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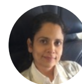
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Molecular Biomethods Handbook

Second Edition

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50

Plant Tissue Culture

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1. Introduction

Plant tissue culture (PTC) is a set of techniques for the aseptic culture of cells, tissues, organs and their components under defined physical and chemical conditions in vitro and controlled environment (**Fig. 50.1**). PTC technology also explores conditions that promote cell division and genetic re-programming in in vitro conditions and it is considered an important tool in both basic and applied studies, as well as in commercial application ***(1)***.

Today, facilities for in vitro cell cultures are found in practically each plant biology laboratory, serving different purposes because tissue culture has turned into a basic asset for modern biotechnology, from the fundamental biochemical aspects to the massive propagation of selected individuals. Today five major areas, where in vitro cell cultures are being currently applied, can be recognized: as a model system for fundamental plant cell physiology aspects, generation of genetic modified fertile individuals, large-scale propagation of elite materials, preservation of endangered species, and metabolic engineering of fine chemicals.

1.1. History of PTC's Development

The theoretical basis for plant tissue culture was proposed by Gottlieb Haberlandt in 1902 ***(2,3)***. He predicted that eventually a complete and functional plant could be regenerated from a single cell. Other studies led to the culture of isolated root tips ***(4,5)***. The approach of using explants with meristematic cells produce the successful and indefinite culture of tomato root tips ***(6)***. The firsts true PTC were obtained by Gautheret ***(7)*** from cambial tissue of *Acer pseudoplatanus*. Several years later White ***(8)*** obtained tumor tissue from a *Nicotiana* × *N. langsdorffii* hybrid and Nobécourt ***(9)*** and Guatheret ***(10)*** produced callus from carrot root tips.

During the following years, the culture of young embryos ***(11)*** and the formation of meristems from callus tissues ***(12)*** were achieved. The discovery of the first cytokinin (kinetin) ***(13)*** led to the recognition that the exogenous balance of both auxin and kinetin in the medium influenced the morphogenic

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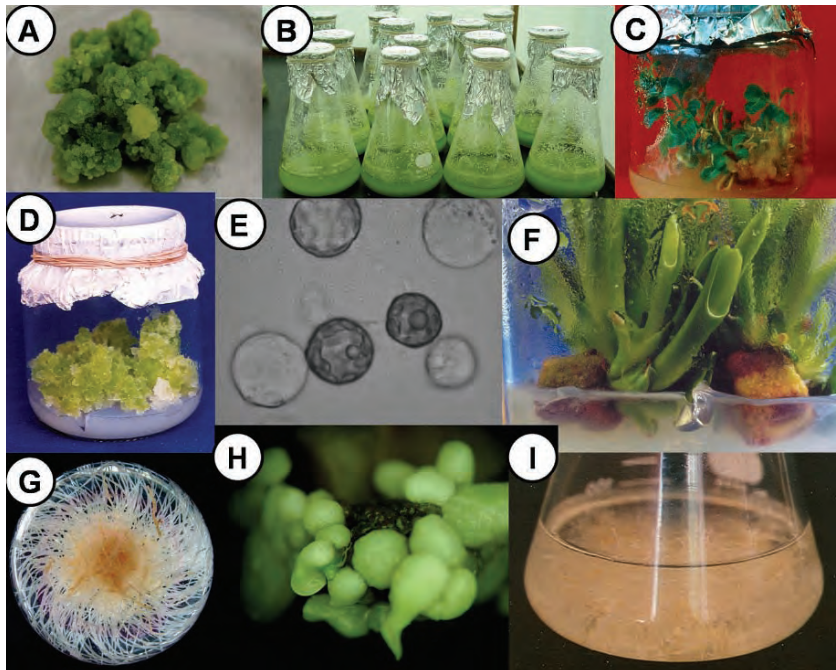

Fig. 50.1. A. Callus from *Catharanthus roseus*. **B.** Suspension culture from *C. roseus*. **C.** Regeneration of plantlets from *C. roseus* callus. **D.** Tumors from *C. roseus*. **E.** Protoplasts from *C. roseus*. **F.** Micropropagation of *Agave tequilana*. **G.** Hairy roots from *C. roseus.* **H.** Somatic embryogenesis of *Coffea canephora*. **I.** Root culture from *C. roseus.* Pictures **A–E, G–I** from the authors' laboratories. Picture **F** from the laboratory of Dr. Manuel Robert all of them at Centro de Investigación Cientifica de Yucatán

fate of callus ***(14)***. A relative high level of auxin to kinetin favored rooting, the reverse led to shoot formation and intermediate levels to the proliferation of callus or wound parenchyma tissue. Several independent groups reported the formation of bipolar somatic embryos ***(15–17)***. The first demonstration of the plant cells' totipotency was carried out by Vasil and Hildebrandt using tobacco cells ***(18)***. This was followed later by the regeneration of plants from protoplasts ***(19)*** and the regeneration of the first interspecific hybrid plants (*Nicotiana glauca* × *N. langsdorffii*) ***(20)*** after Cocking had developed the use of fungal hydrolytic enzymes for the production of protoplasts ***(21)***. More recently, the establishment of commercial cultures for the production of secondary metabolites ***(22,23)*** and the generation of transgenic plants from transformed callus or somatic embryos ***(24,25)*** has opened the field to major basic and commercial applications ***(26–28)***.

1.2. Tissue Culture Media

The nutrition of PTC requires a culture medium. This is formed by both inorganic salts and organic compounds in addition to a carbon source and plant growth regulators ***(29)***. Most of our knowledge about the nutrition of plant cultures comes from the solutions done for the hydroponic system of complete plants. In general, the tissue culture medium must contain the essential elements for plant growth ***(30)***. The addition of "complexes" such as green tomato extract, coconut milk,

orange juice, casein hydrolysate, yeast, and malt extract, to the basic medium frequently resulted in successful growth of the tissues and organs ***(30)***.

The success in the application of PTC is profoundly influenced by the nature of the culture medium used. The most important difference among media may be the overall salt level, mainly the amount and quality of the nitrogen source ***(31–33)***. It is very important when a medium is chosen, to take into account that some of the culture media's components are not only nutrients, but some of them can have a very deep influence either in the growth of the cultures, or in the differentiation process ***(34,35)***. There are several media already published, the choice of one of them will depend on the goal to be reached.

The Murashige and Skoog medium ***(31)*** is currently the most widely used medium; however, this medium has a high content of nitrogen, as well as a high nitrate/ammonium ratio. Other media reported have less total nitrogen and lower nitrate/ammonium ratio ***(32,33,36)***. The Kao and Michayluk medium ***(37)*** is one of the more complex among all 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.

2. Basic Aspects

2.1. Types of Cell Cultures

PTC includes a set of different techniques to manipulate cells. Among the different PTC that can be obtained are callus, suspension cultures, protoplasts, anther and ovule cultures, somatic embryos, and meristem culture ***(30,38–42)***. Depending on particular species employed and the kind of response that is desired, almost every part of a plant can be used as starting material (explant). Among the type of explants frequently used there are leaf portions, isolated meristems, hypocotyls, or root segments among others. For the initiation of the culture three important considerations should be taken into account: a) explant selection, b) election of a suitable culture medium and appropriate environmental conditions for its development, and c) the isolation and maintenance of callus for subsequent experimentation.

2.1.1. Callus

As a first step in many tissue culture experiments, it is necessary to induce callus formation from the primary explant (**Fig. 50.1A**). This explant may be an aseptically germinated seedling of surface-sterilized roots, stem, leaves, or reproductive structures. In the context of PTC, callus is a largely unorganized, proliferating mass of parenchyma cells ***(43)*** that in a wounded tissue is produced in response to injury ***(44)***. Calluses are slow growing, small, and convenient to handle, and hence are a useful means of maintaining and storing germplasm ***(45)***. The growth rate and friability of callus produced can vary widely between explants and even within replicates of the same medium ***(41)***. This heterogeneity is seen in established calluses as differences in color, morphology, structure, growth, and metabolism. Even an apparently uniform callus may contain cells of different ploidy and metabolic capability ***(45)***. Not all cells in an explant contribute to the formation of callus and, more importantly, certain callus cell types are competent to regenerate organized structures. Other callus cell types do not appear to be competent to express totipotency. Early visual selection is usually necessary to select for the cefll type that is regenerable ***(44)***. The level

of plant growth regulators is a major factor that controls callus formation in the culture medium. Culture conditions (temperature, type of jellification agent, light, etc.) are also important in callus formation and development. A wide variety of media compositions have been used with success to induce calluses. These can be maintained on agar, agarose, gelrite, or any other jellification agent. The formation of callus with an explant marks the beginning of successful PTC, and may be used for a variety of experiments ***(43,44)***.

2.1.2. Suspension Cultures

A cell suspension culture could be defined as a rapidly dividing, homogeneous suspension of cells ***(46)*** (**Fig. 50.1B**). These cultures can be used in biochemical and cell physiology research as well as for the study of growth, metabolism, molecular biology, and genetic engineering experiments. Also cell suspension cultures can be used for medium or large scale secondary metabolites and other fine-chemical production.

There is not a standard method to produce a suitable suspension culture. However, in most of the cases the transference of friable callus to a liquid media, such as Murashige and Skoog ***(31)*** or Gamborg media ***(32)*** under agitation during incubation (50–200 rpm), can produce the dispersion of the cells, after several passages. Suspension cultures should ideally consist of single cells, but this is rarely the case and usually small aggregates of 20–100 cells (100–1,000 µm) are found. The suspension cultures grow faster than callus cultures and they are more homogenous; however, the rate of variability also increases producing variability and instability of the cultures. To avoid the problem of instability, the cultures are subcultured when the cells are at the end of the exponential growth phase.

2.1.3. Organ Culture

In addition to callus and suspension cultures, organ culture also has been established. In 1934 Phillip White, one of the pioneers of PTC, developed the first system that allowed indefinite proliferation of roots tips ***(6)***. Since then, root cultures became a standard system in studies of inorganic nutrition, nitrogen metabolism, plant growth regulation, and root development ***(47)***.

Around 20 years ago, the need for cell organization for the biosynthesis of secondary metabolites in PTC was recognized to be fundamental ***(48)***, and encouraged the development of better organ culture systems.

Recent progress on growing roots in isolation has greatly facilitated the study of root-specific metabolism and contributed to our understanding for this remarkable plant organ and showed that they are able to produce the same profiles of natural products as their counterpart in the whole plant ***(49–52)***. Root cultures can be established by cultivating roots isolated from aseptic plant cultivate in vitro (**Fig. 50.1I**). One disadvantage of the roots culture is their slow growth under in vitro conditions. To avoid this problem, Flores and Filner ***(53,54)*** developed a system that involves the generation of fast growing adventitious roots or hairy roots, which are the product of the infection of different tissues with *Agrobacterium rhizogenes* (**Fig. 50.1G**). These hairy root cultures have the same metabolic features as normal root cultures and they produce valuable fine chemicals such as tropane and indole alkaloids among others ***(55)***. On the other hand, shoot cultures also have been established. These cultures can be used to produce natural products in which biosynthetic pathway is located in the aerial part of the plant ***(56–59)***.

Root and shoot cultures have emerged as powerful tools to study the biochemistry and molecular biology of secondary metabolite biosynthetic pathways. The expression of the metabolic pathway can be regulated manipulating the environmental and nutrimental conditions of the cultures. This manipulation also lets the control of the developmental stage of the cultures. Flores and Filner ***(54)*** were capable to demonstrate that *Hyosciamus muticus* hairy roots are able to synthesize hyoscyamine at levels equal to or greater than the roots *in planta*. The biosynthetic capacity of hairy root cultures was strictly correlated with a differentiated state; hairy root cultures that were dedifferentiated to callus lost their capability to produce hyoscyamine. When these callus were differentiated back to hairy roots, synthesis of hyoscyamine returned ***(54)***. This was the first practical demonstration of the differentiation's role in the expression of secondary metabolic pathways.

2.1.4. Protoplasts

Several of the genetic manipulation techniques, such as the induction of somaclonal variation, somatic hybridization, and transformation, require the use of protoplasts. Protoplasts are a powerful tool to study diverse aspects of development, physiology, and genetics of plant cells ***(60)***. Furthermore, protoplasts are basically plant cells without the cell wall (**Fig. 50.1E**). The removal of the cell wall makes it necessary to include osmotic stabilizers into the medium and additional nutritional ingredients to preserve the protoplast and ensure their viability ***(41)***. Although almost any explant of most plant species can be used as a source of protoplasts, and procedures are available to isolate and culture protoplasts from monocotyledons and dicotyledons, the ability to isolate protoplasts capable of sustained division and plant regeneration is still restricted to a limited number of species/plant combinations. Among the different parameters that can influence the isolation and culture of protoplasts are the origin of the explant, culture medium, the osmoticum, duration of enzyme incubation, pH of the enzyme solution, and environmental culture conditions. An emphasis must be made on the influence of tissue physiology to the release of viable protoplasts. Embryogenic cell suspensions have been the preferred source of viable protoplasts in some cultivars such as coffee ***(61,62)***, sugarcane ***(63)***, alfalfa ***(64)*** mango ***(65)***, and wheat ***(65,66)***, among others.

The isolation of protoplast using natural plant cell wall enzymatic degradation activity had lead to multiple applications. Recently, Phillip Benfel and his group ***(67,68)*** used this technique to locate the tissue-specific gene expression in different roots zones. They used five separate transgenic lines expressing the green fluorescent protein (GFP) in stele, endodermis, endodermis plus cortex, epidermal atrichoblast cells, and lateral root cap. After harvesting and protoplasting the root tissue, the protoplasts expressing the GFP were isolated on a fluorescence-activated cell sorter and their mRNA was analyzed with the use of microarrays. This is an elegant method to isolate tissue specific mRNA.

Protoplasts can be fused allowing us to cross natural barriers to produce desirable plant traits that are not possible by sexual means. However, the protoplast fusion is a nonspecific process that can be mediated either by chemicals or by electrical techniques. After the fusion, the heterokaryots (they contain the nuclei of the two parents in a mixed cytoplasm) are isolated and developed into hybrid cells ***(69)***. These hybrid cells are characterized and developed into somatic hybrid plants.

2.2. Morphogenesis

Since the first confirmation of Haberlandt's theory ***(18)*** great effort has been made to understanding the molecular mechanism involved in the stimulation of morphogenesis (from the Greek *morphê* shape and *genesis* creation).

Morphogenesis can be obtained in in vitro plant tissue culture by using synthetic medium supplemented with plant growth regulators among others. However, this "genesis" can also be observed in nature ***(70,71)***. The morphogenesis in vitro can go through two different pathways and they are classified as somatic embryogenesis (**Fig. 50.1C**) and organogenesis (**Fig. 50.1H**), the latter can develop organs such as flowers, shoots, and roots. Both somatic embryogenesis and organogenesis can take place either directly or indirectly; direct or adventitious organogenesis often refers when there is not a callus intermediate stage; by contrast when there is a profusely proliferation of callus, before organ formation, it is called indirect or *de novo* organogenesis ***(72,73)***.

The main factors involve in the stimulation of both embryogenesis or organogenesis and the kind (direct or indirect) of morphogenesis depend on the nature, concentration, and exposure time of the phytohormones employed, status of endogenous phytohormones, the source and physiological state (the ability to respond) of the explant, the medium of culture, and the culture condition used. The interaction between these factors produces the induction and expression of a specific mode of cell differentiation and development ***(74)***.

During morphogenesis' induction three hypothetic phases are recognizable for direct morphogenesis and four for indirect on temporal response caused by the balance of exogenous/endogenous phytohormones (**Fig. 50.2**). In the first phase, the cell can take one of two routes described before. If the cell goes by the direct way, it will change its genetic program to acquire the competence status before it will become a determined cell. In contrast, the cell will pass through a proliferative stage before it gains a competence status. Both routes direct and indirect are a consequence of the response to the physiological status of the explant and hormonal signals. In the second part, the competence cell will get the determined status as a response to influence of phytohormone balance. Afterward, during transition from determined phase into morphogenesis, the cell proceeds independently of the hormonal influence ***(75)***. In general, the somatic embryogenesis pathway depends on high concentration of auxin to pass from somatic to determined stage (from 0 to II), whereas organogenesis

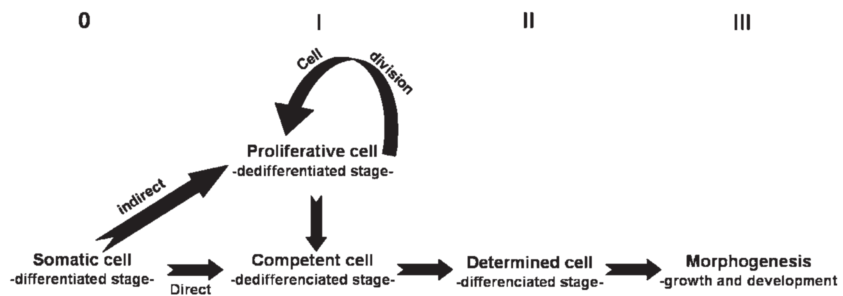

Fig. 50.2. Hypothetic phases of morphogenetic induction from somatic cell to organ or somatic embryo. The numbers represent the morphogetic phases. Adaptated from Sugiyama ***(75)***

pathway is developed mainly by a high ration of cytokinin:auxin, the species that can be easily regenerated using organogenesis are difficult to regenerate through somatic embryogenesis or vice versa *(76)*. Recently, several genes involved in plant morphogenesis have been discovered *(72,76,77)*.

2.3. Micropropagation

The most widely used commercial application for micropropagation is the vegetative propagation of plants, mainly ornamentals (**Fig. 50.1F**) *(30,78–82)*, and medicinal plants (*83*). 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 *(84,85)*. The disadvantage of the axillary bud breaking method is that it produces the smallest number of plantlets; however, they are generally genetically true-to-type. On the other hand, somatic embryogenesis is able to produce the greatest number of plantlets, but it is induced in the lowest number of plant species.

Micropropagation protocols are aimed to the rapid multiplication of plantlets true-to-type to the original material. Meristematic tissues, located either on terminal or axillary buds, are induced to proliferate in response to hormonal treatments. Hypocotyls are also frequently used as the original explant. Culture conditions, mainly nitrogen source, light regime, temperature, and the container's atmosphere can play critical roles in favoring bud development into vitroplants *(86–90)*.

Most micropropagation processes are carried out in small culture vessels containing a culture medium solidified with a gelling agent to create a substrate on which the plant tissues are cultured. In spite of its general use, this method has some disadvantages: the culture conditions are heterogeneous because not all the tissues are in contact with the nutrient medium, different media compositions and growth regulator concentrations are required for each stage of the micropropagation process, which implies that tissues or plants need to be continuously transferred to new containers with fresh medium. The multiplication stage also requires frequent transfers as the biomass increases and fills the culture vessels. Consequently, micropropagation is a labor intensive method that greatly increases the production costs of plants produced in vitro and it is only economically viable on a commercial scale in the case of high value-added species *(91)*.

To simplify the whole process, reducing production costs and making micropropagation available to a larger number of species is necessary to develop simpler and cheaper methods, which can decrease the amount of labor. A first step in this direction was the design of semiautomated bioreactors to culture the plants in liquid media *(92)*. A method that combines the advantages of both semisolid and liquid culture media is the temporary immersion system designed by Teisson and collaborators *(93)*. This system alternates short periods of total immersion in liquid medium with longer ones of complete aeration. Satisfactory results for the propagation of various species have been reported using two bioreactors based on this principle *(93–97)*. A new type of bioreactor for micropropagation has been proposed by Robert et al *(92)*. This device has a number of features specifically designed to simplify its operation and reduce production costs.

2.4. Somatic Embryogenesis

Somatic embryogenesis refers to the process by which somatic cells under induction conditions, generate embryogenic cells, which undergo a series of morphological and biochemical changes resulting in the formation of somatic embryos, which could develop into a plant (**Fig. 50.1H**) ***(98–100)***. Somatic embryogenesis forms the basis of cellular totipotency that is unique to higher plants. Differing from their zygotic counterpart, somatic embryos are easily tractable, culture conditions can be controlled, and lack of material is not a limiting factor for experimentation ***(101)***. These characteristics have made somatic embryogenesis a model for the study of morphological, physiological, molecular, and biochemical events that occur during the onset and development of embryogenesis in higher plants. It also has potentially rich biotechnological applications such as artificial seeds, micropropagation, transgenic plants, etc. ***(73)***. Tissue culture systems have been one of the most useful experimental tools used to understand morphogenesis programs.

The somatic embryo resembles the zygotic embryo in various aspects ***(102,103)*** and it is possible to study diverse subjects related to the embryogenesis process using the somatic embryo system. Nonetheless, other topics cannot be studied, including the moment of fertilization, the differentiation of the endosperm, the absorption of nutrients by the endosperm and its interaction with the embryo, the effect of the mother tissue on embryo's development, the embryo's desiccation, and the embryo's dormancy ***(73)***.

Another "type" of embryogenesis can be obtained from diverse sources, different of somatic embryos; e.g., apomictic embryos are derived from an unfertilized egg cell or from maternal tissue ***(104)***. It is also possible to obtain in vitro "androgenic" embryos from microspores and pollen grains ***(105,106)***. Although somatic embryos are originated from somatic cells ***(98,105,107)***, in nature, foliar embryos are observed in several species such as *Bryophyllum calycinum* ***(71)***, or *Camptosorus rhizophyllus* ***(70)***.

The first report to address somatic embryogenesis came in the late 1950s, in cultures of *D. carota* ***(16,17)*** and *Oenanthe aquatica* ***(15,108)***. Since then somatic embryos have been obtained in many other plant species ***(109)*** even though the carrot has been the most widely used model, mainly owing to its feasibility, fast response, and high yields. Somatic embryos from dicots pass through characteristic morphological stages, which are: globular-shaped, oblong-shaped, heart-shaped, enlarged, torpedo-shaped and cotyledonal ***(110–113)***.

The basic procedure for producing somatic embryo involves the use of a synthetic medium culture supplemented with plant growth regulators, such as auxin, e.g., 2,4-dichlorophenoxy acetic (2,4-D), cytokinin e.g., kinetin (Kin), abscisic acid (ABA), or combination of two or several growth regulators. In the case of carrot somatic embryogenesis, the tissue initially requires an auxin and later the cells must be transferred into a culture medium with low concentration of auxin or without it.

Components of culture media and growth regulators such as 2,4-D are not the only factors controlling somatic embryogenesis ***(80,81)***. It has been demonstrated that other stimuli also induce somatic embryogenesis. For example, stress, including osmotic shock with sucrose or sodium chloride ***(114–119)*** or the presence of heavy metals—cobalt, nickel, zinc, and cadmium—***(115,120)***, and nutrient starvation ***(121,122)***. Other compounds widely used to enhance embryo formation include salicylates (SA) ***(123–125)***. It may be happen via inhibition

of the ethylene biosynthesis ***(123,126–128)***. By contrast, in *Coffea canephora* ethylene is necessary for the induction of somatic embryogenesis ***(129)***.

The exogenous application of H_2O_2 enhances its endogenous levels and promotes somatic embryogenesis ***(130)*** even though Luo et al ***(131)*** using *Astragalus adsurgens* determined that the endogenous increment in H_2O_2 levels, caused by the exogenous application of SA, was critical to enhancing embryo production.

All data referred above suggest a possible connection or an overlapping between embryogenesis and stress response pathways ***(73,103,132,133)***. It has been proposed that the physiological response to stress conditions could depend on two main factors, the physiological state of the cells and the level (time and intensity) of stress condition ***(134)***. When the stress level exceeds cellular tolerance, the cells will die, but if there is low levels of stress, the cells could induce mechanisms of adaptation ***(134)***. The relationship between different stress conditions and embryogenesis is still not understood, but Lee et al. (***122***) have suggested that undifferentiated cell proliferation could be inhibited and, as a consequence, the embryo production would be stimulated; most likely, the cell is driven into the G_0 stage for its differentiation ***(73)***. Indeed, we still do not know the mechanism by which the embryo formation is induced, but the study of such mechanisms may light the understanding of the signalization processes involved in it ***(73)***.

Low molecular mass compounds secreted into culture medium can inhibit ***(135,136)*** or stimulate ***(137,138)*** somatic embryogenesis. The carrot somatic embryogenesis does not proceed at a high cell density ***(139,140)***, it is not due to nutrient uptake or mechanical injury caused by shearing, but factor(s) responsible for the inhibitory effect were found in the culture medium and their molecular masses were estimated under 3.5 kDa ***(135)***.

Two factors have been purified and identified, the first was an alcohol, 4-hydroxybenzyl alcohol ***(141)*** and the other was vanillyl benzyl ether ***(136)***. On the contrary to the inhibitors, one peptide growth factor has been identified that is involved on induction of somatic embryogenesis; it is called α-phytosulfokine. The addition of α-phytosulfokine to the somatic embryogenesis induction medium causes an increment in the number of embryos produced ***(142,143)***.

Most of proteins secreted into the culture medium are glycoproteins ***(144)***. Among them exist a peroxidase that can restore the somatic embryogenesis inhibited by tunicamycin ***(145)***. Another protein, an endochitinase, was able to rescue the embryo beyond the globular stage and complete its development under nonpermissible temperature –32°C ***(146–149)***. Arabinogalacto proteins (AGPs) are proteoglycans with high carbohydrate content and branched structures. These proteoglycans have been detected in cell culture medium of several plant species. When AGPs from embryogenic culture are added to nonembryogenic cultures, they promote and increase somatic embryogenesis ***(150–153)***.

More recently, a number of genes that play specific roles in the initiation of embryogenesis in plants have been identified ***(154)***. An increased expression of somatic embryogenesis receptor-like kinase 1 (AtSERK1), which encodes a leu-rich repeat (LRR) transmembrane receptor-like kinase (RLK), is found in cells acquiring embryogenic competence, in embryogenic cells, and in early somatic embryos up to about the 100-celled globular stage ***(99)***. Ectopic

expression of AtSERK1 confers sustained embryogenic competence to seedlings under in vitro conditions ***(155)***.

LEAFY COTYLEDON (LEC1 and LEC2) genes encode seed-expressed transcription factors. When they were ectopic expressed, both LEC1 and LEC2 promoted somatic embryo formation on the vegetative tissues of the plant ***(156,157)***. BABY BOOM (BBM) encodes a transcriptional factor belonging to an AP2/ERF family and it is preferentially expressed in developing embryos and seeds. The ectopic expression of BBM induces spontaneously somatic embryos formation in *Arabidopsis* and *Brassica* ***(158)***. WUSCHEL (WUS) is a gain-of- funtion mutation, which is responsible of transition from vegetative or somatic cellular stage to embryogenic stage, and eventually somatic embryo formation. WUS gen encodes to a homeodomain protein involves in specifying stem fate in shoot and floral meristems ***(159)***, also it plays a critical role during embryogenesis ***(76)***. Recently, WUS was identified as target of a chromatin-remodeling ATPases -SNF2-class ATPase SPLAYED, known as SYD-through recruiting of SYS by WUS promoter ***(160)***.

The loss-of-function of PICKLE (PKL) in roots was enough to express embryogenic characteristics, and somatic embryos were formed when the roots were cut and placed on medium culture. PKL encodes a chromatin remodeling factor and it suggests that PKL is a repressor of embryogenic program ***(161)***. AGAMOUS like 15 (AGL15) belongs to family of regulatory factors, which binds specific-sequences to DNA. When it was constitutively expressed, it enhanced production of somatic embryos from zygotic embryos ***(162)***.

A higher number of in vitro experimental systems have been developed to elucidate the mechanisms governing the onset and development of morphogenesis; nonetheless, it still remains entirely unknown ***(77)***. Mutants with defects in the biosynthetic pathway or perception of a specific growth regulator will be very useful on understanding plant morphogenesis ***(77)***.

2.5. Somaclonal Variation

During the massive commercial production of plants, it is important to guarantee their genetic integrity, however after micropropagation, or the plants regeneration from calli or somatic embryos, it has been observed the apparition of phenotypic variation among the produced plants ***(163–165)***, such phenomenon has been called somaclonal variation.

Larkin and Scowcroft ***(165)*** have proposed that the origin of this variation could be from the variability already existed into the original cells or a variation generated during the different step of the in vitro culture. The variation detected between the regenerated plants can be epigenetic ***(166,167)*** or heritable ***(168)***. The epigenetic variation in no heritable through sexual propagation. The heritable variation ranges from gross chromosomal abnormalities ***(169)***, changes in the methylation pattern ***(170,171)***, to point mutations ***(172)***. This variation is stable through out the sexual reproduction ***(173)***. The growth regulators, in particular 2,4-D, has been related with the variation produced in tissue cultures ***(174)***.

Because, in some cases, the somaclonal variation can occur at higher frequencies than chemical ***(175)*** or radiation induced mutation ***(176)***, it can be used as alternative tool to introduce variation into breeding programs ***(172)*** and produce commercial varieties with new traits. Among the major traits isolated so far are resistance to pathogens ***(177–180)***, tolerance to

chilling ***(181–183)***, drought tolerance ***(184,185)***, altitude ***(186)***, and salinity tolerance ***(187)***, content of secondary metabolites ***(188–191)***, herbicide tolerant genotypes in *Triticum aestivum* L. ***(192)***, aluminum resistance ***(193,194)*** and submergence tolerance and other characters of agronomic importance ***(195)***.

2.6. Haploid Cultures

Since the discovery by Blakeslee et al. ***(196)***, and Guha and Maheshwari ***(197,198)*** that embryos with a haploid chromosome number can be obtained, plant scientists are using the production of haploid plants for genetic and mutation studies. Haploids originate from a single gamete, and therefore they are sporophytic plants with the gametophytic chromosome number. Because of this trait recessive characteristics are apparent and the haploid plants can be used to produce homozygous diploid plants useful for plant breeding. This technique has the possibility of shortening the time needed to produce completely homozygous lines compared to conventional breeding. This is particularly important in long reproductive cycle plants such as woody plants and fruit crops ***(199)***. Over 200 varieties in 12 species have been developed using doubled haploid methods ***(200)***.

This technique also can be used to improve agronomically important cereal crops, such as maize, which are still problematic to be genetically engineered by current techniques ***(201,202)***.

3. Applications

3.1. Basic Studies

PTC represents a useful system for the study of the physiological, biochemical, and molecular biology processes in plant cells. The effects of a single factor, on a given process, can be monitoring since the culture conditions can be strictly controlled. One of the best examples of the cell cultures' used for such purposes may be the study of the morphogenetic process. The conditions provided by PTC give us an optimum system for the study of the biochemical and molecular aspects associated with plant differentiation. Also, the response of PTC in response to elicitation is an excellent system to study the plant cells' response to the pathogens attack. A number of genes involved in different aspects of such response, including those in perception of the stimulus as well as in the signalling pathway, have been isolated and characterized in cell cultures from different species ***(203)***.

The changes in the membrane's fluidity and the cellular mechanisms for resistance to metals, salinity, or drought among others, can be analyzed without having the interference of tissue organization ***(204,205)***. The mechanism of the plant cell wall biosynthesis has been widely studied using protoplast as the main tool ***(206–208)***.

One of the fields where PTC has been most useful is the study of secondary metabolism; the use of elicitors in cell cultures has led to the identification of enzymes involved in the biosynthesis of different compounds ***(209–212)***. PTC has been the model for the study and elucidation of the purine salvage pathway in higher plants ***(213,214)*** as well as for the study of different aspects of nutrition of plant cells ***(215)***.

3.2. Massive Plant Production

Considerable progress has been achieved to scale up the culture vessel to propagate thousands of uniform plantlets under in vitro conditions of plants of agricultural, horticultural, medicinal, and forestry importance ***(89)***. Micropropagation has several advantages over conventional methods of vegetative propagation. Among the advantages offered by micropropagation are: 1) with few resources large number of plants can be produced, 2) micropropagation of species may be carried out throughout the year, and 3) micropropagated plants are generally pathogen-free material ***(83)***. Therefore, large-scale plant production through cell tissue and embryo cultures using bioreactors is promising for industrial plant propagation ***(216)***.

Automation of micropropagation in a bioreactor has been advanced as a possible way for reducing costs. Bioreactors provide a rapid and efficient plant propagation system for many species, using liquid media to avoid intensive manual handling. These bioreactor-cultures have several advantages compared with agar- based cultures, with a better control of the plant tissue's contact with the culture medium, and optimal nutrient and growth regulator supply as well as aeration and medium circulation, the filtration of the medium and the scaling-up of the cultures ***(217)***. Since the first use of bioreactor for micropropagation ***(218)***, it has been used for the propagation of several species and plant organs including shoots, bulbs, microtubers, corms, and somatic embryos ***(219)***.

To fully achieve the potential to scale-up of propagation in bioreactors for commercial micropropagation, the understanding of the signals and molecular mechanisms that control morphogenesis in liquid media will be reached. Further basic and applied researches will provide the information necessary for an efficient and economic use of bioreactors for massive plant propagation ***(217)***.

3.3. Production of Virus-Free Plants

Plant diseases are caused by fungi, bacteria, viruses, mycoplasma-like organisms, and nematodes ***(220)***. In crop species that are routinely propagated vegetatively there is usually a severe risk of passing on systemic viral infections during the propagation process ***(221)***. Other pathogens can be transmitted during micropropagation but because of the intimate, intracellular association of viruses with plant tissue, viruses constitute by far the largest threat to vegetative propagated crops.

To carry plants through borders, the international trades require that plants be healthy and pathogen-free. In addition, to avoid losses, the production of plants must begin with healthy plants. However, most of the cultivars of different species are contaminated with different pathogenic agents, such as bacteria, fungi and virus. PTC provides a set of techniques to produce plant pathogen-free.

Virus-free plants of many species and/or cultivars have been produced by culture of meristematic tissue ***(220,222)***, somatic embryogenesis ***(223)***, and grafting ***(224)***. The use either one technique or another will depend on several factors, mainly of the specie's regeneration capacity. The widely used technique to produce virus-free plants is the in vitro meristem-tip culture. This protocol can be used either alone or combined with chemo or thermotherapy ***(225)*** from a wide range of plants ***(220)***.

When chemotherapy is used, the chemicals are applied to plants or tip-meristem cultures for several days. Also it is possible, at the same time, to apply

thermotherapy for several weeks. In general, combinations of both treatments give good results. The amount of the chemicals and the duration of the thermotherapy treatments will depend on the infection's severity, the virus present and the plant specie.

In the grafting technique, the shoots tips are excised from virus-infected plants and grafted onto decapitated rootstock seedlings in a green house. By this method, virus-free plants are produced from the mother plants infected by viruses ***(224)***.

In all the cases, regenerated plants (treated with chemo- and thermotherapy) from tip-cultures, somatic embryogenesis or grafted must be indexed for the viruses. Actually, there is a set of assay to test the presence of virus in plant tissues ***(226)***, such as the double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA), and the reverse transcription polymerase chain reaction (RT-PCR) protocols.

3.4. Embryo Rescue and Dangerous Extinction Plants

Plant breeding takes place through hybridization and selection of new plants. The primary objective in plant breeding is to increase the genetic variability and desirable characteristics in crops. It is done by crossing plants to join together traits in offspring from two different plants. However, in many cases the hybridization is not entirely successful because embryo development is arrested in its development or matureness, producing a weak embryo, which will not germinate; in other cases, the endosperm is not properly formed. Under these conditions embryos die (abortion). When embryos are from a desirable genotype, they can be rescued from being aborted by culturing them under suitable conditions on an artificial nutrient medium ***(227–229)***.

The term of embryo rescue is confinable only to those circumstances where embryos need to be saved, otherwise they are endangered and neither germinate nor form seedlings. When embryos are not getting aborted but they are excise and culture, it is named embryo culture ***(227)***. The aim of embryo rescue technique is to promote the development of an immature or weak embryo into a viable plant; the plant embryo could be isolated by excising from maternal tissue, with ovaries or with ovules. The last two cases are done when embryos can not be removed. The application of embryo rescue culture technique is used to produce interspecific and intergeneric hybrids, recover maternal haploids, obtain plants with genes for disease and insect resistance, for earliness and number of flowers per plant, salt tolerance, herbicide-resistance or tolerance, and other favorable agronomic traits. Additionally, general factors should be considered when embryo rescue technique are used. Among them are genotype and developmental stage of the embryo, culture media, temperature and light, time of culture, plant growth regulators and supplements to the culture medium such as different nitrogen or carbon sources ***(227,229,230)***.

The protocols for embryo rescue are simple and carry out an enormous potential in salvage embryo with advantageous characteristics from crossbreeding. On the other hand, embryo culture technique can also be used to save plant species from extinction. Embryo culture is useful when endangered plant produces a few seeds, seeds can be eaten by insect, birds, or any animal, depredation of its inhabit and plant itself. Many techniques have been employed to propagate for example culture axillary buds ***(231)***, organogenesis ***(232)***, and somatic embryogenesis (**233**).

3.5. Germplasm Collections and Seed Conservation

Every year, an important number of plant species disappear, partly owing to the loss of natural habitat. Plants with a complex reproductive biology are particularly endangered given the reduction of their natural habitats, along with the small sizes of their populations and their prolonged life cycle. Furthermore, endangered, asexually propagated plants have to deal with the reduction of their genetic variability, which increases their susceptibility to an abrupt environmental change or to the introduction of new elements into their ecosystem. In vitro culture represents an alternative to preserve and regenerate endangered species' populations through micropropagation techniques.

Not only tropical, but also exotic species are endangered. The use of improved plant varieties have resulted in the diminished use of traditional varieties of several crops, such as maize, potato, tomato, etc. Quite often, these traditional varieties, which may have been bred for hundreds of years, are adapted to very specific environments or conditions, and are still cultivated by farmers of small communities, isolated by distance or geographical conditions. Besides their cultural value, they may represent an unexplored source for resistance genes to pathogens, insects, drought, etc. In vitro culture provides the technology for preservation of such phytogenetic resources, which may not be adapted to flourish either in nurseries or under greenhouse conditions. In vitro cultures may also be used to preserve extended collections of germplasm in reduced areas under strictly controlled environments. This approach is particularly valuable in the case of plants that are vegetatively propagated. Terminal or axillary buds cultured in vitro may also be preserved by cryogenic techniques, thus minimizing the excessive tissue manipulation required. The preservation of valuable tropical genetic resources, deposited in germplasm banks and maintained by means of in vitro techniques, represents a growing trend in tissue culture applications.

3.6. Secondary Metabolites

Higher plants produce a large number of diverse organic chemicals, some of which are of pharmaceutical and industrial interest. Once the technology for culturing plant cells, in the same way as fungal and bacterial cells was available, the production of natural products were among the first applications to be pursued. The first attempt of the use of plant cells for the production of secondary metabolites took place in the 1950s ***(234–236)***. Later, in Germany and Japan in particular, the development of scale-up techniques for suspension cultures led to development of the industrial application of cell cultures commercially ***(22,237,238)***. However, differing from fungi and bacteria, the pattern of natural products yield by plant cells in culture frequently showed variations from those of organized tissues ***(239)***. Despite numerous attempts by several laboratories around the world, in vitro cell cultures have not turned out to be efficient factories of natural products, because since many of the economically important plant products are neither formed in sufficiently large quantities nor at all by plant cell cultures. However, the culture of organs, such as roots or shoots, lead to the production of complex chemicals in amounts equals or higher than those of the mother plant ***(210)***. In addition, cell cultures have proved to be an invaluable source for enzymes and genes involved in the synthesis of these natural products, as well as for establishing the relationship between cell differentiation and secondary metabolism ***(54)***.

Different approaches to enhance yields of secondary metabolites included the induction of cell lines from highly productive tissues or individuals, the cloning and systematic screening of heterogeneous cell populations for strains with a high biosynthetic potential ***(240)***, and the formulation of culture media composition ***(241)***. Another approach involves selection of mutant cell lines that overproduce the desired product ***(242)***. The use of abiotic factors, such as heat or cold, salts of heavy metals, and UV radiation, and the use of biotic elicitors of plant and microbial origin, such as fungi cell walls, methyl jasmonate, salicylic acid, and nitric oxide, has been shown to enhance secondary product formation ***(243–247)***. The use of immobilized cell technology has also been applied successfully ***(248,249)***.

The better understanding of the tight regulation governing secondary metabolism pathways and also, of its close relationship with branches of the primary metabolism, can now be applied through metabolic engineering strategies to promote the accumulation of valuable natural products in in vitro cultures ***(250–252)***. Metabolic engineering is aimed to improve cell processes, by means of recombinant DNA technology, for commercial purposes. Genes coding for enzymes involved in limiting steps in a pathway may be overexpressed in cell cultures favoring carbon flux through it. Alternatively, new enzymatic activities can be introduced, resulting in the formation of new compounds. Recently, the identification of regulatory genes, controlling the coordinated activation of a set of enzymes involved in secondary metabolism, has opened new possibilities for the genetic manipulation of the whole pathway, by means of a single gene.

3.7. Transgenic Plants

The Green Revolution in the mid-1960s saved hundreds of millions of human lives. However, in the last 10–15 years cereal productivity has declined. Among the different factors for this decrease are the salinization of the soil, the quality and amount of water, and the possibility that crops may have reached the physiological limits of their productivity ***(27)***. On the other hand, plants are known for their wide diversity, which allows them to survive in an ever-changing and often stressful environment. A multiplicity of traits encompasses features that are required for optimal growth and reproduction, and includes aspects of stress tolerance, nutrient use, plant morphology, resistance to pathogens, and the production of secondary metabolites ***(253)***. For crops other quality traits are required, such as improved postharvest storage, flavor, nutritional content and color ***(254)***. However, in the case of crops most of these traits need to be transferred to them. Agriculture techniques allow the transferred of some of these characteristics between plants of the same specie but not among members of different species. The chemically induced fusion of plant protoplasts brought a solution to this problem and opened a new research field ***(255)***. This technique established the possibility of the genetic manipulation of plants by bypassing problems of sexual incompatibility ***(256)***.

The first report of the genetic transformation of plant cells was also published by the Cocking group in the United Kingdom ***(257)*** by the direct delivery of DNA into protoplasts of petunia. Only a few years later, the Ti (tumor- inducing) plasmid was used as a vector for gene transference and production of the first transgenic plant ***(258)***.

Actually, transgenic organisms allow scientists to cross the physical and genetic barriers that separate pools of genes among organisms and produce plants with new traits. At the same time transgenic plants are used as an important research tool ***(259)***. Today, all transformation systems for creating transgenic plants require separate processes for introducing cloned DNA into plant cells, for identifying or selecting those transformed cells and for regenerating and recovering fully developed and fertile plants from the transformed cell ***(259)***. Different techniques to introduce foreign genes into plant genomes have been used; these include the *Agrobacterium* system and the bombardment of DNA-covered microprojectiles. Selective markers, such as antibiotic resistance, chromophores, or fluorochromes, are incorporated to distinguish the transformed tissues from those untransformed. The first generation of genetically modified plants suitable for agriculture was largely produced using antibiotic resistance markers for the preparation of plant transformation vectors or for the plant transformation process itself ***(260)***.

Genetically modified plants would rise from individual cells and, because DNA insertion is a random process, an efficient regeneration procedure could increase the probability of recovering a transgenic plant. For this reason, the use of tissues with a high morphogenetic or embryogenic potential is recommended. Protoplasts can also be used; however, they may require a considerable amount of labor before regenerating a new plant, although with better odds of obtaining actual transformants.

In addition to the traits already mentioned genetically modified crops could also manufacture industrial and pharmaceutical compounds as renewable resources with a production system based on solar energy ***(254)***.

4. Future Progress

Plant cell cultures have become an invaluable tool to plant scientists, cell cultures have remained an important tool in the study of plant biology, and today in vitro culture techniques are standard procedures in most of the plant biology's laboratories. Cell cultures will remain as an important tool in the study of morphogenesis. Molecular, physiological, and biochemical studies on somatic embryogenesis and plant regeneration processes will continue lightening the way cells choose any morphogenetic pathway. In addition to *Arabidopsis* model, the isolation of new mutants from PTC will help in this task.

Cell cultures have remained, and will continue, an extremely important tool in the study of primary metabolism, e.g., the use of protoplasts and vacuoles for the study of the mechanisms of toxicity of heavy metals ***(261)***, as well as the production of resistant plants based in PTC technology ***(262)***.

The development of medicinal plant cell culture techniques has led to the identification of complete pathways of alkaloid biosynthesis ***(263)***. Similar information arising from the use of cell cultures for molecular and biochemical studies is generating research activity on metabolic engineering of plant secondary metabolite production ***(264)***.

The helpfulness of this knowledge goes beyond basic research. Massive propagation of plants represents today an economically rewarding enterprise and this will increase in the following years by incorporating new plants into the market, mainly exotic plants with new flower colors and fragrances. Thanks to the development of genomics, proteomics, and metabolomics, plant

biotechnology is experimenting new and exciting advances. These "omics" approaches, with no doubt, will accelerate the discovery, isolation and characterization of genes conferring new agronomic traits to crops.

Successful genetic engineering programs will focused in the development of new plant varieties with traits that increase the quality of the crops to fight undernourishment and in this way the increase in the yields without the use of chemicals in the field will remain an important task. The new plant varieties must also let an increase in the use of the land for agricultural aim by overcoming problems such as salinity, drought and desertification. PTC technique will also allow the production of roots for food in bioreactors ***(265)***, under controlled conditions. Technologies for cell culture in large volumes for the production of fine chemicals in genetically modified cells cultured should be established. This technique presents advantages over their production in field grown plants that normally occupy considerable extensions of land.

The use of in vitro techniques in embryo rescue during plant breeding, to save dangerous extinction plants, and the construction of germplasm banks to preserve plants with valuable traits will help the continuous necessity of genetic improvement programs.

In summary, the advancements made with this technology have gone well beyond what the pioneers lead by Gottlieb Haberlandt could have imagined.

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