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

Somatic Embryogenesis: Fundamental Aspects and Applications

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Springer

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Chapter 1
Somatic Embryogenesis. An Overview

Víctor M. Loyola-Vargas and Neftalí Ochoa-Alejo

Abstract Somatic embryogenesis is one of the most powerful tools in plant biotechnology. It can be used to produce plants commercially, or to carry out basic studies of cell differentiation, gene expression, molecular genetics, and many others. We present here a compilation of the different chapters of this book.

1.1 Introduction

Initially, the necessity of solving important fundamental questions in plant biology, such as the cell theory and totipotency, led to the development of plant cell, tissue, and organ culture (PTC). However, nowadays PTC represents a set of very powerful biotechnological tools. The applications of PTC include commercial micropropagation of agronomically important plant species, production of haploid and double-haploid plants and disease-free plants, rescue of hybrid embryos or somatic cell fusion from intra- or inter-generic sources for the production of novel hybrid plants, induction of genetic or epigenetic variation for the production of variant plants, and more recently the genetic engineering of plants to produce new varieties, resistant to pests and diseases, as well to improve the quality and quantity of a particular product obtained from a plant. Other applications include genetic modification to produce plants that can remove toxic compounds or test its toxicity (bioremediation) (Hannink et al. 2001; Krämer and Chardonnens 2001), and the use

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of micropropagated shoots to maintain plant viruses. Root cultures can be used to study the interaction of roots with nematodes or mycorrhizas. Recently, plants have been modified by genetic engineers with the objective to increase the yield of cellulose or oil for the production of biofuels (Gressel 2008; Stokstad 2012; Takahashi and Takamizo 2012). Somatic embryogenesis is at the core of some of these biotechnological applications and is the focus of this book.

Gottlieb Haberlandt in 1902 (Haberlandt 1902) set the theoretical basis for plant tissue culture. He proposed that a single cell should eventually be capable of giving origin to a complete and functional plant. This theory has been proved to be right. At the core of this proof is the somatic embryogenesis.

1.2 Somatic Embryogenesis

Plant somatic embryogenesis (SE) is a biological process with both amazing basic and applied aspects. SE occurs in nature. At the edges of the leaves of several species of Kalanchoë appear small bipolar structures, some of them produce these structures constitutively, others by the action of environmental stress, and a third group is a combination of both (Garces and Sinha 2009).

In vitro plant cells can undergo dedifferentiation or redifferentiation to enter a new biological program that gives rise to somatic embryos. This process has raised one of the most important biological questions: which signals change the genetic program of a somatic cell and make it an embryogenic cell?

Numerous factors that affect SE have been investigated in order to understand the basis of this process and manipulate it to develop and establish efficient plant regeneration protocols as a fundamental step for its biotechnological application (Loyola-Vargas et al. 2008). A differentiated and specialized somatic plant cell or a group of somatic cells with specific functions must receive a stimulus from a set of plant growth regulators (PGRs), mainly auxins, perceive it, and then initiate the transduction to the nucleus where the specific regulatory and structural genes will be transcribed and subsequently will be translated into proteins involved in the differentiation that ultimately will lead to the formation of a new somatic embryo. All these changes can be followed at morphological, ultrastructural, genetic, physiological, biochemical, and molecular levels.

The idea of this book is to look somatic embryogenesis in an integrative way covering from the historical aspects of somatic embryogenesis to its applications. It is important to know about the history of those researchers whose contributions led to the development of this field. In Chap. 2 we describe the main facts that led to the historical first papers on SE (Miettinen and Waris 1958; Reinert 1959; Steward et al. 1958).

There are several pathways to initiate somatic embryogenesis (Chap. 3). Unlike the initial belief that all plant cells are totipotents, it has been seen that it is necessary to create appropriate conditions for some of them to regain totipotency. Among the several factors that play a role in the induction of SE are the plant

growth regulators, mainly auxins (Altamura et al. 2016). It is interesting that many species require an initial shot of auxins, but thereafter the auxin must be degraded for SE to proceed (Chap. 10) (Altamura et al. 2016). Clearly the onset of SE depends on a complex network of interactions among plant growth regulators, mainly auxins and cytokinins, during the early proembryogenic stages. Ethylene and gibberellic and abscisic acids pass to play a major role during the late stages of development. Together, the PGRs regulate multiple genes temporally and spatially which release the changes in the genetic program of somatic cells, as well as regulating the transition between each embryonic developmental stage.

In addition to phenotype, the origin of the explant, and genetic background of the plant, several stress treatments such as heavy metals, low or high temperature, osmotic shock, among others, might play a crucial role in SE induction, even in the absence of exogenous PGRs (Chap. 9) (Cabrera-Ponce et al. 2015; Ochatt and Revilla 2016; Salo et al. 2016).

An important concern is the fidelity of the somatic embryogenesis-regenerated plants (Chap. 8). There is an epigenetic reprogramming during the SE and the presence of somaclonal variation among the regenerated plants (De-la-Peña et al. 2015; Mahdavi-Darvari et al. 2015; Nic-Can et al. 2015; Solís et al. 2015). This variation can be the result of chromosomal aberrations, genetic alterations, epigenetic regulations, and transposable elements. The variation can be exploited for good, as selecting stress-tolerant somaclones (Bobadilla Landey et al. 2013; Us-Camas et al. 2014).

Beyond the biotechnological application of SE, it can be used to study the very different aspects of its induction and the development of somatic embryos. An aspect that is central to the study of SE is histology. SE has become an appropriate method for studying the morphophysiological and molecular aspects of cell differentiation (Chap. 26). The understanding of the developmental events during the induction phase as well as the development of somatic embryos is essential to regulate and improve each stage of the SE program efficiently. Anatomical and ultrastructural studies may be useful for the development of protocols more efficient for SE induction, as well as for the cellular mechanisms involved in the acquisition of competence for SE (Konieczny et al. 2012; Quiroz-Figueroa et al. 2002).

The molecular aspects of SE have been studied extensively. In this book, several authors have revised the most recent advances in the field. Transcriptomics of several species has been carried out during the induction of SE and the development of the somatic embryos (Chap. 4). Cotton is the species most studied, but the number of species investigated by this technique is growing every day. The pattern that is emerging from these studies suggests a predominant role of auxins during the induction of SE, as well as for genes like *LEC*, *WUS*, *FUS*, and a set of transcription factors (Shi et al. 2016; Tao et al. 2016; Trontin et al. 2016). The Next Generation Sequencing platforms of nucleic acids can be used together with techniques that allow the isolation of a specific cellular type, such as laser-assisted microdissection. Together these two techniques give us a closer approach to the state of the cell in determined space and time (Chap. 27).

The extreme changes required for the transcription of the genome during the change of a somatic cell to an embryogenic cell need a very active participation of transcription factors. In Chap. 5, authors made an extensive analysis of the state of the art in relation to the participation of transcription factors in this process. An interesting finding is that the most frequent transcription factors found active during the induction of SE belong to the pathways of the metabolism of growth regulators, stress, and flower development (El Ouakfaoui et al. 2010; Guan et al. 2009).

Among all the different mechanisms that regulate the expression of the genes, epigenetics also plays an important role (Chap. 6). Different reports suggest that auxins, in conjunction with the in vitro conditions modify the DNA methylation status in the embryogenic cells. These changes in DNA methylation patterns are associated with the regulation of several genes involved in SE, such as *WUS*, *BBM1*, *LEC,* and several others (De-la-Peña et al. 2015).

After the genes are expressed, all the weight of the process is on the proteins. Posttranslational modifications, protein turnover, and protein–protein interactions are common processes associated with the regulation of proteins. All of them are present during the induction of SE and development of somatic embryos. Proteomic studies carried out while the SE has begun to show the deep mechanism that works during the induction of SE (Chap. 7). One key question is if there is a common protein pattern among different species during the induction of SE (Campos et al. 2016; Mukul-López et al. 2012; Tchorbadjieva 2016).

SE has been induced in many different species; many of them crops of commercial interest. In the second part of the book, the SE of several important crops is analyzed: *Agave* spp., *Bixa orellana*, *Capsicum* spp., *Coffea* spp., *Curcuma*, *Musa* spp., *Zea mays*, lipid-producing plants like *Cocus nucifera* and *Jathropha curcas*, conifers such as *Pinus* spp., and model plants as *Arabidopsis thaliana* (Chaps. 11–22).

The two major applications of SE are scale-up propagation (Chap. 24) and genetic engineering (Chap. 23). Among the different systems to scale up the process of SE, the temporary immersion system has some advantages. It can be automatized to reduce labor and costs, and at the same time to produce high-quality plantlets (Fei and Weathers 2014, 2016; Ibaraki and Kurata 2001). The SE process is a very efficient method to regenerate transgenic plants. SE has been used in conjunction with *Agrobacterium* spp., particle bombardment, and chemical-mediated genetic transformation protocols. All the major annual and perennial crops, as well as model plants, have been transformed using efficient SE systems (Arroyo-Herrera et al. 2008; Bouchabké-Coussa et al. 2013; Canché-Moor et al. 2006; Palomo-Ríos et al. 2012).

Another application analyzed is the use of SE to produce secondary metabolites. The production of secondary metabolites by plants requires highly specialized tissues and a fine regulation and coordination in time and development by the plant (De Luca and St Pierre 2000). In nature, several plant species synthesize and store secondary metabolites in the zygotic seed, suggesting that somatic embryos can do it. In Chap. 25 the most recent discoveries related to the accumulation of secondary metabolites by somatic embryos are presented (Aslam et al. 2010, 2011; Sharma et al. 2015).

1.3 Concluding Remarks

PTC in general and SE in particular have turned into an invaluable tool to plant scientists. PTC has been commercialized around the world, and different companies are using plant tissue culture techniques for the massive propagation of plants. The use of PTC and the development of the omics and epigenetics have allowed the understanding of the basic biological process.

The use of SE leads to the understanding of differentiation, as well as to the genetic mechanisms involved in the transition from one stage to the next. Also it has led to the isolation of genes, proteins, and metabolites involved in the cell differentiation process. The combination of SE and genetic engineering will accelerate the discovery, isolation, and characterization of genes involved in different cellular processes.

From the agronomy side, the most important challenges ahead are the generation of resistant plants to pathogens and pests, as well as to abiotic stresses, increments

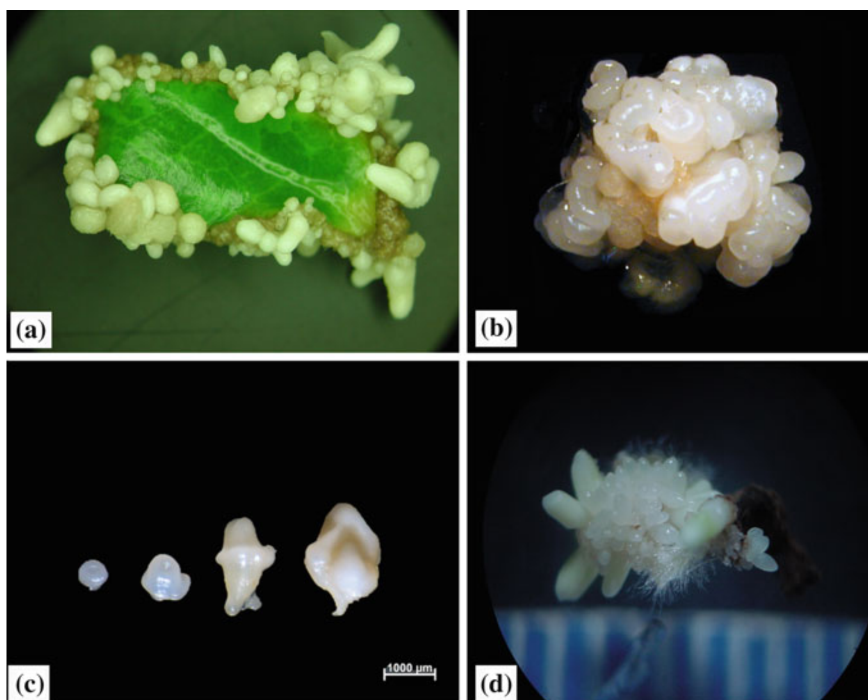

Fig. 1.1 Somatic embryogenesis process in different species. **a** Somatic embryogenesis in a leaf of *Coffea canephora*. **b** Embryogenic mass of *Cocus nucifera*. **c** Different developmental stages of *Musa acuminata* x *Musa balbisiana* genome AAB, subgroup Plantain. **d** Embryogenic mass of *Agave tequilana*. Picture **a** is from the laboratory of Víctor M. Loyola-Vargas. Pictures **b**–**d** are gifts from the laboratories of Carlos Oropeza-Salín, Rosa M. GraciaMedrano from Centro de Investigación Científica de Yucatán and Benjamín Rodríguez-Garay from Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, respectively

in yields in commercial crops, the production of better raw material for biofuel production, as well as the generation of plants capable of absorbing toxic compounds from the environment. In all these cases, SE will play an important role (Fig. 1.1).

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Part I
Basic Aspects

Chapter 2
The History of Somatic Embryogenesis

Víctor M. Loyola-Vargas

Abstract Somatic embryogenesis is used currently as a powerful tool in biotechnology. It is also used to study the development of the embryo. Somatic embryogenesis is a natural phenomenon that was moved from nature to the laboratory by man. The history of the study of somatic embryogenesis is plenty of discoveries of very different natures: from the role of growth regulators, mainly auxins, to the function of the components of the media of culture. In this chapter, a revision of the major contribution to the advance of knowledge of somatic embryogenesis is made.

2.1 Introduction

Embryos, essentials for the reproduction of higher plants, were an important improvement introduced by land plants, even before the development of seeds. This sequence of events probably leads to the production of embryos from different kinds of cells (Radoeva and Weijers 2014). (1) Zygotic embryos originate from gametic cells. (2) Somatic cells give origin to somatic embryos. (3) Cells from the seed primordium can develop into an embryo and subsequently in a seed without fertilization in a process known as apomixis. (4) There is also embryo formation from microspores; in the process called androgenesis, the microspores can form haploid or doubled embryos after the use of different kinds of stresses. (5) In the earliest steps of zygotic embryogenesis, there is a connection between the proper embryo and the maternal tissue, this assembly is known as suspensor. This structure is usually formed by a few cells; however, in some cases suspensor can be generated by thousands of cells and be able to form an embryo (Yeung and Meinke 1993).

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From these different ways to produce embryos, somatic embryogenesis (SE) has become a powerful tool to study and understand the mechanisms of the development of the embryo. In combination with traditional agricultural techniques, SE is used for the propagation of vegetable species of commercial interest and their genetic improvement (Loyola-Vargas et al. 2008). The onset of SE requires a set of interactions between auxins, ethylene, cytokinins, and several other growth regulators to change the genetic program of the cells (Jiménez 2005). Also, other factors can influence the induction and development of somatic embryos, such as the origin of the explant, the physiology of the mother plant, the environmental conditions of the incubation, and the composition of the culture medium (Loyola-Vargas et al. 2008).

A scientific discovery is the result of different efforts, many times from diverse lines of research or thinking. Since the landmark paper by Haberlandt (1902) proposed the theory of totipotency, several research groups were working on the development of plant tissue culture. Almost 60 years later, the discovery of SE was a consequence of the level of development of plant tissue culture at the end of the 50s of the last century. Calli and suspension cultures were well established, and the study of cytokinins (Miller et al. 1955) and auxins (Skoog 1947) was under development. Before the classical papers by Waris (Krikorian and Simola 1999; Miettinen and Waris 1958), Steward et al. (1958b), and Reinert (1959) there were some discoveries that point in the direction of asexual embryogenesis (Levine 1950; Wiggans 1954).

Levine (1950) found that, when indole-3-acetic acid (IAA) was removed from the culture medium, carrot callus regenerated roots and shoots. On the other hand, Wiggans (1954) also found that the carrot cultures were able to produce plantlets when the tissue was transferred to a medium without adenine sulfate, generating buds first and later the roots. Thus, Levine concluded that the removal of auxin led to root formation, followed by whole plantlets, whereas Wiggans deduced that reducing the auxin content of the tissue led to bud formation, followed by plantlets. Both authors concluded that decreasing the IAA in the tissues led to the formation of plantlets through two different routes, root bud in the first case, and viceversa in the second. Its accidental discovery opened an area of research, plenty of challenges, and wonders that have their roots and parallels in the development of plant tissue culture.

During the 30s of the last century, several research groups used *Bryophyllum calycinum* (Crassulaceae), actually known as *Kalanchoë pinnata* Lam. Pers., as a model to study regeneration in plants (Freeland 1933; Howe 1931; Naylor 1932; Yarbrough 1932, 1934). It was observed that at the edges of the leaves of *Kalanchoë* appear small bipolar structures. Several species of this genus can produce somatic embryos constitutively (*K. daigremontiana*). Other species produce plantlets by the action of environmental stress (*K. pinnata*, *K. fedstchenkoi*, *K. strepthantha*, *K. prolifera*, and *K. crenata*) and semi constitutive plantlet-forming species, which produce plantlets both constitutively as well as upon stress induction (*K. gastonis-bonnieri*) (Garces and Sinha 2009). Some additional information of SE in nature was generated from *Tolmiea menziesii* (Yarbrough 1936) and *Crassula multicava* (McVeigh 1938). In all the cases, somatic embryos were found at the edge of the leaves.

SE is a case of accidental codiscovery; in the space of only 15 months, three independent groups published seminal papers on the subject, and a fundamental part of plant tissue culture began its spectacular development. The first of these papers was submitted at the end of 1957 by Harry Waris' laboratory, to the journal Physiologia Plantarum (Miettinen and Waris 1958). Waris worked at the Botanical Institute of the University of Helsinki. The interest of Waris was on the effect of amino acids on the germination of various seeds. He was using amino acids such as alanine, arginine, leucine, α-aminobutyric acid, valine, asparagine, and others. He worked on the hypothesis that the use of different amino acids should change the balance of the proteins synthesis during the development of the new plantlets, and have morphogenetic consequences (Miettinen and Waris 1958; Waris 1957).

Waris found that after 3–4 months in the presence of 13.32 or 53.28 mM glycine the original plantlets almost died. However, new green plants emerged from "minute grains [and were] formed by some root tips of the original, morbid plant" This report was a presentation at the meeting of the Biochemical, Biophysical and Microbiological Society of Finland in March of 1957 (Waris 1957). Later, on November, Waris made a presentation to the Finnish Academy of Sciences entitled "A chemically-induced change in the morphogenesis of a flowering plant"; this talk was published in the Proceedings of the Finnish Academy of Science and Letters. In this talk, Waris presented data of the effect of glycine in carrot. This effect was the production of somatic embryos of carrot (Krikorian and Simola 1999). The next month, and with the help of Dr. Jorma K. Miettinen, a biochemist, submitted a paper to the journal Physiologia Plantarum entitled "A chemical study of the neomorphosis induced by glycine in *Oenanthe aquatica*" (Miettinen and Waris 1958). Waris did not name the structures as "embryos" or something like this, he used the term neomorph and neomorphosis for somatic embryo and SE, respectively. During the next 4 years, Waris published other two papers related to the neomorphosis of the genus *Oenanthe*. The Waris papers not only were among the first papers in SE but also they were the first papers that documented the effect of the nitrogen source on the morphogenesis of plant cells.

At the same time Frederick C. Steward was working at the Department of Botany at Cornell University, on the different ways suspension cultures can grow and multiply. He submitted two papers on June 1, 1958 and they were published back-to-back in the American Journal of Botany (Steward et al. 1958a, b). In the first paper, Steward et al. (1958b) reported the characterization, in particular, the growth, and shape of freely suspended carrot, peanut, and potato cells. The second paper describes the easy formation of roots in the liquid medium, containing coconut milk, from the small aggregates of the suspended cells. After the roots had been cultivated on semisolid medium, they were able to develop shoots opposite to the position of the roots and developed into complete plantlets. Steward et al. (1958a) documented a very ordered process from the sheath of cambium-like cells, to the embryo-like structure. This process is reminiscent of the formation of plantlets from zygotic embryos. Steward observed correctly the formation of vascular tissue before the formation of the root and inferred that such development was characteristic of a "proembryo."

The third paper of this history was published by Jakob Reinert, who was working at the Botanisches Institut der Universität in Tübingen, Germany (Reinert 1959). The paper was submitted on February 12, 1959 to the journal Planta. Reinert was able to induce the formation of carrot shoots, by transferring the callus tissue with roots into a white medium with several organic compounds including inositol, choline, riboflavin, biotin, ca-pantothenate, ascorbic acid, hypoxanthine, and a plethora of amino acids in addition to IAA and 2,4-dichlorophenoxy acetic (2,4-D). The complexity of this "synthetic medium" was antecessor of the complete media published in the next decade (Linsmaier and Skoog 1965; Loyola-Vargas 2012; Murashige and Skoog 1962). Altering this synthetic medium by changing the culture medium, Reinert was able to manipulate the formation of roots and shoots. He deduced, correctly, that the shoots come from "adventitious bipolar embryos."

Confirmations of these landmark papers began to appear in the literature just a couple years later (Kato and Takeuchi 1963; Wetherell and Halperin 1963). At the same time, the first pictures of somatic embryos were published (Kato and Takeuchi 1963; Wetherell and Halperin 1963). The source of the explants extended to *Datura innoxia* anthers (Guha and Maheshwari 1964). In the following years, more species and more different explants were used to study the SE process (Table 1.1). However, *Daucus carota*, had remained the most studied, and become The Model for the study of SE (Fujimura 2014).

Table 1.1 Summary of successful somatic embryogenesis during the first years after its discovery

Species	Explant	Year	Reference
Oenanthe aquatica	Somatic embryos	1958	Miettinen and Waris (1958), Waris (1959)
Daucus carota	Suspension cultures	1958	Steward et al. (1958a)
Daucus carota	Callus	1959	Reinert (1959)
Hordeum vulgare	Zygotic embryos	1961	Norstog (1961)
Cuscuta reflexa	Zygotic embryos	1961, 1962	Maheshwari and Baldev (1961, 1962)
Dendrophthoe falcata	Zygotic embryos	1962	Johri and Bajaj (1962)
Daucus carota	Callus	1963	Kato and Takeuchi (1963)
Daucus carota	Callus	1963	Wetherell and Halperin (1963)
Daucus carota	Seeds	1964	Steward et al. (1964)
Datura innoxia	Anthers	1964	Guha and Maheshwari (1964)
Ranunculus sceleratus	Suspension cultures/stem	1965	Konar and Nataraja (1965a, b)
Nicotiana tabacum	Callus	1965	Haccius and Laksmanan (1965)
Daucus carota *Apium graveolens*	Callus	1966	Reinert et al. (1966)
Cichorium endivia	Callus	1966	Vasil and Hildebrandt (1966a)
Petroselinurn hortense	Callus	1966	Vasil and Hildebrandt (1966b)
Solanum melongena	Callus	1967	Yamada et al. (1967)

2.2 The Effect of Different Factors on the Induction of the Somatic Embryogenesis

A set of various factors such as the genotype and physiology of the donor plant, the explant, the composition and pH of the culture medium, the cell density, the incubation conditions, and the growth regulators, among others, affect the embryogenic response of tissues.

Three important discoveries, few years later, were: (a) the inhibitory effect on the induction of SE by 2,4-D, (b) the necessity to dilute the cell suspension culture, and (c) the effect of ammonium in the induction of SE (Halperin and Wetherell 1965). When the cultures derived from root phloem grew without ammonium, they did not form somatic embryos when the cultures were transferred to the proper conditions for the SE induction. These calli grew very well in the presence of only nitrate, but they were not able to form any embryogenic structure. However, the addition of small amounts of ammonium initiated the SE process (Halperin 1966). It is known that the amount of nitrogen in the medium is important. The relationship between nitrate and ammonium is part of the drive force leading the process of cell differentiation, in particularly SE (Fuentes-Cerda et al. 2001; Reinert et al. 1967; Tazawa and Reinert 1969). Casein hydrolysate was also tested successfully as nitrogen source during the induction of SE (Halperin 1995). However, there was controversy over the role of the nitrogen source on the SE. Reinert et al. (1967) argued that ammonium was no necessary, and that the important thing was the amount of the nitrogen source and not the form of nitrogen. However, the same group found that the *D. carota* cells cultivated in high levels of ammonium, the induction medium for SE, contained increasing levels of this compound; the ammonium was scarcely detected into the cells cultivated in the absence of ammonium which did not induce SE (Tazawa and Reinert 1969). It was speculated that the intracellular ammonium was necessary for the induction of SE. In a more systematic study carried out by Wetherell and Dougall (1976), it was found that glutamine, glutamic acid, urea, and alanine could, each one of them, partially replace ammonium as a supplement to nitrate. Other explanation for the effect of ammonium was that this compound could change the pH of the medium (Smith and Krikorian 1990). The Krikorian's laboratory (Smith and Krikorian 1990) was able to culture a *D. carota* proembryogenic mass on a growth regulators-free semisolid medium containing one mM of ammonium as the only nitrogen source. The titrated of the medium to pH 4 produced the best growth and multiplication and the culture stayed as proembryogenic mass. If the pH was increased to 4.5 or higher the proembryos developed into complete somatic embryos (Smith and Krikorian 1990). However, it was not possible to discard the effect of other factors, such as the potassium present in the medium culture (Brown et al. 1976).

Ammonium is not the only factor-driven SE. The size of the clump seems to be important. Clusters of 20–100 cells (47–81 µm) are more suitable to produce somatic embryos (Fujimura 2014; Halperin 1966). However, Kato and Takeuchi in

Japan (Kato and Takeuchi 1963) were able to regenerate plantlets from somatic embryos originating from friable small callus clumps. When single cells from these clumps were cultivated in fresh medium, no result was obtained. Nevertheless, the presence of a clump of growing cells promotes the complete development of the single cell into a plantlet. We know this process as a nurse culture. There is (are) a factor or factors that are produced by the clump of cells, which is (are) necessary for the growth of the single cell and is (are) diffused into the culture medium.

During the late 90s and earliest 20s, the laboratory of Kobayashi published a series of papers showing that, to induce the SE in *D. carota* suspension cultures was necessary to dilute the cell concentration. After several days of culture, the spent medium contained compounds secreted by the cells. Some of these compounds, such as the 4-hydroxybenzyl alcohol, inhibited the SE (Higashi et al. 1998; Kobayashi et al. 1999, 2000a, b, 2001). Other compounds, with similar inhibitory effect were found during SE induction in *Larix leptolepis* (Umehara et al. 2004). These inhibitory molecules were identified as vanillyl benzyl ether (Umehara et al. 2005) and 4-[(phenylmethoxy) methyl] phenol (Umehara et al. 2007). In our laboratory, we found that several phenolic compounds secreted during the induction of SE in *Coffea arabica*, can inhibit the SE of several species (Nic-Can et al. 2015).

"The auxin factor" or the "auxin paradox" was known very early in the research of the SE. Exposition of the embryogenic callus to concentrations of 2,4-D higher than 0.45 μM did not allow the formation of the somatic embryos (Halperin 1964; Halperin and Wetherell 1965; Reinert and Backs 1968). On the other hand, the presence of auxin was necessary to induce the change in the genetic program of somatic cells (Fujimura and Komamine 1979). Auxin removal was essential to allow the induction of SE and the further development of somatic embryos (Halperin 1964). An important problem of the production of somatic embryos is their heterogeneity. In a callus tissue, it is possible to find a mixture of proembryogenic tissue and somatic embryos at different developmental stages. Nevertheless, it is conceivable to "synchronize" the production of somatic embryos. An early method was the sieving of suspension cultures. The best size range to produce synchronized cultures was between 45–75 μm (Halperin 1964). Other factors, in addition to the size of the clumps, to produce synchronized cultures are the species, a low ammonium content in the induction medium, and the frequent renewing of stock culture (Fujimura 2014).

An early observation during the induction of SE was the changes in the morphology of the cells that occur during the process. Most of the cultures possess two kinds of cells: cells highly vacuolated, which in general do not divide, and meristematic small cells. These cells are spherical, and densely packed with starch (Quiroz-Figueroa et al. 2002). During the induction of SE, there are several ultrastructural changes, including a remarkable increase in free ribosomal content, decrease in endoplasmic reticulum, loss of polyribosomal aggregates, and appearance of microtubules (Halperin and Jensen 1967).

2.3 Molecular Biology of Somatic Embryogenesis

Since a very rapid cell division occurred just before the formation of the globular embryo (Fujimura and Komamine 1982), it is expected a high metabolic activity, including the biosynthesis of macromolecules. The first papers related to the role of protein synthesis, and transcription of genes began to appear at the beginning of the 80s. The laboratory of Raghavan published several articles related to the biosynthesis of proteins and nucleic acids. They found that the rate of RNA synthesis increased in carrot embryogenic cells following their transfer to fresh medium (Sengupta and Raghavan 1980a). The rate of rRNA synthesis in the embryogenic cells was lower than that in the nonembryogenic cells. However, embryogenic cells synthesized mRNA at a higher rate than the nonembryogenic cells during the first 96 h (Fujimura and Komamine 1982; Sengupta and Raghavan 1980b). The ratio of RNA to DNA of both cultures increased in the early stage of the culture. The ratio increase was higher in the embryogenic tissue, suggesting that the embryogenic culture was metabolically more active (Masuda et al. 1984) probably because the replicon size was much reduced in the embryogenic samples (Fujimura and Komamine 1982). The use of wheat germ system to translate the mRNA extracted from embryogenic and nonembryogenic cells, in combination with two-dimensional gel electrophoresis and autoradiograms, was let to determinate that there were small, but clear differences between both systems (Fujimura and Komamine 1982), suggesting a regulation at the transcriptional level.

The group of Dr. Z. Renee Sung at Berkeley made seminal contributions to understand the macromolecular processes during the induction of SE. The comparison of the protein profile, between carrot callus and somatic embryos derived from them, showed the presence of two proteins present only in the embryogenic tissues (Sung 1983; Sung and Okimoto 1981). The switch to turn on and off the biosynthesis of these two proteins was the absence or presence of 2,4-D, respectively. At the beginning of these studies, it was fascinating the fact the proteic profiles were very similar in undifferentiated and nonembryonic cells (Choi and Sung 1984). Using antibodies against an extract of carrot somatic embryos, Choi et al. (1987) was able to detect some proteins produced in the onset of SE. This technique lead to the isolation of three cDNA clones, one of them codified for a 50 kDa protein that was present also in the embryogenic tissues of cassava, peach, and maize (Choi et al. 1987).

Suspension cultures of carrot secreted considerable amounts of protein into the culture medium, most of them glycoproteins. This secretion can depend on the culture conditions and the initial source of the explant, among other factors. Most of the first studies were carried out in carrot. The electrophoretic analysis of the secreted proteins showed the presence of two glycoproteins of 65 (GP65) and 57 (GP57) kDa. The presence of 2,4-D, which inhibits the induction of SE, led to the secretion of GP57 in both embryogenic and nonembryogenic cultures. In the absence of 2,4-D embryogenic cultures formed somatic embryos and secreted GP65

(Satoh et al. 1986). In other study, proteins of 13, 17, 29, 38, and 46 kDa were found in the culture medium during the induction of SE in carrot (De Vries et al. 1988). The secretion of these proteins was reduced or absent in the presence of 2,4-D. On the other hand, the presence of 2,4-D promoted the secretion of a complete different set of proteins with molecular masses of 27, 36, 40, 44, 48, and 72 kDa. The proteins of 29, 46, 72, as well as a 52/54 kDa were high-mannose type glycoproteins (De Vries et al. 1988). Other particular protein secreted by embryogenic cultures was a protease inhibitor with a molecular mass of about 12.8 kDa (Carlberg et al. 1987). This protease inhibitor inhibited trypsin activity. The inhibitor was present in both, nonembryogenic and embryogenic cultures, but was released into the culture medium only by the embryogenic cultures (Carlberg et al. 1987).

SE is completely blocked by inhibitors of the glycosylation, such as tunicamycin and deoxynojirimycin. Tunicamycin treatment resulted in the presence of deglycosylated forms of the 46- and 52/54-kD (De Vries et al. 1988) and the inhibition of SE. The inhibition of SE by tunicamycin can be reverted by addition of extracellular proteins from untreated embryogenic cultures.

The qualitative differences between the electrophoretic protein patterns from nonembryogenic and embryogenic cultures, have also been found in calli from *Oryza sativa* (Chen and Luthe 1987) and embryogenic calli of *Pisum sativum* where two proteins of 45 and 70 kDa were found (Stirn and Jacobsen 1987).

Other proteins, such as acid phosphatase or with α-mannosidase activity, were found in the culture filtrate of both embryogenic and nonembryogenic lines, which suggest that the release of the inhibitor from embryogenic lines was not due to cell lysis.

2.4 Concluding Remarks

SE is a very important and interesting biological process. Scientists have been able to carry this process from nature to the laboratory. The history of this development shows how great discoveries are the result of multiple areas of knowledge, and the necessity of a conceptual framework.

The basic knowledge of the SE process was initially established empirically. The role of growth regulators, majorly auxins, was firmly settled very soon. The role of the nitrogen source, as well as the presence of compounds that are secreted into the culture medium, showed the importance to study all the factors possible involved in the induction of SE. The molecular aspect of SE was later recognized, and showed the importance of studying the biochemical and molecular aspects of the induction of SE.

Today, it is known that there are several factors that can induce SE. Most of these factors are related to stress (De-la-Peña et al. 2015). Among the different kinds of stress that can induce SE are cold, heat, osmotic shock, water deficit, heavy

metals, nutrient starvation, wounding, medium culture dehydration, ultraviolet radiation, and pH (De-la-Peña et al. 2015). The question that raises this knowledge is: what is the common factor that leads to the induction of SE?

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Chapter 3
The Many Ways of Somatic Embryo Initiation

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Abstract It is widely believed that all cells of a plant are totipotent and can regenerate the whole organism. This view is supported by uncountable experimental observations demonstrating the regrowth of whole plants from various explants, even from single cells. However, recent investigations have demonstrated that plant regeneration may proceed via transdifferentiation of meristems or root meristem-like callus tissues due to adult stem cells present all over the plants. These pathways do not start from single totipotent cells. There is a strong argument for plant cell totipotency, however, and that is somatic embryogenesis. During this process, differentiated somatic cells change their fate to develop into an embryo. Animal embryos can develop only from the totipotent zygote and its direct descendants (this cell state can also be artificially produced by injecting a somatic cell nucleus into an egg cell cytoplasm during cloning). Plant cells have to be induced to start somatic embryogenesis and not all of them are competent to respond properly to the induction. In conclusion, plant cells cannot be considered as totipotent per se, but some of them can regain totipotency under appropriate conditions. In addition, accumulating evidence supports the view that even somatic embryo development can follow various initial steps not necessarily requiring cellular totipotency. Although, there are experimental observations to support the progression of somatic embryogenesis through a zygote-like state in certain experimental systems, in other instances the reorganization of several cells into an embryo was described. The direct release/induction of the embryo development program in vegetative plant cells may represent a third pathway of somatic embryo development. In this chapter, a brief literature review is provided to support the above view on plant cell totipotency as well as on the various ways to start somatic embryogenesis.

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