with developing hybrid embryos

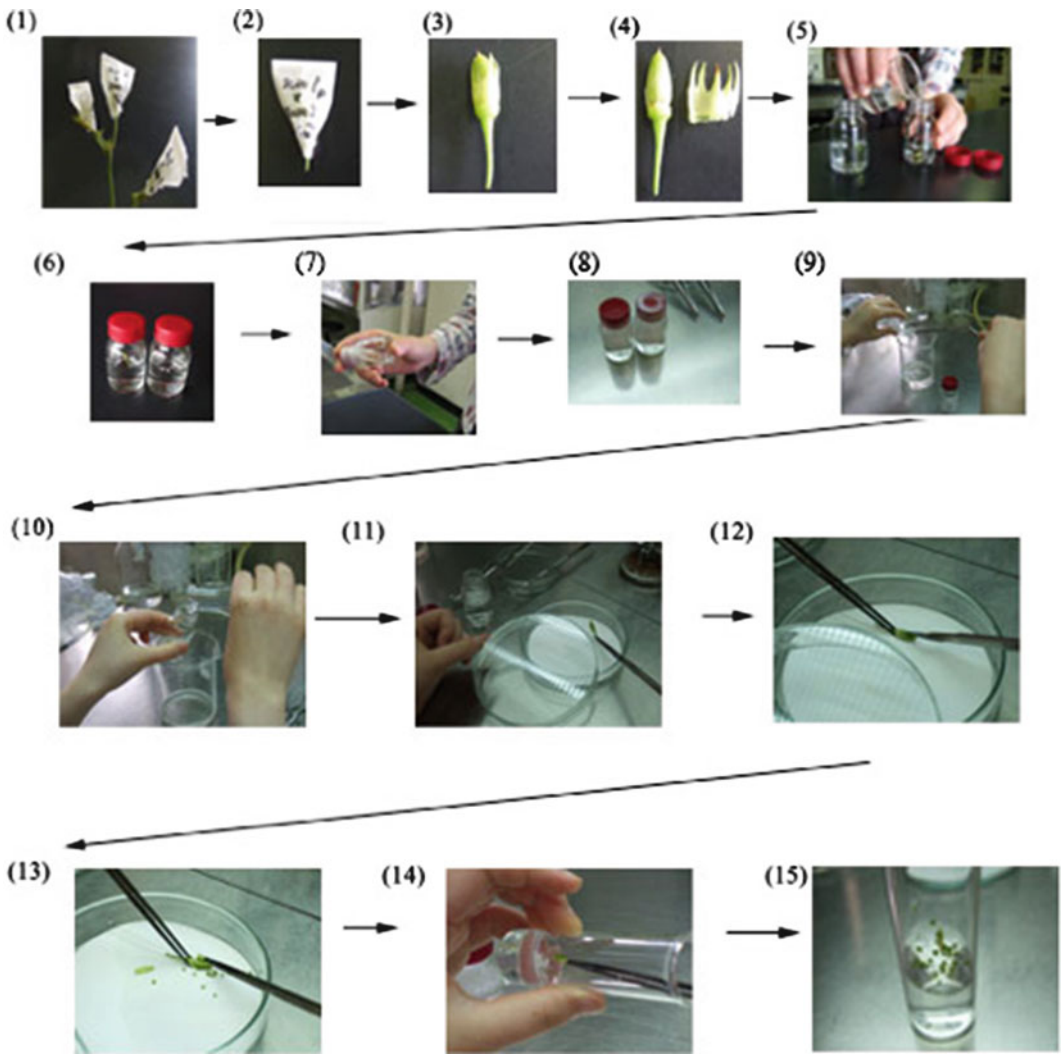


Fig. 2. Procedure of sterile culture.

are collected at an optimal timing after pollination in interspecific crosses (see Fig. 2, 1 and 2). The timing of collection of ovaries is differed according to the cross combinations. Optimum timing should be determined by examining the status of embryo development or simply changing the timing for the culture. After removing sepals and remnant of petals (see Fig. 2, 3 and 4), ovary is put into a vial containing sodium hypochlorite solution (about 15 mL) and the vial is closed with a plastic cap (see Fig. 2, 5 and 6). Then, the ovary is sterilized for 15 min with initial hand shaking for 2 min (see Fig. 2, 7 and 8). In interspecific hybridization of *Kalanchoe*, the ovary or mature seeds are sterilized for 5 min by sodium hypochlorite solution (0.5% available chlorine) with a drop of

Tween 20. In the case of *Cosmos*, tubular florets with enlarged ovaries are collected and surface sterilized by soaking them in 70% (v/v) ethanol for 20 s, followed by 10 min in hypochlorite solution (1% available chlorine) with 0.01% (v/v) Tween 20, and then rinsing them three times in sterilized water (see Note 4). After washing with sterile distilled water twice (see Fig. 2, 9 and 10), the ovary explant is transferred by a forceps onto wet sterilized filter paper with sterilized distilled water (see Fig. 2, 11). Then, ovary wall is carefully removed by using a forceps and a knife to deliver the placenta with ovules (see Fig. 2, 12 and 13). The placenta explant is then plated on culture medium in a tube (see Fig. 2, 14 and 15). The cultures are kept at 20°C under weak light condition (ca 300–500 lx). In the case of *Cosmos*, ovary wall is easy to remove by excising the surface of ovary in a tubular floret by forceps. Then, ovules containing embryos are placed on culture medium in a tube (6, 10, 12). Seedlings or sometimes calluses are obtained from these ovules.

4. Confirmation of Hybridity by RAPD Analysis and PCR-RFLP of ITS Region

4.1. RAPD

DNA is extracted using a commercial kit. For the RAPD analysis (13), the ExTaq DNA polymerase (Takara) is employed, in which a 25 µL reaction solution containing 1 ng of total plant DNA is subjected to analysis according to the manufacturer's protocol. The primers used for RAPD analysis are kits of Operon 10-mer primers (Operon) or arbitrary 12-mer primers (COMMONS primer (14); BEX).

DNA fragments are amplified by repeating 40 cycles of the following thermal treatments: 94°C for 30 s, 42°C for 30 s, and 72°C for 1 min, in a thermal cycler. Electrophoresis of the amplified DNAs is conducted on a 5% acrylamide gel or 1% agarose gel (2, 6). Hybridity of the plant is confirmed by detecting the specific bands derived from both parents (see Note 5).

4.2. PCR-RFLP of ITS Region

The ITS regions are frequently used for making phylogenetic trees because these regions are commonly polymorphic among species. The hybridity of the plants obtained from interspecific crosses can be confirmed by PCR-RFLP of ITS region after digestion with appropriate restriction enzymes.

The ITS region of nr DNA is amplified via PCR. PCR is performed by the procedures of Kita et al. (15) with little modification. Primer sequences are 5'-ACGAATCATGGTCCGGTG AAGGTGTTTCG-3' for ITS AB101 and 5'-TAGAATTCCTCCG TTCGCTCGCCGTTAC for ITSAB102 as reported by Douzery et al. (16). Amplification is carried out using the two-step method by using Takara PCR Personal (TAKARA) with one cycle at 94°C

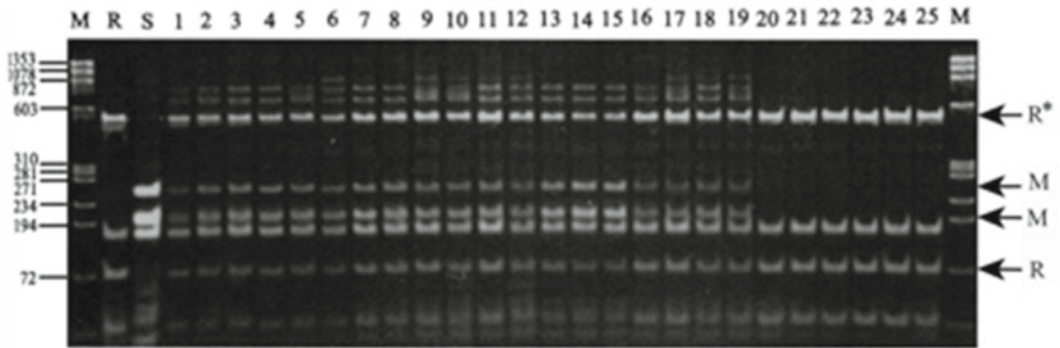


Fig. 3. Hybridity analysis of the plants derived from interspecific crosses between *Primula rosea* and *P. modesta* by PCR-RFLP of ITS region digested by restriction endonuclease HhaI (CGCG). R: *P. rosea*, S: *P. modesta*, 1–19: interspecific hybrids, 20–25: no hybrid plants. M: size marker, arrowhead of R: specific bands of *P. rosea*, arrowhead of M: specific band of *P. modesta*.

for 2 min followed by 40 cycles of 30 s at 94°C and 1.5 min at 68°C (see Note 6). Finger printing of DNA in ITS region is displayed by electrophoresis after digestion of PCR products by specific restricted endonuclease. Because available endonuclease may be differed among cross combinations, trial of several kinds of endonuclease is recommended to confirm the hybridity (see Note 7). In confirmation of hybridity in interspecific hybridization between *Primula denticulata* and *P. modesta* (17) and in backcrossing of 3× hybrid between *P. denticulata* and *P. rosea* with the pollen of *P. denticulata* (18), *Msp* I (Toyobo) and *Hha* I (Takara) are used to detect specific bands of both parents, respectively (Fig. 3).

5. Confirmation of Hybridity and Ploidy Level by FCM

It is common that different species have different DNA contents each other even in the same genus (e.g., *Lilium* (19), *Petunia* (20), and *Primula* (3, 12)). If interspecific hybrids are produced between two species with different DNA contents, they should theoretically show the intermediate value. However, hybrids with unexpected ploidy levels, such as triploid and tetraploid, are rarely formed in interspecific crosses (2, 3). In such cases, it is possible to estimate the genomic combination of the hybrids based on the DNA content of each parental species by using FCM analysis (see Fig. 4).

For FCM analysis using UV detector such as type PA flow cytometer (Partec Co., Germany), the following procedures are usually adopted. Approximately 0.5-cm² segments are excised from a fresh leaf, put into plastic Petri dish with 0.2 mL of nuclei extraction buffer “CyStain UV Precise P” (Partec Co., Germany), and chopped with a razor blade, to which 1 mL solution B of 4',6-diamidino-2-phenylindole (DAPI) staining “High resolution

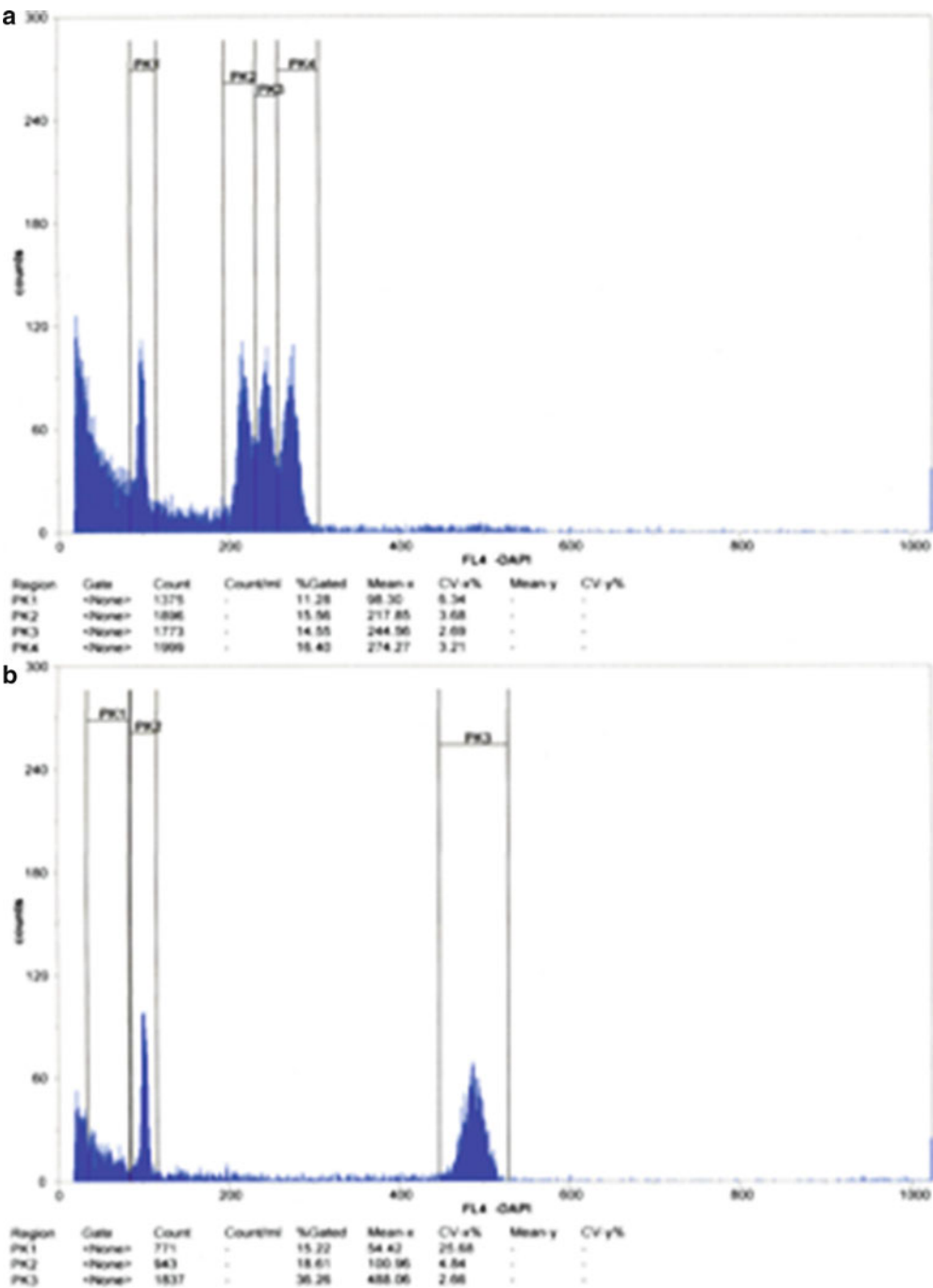


Fig. 4. Flow cytometric profiles of confirmation hybridity and ploidy analysis by Cyflow space (Partec). (a) Interspecific hybrid confirmation between *Primula filchnerae* and *P. sinensis*, (b) tetraploid progenies originated from five-time self-pollinations of polyploidized interspecific hybrid (4×) by somatic chromosome doubling. PK1 of (a) and PK2 of (b): the peaks of internal standards; PK2-4 of (a): the peaks of parents (PK2 and PK 4) and interspecific hybrid (PK3); PK3 of (b): tetraploid progenies.

DNA Kit Type P” (Partec Co., Germany) is added. After a 1- or 2-min incubation, the solution mixture is filtered with a 30- μ m mesh and subjected for measuring DNA content according to the manufacturer’s protocol. For the accurate comparison of the DNA contents, it is recommended to mix the tissue sample of the hybrid with those of the parents and subject to the analysis at the same time. Estimated ploidy level should also be confirmed by counting the chromosome numbers (see Note 8). Some species show peaks with noise, which sometimes makes it difficult to determine the accurate DNA content. When noise peaks are detected, use of leaves with different ages, such as newly developing leaf, might be effective. The addition of 20 mM dithiothreitol (DTT) into nuclei extraction buffer may also solve the problem in case if inhibition of oxidization by DTT is effective.

6. Rescue of Abnormal Seedlings

Seed germination occurs from the ovules on placenta after 1–4 months of culture. Most of the ovules show normal germination and the seedlings grow normally when transplanted after true leaf development onto 1/2 MS medium containing 3% (w/v) sucrose and 0.3% (w/v) gellan gum. However, in the interspecific hybridization between *Primula filchnerae* and *P. sinensis*, only abnormal seed germination such as root growth without shoot formation occurs in all but few ovules and they stop growing (12, 21). However, by transplanting them to 1/2 MS medium supplemented with 3% (w/v) sucrose, 0.3% (w/v) gellan gum, 0.1 mg/L NAA, and 1 mg /L zeatin, these abnormal seedlings are successfully converted into normal plantlets via inducing redifferentiation of plants after callus induction. In the case of *Cosmos*, addition of 0.2 mg/L kinetin into medium is effective to induce redifferentiation (Oku, personal communication). Usually, 0.1–1 mg/L is considered to be suitable for both NAA as an auxin and any one of the cytokinins, such as BA, zeatin (SIGMA), and thidiazuron (SIGMA).

7. Notes

1. In self-incompatibility plants, it is necessary to confirm the strength of self-incompatibility by self-pollination as the strength varies depending on the individuals. In *Primula*, illegitimate crossing, especially pin/pin, sometimes yields hybrid plants (2, 3). In the plants bearing small flowers which are difficult to emasculate before anthesis, emasculation and pollination should be carefully performed immediately after anthesis.

2. The degree of difficulties in obtaining interspecific hybrid is mostly related to the phylogenetic distance between the two species. However, cross combination between distantly related species sometimes shows high crossability, and differences in crossability between cultivars are commonly found in interspecific crosses (2, 3, 22). Therefore, it is necessary to make a test cross using many cultivars in both parental species.
3. Crossing bags used for covering pollinated flowers easily get wet with rain or water, which results in the severe infection with fungi and subsequent difficulty in surface sterilization of the ovary. Therefore, plants used for the crossing must be kept in the glasshouse and watered without causing the bag to get wet.
4. Concentration of available chlorine and sterilization time should be adjusted depending on the objective species because of the difference in susceptibility to chlorine.
5. In RAPD analysis, the annealing temperature of 42°C in our protocol is higher than that in commonly used protocol. If specific marker cannot be obtained from either parental DNA, annealing temperature may be switched to 38°C.
6. Temperature control condition is slightly different among PCR machines. If the two-step program does not work properly to amplify single ITS band, using the three-step program is better.
7. Endonucleases recognizing 4 bps are useful to detect polymorphism. Because polymorphic bands are sometimes too small in PCR-RFLP display, polyacrylamide gel electrophoresis sometimes provides clearer bands than agarose electrophoresis.
8. In flow cytometry analysis, each sample should always be measured with an internal standard since the peak position may fluctuate according to various factors, such as aging of lamp.

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Plant Tissue Culture of Fast-Growing Trees for Phytoremediation Research

José Luis Couselo, Elena Corredoira, Ana M. Vieitez, and Antonio Ballester

Abstract

The ability of plants to remove pollutants from the environment is currently used in a simple and low-cost cleaning technology known as phytoremediation. Unfortunately, little is known about the metabolic pathways involved in the transformation of xenobiotic compounds and the ability of certain plants to tolerate, detoxify, and store high concentrations of heavy metals. Plant cell and tissue culture is considered an important tool for fundamental studies that provide information about the plant-contaminant relationships, help to predict plant responses to environmental contaminants, and improve the design of plants with enhanced characteristics for phytoremediation. Callus, cell suspensions, hairy roots, and shoot multiplication cultures are used to study the interactions between plants and pollutants under aseptic conditions. Many plant species have an inherent ability to accumulate/metabolize a variety of pollutants, but they normally produce little biomass. However, fast-growing trees are excellent candidates for phytoremediation because of their rapid growth, extensive root system, and high water uptake. This chapter outlines the *in vitro* plant production of both somaclonal variants and transgenic plants of *Populus* spp. that exhibit high tolerance to heavy metals.

Key words: Environmental contaminants, Heavy metals, Micropropagation, Phytochelatin synthase, Phytoremediation, *Populus* spp., Somaclonal variation, Transgenic plants

1. Introduction

Phytoremediation is a technology that uses plants to remove pollutants from the environments contaminated by mining, industrial, agricultural, and military activities (1, 2). The most useful strategies for cleaning contaminated soils and waters include (1) phytoextraction, which removes heavy metals and organic compounds from soils by accumulating them in plant biomass; (2) phytostabilization, which reduces the bioavailability of pollutants by binding them to

the soil matrix; (3) phytodegradation, which is the breakdown of contaminants taken up by plants through metabolic processes within the plant; and (4) the breakdown of contaminants external to the plant via the effect of compounds (such as enzymes) produced by the plants. Although phytoremediation includes different simple and low-cost techniques, it is not widely applied, at least in the phytoextraction of metals (3–5). There is still much to learn about the physiological processes involved in maintaining metal homeostasis in plants and in the metabolism of xenobiotics within the plants (1, 6, 7). Molecules, such as glutathione, occupy a central role in plant defences against heavy metals and xenobiotics. Chelating compounds derived from glutathione, such as metallothionines and phytochelatins, are capable of detoxifying metals. Synthesis of these compounds can be induced in plants subjected to the pressure of pollutants (6, 8, 9). Moreover, various enzymes, such as nitroreductases, lactases, dehalogenases, and peroxidases, are involved in the oxidation of xenobiotics, transportation, and further compartmentation processes in plant cells (10, 11), and knowledge of their mode of action is of prime importance for the application of phytoremediation procedures.

The most recent advances in the understanding of the complex interactions between plants and pollutants could not have been achieved without the application of *in vitro* plant cell culture techniques. Application of these techniques in phytoremediation research has recently been reviewed (12). Callus, cell suspensions, hairy roots, and shoot multiplication cultures are currently used as model systems for understanding the uptake, localization, metabolism, toxicity, and tolerance of pollutants under aseptic conditions. *In vitro* cultures can be propagated indefinitely, are available all year around, and their use enables a great reduction in the amount of whole plant material required for research. A rapid response is obtained by the addition to the established culture media of specific additives, such as promoters and/or inhibitors, to the established culture media. The development of transgenic plants to enhance the degradation of xenobiotic compounds or the sequestration and detoxification of heavy metals has recently been highlighted (2, 13–17). Plant tissue culture is a necessary step in most of the genetic transformation applications currently used.

Despite the above considerations, the responses of tissue cultures to the pollutants do not allow the development of a practical phytoremediation technology, as the response of whole plants growing under hydroponic conditions or in the soil may not necessarily be the same as that of tissues cultured *in vitro*. As pointed out in the review by Doran (12), the results derived from the use of tissue culture techniques can be used to predict the responses of plants to environmental contaminants. Nevertheless, *in vitro* tissue culture can help in the production and selection of whole plants with enhanced phytoremediation characteristics. As mentioned

above, the development of transgenic plants that exhibit stable genetic transformation to produce recombinant plants in the field of phytoremediation is a current reality. Unfortunately, there are still considerable concerns as regards the use of genetically modified plants (specifically, trees), so their use would be limited in the short- to medium-term future (13, 18, 19). Even under these specific circumstances, in vitro tissue culture technologies offer a valuable alternative. Somaclonal variation is a phenomenon that may occur spontaneously during in vitro growth and may cause deleterious effects, for example, in clonal propagation. On the contrary, induced somaclonal variation offers the opportunity to develop new germplasm exhibiting specific traits, including those able to enhance phytoremediation properties (20, 21). The only feature that should be clearly demonstrated is the heritable character of the new traits resulting in the new variants identified.

Many plant species, such as *Thlaspi caerulescens* and *Brassica juncea*, have an inherent ability to accumulate a variety of heavy metals. However, these species, which are called metal hyperaccumulators, produce little biomass. For practical purposes, high-biomass plants are preferred for phytoremediation. Fast-growing trees, such as many species of the genus *Populus*, are excellent candidates for phytoremediation because of their rapid growth, extensive root system, and high water uptake. In addition, in vitro tissue culture technology is well defined in many *Populus* species (22–25), which are amenable to genetic transformation and may be considered a model plant for both fundamental and applied research. In addition, somaclonal variation is well documented among *Populus* species and the application of biotechnology to selective tree improvement and the drawbacks and potential benefits of somaclonal variation have been reviewed (26). Heritable somaclonal stability must be confirmed by genetic analysis following sexual reproduction, and the demonstration of stability following vegetative propagation may allow practical exploitation of beneficial traits.

To illustrate the approaches used for plantlet regeneration of forest trees with enhanced properties for phytoremediation, the work carried out with *Populus* spp. is presented. Two protocols are proposed with the aim of regenerating plants capable of tolerating high amounts of heavy metals. In the first protocol, the production of *Populus* somaclonal variants, through the induction of adventitious shoots to enhance heavy metal tolerance, is described. In the second protocol, the production of transgenic *Populus* plants overexpressing the phytochelatin synthase 1 gene of *Triticum aestivum* (*TaPCSI*) and the responses when growing on heavy metal mining soils are described. Although the ultimate goal of the protocols proposed is to select plants with an enhanced capacity for metal accumulation capacity, these protocols may also be suitable for fundamental studies of the relationships between metals and plants under in vitro conditions.

2. Materials

2.1. Induction
of Somaclonal
Variation in *Populus*
spp. to Enhance Cd
Tolerance

1. Plant material: Forced shoots collected from segments of branches from mature trees (see Note 1). Ethanol, sodium hypochlorite solution (0.6% active chlorine), Tween® 80, and sterile distilled water for surface sterilization of shoots used as source of explants.
2. Stock salt formulations for different culture media used for culture initiation, callus induction, adventitious bud induction, shoot multiplication, and rooting, namely, Murashige and Skoog media (MS) (27) and organic additives as outlined in Table 1. In addition, phytohormones including N6-benzyladenine (BA), indolebutyric acid (IBA), thidiazuron (TDZ), 2,4-dichlorophenoxy acetic acid (2,4-D), naphthaleneacetic acid (NAA), sucrose, and Difco bacto agar are all used

Table 1
Culture media used in the different steps of *Populus* spp. plantlet regeneration

Components	Culture initiation	Callus induction	Shoot proliferation	Adventitious bud induction	Rooting
Macronutrients	MS	MS	MS	MS	MS ½
Micronutrients	MS	MS	MS	MS	MS
Fe-EDTA	MS	MS	MS	MS	MS
Myo-inositol (mg/L)	100	100	100	100	100
Glycine (mg/L)	0.3	0.3	0.3	0.3	0.3
Glutamine (mg/L)	1	1	1	1	1
Calcium panthotenate (mg/L)	10	10	10	10	10
Adenine sulphate (mg/L)	0.1	0.1	0.1	0.1	0.1
BA (μM)	2.2	–	0.15	–	–
IBA (μM)	–	–	0.98	–	–
TDZ (μM)	–	–	–	0.023	–
2,4-D (μM)	–	0.5–5.0	–	–	–
NAA (μM)	–	–	0.54	–	–
Sucrose (w/v)	3	2	2	2	2
Agar (g/L) (Sigma A-1296)	7	7	7	7	7

at various stages (see Note 2). The pH of the culture media is adjusted to between 5.7 and 5.8 before the agar is added. Sterilize culture media by steam autoclaving. Supplement the callus induction medium with CdCl_2 from 0 to 300 μM to obtain *Populus* lines derived from adventitious shoots with enhanced metal accumulation capacity.

3. Laminar flow cabinet, forceps, scalpel, glass bead sterilizer, test tubes (20×150 mm, 16.5 mL/tube), and 500-mL glass jars (70 mL/jar) for in vitro tissue culture. Conical forest plastic vessels (170×60 mm) and 2.5-L plastic pots filled with sterile peat–perlite (3:1 v/v) substrate for acclimatization and growth in greenhouse of regenerated plants and metal uptake experiments, respectively. Hoagland's solution (28).
4. For tolerance and toxicity experiments, regenerated plants of *Populus* must be exposed to contaminant solutions from 0 to 1 mM of CdCl_2 supplemented with 0.8 mM KNO_3 , 0.5 mM $\text{Ca}(\text{NO}_3)_2$, 10 μM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 μM FeNaEDTA , 10 μM H_3BO_3 , 0.5 μM KI , 0.1 μM CoCl_2 , 1.0 μM $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.1 μM $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$, 1 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 6 mM NH_4NO_3 . Adjust the pH of the solution to 4.5 (see Note 3).
5. Oven, microwave oven with Teflon PFA vessels, agate ball centrifugal mill, HNO_3 (60%), H_2O_2 (31%), deionized water, 5 mM $\text{CaCl}_2\text{--HCl}$ (pH 3.8), 0.45- μm filters, appropriate standard reference material of *Populus*, ICP-certified elemental standards (at least Zn, Cd, and Pb), ICP internal standards (beryllium, rhodium, and thallium in 2% HNO_3), and inductively coupled plasma mass spectrometer (ICP-MS) for metal determination in plant tissues (see Note 4).

2.2. Genetic Transformation of *Populus* Cultures for Expression of the Phytochelatin Synthase TaPCS1 and Phytoremediation of Contaminated Soils with Pb, Cd, and Zn

1. In vitro stock shoot cultures of *Populus*. Culture medium for adventitious bud induction (see Table 1).
2. Laminar flow cabinet, forceps, scalpel, glass bead sterilizer, tubes for shoot multiplication, and sterile petri dishes (90 mm) for transformation experiments.
3. Strain EHA105 of *Agrobacterium tumefaciens* (29, 30) and binary vector pBITaPCS1 (see Fig. 1). Luria Bertani (LB) medium for *A. tumefaciens* growth: 10 g/L bacto-tryptone, 5 g/L bacto yeast extract, 10 g/L NaCl, 15 g/L agar (31). Antibiotics: carbenicillin, kanamycin, and nalidixic acid. Syringe and 0.2- μm sterile filters to sterilize the antibiotics. Incubator and environmentally controlled shaker for *A. tumefaciens* growth. Spectrophotometer to determine the bacterial optical density at 600 nm (OD_{600}).
4. Polymerase chain reaction (PCR) and southern blot techniques to confirm transformation events. Resources needed to carry out DNA extraction include liquid nitrogen, micropipettes,



Fig. 1. Diagram of a T-DNA region of the plasmid pBITaPCS1: *RB* right border, *LB* left border, *nos-pro* and *nos-ter* promoter and terminator of the nopaline synthase gene, *nptII*, gene-coding region of neomycin phosphotransferase II, *CaMV 35S-pro* 35S gene promoter of the cauliflower mosaic virus, *TaPCS1* coding sequence of the phytochelatin synthase 1 of *T. aestivum*.

DNA extraction kit, centrifuge ($>13,000 \times g$), thermoblock with 1.5-mL/15-mL adapter tubes, nucleic acid electrophoresis reagents and equipment, and UV transilluminator and UV spectrophotometer to determine DNA purity and concentration. For PCR confirmation, a thermocycler, proper oligonucleotide primers (forward: ACCATGGAGGGC; reverse: TTGACAGCACACAGTGGC), thermostable DNA polymerase, $MgCl_2$, dNTPs, and ultrapure water are also needed. For southern blot hybridization, the following are also required: suitable restriction enzymes (XbaI, EcoRI), positively charged nylon membranes, saline sodium citrate buffer (SSC), a southern blot tray—as SSC buffer reservoir, a glass sheet, blotting paper and paper towels, a hybridization oven, DIG Luminescent Detection Kit (detection of digoxigenin (DIG)-labeled nucleic acids by enzyme immunoassay with luminescence), films designed specifically for chemiluminescence purposes, and automatic film processor.

5. Confirm transgene expression by two-step reverse transcription-PCR (RT-PCR). In addition to the reagents and equipment required for PCR, RNA extraction kit with DNase, refrigerated centrifuge ($>13,000 \times g$), reverse transcriptase, RNase inhibitor, oligo-dT primers, intron-specific primers (Fw AGGTGGC TCCTACAAATGC, Rv AACATAGATGACACCGCGC), and housekeeping genes primers are also necessary (18S, Fw AATTGTTGGTCTTCAACGAGGAA, Rv AAAGGGCAGGG ACGTAGTCAA; tubulin alpha-3/alpha-5 chain, Fw AGGT TCTGGTTTGGGGTCTT, Rv TTGTCCAAAAGCACAGCA AC; polyubiquitin (UBQ11), Fw GTTGATTTTTGCTGGG AAGC, Rv GATCTTGCCCTTCACGTTGT).
6. Select appropriate soils for the study of the effect of contaminated soil on plant response. Conical forest plastic vessels (170×60 mm) filled with sterile peat-perlite (3:1 v/v) substrate for acclimatization and growth in greenhouse of regenerated plants. Plastic pots, 2.5 L.
7. Oven, microwave oven with Teflon PFA vessels, agate ball centrifugal mill, HNO_3 (60%), H_2O_2 (31%), deionized water, 5 mM

CaCl₂-HCl (pH 3.8), 0.45- μ m filters, appropriate standard reference material of *Populus*, ICP-certified elemental standards (at least Zn, Cd, and Pb), ICP internal standards (beryllium, rhodium, and thallium in 2% HNO₃), and ICP-MS for metal determination in plant tissues (see Note 4).

3. Methods

3.1. Induction of Somaclonal Variation in *Populus* spp. to Enhance Cd Tolerance

For methods of inducing somaclonal variation in different plant species, including *Populus* spp., see previous reports (20–22, 26, 32, 33).

3.1.1. Explant Preparation, Culture Initiation, and Shoot Multiplication

1. Take branches of the lower part of selected trees growing in the field and prepare cuttings 15 cm long.
2. Arrange the cuttings in moistened perlite in trays and force them to flush in a growth cabinet at 24°C and 90% relative humidity under a 16-h photoperiod (95–100 μ mol/m² s provided by cool-white fluorescent lamps).
3. After 3 weeks, collect shoots 2–5 cm long as the source of explants.
4. Strip off leaves and surface sterilize the collected shoots by successive immersion for 20 s in 70% ethanol and for 5 min in sodium hypochlorite solution (0.6% active chlorine) containing two to three drops of Tween® 80.
5. Rinse the shoots three times with sterile distilled water.
6. Isolate 5-mm-long shoot tips and nodes (primary explants) from the disinfected shoots.
7. Place the explants upright in 20×150-mm culture tubes containing 16.5 mL of initiation medium (see Table 1). Incubate the cultures at 25°C under a 16-h photoperiod, with light provided by cool-white fluorescent lamps at a photon flux density of 50–60 μ mol/m² s.
8. Transfer the explants to fresh medium every 2 weeks for 6 weeks after the initiation of the culture.
9. Isolate the new shoots obtained in vitro and divide them into 0.8–1.0-cm segments (including shoot tips) and subculture them into 500-mL jars (6 explants/jar) containing 70 mL of shoot multiplication medium (see Table 1). Maintain stock shoot cultures of *Populus* by the proliferation of axillary buds with subculture at 4-week intervals (see Note 2).

3.1.2. Callus Induction and In Vitro Selection

1. Isolate internode sections from shoot multiplication cultures at the end of the multiplication cycle (4 weeks) and place them horizontally in 500-mL jars (6 explants/jar) filled with 70 mL of callus induction medium (see Table 1, and Note 5).
2. Incubate the cultures in the dark at 25°C and subculture them every 3–4 weeks onto fresh medium.
3. Transfer the explants onto callus induction medium supplemented with CdCl₂ 0 (control) and 10, 20, 40, 60, 80, 100, 150, 200, and 300 µM.
4. Cultivate the callus on the metal-spiked medium for 4–5 months in the dark with transfers to fresh medium every 4 weeks.
5. Transfer the surviving callus to bud induction medium (see Table 1) and maintain the cultures at 25°C with a 16-h photoperiod (50–60 µmol/m² s).
6. After 4 weeks, isolate the surviving shoots (control and Cd treated) and inoculate them onto shoot proliferation medium devoid of CdCl₂ to achieve the required population (see Note 6).
7. Isolate elongated shoots (1–2 cm) and transfer them to root induction medium (see Table 1). Maintain the cultures at 25°C under a 16-h photoperiod (50–60 µmol/m² s).

3.1.3. Tolerance and Toxicity Experiments (See Note 7)

1. Transfer the rooted plants obtained as outlined in Subheading 3.1.2 into plastic vessels (170×60 mm) filled with sterile peat–perlite (3:1 v/v) for acclimatization in greenhouse. After 1 week, all plants should be supplied weekly with Hoagland's solution at pH 6.7 and allowed to grow for 9 weeks.
2. Select homogeneously rooted and similar sized plants (range of shoot height approximately 15.0±2.0 cm) for subsequent experiments. Place the plants into 2.5-L plastic pots (one plant per pot) filled with sterile peat–perlite (3:1 v/v) substrate (see Fig. 2a).
3. Expose the plants (at least five plants/treatment) to Cd contaminant solution (see Subheading 2). Apply the contaminant solutions weekly and leave the plants to grow in a greenhouse for 18 weeks (see Fig. 2b) (see Note 8).
4. Harvest the plants and divide them into roots and aerial part (stem + leaves). Wash the roots with tap water, then with 5 mM CaCl₂–HCl (pH 3.8) for 15 min, and finally with deionized water (see Note 9). Wash the aerial part with tap water and then with deionized water. The harvested plant material is then blotted dry with tissue paper and oven dried at 70°C until constant weight (48 h).
5. Record the stem length, number of leaves, and biomass, as total dry weight, of roots, stems, and leaves. Calculate the Cd content in these organs as outlined in Subheading 3.2.6.

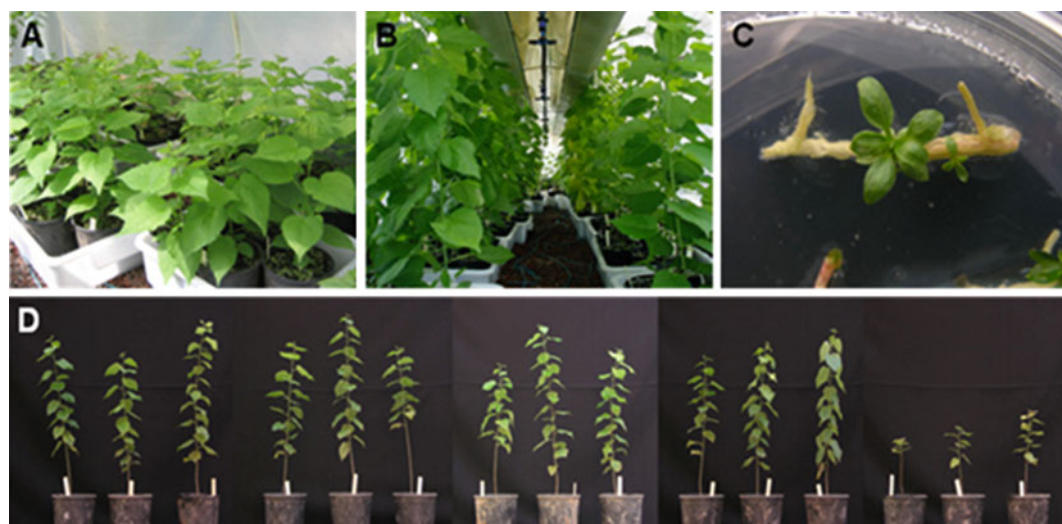


Fig. 2. Tests of tolerance and toxicity to Cd in *Populus* plants. (a) Plants of *Populus* after acclimatization. (b) Plants exposed to Cd contaminant solutions for 18 weeks. (c) Transgenic regenerated shoots from nodal segment growing in selection media for 6 weeks. (d) Different transgenic lines and wild-type plants (the three of the right-hand corner) of *P. tremula* $\times$ *tremuloides* growing in contaminated mining soil.

6. Calculate the bioconcentration factor (BCF) as the metal concentration in the aerial part (stem+leaves) per metal concentration in the contamination solution. Calculate the root concentration factor (RCF) as the metal concentration in the roots per metal concentration in the contaminant solution (see Note 10).

3.2. Genetic Transformation of *Populus* Cultures for Expression of the Phytochelatin Synthase *TaPCS1* and Phytoremediation of Contaminated Soils with Pb, Cd, and Zn

3.2.1. Maintenance of Shoot Cultures

3.2.2. Bacterial Strains and Plasmids

To develop this protocol, we used the hybrid *Populus tremula* $\times$ *tremuloides*, cv. Etrepole (25) (see Note 11). The step-by-step protocol defined by Tzfira et al. (34) was used, but was adapted to the specific characteristics of the plant material. These adaptations should also be followed for any other *Populus* material used.

1. Proliferate stock shoot cultures of *Populus* obtained as defined in Subheading 3.1.1 by subculture in proliferation medium (see Table 1).
1. Use *A. tumefaciens* strain EHA105 (29) carrying the plasmid pBITaPCS1 (see Fig. 1). Prepare the binary vector by replacing the *uidA* gene from pBI121 with the wheat phytochelatin synthase 1 *TaPCS1* (Genebank code No. AF093752) (35). The binary vector also contains an *nptII* marker gene driven by the *nos* promoter for kanamycin selection.

2. Growth bacterial suspension overnight on a horizontal orbital shaker (200 rpm) at 28°C in LB liquid medium containing 50 mg/L of both kanamycin and nalidixic acid. Add the antibiotics to the medium by filter sterilization (0.2- μ m sterile filters).
3. Take 1 mL of bacterial suspension and inoculate it in 300 mL of LB medium with the corresponding antibiotics. Incubate, then, this culture at 28°C (100 rpm) under darkness until an optical density $OD_{600} = 0.3$ is reached.

3.2.3. Transformation, Selection, and Regeneration

1. Take shoots of the *Populus* cultures at the end of the multiplication cycle (4 weeks). Remove the leaves from the stem and prepare nodal sections (approximately 1 cm).
2. Transfer the explants, immediately after cutting, to the diluted bacterial suspension (25 mL; $OD_{600nm} = 0.3$) and allow to sit for 30 min on a horizontal orbital shaker (100 rpm) at 28°C.
3. For co-culture, blot the explants dry on sterile filter paper and lay them, horizontally, in petri dishes (ten explants/dish) filled with 25 mL of adventitious bud induction medium (see Table 1). Let the cultures to grow for 2 days at 25°C in the dark.
4. Place the nodal segments in Erlenmeyer flasks and wash them, with vigorous shaking, three times in deionized water supplemented with 300 mg/L of carbenicillin.
5. Transfer the explants to 90-mm petri dishes with 25 mL of bud induction medium supplemented with kanamycin (50 mg/L) and carbenicillin (300 mg/L) (selection medium) (see Note 12) and let them to grow at 25°C under a 16-h photoperiod at 50–60 μ mol/m² s.
6. For each independent transformation experiment, 50 nodal explants are prepared as a negative control (non-inoculated explants growing in selection medium) and another 50 explants as a positive control (non-inoculated explants growing in bud induction medium). These controls are used to evaluate the regeneration ability and selection efficiency.
7. After 6 weeks of culture in selection medium, isolate the regenerated shoots (see Fig. 2c) and transfer them to proliferation medium (see Table 1) supplemented with kanamycin (50 mg/L) and carbenicillin (300 mg/L).
8. Each successful transformation event constitutes a transgenic line that should be multiplied separately.
9. After the appropriate population of each transgenic line is obtained, transfer elongated shoots (4–5 weeks) to rooting medium (see Table 1) for plant regeneration.

3.2.4. Molecular Analysis

1. To confirm the presence of the *TaPCSI* sequence in each transgenic line, PCR and southern blot hybridization are carried out by standard methods (31).
2. For DNA extraction, collect 100 mg (PCR analysis) or 1 g (southern blot analysis) of fresh leaf from transformed and untransformed lines. Place the material in a mortar and add enough liquid N₂ to cover the tissue. The tissue is then ground thoroughly with a pestle to a fine powder and the manufacturer's instructions for the extraction kit are followed. DNA concentration and purity determination is conducted by measuring the absorbance in a spectrophotometer at 260 and 280 nm, respectively.
3. Use specific oligonucleotide primer pairs for *TaPCSI* amplification by PCR. The reaction mixture (25 μ L) should contain 100 ng of DNA, 200 μ M of dNTPs, 0.6 μ M of each primer, 1.5 mM of MgCl₂, and 1U of thermostable DNA polymerase. The thermocycler conditions include a denaturalization step at 95°C (5 min) followed by 35 cycles of 95°C (1 min), 56°C (90 s), and 72°C (1 min) with final extension step of 72°C (5 min).
4. For southern blot analysis, digest DNA (20–30 μ g) with proper restriction enzymes and then electrophorese the fragments in 1% agarose gel. Place the gels on a glass sheet over southern blot tray. The DNA is transferred by capillarity onto nylon membrane with SSC buffer, blotting paper, and paper towels. Then, cross-link the DNA with UV (254 nm) to fix it to nylon membranes. Hybridize this membrane in a hybridization oven with the DIG-labeled DNA probe of the *TaPCSI*-coding region. The probe is prepared by PCR, following the manufacturer's instructions for the DIG luminescent detection kit. Wash the membranes in SSC buffer under standard ion strength conditions and then expose them to chemiluminescence film. Finally, process the films in an automatic film processor.
5. To confirm *TaPCSI* transcription by two-step RT-PCR, RNA is extracted from fresh leaves, stems, and roots. For total RNA extraction, weigh 100 mg of plant material from transformed and untransformed lines. Soak the tissue in liquid N₂ and grind it thoroughly in a mortar to a fine powder. Add buffer extraction to the powder and RNA is isolated according to the manufacturer's instructions (see Note 13). Measure the absorbance at 260 and 280 nm for RNA concentration and purity determination, respectively. cDNA is synthesized in a total volume of 20 μ L by reverse transcriptase (4 U) from total RNA with oligo-dT as primers (at a final concentration of 1 μ M) at 37°C (60 min) and following the manufacturer's protocol (see Note 14). In a separate tube, perform PCR amplification using 2 μ L of previous RT reaction as template and specific pair of primers for

TaPCSI cDNA. The PCR reaction mixture (20 μ L) contains 200 μ M of dNTPs, 0.6 μ M of each primer, 1.5 mM of MgCl₂, and 1 U of thermostable DNA polymerase. The thermocycler steps of amplification are identical to those for confirmation of the transgene insertion. In cDNA samples, contamination with genomic DNA must be ruled out. This should be confirmed by similar PCR amplification with intron-specific primers. For positive control of transcription, PCR amplification of housekeeping genes with specific primers is also necessary. Transcription of the 18S, tubulin alpha-3/alpha-5 chain, and polyubiquitin (UBQ11) genes can be used as endogenous control (see Note 15).

3.2.5. *Phytoremediation Experiments with Contaminated Soils*

1. Select rooted plantlets from three transgenic lines showing the highest transgene expression and the same number of untransformed (wild type, wt) plants and transfer them to plastic vessels (170×60 mm) filled with sterile peat–perlite (3:1 v/v) for acclimatization in a greenhouse. Supply the plants once with quarter-strength Hoagland’s solution at pH 6.7 and then with tap water when necessary. The plants are allowed to grow for 9 weeks.
2. Select 5 uniform plants per each transgenic line and 15 wt plants and transfer them to 2.5-L plastic pots (1 plant per pot) filled with contaminated M3, M4, or M15 soils (36) with high contents of Cd, Pb, and Zn (see Note 16). Irrigate the plants with only tap water as required and allow them to grow in greenhouse for 22 weeks (see Fig. 2d).
3. Harvest the plants and evaluate the parameters defined in Subheading 3.1.3, steps 5 and 6. Calculate both BCF and RCF as the ratio of metal concentration in, respectively, the aerial and root parts to the available metal concentration in soil.

3.2.6. *Determination of Pb, Cd, and Zn Content*

1. Grind completely dried organs in an agate ball centrifugal mill and use certified reference material of *Populus* for quality assurance of the digestion procedure. Take 0.15-g aliquots of ground material and reference material and digest them with 4 mL HNO₃ and 2 mL H₂O₂ in Teflon PFA vessels in a microwave oven with the operating conditions shown in Table 2

Table 2
Microwave operating conditions for heavy metal determinations in *Populus* samples

Stage	Max. power	% Power	Ramp (min)	Pressure (PSI)	Temp. (°C)	Hold (min)
1	600 W	100	3:00	350	120	5:00
2	600 W	100	10:00	350	200	10:00

- (see Note 17). After cooling, bring the samples to a final volume of 100 mL. Filter all samples through 0.45- μ m filters.
2. Determine the total concentrations of Pb, Cd, and Zn in an ICP-MS equipment by a modified version of EPA method 1638 (37), with beryllium, rhodium, and thallium as internal standards. Run the samples in triplicate and use internal standards, certified standard solutions, and standard reference materials to calibrate the instrument, to quantify the content of Cd, Pb, and Zn in the samples, and to determine the quality of the data collected, respectively (see Note 18).
 3. For each transgenic and wt lines and for each soil tested, the following parameters must be calculated: biomass production (expressed in g, dry weight), concentrations of Pb, Cd, and Zn (in mg/kg), and total accumulation of metals in stems, leaves, and roots (in μ g) (see Note 19).
 4. Calculate BCF and RCF as outlined in Subheading 3.1.3, step 6.

4. Notes

1. The branches are taken from the lower part of the trunk. If available, stump sprouts and root suckers are also good sources of initial explants. Alternatively, the stock plant can be hedged in the greenhouse every 4–6 weeks to stimulate lateral branching to serve as the source of explants (32).
2. Poplars, cottonwoods, and aspens (*Populus* L.) are a group of some 30 species and more than 330 cultivars are being, or have been, cultivated worldwide. Many different culture media and plant growth regulator combinations have been used in in vitro tissue culture of these species (22). For specific species or cultivars, the most appropriate culture medium and subculture period should be selected according to previous reports or experience.
3. Heavy metals differ as regards their solubility in aqueous solutions and this may become altered when the metal is supplied to the plant jointly with a mineral solution, as in the protocol described. To avoid any possible interference, the GEOCHEM-PC simulation application (38) is highly recommended to confirm that the metal in contaminant solutions appears predominantly as soluble ion and does not form EDTA-chelate complexes.
4. According to the range of concentrations to be detected, atomic absorption spectrometry (AAS) or inductively coupled plasma optical emission spectrometry (ICP-OES) analytical techniques can also be used. Standard reference material of

Populus is used as a control for microwave-assisted digestion. Internal standards must be present in all samples, reference standards, and blanks at identical levels. Reagents used to digest plant samples should have a maximum level of purity.

5. Calluses may also be initiated by culturing petiole, leaf, and root sections of plantlets regenerated in vitro. Alternatively, stem internode explants isolated from hedged plants grown in a greenhouse can also be used as source of in vitro callus induction if proper sterilization methods are used (32).
6. Each successful somaclone constitutes, at least theoretically, a different line that should be multiplied and assayed separately for metal tolerance and accumulation. Only then, it will be possible to select the lines that are most tolerant to metals.
7. In vitro experiments can also be carried out to determine the resistance of the regenerated plants to the contaminant (39). In these experiments, leaf discs of regenerated plants are cultured in vitro on media supplemented with different concentrations of the heavy metal assayed. Phytotoxicity is normally evaluated after 20–25 days of culture under aseptic conditions.
8. The protocol described here is related to Cd selection. However, if there is interest in other heavy metals, the concentrations of the metal should be selected according to the results of previous experiments regarding the response of the specific plant material. Generally, only a few somaclones are obtained because of the pressure of the culture medium containing the heavy metal under study. However, this small number should be sufficient to select somaclones that are tolerant to heavy metals.
9. Roots must be exposed to 5 mM CaCl_2 solution (adjusted to pH 3.8 with HCl) to remove the absorbed metals from water-free space prior to analysis, thus avoiding overestimation of the metals' content.
10. BCF and RCF are dimensionless numbers that represent how much of a chemical (Cd, Pb, and Zn in this protocol) is in a tissue, relative to how much of that chemical exists in the environment. Therefore, RCF and BCF are indicators of the plant phytoremediation potential in a particular soil and with respect to a particular metal (Cd, Pb, Zn). The RCF indicates how much metal is retained in the root system and BCF indicates the amount of metal that is translocated from the soil to the harvestable aerial part. Moreover, the ratio between the BCF and RCF indicates the phytoextraction potential ($\text{BCF}/\text{RCF} > 1$) and/or phytostabilization potential ($\text{BCF}/\text{RCF} < 1$).
11. This protocol has been developed specifically to test the phytochelatin synthase *TaPCSI* isolated and identified from *T. aestivum*. However, there are other known phytochelatin (25) isolated from other plant species that, theoretically, are related

to heavy metal metabolism in plants. To study the effect of other phytochelatin, the specific binary vector construction must be available to produce the transformation events. The protocol defined is also valid, with slight modifications, to study the bioremediation capacity of transgenic *Populus* for xenobiotic contaminants, such as 2,4,6-trinitrotoluene (24).

12. The sensitivity of plant tissues to the antibiotics must be tested before starting the transformation experiments. The most appropriate concentration and type of antibiotics that allow the plant growth but not the growth of the bacteria must be selected. The selection culture medium must be prepared by adding the antibiotics by filter sterilization.
13. It is important that the total RNA is free of genomic DNA contamination, which can be removed by treatment with DNase. Commercial kits usually include a DNase treatment, but, if not included, this treatment will be required after the extraction. It is also very important to follow all precautions to prevent RNA degradation (RNase-free water, solutions, tubes, glassware, etc.).
14. The amount of RNA template for the reverse transcriptase depends on the enzyme selected (from less than 50 ng RNA to 2 µg RNA per reaction). The manufacturer's instructions should be followed. RNase inhibitor should be added to RT reaction prior to addition of RNA.
15. Transgenic lines with different levels of expression are required to evaluate the effect of the expression of *TaPCS1* since some researchers have suggested that excess phytochelatin can cause cellular toxicity. If this is true, high levels of expression of *TaPCS1* could induce toxicity and interference with the interpretation of phytoremediation assays. If this is not the case and transgenic lines with high levels of expression are preferred, a selection of transgenic lines can be made according to the levels of transgene expression by quantitative RT-PCR (4).
16. We used mining soils M3, M4, and M15, all of them derived from mining activities (36). The soils used in this type of experiments should be characterized following the standard protocols of international agencies. The physico-chemical characteristics should be known, including the total amount of heavy metals as well as the bioavailable concentrations.
17. Largely, the operational features of the instrument determine the operating conditions of microwave. The conditions outlined in Table 2 are, therefore, for guidance only.
18. The operating conditions of the ICP-MS instrument (nebulizer gas flow rate, ICP RF power, sample uptake rate, acquisition time per measurement, etc.) should be optimized prior to analysis to yield the highest signal intensities. Appropriate

interference standards should be analyzed to provide interference correction factors. Finally, the unknown samples may be analyzed. The quality of the results may be evaluated on the basis of within-run statistics, such as standard deviations, reproducibility of repeated analyses, measured analyte concentrations in blanks, and accuracy of results for any standard reference materials analyzed with the unknown samples.

19. The relationship between biomass production and heavy metal accumulation is very important. In some cases, the concentration of heavy metal found in the tissues may be similar in both transgenic and wild-type plants. However, the biomass production is normally higher in transgenic than in wild-type plants and, consequently, the total amount of heavy metal recorded in these plants is higher than in wild-type plants.

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Removing Heavy Metals by In Vitro Cultures

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Abstract

In vitro roots cultures of *Typha latifolia* and *Scirpus americanus* aquatic plants have the capacity to remove Pb (II), Mn (II), and Cr (III) from the culture medium. Both species remove Cr and Pb by an absorption process, while Mn is mainly adsorbed to the root surface. This chapter describes a protocol for the establishment of in vitro roots cultures (nontransformed) from *T. latifolia* and *S. americanus*, and the procedure for the uptake analysis of Pb (II), Mn (II), and Cr (III) by roots.

Key words: Phytoremediation, Heavy metals, In vitro root cultures, *Typha latifolia*, *Scirpus americanus*, Lead, Chromium, Manganese

1. Introduction

Heavy metals are environmental pollutants, and many of them are toxic even at very low concentrations. They are toxic because they can replace essential metals in pigments or enzymes and cause oxidative stress through the formation of free radicals (1). Unlike organic pollutants, metals cannot be degraded.

The use of plants for remediation of metals offers a very attractive alternative to extract and concentrate several elements from the environment (2–4). Nonetheless, in vitro root cultures and hairy roots have also been successfully employed on phytoremediation studies due to their fast growth in microbe-free conditions, genetic stability, and hormonal auxotrophy. *Thlaspi caerulescens* hairy roots have been found to accumulate Cd (5), and those of *Solanum nigrum* accrue Cd (6) and Zn (7). *Rubia tinctorum* transformed roots build up Cu (8), and *Alyssum bertolonii* hairy roots collect Ni (9). In addition, we have described that in vitro root cultures (nontransformed) from *Typha latifolia* and *Scirpus macrophytes* have the capacity to remove Pb (II), Mn (II), and Cr (III) from the culture medium (10). About 80–90% of Pb and Cr

are absorbed in both cultures, while Mn removal is due mainly to a biosorption process (82–86%) (11).

This chapter describes (1) the protocol to obtain in vitro root cultures from *T. latifolia* and *Scirpus americanus* aquatic plants and (2) the procedure to analyze the removal of Pb, Cr, and Mn by roots.

2. Materials

All solutions and media were prepared using deionized water and analytical grade reagents. The solutions were kept at 4°C.

2.1. Material for In Vitro Culture

1. Digital pH meter.
2. Orbital shaker.
3. Autoclave.
4. Culture flasks. Wash the culture flasks with soap and water and immerse them on HCl 0.1 N for 2 h. Rinse the flasks abundantly with deionized water, dry in oven at 200°C for 2 h, and cover them with three sheets of foil. Sterilize the flasks at 120°C for 30 min and keep them in plastic bags until use.
5. Murashige and Skoog basal medium, MS (12). Weigh 4.43 g of MS medium with micro- and macronutrients (Phyto Technology Laboratories) and dissolve them in 900 mL deionized water; add 30 g sucrose and 2 mL of a stock solution of indoleacetic acid (IAA; 1 mg/L). Adjust the pH to 5.7 using KOH 1 N and complete the volume to 1 L. Sterilize the medium at 120°C for 20 min (15 lb/in²).

2.2. Metal Analysis Components

1. High-Density Polyethylene (HDPE) flasks from 8 to 125 mL.
2. Teflon pincers and spatulas.
3. Flat bottom teflon vessels with caps (7 mL).
4. Antistatic polystyrene hexagonal weighing dishes.
5. Teflon cup for acid digestion with caps and o-ring (Parr).
6. Chromium standard (1 ppm): dissolve 0.94 mg $\text{CrK}(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$ in 100 mL HNO_3 0.02 M (see Note 1).
7. Lead standard (10 ppm): dissolve 0.8 mg $\text{Pb}(\text{NO}_3)_2$ in 100 mL HNO_3 0.02 M.
8. Manganese standard (100 ppm): dissolve 3.6 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ in 100 mL HNO_3 0.02 M.
9. Reference plant material: peach leaves (1547, NIST, USA) and certified water (1643e NIST, USA). Plant material contains certified concentration for 20 trace metals, while reference water has the certified concentration for 29 metals (see Note 2).

10. Aqua regis: 20 mL concentrated HNO_3 and 60 mL concentrated HCl (see Note 3).
11. Atomic absorption spectrophotometer (Varian-Spectr AA 220 FS, Palo Alto, CA) with graphite furnace.

3. Methods

3.1. In Vitro Root Culture

1. Collect adult plants of *T. latifolia* and *S. americanus* aquatic macrophytes with abundant roots.
2. Remove the roots from plants using a scalpel. Use gloves.
3. Wash the roots with tap water to eliminate mod.
4. Transfer the clean roots to a vessel (5 L) containing commercial soap solution and keep them with agitation at 130 rpm for 3 days. Change the solution of soap periodically.
5. Rinse the roots with tap water and immerse the material in 5% (v/v) commercial sodium hypochlorite (Cloralex®, Allen) and 0.2% Tween-20 for 15 min. Wash the roots rapidly with water to avoid oxidative damage caused by the hypochlorite.
6. Transfer the roots to a quarter strength concentration of MS medium ($\frac{1}{4}$ MS) containing 10 mL/L PPM (Plant Preservative Mixture) for 24 h (see Note 4).
7. Rinse the roots with sterile water and place them in 250-mL flasks containing 100 mL of MS liquid medium with IAA (2 mg/L), pH 5.7.
8. Keep the root cultures on an orbital shaker operated at 130 rpm at 25°C and under a photoperiod of 16 h light (white fluorescent lamps; 45 $\mu\text{mol}/\text{m}^2 \text{ s}$)–8 h dark.
9. For root-subculture add 100 mL fresh culture medium to in vitro root cultures and transfer half of material into two sterile flasks. Subculture the roots at 15-day intervals to have active growing cultures (see Fig. 1).

3.2. Metal Removal by In Vitro Root Cultures

1. Place 0.05 g of *T. latifolia* or 0.2 g *S. americanus* roots in 15 flasks (50 mL) containing 10 mL of medium with the corresponding metal. Add 60 μL Pb stock solution (10 ppm), 150 μL Cr stock solution (1 ppm) or 180 μL Mn stock solution (100 ppm) to have a final concentration of 60 $\mu\text{g}/\text{L}$ Pb, 15 $\mu\text{g}/\text{L}$ Cr III, and 1.8 mg/L Mn, respectively.
2. Record the absorbed and biosorbed metal concentrations at 2-day intervals for 8 days as described below.
3. For metal desorption analysis, filtrate the roots on filter paper, transfer the roots to HDPE flasks, and wash the tissue with 3 mL 0.042% HNO_3 for 3 h to desorb metals. Collect

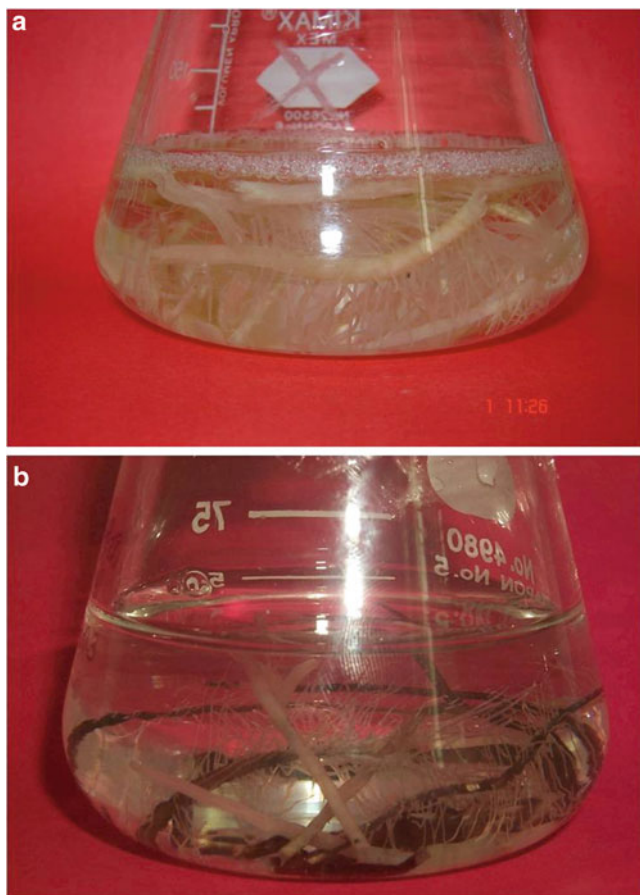


Fig. 1. In vitro root cultures from (a) *Typha latifolia* and (b) *Scirpus americanus* aquatic plants. Cultures are maintained on MS medium containing 2 mg/L IAA, pH 5.7.

supernatants and quantify the adsorbed metal by atomic absorption. Use the roots for absorption analysis.

4. To determine the Cr and Pb absorption, treat the roots with 0.1 mL HNO_3 per 2 mg of tissue at 25°C for 5 days. Keep the treated samples in HDPE bottles and protect them from light and dust with plastic bags.
5. To optimize the oxidation of organic material, add 400 μL H_2O_2 per 2 mg of roots and keep at 25°C. Adjust digestion volume to 5 mL using deionized water.
6. For Mn absorption analysis, add 0.1 mL aqua regis per mg of dry weight of roots at 70°C for 5 h. Make an additional digestion with 0.2 mL HNO_3 per mg of sample for 14 h. These digestions were done on teflon cups with caps and o-ring and using heat plates.

Table 1
Maximum uptake of Pb, Cr, and Mn by *T. latifolia* and *S. americanus* root cultures^a

Metal	<i>T. latifolia</i> (μg/g dry weight)				<i>S. americanus</i> (μg/g dry weight)			
	Absorbed	Adsorbed	Total	CF ^a	Absorbed	Adsorbed	Total	CF ^a
Pb	65.6	3.2	68.8	1,093	131.9	16.4	148.3	2,198
Cr	22.1	0	22.1	1,473	36.5	4.2	40.7	2,433
Mn	219	1,461	1,680	122	755	3,282	4,037	419

^a Concentration factor

- Keep the remaining acidic samples at room temperature for 30 min and redissolve the residue with 5% HNO₃ directly on teflon cups to attain a 25 mL final volume.
- Quantify absorbed and adsorbed metal using an atomic absorption spectrophotometer (Varian-Spectr AA 220 FSYZ, Palo Alto, CA) (see Note 5) with graphite furnace. For the analysis the next parameters were used: current lamp 10 mA; 0.2 nm of wide cleft for Cr and Mn determinations, and 0.5 nm for Pb measurements; wavelength of 357.9 nm, 403.1 nm, and 283.3 nm for Cr, Mn, and Pb quantifications, respectively; 5% HNO₃ as matrix.
- To calculate the factor concentration CF (13) use the formula:

$$CF = \frac{\text{Metal concentration in roots (mg /g dry weight)}}{\text{Metal concentration in the culture medium (mg /g dry weight)}}$$

- Metals accumulated by the in vitro cultures (see Table 1) are greater than the average concentration reported as phytotoxic (>5 μg Pb/g, >1 μg Cr/g, >700 μg Mn/g) by Markert (14). *S. americanus* root cultures are about twice more efficient to remove metals than *T. latifolia* roots. Both plant species capture metals in the following order: Cr>Pb>Mn.

4. Notes

- Dissolving lead, chromium, or manganese salts in deionized water or culture medium promotes a light precipitation of metals. This effect is not observed when metals are dissolved in HNO₃ 0.02 M.
- The concentration of lead, chromium, and manganese in certified water is: 19.63 ± 0.21 μg/L Pb²⁺, 20.40 ± 0.24 μg/L Cr³⁺, and 38.97 ± 0.45 μg/L Mn²⁺. Reference plant material

contains: $0.87 \pm 0.03 \text{ } \mu\text{g/g}$ Pb^{2+} , $1 \pm 0.05 \text{ } \mu\text{g/g}$ Cr^{3+} , and $98 \pm 3 \text{ } \mu\text{g/g}$ Mn^{2+} .

3. Due to the reaction between its components, aqua regis quickly loses its effectiveness. Therefore, its components should be mixed immediately before use. In order to pour the mixture into the sink, first carefully neutralize it with sodium bicarbonate.
4. The PPM is the best choice for asepsis of roots; however, this product is not currently easily available. An optional asepsis includes the following: (a) immersion of root in a biocide mixture (3 g/L benlate, 3 g/L captan, 1 mL/L previcur, 0.5 g/L amoxicilin and 0.4 g/L ketoconazol) for 24 h; (b) treatment with 10% benzalkonium chloride for 24 h; (c) disinfection with 5% (v/v) commercial sodium hypochlorite (Cloralex®, Allen) for 10 min and 70% ethanol for 2 min. It is quite important to wash the roots immediately after treatment with hypochlorite.
5. It is recommended to verify the instrument response with known standards. For the quality control of the metal analysis, determine the levels of Pb, Cr, and Mn in the reference water and plant material. The metal recovery should range between 90 and 110% of certified concentration in the reference material.

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Establishment of a Sanguinarine-Producing Cell Suspension Culture of *Argemone mexicana* L (Papaveraceae): Induction of Alkaloid Accumulation

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Abstract

A protocol for the induction of a cell suspension culture of *Argemone mexicana* is described. This suspension has been kept for over 3 years producing sanguinarine, a benzophenanthridine-type alkaloid. Sanguinarine levels can be increased by exposing these cultures to yeast or fungal elicitation.

Key words: *Argemone mexicana*, Benzophenanthridine, Elicitation, Sanguinarine, Secondary metabolism

1. Introduction

Sanguinarine is a benzophenanthridine-type alkaloid produced by some Papaveraceae plants, where it is mainly accumulated in underground tissues (1). It displays toxic effects since it can be intercalated into DNA, due to its planar structure. Furthermore, its cationic nature allows it to bind to negatively charged membranes, and its reactivity toward thiol groups interferes with a number of cytosolic enzymes (2). Given these toxic properties, sanguinarine is considered part of the plant chemical arsenal against microbes and herbivores (3).

In cell cultures, sanguinarine accumulation is frequently induced upon exposure to different chemical and biological elicitors, such as jasmonates and microbial homogenates (4). This response has been shown to be preceded by the transcriptional activation of several sanguinarine biosynthetic genes, including

tyrosine decarboxylase (TyDC), norcoclaurine synthase (NCS), berberine bridge enzyme (BBE), among others (1). We have developed a cell suspension culture of *Argemone mexicana* L. (Papaveraceae; common name: prickly poppy), which produces sanguinarine in significant amounts, even in the absence of any induction treatment. Maximal sanguinarine accumulation in this *A. mexicana* cell suspension culture (named AMMF_i) is near to 3.0 mg/g DW, which is in the same magnitude order as those from other sanguinarine producing cultures after exposure to conditions that induce secondary metabolism (4). Interestingly, treatment of this *A. mexicana* cell culture with chemical inducers, such as jasmonate or salicylic acid, resulted in a limited increase of sanguinarine content (5). The operation of a sanguinarine detoxification mechanism has been proposed to limit any further increase in these cultures (2, 5). Since plant cell defense responses may evoke the participation of different, although convergent, signaling pathways (6), the exposure to single inducers may not be enough to trigger a full response. Hence, the use of elicitors prepared from cell-free pathogenic microorganism cultures could overcome such limited induction response. These preparations represent complex mixtures of organic compounds, which potentially may trigger the different signaling pathways.

The former part of this chapter describes a protocol for the induction and maintenance of a sanguinarine-producing cell suspension culture of *A. mexicana*. In the latter part, the development of a methodology to induce sanguinarine accumulation in this cell suspension is described.

2. Materials

All media for plant cells must be prepared with deionized water and based on the Phillips and Collins' medium (PC) (7), supplemented with 25 g/L sucrose and adjusted to pH 5.8. For the maintenance of the *Fusarium oxysporum* (see Note 1) culture, potato–dextrose–agar (PDA; Sigma-Aldrich Chemical Co., St. Louis MO) is recommended.

1. Naphthaleneacetic acid (NAA) and 6-benzyladenine (BA), 1.0 mg/mL stocks: Dissolve 100 mg NAA or BA in a 10 mL of 0.1 N KOH and adjust volume to 100 mL with water.
2. Methyl jasmonate (MeJa) 10 mM stock: dilute 22.9 μ L of MeJa (95%, ρ = 1.030 g/mL; Sigma-Aldrich Chemical Co.) in a total volume of 10 mL of ethanol. Sterilize the solution by filtration using 0.2 μ m pore size nylon sterile membranes (Corning, NY).

3. Salicylic acid (SA) 10 mM stock: 13.8 mg of SA (99% Sigma-Aldrich Chemical Co.) in 5 mL of ethanol and adjust volume to 100 mL with water. Sterilize the solution by filtration using 0.2 μ m pore size nylon sterile membranes (Corning, NY).
4. Yeast extract (YE) 400 g/L stock: 2.0 g of YE (Sigma-Aldrich Chemical Co.) dissolved in 5 mL of water. Sterilize the solution by filtration using 0.2 μ m pore size nylon sterile membranes (Corning, NY).
5. Sanguinarine 10 ng/ μ L reference solution: 1 mg of sanguinarine hydrochloride (Sigma-Aldrich Chemical Co.) is dissolved in 1 mL with methanol and 10 μ L of this solution is diluted to 1 mL with methanol (see Note 2).

3. Methods

3.1. Induction of Cell Suspension Cultures

Cell suspension cultures are initiated from *A. mexicana* leaf callus.

1. Collect leaves around 5 cm long from mature plants and soak them in soapy water for 30 min, rinse with tap water and blot on paper towels. Disinfest the excised leaves by subsequent washes in 70% ethanol (3 min), 0.6% of sodium hypochlorite (15 min) and sterile distilled water. Carry out these operations under aseptical conditions (using a laminar air flow cabinet).
2. Cut leaves in 1.0 cm² squares and culture on semisolid PC medium, supplemented with 0.5 and 1.5 mg/L BA and NAA, respectively (hereafter called PC-BN medium). Incubate the tissues at 25 $\pm$ 2°C under continuous light (photon flux density of 40–60 μ mol/m² s), provided by fluorescent lamps (39 W Philips Alto collection, Philips of México, México). After 4 weeks, separate calli formed on explants and transfer them to fresh PC-BN media.
3. Induce cell suspensions by transferring friable calli to PC-BN liquid medium. Maintain suspensions through biweekly subcultures, under continuous illumination and shaking (100 rpm).

3.2. Preparation of the *F. oxysporum* Cell-Free Homogenate

1. Use 30 day-old *F. oxysporum* PDA plate cultures (see Note 1) to initiate liquid cultures. Briefly, transfer sections of mycelium (1 cm²) to 50 mL of PC media without growth regulators and incubate during 6 days at 22°C in the dark and with rotatory shaking (120 rpm).
2. Homogenize mycelia and media with a polytron (Janke-Kunkel, T2) for 2 min, autoclave at 1 kg/cm² for 30 min, and subsequently centrifuge under sterile conditions. Use the supernatant as elicitor (4, 5) and keep it in the dark at 4°C.

3.3. Induction Procedures

Ten-day-old cell cultures, in the active growth phase, are used in all experiments.

1. Add 5 mL of the *A. mexicana* cell suspension (containing between 0.20 and 0.22 g fresh weight/mL) to a 250-mL Erlenmeyer flask, containing 50 mL of PC-BN medium and cultivate for 10 days, as described in previous sections.
2. On the 10th day of culture, induce suspensions by adding either 550 μ L of the MeJa, SA or 500 μ L of YE stock solutions. These volumes will render final concentrations of 100 μ M for MeJa and SA, and 200 mg/L for YE. Also induce cultures with 500 μ L of the cell-free *F. oxysporum* homogenate (final dose 30 mg of glutamate equivalents/L). Mock-induce controls with 500 μ L of water.
3. Collect samples (triplicates) after 0, 24, 48, 72, 96, and 120 h of exposure to the elicitors. Collect cell package by filtration, weigh, freeze in liquid nitrogen, and keep at -80°C until analysis. Keep spent media for analysis (see Note 3).

3.4. Alkaloid Extraction and Quantification

Alkaloids are extracted from freeze-dried tissues and quantified by densitometry after separation by thin layer chromatography (TLC), as reported previously (5).

1. Homogenize 100 mg of freeze-dried tissue with 10 mL of HCl 0.5% in methanol (v/v) and incubate 2 h at 45°C with gently shaking. Separate and eliminate cell debris by filtration and reduce extract volume to dryness at low pressure. Dissolve residue in 1 mL of methanol.
2. Load between 1 and 2 μ L of the extract on silica gel 60 F254 chromatography plates (aluminum supported; Merk Damstadt Germany).
3. In the same plate, load 1 μ L of the sanguinarine dilutions so that a reference plot from 1 to 10 η g is built (see Note 2).
4. Separate alkaloids on the TLC plate using a mobile phase of *n*-buthanol–acetic acid–water (7:1:2 by vol.) (see Note 4).
5. After chromatography, allow solvent to evaporate and visualize alkaloids on the plate using long wave UV light (365 nm). Sanguinarine is identified as a fluorescent red-orange spot with R_f value of 0.52 in this system.
6. Quantify sanguinarine on the plates by *in situ* densitometry using a Shimadzu CS-930 dual wavelength chromatoscanner, equipped with a DR 2 data collector (Kyoto Japan). Set absorbance to 465 nm.

Contents of sanguinarine in the *A. mexicana* cell cultures are comparable to those of plant roots (Table 1). Although elicitation with *F. oxysporum* and YE can double these amounts,

Table 1
Maximal accumulation of sanguinarine in *A. mexicana* cell suspension cultures exposed to different agents

Treatment	Time of exposure (h)	Sanguinarine content (mg/g DW)
Control	— ^a	0.96 (0.11)
Methyl jasmonate	72	2.13 (0.17)
Salicylic acid	— ^a	1.06 (0.83)
Yeast extract	48	3.2 (0.36)
<i>F. oxysporum</i> homogenate	96	3.8 (0.41)

Average of triplicates with standard deviation between brackets
^aSanguinarine contents were kept without significant variation throughout the experiment

they were still at the same magnitude order than those in plants (Table 1). Culture response to MeJa is limited, whereas SA does not produce any response (Table 1).

Argemone mexicana is one of the few species able to accumulate both sanguinarine (a benzophenanthridine) and berberine (a protoberberine). However, berberine is not detected in undifferentiated cultures of this plant, even under induction conditions of secondary metabolism (5). Though, the need of cell organization is well documented for the biosynthesis of several alkaloids, it is not required for the synthesis of this alkaloid in in vitro cultures from other berberine producing species, such as *Berberis stolinifera*, *Coptis japonica*, and *Thalictrum flavum* (1). Comparing alkaloid metabolism in the *A. mexicana* cell cultures to those of different plant tissues may establish the basis for this restriction.

4. Notes

1. The *F. oxysporum* strain was isolated from a *Capsicum chinense* plantation located in Cuzamá Yucatán (20°44'29"N 89°19'6"W; Islas-Flores collection Ref. FPACF4, Centro de Investigación Científica de Yucatán).
2. The equation relating sanguinarine to optical density is $y=5,282x-544$, where y is the amount of sanguinarine in nanogram and x is the optical density at 465 nm. Linear response can be obtained up to 150 ng.

3. Cultures should be continuously monitored during the induction treatment, since occasionally a darkening reaction could develop. If it is detected, allow cell package to settle down and observe media color. A reddish coloration indicates sanguinarine excretion to the media, whereas a brownish one may be related to phenol production. Being this the case, it is recommended to stop the treatment and collect cells shortly after, since it frequently precedes cell death. Use this information to adjust the conditions for the induction treatment, whether by using lower doses or shorter exposure times.
4. The separation obtained in 5-cm plates is sufficient to avoid overlapping of sanguinarine with other alkaloids present in the extract.

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Epigenetics, the Role of DNA Methylation in Tree Development

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Abstract

During development of multicellular organisms, cells become differentiated by modulating different programs of gene expression. Cells have their own epigenetic signature which reflects genotype, developmental history, and environmental influences, and it is ultimately reflected in the phenotype of the cells and the organism. However, in normal development or disease situations, such as adaptation to climate change or during in vitro culture, some cells undergo major epigenetic reprogramming involving the removal of epigenetic marks in the nuclei followed by the establishment of a different new set of marks. Compared with animal cells, biotech-mediated achievements are reduced in plants despite the presence of cell polypotency. In forestry, any sustainable developments using biotech tools remain restricted to the lab, without progressing to the field for application. Such barriers in the translation between development and implementation need to be addressed by organizations that have the power to integrate these two fields. However, a lack of understanding of gene regulation is also to blame for this barrier. In recent years, great progress has been made in unraveling the control of gene expression. These advances are discussed in this chapter, including the possibility of applying this knowledge in forestry practice.

Key words: Aging, Animals, DNA methylation, Histone modification, In vitro tissue culture, Plants, RNAi

1. Introduction

During development of multicellular organisms, cells become differentiated by modulating different programs of gene expression (1). Environmental adaptation coincides with changes in the expression of genes that play a role in the stress response. The spatial and temporal regulation of gene expression is extremely

accurate in cells and tissues, being stable during the adaptation but not static, since expression can be reverted when the stimulus ends. One of the mechanisms that regulate gene expression meeting the characteristics described above is epigenetics, which can be defined as heritable changes in the phenotype caused by mechanisms other than changes in the DNA sequence, usually affecting gene expression.

Each cell has its own epigenetic signature which reflects genotype, developmental history, and environmental influences, and it is ultimately reflected in the phenotype of the cell and the organism. However, in normal development or under adverse environmental conditions, such as those related to climate change or *in vitro* culture, some cells undergo major epigenetic reprogramming involving the removal of epigenetic marks in the nucleus followed by the establishment of a different new set of marks (2). The initiation of new cell fates as well as cell reprogramming to accomplish a new organogenic pathway or to adapt to climate change involving the establishment of distinct cell lines, are both accomplished by changes in the epigenetic marks. These changes normally consist of posttranslational modifications of the core nucleosome histones and transient changes in global DNA methylation, which ultimately determine gene expression reprogramming, which is manifested at the transcriptome, proteome, and metabolome levels.

Although genetic variation plays a role in adaptation to new conditions and the absence or presence of alleles contributes to the surviving phenotypes, the crucial issue is how gene expression is controlled in genetically identical cells. Until now, this has remained an enigma how genetically identical cells of an organism differentiate and reprogram, maintaining strict spatial–temporal coordinates. Plant cell differentiation is the result of different microenvironments in genetically identical nuclei. Therefore, we will not be able to fully understand the genetic expression simply by considering the nucleotide and amino acid sequence information, without taking into account any modification of transcription or translation. Thus, the epigenetic code includes genetic information and the biochemical modifications that take place on the chromatin (3, 4).

During cell differentiation or a metabolic switch, cells undergo profound changes in gene expression. These events are accompanied by complex modifications of chromosomal components and nuclear structures, including covalent modifications of DNA and chromatin up to topological reorganization of chromosomes and genes in the nucleus. To various extents, all these levels of organization appear to contribute to the stability and heritability of transcription programs and define what is meant as the epigenetic control of gene regulation. Most recent data suggest that tissue regeneration and transdifferentiation are controlled by epigenetic functions (5). Thus, the epigenome also provides the molecular basis for the preservation and plasticity of cell identity.

2. Chromatin Regulatory Factors

The chromatin is the form in which DNA is packaged within the eukaryotic cell. Its fundamental unit is the nucleosome, which consists of 146 bp of DNA wrapped around an octamer of core histones, each containing two molecules of H2A, H2B, H3, and H4. The function of this complex is to provide structure (i.e., packaged DNA to fit within the nucleus) and assist in the regulation of gene expression. Far from static, the chromatin structure is very dynamic, both spatially and temporally. This dynamism stems from the properties of the components (DNA and proteins), which are susceptible to a large number and type of biochemical modifications at different sites. These biochemical modifications are DNA methylation and posttranslational modifications (PTMs) of histones and are known as epigenetic marks, which are heritable and may be partly related to specific physiological states and stages.

2.1. DNA Methylation in Plants

DNA deoxycytosine methylation is the methylation of the 5' carbon of the pyrimidine ring of the cytosine (5-mdC). To date this is the only identified DNA epigenetic mark (6, 7). While in mammals it is restricted to CG sites, in plants it also occurs at CNG and CNN sites, where N is any base but G.

Establishment and maintenance of DNA methylation is carried out by DNA methyltransferases (DNMTs), which catalyze the transfer of methyl groups donated by *S*-adenosyl methionine (SAM) to deoxycytosines and are more abundant in plants than in mammals. This, together with the existence of a greater number of sequences susceptible to methylation, allows plants a finer regulation. It is thought that this is a necessity associated with their sessile life form, which restricts them from fleeing adverse conditions.

In plants, DNMTs are classified in four distinct gene families (8):

1. *MET*: This is the most extensively studied of the four gene families in plants. The methyltransferases of this family are responsible for maintenance of methylation especially at CG sites, but also are likely to play a role in maintenance of non-CG sites, as mutant studies indicate (6). Therefore, this family is crucial in epigenetic inheritance and imprinting. It is the homologous group to the *DNMT1* family of mammalian methyltransferases.
2. *DRMs* encoding domain rearranged methyltransferases: These genes are similar to the mammalian *DNMT3* family. Their function is to establish de novo DNA methylation, both CG and non-CG, in the RNA-dependent DNA methylation (RdDM) pathway (9). Thus, this family is very important in the defense against virus infection and in the response to environmental stimuli.

3. *CMTs* encoding chromomethylases, which are plant-specific. Like *DRMs*, *CMTs* also catalyze DNA methylation in non-CG sequences, but are only responsible for their maintenance, not for their de novo methylation.
4. *DNMT2*: the role of this gene family in DNA methylation remains un-elucidated (8).

Removal of DNA methylation is thought to occur in two different ways: replacement of methylcytosines by nonmodified cytosines during DNA replication (passive demethylation) and/or methylcytosines demethylation by DNA glycosylases (active demethylation). Two DNA glycosylases have been reported to date in plants: *DEMETER* (*DME*) and *ROS1*. Recently, a protein *ROS3* containing an RNA recognition motif has been found to bind to *ROS1*. This may be evidence of the existence of a RNA-directed demethylation, possibly as part of the RdDM pathway (10).

Until recently, DNA methylation was considered as a gene silencing mechanism. However, nowadays it is seen as a general gene regulation mechanism (i.e., responsible for both downregulation and upregulation of gene expression) and not independent, but as part of a more complex network (11). Therefore, there are strong indications that DNA methylation has an important role in the regulation of gene expression levels, making it pivotal in plant development and the physiological response to changing environmental conditions.

Clusters of sequences susceptible to methylation, especially CG and CNG, are called methylation islands. They are most often found in association with promoters and first exons, but are less common in 3' regions of genes. The presence of 5-mdC in these regions is usually associated with gene silencing, but the effect is different. Thus, 5-mdCs in the promoter regions are more efficient in repressing gene transcription than those in the coding regions (12). The number of genes regulated by promoter methylation is large in plants. Recent studies have determined that methylation in promoter regions takes place at CG sites and depends on *MET1* for maintenance and *DRM2* for de novo methylation through RdDM (13). The role of DNA methylation in other gene regions are thought to be less determinant in regulation of gene expression, but actually remains unknown (14).

Although the mechanism of DNA methylation is not completely known, it is well established that it can induce further chromatin remodeling by recruitment of methylcytosine-binding proteins such as *KRYPTONITE* (*KYP*), histone H3 (*H3K9*), and *VARIANT IN METHYLATION 1* (*VIM1*), which affect the access of the transcription machinery to DNA, i.e., gene expression (15).

2.2. siRNA

RNA-directed DNA methylation (RdDM) is a nuclear process in which small interfering RNAs (siRNAs) direct the cytosine methylation of DNA sequences that are complementary to the siRNAs.

In plants, double stranded-RNAs (dsRNAs) generated by RNA-dependent RNA polymerase 2 (RDR2) serve as precursors for Dicer-like 3 (DCL3)-dependent biogenesis of 24-nt siRNAs. Plant specific RNA polymerase IV (Pol IV) is presumed to generate the initial RNA transcripts that are substrates for RDR2. siRNAs are loaded onto an argonaute4-containing RISC (RNA-induced silencing complex) that targets the *de novo* DNA methyltransferase DRM2 to RdDM target loci. Nascent RNA transcripts from the target loci are generated by another plant-specific RNA polymerase, Pol V, and these transcripts help recruit complementary siRNAs and the associated RdDM effector complex to the target loci in a transcription-coupled DNA methylation process. Small RNA binding proteins such as ROS3 may direct target-specific DNA demethylation by the ROS1 family of DNA demethylases. Chromatin remodeling enzymes and histone-modifying enzymes also participate in DNA methylation and possibly demethylation. One of the well-studied functions of RdDM is transposon silencing and genome stability. In addition, RdDM is important for imprinting, gene regulation, and plant development. Locus-specific DNA methylation and demethylation, and transposon activation under abiotic stresses suggest that RdDM is also important in stress responses of plants (16).

2.3. Posttranslational Modifications of Histones

As described above, chromatin is a structural and functional complex formed by DNA and proteins, of which the core histones (H2A, H2B, H3, and H4) are the most prominent. Once translated, the core histones are susceptible to a number of biochemical modifications that are known as posttranslational modifications (PTMs) and include methylation, phosphorylation, acetylation, ubiquitination, glycosylation, sumoylation, biotinylation, carbonylation and ADP ribosylation, deimination, and proline isomerization (17).

Core histones are globular proteins, but have an N-terminal tail, in which the PTMs occur most frequently. Therefore, core histones constitute a complex platform for modulating the access of the transcription machinery to chromatin. As various modifications can occur simultaneously in a nucleosome (i.e., at the N-terminal histone tails), these create a specific chromatin transcriptional environment. The set of PTMs that modulate the nucleosome landscape is known as the histone code. Each type of histone PTM is moderated by two enzymes: one that performs the modification and one that counteracts. Enzymes have been identified for acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, deimination, and proline isomerization (17).

Although a transcriptional state depends on the set of PTMs in the nucleosomes, some individual PTMs have been established as primarily responsible. Lysine acetylation is thought to co-occur with transcription activation. The lysine acetylation neutralizes the basic charges and, as a consequence, chromatin becomes unfolded. This modification depends on two antagonistic enzyme families: histone acetyl transferases (HATs) and histone deacetylases

(HDACs). In the model plant *Arabidopsis thaliana*, approximately 15 HATs have been described, classified into several families such as the GNAT, the CBP/p300, and the TAFII family (12). Meanwhile, HDACs in plants belong to three families: RDP-like, HD-tuin, and sirtuin (12). Histone lysine methylation is the most abundant form of histone modification. It is mediated by histone lysine methyltransferases (HKMT families) and, until recently, was thought to be an irreversible modification. However, recent experimental results have shown that enzymes such as FLOWERING LOCUS D (FLD), LSD1-LIKE 1 (LDL1), and LSD1-LIKE 2 (LDL2), involved in the regulation of plant flowering, are histone demethylases (12). Each lysine residue can be mono-, di-, or trimethylated by HKMTs. Lysine trimethylation at H3K4, H3K36, and H3K78 is an epigenetic mark of actively transcribed euchromatin, while trimethylation of H3K9, H3K27, and H4K20 is associated with transcriptional repression and implicated as mediators in the formation of silent heterochromatin (17).

Although the above is generally accepted, it is necessary to point out that the effect of these and other histone PTMs depends on the gene region in which they occur. For example, methylation of H3K36 has a positive effect on transcription when occurring in the coding region and a negative effect when in the promoter. Methylation of H3K9 may have the same effect: restrictive in the promoter and promoting in the coding region (6). These examples make clear that PTMs are not unidirectional, and need to be seen in context. It becomes evident that gene transcription is a balancing act, and the result of various types of modification that are present in the nucleosomes.

2.4. Interplay Between DNA Methylation and Histone PTMs

Although DNA methylation and histone PTMs have been independently presented, both processes are part of the epigenetic network that modulates gene expression. In the literature, it is emerging that they do not act independently, but interact. The two processes may share the same feedback loop model to regulate gene activation or inactivation, as evidenced by factors such as DDM1 (decrease in DNA methylation) jmjC-domain-containing proteins and KYP engaged in both DNA methylation and histone modifications (12). Thus, after DNA replication certain 5-mdC may recruit enzymes and other factors involved in histone modification to establish and maintain the modifications.

3. Tree Development

Plant development in forestry species is achieved through a complex genetic network and regulated by multiple environmental and endogenous cues. Dynamic changes between chromatin states facilitating or inhibiting DNA transcription regulate the expression

of differentiation pathways in response to environmental and developmental signals. The epigenetic code is involved in controlling the functional state of the chromatin and gene expression that underpins the reprogramming of cells necessary for plant development and the survival of species. Efforts made in the field of epigenetics are helping to understand one of the crucial steps during plant ontogenesis that is sexual reproduction. In trees, sexual reproduction starts after a phase change, which allows flowering and seed production in order to ascertain the next generation. The genome-wide distribution of epigenetic marks, known as “epigenome,” has been the subject of extensive studies in recent years, and the “methylome” of *Arabidopsis* was recently presented (11). Although most studies related to epigenetics are carried out in *Arabidopsis*, epigenetics in trees is of great interest since these life forms are long-lived and many tree species involve a breeding program for reasons of economic interest. There is a lack of knowledge of sexual reproduction in trees. The contribution of epigenetic dynamics during flowering, fertilization and embryo development is dawning, with prospects of developing deeper insights.

In trees, the most widely studied epigenetic mark is DNA methylation, playing a role in many physiological topics such as flowering (18), somatic embryogenesis (19), and active growth (20). These processes appear to concur with changes in DNA methylation and/or histone modifications. DNA demethylation is required prior to entering any of these development programs. This variation in DNA methylation and histone modifications associated with development status is known as epigenetic plasticity. Throughout ontogeny, every developmental stage has its characteristic DNA methylation pattern. When this pattern is not followed, aberrant behaviors appear (21).

3.1. Flowering

The molecular aspects of floral initiation are best understood for herbaceous species. Therefore, a brief overview of the control of floral initiation of *Arabidopsis* precedes the discussion of trees and woody plants. Floral initiation includes all development steps necessary for the irreversible commitment by the meristem to produce an inflorescence. Epigenetic reprogramming modifying gene expression was demonstrated during floral transition similar to that defined in other developmental processes in plants and in mammals. The transition from the vegetative to the reproductive phase is arguably the most dramatic development change in the plant's life cycle. The regulation of this important process is achieved by a complex genetic network that monitors the developmental state of the plant as well as environmental conditions such as light and temperature (22, 23). Genetic analysis of flowering-time mutants in *Arabidopsis* has allowed the identification of 80 genes placed in multiple genetic pathways that control floral transition (24). The integration of environmental and developmental parameters

is decisive for reproductive success in plants, and in several studies a relation with epigenetic mechanisms such as DNA methylation and histone modification was demonstrated (25, 26).

Transitions between different developmental phases in the shoot apical meristem involve changes in the pattern of cellular differentiation and organ formation. Meristem cells are organized at different levels, and the central cells remain pluripotent, while cells in the periphery contribute to organ formation and eventually differentiate (27, 28). This zonation is established during vegetative development of the plant; however, DNA replication patterns are extensively altered during floral transition and concurrent reprogramming takes place (22). The cells of the central zone start to divide at a high frequency and the zonation is lost (18). These changes in apical meristem are genetically and epigenetically regulated (18, 29–31). It has become apparent that epigenetic control of transcription is mediated through specific states of chromatin structure (32). Associations of DNA methylation, histone modifications, and specific chromosomal proteins are involved in controlling chromatin states (17, 32, 33). Epigenetic control plays an essential role in the process of cellular differentiation, allowing cells to be reprogrammed in order to generate new differentiation pathway (3, 31, 34).

The regulation of the *FLOWERING LOCUS C* (*FLC*) gene in *Arabidopsis* provides a plant model of how chromatin-modifying systems have emerged as important components in the control of transition to flowering. Genetic and molecular studies have revealed three systems of *FLC* regulation: vernalization, the autonomous pathway, and *FRIGIDA* (*FRI*). All these involve changes in the state of *FLC* chromatin by DNA methylation and/or histone modification (26, 31, 35, 36). There is growing evidence that chromatin remodeling is involved in numerous processes of development and differentiation in plants (17, 37, 38). DNA methylation and histone modification have been revealed as hallmarks that establish the functional status of chromatin domains (39) and confer the flexibility of transcriptional regulation necessary for plant development and adaptive responses to the environment (4, 40, 41).

The results in shrubs (woody plants) indicate that epigenetic mechanisms such as DNA methylation and histone H4 acetylation have opposite and particular dynamics in the apical shoots meristem during the transition from vegetative to reproductive development (42). Global levels of DNA methylation and histone H4 acetylation as well as immunodetection of 5-mdC and acetylated H4, in addition to a morphological study have permitted the delimitation of four basic phases in the development of the *Rhododendron* sp. bud and allowed the identification of a stage of epigenetic reprogramming which showed a sharp decrease of whole DNA methylation (43) similar to that defined in gametogenesis and embryogenic preimplantation in mammals (2). This epigenetic

reprogramming is likely to be needed for totipotency, correct initiation of gene expression during floral transition, and early floral development. The epigenetic control and reorganization of chromatin seem to be decisive for coordinating floral development in *Rhododendron* sp. DNA methylation and H4 deacetylation act simultaneously and coordinately, restructuring the chromatin and regulating the gene expression during shoot apical meristem development and floral differentiation in woody plants.

3.2. Sexual Embryogenesis

In mammals it is well known that epigenetic marks during gametogenesis and early embryo development are erased, and then reestablished according with cell fate (44). Similar to mammals, plants undergo genome-wide demethylation during female gametogenesis leading to the reprogramming needed to control embryogenesis. During *Arabidopsis* sexual reproduction, it has been demonstrated that DNA methylation levels decreased not only in the female gamete (45) but also in the male gamete (46) prior to fertilization. It has been proposed that loss of methylation in the female gamete is due to the dilution of MET1 (METHYLTRANSFERASE1) during cell divisions prior to fertilization (45). Afterward, when fertilization takes place and new developmental patterns are imposed, methylation is reestablished. In chestnut (*Castanea sativa*), recent studies into sexual reproduction have shown that subsequent to fertilization, a transient global DNA demethylation is necessary in order to establish the sexual embryogenic pathway (47). This paper is the first to describe the role of DNA methylation in sexual reproduction in a woody species. Furthermore, it was shown that during sexual embryo development, cotyledons maintain constant DNA methylation levels while the embryonic axis shows an increase in DNA methylation until seed maturity is reached. Similar results were obtained for *Silene latifolia* studying the immunolocalization of 5-mdC during embryo development (18). When the proper pattern is not followed, abnormal plane and cell division during early embryogenesis is observed resulting in reduced viability in mature seeds (48). Loss of function of methyltransferases such as MET1 and CMT3 (CHROMOMETHYLASE3) can mediate the same effects, with purportedly a change in gene expression related to embryo development.

The epigenetic machinery in plants provides a fast response time to external factors. This allows cells to adapt to the biotic or abiotic signals rapidly. DNA methylation is known to play a role in silencing of transgenes, as a protection strategy (49). The phenotypic plasticity of plants could not perform without an efficient and accurate mechanism of gene activation–repression. Part of the DNA methylation marks accumulated in plants throughout development can be transferred to the offspring taking into account that germ lines are formed from somatic cells. Thus, there are evidences of epigenetic memory, defined as “transgenerational inheritance

of epigenetic states” (45). Nevertheless, it is not possible to accumulate epigenetic marks in every generation, which would be incompatible with embryonic development where totipotency of cells is required in order to give rise to valid embryos. However, some steps during gametogenesis and early embryogenesis require expression of genes from one of the parents or a specific ratio of expression, as is the case with endosperm. Epigenetic modifications play a seminal role in this specific control of gene expression. Moreover, transgenerational inheritance of information contained in the epigenome of an individual germ line must go through meiosis and postmeiotic mitosis in order to form the gametophyte, fertilization, embryogenesis and vegetative development of the new generation until the start of a new cycle (50). The accumulation of epigenetic marks and its transgenerational transmission has been suggested to be considered as non-Mendelian inheritance (40), constituting a rapid way of adaptative evolution (51). Methylation of CG is necessary in order to maintain the epigenome stability. When using mutants for conserving CG methylation, the functionality of other epigenetic mechanisms were lost and aberrant phenotypes presented through generations (37).

Imprinting is defined as the “epigenetic modification of maternally and paternally inherited alleles that leads to their differential expression in a parent-of-origin-dependent manner” (52). DNA methylation is, then, an efficient mechanism to control gene expression. This phenomenon was first described in maize (53) and afterward in mammals (54). Evolution of imprinting mechanisms in plants and animals has followed different ways, but converge to control crucial steps of sexual reproduction (55). In contrast to mammals, which reset imprinted genes during gametogenesis, plants maintain the epigenetic status. The default mammal status of imprinted genes is active, while in plants it is repressed (56). This main difference between animals and plants may be due to the tissue fate. While mammal tissues are conserved most of the times in the individual, there are parts of the seed that have a transient function during the ontogenesis. The endosperm, the most studied tissue in which imprinting acts, contains a maternal copy of the *FWA* gene that permanently lost methylation through site-specific DNA glycosylation (57). Since the tissue is destined to die after germination of dicotyledonous species, DNA methylation does not need to be reinstated or maintained.

There are many genes involved in plant sexual embryo development (58). Of the genes related with embryo development, the genes controlling this process epigenetically are predominantly *DEMETER* (a DNA glycosylase gene), *MET1*, *CMT3*, *DOMAINS REARRANGED METHYLASE1* (*DRM1*), and *DRM2*. The interaction between the products of these genes will determine partially the correct expression of the imprinted genes (59). Imprinting is strongly involved in sexual reproduction, specifically

after fertilization in the first steps of the embryo formation when the development pattern is established. Moreover, it is required for the correct formation of the endosperm, which constitutes in many types of seeds a source of energy for the embryo.

3.3. Somatic Embryogenesis

Somatic embryogenesis is the process by which somatic cells develop into plants through characteristic morphological stages that resemble zygotic embryo development. This is an alternative propagation technique to traditional procedures, presenting numerous applications in plant breeding (60). Moreover, production of embryos under in vitro conditions is a key experimental system to study cell division and reactivation, morphogenic competence, or rejuvenation (61). Since the first reports on somatic embryo formation (62, 63), somatic embryogenesis cultures have been successfully achieved for many angiosperms and gymnosperms species (64–69). Embryogenic cultures can be obtained either from explants cells that are already preembryogenically determined (70) or from somatic cells previously dedifferentiated by using stressful conditions such as nonoptimal salt concentration (71), or plant growth regulators (PGRs) such as auxins, of which 2,4-dichlorophenoxyacetic acid (2,4-D) is most widely used.

The induction of somatic embryogenesis entails the end of a current gene expression pattern in the explants tissue, and its replacement with an embryogenic gene expression program (72). Cell differentiation and development are controlled by temporal and spatial activation and silencing of specific genes. Epigenetic changes are a possible mechanism for regulation of current gene expression. During somatic embryogenesis, DNA methylation levels are essential for the acquisition of embryogenic competence and subsequent proliferation and morphogenesis (73–76). Moreover, the treatment of the somatic cultures with 5-azacytidine, a demethylating drug, caused a loss of regeneration capacity in embryogenic lines by arresting the production of somatic embryos (77).

A relationship between the embryogenic potential of somatic cultures and DNA methylation levels has been also established. DNA hypermethylation levels have been reported in nonembryogenic callus of *Eleutherococcus senticosus* compared with embryogenic callus (78) and in *Dactylis glomerata* significant differences in DNA methylation were observed in embryogenic and nonembryogenic calli from the same genotype, cultured under identical conditions (74). In *Pinus nigra* Arn. cell cultures, lower methylation levels were found in the line considered as effectively embryogenic (79). Changes in the methylation pattern observed during development and maintenance of embryogenic cultures show that DNA methylation may be involved in the regulation of the embryogenic program genes at the level of chromatin remodeling under the control of PGRs (80).

3.4. Aging and Maturation

DNA methylation is essential for normal functioning and identity of most somatic cells along lifetime of plants. Recent work demonstrates that loss of both CG and non-CG methylation can render plants unviable in a single generation (46). Multiple abnormalities were observed in methylation mutants from the earliest stages of embryogenesis through reproduction. This makes the hypothesis that DNA methylation is required for proper plant development attractive. An alternative explanation is that severe reductions in DNA methylation alter chromosome stability and function (81).

DNA methylation is maintained by DNMTs during development but the epigenome is dynamic, as it exhibits changes during the aging process. When the first mutants for DNMTs were available the mainstream hypothesis was that DNA methylation selectively shuts down genes whose expression is no longer required as the plant transitions through the vegetative, flowering, and reproductive phases of the life cycle (82). However, this idea has not been investigated in greater depth, and evidence for changes in DNA methylation associated with regulation of a gene's expression is limited. Some studies have compared global DNA methylation levels in plant tissues and observed subtle differences, supporting this hypothesis.

In general, animals have their major epigenetic switches during early developmental stages. Plant cells still have the potency to undergo major changes at later stages, including not only transition from vegetative to floral organs but also root formation from stem and leaves or shoot formation from differentiated organs (83). Maturational changes are particularly persistent and difficult to reverse in conifers, as has been demonstrated in several species (84). Differences in the extent of DNA methylation were found between meristematic areas of juvenile and mature *Pinus radiata* D. Don. trees, whereas differences between differentiated tissues were small (85, 86). Another species of interest is *C. sativa*, of which both the juvenile and mature phases may occur on the same mature tree. The upper parts of a tree exhibiting determinate growth are chronologically younger and often exhibit mature characteristics, whereas the lower, physiologically older parts may retain juvenile attributes. In this system, ontogenic development involves an increase of global DNA methylation in shoot apex during active growth, but the differences are reset with induction of dormancy (87).

The observed changes in extent of DNA methylation during aging and reinvigoration indicate that this form of epigenetic modification modifies the physiological response, with aging and reinvigoration at opposite ends of the scale. Increases in global DNA methylation of vegetative tissues appear incompatible with the activation of some specific genes needed for the reproductive transition in reproductive tissues. However, in chestnut and azalea flowers the differentiation period converges with a mild and temporal decrease of genomic DNA methylation (42, 87).

Global DNA methylation levels are specific for age, species, tissue, and organ in both plants and animals. In the former, the age-dependent demethylation of DNA is evident, and some investigators consider the degree of DNA methylation as a measure of the biological clock that assesses age and forecasts life span. Distortions in DNA methylation may lead to premature aging (7, 88, 89). Until recently, little was known about global DNA methylation with respect to aging and development in plants and information was not conclusive (21, 39, 90–93). Using a method developed for HPCE (85, 94) a gradual increase in global DNA methylation associated with aging was established for several forest tree species (3). By HPLC analysis, immature tomato tissues such as stems, leaves, and roots were showed to be less methylated than mature leaves, fruits, or seeds (95).

In animals aging involves, among other processes, site-specific epigenetic changes such as promoter hypermethylation of CpG islands, associated with gene silencing; and DNA hypomethylation of CpGs dispersed throughout repetitive sequences as well as of some transcriptionally relevant regions. DNA hypomethylation promotes genomic instability, amplification of oncogenes, and also silencing of the genes through an RNAi mechanism (96). Recent analysis of whole genome using Illumina's GoldenGate methylation platform in human tissue samples showed that loci within CpG islands became more methylated with age, but outside these CpG islands methylation decreased with age (97). Despite the well-known differences between animals and plants, certain similarities such as oxidative stress damage and telomere shortening are present among organisms. Aging studies related to global DNA methylation in plants are not available yet and the knowledge about epigenetic events concomitant with plants aging is in its infancy, possibly because of a more complex development. While the outcome of animal embryogenesis is that all organs are initiated and/or developed, plant embryogenesis results only in a bipolar structure: most organs of the mature plant are formed postembryonically. These distinctive developmental strategies of plants and animals concur with different tasks of their stem cells. Whereas the major task of an animal stem cell is to replenish highly specialized body cells with a limited life span, the plant's corresponding meristematic cells provide the material for the continued formation of new organs or in some cases new organisms (98).

AFLP analysis using methylation-sensitive restriction enzymes identified differentially digested fragments among *Arabidopsis* cotyledons, leaves, and flowers (29) and between rice seedlings and adult plants (99). While the significance of these experiments is unclear, they indicate that whole genome methylation profiling is a viable concept, at least for the sequenced genomes, with the potential to identify the sequences that are subject to DNA methylation with aging. Two recent studies have profiled the DNA methylation

pattern of the *Arabidopsis* genome by immunoprecipitating DNA from adult plants with a 5-methylcytosine antibody and hybridizing it to tiling arrays that cover the entire genome (11, 100). Both groups observed high methylation in areas with the highest density of repetitive elements. They also found that approximately one third of genes contain patches of DNA methylation within their transcribed regions, with far fewer genes being subject to DNA methylation in their promoter regions. The distribution of DNA methylation within transposons and genes is distinct; transposons are heavily methylated along the entire length, whereas within genes DNA methylation is present more prominently in 5' regions.

3.5. Dormancy

The phenological event that best describes the seasonal adaptation capacity in perennial plants is bud dormancy. Bud formation and growth cessation are the main phenological variables in the characterization of ecological adaptability, distribution and reproductive success (101). Trees are among the perennials that are strictly bound by these events, since early resumption or late cessation of growth may have severe consequences for these long-lived growth forms. The structures of buds and seeds are designed to overcome environmental conditions that are not suitable for plant growth. These resting organs need to cope with harsh temperatures and restricted water availability for prolonged periods of time, which imposes stress. To handle these difficult conditions, both buds and seeds have developed the ability for dormancy. Although primarily a block to subsequent growth, the function of dormancy is to spread growth across time but in synchrony with the seasons. This makes dormancy an important trait with benefits for survival. Bud dormancy imposition and release are two complex and gradual processes that are induced, imposed, and maintained by exogenous and endogenous factors such as temperature, nutritional state, photoperiod, phytohormones, phytochromes, and others (102).

Important inducing factors during dormancy imposition are hormonal and environmental signals, which exert their effect through activation/inactivation of gene expression programs that dictate para-, eco-, and/or endodormancy (103). The presence of 5-mdC in the promoter regions and coding sequences of specific genes alters the binding of transcription factors and other proteins to DNA (104, 105), thereby impeding transcription and causing gene silencing (106). The highly conserved histone proteins at the heart of the chromatin structure (H3, H4, H2A, H2B, and H1) function as building blocks to package eukaryotic DNA into repeated nucleosomal units, folded into higher-order chromatin. Acetylation of core histone tails, among other forms of posttranslational modification, plays a fundamental role in transcription regulation. Evidence of links between DNA methylation and histone hypoacetylation is accumulating (107). Several proteins that

specifically bind to methylated DNA are associated with complexes that include HDACs. In addition, DNA methyltransferases appear to interact with HDACs (108).

In *Arabidopsis* the WD40 repeat protein HOS15 confers cold tolerance which involves deacetylation of histone H4 and thus inactivation of transcription of downstream genes such as RD29A (109). Cold tolerance is acquired by *C. sativa* trees at the end of the growing season when temperatures drop, and when buds develop and dormancy is induced. There is growing evidence that chromatin remodeling is involved in dormancy progression (110–113). Acetylated H4 increases and methylated DNA decreases during dormancy break in potato tubers (114). A relationship between global DNA cytosine methylation, acetylated H4 histone, and bud dormancy in *C. sativa* was demonstrated (115). Increased methylation levels in the terminal buds coincided with bud set, while decreased methylation levels coincided with bud burst. Intermediate buds with paradormancy were characterized by reduced fluctuation in DNA methylation, particularly during bud burst. Furthermore, using immunodetection, acetylated histone H4 levels from terminal buds were found to be higher during bud burst than during bud set. Thus, global DNA methylation and acetylated histone H4 levels showed opposite patterns and coincided with changes in bud dormancy in *C. sativa*. Not surprisingly, differences in transcription were observed, allegedly as a result of these epigenetic changes. The dormancy transcriptome of *C. sativa* apical buds characterized by generating forward and reverse cDNA subtraction libraries, revealed association of nondormancy with genes in the functional groups for energy; protein with binding function or cofactor requirement; protein synthesis; biogenesis of cellular components; cell cycle and DNA processing. On the other hand, dormancy was associated with stress-related genes; and genes for cell rescue, defense and virulence; interaction with the environment; systemic interaction with the environment. A transcriptome comparison of bud dormancy in *C. sativa* and published data for seed dormancy in *Arabidopsis* demonstrated a core set of genes that are likely to play a principal role (116). These included stress-associated genes and one gene conferring an epigenetic mark (117). A general role for epigenetics in dormancy seems to emerge.

4. Plant Cell Reprogramming

In plants, regeneration of new organs from somatic cells has been known for a long time, but the mechanisms focused to dedifferentiate somatic cells, which are already differentiated, and change their fate enabling the formation of roots, shoots and damage repair are not fully known (118).

Cellular reprogramming involves a rewiring of epigenetic and transcriptional parameters (119). Reprogramming can be induced by molecularly undefined means, using an environment of components or elements that are largely unknown to achieve cellular reprogramming. An example would be fusion of somatic cells or somatic cell nuclear transfer, with unknown factors present in the cytoplasm involved in reprogramming of the nuclei (120). In contrast, direct reprogramming methods use defined genetic or nongenetic elements to induce reprogramming of the cell. Transcription factors and specific culture media with known components such as growth regulators are responsible for this reprogramming (121, 122).

In organ formation of both adventitious and regenerated organs in *Arabidopsis*, a reversion to stem cell niche activity is not necessary for in vivo early-root tip regeneration from developmentally young cells (123). However, the formation of a stem cell niche from competent callus cells obtained from young seedlings during in vitro plant organogenesis (118). Redifferentiation efficiency is much higher in tissues at earlier stages of development (86). Even callus cells from adult tissues show a lower proliferation rate and lower regeneration capacity than those induced from juvenile tissues (124).

4.1. Redetermination

Many pathways and strategies are possible from early to later stages of regeneration in both animals and plants depending on the progenitor cells (125). Despite the diverse regeneration mechanisms, most phenomena in one kingdom seem to have a counterpart in the other. Therefore, another interesting point would be to understand the mechanisms of redetermination of fully differentiated progenitor cells that do not pass through a callus state to regenerate (direct organogenesis), especially in relation to the developmental age of cells and tissues. Propagation of plants through tissue culture can induce a variety of physiological, genetic, and epigenetic changes.

4.2. Molecular Aspects of Reprogramming

In plants, cell plasticity enables somatic cells to begin new developmental programs generating adventitious organ formation in the presence of the appropriate plant growth regulator. Somatic embryogenesis and adventitious shoot formation are practical examples of cell reprogramming (124, 126). Morphogenesis and adventitious organogenesis are complex processes of cell plasticity, and according to Christianson and Warnick (127), they can be divided in three stages: acquisition of competence, induction, and morphological differentiation. In adventitious caulogenesis, somatic cells respond to the hormonal stimulus with a reprogramming process achieving a new cell fate. Cytokinins are the most efficient plant growth regulators in the induction of this process. In *Arabidopsis*, histidine protein kinases are cytokinin receptors,

and histidine phosphotransferases transmit the signal to response regulator in the nucleus activating or repressing transcription (122). Some genes have been already described to be related to the adventitious bud formation process. Examples are *cytokinin response 1* (*CRE1*) (128) taking part in the cytokinin hormone receptor; *enhancer of shoot regeneration 1* (*ESR1*) allowing the development of cytokinin independent shoots (129); the gene family *NO APICAL MERISTEM* (*NAM*); *CUP SHAPE COTYLEDON* genes (*CUC1* and *CUC2*) that have an additional function related to cotyledon separation (130–132); and *PkMADS1* (133) for which expression is only observed in the apex and in tissues producing adventitious shoots. Global programs of gene expression during shoot and root development involved in cytokinin and auxin signaling have been revealed by microarray hybridization demonstrating the effects of gene families and their interaction (134, 135). Regarding somatic embryogenesis several biological studies show a different effect of growth regulators during the process (19, 60), and different gene families such as *LEAFY COTYLEDON* (*LEC*) or *AGAMOUS-LIKE* (*AGL*) are overexpressed, while ectopic expression is sufficient to promote somatic embryogenesis (136).

4.3. Genetic Stability of Clones

Somaclonal variation is the genetic variation that is observed when plants are regenerated from cultured somatic cells, and was first detected by the high frequency of qualitatively segregating phenotypes observed among progeny of plants that were expected to be genetically identical (137). It has been used to generate desirable traits, but the results have not met the expectations (138). This genetic variation is heritable and usually reduces the commercial value of micropropagation of valuable elite clones, which is a problem in breeding and propagation techniques that involves adventitious regeneration. Various types of mutations have been described in somaclonal variants including point mutations, gene duplication, chromosomal rearrangements, and chromosome number changes (139). Somaclonal variation takes place in plants generated via adventitious shoot or somatic embryo formation, in particular after an intermediate callus phase (140, 141). Micropropagation and somatic embryogenesis, via suspension cultures, may also suffer from somaclonal variation (142). A number of different molecular techniques are currently available to detect sequence variation between closely related genomes such as those between source plants and somaclones. Main techniques include random amplified polymorphic DNAs (RAPDs) and amplified fragment length polymorphisms (AFLPs) (87, 143, 144). Both techniques are useful in comparing the DNA from any number of different samples for the differentiation of plants because of sequence variation by identifying random polymorphisms. There are also some approaches to measure somaclonal variation by statistical analysis for in vitro culture (145).

4.4. Epigenetic Stability of Clones

Genetic alterations can cause epigenetic changes which are not hereditary and which confer stable phenotypes and persist even in the absence of an agent or inductive condition. These epigenetic changes are caused by alterations in DNA methylation, in histones, or through other epigenetic modifications. Usually, these modifications last until the effect of the inducing condition disappears (146), but there are several reports stating that these changes may be transferred by sexual propagation and even have the capability to persist during in vitro culture (147, 148). In a similar way, especially in forestry species, a prior modification of the DNA methylation levels sometimes allows increased success in the in vitro culture. This situation happens in a reinvigoration process where a decrease in DNA methylation takes place (85).

It has been suggested that changes in the epigenetic status of a plant tissue cultured in vitro could be caused by an incomplete resetting of the epigenetic marks, but stress is also known to provoke epigenetic changes such as transposon activation and activation or deactivation of the methylation status of loci (16, 149, 150). Even using different plant growth regulators showed big genetic differences and one might wonder if the differences in methylation in plants cultured in vitro is the result of culturing or of additional processes such as wounding, asepsis, or hormone application.

5. Tissue Culture-Induced Phenotypic Variations and DNA Methylation

One of the molecular mechanisms responsible for in vitro culture-associated phenotypic changes is DNA methylation which generates somaclonal variation by an indirect pathway; that is, without involvement of genetic changes. Low levels of DNA methylation are known to affect normal plant development (21), and have an effect on the gene–environment interaction (151). Furthermore, variation in DNA methylation of specific genes causes different patterns of flower symmetry (152).

The effects of somaclonal variation are detectable in various phenotypic traits, from bushiness in the ornamental crop *Zantedeschia* to leaf shape in begonia. The most studied species with aberrant development as a result of somaclonal variation is the crop *Elaeis guineensis*, because of its economic importance. Oil palm in vitro propagated may show aberrant flowers, also known as mantled, because they develop a second whorl of carpels instead of stamens (153). Most of the genes found related to this change are members of the MADS box transcription factor family. (154) have found epigenetically regulated genes related to auxin response, although no direct correlation was found for mantled flowers and methylation of specific genes. A similar issue was observed in cauliflower, but this seemed to be more related to nuclear DNA content rather than to DNA methylation (155).

Results obtained with MADS box genes and with genes related to hormone response have an important effect in somaclonal variation because of the role played by cytokinins (156), encouraging scientists to study epigenetic mechanisms that might explain the observed changes.

6. Conclusions

DNA methylation is implicated in the correct transition between stages of plant development (i.e., flowering, dormancy, etc.). Plants have common and distinct DNA methylation mechanisms compared with animals, summarized in three different classes of DNA methyltransferases: the *MET1* family, which acts as the homologue to the mammalian *DNMT1* family; the chromomethylase family, which preferentially methylates CpNpG sequences; and de novo methyltransferases, which are homologous to the mammalian *DNMT3* family. DNA methylation is stable during development, albeit not static, and contributes to cellular plasticity. Cellular differentiation is associated with an initial DNA demethylation, which permits later cellular reprogramming. Specific DNA demethylation is related to flowering, stem cell differentiation, etc. The formation of germ-line cells late in development allows the transmission of stable epigenetic information acquired during the life cycle. This involves several different epigenetic mechanisms including DNA methylation, histone modifications, and siRNA.

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The Potential Roles of microRNAs in Molecular Breeding

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Abstract

microRNAs (miRNAs), a class of small, endogenous, noncoding RNAs, are uncovered to play greatly expanded roles in a variety of plant developmental processes by gene silencing through inhibiting translation or promoting the degradation of target mRNAs. In virtue of their ability to inactivate either specific genes or entire gene families, artificial miRNAs function as dominant suppressors of gene activity when brought into a plant. Moreover, artificial target mimics are applied for the reduction of specific miRNA activity. Consequently, miRNA-based manipulations have emerged as promising new approaches for the improvement of crop plants. This action includes the development of breeding strategies and the genetic modification of agronomic traits. Herein, we describe the current miRNA-based plant engineering approaches, and their advantages and challenges are also stated.

Key words: microRNA, Molecular breeding

1. Introduction

Due to an increasing world population, as well as scarcity of arable lands and water, global climate changes, and the demands for biofuels as substitutive energy sources, sufficient future global production of food from crop plants is of serious concern (1). To overcome these problems, new agricultural technologies will be needed to ensure global food supply and security, with no regard to the efforts of conserving water and lands (2). Plant molecular breeding seems to be an efficient and economic means of tailoring crops to improve their qualitative and quantitative traits including tolerance to biotic and abiotic stresses. The rapid advance in knowledge on genomics and proteomics will certainly be beneficial to fine-tune the molecular breeding approaches so as to achieve a significant progress in crop improvement in the near future.

Recent studies have begun to reveal powerful and unexpected roles for microRNAs (miRNAs) in controlling plant growth by native gene silencing. miRNAs have been reported to modulate various aspects of plant development, including leaf morphogenesis and polarity, root initiation and development, floral differentiation and development, vascular development and transition of plant growth from vegetative growth to reproductive growth (3, 4). Moreover, miRNAs are also involved in phytohormone signal transduction (5) and signal transduction in response to environmental stress and pathogen invasion (6, 7). These regulatory miRNAs stimulate the idea of developing artificial miRNAs to silence specific gene(s), allowing for the direct molecular modulation of plant traits, which can be applied to crop species for breeding.

Additionally, another layer of modulation in miRNA action has been reported by Franco-Zorrilla and his colleagues when studying phosphate starvation (8). The *IPSI* sequence which encodes a noncoding RNA, contains a three-nucleotide insertion in the motif that is highly complementary to the sequence of miR399. This characteristic prevents the normal cleavage of *IPSI* and thus sequesters the action of miR399. Based on these features, artificial target mimics were born to exceptionally reduce the miRNA activity (9). This method can be applied to study the function of miRNA and reversely modulate agronomically related miRNAs to facilitate molecular breeding. Here, we summarize the already known miRNA-based approaches in plant engineering and lastly consider the modulation feasibility and future challenges for putting these approaches into practical use in breeding program to increase crop yield.

2. miRNAs Modulation as an Innovative Approach to Plant Engineering

Some lessons learned from existing antisense technology and gene engineering approaches can be adapted to manipulate specific genes or miRNAs levels in vivo (Fig. 1). To date, there are two methods available to selectively target miRNA pathways. Presently, short-hairpin RNAs based on miRNA precursor backbones (artificial miRNAs (amiRNAs)) are the most successful expression cassettes to induce highly specific gene silencing in plants (10). Expression of amiRNA precursors can generate mature amiRNAs with high specificity similar to natural miRNAs to cleave their target genes (11) (Fig. 1b). Moreover, target mimics, consisting of a noncleavable RNA that forms a nonproductive interaction with a complementary miRNA, provide a new policy that can be used to inhibit the activity of specific miRNAs (8, 9) (Fig. 1c).

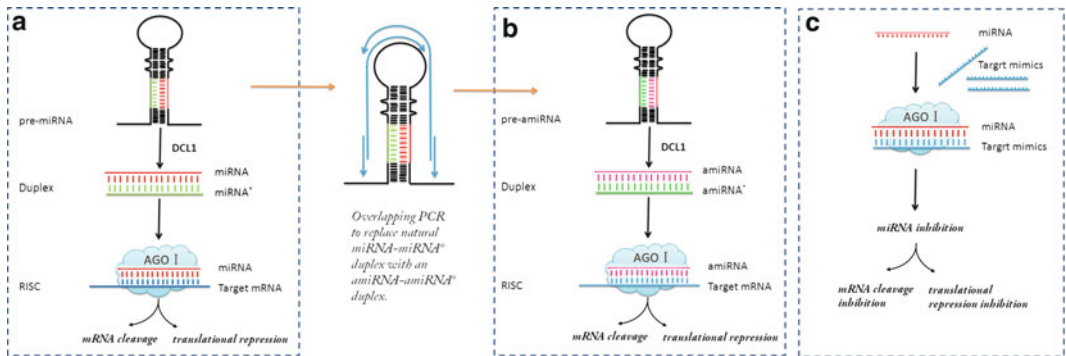


Fig. 1. miRNA-based technologies to regulate gene expression in vivo in plants. (a) Following transcription, the pri-miRNA is processed by DCL1, perhaps with the aid of HYL1 and other factors, to a miRNA:miRNA* duplex. The mature miRNA is incorporated into a silencing complex that includes AGO1 which then mediated the degradation of mRNA or its translational repression. (b) AmiRNA is engineered into a miRNA precursor using overlap PCR to replace the endogenous miRNA sequence. The pri-amiRNA is processed by DCL1 and function as normal miRNA. (c) Artificial target mimic is a noncleavable RNA which is not cleaved but instead sequesters a complementary miRNA. The perfect sequence complementarity allows the target mimic to bind to the miRNA and interfere with miRNA function.

2.1. Artificial miRNAs

RNA silencing is a natural regulatory mechanism of eukaryotes modulating endogenous gene expression. Generally, double-stranded RNAs (dsRNAs) or hairpin RNAs (hpRNAs) are processed into small interfering RNA (siRNA) or microRNA (miRNA) duplexes by specific enzymes (12). Then these small RNAs are incorporated into the RNA-induced silencing complex (RISC) to guide the degradation or translation repression of mRNA targets. Thus, miRNAs and siRNAs can affect gene expression in animals and plants by posttranscriptional gene silencing.

Recently, more and more reports have demonstrated that the alteration of several nucleotides within the miRNA sequence does not affect its biogenesis and the positions of matches and mismatches within the precursor stem loop remain unaffected. Because of these features, artificial miRNAs can be created by exchanging the miRNA/miRNA* sequence within miRNA precursor genes. Furthermore, the genome-wide gene expression analyses have also demonstrated that plant amiRNAs have similarly high specificity as endogenous miRNAs. Thus, miRNA sequences can easily be optimized to knock down a single gene or highly conserved gene families without affecting the expression of other genes (11, 13). Based on these results, for gene engineering to improve agronomical traits, amiRNAs can be designed to target any gene in need with great specificity.

AmiRNAs share many properties with siRNAs. However, differences still exist. Particularly, a diverse set of siRNAs can be produced from the complete dsRNA; thus, siRNAs not only target

candidate RNAs but also can affect RNAs that are not perfectly complementary, generally considered off-targets, while an amiRNA precursor gives rise to only a single small RNA species without extensive homology to any plant genes that can be optimized to avoid off-target effects (10, 13). Besides, it has been reported that transgene-derived or viral-induced siRNAs are able to move from cell to cell, indicating that their functions are nonautonomous, whereas miRNAs are not mobile and act cell autonomously (13, 14). Finally, amiRNA-mediated method has no environmental biosafety problem when applied in agriculture (10). In summarize, these predominances make the application of amiRNAs credible for plant molecular engineering to a large extent.

Nowadays, the amiRNA-based gene silencing technique has been used successful as expression cassettes to induce highly specific gene silencing not only in higher plants (*Arabidopsis thaliana*, *Oryza sativa*, *Populus trichocarpa*, *Lycopersicum esculentum*, and *Nicotiana tabacum*) but also in lower plants (*Physcomitrella patens*), as well as unicellular organisms (*Chlamydomonas*) (10, 11, 13, 15–18). The results showed that custom-made, synthetic miRNAs vectored by endogenous pre-miRNA backbones produced phenocopies of multiple mutant combinations of genes that are not naturally regulated by miRNA, suggesting the feasibility that amiRNAs can be used successfully to target any candidate genes other than the one which are natural targets of endogenous miRNAs (2).

As a model plant for monocots, rice is also one of the most important staple crops in the world (16). Fortunately, the successful use of amiRNAs for the specific downregulation of genes was also shown for rice. This action has been utilized effectively to improve the significant agronomic traits and at last increases the crop yields (16). Besides, the successful application of amiRNAs to mediate specific gene silencing was demonstrated not only in indica rice but also in japonica rice. Most importantly is that the transgenes are stably inherited and they remain effective in the progeny. This remarkable characteristic paves a way for amiRNAs-based gene silencing to regulate agronomically important traits in varieties used in modern breeding, with the ultimate intention of improvement of agronomic performance and nutritional value (2, 16). Lastly, since rice shares excellent gene colinearity with other monocots such as wheat and maize, amiRNAs-mediated gene breeding strategy in rice can also be applied to other species to a large extent (16).

In the entire life of plant development, plants have to deal with a variety of environmental stresses such as salinity, drought, and pathogen invasion. Generally, RNA silencing in plants is a natural defense system against foreign genetic elements including viruses. Furthermore, it has been reported that short-hairpin RNAs based on miRNA precursors to express the artificial miRNAs (amiRNAs) can specifically target transcripts originally not under miRNA control to induce gene silencing (11, 15). Based on these results, amiRNAs

appears to be a promising tool for conducting plant virus resistance. So far, the use of amiRNAs in engineering plant virus resistance has been extensively explored. The results declare that the expression of virus-specific artificial miRNAs has been verified to be an effective and credible new approach to engineering plant resistance to CMV (Cucumber mosaic virus) and TuMV (HC-Pro of turnip mosaic virus), and possibly, to other plant viruses as well in the near future (10, 15).

To sum up, amiRNA-based approach has been proved to be a highly effective tool to modulate agronomically important traits in different plant varieties used in modern breeding, the ultimate aim of which is to improve the agronomic performance and crop yield (2). With regard to the genes which confer significant agronomic traits and perform as negative regulators, amiRNAs can be engineered to silence these specific genes to satisfy the desirable traits in crop plants. We believe that as long as we expend sufficient effort into this approach, the aim of putting amiRNAs to practice is highly likely to be achieved in plant molecular breeding as soon as possible (2).

2.2. Artificial Target Mimics

In plants, most miRNA targets are cleaved and show much higher complementarity with the miRNAs around the cleavage sites in the coding regions. This characteristic accounts for the generation of target mimicry, which is considered to be composed of a noncleavable RNA which is not cleaved but instead sequesters a complementary miRNA (8). Target mimicry represents an additional layer of regulation in miRNA action by counteractive impact and it was found while studying Pi homeostasis, a critical determinant of growth performance and one process regulated by miR399 (8, 19). *IPS1* (*INDUCED BY PHOSPHATE STARVATION1*) RNAs is involved in response to Pi starvation and contains a region of complementarity with miR399. In comparison to regular miRNA target sites, the *IPS1* sequence contains a mismatched loop at the expected miRNA cleavage site, and this bulge in the miRNA–target pair prevents endonucleolytic cleavage of *IPS1* transcripts (8). Thus, *IPS1* RNA is not cleaved but instead sequesters miR399, leading to a reduction of miR399 activity.

More significantly, the *IPS1* family members represent an example of natural target mimicry and this mechanism can be exploited to study the effects of inhibiting miRNAs other than miR399 as well as even the entire miRNA families. The nearest report indicated that a large-scale collection of knockdowns for *Arabidopsis thaliana* miRNA families has been achieved using artificial miRNA target mimics (9). Conceptually, target mimics share much similarity with miRNA sponges which were used to reduce miRNA actions in animals. Both of them are transcripts containing multiple miRNA binding sites that compete with endogenous target mRNAs, thereby reducing the efficiency of the corresponding

miRNA (9, 20). The difference is that the miRNA cleavage sites of target mimics must be modified to prevent cleavage, but to still allow miRNA binding. Therefore, the plant target mimics used for research are modified from the 23 nucleotides of miR399-complementary motif in *IPS1* (9). They were designed to knock-down the majority of *Arabidopsis thaliana* miRNA families. The results showed that one fifth of these transgenic plant lines have obvious morphological defects. Additionally, phenotypes of plants expressing artificial target mimics directed against miRNAs involved in development were in several cases consistent with previous reports on plants expressing miRNA-resistant forms of individual target genes, demonstrating that a limited number of targets mediate most effects of these miRNAs (9). In conclusion, artificial target mimics can be applied successfully to reduce miRNA activity to some extent in plants.

According to previous reports, miRNAs have been proved to influence almost every aspect of plant growth and development, with a diverse set directly involved in the modulation of agronomic traits, such as miR164 in lateral root formation and miR165/miR166 in shoot branching (2). Naturally, miR164 negatively modulate *NAC1* mRNA to repress lateral root formation and reduced lateral roots affect the ability of a plant to secure edaphic resources, which ultimately influence crop yields. Engineering target mimics to sequester miR164 is capable for plants to express more lateral roots to sufficiently secure edaphic resources. Similarly, target mimics can also be applied for miR165/miR166 to increase tillers and consequently improve grain yield. As a result, plant artificial target mimics represents a novel technique to modulate endogenous miRNAs levels to improve the agronomic traits in crop plants, with the eventual intention to improve crop output (2).

3. Challenges for Improving the Efficiency of miRNA-Based Approaches

Compared to the conventional targeted gene knockout approaches, though still a relatively new method, the application of amiRNA seems to be at least as effective and versatile as conventional hpRNAi while at the same time promising greater specificity and safety (2, 21). More and more researchers have adopted amiRNA to silencing specific target genes (22). However, limitations are still existed. It is reported that the overall success rate of amiRNA-based gene silencing is close to 75%, suggesting that amiRNAs design is not maximally optimized (21, 23). Recent work have indicated that the amiRNA design incorporating perfect complementary to a target gene could be favorable in increasing gene silencing efficiency, and manipulation of amiRNA structure and sequence proves also beneficial to further enhance the

effectiveness of amiRNA in plants (2, 23). Therefore, progression in the understanding of biogenesis and action of endogenous miRNAs, which appears to be a very active field of scientific inquiry within a long time, should allow further improvement and strengthened sophistication and application of the amiRNA approach (2).

From the above indication, we get the knowledge that the application of target mimics to reduce activity for most of the known miRNA families in *A. thaliana* was proved to be feasible, with 14 out of 71 miRNA families with target mimics led to morphological abnormalities, agreeing with former reports in which miRNAs were overexpressed, miRNA target genes were mutated, or miRNA genes were inactivated by conventional knockouts (9). However, it has to be mentioned that when compared to the conventional gain-of-function or loss-of-function of miRNA mutants, the defects of target mimics transgenic plants were often weaker. For example, plants overexpressing miR172 target mimics displayed absence of altered floral phenotype. One of the reasons may be that many miRNAs affect their targets only in a small set of cells and assaying expression in whole organs would obscure the effects of miRNA downregulation on mRNA levels (9). Moreover, for miRNAs required for embryonic development, only lines with relatively weak expression of the artificial target mimic might have survived. Such defects could be overcome by tissue-specific or inducible expression of target mimics. Nonetheless, though artificial target mimics have limitations, they possess the advantage that they affect all targets simultaneously (8, 9).

4. Concluding Remarks

Gene silencing, a powerful genetic tool for knocking down gene expression, is commonly employed to clarify and manipulate biological function of novel, agronomically important genes (23). Plant miRNA, a class of endogenous small RNA, has been demonstrated to play a pivotal role in plant growth and development also by gene silencing using mRNA degradation or mRNA translational inhibition. This station led to the generation of miRNA-based approaches—artificial miRNA and artificial target mimics, used for gene function exploration or modern molecular breeding. The amiRNA technology exploits endogenous miRNA precursors as structural backbones but replacing the stem-loop region with a specific amiRNA sequence, designed to specifically target a selected gene(s) of interest or groups of closely related genes (21). This method could produce amiRNA that have similarly high specificity to naturally endogenous miRNA and guide the RISC to cleave their target genes (11, 21). Thus, the amiRNA sequences can be easily optimized to silence one or several target genes without

affecting the expression genes (21). Artificial target mimics were generated by the modification of miR399-complementary sites in *IPSI* to sequester specific miRNA or the entire miRNA families (9). The phenotypes caused by target mimics share extensive similarities with the ones by expressing resistant forms of individual targets, suggesting the feasibility of using target mimics to reduce the activity of miRNA (9).

More intriguingly, the researchers also shown that when mimic-like sites are introduced into the 3'-UTR of a protein-coding gene, they not only are active in sequestering the targeted miRNA but can also reduce protein levels produced by the mRNA linked in *cis* (9). Since mimic sites are not subject to miRNA slicing, the reduction is thought to occur at the translational level (8, 9). These results give us an intriguing scenario which exhibits that mRNAs containing mimic-like sites, or possibly other sites with reduced complementarity with miRNAs, are regulated by miRNAs exclusively through translational inhibition (9).

In conclusion, the miRNA-based approaches could be designed to silence specific genes or miRNAs so as to validate the function of candidate genes, which are responsible for advantageous agronomical traits, and contrarily broaden the usage of these approaches to improve agronomic performance. In a word, amiRNA and artificial target mimics appear as promising methods that are being developed for identifying agronomical trait-relevant genes and putting them to practical use in crop improvement (2).

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Determination of Histone Methylation in Mono- and Dicotyledonous Plants

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Abstract

Epigenetics includes DNA methylation and histones posttranslational modifications such as methylation, acetylation, phosphorylation among others. One of the most abundant modifications in histone tail is the methylation. It has been found that the methylation pattern in the histone H3 may provide understanding of the process involved in cell differentiation, adaptation, and evolution in plants. In this work, we detail a method for isolation of nuclear proteins from small amount of sample to identify global changes in different lysines of the histone H3 tail by using immunodetection.

Key words: *Agave fourcroydes*, *Coffea canephora*, Epigenetics, Histone methylation

1. Introduction

1.1. Patterns of Histone H3 Methylation

The nucleosome is the fundamental unit of the chromatin; it is composed of 146 pb of DNA wrapped on a histone octamer which is formed by two copies each of the cores histone proteins H2A, H2B, H3, and H4 (1). The chromatin is a dynamic molecule regulated by multiple epigenetic mechanisms, including DNA methylation, histone modification and by noncoding RNA, which together with the former mechanisms allows the chromatin to relax or to compact creating euchromatin (actively transcribed region) or heterochromatin (transcriptionally silent region), respectively (2, 3).

Histone posttranslational modifications (PTMs) play a central role in the epigenetic regulation of gene expression in eukaryotic organisms. These modifications reorganize the chromatin structure in which the DNA is packaged within the cell, controlling cell differentiation and development. Histone PTMs occur mainly on the N-terminal tails of histones. Histone methylation together with

other modifications (acetylation, phosphorylation, ubiquitylation, sumoylation, ADP-ribosylation, deamination, proline isomerization) forms a sophisticated code which is catalyzed by different enzymes at specific amino acids residues (4, 5).

Methylation is only found in the lysine (K) residues of the histones H3 (K4, K9, K27, K36, and K79) and H4 (K20), being more abundantly in H3. Lysine methylation can be found as mono, di, or trimethylated, which is controlled by different histone methyltransferases and demethylases (6, 7). Histone lysine methylation is involved in the activation or repression of gene transcription depending on both the position of the lysine that is methylated and the degree of methylation (8).

Recent studies in *Arabidopsis* have shown that trimethylation of histone H3 at K4 is associated with transcribed regions, while di- or trimethylation of histone H3 at K36 is linked to transcription elongation (7, 9). On the other hand, the dimethylation at K9 represses gene expression, and it has been observed that this epigenetic mark is concentrated in heterochromatin regions (10). Another repressor mark of the transcriptional activity well studied is the methylation at K27 in the histone H3. Trimethylation of histone H3 at K27 has been implicated in development regulation to maintain the transcriptional repressed stage of target genes during cell division in *Arabidopsis* (8, 11, 12). Unlike the dimethylation at K9, the monomethylation of K27 mark is independent of DNA methylation, and it has been observed in heterochromatic regions, whereas di- or trimethylation at K27 is mainly present in euchromatic regions (13).

1.2. Methods for Detection of Histones Posttranslational Modifications

The study of chromatin and epigenetics has grown rapidly in the past few years, and now it has become one of the most outstanding biology areas for plant development research. The genome-wide distribution of the different marks in histones can be determined by mass spectrometry analysis (14), high performance liquid chromatography (HPLC), chromatin immunoprecipitation (ChIP) or ChIP coupled to gene array technology (ChIP-ChIP) (15), and covalent attachment of tags to capture histones and identify turnover (CATCH-IT) (16). Other techniques available are used to examine the distribution of histone variants or histone PMTs, e.g., fluorescent in situ hybridization (FISH) or immunodetection by western blot (2, 17, 18). These and other approaches can be used to understand plant development in response to environmental changes. However, some of them, although they have many molecular advantages, are very expensive to use or require sophisticated software. In the present chapter, we describe a less expensive method to determine histone modifications by western blot which has been modified from current published methods for nuclear proteins (2, 19). This method details the steps used for nuclear proteins extraction from a very small amount of tissue, the transference of nuclear proteins to either a PVDF or a nitrocellulose membrane,

the incubation protocol of different antibodies to determine the different PTMs in the histone H3 in mono- and dicotyledonous plants, as well as the detection method.

1.3. Analysis of the Epigenetic Changes in Mono- and Dicotyledonous In Vitro Systems

During plant development, transcription regulation is carried out by epigenetic mechanisms (20–22). These epigenetic mechanisms are mainly DNA methylation and histone posttranslational changes that modify the chromatin structure. The histone PTMs are a crucial step to change the structure of chromatin for the reprogram of plant development for acquisition of pluripotency, totipotency or for maintaining the integrity of the genome (23). Recent reports indicated that epigenetic mechanisms are similar in both mono- and dicotyledons; for example, in *Arabidopsis thaliana* the overexpression of SET1 domain proteins decreases in both root and leaf growth, whereas in *Oryza sativa* it leads to retarded plant growth (24, 25). Furthermore, it has been observed that KRYPTONITE of *Arabidopsis* and SDG714 of *O. sativa*, both methyltransferases of the H3K9, play a similar role repressing the expression of transposable elements as *Ta3* and *Tos17*, and in both species the loss of this mark increases gene expression (10, 26). This mechanism of methylation at the H3K9 indicates that the pattern of histone methylation works in the same way to program, establish, maintain, and protect the integrity of the genome in plants.

1.4. Method Advantages

The method presented in this chapter has the advantage of good extraction compared with previous studies (2, 10). This method increases the quantity of protein extracted. By using a small amount of sample, reducing it from 3 g (19) to only 500 mg of tissue, we are able to obtain approximately 200–250 µg of total protein that is sufficient to perform more than 50 blots. Five micrograms of total nuclear protein is enough to transfer to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P) in 3.5 h. The blocking solution used is nonfat milk, and the incubation period with primary and secondary antibodies is 60 and 30 min at room temperature, respectively. The detection method used is chemiluminescence. The method described here for the analysis of histone H3 methylation pattern has been tested with mono- and dicotyledonous species, obtaining reproducible results. The methodology described here is simple, easy, efficient, and reproducible. Furthermore, the membrane can be nitrocellulose or PVDF and be retested with different antibodies to see different marks in the same sample.

2. Materials

2.1. In Vitro Plantlets

The plant material used was embryogenic tissue of the dicotyledonous plant *Coffea canephora* obtained as described by Quiroz-Figueroa et al. (27). The monocotyledonous plant *Agave fourcroydes*

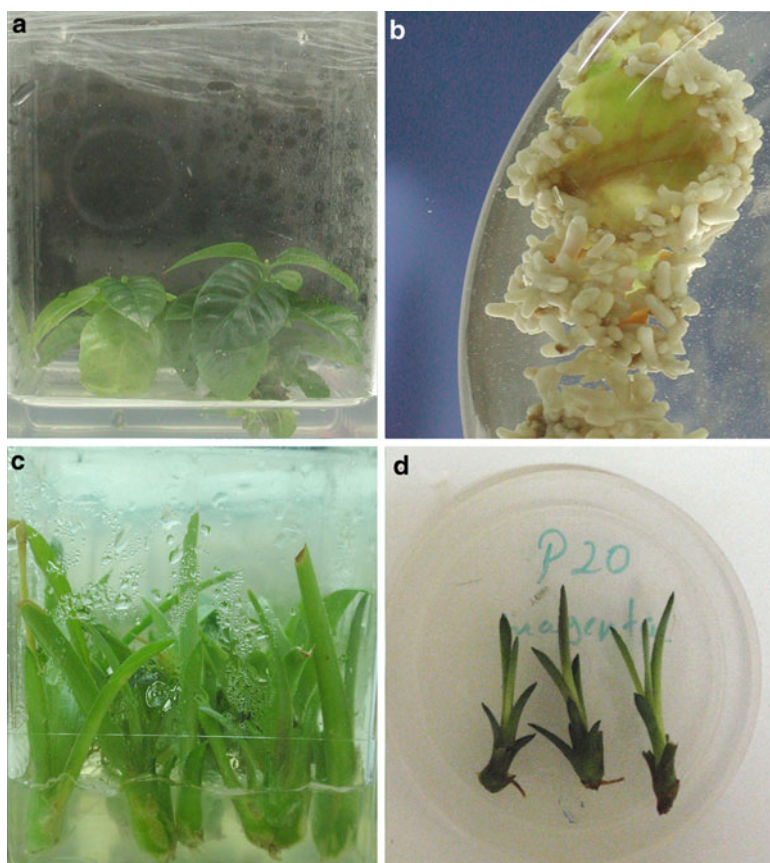


Fig. 1. (a) Plantlets of *Coffea canephora* in vitro. (b) Embryogenic system of *C. canephora* after 2 months of induction. (c) Micropropagation of *Agave fourcroydes* in vitro. (d) Plantlets of *A. fourcroydes* obtained in vitro ready to transplant into soil.

was grown at $25 \pm 2^\circ\text{C}$ under a 16/8-h (light/darkness) photoperiod (see Fig. 1).

2.2. Histone Isolation

1. Nuclei isolation buffer (NIB): 15 mM PIPES pH 6.8, 5 mM MgCl_2 , 60 mM KCl, 0.25 M sucrose, 15 mM NaCl, 1 mM CaCl_2 , 0.8% Triton X100, and as inhibitors of proteases 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 $\mu\text{g}/\text{mL}$ aprotinin (see Note 1).
2. 0.4 M H_2SO_4 .
3. Cold acetone (maintained at -20°C).
4. 4 M urea.
5. Liquid nitrogen.
6. Mortars and pestle.
7. Falcon tubes (50 mL).
8. Microcentrifuge tubes.
9. Sterile cheesecloths.

Table 1
Solutions for SDS PAGE 15%

4× separating buffer	2.5 mL	4× stacking buffer	2.3 mL
Acrylamide (30%)	5 mL	Acrylamide (30%)	0.670 mL
Water	2.5 mL	Water	2.5 mL
APS (10%)	50 µL	APS (10%)	30 µL
TEMED	25 µL	TEMED	15 µL

2.3. SDS PAGE

1. 4× separating buffer: 1.5 M TRIS–HCl, 0.4% (w/v) SDS, pH 8.8. Store at 4°C (see Table 1).
2. 4× stacking buffer: 0.5 M TRIS–HCl, 0.4% (w/v) SDS, pH 6.8. Store at 4°C (see Table 1).
3. 30% (w/v) Acrylamide-bisacrylamide (29.2% (w/v) Acrylamide, 0.8% (w/v) N'N'-bis-methylene-acrylamide).
4. 10% ammonium persulfate (APS). Prepare a fresh solution every time.
5. N,N,N',N'-Tetramethylethylenediamine (TEMED).
6. 2× SDS sample buffer: 25% of 4× stacking buffer, 20% of glycerol, 10% (w/v) SDS, 5% β-mercaptoethanol, 0.1% (w/v) bromophenol blue.
7. 10× running buffer: 250 mM TRIS base (adjusted pH at 8.5 with HCl), 2 M glycine, 1% (w/v) SDS.
8. Protein molecular weight standard prestained (Bio-Rad) or compatible with other colorimetric and fluorescent detection method.
9. Vertical polyacrylamide gel apparatus Mini Protean System (Bio-Rad) or equipment equivalent and power supply Power Pac HV (Bio-Rad) or equivalent.

2.4. Western Blot

1. Transfer membranes: PVDF (Millipore Immobilon-P).
2. 15% SDS-PAGE gel containing the resolved proteins.
3. Methanol 100%.
4. Pads or equivalent sponge.
5. Filter paper.
6. Transfer buffer: 25 mM TRIS base, 192 mM Glycine, 10% methanol, pH 8.3 (see Note 2).
7. Electroblothing apparatus (Bio-Rad).
8. Power supply Pac HV (Bio-Rad) or equivalent.

- 9. 10× PBS buffer (see Table 2).
- 10. Ponceau-S red 0.005% (Sigma) or any reversible stain.
- 11. Blocking reagents: non fat dry milk or bovine serum albumin (BSA) or Casein.
- 12. Antibodies (see Table 3).
- 13. Chemiluminescent HRP substrate (Millipore).
- 14. Plastic wrap or plastic bag.
- 15. Developing cassette.

Table 2
Preparation of Phosphate Buffered Saline (PBS)
and Phosphate Buffered Saline Tween-20 (PBST)

Reagent	1× PBS	TPBS
Sodium chloride (1.4 M)	8 g	8 g
Potassium chloride (26.8 mM)	0.2 g	0.2 g
Sodium phosphate dibasic (77.5 mM)	1.1 g	1.1 g
Potassium phosphate (17.6 mM)	0.24 g	0.24 g
Tween-20 (0.5%)		5 mL
pH 7.4		

The pH of PBS and TPBS solutions is adjusted with 1 N NaOH. TPBS buffer is used to prepare the blocking solution by adding 3–5% of nonfat dry milk and to prepare antibodies dilution. For washing the membranes, use only TPBS and PBS buffer

Table 3
Dilution of antibodies specific directed against histone
H3 methylation at four residues of lysine and respective
detection times

	Catalogue number	Dilution in PBST	Detection time
<i>Primary antibody</i>			
Anti-Histone H3	07-690	1:10,000	5–15 s
Anti-dimethyl-Histone H3 (Lys4)	07-030	1:10,000	20–60 s
Anti-trimethyl-Histone H3 (Lys4)	05-745R	1:1,500	3–5 min
Anti-dimethyl-Histone H3 (lys9)	07-441	1:1,000	3–5 min
Anti-trimethyl-Histone H3 (Lys27)	07-449	1:1,000	5 min
Anti-dimethyl-Histone H3 (Lys 36)	07-274	1:1,000	3–5 min
<i>Secondary antibody</i>			
Goat Anti-Rabbit IgG, HRP-conjugate	12-348	1:20,000	

All these antibodies are available from Millipore

16. X-ray film (Thermo Scientific).
17. Developing Reagents. Solution A: GBX developer, Solution B: GBX fixer (Sigma).
18. Dark room.
19. Gel documentation system (BioRad).

3. Methods

3.1. Histone Isolation

1. Grind 300–500 mg of tissue in N_2 liquid into a fine powder using a mortar and pestle and transfer to a Falcon tube and add 10 mL of NIB and maintain for 5 min on ice.
2. Filter the slurry through three layers of sterile cheesecloth and centrifuge the filtrate at $10,000 \times g$ for 20 min at $4^\circ C$.
3. Resuspend the pellet with 2 mL of 0.4 M H_2SO_4 and vortex for 20 s; centrifuge the homogenate at $10,000 \times g$ for 10 min at $4^\circ C$.
4. Transfer the supernatant to a new tube (the pellet is extracted twice; repeat step 3).
5. Precipitate proteins with 10–12 volumes of cold acetone and keep at $-20^\circ C$ for 12 h (see Note 3).
6. Centrifuge the precipitate at $13,000 \times g$ for 30 min at $4^\circ C$ and carefully discard the supernatant and air-dry the pellet to remove the residual acetone.
7. Resuspend the pellet with 200 μL of 4 M Urea by vortexing until the pellet is dissolved.
8. Take 5 μL of sample to quantify total protein and store the samples at $-20^\circ C$ until used.

3.2. Protein Separation and Transfer

1. Five microgram of protein with 10 μL of $2\times$ SDS buffer must be heated at $100^\circ C$ for 5 min. The proteins are resolved by electrophoresis in a 15% SDS-PAGE gel. One of the gel wells should include a protein molecular mass marker and a positive control for histones such as calf thymus histone. The electrophoresis is carried out at 100 V for 90 min (see Table 1 for details SDS-PAGE and see Note 4).
2. After the electrophoresis has been completed, remove the gel from the cassette and remove the stacking gel.
3. Take the gel and immerse it in the transfer buffer to equilibrate the gel for 10 min (see Note 5).
4. For each gel, cut the blotter paper to fit the transfer cassette.

5. Prepare the membrane for transfer:
 - (a) PVDF membrane: cut the membrane to fit in the transfer cassette and soak it in 100% methanol for 15–20 s.
 - (b) To remove the excess of methanol, place the membrane for 2 min in distilled water.
 - (c) Nitrocellulose membrane: do not soak in methanol.
 - (d) Place both types of membranes in the transfer buffer for 5–10 min.
6. Transfer cassette assembly (see Fig. 2a and Note 6):
 - (e) Open the cassette holder.
 - (f) Place a pad on one side of the cassette.
 - (g) Place two layers of blotter paper previously wet in transfer buffer.
 - (h) Carefully place the gel with the proteins resolved and the molecular marker standards at the right side.
 - (i) Carefully place the membrane on top of the gel.
 - (j) Place two layers of blotter paper on top of the stack.
 - (k) Place a second pad on top of the blotter paper and close the cassette holder.
7. Place the cassette holder containing the gel and membrane into electroblotting apparatus with the membrane side to the anode (+) and fill the tank with the transfer buffer.
8. The transference of the proteins was done in 3.5 h at 265 mA at 4°C.

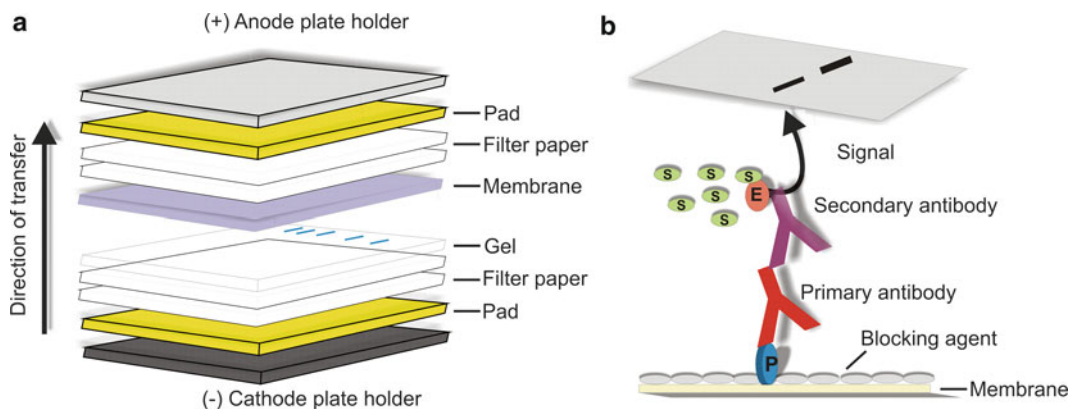


Fig. 2. Transfer protein and chemiluminescence principle. **(a)** The acrylamide gel where the proteins are resolved and the membrane are placed in the middle of two layers of blotter papers and two pads. The gel is kept faced to the anode side and then the whole assembly is placed in the electroblotting apparatus and the transfer carried out at 265 mA for 3.5 h at 4°C. **(b)** After protein (P) has been transferred to the membrane, all the unoccupied spaces in the membrane need to be blocked by a blocking agent (nonfat milk). The membrane is then exposed to an antibody which recognizes a specific protein in the membrane, and then the blot is probed with a second antibody that recognizes the first antibody. This second antibody is coupled to an enzyme (E) which converts the substrate (S) into a luminescence signal that produces a mark in the film that indicates the presence, abundance, and position of the protein of interest.

9. After the transference has been completed, remove the cassette holder from the tank and disassemble.
10. Carefully rinse the membrane with distilled water to remove gel remains.
11. Stain the membrane to verify the transfer efficiency:
 - (l) Ponceau-S red: dissolve 0.5 g in 1 mL glacial acetic acid and bring to 100 mL with distilled water.
 - (m) Soak the membrane with the Ponceau-S red solution for 1 min or until the proteins are visible.
 - (n) Remove the membrane and rinse thoroughly with water until the desired contrast and document.
 - (o) Remove the stain with 0.1 N NaOH and wash the membrane two times with PBS buffer and continue with the immunodetection.

3.3. Immunodetection (Antibody Incubations)

1. The dilution of antibodies is detailed in the Table 3.
2. Place the membrane blocking buffer (1× PBS, 0.5% Tween-20, 3–5% non fat dry milk) and incubate it for 1 h with slow agitation at room temperature.
3. Wash the membrane three times with TPBS for 5 min each (see Table 2).
4. Incubate the membrane in the primary antibody solution for 1 h at room temperature or overnight at 4°C with constant agitation.
5. Wash the membrane at least two times for 5 min with PBST and one time with PBS.
6. Incubate the membrane in the secondary antibody solution for 30 min at room temperature with constant agitation.
7. Wash the membrane three times with PBST for 5 min each and one time with PBS. Additional washes may further background.
8. Proceed with the chemiluminescent detection (see Fig. 2b).

3.4. Chemiluminescent Detection

1. Prepare the agent chemiluminescent substrate following the manufacturer's instructions (Chemiluminescent HRP substrate of Millipore).
2. Remove the PBS and add the substrate to completely cover the membrane.
3. Drain the excess substrate and cover the membrane with plastic wrap, avoiding bubble formation.
4. Expose the membrane to X-ray film and place it into the film cassette in the dark room. A red light could help inside the dark room. The times of exposure is detailed in Table 3.
5. Remove the film cassette and take out the film.

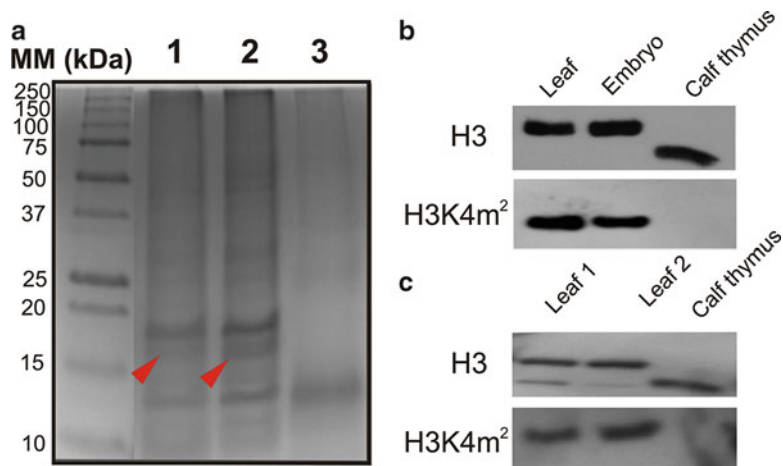


Fig. 3. Analysis of histone H3 methylation. (a) Separation of total nuclear proteins on a 15% SDS-PAGE gel stained with Coomassie R-250. Line 1: leaf of in vitro *Agave fourcroydes*, Line 2: leaf of in vitro *Coffea canephora*, Line 3: Positive control of histone H3 from calf thymus. The *head rows* indicate the histone H3 in the samples. (b) Immunodetection of histones H3 (*top*) and H3K4m2 (*bottom*) isolated from leaf and embryo of *C. canephora*. (c) Immunodetection of histones H3 (*top*) and H3K4m2 (*bottom*) isolated from leaves of two different plants of *A. fourcroydes*.

6. Place the film in a developer solution to obtain the desired contrast, then wash quickly in water and soak the film in the fixative solution, and then wash the film again in water (see Note 7).
7. Photodocument the film (see Fig. 3).

3.5. Membrane Stripping and Retesting

1. After exposing the membrane to the film, wash the membrane twice with TPBS for 5 min each.
2. Place the membrane in stripping solution (25 mM glycine, 1% SDS, adjust pH to 2 with HCl), gently shaking for 15 min at room temperature (two times).
3. Wash the membrane two times with TPBS for 5 min each.
4. Repeat the steps 3–9 of Subheading 3.3 and all Subheading 3.4.
5. The membrane retesting can be done up to five times.

4. Notes

1. All stock solutions of NIB should be kept at 4°C. All the extraction steps should be carried on ice. PMSF stock: prepare a 200 mM solution in ethyl alcohol absolute and store it at 4°C. Aprotinin stock: prepare 1 µg/mL solution in water and store it at –20°C.

2. This solution can be prepared as 10× stock and adjust the pH to 8.3. Add the methanol prior to use.
3. This step guarantees the best histone precipitation, particularly when the sample is small.
4. The proteins markers prestained can help to visualize the transference into the membrane and the molecular mass of the histones after the film has been exposed.
5. Gel equilibration prevents a change in the gel size during the transference time and avoids formation of bubbles during the transference.
6. Always use gloves when manipulating gels and use sterile forceps for the membrane or blotter paper to avoid false positives. To ensure an even transference to the membrane, remove any air bubbles between layers.
7. If a high background exists in the film, it is needed to increase the concentration of the blocking reagent, extend the washings time, or decrease the concentration of the secondary antibody.

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Chapter 25

Basic Procedures for Epigenetic Analysis in Plant Cell and Tissue Culture

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Abstract

In vitro culture is one of the most studied techniques, and it is used to study many developmental processes, especially in forestry species, because of growth timing and easy manipulation. Epigenetics has been shown as an important influence on many research analyses such as cancer in mammals and developmental processes in plants such as flowering, but regarding in vitro culture, techniques to study DNA methylation or chromatin modifications were mainly limited to identify somaclonal variation of the micropropagated material. Because in vitro culture is not only a way to generate plant material but also a bunch of differentially induced developmental processes, an approach of techniques and some research carried out to study the different changes regarding DNA methylation and chromatin and translational modifications that take place during these processes is reviewed.

Key words: Bisulfite sequencing, ChIP, Chromatin, DNA methylation, Histone PTMs, In vitro tissue culture

1. Introduction

In plants, cell plasticity enables somatic cells to begin new developmental programs generating adventitious organ formation in presence of the appropriate plant growth regulators. Somatic embryogenesis and adventitious shoot formation are practical examples of cell reprogramming (1, 2), which are negatively affected by ontogenic and physiological age, both constitute important tools to propagate selected genotypes in breeding programs.

Plant cell culture techniques are a source of epigenetic variation, especially those which involve and indirect organogenesis through a callus stage (3, 4), causing transposon reactivation and epigenetic changes focused on genes, such as epiallele formation (5, 6), leading to heritable changes in gene expression (7). Understanding of mechanisms that regulate cell plasticity is essential from a basic point of view of developmental processes and from an applied point of view regarding the biotechnological importance of morphogenesis manipulation. These processes involve the establishment of new gene expression patterns and changes in epigenetic plasticity, making this process a suitable experimental system to study loss of competence regarding development as well as to analyze epigenetic changes regarding ontogenic state (8).

Epigenetics is defined as “heritable changes in gene expression that occur without a change in DNA sequence” (9), and can be understood as a system to selectively regulate genome information through activating or inactivating gene expression. At a molecular level, DNA methylation, posttranslational histone modifications, chromatin remodeling factors, transcriptional factors, and chromosomal proteins cooperate together. Although the mechanisms of chromatin regulation are less studied in plants, advances in their research have shown that they share similarities with other eukaryotes in chromatin remodeling factors, histone acetylation/deacetylation and methylation/demethylation, and DNA methylation/demethylation and at a gene level as well (10).

Chromatin is not just a static method for packing DNA but also a dynamic strategy that cells use to respond to environmental stimuli. The precise control of chromatin modification in response to developmental and environmental cues determines the correct spatial and temporal expression of genes.

The differences among epigenetic modifications are a precise cross talk; variations in DNA methylation have an effect in histone deacetylation and methylation affecting chromatin structure in animals (11, 12) and in plants (13). To understand their influence on a specific physiological situation, an overview of the different modifications with a correct interpretation of the individual data must be done. In this chapter, we show the main techniques used for the analysis of DNA methylation (global and site-specific) and chromatin modifications from histones and transcription factors focusing more on the results than on the techniques achieved in plant cell and tissue culture as well as on several developmental processes by Epiphysage Research Group.

2. DNA Methylation

Methylation at position 5 of cytosines is the main epigenetic modification in eukaryotes, and alterations in DNA methylation are frequently involved in transcriptional gene regulation. Its presence and quantification was first time described in mammals and plants in the 1950s (14, 15). Methylation generally represses transcription in two ways: blocking transcription factors binding and modifying nearby histones and therefore chromatin structure (16).

Cytosine is primarily methylated in the CG dinucleotide called CG islands, but plants unlike animals have a high amount of 5-methylcytosine, suggesting that methylation is not restricted to the CG sequence, and it was also found that CNG and, less abundantly, CNN sequences were susceptible of cytosine methylation (17).

Recent advances in whole genome methylation profiling technologies have caused a great interest in analyzing cytosine methylation levels and distribution within the genome, allowing us to better understand various developmental processes, like in vitro plant culture. Some reviews about DNA methylation analysis techniques have been done (18, 19), but the increasing number of scientific research about DNA methylation in plant development, and to a lesser extent in plant in vitro culture, encouraged us to see the influence of DNA methylation on this process.

2.1. Global DNA Methylation

It is widely accepted that DNA methylation controls differentiation maintaining cell status stability (20), and global methylcytosine content in genomic DNA varies widely across species, organs, and developmental states. Epigenetic modifications, and in particular DNA hypomethylation, are suspected of being responsible for somaclonal variation (6), being growth treatments and environmental stress proposed as main factors in this variation (21). Although genetic stability is certified by conventional DNA markers, no uniform clone regenerants are often obtained, being this variation caused by different DNA methylation levels. The analysis of global methylated DNA can be measured by the content of 5-mdC or by its distribution along the tissue.

2.1.1. HPLC-MS and HPCE-MS (High-Performance Liquid Chromatography-Mass Spectrometry, High-Performance Capillary Electrophoresis-Mass Spectrometry)

Quantification of 5-methyldeoxycytosine genomic DNA methylation establishes certain degrees of global DNA methylation which can be used as markers for developmental or physiological processes (22). The analysis of global DNA methylation can be performed by HPCE (23, 24) or by HPLC (25, 26); both methods share the enzymatic hydrolysis of the DNA strands and a phosphatase alkaline treatment, to obtain deoxynucleosides; these deoxynucleosides are run in a HPCE or HPLC system, and the peaks obtained are measured by the following formula % 5-mdC = 5-mdC peak area / (5-mdC + 5-dC peak areas; Fig. 1).

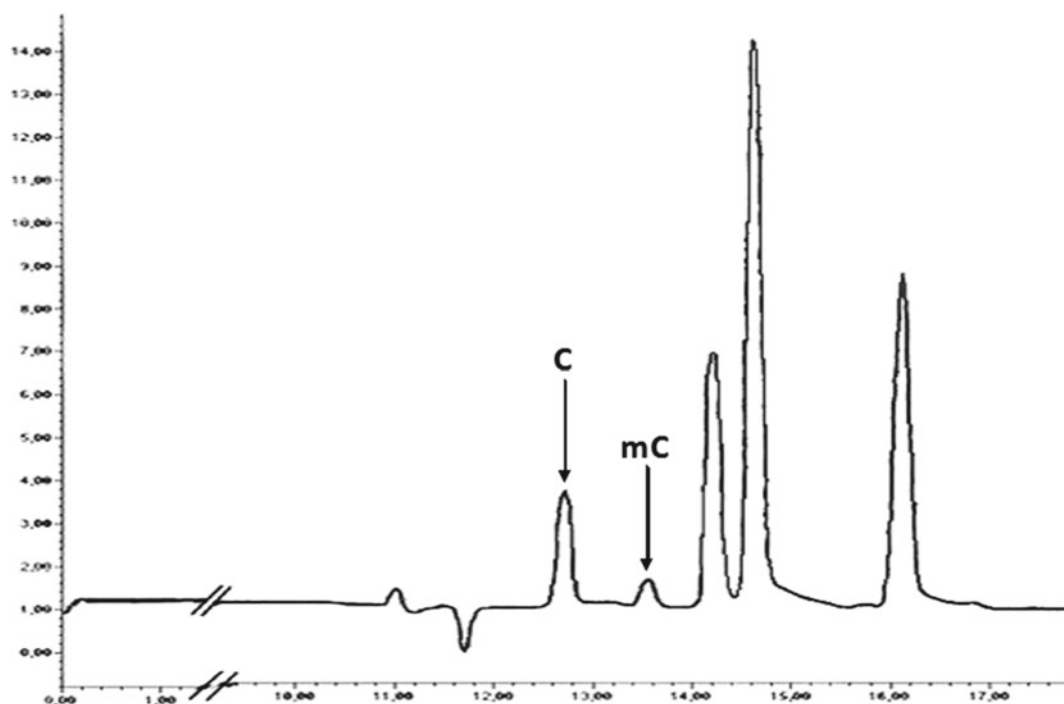


Fig. 1. High-performance capillary electrophoresis electropherogram of *Pinus radiata* genomic DNA. Each peak belongs to a different nucleoside. *C* corresponds to 5-dC and *mC* corresponds to 5-mdC.

It has been shown that different levels in global DNA methylation are essential for a correct response of plant material to in vitro culture (27), and it seems that both 5-mdC amount and physiological state of the plant have a key influence on the success of the culture, even different tissues in the same organ (28–30). Global DNA methylation has been proposed as an epigenetic marker in different developmental situations (31, 32), although there is some disagreement about the levels of DNA methylation and the age of the explants depending on the species (33–35).

2.1.2. Antibody-Based Detection

Global DNA methylation can be also quantified by ELISA, and many commercial kits are available. In our experience, these kits are quite inaccurate because the standards technical design is based on mammal DNA, so we focus on spatial distribution of DNA methylcytosine along the tissue. It is also necessary to know which cells contain methylated DNA, the number of cells, their location, and the evolution of the DNA methylation pattern of the tissue during ontogenic development. Histological analyses based on DAPI staining of cell nucleus and fluorescent immunolocalization of 5-mdC is used (36).

There is no bibliography related to in vitro culture, but several studies related to which organ response to organogenesis and the epigenetic status of the tissues is available. In *P. radiata* needle development, responsive immature needles showed less labeling in palisade layers, while mature needles are strongly labeled.

Similar responses are also observed in other organ maturation processes, such as flower development, in which 5mdC layout increases with the differentiation of their productive organs (36–39) or during bud dormancy in *C. sativa*, in which 5mdC layout increases with the bud dormancy and decreases during bud burst (40). Some research in adventitious caulogenesis in *P. radiata* zygotic embryos done in our research group, shows that main changes in DNA methylation labeling are focused in meristematic tissues in the early steps of the process but the tissues responding to the treatment are affected in the later steps (Fig. 2a).

2.2. Restriction Enzyme DNA Methylation Patterns

Analysis of DNA by restriction enzyme has been widely used to identify somaclonal variation in micropropagated species and many molecular markers has been used (41, 42). These variations have been also studied using methylation-sensitive restriction enzymes, Hpa II and Msp I; isoschizomers are usually the most used enzymes to make these analysis, having one common procedure by an adaptor ligation and a later PCR amplification (6).

2.2.1. Microarray-Based DNA Methylation Profiling

These methods are high-throughput DNA methylation screening tools, which performs a methylation-sensitive restriction enzyme treatment and a hybridization of a CpG island microarray to make a profile of methylated fragments (43). This technique is widely used in human cancer research because of the availability of CpG island databases that allow you to design a custom microarray or use a commercial one (44, 45).

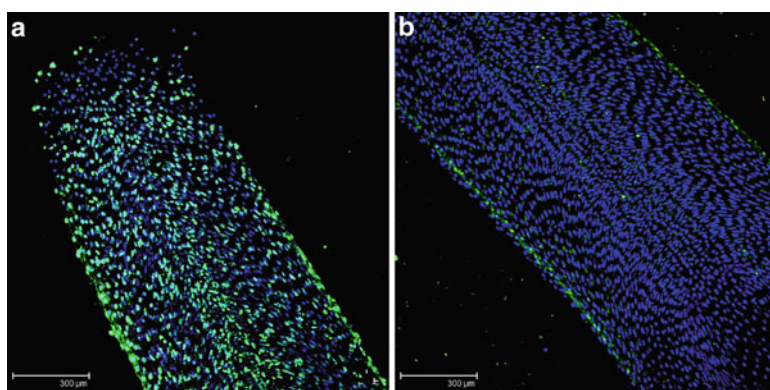


Fig. 2. Merged image of indirect immunolocalization in nuclei of *Pinus radiata* embryos of 5-methyl cytosine (a) and acetylated histone H4 (b) (green color) and nuclei stained with DAPI as a positive control (blue color). Light blue corresponds to overlaid secondary antibody and DAPI signals. Scale bar 300 µm.

2.2.2. MS-AFLP, HELP, MS AP PCR/MSRF, MS-RDA, MCA-RDA, AIMS, and RLGS (Methylation Sensitive Amplified Fragment Length Polymorphism/HpaII Tiny Fragment Enrichment by Ligation-Mediated PCR, Methylation-Sensitive Arbitrarily Primed PCR/ Methylation-Sensitive Restriction Fingerprinting, Methylation-Sensitive Representational Difference Analysis/ Methylated CpG Island Amplification-Representational Difference Analysis/ Amplification of Intermethylated Sites, Restriction Landmark Genomic Scanning)

2.3. Genome-Wide DNA Methylation

2.3.1. MeDIP, MIRA, MBD Columns (Methylated DNA Immunoprecipitation/ Methylated CpG Island Recovery Assay)

2.4. Individual CpG Analysis

These techniques and many others such as MS-RFLP (46) are based on the band amplification profile after PCR. Depending on the technique and the primers used, the profile of bands can vary from a few bands to many of them (47, 48). A later on analysis of the data reveals methylation and demethylation events because of the difference in the experimental situation. They have been used mainly in plant in vitro culture to make an epigenetic certification of micropropagated plant (29) and to identify DNA sequences that suffer changes in its promoter region, related probably to the in vitro culture process (49, 50).

5mC marks can selectively be detected through the genome using specific antibodies and proteins that selectively bind 5mC (51, 52). These methods are relatively straightforward and do not require either digestion of genomic DNA or bisulfite treatment. The basis of these methods is the enrichment of a fraction of DNA in methylated DNA; this population of DNA fragments can follow two main analysis strategies identifying the sequences of the enriched fraction by mass sequencing and hybridizing a microarray seeing the differential amount of each sequence.

For this kind of analyses, DNA is fragmented in a size range about 200–1,000 bp by enzyme restriction or by mechanical methods, and a later precipitation is done using a 5-methyl cytosine antibody or a methylated-CpG binding domain (MBD) protein, being MBD2B, the shorter protein isoform of MBD2 family which possesses the highest affinity to methylated DNA among the MBD proteins the most used (53, 54).

The study of known promoters of sequences of interest can be tackled by quantitation of methylation at individual CpG sites (47, 55). These techniques rely on the fact that restriction digestion can be used to reveal DNA methylation-dependent sequence differences in PCR-amplified bisulfite-treated genomic DNA and that these differences can be applied to specific loci. Methylation levels

in the original DNA sample are represented by the relative amounts of digested and undigested PCR product in a linearly quantitative fashion across a wide spectrum of DNA methylation levels.

2.4.1. COBRA, MS-SNuPE, MSO, and Real-Time MSP (Combined Bisulfite Restriction Analysis, Methylation-Sensitive Single Nucleotide Primer Extension, Methylation-Specific Oligonucleotide Array, Methylation-Specific PCR)

These techniques are the first ones which involve a DNA modification in the sequence, by a modification of unmethylated cytosine into uracil, while methylated cytosine (both 5mC or 5hmC) remains unchanged (56). This change in the DNA sequence following bisulfite conversion can be detected by two different approaches, a combination with methylation sensitive enzymes or by direct sequencing. The use of bisulfite-converted DNA for DNA methylation analysis has surpassed almost every other methodology for DNA methylation analysis of site-specific CpG islands. This sequence conversion can lead to the methylation dependent creation of new restriction enzyme sites or old sites can still remain in case of cytosine is methylated. Specific primer design can show us an amplification of a mixture of methylated and unmethylated CpGs that will be resolved in a polyacrylamide gel (55, 57) or hybridizing an oligonucleotide microarray with specific sequences that match for unmodified cytosines and for the modified ones, calculating the relative amount of each (58). Methylation Specific PCR differs from the other techniques in the absence of restriction enzymes and in the design of the primers, in this case the primer is located in the region where CpG are present and next to the 3' end to provide a higher degree of mispairing in the PCR (59).

2.4.2. MethyLight, QAMA (Quantitative Analysis of Methylated Alleles)

These techniques are claimed to be high-throughput methylation analysis and are based on the same principles as previous ones, that is to say, sodium bisulfite treatment and a PCR reaction. The difference relies in that this method is a sensitive, fluorescence-based real-time PCR technique that is capable of quantitating DNA methylation at a particular locus. The use of a fluorescent probe inside the PCR product sequence and the 5' to 3' exonuclease activity of the polymerase, release the fluorophore that is next to a quencher already present in the probe. Both the primers and the probe have CpG dinucleotides in their sequence (60). However, the availability of a new kind of probes, which differentiate a single base pair change, based on the minor groove binder technology (61) allows the quantification of methylated alleles more accurately. The use of two probes, one of them designed for unmethylated DNA and the other designed for methylated DNA, is the difference with MethyLight (62).

2.5. Sequencing DNA Methylation

2.5.1. Sanger Sequencing, Pyrosequencing

Direct bisulfite sequencing was first time used by Frommer in 1992 (63) to identify the nucleotides resistant to bisulfite conversion. After DNA conversion, a suitable couple of primers must be designed free of CpG dinucleotides (64); however, all programs for bisulfite primer design are reduced exclusively to mammal DNA and therefore to CpG dinucleotide, so primer design for plant DNA could be a cumbersome job sometimes. This technique requires cloning of the

PCR product prior to sequencing for adequate sensitivity because a mixture of different populations of differential methylated cytosines along the sequence is present. In the chromatogram results all sites of unmethylated cytosines are displayed as thymines in the resulting amplified sequence and methylated ones remain being cytosines. This technique has been widely used in plant tissue culture to control transposon activation and monitor specific promoter methylation status of interest genes (65, 66).

After DNA bisulfite modification and PCR amplification of the region of interest, pyrosequencing is used to determine the bisulfite-converted sequence of specific CpG sites in the region. The technique detects the amount of C and T incorporated during the sequence extension being very precise with the relative amount of each one (67). The main limitation of this method is that only around 50 nucleotides are sequenced accurately, so a low number of CpGs are analyzed.

2.6. Regional DNA Methylation Levels

2.6.1. Denaturing HPLC

Denaturing high-performance liquid chromatography (DHPLC) (68) analyzes the overall degree of methylation of a region through differential elution profiles based on temperature-dependent resolution of heteroduplexes from homoduplexes; for DNA methylation analyses, the modification with sodium bisulfite provides the heteroduplexes to analyze. This method does not give any information about the methylation status of individual CpGs, but several improvements have been done to detect other molecular markers at the same time (69).

DNA methylation plasticity is a major factor involved in plant development and in vitro culture response. Methylation increases with age, phase change, or culture period and is normally associated with a lack of organogenesis potential. Its analysis has undergone a revolution, and enzyme-based and affinity enrichment-based DNA methylation analysis techniques have provided a lot information in genome-wide analysis.

High throughput sequencing has become a revolutionary tool for the analysis of methylated DNA. In nonrepeat regions, 5mC can now be mapped with precision by genome-wide bisulfite sequencing revealing the role of DNA methylation along gene promoter or coding sequence. The next step in methylation analysis is to identify the methylome in specific physiological situations and compare the data to that obtained by transcriptomic or proteomic techniques.

3. Chromatin Modifications

Chromatin is the state in which DNA is packaged within the cell. The core histones present in the nucleosome, are predominantly globular except for their N-terminal tails, chromatin remodeling factors in histone modifications act by covalently altering the charge

of hydrophobicity of specific amino acids on histone tails. These modifications alter *cis*-regulatory elements binding to DNA and act by modifying other chromatin regulators (70). There is a myriad of possible modifications carried out by a gene complex network (71), but all of them, because of the different responses they cause, cannot be present at the same time, so its variations along a process determine a specific chromatin state (72). To study chromatin, there are several protocols depending on the nucleosome distribution, proteins involved, or their modifications (18).

3.1. Genomic Profile of Histone PTMs

3.1.1. Global Quantification

A general overview of a determined histone modification is essential to know its possible role along a process and along the tissue. Up to date, these techniques allow us to identify from a single histone modification to a group of modifications in different histones (73, 74).

3.1.2. HPLC-MS, Antibody-Based Detection

Genome-wide histone analysis, as well as global DNA methylation levels, reveals a general behavior of the studied characteristic and uses it as a marker in the physiological process. Mass spectrometry is a chemical analysis technique that exploits the physical properties of ions to determine their mass to charge ratio (m/z) and a variety of methods can be used to make the analysis after HPLC procedure (75). Histones can be easily extracted from cells, and reproducible separation of histone family members can be achieved on C8- or C18-based columns using reverse-phase high-performance liquid chromatography (RP-HPLC) (76). Individual fractions of the histones are enzymatically digested and analyzed by PMF (Peptide Mass Fingerprint), MS/MS (tandem MS), CAD (Collisionally Activated Dissociation), and ETD (Electron Transfer Dissociation) (77–79).

Western blot histone PTMs have been widely analyzed, and in the field of plant *in vitro* culture we have many references mainly devoted to monitor chromatin remodeling (3, 49). On the other hand, to study the distribution in the different tissues of an organ, immunolocalization is an easy and an informative technique (Fig. 2b) (27, 37).

3.1.3. Genome-Wide Analysis

Chromatin Immunoprecipitation ChIP

Cells of multicellular organisms are genetically identical but structurally and functionally different because of the differential expression of their genes. These differences are, in part, consequence of an epigenetic control. DNA methylation and histone PTMs are among the best studied epigenetic regulators. Gene expression depends on DNA–protein interactions, by itself and by the potential possibility that it happens related to DNA accessibility. For this reason, developing methods and techniques to study protein–DNA interactions have been, are and will be focused to the study different genomic processes, such as DNA replication, recombination, repair, and transcription. In this way, chromatin immunoprecipitation is one of the most powerful tools in the characterization of protein modifications and *in vivo* DNA–protein interactions (80).

ChIP procedure consists of discrete steps including in vivo cross-linking of proteins to DNA with formaldehyde, isolation of chromatin from isolated nuclei, shearing of chromatin into small fragments from 200 pb to 800 pb by ultrasound or micrococcal nuclease (MNase) digestion, immunoprecipitation with specific antibodies to DNA-bound proteins, release of the coprecipitated DNA, and finally, PCR amplification with specific primers to determine whether DNA sequences of interest have been precipitated. The latter steps involve comparison of the signal obtained from RT-qPCR signals from the precipitated template with positive and negative controls (80–83).

Nonetheless, ChIP has inherent limitations that must be known. Thus, utilization of highly specific antibodies is critical, as it can give false-negative signals. Another source of false-negative signals can be epitope disruption during chromatin cross-linking and shearing. On the other hand, false-positive signals may arise because of formaldehyde may fix transiently, or even nonspecifically, adjacent proteins. As a result, cross-linking fit and calibration curves build to determine the optimal amounts of antibody and chromatin to be used are recommended. Previous recommendations show that ChIP procedures are very sensitive (80).

First protocols for ChIP assays were developed and described for mammals, yeast, and *Drosophila*, but structural anatomical differences between plant and animal cells make impossible the direct application of animal ChIP protocols to plants. Some modifications and additional procedures, such as vacuum fixation, that has been introduced to ensure penetration of the DNA–protein cross-linking solution into plant cells, are needed. The presence of large vacuoles in mature plant cells is another capital item, giving as result a relatively low yield of isolated nuclei per gram of starting tissue. They are also a source of proteolytic enzymes, so special attention during isolation procedures and use of healthy fresh plant tissues are required. As a result, plant-specific ChIP protocols have been developed and used for analyzing a large number of epigenetically controlled genes in plants, as it is the case of our research group, EPIPHYSAGE (<http://www.uniovi.es/epiphysage/>), in which we use ChIP approach in gene-specific epigenetic regulation studies related to *P. radiata* cell suspension cultures and abiotic stress.

SAGE, SACO,
GMAT, PET, Seq
(Serial Analysis of Gene
Expression, Serial Analysis
of Chromatin Occupancy,
Genome-Wide Mapping
Technique, Paired-End
Ditag Sequencing, Massive
Sequencing)

In the past few years, various sequencing-based protocols have been developed to analyze ChIP samples (84). They are known as ChIP downstream protocols, combining ChIP with gene expression and sequencing analysis. The most commonly used techniques are ChIP + serial analysis of gene expression (ChIP-SAGE, GMAT) (85, 86), ChIP + serial analysis of chromatin occupancy (ChIP-SACO) (87, 88), ChIP + genome-wide mapping technique (ChIP-GMAT), and ChIP combined with paired-end ditag sequencing (ChIP-PET) (89). The most recent technique combines ChIP

with massively parallel sequencing (ChIP–Seq). It allows for genome-wide analysis in less time and high-throughput studies, which is going to unveil new aspects of plant development biology in the next years (90).

Application of these techniques has led to great advances in our understanding of how epigenetic phenomena are regulated and how they affect gene expression. Nowadays, the most useful technique used to map histone modifications at a genomic scale has been ChIP combined with DNA microarrays (ChIP–chip) (91), in which chromatin fragments are differentially immunoprecipitated with a specific antibody and then are amplified and fluorescently labeled to be hybridized in a DNA microarray. The first ChIP–chip studies of histone modifications in *Saccharomyces cerevisiae* and *Drosophila melanogaster* suggested that histone modifications are associated with distinct genomic regions and with distinct transcription states. In plants, ChIP–chip approach has been used with similar purposes (92), but in many species the inexistence of complete sequenced genome is a limitation.

Another downstream ChIP technique that combines this with SAGE is GMAT, which is also known as ChIP–SAGE (<http://www.sagenet.org>). Briefly, first the procedure begins with already mentioned ChIP procedure, second a biotinylated universal linker is ligated to DNA ends, which are bound to streptavidin beads, third DNA is digested with *Nla*III and ligated to linkers containing recognition sequence of *Mme*I, and fourth *Mme*I digestion produces 21–22 pb sequence tags of the immunoprecipitated DNA, which are concatenated, cloned in a vector, sequenced, and mapped by hybridization with a reference genome, correlating directly the number of tags detected at a genomic region with the modification level of this. Thus, the results obtained from GMAT might be more quantitative than ChIP–chip, though these two techniques have not yet been directly compared (84).

ChIP–Seq is a recently developed technique for analyzing ChIP DNA using a high-throughput massively sequencing technique and was one of the early applications of the technology called “next-generation sequencing” (93). The immunoprecipitated DNA is ligated to a pair of adaptors and hybridized on a solid surface with covalently bonded oligos, which are complementary to the adaptor sequences. A sequence ranging 35 bp is generated by a “sequencing-by-synthesis” technique, a modified Sanger sequencing procedure. In this technique, the number of sequenced reads that are mapped to a genomic locus is directly proportional to its modification level. Obtained ChIP–Seq results are more quantitative than those from ChIP–chip.

Each of the methods discussed above relies on ChIP experiments to examine specific modifications, but several points need to be kept in mind when interpreting ChIP results. Antibody specificity

depends on immunoprecipitation results, the different methods used for chromatin preparation such as sonication or micrococcal nuclease digestion must be considered, and finally, ChIP requires high amounts of DNA, using hundreds of thousands or even millions of cultured cells. As well as a transcriptome, results obtained are a snapshot of the histone modification status, which varies along time and experimental situation, making difficult to work with heterogeneous tissues.

3.2. Genomic Profile of Nucleosome Distribution

Nucleosomes are essential in eukaryotes for DNA packaging and telomere function (94, 95). The DNA strand is wrapped around this protein complex, form by two copies of histones H2A, H2B, H3, and H4. Their distribution in the chromatin, the histone Posttranslational modifications, and DNA methylation determine the accessibility for the expression of the regions of the genome under its control (96). Thus, chromatin status (euchromatin or heterochromatin) and epigenetic modification will lead to gene expression or repression, respectively. Regarding the position of the nucleosomes along the genome, it is believed that less amount of them have a fixed position predicted by DNA sequence, and the rest does not follow this pattern (90); some authors relate these fixed nucleosomes to low plasticity genes and others to high plasticity genes (97). Recently, studies in Arabidopsis related to gene expression show a correlation between nucleosome status and plant heat responses. It seems that modifications of chromatin led by nucleosome density (98) and substitution of histone H2A by Histone H2A.Z (99) play an important role in heat-related gene expression.

Nucleosomes are an essential actor by taking part in the epigenetic regulation of genome expression and chromatin packaging during cell division; no studies have been carried out focusing into its relation with the control of gene expression during in vitro culture.

3.2.1. Micrococcal Nuclease Analysis of Chromatin Structure

This technique is used to map nucleosome position and spacing along the genome, this enzyme is known to cut between nucleosomal spaces. There are two ways to isolate nucleosomes after cutting cross-linked DNA: The first of them is an immunoprecipitation with an antibody against a histone, which has been already described in the previous paragraphs, and the second one is based on the isolation of mononucleosomal DNA bands from a low melting point agarose gel (100).

3.3. Transcription Factor PTMs

There are many transcription factors related to in vitro culture process, and the techniques to analyze them are similar to the previously described immunoprecipitation techniques such as ChIP but against a transcription factor; however, no results have been found

related to plant in vitro culture, although the production of specific antibodies against new transcription factors will allow researchers make these studies soon. Identifying the position where protein complexes join DNA can be detected with this technique (101), and there is a statistical approach based on Kernel Density Estimation approach, which utilizes ChIP-Seq data, named QuEST (102).

4. Small RNAs (miRNA and siRNA)

The Small RNAs (sRNAs) constitute a family of regulatory non-coding RNAs (ncRNAs) of 19–28 nt in length, derived from double-stranded RNAs (dsRNAs) and are highly conserved evolutionarily across species. There are two main classes of small RNAs involved in RNA silencing: microRNAs (miRNAs) and small interfering RNAs (siRNAs); the classification depends on the biogenesis and not on their action (103, 104).

These sRNAs come from the long single-stranded RNA (ssRNAs) and are processed by RNase III proteins. They are involved in several processes including DNA elimination, heterochromatin assembly, mRNA cleavage, and translational repression related to developmental processes and response to stress (105).

To study sRNAs, there are many protocols and commercial kits to extract them, and the identification of the different families is in progress (106). Because these sequences have a homology with their targets, there are different procedures to predict them, giving as result conserved targets among species (107).

Regarding in vitro culture, there are some research related to transposable elements and the induction of transcription of siRNAs during cell suspension cultures (108), and new research has been done in relation to expression of miRNAs climatic adaptation and epigenomic imprinting even through an in vitro culture step probably related to DNA methylation (109).

5. Conclusions

Epigenetics is an important factor with a huge influence on many development processes, and its analysis appears to be essential in their regulation. In vitro culture as a developmental process is also affected by the different epigenetic modifications, and although several techniques have been used to identify somaclonal variation or global methylation content widely, recent techniques open a gate to unravel the function of specific genes or gene families and their regulation during this complex process.

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Plant Tissue Culture and Molecular Markers

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Abstract

Tissue culture can be used to propagate elite material or to generate new variability by employing somaclonal variation. Genetic stability of the process must be evaluated analyzing DNA profiles by the use of molecular markers. Several techniques have been reported for the screening of genetic variation on tissue culture derived material; however, a highly informative and good relation among the time–cost–information is obtained using Amplified Fragment Length Polymorphism (AFLP) in automatic sequencer. This technique involves a double-digestion of DNA with restriction enzymes, ligation of adapters at both extremities of the restriction fragments, and finally, selective polymerase chain reaction (PCR) amplification of the fragments. A semiautomatic process for the analysis could be used, but several considerations must be taken into account before such a use.

Key words: AFLP, Fingerprint, Genetic stability, Genetic screening, In vitro variability, Somaclonal variation

1. Introduction

Tissue culture can be used to propagate elite material or generate new variability by somaclonal variation (SV), a term used to define the variability induced by tissue culture (1) which is a result of a combination of both genetic and epigenetic variation (2). The level of such variability can be evaluated using molecular markers. Several techniques of molecular markers can be mentioned such as AFLP, RAPD, SSR, SSAP, etc.; all of them evaluate changes at the DNA level based on PCR amplification using specific primers, and in some cases addressed to amplify repetitive elements on the genome. Amplified fragment length polymorphism (AFLP) is a widely used technique to detect variability comparing the regenerated plants with the parental material, involving a process that includes the

double-digestion of DNA with restriction enzymes, ligation of adapters at both extremities of the restriction fragments, and finally, selective PCR amplification of the fragments to generate a fingerprint specific to individuals, varieties, or species (3). It has number of potential applications such as monitoring inheritance of agronomic traits in plant and animal breeding, diagnostics of genetically inherited diseases, pedigree analysis, forensic typing, parentage analysis, screening of DNA markers linked to genetic traits, and microbial typing (4). The AFLP technique has several advantages over other DNA fingerprinting systems. The most important of these are the capacity to examine an entire genome for polymorphism and its reproducibility. Additionally, it can be applied to any DNA samples including human, animal, plant, and microbial DNAs, giving it the potential to become a universal DNA fingerprinting system (4). We argue here that an AFLP-based method, which exploits a pair of enzymes and selective PCR amplification, provides an effective means to evaluate and quantify induced variation by the tissue culture and that the use of an automatic sequencer would increase the capability of processing to analyze high number of samples simultaneously. Several considerations presented here must be taken into account prior to applying such technology in order to obtain the best results and feasible profiles for the analysis.

2. Materials

1. 1.5-mL centrifuge tubes.
2. 0.2/0.5-mL thin-walled centrifuge tubes (depending on thermal cycler).
3. Automatic pipettes capable of dispensing 1–20 μ L and 20–200 μ L.
4. Autoclaved, aerosol-resistant tips for automatic pipettes.
5. Centrifuge capable of generating a relative centrifugal force of $14,000 \times g$.
6. 37°C, 65°C, 70°C, 90°C water baths or programmable thermal cycler gradient.
7. Thermal cycling plates and caps.
8. Submerged horizontal electrophoresis system for agarose gels.
9. CEQ 8000 Series genetic analysis system (Beckman) or another system of capillary electrophoresis.
10. Gel documentation system.

3. Reagents and Solutions

1. DNA isolation:

All solutions should be made using double sterile and deionized water. Prepare and store all reagents at room temperature unless otherwise stated.

- (a) 1 M Tris pH 8: weigh 121.1 g of Tris-Base (FW 121.1) and adjust to 1 L of final volume (see Note 1).
- (b) 0.5 M EDTA: weigh 186.12 g of EDTA (FW 372.24) and adjust to a final volume of 1 L (see Note 2).
- (c) 5 M NaCl: weigh 292 g NaCl (FW 58.4) and adjust to a final volume of 1 L.
- (d) DNA isolation buffer: 100 mM Tris pH 8, 50 mM EDTA, and 500 mM NaCl.
- (e) 100 mM β -Mercaptoethanol solution: take 700 μ L of β -mercaptoethanol (FW 78.13) molecular biology grade and dilute with water to a final volume of 100 mL, in fume hood.
- (f) Solution 20% (w/v) sodium dodecyl sulfate (SDS): weigh 20 g of SDS and dilute using hot water to reach 100 mL (see Note 3). The heating before use at 65°C is recommended.
- (g) 5 M potassium acetate: 245.35 g of potassium acetate (FW 98.14) in 500 mL using water. Once prepared, maintenance at -20°C is recommended.
- (h) Silicon dioxide 2 g/L (Sigma® Cat. # S5631-500 G): The silicon dioxide (FW 60.09) is prepared in corning tubes of 50 mL, by weighing 2 g of silicon dioxide and diluting to a final volume of 50 mL using water (see Note 4).
- (i) Ethanol 70% (v/v): mix 70 mL of ethanol (grade molecular biology) in 30 mL of water. Once prepared, maintain at -20°C.

2. Agarose gels.

- (a) 10× TBE buffer: 0.9 M Tris, 0.9 M boric acid and 20 mM EDTA. Weigh 109 g of Tris-Base (FW121.1), 55.6 g of boric acid and dilute in water. Add 40 mL of 0.5 M EDTA. Adjust to a final volume of 1 L using water.
- (b) 1× TBE buffer; dilute 100 mL of 10× TBE in 900 mL of water.
- (c) Agarose gels 1 or 1.5%: weigh 1 or 1.5 g of agarose, respectively, and dissolve in 100 mL of 1× TBE buffer. Boil until agarose is completely dissolved producing a clear

solution (see Note 5) and to fill the container with the comb according with necessities.

- (d) Stock of ethidium bromide (EtBr) (10 mg/mL); weigh 50 mg of EtBr (FW 394.31) and dilute in 5 mL of water (see Note 6).
 - (e) Glycerol-dye bromophenol blue (BPB): 2 mg/mL BPB, 50 mM Tris pH8, 5 mM EDTA, v/v and 25% glycerol; weigh 100 mg of BPB (FW 691.94) and add 2.5 mL of 1 M Tris-base pH 8, 0.5 mL of 0.5 M EDTA, 12.5 mL of glycerol (FW 92.09) and then add water to a final volume of 50 mL and mix well.
 - (f) Hyper ladder I (BIOLINE® Cat. # H1K5-1006) is a marker for size and mass for DNA display that contain 14 bands in a range of 200–10,000 pb.
 - (g) 100 bp ladder (Invitrogen® Cat. # 15628-019): it consists of 15 fragments between 100 and 1,500 bp in multiples of 100 bp and an additional fragment at 2,072 bp.
3. Double digestion using restriction enzymes
- (a) Enzymes Eco RI and Mse I (Biolabs Cat # R0101S and Cat # R0525S). Commercially, the concentration is 10 U/μL of each restriction enzyme in a buffer that contain 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mg/mL BSA, 50% (v/v) glycerol, 0.1% Triton® X-100. They should be stored at -20°C all the time.
 - (b) 10× NEBuffer 4 for Mse I (Biolabs Cat # B7004S) containing 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9. Store at -20°C (see Note 7).
 - (c) Bovine serum albumin (BSA) (Biolabs Cat # B9001S). 10 mg/mL BSA, 20 mM KPO₄, 50 mM NaCl, 0.1 mM EDTA, 5% glycerol, pH 7.0. Store at -20°C.
4. Ligation
- (a) Annealing buffer: 10 mM Tris, pH 7.5–8.0, 50 mM NaCl, 1 mM EDTA.
 - (b) Double strain Eco RI adapter should be at 5 pmol: To prepare, mix 20 μL of Eco RI adapter-1 (1 nmol/μL, 5'-CTC GTA GAC TGC GTA CC-3') and 20 μL of Eco RI adapter-2 (1 nmol/μL, 5'AAT TGG TAC GCA GTC TAC3') in 20 μL of annealing buffer. Adjust to a final volume of 200 μL and incubate at 65°C for 10 min. Slow cooling to room temperature should take place for 45–60 min. The final concentration of the mixture is 100 pmol. A dilution 1:20 must be prepared by taking

1 μL of 100 pmol stock solution and diluting up to 19 μL with water. Store at -20°C .

- (c) Double strain Mse I adapter should be at 50 pmol: To prepare, mix 20 μL of Mse I adapter-1 (1 nmol/ μL , 5'-GAC GAT GAG TCC TGA G-3') and 20 μL Mse I adapter-2 (1 nmol/ μL , 5'-TAC TCA GGA CTC AT-3') and 20 μL annealing buffer. Adjust to a final volume of 200 μL and incubate at 65°C for 10 min. Slow cooling to room temperature should take 45–60 min. The final concentration of the mixture is 100 pmol/ μL . A dilution of 1:2 must be prepared taking 1 μL of 100 pmol stock solution and diluting up to 1 μL with water. Store at -20°C .
 - (d) T4 DNA ligase (Invitrogen Cat # 15224-017): 1 unit/ μL in 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 50 mM KCl, 50% glycerol (v/v). Store at -20°C .
 - (e) 5 $\times$ buffer of T4 DNA ligase: 250 mM Tris-HCl pH 7.6, 50 mM MgCl_2 , 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000. Store at -20°C .
 - (f) TE buffer: 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA.
5. Primers for AFLPs: AFLP primers consist of three parts: a core sequence, an enzymatic specific sequence (ENZ), and a selective extension (EXT) (3). This is illustrated bellow for Eco RI and Mse I primers with three selective nucleotides (represented by NNN) and they could correspond to any of the sequence described in Table 1:

	CORE	ENZ	EXT
Eco RI	5-GACTGCGTACC	AATTC	NNN-3
Mse I	5-GATGAGTCCTGAGTAA	NNN-3	

6. PCR. Preamplification.

For selective preamplification, a specific primer combination of Eco RI and Mse I is used. Both primer Eco RI (5'-GACTGC GTACCAATTCN-3') and primer Mse I (5'-GACTGCGT ACCAATTCN-3') must be prepared at 20 pmol/ μL .

- (a) To prepare a stock solution (100 pmol/ μL) of each primer, the stock of 100 pmol/ μL it is used to make a 1:5 dilution (take 1 μL of 100 pmoles each stock and dilute 5 μL of water ultrapure). Final concentration of each primer should be 20 pmol/ μL . Store at -20°C .
- (b) 10 mM dNTP (Invitrogen Cat # 18427-013). Mix dATP, dCTP, dGTP, and dTTP (2'-deoxynucleoside 5'-triphosphates) at a concentration of 10 mM in Tris-HCl (pH 7.5). Store at -20°C .

Table 1
AFLP primer combinations. Primer combinations that can be used for different organisms that contain a range of genomic DNA content between 2 and 7 pg per haploid genome. First nucleotides on the primers are A and C for Eco RI and Mse I, respectively, and they are used on the previous preamplification step

				Mse I selective nucleotides															
				C															
				A				C				G				T			
				A	C	G	T	A	C	G	T	A	C	G	T	A	C	G	T
Eco RI selective nucleotides	A	A	A																
			C																
			G																
			T																
		C	A																
			C																
			G																
			T																
		G	A																
			C																
			G																
			T																
		T	A																
			C																
			G																
			T																

- (c) 10× PCR buffer: 200 mM Tris–HCl (pH 8.4), 500 mM KCl. Store at –20°C.
- (d) *Taq* DNA polymerase (Invitrogen Cat # 11615-025): 10 U/μL of *Taq* DNA polymerase, 20 mM Tris–HCl (pH 8.0), 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol, and stabilizers. Store at –20°C.
- (e) 50 mM MgCl₂.

7. PCR. Selective amplification.

- (a) Primer Eco RI (5′-GACTGCGTACCAATTCNNN-3′) at 2.5 pmol/μL. Prepare a stock solution of 100 pmol/μL, and then take 1 μL of 100 pmoles stock and dilute in 40 μL of water ultrapure.

- (b) Primer Mse I (5'-GATGAGTCCTGAGTAANN-3') at 6.7 pmol/ μ L. Prepare a stock of 100 pmol/ μ L and dilute by taking 1 μ L of the stock in 15 μ L of water.
- 8. Fragment analysis
 - (a) DNA size standard kit 400 (Beckman Cat # 608098). Includes DNA fragments of the following sizes labeled with WellRED fluorescent dye: 60, 70, 80, 90, 100, 120, 140, 160, 180, 190, 200, 220, 240, 260, 280, 300, 320, 340, 360, 380, 400, and 420 nucleotides.
 - (b) Separation gel 20 mL for 8800 Series (Beckman Cat # 391438). Linear polyacrylamide (LPA) denaturing gel assures high-resolution separations.
 - (c) Separation buffer (Beckman Cat # 608012).
 - (d) Samples loading solution, SLS (Beckman Cat # 608082).
 - (e) WellRED Dyes-labeled primers.

4. Methods

1. Isolation of DNA:

The most critical requirement for an efficient PCR amplification is a high quality DNA which must be free of contaminant compounds that could inhibit the enzymatic reaction catalyzed by the *Taq* DNA polymerase. Thus, an easy, fast, and efficient protocol for DNA extraction reported by Echevarria-Machado I et al. (5) is suggested for the vast application on several plant species as follows:

500 mg of leaf is chopped into small pieces and placed into a mortar and frozen by adding liquid nitrogen (see Note 8).

The powder is transferred to a 2.0-mL Eppendorf tube containing 1 mL of a prewarmed (65°C) DNA isolation buffer containing 100 μ L of 100 mM β -mercaptoethanol solution. The suspension must be shaken briefly to obtain a homogeneous suspension.

Add 100 μ L of SDS 20% and shake it vigorously for several seconds and incubate at 65°C for 10 min.

To this, add 500 μ L acetate potassium 5 M and shake vigorously for several seconds and incubate on ice for 20 min.

The samples must be centrifuged immediately for 5 min at 13,800 $\times g$.

The supernatant is transferred to a 1.5-mL Eppendorf tube and 300 μ L of silicon dioxide is added and mixed slowly, and centrifuged immediately for 5 min at 13,800 $\times g$ (see Note 9).

The supernatant is discarded and the pellet washed carefully with 0.5 mL of 70% ethanol for three times, after which a

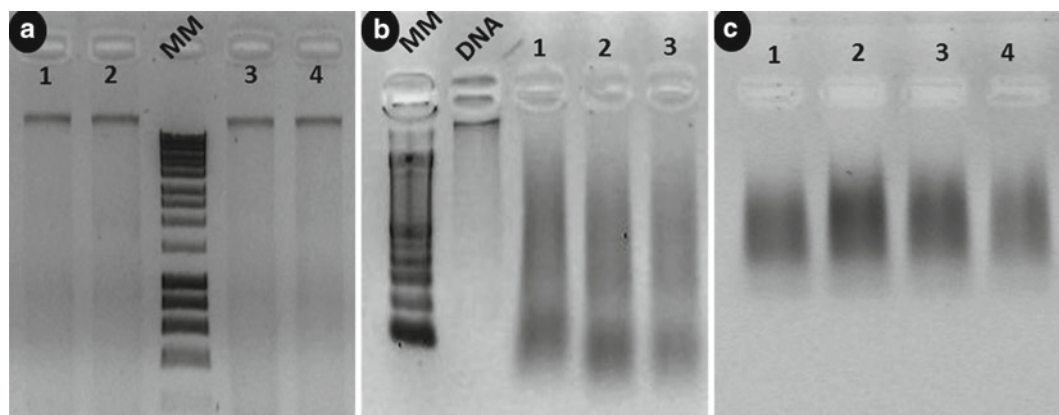


Fig. 1. Quality control evaluation by electrophoresis during several steps of the AFLP process. (a) Genomic DNA of several individuals (1–4) of *Agave fourcroydes* run on 1% agarose gel. (b) Double restriction-ligation of genomic DNA of several individuals on 1% agarose gel. DNA represents a nondigested sample. (c) Pre-amplification profile of several individuals (1–4) on 1.5% agarose gel. MM corresponds to 1 kb ladder.

spin for 2 min at $900\times g$ is needed to remove the remaining ethanol (see Note 10).

- (a) The pellet is air-dried overnight and dissolved in 50 μL of sterile water pH 7.4.
- (b) The mix must be centrifuged immediately for 5 min at $13,800\times g$. The supernatant is transfer carefully into a new 1.5-mL Eppendorf tube (see Note 10).

2. Integrity and concentration of DNA.

- (a) To check the integrity of the DNA, 5 μL of the solution of DNA are mixed with 2 μL of the glycerol dye BPB and electrophoresed on a 1.0% agarose gel at 80 V for approximately 25 min. A predominant and intense band is expected (see Fig. 1a).
- (b) The concentration of DNA can be checked by spectrophotometry (with a NanoDrop 1000 spectrophotometer) at 260 nm/280 nm.
- (c) A dilution of each DNA is prepared at 100 ng/ μL , in order to simplify the preparation and dispend of the mixtures for the next steps.

3. Double digestion of DNA using *EcoRI* and *MseI* restriction enzymes.

- (a) Five microliters of genomic DNA (100 ng/ μL) must be double digested with 0.4 U *EcoRI* (10 units/ μL), 0.1 U *MseI* (10 units/ μL), in 20 μL final volume that contain 2 μL 10 $\times$ restriction buffer *MseI*, 0.2 μL BSA (10 mg/mL), and 12.3 μL H_2O (see Note 11).
- (b) The restriction mixture is incubated for 3 h at 37°C .

- (c) The enzymes must be inactivated by incubation at 65°C for 15 min.
 - (d) The ligation of adapters could be done immediately; otherwise, it is recommended to store the restriction mixture at -20°C up to use.
4. Linker addition.
- Following to the heat inactivation of the restriction endonucleases, the genomic DNA fragments should be ligated to EcoR I and Mse I adapters to generate a template DNA for amplification based on the recognition by the specific primers to the adapter regions (see Note 12).
- (a) The restricted DNA is incubated with 10 µL of a mix containing 1.0 µL EcoRI adapter (5 pmol), 1.0 µL MseI adapter (50 pmol), 6.0 µL 5× buffer de T4 DNA ligase, 1.0 U T4 DNA ligase, and 1.0 µL H₂O.
 - (b) Mix gently at room temperature and centrifuge briefly to collect contents at the bottom of the tube, and incubate for 16 h at 20°C (heatblock or PCR machine).
 - (c) In order to check the preamplification reaction, aliquots of 5 µL of the preamplification are mixed with 2 µL of glycerol loading dye BPB and loaded into the slots, when the 1.5% agarose gel has been solidified, and the electrophoresis is carried out at 80 V for approximately 15 min. The profile is observed using a UV-transilluminator (Fig. 1b).
5. Preamplification with primers that contain a single selective nucleotide.
- (a) The ligated DNA from the previous step is diluted 1:10 using TE and then 5 µL of this dilution is used for the preamplification reaction in a mixture that contain 1.0 µL EcoRI primer E01 (20 pmol), 1.0 µL MseI primer M01 (20 pmol), 2.0 µL 10× PCR buffer, 0.4 µL 10 mM dNTPs, 0.6 µL 50 mM MgCl₂, 0.1 µL Taq polymerase (5 units/µL), and 9.9 µL H₂O to reach 20 µL of final volume.
 - (b) The PCR conditions differed depending on the nature of the selective extensions of the AFLP primers used for amplification. The condition for the PCR include an initial denaturation for 5 min at 94°C, followed by 20 cycles consisting of a denaturation step by 30 s at 94°C, an annealing step for 1 min at 56°C, and an extension step for 1 min at 72°C. A final extension step at 72°C at 7 min is needed.
 - (c) In order to check the preamplification reaction, aliquots of 5 µL of the preamplification are mixed with 2 µL of glycerol loading dye BPB and loaded into the slots, when the 1.5% agarose gel was solidified, and the electrophoresis is

carried out at 80 V for approximately 15 min. The profile is observed using a UV-transilluminator (see Fig. 1c)

- (d) To prepare the reaction for selective amplification, dilute 3 μL of the preamplification products in 147 μL H_2O (1:50 dilution) (see Note 13)

6. Selective amplification

PCR reactions must be prepared within a thin wall microplate or PCR tubes. All reagents should be kept on ice while preparing the PCR reactions. Final concentration of the *EcoRI* labeled primers in the PCR reaction should be 0.25 μM . The WellRED-Labeled primers are designed specifically for use with the CEQ series Genetic 8800 analysis systems and are excited to fluorescence using diode lasers (D2, D3, and D4) (see Note 14).

- (a) The reaction contain 5 μL of preamplified DNA diluted 1:50, 1.0 μL *EcoRI* labeled primer+3, 2.25 μL *MseI* primer+3 (6.7 pmol/ μL), 0.2 μL 10 mM dNTPs, 1.0 μL 10 $\times$ PCR-buffer, 0.3 μL 50 mM MgCl_2 , 0.1 μL *Taq* DNA polymerase (5 units/ μL), and 0.15 μL H_2O in 10 μL final volume (see Note 15).
- (b) AFLP reactions with selective primers were performed with a touchdown program that works well for most primer sets with an initial denaturation step for 5 min at 94°C, followed by 12 cycles with denaturation step for 30 s at 94°C, 30 s for annealing step at 65°C and 1 min extension step at 72°C. In each cycle, a decrease of 0.7°C in the annealing temperature is done. Final annealing temperature reaches 56°C which is used for the next 24 cycles, with final extension step at 72°C for 7 min.
- (c) For sample preparation, load into the automatic sequencer 0.25–3.0 μL of PCR product added to the SLS–size standard mix. For optimal analysis results, do not saturate the detection system by overloading the sample separation with labeled PCR products. A maximum of three different labeled primers (Different dyes) can be used simultaneously to separate on the sequencer with a prior individual characterization of each profile.
- (d) For more consistent pipetting, the samples should be diluted in SLS and loaded in a larger volume. For example, for 0.25 μL of the original sample, load 2.5 μL of a 1:10 dilution.
- (e) The relative signal strengths of the amplification products vary depending on the dye label. As a guide, roughly equal signal strengths can be obtained by adding the following relative volumes of PCR reaction products: 0.1 μL D4 PCR reaction, 0.2 μL D3 PCR reactions, and 0.4 μL D2 PCR reactions. Dilute the reactions appropriately to

increase pipetting accuracy. If large volumes of diluted reactions are used, compensate to reach a final injection volume of approximately 40 μL .

- (f) The amount of PCR product used will depend upon the efficiency of the PCR reaction. The volumes given above are a recommended starting point.
- (g) Fill each well of one row of the sample plate with 25 μL of the prepared test sample/size standards mix.
- (h) Add one drop of mineral oil to each of the 8 wells and load onto the instrument.
- (i) Run the prepared sample on the instrument using the “FragTest” or Frag-1 and Frag-3 run method. Several different separation methods and analysis parameter sets are provided with the System. The Frag-1 and Frag-3 separation methods are appropriate for use with the size standard 400 ladder. Once the run is over, it is necessary to verify that the six peaks of the Fragment Analysis Test Sample have been identified and their sizes estimated, as listed above. The Size Standard-400 contains fragments of the following sizes, labeled with D1 (red) dye: 60, 70, 80, 90, 100, 120, 140, 160, 180, 190, 200, 220, 240, 260, 280, 300, 320, 340, 360, 380, 400, and 420 nucleotides.
- (j) To analyze the profiles (Fig. 2), a binary matrix (1 = band present, 0 = band absent) is prepared based on the pattern of AFLP bands. A polymorphic band is defined as a band (Pick) that is absent from at least one individual. Genetic similarity index (GSI) could be determined according to Nei and Li (6). The resulting dendrogram could be obtained using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), using the SAHN (Sequential

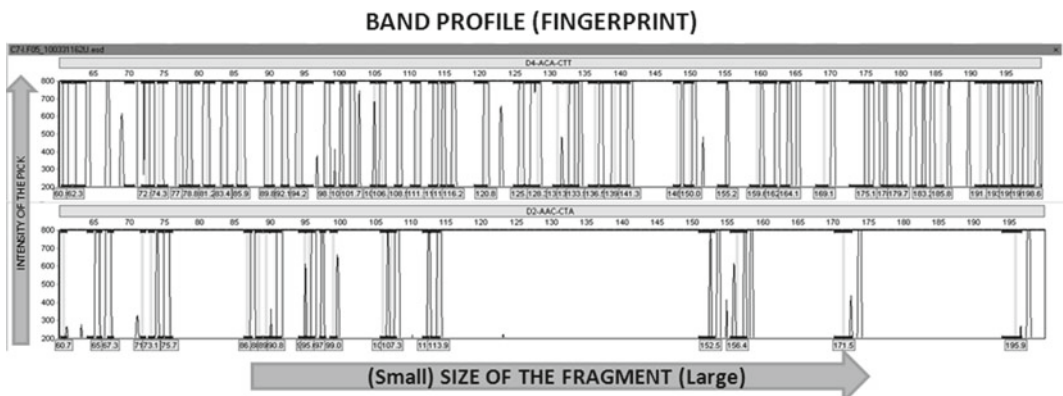


Fig. 2. AFLP profiles of *A. angustifolia* using two primer combinations in an automatic sequencer. Several primer combinations produce different fingerprint profiles.

Agglomerative Hierarchical Nested Cluster Analysis) statistical module of NTSYS (Numerical Taxonomy and Multivariate Analysis System) software version 2.02.

5. Notes

1. To adjust the pH use concentrated HCl (12 M) when around pH 8 if you prefer adjusted with series of the 6 N or 1 N HCl. The concentrate HCl is toxic and dangerous for the environment. For manipulation use face shields, full-face respirator, gloves, goggles, multipurpose combination respirator cartridge. Before use, check the risk and safety statements.
2. To prepare EDTA, it is necessary to adjust to pH 8 in order to increase the solubility; add gradually the reactant to avoid lumps. Use NaOH 1 N to adjust pH.
3. Prewarm water up to 65°C and add gradually, and shaking gently to prevent foaming. For manipulation, use eye shields, face shields, mask, gloves. Before use check the risk and safety statement.
4. Before use, it is necessary to remove small particles of silica (to prevent DNA lost) by washing for at least three times, mixing the solution with 500 mL of water and shaking and after that centrifuging at $17,000\times g$ for 5 min and discarding supernatant. Repeat the process until the supernatant appears clear.
5. It is possible to use a microwave oven; to avoid air bubbles, turn off the microwave immediately after boil. Wait for it to cool before discarding into the mold, avoiding bubbles.
6. Ethidium bromide intercalates double-stranded DNA and RNA and acts as a mutagen. EtBr is toxic for use. Utilize eye shields, face shields, mask, and gloves. It is important to designate a special area and management to work with the materials containing EtBr. Check the risk and safety statement.
7. The use of 10× NEBuffer 4 Mse I guarantees a cutting efficiency of 100% for the enzyme Mse I and Eco RI at 37°C.
8. The tissue should not be thawed during the process. It is necessary to add liquid nitrogen several times.
9. Mix by inversion or vortex the silicon dioxide until the pellet disappears.
10. Without removing the tubes from silicon dioxide, carefully aspirate and discard the supernatant without disturbing the pellet by angling the pipette such that the tip is pointed away from the pellet.

11. The AFLP technique is based on the amplification of subsets of genomic restriction fragments using PCR. DNA is cut with restriction enzymes, and double-stranded (ds) adapters are ligated to the ends of the DNA fragments to generate template DNA for amplification. The sequence of the adapters and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments. Selective nucleotides are included at the 3' ends of the PCR primers, which therefore can only prime DNA synthesis from a subset of the restriction sites. Only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified (3).
12. Depending on the intensity of the amplified fragments, the dilution will be at 1:5 or 1:10.
13. Depending on the intensity of the amplified fragments, the dilution will be at 1:25 or 1:50
14. Storage of all Fragment Analysis reagents (Size Standards, Test Sample, and Mobility Calibration Standard) must be in a -20°C non-frost-free freezer. Certain primers may work better at higher or lower concentrations because of primer sequence specific factors. It is recommended to start at $0.025\text{ }\mu\text{M}$ for the System, and adjust as required.
15. The most important factor in determining the number of restriction fragments amplified in a single AFLP reaction is the number of selective nucleotides in the selective primers (Fig. 2). The selective primers in the AFLP are three selective nucleotides used with plants having genomes ranging in size from 5×10^8 to 6×10^9 bp; the number of fragments amplified per sample per primer pair averages 50, but may range from as low as 10 to ~ 100 depending on the sequence context of the selective nucleotides, and the complexity of the genome (7, 8).

Acknowledgments

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Chapter 27

Biolistic- and *Agrobacterium*-Mediated Transformation Protocols for Wheat

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Abstract

After rice, wheat is considered to be the most important world food crop, and the demand for high-quality wheat flour is increasing. Although there are no GM varieties currently grown, wheat is an important target for biotechnology, and we anticipate that GM wheat will be commercially available in 10–15 years. In this chapter, we summarize the main features and challenges of wheat transformation and then describe detailed protocols for the production of transgenic wheat plants both by biolistic and *Agrobacterium*-mediated DNA-delivery. Although these methods are used mainly for bread wheat (*Triticum aestivum* L.), they can also be successfully applied, with slight modifications, to tetraploid durum wheat (*T. turgidum* L. var. *durum*). The appropriate size and developmental stage of explants (immature embryo-derived scutella), the conditions to produce embryogenic callus tissues, and the methods to regenerate transgenic plants under increasing selection pressure are provided in the protocol. To illustrate the application of herbicide selection system, we have chosen to describe the use of the plasmid pAHC25 for biolistic transformation, while for *Agrobacterium*-mediated transformation the binary vector pAL156 (incorporating both the *bar* gene and the *uidA* gene) has been chosen. Beside the step-by-step methodology for obtaining stably transformed and normal fertile plants, procedures for screening and testing transgenic wheat plants are also discussed.

Key words: *Agrobacterium*, Biolistics, Cereals, Embryogenic cultures, Genetic transformation, *Triticum aestivum* L., Wheat

1. Introduction

Wheat forms the staple food of the human diet in over 60 countries and provides 10–20% of the daily calorific intake for almost half the world's population. Wheat flour is unique in its ability to be processed and baked into leavened bread, but it is also a major constituent of many other foods including biscuits, cakes, breakfast cereal, pasta, and noodles. In addition, low-grade wheat and industrial

wheat by-products are incorporated into animal feed products and industrial products such as inks, paper, textiles, and pharmaceuticals, among others.

Wheat became the last cereal crop to be successfully genetically transformed in 1992 (1), and aspects of this technology have been reviewed previously (2–11). The transformation of elite or model wheat genotypes provided the opportunity to study the role of specific gene sequences in wheat and to introduce important agronomic traits into different varieties. Agronomically important properties such as resistance to biotic (pests, pathogens) and abiotic (drought, soil salinity, heat or cold) stresses have been investigated (12–16). In addition, this technique was successfully used for the modification of the end-use quality of several wheat varieties by increasing dough strength, focusing on one specific group of polymeric gluten proteins, called high-molecular-weight glutenin subunits (HMW-GS) (17–25). Field trials revealed no differences between the transgenic and control lines in terms of relative stability of expression of the endogenous and transgenic forms of the HMW subunit genes and inheritance between generations at several locations (23, 26–29). The effects of another storage protein, the low-molecular-weight glutenin subunit (LMW-GS), on the dough quality were also studied in transgenic wheat lines, and it was also demonstrated that transgenic approach could be used to fine-tune the properties of wheat lines for different end-use purposes (30–32).

Improvements to the nutritional quality of the wheat flour and dough were also seen as relevant goals for the overall sustainability of wheat as a food crop (*reviewed by* Shewry and Zhu et al. (33, 34)). Wheat whole grain contains several important vitamins, minerals, a high amount of fibre, complex carbohydrates, and a substantial amount of proteins, but in the white flour some of these components are lost due to the milling process. To improve indices of large-bowel health in rats, Regina et al. (35) generated high-amylose wheat by RNA interference. To improve the nutritional quality of the wheat flour and the dough, Tamas et al. (36) expressed an amarant albumin (Ama1) gene in transgenic wheat endosperm increasing the content of some essential amino acids (tyrosine, threonine, and lysine) between 3.8 and 7.3% in the flour. To modify the protein content and quality attributes of the endosperm, it requires tissue specific promoters that underline all the efforts carried out to characterize storage protein promoters and improve their strength to enhance the recombinant protein yield in the transgenic wheat grain (37–40).

Wheat was also used to study if the endosperm tissue can be considered as a seed based production platform of pharmaceuticals, expressing medically important antibodies, after the introduction of the appropriate genes into the genome (41). The success in the plant biopharmaceutical production and molecular farming

efforts may have more of an impact on the social acceptance of the GM technology.

The protocols described below are adapted from reliable and widely used methods of wheat transformation (4, 36, 42–44) and have been augmented by a Note section (Subheading 4) explaining our detailed experiences in generating viable and stable transgenic lines. Regardless of the DNA-delivery methods and specific regeneration methods, the herbicide selection system, based on the *bar* gene and the phosphinothricin (PPT) selection agent, was applied to distinguish between transgenic and non-transgenic cell lines. For particle bombardment, the plasmid construct pAHC25 (45) was used, carrying the selectable marker gene (*bar*) and the reporter gene (*uidA*) both under the control of the constitutive ubiquitin promoter (*Ubi1*) from maize. The *Agrobacterium*-mediated transformation exploited the *Agrobacterium tumefaciens* strain AGL1 (46). This super-virulent strain contains the plasmids pAL154/pAL156 based on the plasmid pSoup/pGreen (47). The single T-DNA in binary vector pAL156 incorporates the *bar* gene and a modified *uidA* gene with an intron within the open reading frame to prevent its expression in *A. tumefaciens* itself; both genes are driven by the promoter of the maize ubiquitin gene plus the first intron of it (45). The *bar* gene is located next to the left border, and *uidA* gene is adjacent to the right border. The plasmid pAL154 helps the replication functions for pAL156 *in trans* and contains the 15 kb Komari fragment (48, 49) supplying extra *vir* genes.

The following Methods section (Subheading 3) is divided into three sections: the first covers tissue culture conditions and media composition, the second covers selection and growth of healthy, fertile transgenic plants, and the third section describes the screening of T₀ lines at molecular level by PCR and provides methods to measure the enzyme activity of the reporter and selectable marker genes. The action of β -glucuronidase enzyme, encoded by the *uidA* gene, is demonstrated in a non-quantified way by histochemical GUS staining, while the activity can be quantified using the MUG fluorescent assay. The *bar* gene expression is demonstrated either by herbicide leaf painting or using the ammonium assay to determine the activity of phosphinothricin acetyltransferase (PAT) enzyme.

2. Materials

2.1. Materials for Biolistics Transformation

2.1.1. Plant Materials

Wheat (*Triticum aestivum* L.) plants (cv. Cadenza, Fielder, Florida, and different elite varieties) and tetraploid durum wheat (*T. turgidum* L. var. *durum*) are grown under controlled conditions in temperature growth rooms or phytotron chambers. Artificial light is provided by banks of HQI lamps 400 W (Osram Ltd., UK) in

growth rooms [intensity $\sim 700 \mu\text{mol}/\text{m}^2 \text{ s}$ photosynthetically active radiation (PAR) for a 16-h photoperiod, and the day–night temperature regime is 18/14°C). Prior to transfer to these conditions, winter wheat seeds are imbibed and vernalized for 6 weeks at 4°C. To ensure continuous production of immature embryos, batches of seeds are planted every 1 or 2 weeks. Particular care should be given to maintain growing conditions at the same level (see Note 1).

2.1.2. Sterilization

Materials for Wheat Spikes and Caryopses

1. 70% (v/v) aqueous ethanol.
2. Sodium hypochlorite (NaOCl, 1.3%) aqueous solution with a drop of surfactant (Triton X-100 or Tween-20) (see Note 2).
3. Sterile distilled water (reverse osmosis, purity of 18.2 MΩ/cm).

2.1.3. Stock Solutions

for Tissue Culture Media

The final tissue culture media (Subheading 2.1.4) are prepared from stock solutions detailed below. Prepare all solutions using ultrapure water (purity of 18.2 MΩ/cm) and analytical grade reagents.

1. Ferrous sulphate chelate solution (100×): buy a ready-to-use solution (Sigma-Aldrich) or dissolve 3.725 g/L EDTA- $\text{Na}_2 \cdot 2\text{H}_2\text{O}$ (ethylenediaminetetraacetate disodium salt) and 2.785 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (see Note 3). Autoclave at 121°C for 20 min, store at 4°C for maximum 2 months.
2. MS macrosalts (10×): 16.5 g/L NH_4NO_3 , 19.0 g/L KNO_3 , 1.7 g/L KH_2PO_4 , 3.7 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.4 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (see Note 3). Autoclave at 121°C for 20 min, store at 4°C for maximum 2 months.
3. MS Vitamins (-glycine) (1,000×): 100 mg/L thiamine·HCl, 500 mg/L pyridoxine·HCl, 500 mg/L nicotinic acid. Prepare 100 mL at a time (see Note 3), filter-sterilize (see Note 4), and store at 4°C for maximum 2 months.
4. L7 macrosalts (10×): 2.5 g/L NH_4NO_3 , 15.0 g/L KNO_3 , 2.0 g/L KH_2PO_4 , 3.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.5 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (see Note 3). Autoclave at 121°C for 20 min and store at 4°C for maximum 2 months.
5. L7 microsalts (1,000×): 15.0 g/L MnSO_4 (various hydrated states alter the required weight), 5.0 g/L H_3BO_3 , 7.5 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.75 g/L KI, 0.25 g/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.025 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.025 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$. Prepare 100 mL at a time (see Note 3), filter-sterilize (see Note 4), and store at 4°C for maximum 2 months.
6. Silver nitrate (AgNO_3) solution (used to promote embryogenesis): 20 mg/mL in water. Prepare 10 mL at a time in graduated plastic tube. Mix well, filter-sterilize (see Note 4), and aliquot into 1 mL volumes. Store at -20°C in the dark.

(see Note 5). Should remain effective for at least a year, provided no freezing–thawing has occurred.

7. Copper sulphate (CuSO_4) solution: 0.1 M (16 mg/mL) (24.97 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ /L) in water. Prepare 10 mL at a time in graduated plastic tube. Mix well, filter-sterilize (see Note 4), and store at 4°C in 1 mL aliquots for maximum 2 months.
8. L7 vitamins/inositol (200×): 40.0 g/L myo-inositol, 2.0 g/L thiamine·HCl, 0.2 g/L pyridoxine·HCl, 0.2 g/L nicotinic acid, 0.2 g/L Ca-pantothenate, 0.2 g/L ascorbic acid. Prepare 100 mL at a time (see Note 3). Filter-sterilize (see Note 4) and store at –20°C in 10 mL aliquots (should remain effective for at least a year).
9. Amino acids 3AA (25×): 18.75 g/L glutamine, 3.75 g/L proline, 2.5 g/L asparagine. Prepare 200 mL at a time (see Note 3). Store solution at –20°C in 40 mL aliquots (should remain effective for at least a year).
10. 2,4-dichlorophenoxyacetic acid (2,4-D) (auxin type hormone): 1 mg/mL in ethanol/water. Dissolve 10 mg powder in 2 mL ethanol in graduated plastic tube and then add water to volume of 10 mL. Mix well, filter-sterilize (see Note 4) and store at –20°C in 1 mL aliquots (should remain effective for at least a year).
11. Zeatin mixed isomers (cytokinin type hormone): 10 mg/mL in HCl/water. Dissolve 100 mg powder in 3 mL 1 M HCl in graduated plastic tube and then add water to volume of 10 mL. Mix/vortex well, filter-sterilize (see Note 4) and store at –20°C in 1 mL aliquots (should remain effective for at least a year).
12. PPT (synthetic l-phosphinothricin) selection agent: 10 mg/mL in water (see Note 6). Prepare 10 mL at a time in a graduated plastic tube. Mix/vortex well, filter-sterilize (see Note 4), and store at –20°C in 1 mL aliquots (should remain effective for at least a year).
13. Agargel (2×): 10 g/L in water. Add 4 g gel powder to 400 mL water in a 500-mL bottle and sterilize it at 121°C for 20 min in an autoclave. Shake well both before and after autoclaving to avoid non-uniform solidification which leads to difficulties when re-melting. Store at room temperature and melt in microwave before use.

2.1.4. Tissue Culture Media for Wheat Immature Scutella

Tissue culture media are prepared at double strength to allow the addition of an equal volume of double-strength gelling agent (Agargel) melted (see Notes 7–9).

Callus Induction Media

1. Callus Induction Media (CI/Biol) (2×): 20 mL/L ferrous sulphate chelate solution (see Note 7), 200 mL/L MS macro-salts, 2 mL/L L7 microsals, 2 mL/L MS (-glycine) vitamins,

200 mg/L myo-inositol, 40 mL/L 3AA amino acids, 180 g/L (9% final concentration) sucrose (see Note 10). Adjust pH to 5.8 with 5 M NaOH or KOH and filter-sterilize (see Note 4). Prepare in 400 mL volumes (see Notes 3 and 8) and store at 4°C for maximum 2 months.

2. CI/Biol: Mix an equal volume of CI/Biol (2×) with sterile, melted Agargel (see Note 9). Add 0.5 mg/L 2,4-D, 10 mg/L AgNO₃, mix by rotating the bottle gently, and pour into 9 cm diameter Petri dishes (26–30 mL per dish). Store at 4°C in the dark (see Note 5) for maximum 3 weeks.

Regeneration Media

1. R (2×): 20 mL/L ferrous sulphate chelate solution (see Note 7), 200 mL/L L7 macrosalts, 2 mL/L L7 microsals, 10 mL/L L7 vitamins/inositol, 60 g/L maltose. Adjust pH to 5.8 with 5 M NaOH or KOH and filter-sterilize (see Note 4). Prepare in 400 mL volumes (see Notes 3 and 8) and store at 4°C for maximum 2 months.
2. RZD + 1/2Cu (for stage 1. regeneration without selection): mix an equal volume of R (2×) medium with sterile, melted Agargel (see Note 9). Add 5 mg/L zeatin, 0.1 mg/L 2,4-D, and 0.05 mM (8 mg/L) CuSO₄, mix by rotating the bottle gently, and pour into 9-cm Petri dishes (26–30 mL per dish). Store at 4°C for maximum 3 weeks.
3. RZ (for controls at stage 2. regeneration): mix an equal volume R (2×) with sterile, melted Agargel (see Note 9). Add 5 mg/L zeatin, mix by rotating the bottle gently, and pour into 9-cm Petri dishes (26–30 mL per dish). Store at 4°C for maximum 3 weeks.
4. R0 (for controls at stage 3. regeneration and for root regeneration): mix an equal volume R (2×) with sterile, melted Agargel (see Note 9). Pour into 9-cm Petri dishes (26–30 mL per dish) or GA-7 Magenta vessels (Sigma-Aldrich) (55–60 mL per vessel). Store at 4°C for maximum 3 weeks.

Selection Media

1. RZPPT2-3 (for stage 2. regeneration with selection): mix an equal volume of R (2×) with sterile, melted Agargel (see Note 9). Add 5 mg/L zeatin and 2–3 mg/L PPT (see Note 11). Mix by rotating the bottle gently and pour into 9-cm Petri dishes (26–30 mL per dish). Store at 4°C for maximum 3 weeks.
2. RPPT3-4 (for stage 3. regeneration and for root regeneration with selection): mix an equal volume of R (2×) with sterile, melted Agargel (see Note 9). Add 3–4 mg/L PPT (see Note 11). Mix by rotating the bottle gently and pour into 9-cm Petri dishes (26–30 mL per dish) or GA-7 Magenta vessels (55–60 mL per vessel). Store at 4°C for maximum 3 weeks.

2.1.5. Materials and Consumables for Bombardment

1. 3 M Calcium chloride: dissolve 4.40 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 10 mL water. Mix/vortex well. Filter-sterilize (see Note 4) and store at -20°C in 50 μL aliquots (should remain effective for at least a year).
2. 0.1 M spermidine free-base: prepare 1 mL of 1 M stock solution from powder in sterile ultrapure water and store at -80°C in 20 μL aliquots. Prepare the 0.1 M working solution by dilution of stock in sterile ultrapure water under sterile conditions. Mix/vortex well and store at -20°C in 10 μL and 20 μL aliquots for 6 and 12 shots, respectively. If once thawed, it should not be used again.
3. Gold particles: 0.6 μm (sub-micron) gold particles (Bio-Rad Laboratories) as the smaller, more uniform size of the particles is preferable for small wheat cells. For gold suspension (40 mg/mL) preparation see Subheading 3.1.3.
4. Macrocarriers, stopping screens, 650 or 900 psi rupture discs (all Bio-Rad Laboratories).
5. Plasmid DNA: prepare high purity DNA sample, using for example Qiagen kit (Qiagen Ltd.), adjust the concentration to 1 $\mu\text{g}/\mu\text{L}$ in sterile TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and store at -20°C in 20 μL aliquots.

2.2. Materials for *Agrobacterium*-Mediated Transformation

2.2.1. Plant Material and Sterilization Chemicals for Wheat Spikes and Caryopses

1. Plant material: as described in Subheading 2.1.1 for biolistic transformation.
2. Sterilization materials for wheat spikes and caryopses: as described in items 1–3 in Subheading 2.1.2 for biolistic transformation.

2.2.2. Growing *A. tumefaciens*

1. Standard glycerol inoculum of *A. tumefaciens* strain AGL1.
2. MG/L medium according to (50): Dissolve 5 g/L mannitol, 1 g/L L-glutamic acid, 250 mg/L KH_2PO_4 , 100 mg/L NaCl, 100 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g/L tryptone, and 2.5 g/L yeast extract in ultrapure water (see Note 3). Adjust pH to 7.0 with 5 M NaOH or KOH. Autoclave at 121°C for 20 min. At room temperature add 1 μg biotin (100 μL from sterile stock solution at 1 mg/100 mL) to 1 L medium. Store at 4°C in 10 mL aliquots for maximum 2 months.

2.2.3. Stock Solutions for Tissue Culture Media

The final tissue culture media (Subheading 2.2.4) are prepared from stock solutions as it is given in details below. Prepare all solutions using ultrapure water (purity of $18.2 \text{ M}\Omega/\text{cm}$) and analytical grade reagents.

1. Ferrous sulphate chelate solution (100×): as described in item 1 in Subheading 2.1.3 for biolistic transformation.
2. MS macrosalts (10×): as described in item 2 in Subheading 2.1.3 for biolistic transformation.
3. L7 microsals (1,000×): as described in item 5 in Subheading 2.1.3 for biolistic transformation.
4. MS vitamins (-glycine) (1,000×): as described for biolistic transformation in item 3 in Subheading 2.1.3.
5. L7 vitamins/inositol (200×): as described in item 8 in Subheading 2.1.3 for biolistic transformation.
6. Acetosyringone (3',5'-dimethoxy-4'-hydroxyacetophenone): 10 mg/mL (50 mM) stock solution in 70% ethanol. Prepare 10 mL at a time in graduated plastic tube. Mix well, and store at -20°C in 500 µL aliquots (should remain effective for at least a year), or prepare fresh.
7. 2,4-dichlorophenoxyacetic acid (2,4-D) (auxin type hormone): as described in item 10 in Subheading 2.1.3 for biolistic transformation.
8. Zeatin mixed isomers (cytokinin type hormone): as described in item 11 in Subheading 2.1.3 for biolistic transformation.
9. Picloram (plant cell culture tested): 1 mg/mL in ultrapure water. Prepare 10 mL at a time in a graduated plastic tube. Mix well, filter-sterilize (see Note 4), and store at -20°C in 2 mL aliquots (should remain effective for at least a year).
10. Timentin antibiotic (15:1 ratio of ticarcillin disodium and clavulanate potassium): 300 mg/mL in ultrapure water. Prepare 10 mL at a time in a graduated plastic tube. Mix well, filter-sterilize (see Note 4), and store at -20°C in 1 mL aliquots (should remain effective for at least a year).
11. PPT selection agent: as described in item 12 in Subheading 2.1.3 for biolistic transformation.
12. Silwet L-77 (Lehle seeds, USA), a 100% non-ionic surfactant: prepare 1% (v/v) solution in water. Filter-sterilize (see Note 4) and store at 4°C in 0.5 mL aliquots.
13. Phytigel (2×): 4 g/L in water. Add 1.6 g gel powder to 400 mL water in a 500-mL bottle and sterilize it at 121°C for 20 min in an autoclave. Shake well both before and after autoclaving to avoid non-uniform solidification which leads to difficulties when re-melting. Store at room temperature and melt in microwave before use.
14. Agargel (2×): as described in item 13 in Subheading 2.1.3 for biolistic transformation.

2.2.4. Media for Plant Tissue Culture

Tissue culture media are prepared at double strength to allow the addition of an equal volume of gelling agent at double strength (see items 13 and 14 in Subheading 2.2.3) melted (see Notes 7–9). Phytigel is used for inoculation and induction media, while Agargel is used for regeneration- and selection media (R/inDTZ, R/inT, and R/inT-DTT) (see below in this Subheading).

Inoculation Media

1. Double-strength inoculation medium (IM $\times$ 2): 20 mL/L ferrous sulphate chelate solution (see Note 7), 200 mL/L MS macrosalts, 2 mL/L L7 microsals, 2 mL/L MS (-glycine) vitamins, 200 mg/L myo-inositol, 1 g/L glutamine, 200 mg/L casein hydrolysate, 3.9 g/L MES monohydrate buffering agent, 20 g/L glucose, 80 g/L maltose. Adjust pH to 5.8 with 5 M NaOH or KOH and then filter-sterilize (see Note 4). Prepare in 400 mL volumes (see Notes 3 and 8), and store at 4°C for maximum 2 months.
2. Single-strength liquid inoculation medium (for re-suspending *Agrobacterium* cells in step 3 in Subheading 3.2.2): mix double strength inoculation medium with same volume of sterile, ultrapure water.
3. Semi-solid inoculation medium: mix an equal volume of double-strength inoculation medium with sterile, melted Phytigel (see Note 9). Add 2.0 mg/L 2,4-D, 2.0 mg/L picloram, 200 μ M (or 400 μ M, see Note 31) acetosyringone, mix by rotating the bottle gently and pour into 5.5 or 9 cm Petri dishes (26–30 mL per dish). Store at 4°C for maximum 3 weeks.

Callus Induction Media

1. Callus Induction Media (CI/Agr) (2 $\times$): 20 mL/L ferrous sulphate chelate solution (see Note 7), 200 mL/L MS macrosalts, 2 mL/L L7 microsals, 2 mL/L MS (-glycine) vitamins, 200 mg/L myo-inositol, 1 g/L glutamine, 200 mg/L casein hydrolysate, 3.9 g/L MES monohydrate, 80 g/L maltose. Adjust pH to 5.8 with 5 M NaOH or KOH and then filter-sterilize (see Note 4). Prepare in 400 mL volumes (see Notes 3 and 8), and store at 4°C for maximum 2 months.
2. Semi-solid CI/Agr: mix an equal volume of double-strength induction medium with sterile, melted Phytigel (see Note 9). Add 0.5 mg/L 2,4-D, 2.0 mg/L picloram, 150 mg/L timen-tin, mix by rotating the bottle gently, and pour into 9 cm Petri dishes (26–30 mL per dish). Store at 4°C for maximum 3 weeks.

Regeneration Media

1. R/in (2 $\times$): 20 mL/L ferrous sulphate chelate solution (see Note 7), 200 mL/L MS macrosalts, 2 mL/L L7 microsals, 10 mL/L L7 vitamins/inositol, 200 mg/L myo-inositol, 60 g/L maltose. Adjust pH to 5.7 with 5 M NaOH or KOH

and then filter-sterilize (see Note 4). Prepare in 400 mL volumes (see Notes 3 and 8), and store at 4°C for maximum 2 months.

2. R/inDTZ (for stage 1. regeneration without selection): mix an equal volume of double-strength R/in medium with sterile, melted Agargel (see Note 9). Add 0.1 mg/L 2,4-D, 150 mg/L timentin, 5 mg/L zeatin, mix by rotating the bottle gently, and pour into 9 cm Petri dishes (26–30 mL per dish). Store at 4°C for maximum 3 weeks.
3. R/inT (for controls at stage 2–3. regeneration and for strengthening root system): mix an equal volume of double-strength R/in medium with sterile, melted Agargel (see Note 9). Add 150 mg/L timentin, mix by rotating the bottle gently, and pour into 9 cm Petri dishes (26–30 mL per dish) or GA-7 Magenta vessels (55–60 mL per vessel). Store at 4°C for maximum 3 weeks.

Selection Media

1. R/inT-PPT (for stage 2–3. regeneration and for root regeneration with selection): mix an equal volume of double-strength R/in medium with sterile, melted Agargel (see Note 9). Add 150 mg/L timentin, 2–4 mg/L PPT (see Note 11), mix by rotating the bottle gently, and pour into 9 cm Petri dishes (26–30 mL per dish) or GA-7 Magenta vessels (55–60 mL per vessel). Store at 4°C for maximum 3 weeks.

2.3. Materials for Testing Transient and Stable Gene Expression

In the following subsections (Subheadings 2.3.1–2.3.3) the materials for detecting the *bar* and the *uidA* genes are provided because for biolistic transformation the plasmid constructs pAHC25 (see Note 12), while for the *Agrobacterium*-mediated transformation the binary vector pAL156 (both incorporating the *bar* gene and the *uidA* gene) were applied.

2.3.1. Screening the Primary Regenerated Plantlets by PCR

1. DNA extraction reagents and equipment (see Note 14).
2. PCR (polymerase chain reaction) and electrophoresis solutions and equipment.
3. Primers (see Subheading 3.3.1).

2.3.2. Histochemical GUS Assay for Transient and Stable Gene Expression

GUS assay can be conducted in transgenic plants since both the plasmid construct pAHC25 and the binary vector pAL156 in *A. tumefaciens* strain AGL1 contain not only the selectable marker gene *bar* but the *uidA* reporter gene as well (see Notes 12 and 13).

1. X-Glca (5-bromo-4-chloro-3-indolyl- β -D-galacturonide) (or X-Gluc, 5-bromo-4-chloro-3-indolyl- β -D-glucuronide) stock solution (50 $\times$): 1 mg/mL (100 mM) in *N,N*-dimethylformamide (DMF). Prepare 10 mL at a time in graduated plastic tube.

2. X-Glca (or X-Gluc) working solution: 100 mM phosphate buffer (pH 7.0), 10 mM EDTA, 0.8 mM potassium ferricyanide, 0.8 mM potassium ferrocyanide, 0.1% (v/v) Triton X-100, and 2 mM X-Glca in ultrapure water. Prepare 10 mL at a time in graduated plastic tube from stock solutions (1 M phosphate buffer, 100 mM EDTA, 40 mM potassium ferri- and ferrocyanide, 1% Triton X-100, and 100 mM X-Glca or X-Gluc). Filter-sterilize and store in 2 mL aliquots at -20°C .
3. 25-multiwell microtitre plates.

*2.3.3. Materials
for Analysis of Stable
Expression of the bar Gene*

The *bar* gene encodes the enzyme phosphinothricin acetyltransferase (PAT) which confers resistance to phosphinothricin (PPT) and glufosinate ammonium-based herbicides.

*2.3.3.1. Leaf Painting
Bioassay*

1. 0.1% (v/v) Tween-20 solution.
2. Glufosinate ammonium-based herbicide solutions containing 0.2 g/L and 2 g/L PPT (see Note 6).
3. Ballpoint pen and cotton buds.

2.3.3.2. Ammonium Test

1. Ammonium assay incubation medium: 50 mM potassium-phosphate buffer pH 5.8, 2% sucrose, 1.0 mg/L 2,4-D, 25 mg/L glufosinate ammonium, and 0.1% (v/v) Tween-20.
2. Ammonium assay Reagent 1: 34 g/L sodium salicylate, 25 g/L trisodium citrate, 25 g/L sodium tartrate, and 0.12 g/L sodium nitroprusside (see Note 15). Store at 4°C in the dark.
3. Ammonium assay Reagent 2: 30 g/L sodium hydroxide and 0.52 g/L sodium dichloroisocyanurate (see Note 16). Store at 4°C for maximum 2 months.
4. Ammonium chloride: 1 g/L (1,000 mg/L) in ultrapure water. Prepare 100 mL at a time. For 10 mL working solutions (2, 4, 6, 8, and 10 mg/L) pipette 20, 40, 60, 80, and 100 μL , respectively, from the stock into 10 mL ultrapure water in plastic tubes.

*2.3.3.3. Herbicide
Resistance (PAT) Assay
by Spraying Putative
Transgenic Plants*

1. Glufosinate ammonium-based herbicide solution containing 0.1% (m/v), 1 g/L PPT (see Note 6).
2. Spraying bottle with a volume of 1 or 2 L.

3. Methods

**3.1. Method
for Biolistic
Transformation**

This is an optimized method for transformation of immature scutella, but immature inflorescences can also be used as explants (51–54). Carry out all procedures at room temperature unless otherwise specified.

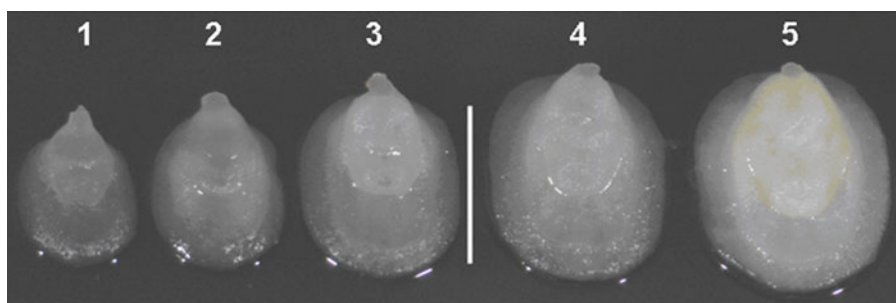


Fig. 1. Immature embryos prepared from caryopses at 12–16 days post anthesis. The embryos with slightly translucent scutellum and axis turning opaque (No 3) are generally most responsive. The size of the scutella is 0.8–1.2 mm in length; however, genotypic variations can be observed. Smaller and/or larger embryos may respond but with lower efficiencies. White bar represents 1 mm (The photo was taken by Terese Richardson and used with her kind permission).

3.1.1. Collection and Sterilization of Wheat Caryopses

1. Collect spikes from phytotron chambers-grown plants at around 11–12 weeks after sowing (see Subheading 2.1.1 for growing condition). Embryos at the correct stage are usually found 12–14 days post anthesis (DPA). A few grains can be checked at the time of collection to determine the size of the embryos within them (for proper size see step 1 in Subheading 3.1.2 and Fig. 1).
2. Sterilize spikes by rinsing in 70% (v/v) aqueous ethanol for 30 s before removing the immature grains from the panicles. Collect caryopses only from the middle half of each spike and avoid using the inner caryopses of the spikelet that generally contain smaller embryos due to asynchronous development.
3. Surface sterilize the caryopses by soaking them for 15–20 min in sodium hypochlorite (1.3%) aqueous solution with a drop of surfactant (see Note 2) while shaking gently on a platform shaker (~60 rpm).
4. Rinse in sterile distilled water at least three times under aseptic conditions. Maintain the sterile caryopses in moist conditions, but do not keep immersed in water.

3.1.2. Isolation and Pre-culture of Immature Scutella

1. Isolate the embryos microscopically in a sterile environment while removing the embryo axis with a sharp scalpel, to get scutella and prevent precocious germination. Embryos of size 0.7–1.2 mm (slightly translucent immature embryos) are generally the most responsive, but there is genotypic variation (see Note 17 and Fig. 1).
2. Place 30 scutella (5×6) within central (approximately 2 cm diameter) target area in a 9 cm Petri dish containing induction medium (MS9%0.5DAg). Orientate them with the uncut scutellum up, i.e. the uncut side is bombarded.
3. Pre-culture the prepared donor material in the dark at $23 \pm 1^\circ\text{C}$ for 36–48 h prior to bombardment. The pre-culture allows to

recover the tissues from the isolation procedure and to discard any contaminated explants.

3.1.3. Gold Suspension Preparation

1. Weigh 40 mg gold particles (0.6 μm) in a 1.5-mL Eppendorf tube, add 1 mL 100% ethanol, vortex, and sonicate in water bath for 90 s. Pulse-spin in a microfuge for 3 s and remove the supernatant. Repeat this ethanol wash once in 100% ethanol and once in 70% ethanol.
2. Add 1 mL sterile water, vortex, and sonicate for 90 s. Pulse-spin in a microfuge for 3 s and discard the supernatant. Repeat this step once.
3. Re-suspend gold particles fully by vortexing in 1 mL sterile water. Aliquot 50 μL suspension into sterile 1.5-mL Eppendorf tubes. Do not forget to vortex between each pipetting to ensure an equal distribution of particles. Store the aliquots at -20°C for maximum 6 months.

3.1.4. Coating the Gold Particles with DNA for Bombardment

Each step of the procedure is carried out on ice in a sterile environment.

1. Allow a 50 μL aliquot of prepared gold (see Subheading 3.1.3) to thaw at room temperature. Vortex and then sonicate for 90 s (see Note 18). If the aliquots are going to be subdivided for smaller preparations (fewer shots), vortex in between taking aliquots is advisable. Volume(s) of solutions added later are scaled down accordingly.
2. Add 5 μg DNA (5 μL of 1 mg/mL in TE) or 5 μL sterile water (to replace DNA for bombarded controls) and vortex briefly to ensure good contact of DNA with the particles (see Note 19).
3. Place 44 μL 3 M CaCl_2 and 20 μL 0.1 M spermidine first onto the side of the Eppendorf tube and mix together using the pipette tips. It helps to make coating as even as possible because DNA binds with them very rapidly. Mix the solution with the gold+DNA suspension by vortexing briefly. Pulse-spin twice for 3–5 s in a microfuge to pellet the DNA-coated particles. Discard the supernatant.
4. Add 150 μL 100% ethanol to wash the particles. Re-suspend them as fully as possible by scraping the side of the tube with the pipette tip to remove clumps, and drawing up and expelling the suspension repeatedly. This is a crucial step since clumps cannot be removed later by vortexing. Pulse-spin twice for 3–5 s in a microfuge to pellet the DNA-coated particles and discard the supernatant. Repeat this step once.
5. Re-suspend the particles completely in 85 μL 100% ethanol and keep the tube(s) on ice. Use the DNA-coated gold suspension as soon as possible or keep on ice for no longer than 1 h.

In this latter case, seal the Eppendorf lid(s) with Parafilm to reduce evaporation of the ethanol (see Note 20).

*3.1.5. Particle
Bombardment Using the
PDS-1000/He Particle Gun
(Bio-Rad)*

When operating the gun, appropriate safety precautions should be taken as the delivery system involves the use of helium gas at high pressure to accelerate gold particles at high velocity.

Include various control plates (non-bombarded controls (NBC) and bombarded controls (BC)) within each experiment to monitor regeneration and selection efficiencies (see Note 21).

1. Deliver the DNA-coated gold particles (see Subheading 3.1.4) into the target tissue according to the manufacturer's instructions (Bio-Rad). Standard settings in the procedure are the gap (distance between rupture disc and macrocarrier, 2.5 cm), stopping plate aperture (distance between macrocarrier and stopping screen, 0.8 cm), target distance (between stopping screen and target plate, 5.5 cm). The vacuum is 27–28 in.Hg (91.4–94.8 kPa), vacuum flow rate 5.0, vent flow rate 4.5 (54).
2. Sterilize the gun's chamber and all the component parts by spraying with 70% (v/v) ethanol. Allow the ethanol to evaporate completely (~5 min).
3. Sterilize macrocarrier holders, macrocarriers, stopping screens, and rupture discs by dipping in 100% ethanol. Do not soak rupture discs in ethanol for more than 5 min or the laminate layers may become separated. Allow the alcohol to evaporate completely on a mesh rack or on Petri dishes in a flow hood. Place three or four macrocarrier holders into a sterile 9-cm Petri dish. Put one dry macrocarrier into each holder and push it down using the red seating tool. The edge of the macrocarrier should be securely inserted under the lip of the macrocarrier holder.
4. Briefly vortex the coated gold particles (see Subheading 3.1.4), take 5 μ L and spread evenly over the central 1 cm of the macrocarrier membrane in the holder. Load only three or four macrocarriers at one time and cover the Petri dish with its lid immediately. Allow gold suspension to dry naturally outside of the flow hood but on a non-vibrating surface in order to avoid particle agglomeration (see Note 22).
5. Load a rupture disc (either 650 or 900 psi; 4.484 or 6.209 MPa) into the rupture disc retaining cap and screw into place on the gas acceleration tube. Tighten it firmly using the mini torque wrench (see Note 23).
6. Place a stopping screen into the fixed nest. Invert the macrocarrier holder containing macrocarrier with DNA-coated gold particles and put over the stopping screen in the nest (dried particles should be facing down toward the stopping screen). Replace the macrocarrier cover lid on the assembly and turn

clockwise until snug, but do not over-tighten. Put the fixed nest assembly onto the second shelf from the top to give a gap of 2.5 cm.

7. Place the Petri dish carrying the tissue samples on the appropriate shelf to give the required distance (5.5 cm, fourth shelf from the top) from the stopping screen.
8. Draw a vacuum of 27–28 in.Hg (91.4–94.8 kPa) and fire the gun. The helium pressure builds up, breaks the rupture disc, moves the macrocarrier onto the stopping plate, and thus the DNA-coated particles are released and dispersed. Monitor the actual pressure at which the rupture disc bursts to ensure successful shot (may affect transformation efficiency).
9. Release the vacuum, remove the tissue samples (put the lid to the Petri dish first), and disassemble the component parts. Collect or discard the ruptured disc and macrocarrier (see Note 24).
10. Place the macrocarrier holder and stopping screen in 100% ethanol to re-sterilize if they are to be reused for further shots.
11. Remember to bombard two to three Petri dishes (BC) with gold (no DNA) to monitor the effect of bombardment and the selection efficiency later (see Note 25).

3.1.6. Induction of Embryogenic Calli from Immature Scutella Following Bombardment

1. After bombardment, spread the scutella evenly across the induction medium (CI/Biol, see Subheading 2.1.4) in 9-cm Petri dishes. To prevent competition for nutrients, divide each replicate between three plates (one is the original) (10 scutella per plate).
2. Seal the plates with plastic film (Parafilm or PE film) and incubate for 3 weeks at $23 \pm 1^\circ\text{C}$ in the dark for induction of embryogenic calli. Check regularly for contamination. After 2–3 days, transient assays e.g. histochemical GUS assay, can be carried out (see Subheading 3.3.2). Using stereomicroscope, observe calli randomly for visible nodular outgrowth (called embryogenic calli).

3.1.7. Regeneration and Selection of Transgenic Plants

1. Stage 1. Regeneration, without selection: after 3 weeks on callus induction medium, transfer calli bearing somatic embryos to regeneration medium (RZD + $1/2\text{Cu}$, see Subheading 2.1.4) in 9 cm Petri dishes. Discard any hydrated and necrotic ones. Transfer whole calli without division (7–8 calli per plate) and incubate at $23 \pm 1^\circ\text{C}$ in the light for 3 weeks (see Notes 26 and 27). By the end of the third week, shoots and green structures should appear.
2. Stage 2. Regeneration with selection: after 3 (or maximum 6) weeks, transfer calli bearing shoots without division onto RZ+selection media in 9 cm Petri dishes with RZPPT2

medium (see Note 11). Incubate at $23 \pm 1^\circ\text{C}$ in the light for 3 weeks. Culture 7–8 calli per Petri dish of controls (NBC, BC) on media with and without (RZ, see Subheading 2.1.4) selection agent too. Apply selection from now on, until all control plantlets have been killed (see Note 11).

3. Stage 3. Regeneration with selection: after a further 3 weeks, and every subsequent 3 weeks, transfer surviving (healthy and responsive) calli with shoots to regeneration medium + selection agent but without hormones (RPPT3-4, see Note 11) in 9-cm Petri dishes (5–6 calli per plate). Discard any unresponsive calli. Transfer calli with shoots from control plates onto R0 and RPPT3-4 in Petri dishes. Incubate them in the light at $23 \pm 1^\circ\text{C}$ or at $25\text{--}26^\circ\text{C}$ to shorten tissue culture period, provided another plant room is available.
4. Root regeneration with selection: separate 3–4 cm long plantlets with small or definite roots from the callus and transfer to RPPT4 media but in GA-7 Magenta vessels. Place only 3–4 plantlets per Magenta and incubate in the light (see step 3 in Subheading 3.1.7).
5. Transfer plantlets (>10 cm in length) with established root system to Jiffy pots (see Note 28) or vernalize winter wheat varieties on R0 media in Magenta vessels at 4°C for 6 weeks. Remove plantlets carefully from the Agargel (solidified medium). If they are to be planted into Jiffy pots, rinse the roots with tap water before potting. Grow them in a GM containment chamber, initially within a propagator (or in plastic bags) to provide high humidity for 12–14 days to acclimatize from tissue culture (see Note 29).
6. Pot the 3 and 4 leaf stage plants to 13 cm diameter pots and grow them to maturity in separate growth chambers for GM but under conditions similar to those used for donor plants. Plants should reach maturity in 3 months (it takes 4 and a half months for winter wheat varieties).

3.2. Method for *Agrobacterium*- Mediated Transformation

3.2.1. Growth of Donor Plants

3.2.2. Preparation of *Agrobacterium* Cells for Inoculation

Wheat (*T. aestivum* L.) plants (cv. Cadenza, Fielder, Florida, and different elite varieties) and tetraploid durum wheat (*T. turgidum* L. var. *durum*) are grown under controlled conditions in phyto-tron chamber as it is described in Subheading 2.1.1.

1. Initiate *Agrobacterium* liquid cultures by adding ~200 μL of a standard glycerol inoculum to 10 mL MG/L (see item 2 in Subheading 2.2.2) plus antibiotics (see Note 30). Prepare as many 10 mL cultures as plates (Petri dishes with 50 scutella in each) to be treated.

2. Incubate at 28–29°C, shaking (250 rpm) for 16–20 h (usually overnight) to reach an OD > 1 (optical density). Measure absorbance at 600 nm.
3. Pellet the *Agrobacterium* culture at 4,500 × *g* for 10 min and re-suspend the bacteria in 4 mL single-strength inoculation medium (see item 2 in Subheading 2.2.4) supplemented with 200 μM acetosyringone (see Note 31) for each culture (originally 10 mL).
4. Put the suspended cultures back to the shaker until required, but they should be used within 3 h.

3.2.3. Collection and Sterilization of Wheat Caryopses

Collect spikes from phytotron chambers-grown plants 12–14 days post anthesis (DPA) and sterilize as it is described in steps 1–4 in Subheading 3.1.1.

3.2.4. Isolation of Immature Scutella and Inoculation with *A. tumefaciens*

1. Isolate immature scutella microscopically in sterile environment with sharp scalpel while removing the embryo axis (see step 1 in Subheading 3.1.2).
2. Place 50 scutella with the axis side (now removed) down onto semi-solid inoculation medium (see Subheading 2.2.4) in 5.5 or 9 cm Petri dish, within the central area of ~3 or 6 cm diameter. Before isolating scutella for the next plate, it is important to inoculate each plate of 50 scutella with *A. tumefaciens*, as described below (see steps 3–5).
3. Take the re-suspended *Agrobacterium* out of the shaker, add 60 μL 1% Silwet, mix, and pour the whole 4 mL over a batch of 50 plated scutella.
4. Incubate for 1–2 h in the dark at room temperature while preparing more scutella for inoculation as described in step 1 in Subheading 3.1.2 and step 2 in Subheading 3.2.4.
5. Pipette off the *Agrobacterium* cells. Transfer scutella without blotting, keeping the ex-axis side down, onto fresh inoculation medium in Petri dishes. Spread 12–13 calli per plate. Allow to co-cultivate in the dark at 23 ± 1°C for 3 days.
6. Within each experiment, prepare scutella (30/dish) for negative control plates to monitor regeneration and selection efficiencies.

3.2.5. Induction of Embryogenic Calli, Regeneration and Selection of Transgenic Plant

Use antibiotic timentin (150 mg/L) to inhibit the growth of *A. tumefaciens* cells during the embryogenic callus phase, and in all the subsequent media including regeneration and two rounds of selection, each for 3 weeks.

1. Callus induction: after 3 days of co-cultivation in the dark, transfer all scutella (12–13 calli per plate) to semi-solid induction medium (see Subheading 2.2.4) and continue to incubate in the dark at 23 ± 1°C for 18 days.

2. Stage 1. Regeneration, without selection: after 18 days, transfer embryogenic calli to regeneration medium (R/inDTZ, see Subheading 2.2.4). Discard any hydrated and necrotic ones. Incubate 7–8 calli only per plate at $23 \pm 1^\circ\text{C}$, but in the light (see step 1 in Subheading 3.1.7). Keep embryogenic calli derived from the same immature embryo intact without breaking up.
3. Stage 2–3. Regeneration with selection: after 3 weeks, transfer embryogenic calli and plantlets from Stage 1 to selection medium, called R/inT-PPT (see Note 11). Put only 5–6 calli with shoots onto one plate. Discard any unresponsive one (see Note 32). Culture 7–8 calli per Petri dish of negative controls on media with and without (R/inT, see Subheading 2.2.4) selection agent too. Apply selection from now on (see Note 33), until all control plantlets have been killed (see Note 11).
4. Continue transferring calli and plantlets to fresh R/inT-PPT media in Petri dishes for shoot regeneration, and in Magenta vessels for root regeneration and strengthening, respectively. Subculture every 3 weeks until PPT tolerant plantlets are ready to be potted to Jiffy pots, then to soil (see Note 34). (See also steps 5 and 6 in Subheading 3.1.7 at the biolistic transformation method).

3.3. Analysis of the Regenerated Transgenic Wheat Plants

Transgenic plants can be analyzed in a number of ways, but below we show only how the reporter/marker gene expression can be assessed. The histochemical GUS test (see Subheadings 3.3.2 and 3.3.3) was used to monitor the *uidA* gene expression. For *bar* gene expression the herbicide leaf paint assay, the ammonium test and/or the PAT assay (spray test) were used (see Subheading 3.3.4).

The presence of recombinant DNA can be determined using PCR method (see Subheading 3.3.1); however, it should be noted that PCR does not confirm stable transgene integration into the plant genome. Additional analyses can be performed to identify the site of genomic integration, estimate copy number, confirm inheritance/segregation using, for example, southern analysis, fluorescent in situ hybridization (FISH) and/or qPCR, but these methods are not described here.

3.3.1. Screening the Primary Regenerated Plantlets (T_0 Transgenic Plants) by PCR

When plants are large enough (3 and 4 leaf stage) collect 25–40 mg leaf material, and extract genomic DNA using any commercially available extraction kit (see Note 14). Both the *bar* and the *uidA* genes can be detected by PCR using the primers and conditions detailed below.

Detecting the *bar* Gene

Forward primer: 5' GTCTGCACCATCGTCAACC 3'

Reverse primer: 5' GAAGTCCAGCTGCCAGAAAC 3'

Product size: 444 bp.

Carry out the PCR analysis in 10 μ L reaction mixture containing one volume Taq PCR buffer, 0.1 mM dNTPs, 0.5 μ M of each primer, 1.25 U Go Taq polymerase (Promega), and ~80 ng genomic DNA. After initial denaturation (95°C for 5 min), apply the following cycling conditions: 36 cycles at 95°C for 30 s (denaturation), 60°C for 60 s (annealing), 72°C for 30 s (elongation), followed by a final extension at 72°C for 5 min. Analyze PCR products (444 bp) by electrophoresis in 1.0% agarose gel.

Detecting the *uidA* Gene

Forward primer: 5' TACGTATCACCGTTTGTGTGAAC 3'

Reverse primer: 5' CCGCTTTGGACATACCATCCGTA 3'

Product size: 1,042 bp.

Carry out the PCR analysis as it is described for detecting *bar* gene; however, use 1 min as elongation time.

3.3.2. Histochemical GUS Assay for Transient Expression

The *uidA* gene encodes the β -glucuronidase (GUS) enzyme whose activity can be measured using a histochemical (GUS) assay (see Note 35).

1. Two to three days after bombardment (time depending on the construct i.e. strength of the promoter) remove two scutella from four dishes each of control and treated with DNA. Place scutella separately into the wells of a microtitre plates with X-Glca (or X-Gluc) solution (50–80 μ L in each well).
2. Seal the plate with Parafilm and incubate overnight in a thermostat at 37°C.
3. Place the samples (scutella) under a stereomicroscope to visualize and count the blue, GUS-expressing spots.

3.3.3. Stable Expression of *uidA* Gene (GUS Activity Assay)

Leaf Segment Test

At the end of the first round of selection in Petri dishes, some of the T₀ transgenic plants may be identified by GUS assay.

1. Place ½ cm long leaf fragments into the wells of a microtitre plate with 100 μ L X-Glca (or X-Gluc) solution in each well (see Note 36).
2. Seal the wells and incubate the samples overnight in a thermostat at 37°C. If necessary, incubate at 25°C for a further 24 h for the colour to fully develop.
3. After incubation, remove the chlorophyll in leaf fragments by replacing X-Glca (or X-Gluc) with 70% ethanol under light until the green colour faded and blue diXH-indigo product is clearly visible.
4. The plantlets tested positive and with good strong roots may be transferred to Jiffy pots, then to soil. Winter wheat varieties should be put into the vernalization room first. Plantlets with shoots only or weak root system should be transferred to R medium without PPT selection agent in Magenta vessels for root strengthening.

Testing GUS Activity in Dry Seed Segments

The stable expression of *uidA* gene by the method described above can be performed on a thin cross section of dry seeds, as well.

1. Cut 1–1.5 mm cross sections of the dry seeds opposite to the embryo side (see Note 37) and put them into the wells of a marked microplate (one in each well).
2. Incubate the tissues fully immersed in X-Glca (or X-Gluc) solution (150–200 μ L) in conditions as for leaf segment test.
3. Germinate the embryo-containing part of the seed if the correlating segment tested positive (see Note 37).

3.3.4. *Stable Expression of the bar Gene: PAT Assay for the Selection of Herbicide Resistant*

Resistance to herbicides can be evaluated in established primary transgenic plants (T_0 generation) and their progeny (T_1 and subsequent generations).

Transgenic Lines

Leaf Painting Bioassay

This approach for qualitative expression of the *bar* gene is simple and inexpensive and can be carried out on plants in situ.

1. Prepare dilutions of glufosinate ammonium-based herbicide using 0.1% Tween-20 to give final concentrations of the PPT solution of 0.2 g/L and 2 g/L (see Note 6).
2. Select healthy wheat plants at the tillering phase of growth. For each plant to be tested, select 3 approximately equal sized, healthy leaves, except for the flag leaf. Water the plants before herbicide application.
3. Label the three chosen leaves on the stem right below the leaf to be treated: “Tween only” for controls, 0.2 g/L PPT, and 2 g/L PPT.
4. Mark each leaf with a ballpoint pen halfway along its length and paint the upper surface of the distal half of the leaf with the appropriate solution using a cotton bud (see Note 38). Paint all the control leaves first (Tween only), followed by the lower concentration (0.2 g/L), then the higher concentration (2 g/L) of herbicide to minimize carry-over of the herbicide to other leaves.
5. Assess the herbicide resistance of each plant 7 days after application by scoring each treated leaf according to the percentage desiccation/browning over the painted area and the percentage of the proximal region of the leaf that has been affected by the spread of the herbicide (see Note 39).

Qualitative Ammonium Test

1. Place 4 $\times$ 8 mm leaf pieces in 25-multiwell plates containing 1 mL incubation medium (see Subheading 2.3.3) per well, leaving 1 or 2 wells without tissue as negative controls (see Note 40).

2. Incubate at 24°C under a photosynthetic photon flux density of 250 $\mu\text{mol}/\text{m}^2 \text{ s}$ for 5 h (see Note 41).
3. Take out 200 μL of the incubation medium of each sample and add to 1 mL of Reagent 1 (see Note 42).
4. Add 1 mL of Reagent 2 to the mix and vortex it.
5. Incubate at 37°C for 15 min, followed by 15 min incubation at room temperature.
6. Evaluate the result according to the colour developed in the solutions (see Note 43).

Quantitative
Ammonium Test

1. Place 4×8 mm leaf pieces in 25-multiwell plates. Proceed as for the qualitative assay.
2. Prepare a standard curve with increasing concentrations of ammonium chloride by adding 200 μL of standard solutions of ammonium (from 0 to 10 mg/L) to the mixture of 1 mL Reagent 1 plus 1 mL Reagent 2 (see Note 42), then incubate as in step 5 for the qualitative assay.
3. Measure the absorbance of each solution at 655 nm.
4. Determine the concentration of ammonium ion in each sample using the standard curve.

PAT Assay (Spraying
Transgenic Plants at Their
Five- or Six-Leaf Stage)

Transgenic plants resistant to the herbicide can also be identified by a spraying test (see Note 44).

1. Spray 12 (or 16) plants for each line twice (2 days in between) at their five- or six-leaf stage with 1 g/L glufosinate ammonium-based solution (see Note 6).
2. Assess (i.e. count the number of survived plants) the herbicide resistance of the plants 2 weeks after the treatment. Let healthy strong plants to grow and to set seeds.

4. Notes

1. It is important to avoid any stress to the donor plants. Apply routine pesticide and fertilizing treatments in every 15 days/ fortnightly, but it should be avoided from pollination to harvest time.
2. Commercially available 10% (v/v) aqueous Domestos solution can be applied without surfactant as it contains detergent as well.
3. Weigh solutes in weighing boats and then transfer each component separately to a glass beaker containing 600 mL ultra-pure water. Allow solutes to dissolve completely one after the

other on magnetic stirrer. Note that CaCl_2 dissolves slowly. Pour solution into a graduated cylinder and then add ultrapure water to a volume of 1 L. If 100–400 mL of solutions are going to be prepared, scale-down the amounts of each component accordingly.

4. Filter sterilization is carried out using Steritop 0.22- μm filters, Millipore. For small volumes of solutions (5–50 mL), use disc syringe filter. For larger volumes (0.2–2 L) use for example MediaKap[®] filters which are operated by gravity or under pressure, using a peristaltic pump or pressure vessel.
5. Silver nitrate (used to promote embryogenesis) is photosensitive, so stock solution and all media plates containing it must be stored in the dark.
6. For selection media preparation it is recommended to use synthetic l-phosphinothricin (PPT) selection agent at the concentration of 2–4 mg/L, which provides successful selection. For analysis of stable expression of the *bar* gene (PAT assay, see Subheading 3.3.4), glufosinate ammonium-based solutions are recommended. Glufosinate ammonium is a synthetically produced PPT bound to ammonium and is the active component in herbicides such as Basta[™], Bialaphos, or FINALE. Be aware of that the various commercially available formulations contain different concentrations of the active ingredient.
7. When preparing double-strength tissue culture media add the ferrous sulphate chelate stock solution first (or ethylenediaminetetraacetate disodium salt, see item 1 in Subheading 2.1.3) to the distilled water to keep other components dissolved.
8. Filter-sterilize only 400 mL double-strength tissue culture medium into a sterile 1 L bottle to give space for the addition of melted double-strength Agargel (or Phytigel) when preparing the working medium later.
9. Allow to cool the melted gelling agent to 50°C in a water bath. Warm up the double-strength tissue culture medium in the water bath too, before mixing it with the double-strength melted gelling agent.
10. At this high concentration (9%) of sucrose in the medium, the walls of the cells shrink due to the loss of water through osmosis during pre-culture, which may allow them to better withstand bombardment. But 3% sucrose is often suitable e.g. for *T. turgidum* ssp. *durum* scutella (42).
11. The concentration of PPT selection agent (2–4 mg/L) applied in the medium depends on the wheat variety to be transformed. Trial experiments are needed to assess the level of selection agent that is studied to fully inhibit the growth of non-transformed explants. Above all, it is recommended to use the selection agent at the lowest concentration (2 mg/L) in

the first round of selection, then increase it to the maximum of 4 mg/L (usually 3–4 mg/L).

12. The transformation cassette, carried in the plasmid pAHC25, contains the selectable marker gene *bar* and the *uidA* reporter gene, both driven by the constitutive promoter ubiquitin1, also containing the ubiquitin1 intron from maize.
13. For *Agrobacterium*-mediated transformation the AGL1 *A. tumefaciens* strain was used (46). This super-virulent strain contains the plasmids pAL154/pAL156 based on the plasmid pSoup/pGreen (47). The single T-DNA in binary vector pAL156 incorporates the *bar* gene and a modified *uidA* gene with an intron within the open reading frame to prevent its expression in *Agrobacterium* itself; both genes are driven by the maize ubiquitin1 promoter also carrying the ubiquitin1 intron (45). The *bar* gene is located next to the left border, and *uidA* is adjacent to the right border. The plasmid pAL154 helps the replication functions for pAL156 in *trans* and contains the 15 kb Komari fragment (48, 49) supplying extra *vir* genes.
14. To purify DNA for PCR, kits such as AquaGenomic, Extract-N-Amp, QIAGEN, or Wizard® Genomic DNA Purification Kit may also be used. If the DNA is used for southern blot analysis as well, the CTAB method (55) is recommended (not provided in this Chapter).
15. Store sodium nitroprusside containing solutions in the dark, as it slowly breaks down to release cyanide ions, especially upon exposure to UV light.
16. Sodium dichloroisocyanurate is a stabilized chlorine donor containing cyanuric acid and used as a disinfectant. Store the solution for maximum 2 months to keep it effective.
17. Smaller and larger embryos than 0.7–1.2 mm may respond but with much lower efficiencies. Better to keep scutella from this size of embryos on separate Petri dishes.
18. After efficient sonication, gold particles should be re-suspended in the liquid rather than be present as a pellet in the base of the tube, but either shorter than 60 s or longer than 2 min sonication could promote agglomeration of gold particles.
19. For co-bombardment, use equimolar amounts of DNA not more than a total of 5 µg for the two cassettes, which may reduce clumping of gold particles.
20. Although 85 µl of gold suspension should be sufficient for 16–17 shots (5 µL/shot), it is generally enough for only 10–12 shots because of evaporation during the work.
21. Non-bombarded controls (NBC) monitor the development/regeneration of donor tissue. Bombarded controls (BC) with

gold only (no DNA) without selection are used to monitor the tissue culture response following the bombardment. Bombarded controls with gold only (no DNA) with selection monitor the effects of the selection on regeneration.

22. Let the ethanol evaporate for not longer than 10 min; otherwise, the coated gold particles would adhere to the macrocarrier too strongly to release them at shooting. Check macrocarriers microscopically prior to bombardment for the uniformity of particles (spread of particles) and discard any that may have clumps of gold.
23. Remember to set the helium pressure on the cylinder to approximately 200 psi (1.38 MPa) more than the intended rupture pressure, which is genotype dependent and should be studied empirically.
24. Check the macrocarriers under a stereomicroscope after the shot, to visualize the mesh pattern left by the stopping screen and check how much gold has been released/retained.
25. Do not bombard 2 Petri dishes (NBC) out of 10 or 15 dishes to monitor regeneration efficiency of target tissue.
26. Light source should be white cool fluorescent tubes ($20 \mu\text{mol}/\text{m}^2 \text{ s}$ PAR for a 16-h photoperiod). Covering the plates with 10–15 layers of PE film (thickness of $25 \mu\text{m}$) during the first week may reduce the light shock to the tissues and may increase the number of leafy structures.
27. If the regeneration response is poor, i.e. if no shoots appear, repeat one round of regeneration (up to 3 weeks) on fresh medium without selection agent (RZD + 1/2Cu).
28. It takes usually at least 3 months from bombardment to get plantlets ($>10 \text{ cm}$ in length) with an established root system.
29. Small plantlets have little or no waxy cuticle and are particularly prone to desiccation.
30. The antibiotics used depend on the selectable markers in the *Agrobacterium* strain and binary vectors.
31. Add $16 \mu\text{L}$ of the stock solution (50 mM) (see item 6 in Subheading 2.2.3). Use $400 \mu\text{M}$ acetosyringone for tetraploid durum wheat (*T. turgidum* L. var. *durum*; cv. Ofanto) (44).
32. At this point, the regenerating callus pieces may be divided into defined shoots/roots (or accidentally break into pieces) when transferred, but it is important to keep these together, or mark them clearly, as there is a possibility that these may be clones.
33. Some of the transgenic plants may be identified by GUS assay on leaf fragments at the end of the first round of selection (see Subheading 3.3.3).

34. If they have good strong roots, they may be transferred to soil or put into the vernalization room immediately. Otherwise, transfer them to R/inT medium without PPT for root strengthening.
35. The GUS enzyme (expressing in transgenic plant tissues) hydrolyzes the X-Gluc (or X-Glca) substrate to give an insoluble and highly coloured indigo dye. This blue precipitate can be visualized at the site of enzyme activity or easily detectable by quantitative (fluorometric or spectrophotometric) analysis.
36. With this assay, the stable expression of the *uidA* gene can be monitored not only in embryogenic calli and leaf fragments but in roots, anthers, pollen, and ovaries as well.
37. Keep the embryo parts of the tested seeds separately in marked microplates. If the segments were tested positive, germinate the corresponding embryo-containing parts of the seeds. Transfer plantlets into soil then grow in environmentally controlled growing chamber to set seeds.
38. The application should be firm to ensure coating and some penetration of the solution into the leaf tissue.
39. The application of the herbicides based on glufosinate-ammonium causes desiccation and browning in control plants, whereas plants carrying the *bar* gene and synthesizing the appropriate enzyme are resistant and remain green.
40. Choose young, green leaves for this assay. Set incubations up in a laminar flow hood to avoid contamination. Ensure that leaf pieces are fully immersed in the incubation medium; this can be achieved by briefly shaking the plates by hand.
41. Tissue culture room is adequate for this purpose.
42. The assay is performed in either microplate or cuvette, but having large number of samples to test, microplate is recommended.
43. The incubation medium with plant samples not expressing the *bar* gene and the negative controls will develop an emerald green to dark-blue colour due to the presence of ammonium ions. The medium of explants from transgenic plants expressing the enzyme coded by the *bar* gene will develop a light green or yellow colour that occurs in the absence of ammonium ions.
44. When the transformation is done by co-bombardment of the selectable marker and gene of interest on separate plasmids, selection pressure will kill plants that have taken up only the gene of interest, as they are not resistant to herbicide. In the experience of the authors, only about 10–20% of independent transgenic plants possess only one of the plasmids in a co-bombardment experiment.

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Improved Genetic Transformation of Cork Oak (*Quercus suber* L.)

Rubén Álvarez-Fernández and Ricardo-Javier Ordás

Abstract

An *Agrobacterium*-mediated transformation system for selected mature *Quercus suber* L. trees has been established. Leaf-derived somatic embryos in an early stage of development are inoculated with an AGL1 strain harboring a kanamycin-selectable plasmid carrying the gene of interest. The transformed embryos are induced to germinate and the plantlets transferred to soil.

This protocol, from adult cork oak to transformed plantlet, can be completed in about one and a half years. Transformation efficiencies (i.e., percentage of inoculated explants that yield independent transgenic embryogenic lines) vary depending on the cork oak genotype, reaching up to 43%.

Key words: AGL1, *Agrobacterium tumefaciens*, Cork oak, Fagaceae, Herbicide resistance, Kanamycin resistance, *Quercus suber*, Somatic embryogenesis, Tree genetic transformation

1. Introduction

Biotechnology has become a powerful tool for the introduction of foreign genes into long-lived perennials and for fundamental studies of gene expression (1–3). It can help to minimize difficulties associated with traditional breeding and reduce the time necessary to introduce traits, a very time-consuming process particularly in woody species. Moreover, this technology allows for the introduction of these traits without compromising the genetic background of the elite clone in the way classical breeding does, i.e., in one generation and avoiding the likely need for backcrosses.

Biotechnological approaches have been successfully applied in the family Fagaceae, which includes financially important forest species that play a role in soil and environment conservation, CO₂ assimilation, wood production and as energy resources. Genetic transformation protocols have been developed for the species this

chapter is devoted to, *Quercus suber* L. (4–6), and also for a few other Fagaceae: *Castanea dentata* (7–11), *Castanea sativa* (12–16), *Nothofagus alpina* (17), and *Quercus robur* (18–20).

Cork oaks (*Q. suber* L.) grow in the Mediterranean area (see a review on cork oak woodlands in (21)). These trees are among the most important ones in the dehesa (22), a managed ecosystem that also produces acorns—used to feed pigs—honey and, very importantly, cork.

Many efforts have been done in cork oak breeding, but classical approaches in this species are constrained by its reproductive characteristics: a long juvenile period of up to 30 years, self-incompatibility and a high degree of heterozygosis, among others. This explains that, like other trees, it has undergone little domestication. However, in the past few years biotechnology has made available a very useful tool: somatic embryogenesis.

A prerequisite for the production of transgenic plants is the availability of a method to regenerate complete individuals from the transformed cells. Somatic embryo cultures (23, 24) meet this requirement and offer an excellent starting point for genetic engineering. Cork oak somatic embryo cultures have low manipulation requirements, high proliferation rates, and plantlets can be obtained from embryogenic lines either initiated from zygotic embryos (25, 26) or induced in leaves from seedlings (27, 28).

Desired traits are commonly expressed at maturity, so an effective selection can only be performed on mature trees. Perfectly fitted for this purpose, repetitive embryogenic lines can also be induced from mature individuals of this species (29, 30), opening the way to manipulation and cloning of desired genotypes. In fact, plants have been regenerated from several selected trees (31) and they do not seem to show significant differences with plants obtained from zygotic embryos (32). Moreover, genetic stability is a very valuable factor, and it has been shown that true-to-type plants can be obtained by somatic embryogenesis (33–35), and from cryopreserved embryogenic lines (36).

Other recent advances in cork oak biotechnology include the use of anther culture to obtain haploid embryos (37) and the production of synthetic seeds (38).

This chapter on cork oak genetic transformation follows a previous one (39), revised and updated accordingly to the findings in (6, 40, 41). Here, it is shown that transformation efficiencies (i.e., percentage of inoculated explants that yield independent transgenic embryogenic lines) of up to 43% can be obtained. Although still highly dependent on the genotype, which is a critical variable, correct choices of explant, cocultivation period, and inoculum density enable a significant improvement over the previous protocol.

This chapter is focused on the genetic transformation step. However, the complete protocol is provided for convenience.

From the collection of branches from a mature cork oak between December and May, to the transformed plantlet growing in the nursery, the protocol can be completed in about one and a half years. It involves the following:

- Collection of branches and induction of somatic embryogenesis: at least 4 months.
- Proliferation until enough materials are obtained to start the transformation: at least 3 months.
- Genetic transformation and selection of kanamycin-resistant lines: at least 4 months.
- Proliferation until enough materials are obtained for the germination stage: at least 3 months.
- Germination protocol: 3 months.
- Plantlet establishment: 1 month.

2. Materials

2.1. Plant Materials

If you wish to obtain embryogenic lines from trees: branches of 1–4 cm in diameter from the crown of the selected *Q. suber* L. tree, cut preferably between December and May.

If you are starting the transformation protocol straightaway: *Q. suber* L. somatic embryos and clusters from a cork oak embryogenic line (see Note 1).

2.2. Bacterial Strain and DNA Construct

AGL1 *Agrobacterium tumefaciens* strain (42) harboring a suitable plasmid with kanamycin resistance (see Notes 2 and 3).

2.3. Culture Media

If you wish to obtain embryogenic lines from trees:

1. Basic medium: microelements, vitamins, and Fe-EDTA from the MS medium (43).
2. Preconditioning medium (GMS): basic medium plus $\frac{1}{2}$ PRL-4-C macroelements [PRL-4-C (1 $\times$) contains 90 mg/L $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 30 mg/L Na_2HPO_4 , 300 mg/L KCl, 200 mg/L $(\text{NH}_4)_2\text{SO}_4$, 250 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1000 mg/L KNO_3 , and 150 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$] (44), and 1% (w/v) sucrose.
3. Primary induction medium (MSSH1): basic medium plus SH macroelements [SH (1 $\times$) contains 2500 mg/L KNO_3 , 200 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 400 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 300 mg/L $\text{NH}_4\text{H}_2\text{PO}_4$] (45), 3% (w/v) sucrose, 10 μM benzyladenine (BA), and 50 μM 1-naphthaleneacetic acid (NAA).
4. Secondary induction medium (MSSH2): basic medium plus SH macroelements, 3% (w/v) sucrose, 0.5 μM BA, and 0.5 μM NAA.

5. Expression/proliferation medium (MSSH): basic medium plus SH macroelements and 3% (w/v) sucrose.

The above-mentioned media (GMS, MSSH1, MSSH2, and MSSH) were pH-adjusted at 5.7 and 0.6% (w/v) agar added prior autoclaving at 120°C for 20 min.

If you are starting the transformation protocol straightaway:

6. Expression/proliferation medium (MSSH): basic medium plus SH macroelements and 3% (w/v) sucrose, pH 5.7 and 0.6% (w/v) agar.
7. YEP medium [YEP (1×) contains 10 g/L bacteriological peptone, 5 g/L NaCl, and 10 g/L yeast extract. Adjust pH to 6.9] (46). Alternatively, Lysogeny Broth [LB; (1×) contains 10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract] can be used. Add 1.5% (w/v) agar for solid medium (store indefinitely at room temperature and always check the medium for contamination before use) (see Note 4).
8. Liquid MSSH medium.
9. Embryo maturation medium (see Subheading 3.6, Protocol 2, SOM medium): basic medium plus Sommer's Medium 1 macroelements [Sommer's M1 (1×) contains 200 mg/L $(\text{NH}_4)_2\text{SO}_4$, 150 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 250 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1000 mg/L KNO_3 , 300 mg/L KCl, 0.75 mg/L KI, 90 mg/L $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 30 mg/L NaHPO_4] (47), 3% (w/v) sucrose, 1% activated charcoal, and 0.8% (w/v) agar, pH 5.6.
10. Embryo germination medium (Protocol 2, SOMG): SOM with sucrose concentration reduced to 1.5% (w/v), no activated charcoal, agar concentration increased to 1% (w/v) and supplemented with 0.22 μM BA and 0.50 μM indole-3-butyric acid (IBA).

The above-mentioned media were pH-adjusted and then autoclaved at 120°C for 20 min.

2.4. Stock Solutions

If you wish to obtain embryogenic lines from trees:

- For the asepsia: fungicides (e.g., Propamocarb and Carbendazyme), 70% ethanol, commercial bleach, Tween 20, and sterile water.
- Plant growth regulators: BA, NAA, and IBA (see Note 5).

2.4.1. Antibiotics (see Note 6)

1. Kanamycin (100 mg/mL stock in water, sterile filtrated. Store at -20°C).
2. Rifampicin (10 mg/mL stock in DMSO or methanol. Light sensitive. Store at -20°C).
3. Cefotaxime (250 mg/mL stock in water, sterile filtrated. This antibiotic is light and temperature sensitive. Store at -20°C).

2.4.2. Other Stocks

1. 10 mM MgSO₄ (sterilize by autoclaving at 120°C for 20 min and store indefinitely at room temperature).
2. Activated charcoal, acid washed.

If a β-glucuronidase assay is to be performed, prepare a buffer containing:

- 100 mM phosphate buffer 7.0 (see Note 7)
0.5 mM K₃Fe(CN)₆
0.5 mM K₄Fe(CN)₆ (see Note 8)
10 mM Na₂EDTA
0.1% Triton X-100
1 mM 5-bromo-4-chloro-3-indolylglucuronide (X-Gluc) in dimethylformamide. Store at −20°C for up to a few weeks.
Adjust to the final volume with water (see Note 9).

2.5. Other Supplies

1. 250-mL baby food jars or other appropriate containers for in vitro culture.
2. Sterile consumables: 60- and 90-mm Petri dishes, 50-mL centrifuge tubes, and filter paper (see Note 10).
3. Parafilm®.
4. Sterile water.
5. Forest containers (180 mL, Arnabat SA, Spain) (see Note 11).
6. Substrate: pine bark–peat–sand 3:1:1 (v/v/v).
7. REDExtract-N-Amp™ Plant PCR Kit (Sigma®; see Note 12).
8. DNeasy® Plant Mini Kit for DNA isolation from plant tissue (Quiagen®; see Note 13).
9. Primers:

Gene	GenBank	Forward primer (5'–3')	Reverse primer (5'–3')	Amplified fragment (bp)
<i>nptII</i>	V00618.1	GAGGCTA TTCGGC TATGACTG	ATCGGGAGC GGCGATAC CGTA	700
<i>nidA- PIV2</i>	–	GGTGGGA AAGCGC GTTACAAG	GTTTACGC GTTGCTTC CGCCA	1,199
<i>virG</i>	X62885.1	AAGGTGA GCCGTT GAAACAC	ATCTCAA GCCCA TCTTCACG	199

10. Laboratory equipment, including the following: water purification system, autoclave, tabletop centrifuge (capable of spinning 50 mL tubes at 3,000×g), flow hood, culture chamber

(illumination of 120–180 $\mu\text{mol}/\text{m}^2 \text{ s}$), sterile equipment (scalpels and forceps), 30°C oven, orbital shaker (capable of keeping a temperature below 30°C), PCR thermocycler, and agarose gel electrophoresis equipment.

3. Methods

3.1. Induction of Embryogenesis in Leaves from Adult *Q. suber*

For a more complete description of this protocol, or if you wished to induce embryogenesis in zygotic embryos or leaves from seedlings, please see the original references (30, 48).

1. Cut 1–4 cm diameter branches from the crown of the selected tree, preferably between December and May. Remove lateral branches and leaves, washing and brushing thoroughly, and culture segments up to 15 cm long in a perlite substrate in appropriate available containers (e.g., 5,000 cm^3) at $25 \pm 5^\circ\text{C}$ and 80–95% relative humidity in a greenhouse or climatic chamber. Spray them weekly with fungicides (e.g., 1.8 g/L Propamocarb and 60% Carbendazyme) to prevent fungal infections.
2. Collect the expanding leaves (0.5–1.5 cm from the base to the apex) with small petioles from the growing epicormic shoots (see Fig. 1a) and surface-sterilize them by vigorously hand-shaking in 70% (v/v) ethanol for 30 s. Transfer them to 10% commercial bleach (3.5% active chlorine) plus two drops of Tween 20 for 10 min, preferably on a magnetic stirrer (see Note 14). Finally, rinse them three times with sterile distilled water.
3. Place two surface-sterilized leaves with the abaxial surface on GMS in each 60-mm Petri dish, seal it with Parafilm, and culture at 25°C in the dark for 7 days.
4. Transfer the leaves to the MSSH1 and incubate in the dark at 25°C for 30 days.
5. Transfer the leaves to the MSSH2 and incubate in a 16-h photoperiod (mixed Sylvania Gro-Lux and Philips cool white fluorescent tubes, 120–180 $\mu\text{mol}/\text{m}^2 \cdot \text{s}$) at 25°C for 30 days.
6. Transfer the leaves onto MSSH and subculture every 30 days in the conditions of the previous step until embryo clusters appear (see Fig. 1b).
7. Isolate embryo clusters and keep on subculturing them without changing the conditions every 20–40 days. The shorter the subculture period, the faster the growth and the lower the number of embryos that will reach maturity.

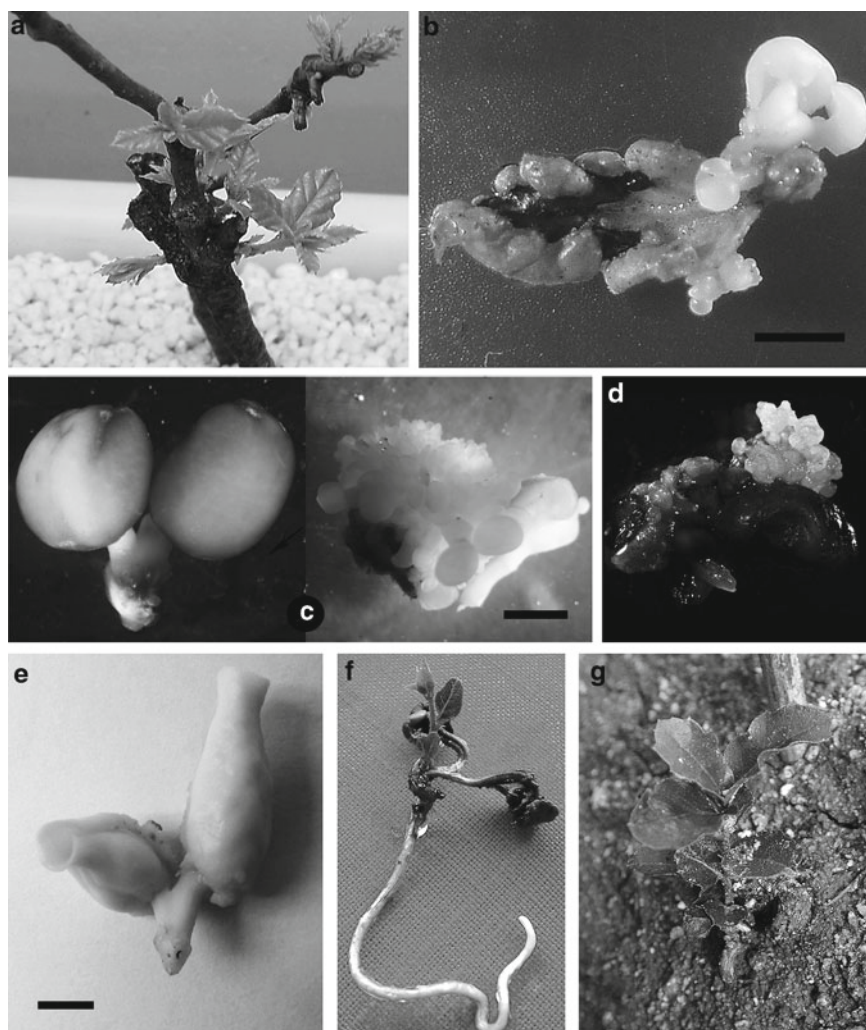


Fig. 1. Stages of the transformation procedure. (a) Epicormic shoots with leaves, sprouted in a fragment of branch in perlite substrate. (b) Somatic embryogenesis induction on a treated expanding leaf. The somatic embryos spontaneously undergo secondary embryogenesis. Bar, 4 mm. (c) Cotyledonary embryo (*left*) and cluster of embryos in early stages of development (*right*). Bar, 1 mm. (d) White putatively transformed embryo mass, proliferating on a necrotic cluster in presence of kanamycin. (e) Appearance of an embryo selected to be cold-treated. Bar, 4 mm. (f) Germinated somatic embryo. (g) Cork oak plantlet growing in field conditions. Partially reproduced from (55) with kind permission from Springer.

3.2. Bacterial Strain Culture and Preparation

1. Streak the -80°C preserved *Agrobacterium* strain on agar-YEP with 100 mg/L kanamycin and 20 mg/L rifampicin in the dark at 28°C for 2–3 days (these cultures can be stored for up to a month at 4°C , see Notes 6 and 15).
2. Pick an isolated colony and grow it overnight in liquid YEP with 50 mg/L kanamycin in an orbital shaker at 28°C and 250 rpm (see Note 16).
3. Measure the OD_{600} of the culture and take the necessary amount to prepare 20 mL of a final OD_{600} of 1 (see Note 17).

4. Pellet the bacteria ($3,000\times g$ for 10 min) and resuspend the pellet in 10 mL of 10 mM MgSO_4 .
5. Pellet again and resuspend to a final OD_{600} of 1 in liquid MSSH medium.

3.3. Preculture, Inoculation, and Coculture of the Embryo Clusters

1. Collect embryo clusters (see Fig. 1c) 20 days after the last subculture (see Note 18). Do it in liquid MSSH to prevent desiccation.
2. Halve the clusters with a scalpel and inoculate them with the bacteria–MSSH solution for 20 min with gently shaking (e.g., up to 100 rpm in an orbital shaker) (see Notes 17 and 19).
3. Blot the embryos on sterile filter paper to eliminate excess of bacteria and transfer them (20–30 embryos per Petri dish) to solid MSSH medium without antibiotics.
4. Coculture for 2 days at 25°C in the dark (see Note 20).
5. Wash the embryos in liquid MSSH medium with 500 mg/L cefotaxime to eliminate excess of bacteria, blot on sterile filter paper and transfer them (10–15 embryos per baby food jar) to MSSH medium with 100 mg/L kanamycin and 500 mg/L cefotaxime (see Note 21).

3.4. Selection of Transformed Embryogenic Lines

1. Subculture the inoculated and washed embryos in MSSH medium with 100 mg/L kanamycin and 500 mg/L cefotaxime (selective medium) every 7–10 days for the first month, and at 15–20 day intervals thereafter. Keep the cultures at $25 \pm 1^\circ\text{C}$ under a 16-h photoperiod (see Note 22).
2. Isolate white proliferating embryogenic masses emerging from the initial explants (see Fig. 1d) and keep on subculturing them on selective medium for about three more months (see Note 23).
3. As soon as you have enough material, check the selected explants for the presence of *nptII*, your gene of interest and the *Agrobacterium* helper plasmid gene (*virG*) by PCR or southern blot. Then check for the expression of your gene of interest by reverse-transcription PCR, northern blot or a histochemical assay (see *next section* and Note 24).
4. Maintain the kanamycin-resistant embryogenic masses by subculturing them on proliferation (MSSH) medium without antibiotics in standard conditions ($25 \pm 1^\circ\text{C}$ and 16-h photoperiod) every 20–40 days.

3.5. Molecular and Histochemical Analyses

For PCR, the DNA can be extracted using either the REDExtract-N-Amp™ Plant PCR Kit from Sigma® or a standard procedure. For southern blot, the DNA can be extracted and purified using the DNeasy® Plant Mini Kit for DNA isolation from plant tissue (Quiagen®) or a standard procedure (see Note 13).

3.5.1. PCR Amplification

1. Both *uidA* and *nptII* genes can be amplified in the same PCR tube in the following conditions: 4 min 95°C, 35x(20 s 95°C, 30 s 58°C, 60 s 72°C), 5 min 72°C.
2. PCR conditions for *virG*: 4 min 95°C, 35x(20 s 95°C, 30 s 56°C, 15 s 72°C), 5 min 72°C.

3.5.2. Southern Blot Analysis

Southern blot analyses (4, 49) should be performed to confirm the stable integration of the transgenes.

3.5.3. Histochemical (β -glucuronidase) Analysis of Transformants

If a *uidA*-harboring plasmid such as the pBINUbiGUSint is used, a β -glucuronidase assay can be performed to assess protein functionality. To do so:

1. Take embryos proliferating in presence of kanamycin, not exceeding 2–3 mm in size.
2. Incubate them in the dark at 37°C for at least 2 h (see Note 25) in the solution of GUS buffer plus X-Gluc (50).

After transgene integration has been confirmed, expression should be assessed by RT-PCR or northern blot. For standard procedures see (49).

3.6. Embryo Germination**3.6.1. Protocol 1 (Extracted from (31, 48))**

1. Select embryos that undergo spontaneous maturation (white opaque, 15–20 mm in length, average fresh weight of 225 mg and without signs of secondary embryogenesis, see Fig. 1e) and transfer them to fresh MSSH medium.
2. Cold store the cultures in the dark at 4°C for 2 months.
3. Transfer the cultures to 16-h photoperiod (100 $\mu\text{mol}/\text{m}^2\cdot\text{s}$) at 25°C. About 15 days after, they will start germinating (see Fig. 1f).

3.6.2. Protocol 2 (Extracted from (41))

1. Select immature translucent embryos at the cotyledonary stage (about 3–4 mm long and 30 mg fresh weight).
2. Culture the embryos in 90-mm Petri dishes containing 25 mL SOM medium for 1 month at 25°C in the dark.
3. When they are about 1.5–2 cm long and 700–1,000 mg fresh weight, transfer them to fresh SOM and store the cultures for 2 months at 4°C in the dark.
4. After the cold stratification, imbibe the embryos in 10-mL tubes with sterile distilled water for 24 h in the dark.
5. Germinate the embryos in SOMG medium at 25°C in a 16-h photoperiod (100 $\mu\text{mol}/\text{m}^2\cdot\text{s}$).

3.7. Plant Conversion

1. Once germinated, transfer the embryos to 180-mL forest containers filled with pine bark–peat–sand 3:1:1 (v/v/v) substrate (see Note 26). Cover them with inverted glass beakers and

keep them in the growth chamber (alternatively you can keep them in a chamber with a fog system, gradually reducing the humidity for a month before transferring the plants to the nursery).

2. After 2 months, remove the beakers for 1 h/day for 1 month.
3. Move the plants to the nursery, under shade (see Fig. 1g).

4. Notes

1. Our research suggests that the parental tree genotype strongly influences transformation efficiency (6). The embryogenic lines with lower proliferation efficiency were less transformation-responsive. We suggest that a treatment to increase the proliferation rate would likely increase the transformation competence of these lines as well.
2. Our research suggests that the *A. tumefaciens* strain strongly influences transformation efficiency (4).
3. Have your gene of interest sequenced from the final construct. A size confirmation by PCR is not safe enough, since the cloned gene may have mutations that could produce aberrant proteins. Therefore, make sure you start this lengthy transformation protocol with the right construct.

We have successfully used the pBINUbiGUSint binary vector (51) to transform explants with the *uidA* gene (6), and the pBINUbiBar to introduce herbicide resistance with the *bar* gene in cork oak (40). Both plasmids are derivatives of pBIN19 (52) and carry the neomycin phosphotransferase II gene (*nptII*), which confers resistance to kanamycin. Hygromycin resistance has also been successfully used to obtain cork oak transgenic embryogenic lines (5).

4. The smaller the agar-media stocks, the lower the time required to melt and thaw them. Therefore, 50–200 mL stocks are recommended. Melt the medium in a microwave oven, cool the medium down till about 50°C (at this temperature it can be held with bare hands) and then add the appropriate sterile-filtered antibiotics.
5. Prepare the plant growth regulators as 1 mg/mL stocks. Dissolve the powder in NaOH 1 M (no more than 5% of the final volume), dilute them with milliQ water up to the final volume, sterile-filter, and transfer them to either Pyrex glass or plastic. If you store them in regular glass, you will probably observe silicate precipitates after a few days or weeks due to the presence of NaOH. Store in the fridge for a few weeks or at

–20°C for a few months (indole-3-butyric acid liquid stocks should be always stored below 0°C). In the original articles, plant growth regulators are added before autoclaving.

6. You can store the antibiotics as 1-mL aliquots. The AGL1 *Agrobacterium* strain has a chromosomal resistance to rifampicin and carbenicillin, and the pBINUbiGUSint plasmid confers resistance to kanamycin. Carbenicillin resistance confers cross-resistance to ticarcillin (both of them are beta-lactam antibiotics), so the latter will not effectively eliminate AGL1 from the cultures. Cefotaxime is a cephalosporin antibiotic with bacteriostatic properties which can be used to eliminate AGL1.
7. The phosphate buffer pH 7 can be prepared by adding 61 mL of 200 mM Na_2HPO_4 (weak base) to 39 mL of 200 mM NaH_2PO_4 (weak acid). If the pH is not right, it can be adjusted with either the weak base or the weak acid. Store these stocks at room temperature and sterile-filter to avoid precipitation and contamination.
8. These cyanides are catalysts. If you do not add them to the buffer you may obtain false positives due to the activity of cell peroxidases. For convenience, prepare them separately as 50 mM stocks in water and store refrigerated in the dark.
9. X-Gluc can be prepared separately as a 20 mM stock, and both X-Gluc and buffer stored at –20°C for up to a few weeks. Just mix them before use to make up the right concentrations. Large GUS assays can be conveniently performed and screened, e.g., in 96-well plates.
10. Sterilize filter paper by autoclaving instead of using an oven (100–150°C). After some time in the oven the paper starts scorching, turns brittle, and loses its ability to absorb water. In addition, phenols are produced when scorching and can interfere with the cultures.
11. Square-section forest containers are better than their circle-section counterparts. Roots grow straight to the bottom in the square containers, but in spiral manner along the container wall in the circular ones. The straight-growing roots provide higher conversion rates (% survival of greenhouse plants when transferred to field), since a coiled root system usually cannot support the weight of the aerial part and the plant is more prone to fall down.
12. The Sigma® REDExtract-N-Amp™ Plant PCR is a convenient and low-cost kit. It provides DNA extraction buffers and PCRMix (*Taq* polymerase plus buffer, dNTPs, and MgCl_2), and no DNA purification is needed. If a classical PCR reaction is preferred, extract or purify DNA with an appropriate method (e.g., (53)) and set up a PCR reaction containing DNA

(100 ng), 250 μ M of each dNTP, 10 pmol of each primer, *Taq* DNA polymerase, and 2 mM MgCl_2 .

13. For southern analysis, purification of the DNA is required for the digestion with restriction enzymes to work well. Ten micrograms of DNA can be easily obtained with the DNeasy® Plant Mini Kit for DNA isolation from plant tissue (Quiagen®) starting from 100 mg of embryogenic tissue (fresh weight). If a column-based kit is not available a CTAB-based DNA purification protocol is recommended, as a phenol-based one would probably work poorly due to the high carbohydrate content of the embryos. Pick up clusters or embryos in early stages of development, since cotyledonary embryos consist of highly vacuolated cells that can result in a poor DNA extraction rate.
14. The shake-in-ethanol step kills some microorganisms and removes mainly waxes and dust, facilitating the bleaching that follows. Stirring or shaking is strongly recommended as it improves the efficiency of the asepsia by removing bubbles and brushing the leaf surface. Do not increase stirring speed until a vortex is formed because air bubbles hinder the asepsia; just keep the leaves in a reasonable motion.
15. *Agrobacteria* strains can be cryopreserved and maintained at -80°C for about 10 years. To do so, grow bacteria to the exponential phase ($\text{OD}_{600} \sim 0.4$), spin, redissolve the pellet in 0.5 mL of sterile YEP with 15% glycerol in suitable tubes, and flash-freeze in liquid nitrogen. If you do not flash-freeze them their viability will be reduced, but they will still be fine for a few years at -80°C . For short-term preservation up to a few months, pick a colony and stab it in a microcentrifuge tube with 1 mL of sterile agar-YEP. Keep this so-called “stab culture” at room temperature or in the fridge.
16. Cultures require 16–24 h to grow in 20–30 mL of YEP medium. If the bacteria have been for some time in the fridge, it is recommended to start a preinoculum in 1 mL of medium without antibiotics, which after 4–8 h is diluted with medium with 50 mg/L kanamycin.
17. 20 mL of bacterial suspension are enough to inoculate 100–150 embryo clusters in a 50-mL sterile tube. 10 mL ($2 \times \text{OD}_{600}$) of bacterial suspension can be used and mixed with the 10 mL of liquid MSSH employed to gather the embryos while they are collected. By doing this, phenols released by the embryos could induce bacterial *vir* genes. Inoculum density is an important factor (6), but the density could be reduced to $\text{OD}_{600} = 0.5$ if bacterial regrowth after coculturing is too persistent.
18. The 20-day preculture period is a key factor (6). A preculture longer than 27 days is likely to result in poor transformation efficiencies.

19. Collection can be performed while preparing bacteria, saving time. Shaking rates above 100 rpm can result in damage for the embryos.
20. Although a cocultivation at 22°C yielded the highest transformation efficiency, the temperature in the range of 22–26°C did not significantly affect this efficiency. Moreover, a cocultivation period longer than 2 days significantly reduced the efficiency (6). Interestingly, *vir* gene induction is maximal at 25–27°C, while the pilus of some *Agrobacterium* strains is most stable at lower temperatures (18–20°C) and unstable over 28°C (references in (54)). Coculturing in the light might affect the transformation efficiency (Alvarez et al. unpublished results).
21. This step is *strongly* recommended. We have observed that the embryos looked healthier during the subsequent subculture periods when they were washed to eliminate excessive *Agrobacterium*. More importantly, this step reduces the number of explants that suffer bacterial regrowth. Given that embryogenic clusters are irregularly shaped, vacuum infiltration of antibiotics could be performed to improve elimination of bacteria.
22. Cefotaxime is photo- and thermolabile; therefore, frequent subculture is recommended to avoid regrowth of bacteria. As commented in (30), embryos grow irrespective of the light conditions, so culturing them in dim light or darkness is also a good option to mitigate cefotaxime activity loss. Picking and discarding dead cotyledons is a good practice, since bacteria usually regrow on them.
23. This continuous subculture in selective medium allows obtaining nonchimeric cultures (4).
24. Bear in mind that *presence* of a gene does not always imply *expression*. If your transgenic plant shows no phenotype, this might mean for instance that the gene has been silenced or that it is truncated and therefore not expressed correctly.
25. Whitish embryos that have grown in the dark are the preferred samples for a GUS assay. Under light conditions some embryos become green or develop black necrotic areas, which can be misleading when trying to see blue spots. Blue staining appears usually within 4 h of incubation, but overnight incubations can be required for instance in transient expression analysis, since the GUS spots will be very small. A low vacuum can be applied to facilitate penetration of the X-Gluc into the embryos. The smaller the samples, the faster the staining.
26. Do not wait for the radicle to elongate too much before the transfer, since this reduces conversion rates.

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Organelle Transformation

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Abstract

The source of genetic information in a plant cell is contained in nucleus, plastids, and mitochondria. Organelle transformation is getting a lot of attention nowadays because of its superior performance over the conventional and most commonly used nuclear transformation for obtaining transgenic lines. Absence of gene silencing, strong predictable transgene expression, and its application in molecular pharming, both in pharmaceutical and nutraceuticals, are some of many advantages. Other important benefits of utilizing this technology include the absence of transgene flow, as organelles are maternally inherited. This may increase the acceptability of organelle transformation technology in the development of transgenic crops in a wider scale all over the globe. As the need for crop productivity and therapeutic compounds increases, organelle transformation may be able to bridge the gap, thereby having a definite promise for the future.

Key words: Organelle transformation, Plastids, Biolistics, Gene expression

1. Introduction

Even after quite a few decades of successful production of transgenic crops, several countries are still skeptical about the final outcome or long-term effects of transgenic crops or, more specifically, transgenes. The perceived threat is with the outflow of transgenes in the environment, thereby contaminating crops. At the same time, with the ever-increasing pressure on land, climate change, and the need to feed the ever-increasing human populations and their livestock, there is an increasing demand towards developing crop varieties which can sustain on minimal inputs, survive in extremities of climate, disease, and pest infestation, and yet produce higher yield. Again, the burning issue of food versus fuel has added pressure to our limited resources to increase and sustain food production. Most of the concern against GM technology pertains to regulation of

transgene activity, gene flow across species and gene contamination, and as such. However, nuclear transformation has several drawbacks including, but not restricted to, unpredictable expression of gene of interest and gene silencing (1, 2). This has restricted the acceptance of transgenic technology and a number of trials have to be done before release of a particular variety. Sometimes described as good management practices, it costs millions of dollars to get to the approval process. This led researchers to look beyond nuclear transformation, the method of choice until now.

Finally, scientists looked at other organelle within the plant cell for developing effective transformation strategy (3). The mitochondria and chloroplast became the targets of choice. Briefly, during the evolution of land plants, chloroplast had an independent existence, as prokaryotic organism (4). Somehow, a symbiosis was established over millions of years of evolution of early primitive plants and chloroplasts, which finally may have led to loss of several genes from the chloroplast genome, which typically changed from parasitic to symbiotic mode, required for independent existence (5). Thus, the chloroplast became part and parcel of cell. The same theory has been hypothesized for mitochondria. The only limitation is that nongreen cells do not contain chloroplast. This is where mitochondrial transformation is of importance. Further, plastids can be categorized as chloroplast, chromoplast, and amyloplast behind several others (6).

1.1. Chloroplast Transformation

The chloroplast genome is comparatively very small, 150 kb on an average, and is normally similar across species, compared to the nuclear genome, 125 Mb in *Arabidopsis* to about 2,800 Mb in sugarcane. Chloroplast genome replicates independently of the nuclear genome. Chloroplast genome is of prokaryotic origin and circular in nature. Therefore, specific constructs are prepared with the gene of interest and flanking regions similar to chloroplast genome are included, specific for specific crops (7, 8). When the construct is delivered to the plant cell, these flanking regions guide the gene of interest to the exact place within the chloroplast genome, and thereby targeted delivery of genes can be done (8). This results in enhanced expression of transgene, avoidance of integration of multiple copies of gene of interest, and as such. Besides, chloroplasts are inherited maternally and therefore, transgene escape is avoided.

1.2. Mitochondria Transformation

Unlike chloroplast transformation, mitochondria have not been exploited to that extent. There are only a few thematically published papers like those of (9–11) on this subject. Till date, there is hardly any report that proved commercial application of such technology. Mitochondria genome is about 1,800 kbp, which is ten times larger than chloroplast genome but very small compared to nuclear genome. Hopefully, in the coming years, mitochondria transformation technology will be fine-tuned to suit for a variety of crops.

2. Illustration of the Method

2.1. Overview

2.1.1. Bioinformatics

Flanking regions suitable for any chloroplast transformation experiment from the chloroplast genome database should be identified from the freely available gene data available at NCBI, AtESEMBL, and as such. Please note that several researches have noted that cross-species flanking regions do not work properly on several occasions when performing chloroplast transformation (6).

On this note, as demand for DNA sequencing increased, new technologies were invented to reduce cost and the time involved and increase reliability. From the traditional Sanger sequencing that could read 70 kbp per run, sequencing technologies have revolutionized to the modern-day Illumina sequencing (Solexa) or AB gene (SOLiD) instrument which can read up to 3 Gbp reads per run. These have set the stage for low-cost rapid sequencing techniques with greater coverage for other cultivated crop species beyond the scope of first-generation sequenced model crop plants and that fasten the pace for trait discovery. So, in the coming years, many more chloroplasts from several crop species will be sequenced from the current 40 crops species and contribute towards developing better constructs for chloroplast transformation.

2.1.2. Cloning the Gene of Interest

Let us say, we have a gene *A*, which we want to express and obtain a certain compound, say *B*. Therefore, we have to express *A* in an organism, say plant's chloroplast. In order to do so, we have to find machinery, which will produce the gene in multiple copies and then we can use the construct (Vector+Insert) to transform a plant, i.e., chloroplast.

- (a) The gene of interest is PCR amplified or isolated and inserted into circular DNA molecule (Vector) which involves cutting the molecule at specific point using suitable restriction enzymes, confirming DNA (product) size, ligating the fragment to the vector, multiplication in *E. coli*, and restriction digest to confirm the size of the insert. Now, the resultant construct is a hybrid/chimera containing the gene as an insert. The gene is said to be cloned.
- (b) This construct carrying the cloned gene is used to transfer the Insert (which is the gene of interest) into the target organism.
- (c) Before doing so, it is essential to transfer the construct into a bacterium, which can make multiple copies.

2.2. Biolistics

For biolistics transformation, most constructs will work. Unlike *Agrobacterium*-mediated transformation, cross-species incompatibility of plasmids is not a problem with biolistics. The target gene is coated on to gold particles and fired into the plant cell (refer standard protocol). The transformed cells will regenerate in a

suitable antibiotic media, which helps to kill or suppress the growth of nontransformed cells, thus differentially helping to enhance the growth of transformed cells only (see Note 1).

Few points to be noted for particle preparation: Please take proper advice, care, and precautions before conducting experiments. Avoid contamination during the whole process.

Key points

1. Take 60 mg of 0.6–1.0- μm gold particles, (this holds good for 120 bombardments), and vortex with 1 mL 100% ETOH. Repeat four times.
2. Centrifuge and remove supernatant. Add 1 mL sterile water (dH_2O) and resuspend in sterile water. Wash twice with 1 mL sterile water. This preparation can be kept for up to 1 month at 4°C . This is good for 120 bombardments (500 ng gold/shot). However, we have seen that reducing gold concentration to 50 ng per shot did not bring about any drastic change in *gfp* expression in optimization trials. Vary plasmid DNA concentration 75–750 ng/shot. Optimization of protocol for specific experiments must be made for each new crop and variety.
3. For coating DNA, use 2.5 M CaCl_2 (can be stored at -20°C for 6 months) and 0.1 M spermidine (prepare fresh or once prepared, store aliquot into 0.5-mL Eppendorf tubes at -20°C for about a month at the most and use each vial only once, i.e., once thawed, discard vial) as described in the Bio-Rad protocol.
4. Bombardment pressure: 1,800 psi or as applicable (adjust) to particular experiments.
Distance between the sample platform and launch assembly: 5 cm; but we have found that 5 cm works better than 8 or 11 cm. This will vary between experiments.
5. Tissue culture protocol and explant type depend on particular crop and species. Generally, green callus is the preferred type for delivering transgene through chloroplast transformation (see Fig. 1).

3. Conclusion

Organelle transformation, i.e., chloroplast transformation, provides new venues for enhancing crop production. Briefly, low copy number of genes in the chloroplast and presence of several origin of replication make it an attractive target for genetic engineering. Plant breeding alone is not able to deliver the goal of increased and sustained crop production. Today, GM crops are being grown in increasing acreage year after year across different countries and it is evident that GM technology will regain its lost status in the coming

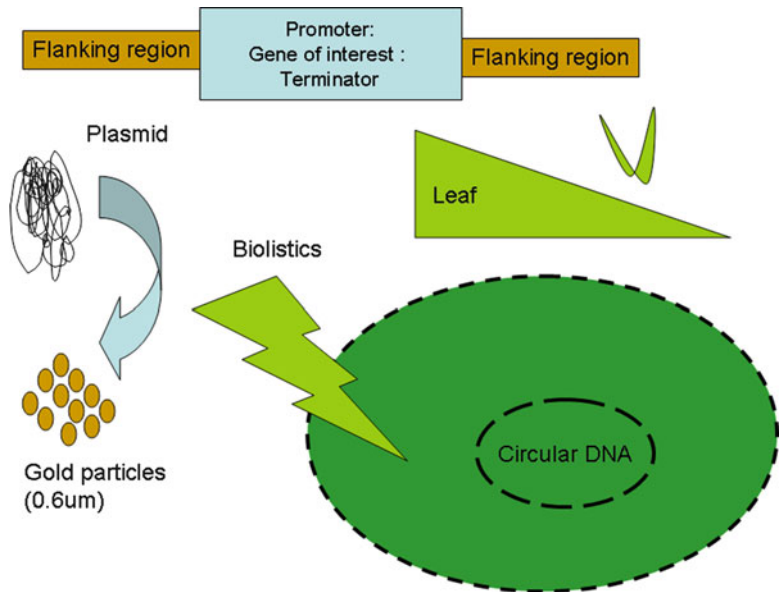


Fig. 1. Simplified representation of plasmid transformation. The gold particles are coated with DNA and fired in the chloroplast using gene gun. The flanking sequences help to direct the DNA in order to integrate at specific position in the circular DNA of the chloroplast.

years. Chloroplast transformation technology provides viable clean GM technology. There are several advantages of adopting chloroplast transformation as chloroplast divides independent of the nucleus and precision genetic combination by targeted homologous combination at specified sites is a possibility, thus enhancing the value of chloroplast transformation. With many more plasmids being sequenced nowadays, large amount of data is available for data mining and developing new flanking regions (12). Organelle transformation is superior to nuclear transformation because of trans-silencing, stable transformation, absence of cis-, high level of directed gene expression, and absence of transgene flow (13). However, organelle transformation is still in infancy and better approach must be taken to increase the efficiency of transformation in a wide variety of crops, particularly those that are recalcitrant to transformation (14, 15).

4. Note

1. Please refer standard Bio-Rad protocol for sample preparation and Gene gun operation. Please ensure that all safety measures are taken into consideration. The authors are not responsible for any situation arising thereof.

Disclaimer

Protocols described in this chapter are merely for descriptive purposes and we have no affiliation with any company including Bio-Rad for any commercial interest. The readers/users should read the user manual by manufacturer carefully and discuss all the procedures with experts/trained personnel before using any instruments or conducting any experiment. The readers will indemnify the contributors against any costs, expenses, or damages that may incur or for which contributors may become liable as a result of breach. These representations and warranties hold good for third parties also.

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Chapter 30

Appendix A: The Components of the Culture Media

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Abstract

The success in the technology and application of plant tissue culture is greatly influenced by the nature of the culture medium used. A better understanding of the nutritional requirements of cultured cells and tissues can help to choose the most appropriate culture medium for the explant used. It is also important to pay attention to a number of inaccuracies and errors which have appeared in several widely used plant tissue culture basal medium formulations.

Key word: Culture media

A medium is defined as a formulation of inorganic salts and organic compounds (apart from major carbohydrate sources and plant growth regulators) used for the nutrition of plant cultures (1). The success in the technology and application of plant tissue culture is greatly influenced by the nature of the culture medium used. A better understanding of the nutritional requirements of cultured cells and tissues can help to choose the most appropriate culture medium for the explant used.

Most of our knowledge of the nutrition of plant cultures comes from the solutions developed for the hydroponic culture of intact plants during the last part of the nineteenth and beginning of the twentieth centuries. The major changes made in the composition of the media since the early days have been the introduction of ammonium as nitrogen source, together with the higher amount of nitrate and potassium, as well as the use of organic additives, mainly vitamins and amino acids.

Plant tissue culture provides major (*macro*-), minor (*micro*-), a carbon source, and trace amounts of certain organic compounds, notably vitamins, amino acids, and plant growth regulators (Tables 1–8). In general, the tissue culture medium must contain the 16 essential elements for plant growth (2).

Table 1
Murashige and Skoog (5) media composition

Macroelements	mg/L	mM
NH ₄ NO ₃	1,650	20.60
KNO ₃	1,900	18.80
CaCl ₂ ·2H ₂ O	440	3.00
MgSO ₄ ·7H ₂ O	370	1.50
KH ₂ PO ₄	170	1.25
Na ₂ EDTA	37.30	0.10
FeSO ₄ ·7H ₂ O	27.80	0.10

Microelements	mg/L	μM
H ₃ BO ₃	6.20	100.0
MnSO ₄ ·4H ₂ O	22.30	100.0
ZnSO ₄ ·7H ₂ O	8.60	30.0
KI	0.83	5.0
Na ₂ MoO ₄ ·2H ₂ O	0.25	1.0
CuSO ₄ ·5H ₂ O	0.025	0.1
CoCl ₂ ·6H ₂ O	0.025	0.1

Organic components	mg/L	μM
<i>myo</i> -Inositol	100.0	555.10
Nicotinic acid	0.5	4.06
Pyridoxine·HCl	0.5	2.43
Thiamine·HCl	0.1	0.30
Glycine	2.0	26.60
Sucrose	30,000	87.64 mM
pH	5.7–5.8	

Table 2
Linsmaier and Skoog (7) media composition

Macroelements	mg/L	mM
NH ₄ NO ₃	1,650	20.60
KNO ₃	1,900	18.80
CaCl ₂ ·2H ₂ O	440	3.00
MgSO ₄ ·7H ₂ O	370	1.50
KH ₂ PO ₄	170	1.25
Na ₂ EDTA	37.30	0.1
FeSO ₄ ·7H ₂ O	27.80	0.1

Microelements	mg/L	μM
H ₃ BO ₃	6.20	100.0
MnSO ₄ ·4H ₂ O	22.30	100.0
ZnSO ₄ ·4H ₂ O	8.60	30.0
KI	0.83	5.0
Na ₂ MoO ₄ ·2H ₂ O	0.25	1.0
CuSO ₄ ·5H ₂ O	0.025	0.1
CoCl ₂ ·6H ₂ O	0.025	0.1

Organic components	mg/L	μM
<i>myo</i> -Inositol	100.0	555.10
Thiamine·HCl	0.4	1.20
Sucrose	30,000	87.64 mM
pH	5.6	

Table 3
Gamborg et al. (8, 9) media composition

Macroelements	mg/L	mM
(NH ₄) ₂ SO ₄	134	1.0
KNO ₃	2,528	25.0
CaCl ₂ ·2H ₂ O	150	1.0
MgSO ₄ ·7H ₂ O	250	1.0
NaH ₂ PO ₄ ·H ₂ O	150	1.1
Na ₂ EDTA	37.30	0.1
FeSO ₄ ·7H ₂ O	27.80	0.1

(continued)

Table 3
(continued)

Microelements	mg/L	μM
H ₃ BO ₃	3.0	50.0
MnSO ₄ ·H ₂ O	10.0	60.0
ZnSO ₄ ·7H ₂ O	2.0	7.0
KI	0.75	4.5
Na ₂ MoO ₄ ·2H ₂ O	0.25	1.0
CuSO ₄ ·5H ₂ O	0.025	0.1
CoCl ₂ ·6H ₂ O	0.025	0.1

Organic components	mg/L	μM
<i>myo</i> -Inositol	100.0	555.10
Nicotinic acid	1.0	8.12
Pyridoxine·HCl	1.0	4.86
Thiamine·HCl	10.0	30.0
Sucrose	20,000	58.42 mM
pH	5.5	

Table 4
Phillips and Collins (10) media composition

Macroelements	mg/L	mM
NH ₄ NO ₃	1,000	12.5
KNO ₃	2,100	20.8
CaCl ₂ ·2H ₂ O	600	4.1
MgSO ₄ ·7H ₂ O	435	1.8
KH ₂ PO ₄	325	2.4
NaH ₂ PO ₄ ·H ₂ O	85	0.6
FeSO ₄ ·7H ₂ O(EDTA)	25.0	0.1

(continued)

Table 4
(continued)

Microelements	mg/L	μM
H ₃ BO ₃	5.0	82.0
MnSO ₄ ·H ₂ O	15.0	90.0
ZnSO ₄ ·7H ₂ O	5.0	17.5
KI	1.0	6.0
Na ₂ MoO ₄ ·2H ₂ O	0.4	1.7
CuSO ₄ ·5H ₂ O	0.1	0.4
CoCl ₂ ·6H ₂ O	0.1	0.4

Organic components	mg/L	μM
<i>myo</i> -Inositol	250.0	1,400.0
Pyridoxine·HCl	0.5	2.43
Thiamine·HCl	2	6.0
Sucrose	25,000	73 mM
pH	5.8	

Table 5
White (11, 12) media composition

Macroelements	mg/L	mM
KNO ₃	80	0.79
Ca(NO ₃) ₂ ·4H ₂ O	300	1.27
KCl	65	0.87
CaCl ₂ ·2H ₂ O	440	3.00
MgSO ₄ ·7H ₂ O	720	2.92
NaH ₂ PO ₄ ·H ₂ O	19	0.13
Na ₂ SO ₄	200	1.40
Fe ₂ (SO ₄) ₃	2.5	0.006

(continued)

Table 5
(continued)

Microelements	mg/L	μM
H ₃ BO ₃	1.5	24.2
MnSO ₄ ·4H ₂ O	7.0	31.3
ZnSO ₄ ·7H ₂ O	3.0	10.4
KI	0.75	4.5
MoO ₃	0.0001	0.007
CuSO ₄ ·5H ₂ O	0.001	0.004

Organic components	mg/L	μM
Nicotinic acid	0.5	4.0
Pyridoxine·HCl	0.1	0.5
Thiamine·HCl	0.1	0.3
Glycine	3.0	40.0
Sucrose	20,000	58.42 mM
pH	5.5	

Table 6
Nitsch and Nitsch (13) media composition

Macroelements	mg/L	mM
NH ₄ NO ₃	720	9.0
KNO ₃	950	9.4
CaCl ₂	166	1.5
MgSO ₄ ·7H ₂ O	185	0.75
KH ₂ PO ₄	68	0.5
Na ₂ EDTA	37.2	0.1
FeSO ₄ ·7H ₂ O	27.8	0.1

Microelements	mg/L	μM
H ₃ BO ₃	10	161.7
MnSO ₄ ·4H ₂ O	25	112.1
ZnSO ₄ ·7H ₂ O	10	42.8
Na ₂ MoO ₄ ·2H ₂ O	0.25	1.0
CuSO ₄ ·5H ₂ O	0.025	0.1

(continued)

Table 6
(continued)

Organic components	mg/L	μM
<i>myo</i> -Inositol	100.0	555.10
Nicotinic acid	5.0	40.6
Pyridoxine·HCl	0.5	2.43
Thiamine·HCl	0.5	1.50
Glycine	2.0	26.60
Folic acid	0.5	1.1
Biotin	0.05	0.2
Sucrose	20,000	58.42 mM
pH	5.5	

Table 7
Schenk and Hildebrandt (14) media composition

Macroelements	mg/L	mM
KNO ₃	2,500	24.72
MgSO ₄ ·7H ₂ O	400	1.63
NH ₄ H ₂ PO ₄	300	2.60
CaCl ₂ ·2H ₂ O	200	1.36
Na ₂ EDTA	20	0.053
FeSO ₄ ·7H ₂ O	15	0.054
Microelements	mg/L	μM
MnSO ₄ ·H ₂ O	10.0	59.17
H ₃ BO ₃	5.0	80.86
ZnSO ₄ ·7H ₂ O	1.0	3.47
KI	1.0	6.02
CuSO ₄ ·5H ₂ O	0.2	8.00
Na ₂ MoO ₄ ·2H ₂ O	0.1	0.41
CoCl ₂ ·6H ₂ O	0.1	0.42

(continued)

Table 7
(continued)

Organic components	mg/L	μM
<i>myo-Inositol</i>	1,000.0	5,500.6
Nicotinic acid	5.0	40.6
Thiamine·HCl	5.0	14.8
Pyridoxine·HCl	0.5	2.43
Sucrose	30,000	58.42 mM
pH	5.9	

Table 8
Kao and Michayluk (6) media composition. This medium is filter sterilized

Macroelements	mg/L	mM
NH ₄ NO ₃	600	7.49
KNO ₃	1,900	18.80
CaCl ₂ ·2H ₂ O	600	4.08
MgSO ₄ ·7H ₂ O	300	1.21
KH ₂ PO ₄	170	1.25
Sequestrene® 330Fe	28	–

Microelements	mg/L	μM
H ₃ BO ₃	3.00	48.5
MnSO ₄ ·H ₂ O	10.00	59.2
ZnSO ₄ ·7H ₂ O	2.00	7.0
KI	0.75	4.5
Na ₂ MoO ₄ ·2H ₂ O	0.25	1.0
CuSO ₄ ·5H ₂ O	0.025	0.1
CoCl ₂ ·6H ₂ O	0.025	0.1

(continued)

Table 8
(continued)

Vitamins	mg/L	μM
<i>myo</i> -Inositol	100.0	555.10
Nicotinamide	1.0	8.19
Pyridoxine·HCl	1.0	4.86
Thiamine·HCl	1.0	3.00
Calcium d-pantothenate	1.0	4.20
Folic acid	0.4	0.90
<i>p</i> -Aminobenzoic acid	0.02	0.15
Biotin	0.01	0.04
Choline chloride	1.00	7.16
Riboflavin	0.20	0.53
Ascorbic acid	2.00	11.35
Vitamin A	0.01	0.03
Vitamin D ₃	0.01	0.02
Vitamin B ₁₂	0.02	0.01
Organic acids	mg/L	μM
Sodium pyruvate	20.0	181.8
Citric acid	40.0	208.2
Other sugars and sugar alcohols	mg/L	mM
Fructose	250.0	1.38
Ribose	250.0	1.66
Xylose	250.0	1.66
Mannose	250.0	1.38
L-Amino acids	mg/L	μM
All are used at a concentration of 0.1 mg/L, except:		
Glutamine	5.6	38.3
Alanine	0.6	6.7

(continued)

Table 8
(continued)

L-Amino acids	mg/L	μM
<i>Nucleic acid bases</i>		
Adenine	0.10	0.74
Guanine	0.03	0.20
Thymine	0.03	0.24
Uracil	0.03	0.27
Hypoxanthine	0.03	0.22
Xanthine	0.03	0.19

Other	mg/L	μM
Vitamin-free casamino acid	250.0	–
Coconut water ^a	20.0 mL/L	–
Sucrose	20 g/L	58.40 mM
Glucose	10 g/L	55.49 mM
pH	5.6	

^aFrom mature fruits; heated to 60°C for 30 min

The most important difference among media may be the overall salt level. There seems to be basically three different media types by this classification: high salt (e.g., Murashige and Skoog medium, Table 1), intermediate level (e.g., Nitsch and Nitsch, Table 6), and low salt media (e.g., White, Table 5).

Researchers quickly found that the addition of “complexes” to the basic medium frequently resulted in successful growth of the tissues and organs. Some of these complexes have included green tomato extract, coconut milk, orange juice, casein hydrolysate, yeast, and malt extract (2).

It is very important when a medium is chosen; take into account that some of the components of the culture media are not only a nutriment: some of them can have a very deep influence not only in the growth of the cultures, but also in the differentiation process. Another important fact is that vigorous colonies of callus tissue required more nutriments than slowly growing ones, while the situation can be reverse for other nutriments.

It is also important to pay attention to a number of inaccuracies and errors which have appeared in several widely used plant tissue culture basal medium formulations (3, 4). The exact hydration of the iron salt and molar equivalence of iron and its chelating agent since inconsistencies exist in popular commercial preparations for several common basal media, over and above those found in the primary literature. Even the primary literature can

have some mistakes (5) or the same name is used to designate the same medium. There have been many different versions presented in print either by White or his coworkers (4).

Minor variations in medium composition can determine the success or failure of certain protocols. The excesses of chelating agent, although small, may influence micronutrient availabilities. Investigators should examine original papers carefully and compare them with commercial formulations, when seeking details on a given nutrient or medium.

In relation with the Kao and Michayluk medium, the presence of vitamin-free casamino acid and the coconut water is essential for the culture of protoplasts, but they are not necessary for the culture of cells. This medium is one of the most complex between all the media used in plant tissue culture. It is used mainly for the growth of very low cell density cultures, as well as protoplasts in liquid media (6).

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Chapter 31

Appendix B: Plant Biotechnology and Tissue Culture Resources in the Internet

Víctor M. Loyola-Vargas

Abstract

This appendix compiles a list of useful Internet sites for cell culture scientists. A total of more than 100 sites have been selected, based on the quality of the information offered in them, as well as on their users' friendliness. We anticipate that some of these sites will be included among the reader's favorites (if they are not already).

Key words: Internet, Plant tissue culture

1. General resources

- Access excellence (<http://www.accessexcellence.org/LC/ST/st2bgplant.php>)
- American Ag-Tec Potato Mini-Tuber Production (<http://www.ag-tec.com/potato.htm>)
- Biotechnology Information Center, National Agricultural Library (http://riley.nal.usda.gov/nal_display/index.php?info_center=8&tax_level=2&tax_subject=8&topic_id=1067&placement_default=0)
- Soybean Tissue Culture and Genetic Engineering Center (<http://mulch.cropsoil.uga.edu/soy-engineering/>)
- Cevie asbl (<http://www2.ulg.ac.be/morphovg/main.htm>)
- Ethical, Legal and Social Aspects (ELSA) of biotechnology (<http://ec.europa.eu/research/life/elsa/index.html>)
- Fruit Tree Research Institute, Italy (<http://www.propag.org/>)
- Office of Biotechnology, Iowa State University (http://www.biotech.iastate.edu/ed_resources/Laboratory_protocols.html)
- Iowa State University Plant Transformation Facility (<http://www.agron.iastate.edu/ptf/>)

- Kitchen Culture Kits (<http://www.kitchenculturekit.com/begin.htm>)
- Listserv: PLANT-TC (<http://plant-tc.cfans.umn.edu/listserv/>)
- National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>)
- National Botanical Research Institute (<http://www.nbrienvs.nic.in/>)
- National Research Council of Canada/Plant Biotechnology Institute (<http://www.nrc-cnrc.gc.ca/eng/ibp/pbi.html>)
- NCGR-Corvallis Information—Tissue Culture (<http://www.ars-grin.gov/cor/tc.html>)
- Oklahoma Plant Transformation Facility (<http://www.ptf.okstate.edu/>)
- Plant Hormones (<http://www.plant-hormones.info/>)
- Plant Tissue Culture Research at the University of Minnesota (<http://plant-tc.cfans.umn.edu/>)
- Plant World Explorations (<http://members.ozemail.com.au/~mhempel/index.html>)
- Succulent Tissue Culture (<http://www.succulent-tissue-culture.com/EN>)
- Institute for Plant Genomics and Biotechnology (<http://ipgb.tamu.edu/>)
- Texas A&M Plant Tissue Culture Information Exchange (<http://aggie-horticulture.tamu.edu/tisscult/tcintro.html>)

2. Micropropagation

- Flora laboratories (<http://home.alphalink.com.au/~andre/>)
- Growmore Bio tech (<http://www.growmorebiotech.com/>)
- Micropropagation of Orchids (<http://www.angelfire.com/on4/angelorchids/PropagatingOrchidsnew.htm>)
- In Vitro Propagation (http://www.alt.med-rz.uni-sb.de/med_fak/physiol2/disa/invitro/000.htm)
- Meyers Conservatory (<http://www.troymeyers.com/>)
- Micropropagation unit at Royal Botanic Gardens, Kew (<http://www.kew.org/science/micropropagation.html>)
- Oglesby Plant Laboratories (<http://www.oglesbytc.com/>)
- Orchid Species Culture (<http://www.orchidculture.com/>)
- The Micropropagation of Aroids (<http://www.aroid.org/horticulture/tculture.html>)
- Tissue culture in the home kitchen (<http://www.omnisterra.com/botany/cp/slides/tc/tc.htm>)

3. Databases

- AGRICOLA (<http://agricola.nal.usda.gov/help/aboutagricola.html>)
- Angiosperm DNA C-Values Database (<http://data.kew.org/cvalues/CvalServlet?querytype=2>)
- Expasy (<http://ca.expasy.org/>)
- Internet Directory for Botany (<http://www.ou.edu/cas/botany-micro/idb-alpha/botany.html>)
- Protein data bank (<http://www.rcsb.org/pdb/home/home.do>)
- Terminology Associated with Cell, Tissue and Organ Culture, Molecular Biology and Molecular Genetics (<http://www.springerlink.com/content/0g83p23206428708/>)
- The Arabidopsis Information Resource (<http://www.arabidopsis.org/>)
- The WWW Virtual Library—BioScience Resources (<http://mcb.harvard.edu/BioLinks.html>)
- The WWW Virtual Library—Plant Science Resources (<http://www.ou.edu/cas/botany-micro/www-vl/>)

4. Books

- Agritech Publications (<http://agritechpublications.com/>)
 - Media and Techniques for Growth, Regeneration and Storage 2002–2005. Volume 9 of Recent Advances in Plant Tissue Culture (<http://www.agritechpublications.com/rec9book.htm>)
 - Micropropagation Systems and Techniques 2002–2006. Volume 10 of Recent Advances in Plant Tissue Culture (<http://www.agritechpublications.com/rec10book.htm>)
 - Microbial Contaminants in Plant Tissue Culture III 2003–2007. Volume 11 of Recent Advances in Plant Tissue Culture (<http://www.agritechpublications.com/rec11book.htm>)
 - Media and Techniques for Growth, Regeneration and Storage 2005–2008. Volume 12 of Recent Advances in Plant Tissue Culture (<http://www.agritechpublications.com/rec12book.htm>)
 - Micropropagation Systems, Techniques and Applications 2006–2010. Volume 13 of Recent Advances in Plant Tissue Culture (<http://www.agritechpublications.com/rec13book.htm>)
 - Secondary Metabolite Production. Volume 14 of Recent Advances in Plant Tissue Culture (<http://www.agritechpublications.com/rec14book.htm>)

- Bibliography of Plant Micropropagation and Regeneration 2005–2009 (<http://www.agritechpublications.com/bibliography.htm>)
- Plant Culture Media. Vol 1. Formulations and Uses, 1999 (<http://agritechpublications.com/media1.htm>)
- Plant Culture Media. Vol 2. Commentary and Analysis, 1999 (<http://agritechpublications.com/media1.htm>)

5. Editorials

- Annual Reviews (http://arjournals.annualreviews.org/?jsessionid=iBIj52VDf_R8)
- Cambridge University Press (<http://www.cup.cam.ac.uk/>)
- CRC Press (<http://www.crcpress.com/>)
- Elsevier (http://www.elsevier.com/wps/find/homepage.cws_home)
- Humana Press (<http://www.humanapress.com/Index.pasp>)
- Kluwer (<http://www.kluweronline.com/>)
- Springer-Verlag (<http://www.springeronline.com/sgw/cda/frontpage/0,0,0-0-0-EAST,0.html>)
- Timber Press (<http://www.timberpress.com/books/index.cfm>)
- Vedams (<http://www.vedamsbooks.com/newbot.htm>)

6. Journals and newsletters

- Agricell Report (<http://www.agritechpublications.com/>)
- American Journal of Botany (<http://www.amjbot.org/current.shtml>)
- Biologia Plantarum (<http://www.kluweronline.com/issn/0006-3134/>)
- Biotechnology Letters (<http://www.kluweronline.com/issn/0141-5492/contents>)
- Biotechnology Process (<http://pubs3.acs.org/acs/journals/toc.page?incoden=bipret>)
- Canadian Journal of Botany (http://pubs.nrc-cnrc.gc.ca/cgi-bin/rp/rp2_desc_e?cjb)
- Crop Science (<https://www.crops.org/publications/cs>)
- Current Opinion in Biotechnology (<http://www.current-opinion.com/jbio/about.htm?jcode=jbio>)
- Current Opinion in Cell Biology (<http://www.sciencedirect.com/science/journal/09550674>)
- Current Opinion in The Plant Biology (<http://www.current-opinion.com/jpbl/about.htm?jcode=jpbl>)

- Electronic Journal of Biotechnology (<http://www.ejbiotechnology.info/>)
- Euphytica (<http://www.kluweronline.com/issn/0014-2336/>)
- In Vitro Cellular and Developmental Biology—Plant (<http://www.springerlink.com/content/120502/>)
- Journal of Experimental Botany (http://www.oxfordjournals.org/our_journals/exbotj/about.html)
- Journal of Phytopathology ([http://onlinelibrary.wiley.com/journal/10.1111/\(ISSN\)1439-0434/issues](http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1439-0434/issues))
- Journal Plant Physiology (<http://www.elsevier-deutschland.de/jpp>)
- Nature Biotechnology (<http://www.nature.com/biotech/>)
- New Phytologist ([http://onlinelibrary.wiley.com/journal/10.1111/\(ISSN\)1469-8137/issues](http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1469-8137/issues))
- Physiologia Plantarum ([http://onlinelibrary.wiley.com/journal/10.1111/\(ISSN\)1399-3054/issues](http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1399-3054/issues))
- Plant Biotechnology Journal ([http://onlinelibrary.wiley.com/journal/10.1111/\(ISSN\)1467-7652/issues](http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1467-7652/issues))
- Plant Breeding ([http://onlinelibrary.wiley.com/journal/10.1111/\(ISSN\)1439-0523/issues](http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1439-0523/issues))
- Plant Cell (<http://www.plantcell.org/>)
- Plant Cell and Environment ([http://onlinelibrary.wiley.com/journal/10.1111/\(ISSN\)1365-3040/issues](http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1365-3040/issues))
- Plant Cell Physiology (<http://pcp.oupjournals.org/>)
- Plant Cell Reports (<http://www.springerlink.com/content/100383/>)
- Plant Cell, Tissue and Organ Culture (<http://www.springerlink.com/content/100327/>)
- Plant Growth Regulation (<http://www.springerlink.com/content/100329/>)
- Plant Journal (<http://www.wiley.com/bw/journal.asp?ref=0960-7412>)
- Plant Molecular Biology (<http://springerlink.metapress.com/content/100330/>)
- Plant Physiology (<http://www.plantphysiol.org/>)
- Planta (<http://www.springerlink.com/content/100484/>)
- Theoretical and Applied Genetics (<http://www.springerlink.com/content/100386/>)
- Trends in Biochemical Sciences (http://www.sciencedirect.com/science?_ob=PublicationURL&_cdi=5180&_auth=

y&_acct=C000059356&_version=1&_urlVersion=0&_userid=2974920&_pubType=J&md5=1b4a07b34d6fe15d dacb8cd1fb738380)

- Trends in Biotechnology (http://www.sciencedirect.com/science?_ob=PublicationURL&_cdi=5181&_auth=y&_acct=C000059356&_version=1&_urlVersion=0&_userid=2974920&_pubType=J&md5=9efcee93233c50cb1b127c9b14cada8d)
- Trends in Cell Biology (<http://www.cell.com/trends/cell-biology/>)
- Trends in Genetics (http://www.sciencedirect.com/science?_ob=PublicationURL&_cdi=5183&_auth=y&_acct=C000059356&_version=1&_urlVersion=0&_userid=2974920&_pubType=J&md5=d57158b47005b4a548870531924e4cf2)
- Trends in Plant Science (http://www.sciencedirect.com/science?_ob=PublicationURL&_cdi=5185&_auth=y&_acct=C000059356&_version=1&_urlVersion=0&_userid=2974920&_pubType=J&md5=79e9758da7316889alc1ac01ec45414f)

7. PTC inside the classroom

- Access Excellence (<http://www.accessexcellence.org/>)
- Cloning Plants By Tissue Culture (<http://csm.jmu.edu/biology/renfromh/pop/pctc/cloning.htm>)
- Plant Cell Protoplasts (<http://agronomy.unl.edu/815/cropt.htm>)
- TNAU Agritech Portal (http://agritech.tnau.ac.in/bio-tech/biotech_tc_jainirrigation.html)

8. Societies

- American Phytopathological Society (<http://www.apsnet.org/Pages/default.aspx>)
- American Society for Horticultural Science (<http://www.ashs.org/>)
- American Society of Plant Physiologists (<http://my.aspb.org/members/>)
- Botanical Society of America (<http://www.botany.org/>)
- International Association for Plant Tissue Culture and Biotechnology (IAPTC&B) (<http://www.danforthcenter.org/iapb-stl/>)
- International Association of Sexual Plant Reproduction Research (IASPRR) (<http://www.iaspr.org/>)
- International Plant Propagators' Society (<http://www.ipps.org/>)

- Society for In Vitro Biology (<http://www.sivb.org/>)
- The American Orchid Society (<http://www.aos.org//AM/Template.cfm?Section=Home>)

9. Design and layout of a micropropagation facility

- Design and Layout of a Micropropagation Facility (<http://aggie-horticulture.tamu.edu/tisscult/microprop/facilities/microlab.html>)

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