REVIEW

REGULATION OF *IN VITRO* SOMATIC EMBRYOGENESIS WITH EMPHASIS ON THE ROLE OF ENDOGENOUS HORMONES

VÍCTOR M. JIMÉNEZ[1]

CIGRAS, Universidad de Costa Rica, 2060 San Pedro, Costa Rica.

ABSTRACT - Different aspects of *in vitro* somatic embryogenesis regulation are reviewed in this paper. General aspects, such as terminology, uses, stages of development and factors associated with the somatic embryogenesis, are described. Although a brief description of the effects of the addition of different plant growth regulators to the culture medium is given, the article is centered on the effect that endogenous hormone concentrations in the initial explants and in the tissue cultures derived from them could play in the induction and expression of somatic embryogenesis. It is significant that few systematic studies have been conducted, in which different species and hormone groups were compared in cultures with and without embryogenic capacity. Moreover, the lack of correlation between the results presented in different studies indicates that the hormone content of the cultures is not the only factor involved.

ADDITIONAL INDEX TERMS: *In vitro* morphogenesis, embryo regeneration, embryogenic competence, plant growth regulators, hormones.

ABBREVIATIONS - 2,4-dichlorophenoxyacetic acid (2,4-D); abscisic acid (ABA); gibberellic acid (GA_3); indole-3-acetic acid (IAA); N^6-(Δ^2-isopentenyl) adenine (iP); N^6-(Δ^2-isopentenyl) adenosine (iPA); proembryogenic mass (PEM); zeatin (Z); zeatin riboside (ZR).

REGULAÇÃO DA EMBRIOGÊNESE SEMÁTICA *IN VITRO* COM ÊNFASE DO PAPEL DE HORMONIOS ENDÓGENOS

RESUMO - Neste trabalho se faz uma revisão de diversos aspectos da regulação da embriogêneses somático in vitro. Vários aspectos gerais a este fenômeno tem sido discutidos, tais como a definição de terminologia, descrição de eventuais aplicações, seus estados de desenvolvimento e outros fatores associados com sua indução e expressão. Embora se faça uma breve descrição do efeito da adição de diferentes reguladores de crescimento ao meio de cultivo, o artigo está centrado no efeito que as concentrações hormonais endogênas nos explantes iniciais e nos cultivos in vitro derivados deles podem ter na indução e expressão da embriogênese somática. Tem de se fazer ênfase na pouca quantidade de estudos sistemáticos realizados neste tema que comparem em várias espécies e diferentes grupos hormonais em cultivos com e sem competência embriogênica. Finalmente, indica-se que a falta de correlação entre os resultados destes poucos trabalhos parece indicar que os conteúdos hormonais endôgenos não são os únicos fatores envolvidos neste fenômeno.

TERMOS ADICIONAIS PARA INDEXAÇÃO: Morfogêneses in vitro, regeneração de embriões, competência embrogênica, reguladores de crescimento, hormônios.

Received: 18/12/00 - Accepted: 11/06/01

1. CIGRAS, Univesidad de Costa Rica, 2060 San Pedro, Costa Rica. e-mail: vjimenez@cariari.ucr.ac.cr

INTRODUCTION

There are two alternative mechanisms by which an explant can regenerate an entire plant, namely organogenesis and somatic embryogenesis. Generally, in the first case, shoots and roots form sequentially and in response to appropriate culture conditions (mainly to the type and concentration of plant growth regulators present in the culture medium). This type of development is also characterized by the presence of vascular connections between the mother tissue and the regenerating section (Terzi & Lo Schiavo, 1990).

On the other hand, somatic embryogenesis can be described as the process by which haploid or diploid somatic cells develop into structures that resemble zygotic embryos (i.e., bipolar structureswithout any vascular connection with the parental tissue) through an orderly series of characteristic embryological stages without fusion of gametes (Williams & Maheswaran, 1986; Emons, 1994; Raemakers *et al.*, 1995). One striking characteristic of the somatic embryo is its continuous growth resulting from the absence of developmental arrest (Faure *et al.*, 1998). Both processes, organogenesis and somatic embryogenesis, have been reported to occur in the same explant (He *et al.*, 1990), but originate from particular tissue layers or cells within explants (Osternack *et al.*, 1999).

A number of specialized examples of somatic embryogenesis have been reported to occur *in vivo*, from both reproductive tissues such as the nucellus and synergid cells, and somatic tissues such as somatic cells in ovules (apomixis) and leaf margins (Williams & Maheswaran, 1986; Merkle *et al.*, 1990, and also reviewed by Sharma & Thorpe, 1995). However, somatic embryogenesis is nowadays best known as a pathway to induce regeneration from *in vitro* tissue cultures.

Although it is widely accepted that the first descriptions of *in vitro* somatic embryo production were carried out independently by Steward *et al.* (1958) and Reinert (1959) working with carrot, recently Krikorian & Simola (1999) underlined the less noticed pioneer role of Harry Waris, working with *Oenanthe aquatica* (Umbelliferae). These early studies were very significant, because they confirmed Haberlandt's prediction that embryos can arise from single cells in culture (i.e., cellular totipotency) (Kiyosue *et al.*, 1993; Höxtermann, 1997). The early history of somatic embryogenesis has been reviewed elsewhere (Raghavan, 1986; Halperin, 1995; Krikorian & Simola, 1999).

Since these first studies, the number of higher plant species from which somatic embryos could be obtained and regenerated has continuously increased. This phenomenon has been documented in at least 200 Gymnosperm and Angiosperm species (reviewed by Raemakers *et al.*, 1995). Some species, however, are more recalcitrant than others regarding both initiation of embryogenic cultures and regeneration of plants (Rao, 1996).

Finding the right conditions to induce somatic embryogenesis in different species and cultivars is yet, for the greater part, based on trial and error experiments (Jacobsen, 1991; Henry *et al.*, 1994): analyzing the effect of different culture conditions and media and modifying especially the type and levels of plant growth regulators. However, the role that the genotype and its physiological condition play in this process has seldom been studied. This is a limitation for improvement of long-term tissue cultures, to the point that it restricted the development of protoplast fusion techniques and delayed for years the development of genetic transformation techniques beneficial for plant breeding of monocots (Henry *et al.*, 1994). Only recently, by employing modern methods of hormonal manipulations (transgenic and habituated callus) in combination with precise and sensitive methods for hormone determination, has some progress been achieved in this area (Bangerth, 1993). Most of the success achieved so far in understanding the mechanisms that govern the efficient regeneration of plants through somatic embryogenesis has been accomplished with model plant species and the transfer of these new technologies to major crop species has been slow and difficult (Vasil, 1987). The successful induction of somatic embryos and

subsequent recovery of viable plants is not routine or efficient for the majority of species (Merkle *et al.*, 1995). The ability to understand the mechanisms involved in the induction and expression of somatic embryogenesis in different species will increase the number of genotypes capable of regeneration by this process.

APPLICATIONS OF SOMATIC EMBRYOGENESIS

Somatic embryogenesis is a very valuable tool for achieving a wide range of objectives, from basic biochemical, physiological and morphological studies, to the development of technologies with a high degree of practical application.

One of the main uses of somatic embryogenesis constitutes its employment as an approach to investigate the initial events of zygotic embryogenesis in higher plants. Perhaps the primary reason for the limited progress in understanding the developmental events in plant embryos is that zygotic embryos of higher plants consist of several tiny cells that grow within maternal tissues, such as the flowers or immature fruits, and it is quite difficult to collect sufficient embryos for biochemical, physiological and morphological analyses of the biological events that occur early in the developmental process. Somatic embryos provide a good model system by which such problems could be circumvented. The knowledge of many of the events that occur during the early embryogenesis has resulted from experiments on somatic embryogenesis of a few plant species (de Jong *et al.*, 1993; Kiyosue *et al.*, 1993; Zimmerman, 1993).

The mass propagation of plants through multiplication of embryogenic propagules is the most commercially attractive application of somatic embryogenesis (Merkle *et al.*, 1990). Somatic embryogenesis has many advantages over organogenesis in this respect: (a) it permits the culture of large numbers of 'reproductive units'– e.g., 60,000 to 1.35 million somatic embryos per liter of medium– with the presence of both root and shoot meristems in the same element; (b) the modeof culture permits easy scale-up transfers with low labor inputs since embryos can be grown individually and freely floating in liquid medium; (c) unlike shoots, somatic embryos frequently originate from single cells and the embryogenic cultures can be synchronized and purified so that one can deal with practically pure cultures of homogeneous material; and, (d) plants derived from somatic embryos are less variable than those derived by way of organogenesis (Ammirato, 1987; Merkle *et al.*, 1990; Terzi & Lo Schiavo, 1990; Osuga *et al.*, 1999). The last point mentioned above could be explained by an intolerance of somatic embryos to mutations in any of the numerous genes that must be necessary for a successful completion of ontogeny (Ozias-Akins & Vasil, 1988), while vegetative meristems may be more tolerant to mutations and epigenetic changes (Merkle *et al.*, 1990).

Another application is in the production of plants with different levels of ploidy; i.e., obtaining haploid embryos by cultivating anthers and raising triploids from endosperm have been suggested and, to a very limited extent, exploited (Terzi & Lo Schiavo, 1990). Also, success in inducing dormancy and the accomplishment of long-term storage, together with the achievement of encapsulation of somatic embryos, has opened up the possibility for their use in the synthetic seed technology (Gray & Purohit, 1991; Gray *et al.*, 1995; Litz & Gray, 1995).

The use of embryogenic callus and cell suspension cultures, as well as somatic embryos themselves as a source of protoplasts, has been exploited for a range of species, taking advantage of the totipotency of these embryogenic cultures (Merkle *et al.*, 1990). Embryogenic cultures have proven to be especially valuable in providing a source of regenerable protoplasts in graminaceous species (Finch *et al.*, 1991; Chang & Wong, 1994; Lyznik & Hodges, 1994; Funatsuki *et al.*, 1996), *Citrus* species (Jiménez, 1996), and forest trees (David, 1987; McCown & Russell, 1987).

Gene transfer into embryogenic plant cells is already challenging conventional plant breeding, and has become an indispensable tool for

crop improvement. One of the most important prerequisites for genetic manipulation of plants *in vitro* is the ability to grow somatic cells in sterile plant growth medium and to regenerate viable plants from these cultures. Somatic embryogenesis, therefore, is a more efficient pathway for studies involving production of genetically transformed plants (Litz & Gray, 1995; Vicient & Martínez, 1998).

Secondary or recurrent embryogenesis, which is reported in at least 80 species (reviewed by Raemakers *et al.*, 1995), offers a great potential for *in vitro* production of embryo metabolites, such as lipids and seed storage proteins. However, since the production costs are still higher than the extraction from natural seeds, this technology is not yet commercially viable (Merkle *et al.*, 1990).

Finally, the embryogenic development of somatic cells appears to be more sensitive to the application of exogenous chemical compounds than the growth of whole plants or even callus cultures. This offers the possibility of using *in vitro* screening and selection procedures to identify plant genotypes resistant to certain factors, such as aluminum toxicity or toxins produced by pathogens (Merkle *et al.*, 1990).

DEVELOPMENT OF SOMATIC EMBRYOGENESIS

Somatic embryogenesis development has been divided into two main phases, namely, the one whereby differentiated somatic cells acquire embryogenic competence and proliferate as embryogenic cells, and the phase whereby the embryogenic cells display their embryogenic competence and differentiate into somatic embryos. Both processes appear to be independent from each other and thus to be influenced by different factors. The former phase, called 'Phase 0' by Komamine *et al.* (1992), 'determination phase' by Rao (1996) and'induction phase' by Dodeman *et al.* (1997), has no direct counterpart in zygotic embryogenesis (Emons, 1994). In this work, the term 'induction' will be used to designate the first phase and 'expression' for the second one.

The term 'embryogenic cell' is restricted to those cells that have completed their transition from a somatic (non-embryogenic) state to one in which no further exogenously applied stimuli, such as the application of growth regulators, are necessary to produce the somatic embryo (Komamine *et al.*, 1992; de Jong *et al.*, 1993). The cells that have reached this transitional state and have already started to become embryogenic, but that still require exogenously applied stimuli, are designated as competent cells (Mordhorst *et al.*, 1997).

Induction of somatic embryogenesis

Induction of embryogenic growth in carrot cultures, as well as in cultures of many other species, appears to occurin one of two ways. Somatic embryos can form directly on the surface of an organized tissue such as a leaf or stem segment, zygotic embryo, from protoplasts or from microspores (i.e., the cells may be considered as already determined for embryogenic development, needing only permissive conditions to allow its expression). They can also form indirectly via an intermediary step of callus or suspension culture (in these cases a more complex medium should be used, including additional factors to induce dedifferentiation and reinitiation of cell division of already differentiated cells before they can express embryogenic competence) (Williams & Maheswaran, 1986; Ammirato, 1987; Emons, 1994).

Direct and indirect somatic embryogenesis have also been considered as two extremes of a continuum (Williams & Maheswaran, 1986; Carman, 1990). Once induction of embryogenic determined cells has been achieved, there appear to be no fundamental differences between indirect and direct somatic embryogenesis (Williams & Maheswaran, 1986). Emons (1994) argued that in many systems in which embryogenesis has been designated as indirect, the embryogenic callus is composed of young embryos (pre-embryogenic masses or (pre-)globular embryos) and that their further development dependson the duration of the application of the inductive stimulus. If that period is relatively

short, the process will be direct and if it is long, then the process will be indirect. On the other hand, in other studies, direct embryogenesis has beenused to describe the formation of an embryo from a single cell without an intervening callus stage, although the embryo has arisen by means of the dedifferentiation of a differentiated cell within the explant (c.f. references in Ammirato, 1987).

In the carrot model described by Komamine *et al.* (1992), competent single cells (State 0) formed embryogenic cell clusters (State 1) in the presence of auxin. These single cells are considered as predetermined for embryogenesis. During this phase, the cell clusters gained the ability to develop into embryos when auxin was removed from the medium, leading to the development of State 1 cell clusters (Nomura & Komamine, 1985; Komamine *et al.*, 1992). Embryogenic cells are unique: superficially they resemble meristematic cells, though they generally are smaller, more isodiametric in shape, have larger, more densely staining nuclei and nucleoli, and have a denser cytoplasm (Williams & Maheswaran, 1986; Carman, 1990).

Expression of somatic embryogenesis

Once the inductionof an embryogenic state is complete, the mechanisms of pattern formation that lead to the zygotic embryo are common to all other forms of embryogenesis (Mordhorst *et al.*, 1997). Thus, somatic and zygotic embryos share similar gross ontogenies, with both typically passing through globular, heart-shaped and torpedo-shaped stages in dicots, or globular, scutellar (transition), and coleoptilar stages in monocots (Gray *et al.*, 1995; Toonen & de Vries, 1996). Schiavone & Cooke (1985) described an intermediate growth stage between globular and heart-shaped embryos, that they termed the oblong embryo. Although heart- and torpedo-shaped embryos have traditionally been defined as separate stages of the embryo development, the distinction between them is apparently based on the difference in size (Schiavone & Cooke, 1985). These shape-based differences have their origin in the particular development of each of the two major groups of flowering plants, i.e., monocot embryos initiate only a single cotyledon and consequently do not proceed through a heart-shaped stage (Kaplan & Cooke, 1997). Yet another type of embryo development takes place in conifers (Tautorus *et al.*, 1991), which includes three stages: globular, early cotyledonary and late cotyledonary embryos (Dong & Dunstan, 2000).

In the aforementioned model of Komamine *et al.* (1992), describing the early process of embryogenesis, 'Phase 1' is designated as the first phase in the expression of somatic embryogenesis. This phase is induced by the transfer of State 1 cell clusters (already induced to express embryogenic development) to auxin-free medium. During this phase, cell clusters proliferate slowly and apparently without differentiation. After this phase, rapid cell division occurs in certain parts of cell clusters, leading to the formation of globular embryos (designated as 'Phase 2'). In the following phase (Phase 3), plantlets develop from globular embryos through heart- and torpedo-shaped embryos.

Expression of somatic embryogenesis might be triggered by different factors, depending on species, cultivar, physiological conditions of the donor plant and so on. However, as already mentioned, the most common procedure is the exclusion or reduction of the auxin (mainly 2,4-dichlorophenoxyacetic acid [2,4-D]) concentration in the culture medium of embryogenic cultures induced with this plant growth regulator.

FACTORS ASSOCIATED WITH EMBRYOGENIC COMPETENCE

To take advantage of the previously mentioned potentialities that somatic embryogenesis offers, it is of great importance to understand the mechanisms underlying the transition from a somatic or a gametophytic cell to an embryogenic cell and, in most cases, to be able to regenerate plants from it (Mordhorst *et al.*, 1997). At the same time, it is desirable to extend these induction and expression capacities to those

species and cultivars that are recalcitrant to somatic embryogenesis, and are also of agronomic importance. When studying the factors involved in the control of somatic embryogenesis, it is very important to differentiate between those related to the induction and those related to the expression of this event (Ermakov & Matveeva, 1994). Somatic embryogenesis induction can be spontaneous at one end of the spectrum or may demand long and complex treatments at the other end (Toonen & de Vries, 1996). Embryogenic competence is the term used for describing the relative ease of inducing somatic embryogenesis from tissue cultures (Carman, 1990).

Explant (non-embryogenic) cells can be induced to an embryogenic state by a variety of procedures that usually include exposure to plant growth regulators, pH shock, heat shock or treatment with various chemical substances. However, it is still not clear whichchanges a somatic cell must undergo in order to become an embryogenic cell capable of forming an embryo. There appears to be no single, universally applicable signal that renders cells embryogenic (Mordhorst *et al.*, 1997). Moreover, in general only a very limited number of cells in any given explant responds by becoming embryogenic (Toonen & de Vries, 1996).

Structural factors

The initiation of polarity in embryos is often regarded as the first step in embryogenesis (Warren Wilson & Warren Wilson, 1993). In carrot and *Medicago* it appears that the first division has to be asymmetric, producing two cells of different sizes, in order to confer embryogenic competence to the individual cells (Dudits *et al.*, 1991). In the case of carrot, the first division of single suspension cells capable of forming embryogenic cells is unequal (Komamine *et al.*, 1992), and only the smaller daughter cell will ultimately develop into an embryo (de Jong *et al.*, 1993). Komamine *et al.* (1992), Tsukahara & Komamine (1997) and Sato-Nara & Fukuda (2000) reported a polarized DNA synthesis in these cells. In most species showing embryogenic capacity, the asymmetric division does not form an embryo directly, but forms a proembryogenic mass (PEM), in which only one or a few cells can subsequently develop into an embryo (Komamine *et al.*, 1992; Nuti Ronchi & Giorgetti, 1995). The rest of the PEM cells are probably eliminated through a cycle of programmed cell death, as observed in Norway spruce (Filonova *et al.*, 2000).

In a close relationship with *in vitro* embryogenesis, the orientation of the first cell division has also been considered to be essential to the establishment of the basic polarity of the zygotic embryo. However, a more extensive survey of the embryological literature turned up a minimum of 19 different dicotyledonous families in which the zygotes of at leastone species undergo longitudinal, oblique, or even variable first divisions instead of the expected transverse division (Kaplan & Cooke, 1997).

In general, it can be concluded that a correct asymmetrical first division is not an essential requirement for somatic or for zygotic embryogenesis. This does not imply that an asymmetrical distribution of intracellular determinants is not important in the early stages of plant embryogenesis, but rather that such a distribution is not necessarily visibly fixed by an asymmetrical first division. There are some other structural factors that could influence the capacity forembryogenic induction in certain cells: microtubule organization and cell wall (cell size appears to play an important role in somatic embryogenesis) (Toonen & de Vries, 1996). McCabe *et al.* (1997) recently observed that a cell-wall antigen on cells destined to form embryos segregates asymmetrically during a formative division, producing one daughter cell with a cell wall antigen recognized by the antibody JIM8 and the other without, and the epitope-free cells ultimately form somatic embryos.

Another important point is the necessity of the cells subjected to embryogenic induction, for physical isolation from the surroundings. This is the case with somatic embryos formed in suspension cultures. This physical isolation leads to varying degrees of physiological isolation

caused by loss of plasmodesmata between surrounding cells, interrupting symplastic continuity and reducing electrical coupling (Warren Wilson & Warren Wilson, 1993).

Physiological factors

Although plant growth regulators play a key role in inducing somatic embryogenesis, there are many other factors that have been found which could affectthe disposition of a particular tissue to undergo somatic embryogenesis. The range of possible induction treatments suggests that it is unlikely that a single inducing molecule is responsible (Toonen & de Vries, 1996).

Examples of these other factors that can direct the transition from somatic cells to cells able to form embryo-like structures are: in *Citrus* suspension cultures a change in carbon source from sucrose to glycerol (Ben-Hayyim & Neumann, 1983; Gavish *et al.*, 1991; Jiménez & Guevara, 1996); in carrot the NH_4^+ concentration (Smith & Krikorian, 1989) and pH changes (Smith & Krikorian, 1990, 1992) in the culture medium; in *Araujia sericifera* the light quality (Torné *et al.*, 2001), in *Brassica* microspores a temperature shock (Pechan & Keller, 1988); also pre-treatment of donor plants and subculture duration (Mórocz *et al.*, 1990), to name only a few factors. A more detailed description of some of these factors was given by Tisserat *et al.* (1979), Sharp *et al.* (1980), Tulecke (1987) and Harada (1999).

Culture density of cell suspensions could be another important factor that affects somatic embryogenesis. While a high cell density (10^5 cells/ml) is required for the formation of embryogenic cell clusters from single cells (Nomura & Komamine, 1985), a lower cell density (2×10^4 cell/ml) favors the development of embryos from embryogenic cells (Fujimura & Komamine, 1979). This may be related to the secretion of proteins and/or other cellular factors into the culture medium. In fact, secreted (extracellular) and constitutive (intracellular) proteins are also considered to play a very important part in induction of somatic embryogenesis (de Vries *et al.*, 1988a,b; Gavish *et al.*, 1991, 1992). The addition of arabinogalactan proteins, isolated from the culture medium of embryogenic carrot lines and from dry carrot seeds, was capable of promoting the formation of PEMs, even in previously non-embryogenic carrot cell lines. Similar proteins, but isolated from the medium of non-embryogenic lines, acted negatively on the formation of PEMs (de Jong *et al.*, 1993). McCabe *et al.* (1997) found that cells competent to become embryogenic require a soluble signal from other cells to trigger somatic embryogenesis, and postulated that signal is an arabinogalactan protein. The way in which secreted proteins influence somatic embryogenesis is not known, but it is reasonable to postulate that their function can be explained in terms of an effect on particular cell wall polymers (van Holst *et al.*, 1981). For a review of the role of these proteins in this process, refer to de Jong *et al.* (1993), Kiyosue *et al.* (1993) and Schmidt *et al.* (1994).

Meijer *et al.* (1999) observed that co-culture of *Arabidopsis thaliana* suspension culture aggregates interferes with the development of carrot somatic embryos beyond the globular stage. These authors attribute this effect to the release of previously accumulated 2,4-D by the *Arabidopsis* cultures. Moreover, Kobayashi *et al.* (2000) found that somatic embryogenesis is strongly inhibited in cultures of carrot cells when the cell density is high, and successfully isolated and identified the inhibitory factor 4-hydroxybenzyl alcohol, which was found to strongly inhibit the formation of somatic embryos when added to the culture medium at a concentration equal to that found in high-cell-density cultures. Differences in the contents of reducing sugars and starch have been reported to be characteristic of embryogenic and non-embryogenic calli from *Medicago arborea*, with higher sugar concentrations and lower starch content in the embryogenic cultures than in the non-embryogenic cultures (Martin *et al.*, 2000).

Gene expression

Plant development and differentiation are regulated directly or indirectly by changes of gene

expression, especially during embryogenesis (Dong & Dunstan, 2000). Molecular studies on plant embryogenesis began in the 1980's, and since then they have evolved in an interesting way. Initial studies of zygotic embryogenesis were generally designed to estimate the number of different RNAs present in developing seeds, to examine the spatial and temporal distribution of distinct RNA species, to isolate and to characterize the genes that code for abundant seed proteins, particularly the seed-storage proteins, and to identify the *cis*-acting regulatory sequences and *trans*-acting DNA-binding proteins that regulate expression of seed-specific genes (Meinke, 1995).

However, the basic experimental strategy for molecular analysis of somatic embryogenesis has mostly relied on comparing genes and proteins being expressed in embryogenic and non-embryogenic cells as well as in the different stages of embryogenesis (Rao, 1996). Gene expression studies during the different stages of this process have been carried out (reviewed by Henry *et al.*, 1994; Kawahara and Komamine, 1995; Meinke, 1995; Wilde *et al.*, 1995 and Dong & Dunstan, 2000) and suggest that the number of genes specifically expressed during these events is rather limited (Komamine *et al.*, 1992; Ermakov & Matveeva, 1994; Dodeman & Ducreux, 1996; Schrader *et al.*, 1997), and that changes in protein patterns are highly regulated posttranscriptionally, at the mRNA level (Komamine *et al.*, 1992; Wilde *et al.*, 1995). Additionally, Dodeman & Ducreux (1996) indicate that changes in hormonal levels in tissue cultures may modify the synthesis of some somatic embryogenesis-specific-proteins. Recently, Kitamiya *et al.* (2000) succeeded in isolating two genes that were induced after exposure of carrot hypocotyls to high concentrations of 2,4-D for 2 hours, a treatment that initiated somatic embryogenesis directly on these explants.

Cell cultures of carrot that are growing as unorganized callus in the presence of 2,4-D have been used to study genetics during expression of somatic embryogenesis, the latter of the two phases in which, as previously described, this process has been divided. On the other hand, some mutations of *Arabidopsis thaliana* have been used to characterize the induction phase, i.e., the first stage during somatic embryogenesis.

Most genes expressed differentially during somatic embryogenesis belong to the late embryo-abundant (*lea*) genes. Proposed functions for the products of this family of genes arethe protection of cellular structures in mature embryos during seed desiccation and prevention of precocious germination of the zygotic embryos during seed development (reviewed by Wilde *et al.*, 1995 and Dong & Dunstan, 2000). Several genes expressed in carrot somatic embryos code for secreted extracellular proteins. One gene product (EP1), with homology to *Brassica* S-locus glycoproteins, is present in nonembryogenic callus but not in somatic embryos themselves. Another gene that produces a lipid transfer protein (EP2) has been particularly useful as a marker for epidermal cell differentiation during embryogenesis. The precise role of these extracellular proteins remains to be established, but they may be involved in the regulation of cell expansion and the maintenance of biophysical features required for morphogenesis. Perhaps the most unexpected finding involves a secreted glycoprotein (EP3) that rescues a temperature-sensitive mutant of carrot (*ts11*) that fails to complete the transition from a globular to heart stage of somatic embryogenesis (Meinke, 1995; Sugiyama, 2000). Another group of genes that are developmentally regulated in carrot suspension cultures is constituted by the *Dc3, Dc8, J4e* and *ECP31* genes. They are expressed at different momentsduring embryo development and localized in different cell groups within the PEMs and embryos (Wilde *et al.*, 1995). Dudits *et al.* (1995) indicate that the gene expression is expected to be different during the processes of embryogenic commitment in primary explants or fully differentiated somatic cells from those that act in suspension cultures with proembryogenic structures, such as in the case of carrot.

In recent years, there has been a shift toward combining the tools of molecular biology

with the power of genetics. Future research in plant molecular biology must focus not simply on isolating and characterizing large numbers of genes expressed during plant embryo development, but also on determining the biological significance of these genes by demonstrating what happens when their function is disrupted. This can be accomplished either by creating transgenic plants that express an antisense construct or by working with genes that have already been disrupted through loss-of-function mutations (Meinke, 1995).

The general strategy in genetic analysis is to use mutants either as markers of cell lineages or as vehicles for the identification of essential genes. Genetic analysis has played an increasingly important role in recent studies of plant development. However, not all genes can be identified readily by recessive loss-of-function mutations. Genes that are duplicated in the genome, genes that are required for early stages of gametogenesis, and genes that perform functions that are redundant, nonessential, or detectable only in unique circumstances often escape detection in mutant screens. The power of genetics is not that it leads to the identification of every transcriptional unit within the genome, but rather that it allows one to focus on genes that must be expressed in order for growth and development to proceed in a normal manner (Meinke, 1995).

This strategy has been used more readily to try to understand the events related to zygotic embryogenesis. Several different classes of embryonic mutantshave been identified in higher plants. Many mutants are defective in early stages of cell division and morphogenesis. Others fail to accumulate pigments and storage materials during embryonic maturation. Still others are disrupted in the preparation for dormancy and germination. Many of these mutants are likely to be defective in genes with housekeeping functions that first become essential during embryo development. Although this information may be discouraging to developmental biologists interestedin finding genes that play a direct role in the regulation of plant embryogenesis, it should be very useful for biochemists, physiologists, and cell biologists, many of whom could use mutants defective in basic cellular processes. Embryonic mutants also differ in the initial site of gene action. The primary defect in some embryonic mutants is limited to the endosperm tissue. Altered development of the embryo in these mutants is often an indirect consequence of endosperm failure. The primary defect in other mutants appears to be restricted to the embryo proper. Although the most extensive studies of embryonic mutants have dealt with maize and *A. thaliana*, related mutants have also been described in barley, carrot, rice, and peas. Most of these recessive loss-of-function mutations were induced with chemical mutagens, X rays, transposable elements, or T-DNA from *Agrobacterium tumefaciens* (Meinke, 1995).

Molecular markers

The search for markers of plant embryogenesis is an important aspect of modern plant breeding (Schel *et al.*, 1994). Several physiological, biochemical and molecular markers associated with embryogenic competence of cells have been reported, including isozymes and molecular markers, to be discussed in the following paragraphs, and plant hormones, which will be discussed later.

Isozyme patterns are helpful tools for a better understanding of the basic mechanisms of cellular differentiation and further plant development. Apart from the classical studies in which isozymes were used as markers for events at the later stages of the growing plant (e.g., organogenesis or seed germination), during the last twenty years their usefulness as markers during early development of the plant (i.e., early embryogenesis) was also demonstrated.Tissue culture techniques and, more specifically, somatic embryogenesis allowed the application of isozyme analysis to the embryogenic process, because they permitted the availability of relatively high amounts of plant material in the desired developmental stage.

Coppens & Dewitte (1990) found the esterase system to be very sensitive for detection of

embryogenesis in barley callus before somatic embryos are formed. In carrot, two esterase isozyme systems are differentially expressed in embryogenic and non-embryogenic cells (Chibbar *et al.*, 1988). Tchorbadjieva & Odjakova (2001) recently found an acidic-esterase isozyme (36 kDa and an isoelectric point of 3.8) present in embryogenic suspension cultures of *Dactylis glomerata* but not in non-embryogenic cultures. Also, it has been observed that the pattern of acid phosphatase showed qualitative differences between embryogenic and non-embryogenic coffee callus cultures (Menéndez-Yuffá & García, 1996). An extensive review on the use of isozymes as biochemical markers in somatic embryogenesis has been already published (Schel *et al.*, 1994).

There are several candidate genes that could be used as molecular markers of single competent cells (Schmidt *et al.*, 1997). One of these genes, the Somatic Embryogenesis Receptor-like Kinase (SERK) gene, was found to mark single *Daucus* and *Dactylis* suspension cells that are competent to form somatic embryos (Schmidt *et al.*, 1997; Somleva *et al.*, 2000).

EFFECT OF EXOGENOUSLY APPLIED PLANT GROWTH REGULATORS ON SOMATIC EMBRYOGENESIS

Although auxins, which are known to mediate the transition from somatic to embryogenic cells, are the agents generally used to induce embryogenesis, the effect of other plant growth regulators on this phenomenon must not be overlooked. While in Angiosperm monocots, primary embryogenesis was exclusively induced by auxin-supplemented media, there is a large variation of growth regulators used to induce somatic embryogenesis in dicot species. From a list of 65 dicot species reviewed by Raemakers *et al.* (1995), somatic embryogenesis was induced in 17 species on hormone-free media, in 29 species on auxin-containing media and in 25 species on cytokinin-supplemented media. Among auxins, the most frequently used was 2,4-D (49%) followed by naphthalene acetic acid (27%), indole-3-acetic acid (IAA) (6%), indole-3-butyric acid (6%), Picloram (5%) and Dicamba (5%). In the case of cytokinins, N^6-benzylaminopurine was used most often (57%), followed by kinetin (37%), zeatin (Z) (3%) and thidiazuron (3%).

In those cases in which the exogenous application of auxins has proved to be the most efficient treatment to induce somatic embryogenesis, further development of the existing somatic embryos has been achieved by reducing or removing auxin fromthe culture media. Although the process of embryo induction from cells in culture is not fully understood, it is now generally believed that, in the continued presence of auxin, a differential change in gene expression (probably associated with increased demethylation of DNA; Lo Schiavo *et al.*, 1989) in PEMs occurs (Litz & Gray, 1995). Under these circumstances, the PEMs within the culture synthesize all the gene products necessary to complete the globular stage of embryogenesis. At that point, the PEMsalso contain many other mRNAs and proteins whose continued presence generally inhibits the continuation of the embryogenic program. The removal of auxin results in the inactivation of a number of genes so that the embryogenic program can now proceed. The observation that some carrot cell lines are able to develop to the globular stage, but not beyond in the continued presence of auxin, suggests that new gene products are needed for the transition to the heart stage and that these new products are synthesized only when exogenous auxin is removed (Zimmerman, 1993). However, as stated earlier in this work, the number of genes directly involved in development of somatic embryos seems to be rather limited.

The effect of the addition of other plant growth regulators is not so well documented. It has been observed that the addition of abscisic acid (ABA) inhibits the precocious germination of the somatic embryos and allows them to mature into 'normal-shaped' plants, as observed in grapevine (Rajasekaran *et al.*, 1982; Goebel-Tourand *et al.*, 1993). Bell *et al.* (1993) found that explants of embryogenic genotypes of *Dactylis glomerata* responded to the inclusion of ABA in the culture medium by increasing the number of somatic

embryos formed. Nishiwaki *et al.* (2000) observed that seedlings of carrot formed somatic embryos when cultured on medium containing ABA, with the number of embryos originated per explant dependent on the ABA concentration employed. Despite the wide range of physiological effects of gibberellins, their effect when added to culture media, primarily as gibberellic acid (GA_3), has been minimal (Krikorian, 1995). Exogenous application of GA_3 has been reported to inhibit somatic embryogenesis and somatic embryo development in several species; but it was also reported that this substance is required for germination of the mature somatic embryos if chilling is not applied (Takeno *et al.*, 1983 and references therein). Even in those cases in which the addition of cytokinins as the sole plant regulator is effective in inducing somatic embryogenesis„ the stimulatory effect of these substances is not universal, and their addition should often be coupled with that of auxins to obtain the desired effect (reviewed by Merkle *et al.*, 1995).

Effect of endogenous hormones on somatic embryogenesis

It has been frequently observed that embryogenic competent and incompetent callus sections are produced in the same explant, indicating that even genetically identical cells respond differently to a particular stimulus, with a minority of the cells being responsive. Several changes should occur for reprogramming a cell to an embryogenic competent state, the first one being the termination of the current gene expression patterns, permitting their replacement with an embryogenic program, which does not occur in all cells at the same time, and in some cells does not occur at all (Merkle *et al.*, 1995). Anatomical and physiological differences between embryogenic and non-embryogenic cultures are expressed when such changes occur. Among these differences, the endogenous hormone levels should be of great importance, since they regulate the processes of explant differentiation in culture (Grieb *et al.*, 1997) and are postulated to be the main difference between genotypes with various grades of competence (Bhaskaran & Smith, 1990).

Fot this reason the endogenous hormone levels and their relation to the embryogenic competence of the explants may be the key to induction and expression of somatic embryogenesis in recalcitrant genotypes through amendments to the culture medium, with substances that may mimic the inductive condition (supplying a deficiency or counteracting an excess), to develop or optimize *in vitro* protocols for somatic embryo production, maturation, and conversion to plantlets (Merkle *et al.*, 1995). At the same time, the characterization of the differences between embryogenic and non-embryogenic cells is necessary if the mechanisms involved in the induction and maintenance of embryogenic competence of somatic cells are to be elucidated (Kiyosue *et al.*, 1993).

As previously stated in this work, in most early studies on hormonal regulation of physiological processes in plants, especially in *in vitro* cultures, more attention was paid to the effects of the addition of plant growth regulators than to the possible role of endogenous hormonal levels in the tissues. Very little is known about the possible interactions of an endogenous phytohormone system with the exogenous growth regulators supplied to the nutrient medium. It seems probable that the observed responses of cell culture systems, after a growth regulator supplement, are related to such interactions (Neumann, 1988; Carman, 1990). Evidence for this has been reported by Liu *et al.* (1998), who observed an accumulation of endogenous IAA in soybean hypocotyl explants after their treatment with napththaleneacetic acid and indole-3-butyric acid (two exogenously applied auxins).

There are some reports that relate the endogenous hormone levels in the initial explants (Carnes & Wright, 1988; Kopertekh & Butenko, 1995; Hess & Carman, 1998) and in the callus or cell suspension cultures derived from them (Epstein *et al.*, 1977; Fujimura & Komamine, 1979; Rajasekaran *et al.*, 1982; Takeno *et al.*, 1983; Li &

Neumann, 1985; Rajasekaran *et al.*, 1987b; Kiyosue *et al.*, 1992; Michalczuk *et al.*, 1992a; Sasaki *et al.*, 1994; Kopertekh & Butenko, 1995; Hess & Carman, 1998) to the morphogenetic competence of a particular genotype. However, most of these studies were limited to only one or a few hormones, and to one plant species. Usually, information presented in different articles can not be put together and used to obtain a general view of the role of endogenous hormone levels in somatic embryogenesis, because distinct genotypes were used and also because the analytic methods were not the same in the different studies. It is very important to compare different plant species simultaneously, using the same analytical procedure, because from the study of a single organism, it is impossible to determine whether the particular features are unique to that organism or generally applicable to other species (Kaplan & Cooke, 1997).

Endogenous hormone levels in the initial explants

There are contrasting reports in relation to the effect of the endogenous hormone levels in the initial explants on determining the ability of a particular genotype to conduct somatic embryogenesis. Whereas differences in hormone levels among competent and non-competent plant genotypes have been documented, other investigations reported no discrepancy, or even that the differences found did not account for variations in the degree of competence.

In many cereal species, immature zygotic embryos are the primary source of explants employed to establish embryogenic cultures. When analyzing wheat immature embryos of two cultivars differing in their degree of competence, Kopertekh & Butenko (1995) found higher IAA and ABA and lower cytokinin levels in the most competent genotype. However, in a similar experiment, but using another set of wheat genotypes also differing in their embryogenic competence, Jiménez & Bangerth (2001b) found higher ABA levels in the competent one as the unique difference among them. The latter authors related this finding to a reduction in the precocious germination rate. Precocious germinationand callus formation have been frequently reported as alternative responses of immature embryos to *in vitro* culture (i.e. the occurrence of one diminishes the chances of the other to occur) (Carman, 1988; Qureshi *et al.*, 1989). Thus, a reduction in precocious germination would favor, indirectly, callus formation. Working with maize, Jiménez & Bangerth (2001c) did not find any difference in the levels of five hormones analyzed between two genotypes differing in their degree of competence to conduct somatic embryogenesis.

In similar studiesanalyzing the whole grains of wheat instead of isolated zygotic embryos, Hess & Carman (1998), working with wheat, found lower levels of IAA, ABA, N^6-(Δ^2-isopentenyl) adenine (iP) and N^6-(Δ^2-isopentenyl) adenosine (iPA) and Carnes & Wright (1988) found higher levels of total IAA and lower levels of Z and zeatin riboside (ZR) in the most competent of two maize varieties. However, it has been observed that the whole kernel hormone level poorly reflects the levels in the immature embryos since the endosperm constitutes the majority of kernel dry matter, and, as was demonstrated by Jiménez & Bangerth (2001b, 2001c), hormone levels in the endosperm might vary greatly in relation to those of the immature embryos.

In three other monocot systems, *Pennisetum purpureum* (Rajasekaran *et al.*, 1987a), *Dactylis glomerata* (Wenck *et al.*, 1988) and *Medicago falcata* (Ivanova *et al.*, 1994), in which leaf sections were used as the initial explants, higher IAA levels were found in embryogenic than in non-embryogenic explants. Ivanova *et al.* (1994) also found a positive correlation between the ability to respond to a 2,4-D induction treatment and endogenous IAA levels. The line with the shortest induction phase had the highest IAA level. The other embryogenic line needed an induction period 4 to 5 times longer and had an IAA content 4 times lower. In the same study, a negative relation between the endogenous ABA level and the embryogenic potential was observed. However, in *Pennisetum purpureum* leaves, higher

levels of ABA (3- to 4-fold) were found in the more embryogenic sections than in the less embryogenic (Rajasekaran *et al.*, 1987b). The same group found that treatments which lowered ABA levels also inhibited somatic embryogenesis, and that this inhibition could be overcome by exogenous ABA in the medium (Rajasekaran *et al.*, 1987a).

There are also contrasting reports in relation to the role that endogenous gibberellin levels in the initial explants could play in determining the embryogenic competence. As previously mentioned, Jiménez & Bangerth (2001b, 2001c) did not find any difference in the endogenous gibberellins levels ($GA_{1,3,20}$) between embryogenic and non-embryogenic genotypes of wheat and maize. In another study (Jiménez & Bangerth, 2001d), the same authors indicate that differences in the endogenous levels of the same substances in two genotypes of barley did not condition the response to the culture conditions evaluated. Also, Rajasekaran *et al.* (1987a) observed that neither paclobutrazol nor the reduced levels of gibberellins, which may have resulted from its application, altered the embryogenic character of the explants. In contrast, Hutchinson *et al.* (1997) reported that both exogenously supplied as well as endogenous gibberellins play a negative role in the induction of somatic embryogenesis in geranium (*Pelargonium* x *hortorum*) hypocotyl explants.

In contrast to the results obtained in alfalfa (Ivanova *et al.*, 1994), wheat (Jiménez & Bangerth, 2001b) and maize (Jiménez & Bangerth, 2001c), which indicate that the endogenous cytokinin levels in the initial explants do not apparently determine their embryogenic competence, Wenck *et al.* (1988) reported that low endogenous levels of cytokinins were characteristic of leaves of *Dactylis glomerata* with high embryogenic capacity. Supporting the latter finding, Rajasekaran *et al.* (1987a) reported that both non-embryogenic leaf regions and callus of *Pennisetum purpureum* contained higher levels of cytokinins than the highly embryogenic material. Centeno *et al.* (1997) found similar contents of total cytokinins in cotyledons of three *Corylus avellana* genotypes with different levels of embryogenic capacity; however, they showed a very different iP-type/Z-type cytokinin ratio. They reported higher endogenous levels of iP and lower of Z, correlated with higher levels of embryogenic competence, when compared to those of less competent genotypes.

The contrasting results cited above seem to indicate that differences in the endogenous hormone levels in the initial explants of cereals are not indicative of their embryogenic competence. Supporting this conclusion, Jiménez & Bangerth (2001d) reported marked differences in the IAA and gibberellins levels in immature zygotic barley embryos, differences that did not affect their degree of competence.

Endogenous hormone levels in embryogenic and non-embryogenic cultures

Compared to the few studies available on the effect of the hormone status of the initial explant on embryogenic competence, more research has been carried out in relation to the endogenous hormone levels in embryogenic and non-embryogenic callus cultures. Several reports indicate that higher endogenous free IAA levels are characteristic of embryogenically competent callus lines, as observed in carrot (Li & Neumann, 1985; Sasaki *et al.*, 1994, Jiménez & Bangerth, 2001a), *Pennisetum purpureum* (Rajasekaran *et al.*, 1987b), sugarcane (Guiderdoni *et al.*, 1995), wheat (Jiménez & Bangerth, 2001b) and maize (Jiménez & Bangerth, 2001c). On the other hand, Michalczuk *et al.* (1992a) did not find differences in free and conjugated IAA between embryogenic and non-embryogenic carrot cultures, and Besse *et al.* (1992) did not observe great differences in the IAA content of callus lines of *Elaeis guineensis* that differ in their embryogenic response.

Rajasekaran *et al.* (1987a) found that low auxin levels coincided with high cytokinin levels in non-embryogenic callus cultures of *P. purpureum* and postulated that the lower levels of IAA in non-embryogenic callus may be a consequence of the uptake and accumulation of

cytokinins, which, at optimal concentrations, have been shown to stimulate the formation of isoperoxidase and IAA oxidase, enzymes responsible for irreversible degradation of IAA (Lee, 1971). However, Jiménez & Bangerth (2001a, 2001b, 2001c) could not confirm this hypothesis since they did not find any coincidence between low levels of free IAA and high levels of cytokinins in embryogenic and non-embryogenic callus cultures of carrot, wheat and maize.

A reduction in the embryogenic capacity of the explants with time in culture under inductive conditions (specially on medium containing 2,4-D) has been reported by Smith & Street (1974) and Filippini *et al.* (1992). Rajasekaran *et al.* (1987b) in *P. purpureum*, Kopertekh & Butenko (1995) and Jiménez & Bangerth (2001b) in wheat and Jiménez & Bangerth (2001c) in maize found that decreased embryogenic competence was characterized by a reduction in the endogenous free IAA, practically to the levels present in the non-embryogenic lines.

Higher ABA levels in embryogenic callus lines, when compared to non-embryogenic lines, were reported by Rajasekaran *et al.* (1987b) in *Pennisetum purpureum*, Kiyosue *et al.* (1992) and Jiménez & Bangerth (2001a) in carrot, Guiderdoni *et al.* (1995) in sugarcane and Jiménez & Bangerth (2000) in grapevine. Kiyosue *et al.* (1992) postulated that high endogenous ABA levels could be necessary to induce or maintain somatic embryogenesis in carrot. Contrary to these results, Etienne *et al.* (1993) found that high levels of ABA accumulated in non-embryogenic calli of *Hevea brasiliensis*, but remained at a low level in the embryogenic callus. These latter authors postulated that high ABA levels are incompatible with the initiation and then the development of somatic embryos.

No defined pattern was observed in the endogenous levels of gibberellins when comparing embryogenic with non-embryogenic callus cultures. While Jiménez & Bangerth (2001a) in carrot, Jiménez & Bangerth (2001b) in wheat and Jiménez & Bangerth (2000) in grapevine did not find any difference, Jiménez & Bangerth (2001c) found higher gibberellin levels in embryogenic maize lines and, in contrast, Noma *et al.* (1982) related high levels of polar gibberellins (probably GA_1) to an absence of embryogenic competence in carrot.

Concerning the endogenous levels of cytokinins, Ernst *et al.* (1984) and Ernst & Oesterhelt (1984, 1985), working with anise, concluded that the cytokinin levels in the callus cultures seemed to be more related to the growth rate of the calli than to their embryogenic competence. Similar conclusions were postulated by Jiménez & Bangerth (2000) in grapevine. However, Rajasekaran *et al.* (1987a) found levels of cytokinins in non-embryogenic callus at least two times higher than in embryogenic callus, after 10 days of culture of *Pennisetum purpureum* leaf explants. Guiderdoni *et al.* (1995) reported higher levels of iP and iPA in embryogenic calli than in the non-embryogenic calli of sugarcane, the opposite for Z, andno differences in the ZR levels.

Habituated cultures and endogenous hormones

Habituated cultures are those that can proliferate in a culture medium without the supplement of exogenous plant growth regulators (Meins, 1989). There is some controversy in relation to the hormone physiology of habituated and non-habituated cells. While similar concentrations of hormones in both habituated and non-habituated cell types have been reported (Kevers *et al.*, 1981), higher hormone concentrations were also found in habituated than in non-habituated cells (du Plessis *et al.*, 1996 and references therein). It has been argued that habituation may be due to the elevated levels of cytokinins in the habituated cells (du Plessis *et al.*, 1996). However, Das and Saha (1995), analyzing the endogenous cytokinin levels in habituated and non-habituated nucellar callus of sweet orange, found higher levels of Z, ZR and Z glucoside in the second callus type.

It was previously mentioned that embryogenic callus cultures developed on culture medium containing 2,4-D are characterized by higher endogenous IAA contents than those of the

non-embryogenic cultures. Since Michalczuk *et al.* (1992b) postulated that the 2,4-D in the culture medium is the cause of this increase in the endogenous IAA levels, it is conceivable to think that in the habituated cultures, grown on a hormone free medium, this increase does not occur. In grapevine, Jiménez & Bangerth (2000) reported that competent callus cultures did not show this characteristic pattern of higher IAA levels, instead the levels were similar in embryogenic and non-embryogenic cultures. Working with habituated *Citrus* callus and cell suspension cultures, Jiménez *et al.* (2001) found that the treatment that stimulated the further development of the somatic embryos also stimulated auxin and cytokinin accumulation. However, these authors did not make comparisons between embryogenic and non-embryogenic cultures.

Limanton-Grevet *et al.* (2000) analyzed the endogenous levels of IAA, ABA, Z, ZR, iP and iPA in eight embryogenic habituated callus lines derived from six different *Asparagus officinalis* genotypes, maintained on hormone-free medium for more than one year. Even when a great variaton in the intensity of the secondary embryogenesis was observed between lines, this could not be related to the hormone metabolism, since no significant differences were found between distinct embryogenic lines.

PROPOSED ROLE FOR EACH HORMONE GROUP DURING SOMATIC EMBRYOGENESIS

Although the endogenous hormone levels by themselves do not account for all the phenomena observed during induction and development of somatic embryogenesis, a general view of the importance of each hormone group can be postulated.

Auxin is considered to be the most important hormone in regulating somatic embryogenesis (Cooke *et al.*, 1993). Both the endogenous contents and the application of exogenous auxins are determining factors during the induction and expression phases of somatic embryogenesis. It has been reported that the culture of explants in 2,4-D-containing medium, the classic induction treatment for many species, increases the endogenous auxin levels in the explants (Michalczuk *et al.*, 1992b).

It has also been observed that polar transport of auxin is essential for the establishment of bilateral symmetry during embryogenesis in dicotyledonous somatic (Schiavone & Cooke, 1987) and zygotic (Liu *et al.*, 1993) embryos, and more recently it was also demonstrated for monocotyledonous zygotic embryos (Fischer & Neuhaus, 1996). For this gradient to be established, relatively high levels of free IAA may be necessary in the competent tissues. However, once the stimulus for the further development of the somatic embryos is given (i.e., through reduction or removal of 2,4-D from the culture medium), those levels must be reduced, to allow the establishment of the polar auxin gradient. If the levels are extremely low or high, or if they do not diminish after the induction treatment, the gradient cannot be formed, and thus somatic embryogenesis cannot be expressed. Indoleacetylaspartate as been found in high levels in embryogenic callus of 'Shamouti' orange (Epstein *et al.*, 1977), and it was suggested that through this mode of IAA conjugation, auxin levels in embryogenic callus are reduced to a level inductive to active embryogenesis.

Endogenous levels of ABA also appear to be significant in some monocots for initiation of embryogenic cultures (Bhaskaran & Smith, 1990). Its role as an inhibitor of precocious germination when immature zygotic embryos are used as initial explants, thus favoring indirectly callus formation, was discussed by Jiménez & Bangerth (2001b). The role of ABA in somatic embryogenesis may be exerted through regulation of certain genes (e.g., DC8) that are thought to be involved in desiccation and maturation phases of embryogenesis (Hatzopoulos *et al.*, 1990). Rajasekaran *et al.* (1987a,b) proposed that ABA could exert its role on somatic embryogenesis by regulating carbohydrate metabolism, *via* inhibition of α-amylase activity. There are several instances where applied ABA has been shown to stimulate

cell division and DNA synthesis, callus production and shoot morphogenesis, and to inhibit peroxidase activity, an enzyme that, as previously mentioned, causes an irreversible degradation of IAA (Rajasekaran *et al.*, 1987a and references cited therein). Senger *et al.* (2001), using a homozygous transgenic line of *Nicotiana plumbaginifolia* constitutively expresing an anti-ABA single chain fragment variable antibody in the endoplasmic reticulum, clearly demonstrated that ABA is important for the formation of pre-globular structures.

The role of exogenous and endogenous gibberellins in somatic embryogenesis has not been studied in detail. The results of different reports seem to indicate a minor role for endogenous gibberellin levels, both in the initial explants and the callus cultures (Jiménez & Bangerth, 2001a,b,c,d).

There is support for the concept that cytokinins, in general, are important during the initial cell division phase of somatic embryogenesis, but not for the later stages of embryo development and maturation in carrot (Fujimura & Komamine, 1980), anise (Ernst *et al.*, 1984; Ernst &Oesterhelt, 1985) and orchardgrass (Wenck *et al.*, 1988). This suggests that cytokinins may have a role in cell division, but not in embryo differentiation (Danin *et al.*, 1993). When analyzing the individual role of the iP- and Z-type cytokinins, Centeno *et al.* (1997) postulated that the role the first group in the embryogenic response might be as biosynthetic precursors to supply the large amount of active cytokinins required for the stimulation of cell division prior to somatic embryogenesis. In the general proposed pathway of cytokinin biosynthesis in prokaryotes (Morris, 1995) and in higher plants (Einset, 1986), iP acts as a precursor of Z, probably the most active endogenous cytokinin (Kamínek *et al.*, 1997; Strnad, 1997).

HORMONE CONCENTRATION VS. SENSITIVITY

Several investigations, in which a lack of correlation between the endogenous hormone concentration and the response of the explants was observed, led Trewavas (1981) to postulate that the sensitivity of the tissues to a change in the hormone concentration is more important than the change in the concentration itself. This proposal generated a lot of controversy at that time, but it was supported by several observations, such as the fact that immature flowers and fruits did not respond to ethylene application, but the mature ones did (Davies, 1995).

Several reports, such as those in which no differences between the endogenous levels of most hormones evaluated in the initial explants of competent and non-competent genotypes were found (Jiménez &Bangerth, 2001b, 2001c) or that in which differences in the hormone levels did not seem to play any role in determining the morphogenetic capacity of the explants (Jiménez & Bangerth, 2001d), demonstrate that there are, certainly, other factors, besides the endogenous levels of plant hormones, that determine the competence of the tissues to develop embryogenic cells. This kind of result seems to support the postulate of Trewavas; however, now it is widely accepted that both the sensitivity of the tissues to the plant hormones and the concentration of the hormones, are important and modulate the responses observed in most cases. Sensitivity has been shown to contribute in a higher degree to, at least, gravitropic responses, vascular tissue regeneration, cambial growth, seed dormancy, germination, cell division, cell extension, ripening, senescence, abscission, stomatal aperture, flowering and amylase formation (reviewed by Trewavas, 1991).

There is some evidence that sensitivity may be important in conferring embryogenic competence to tissue culture explants. For example, the phytohormone content of the culture medium is a major factor regulating growth and differentiation of plant tissue *in vitro* only if a responsive tissue is taken as starting material (Bell *et al.*, 1993; Somleva *et al.*, 1995). Besides, Bögre *et al.* (1990) postulated that the differences between embryogenic and non-embryogenic alfalfa lines are based on their sensitivity to 2,4-D, with

the first line being more sensitive. It has also been observed that 2,4-D can modulate the level of auxin-binding proteins in the membranes of carrot cell suspension cultures (Lo Schiavo *et al.*, 1991; Filippini *et al.*, 1992; Lo Schiavo, 1995) and in consequence may increase the sensitivity to this class of hormones.

Guzzo *et al.* (1994) proposed the following model, which links together auxin response, asymmetric division and totipotency: upon environmental stimuli, cells can be made competent to respond to auxin in a morphogenetic way; if the cells have, or could be induced to produce the proper receptors, complete embryogenesis would follow; if the receptors are not the proper ones, only organogenesis or unorganized proliferation will occur. Even when the importance of cytokinin concentration in sensitivity has also been discussed, the understanding of the molecular basis of cytokinin action lags behind that of other hormones (Hare & van Staden, 1997).

CONCLUDING REMARKS

In spite of the relatively large number of studies related to the hormonal regulation of somatic embryogenesis, the results cited seem to have a low degree of concordance among them. As previously mentioned, there are few reports in which the different hormone groups were analyzed systematically in the different stages of somatic embryogenesis.

With reference to induction, the initial stage of somatic embryogenesis, it could be concluded that, in spite of several reports that relate certain endogenous hormone levels with embryogenic competence of a particular genotype (Rajasekaran *et al.*, 1987a; Wenck *et al.*, 1988; Ivanova *et al.*, 1994; Kopertekh & Butenko, 1995), few, if any, differences were observed between competent and non-competent genotypes in a series of systematic studies, in which the same methodology of analysis was employed for determining these levels in the initial explants of wheat and maize (Jiménez & Bangerth, 2001b, 2001c). Moreover, differences found between barley genotypes had no effect on determining their embryogenic competence (Jiménez & Bangerth, 2001d). Therefore, the endogenous hormone contents seem inadequate as markers of embryogenic potential in those initial explants whose *in vitro* behavior is unknown. There must be other factors involved in determining the competence of these explants, as previously discussed.

In all species reported in the literature in which embryogenic and non-embryogenic callus lines could be obtained in the presence of 2,4-D, it was observed that the embryogenic calli contained higher levels of free IAA than the non-embryogenic ones. These higher levels could be important in the establishment of polar auxin transport, which is postulated to be determinant in somatic embryogenesis development. In fact, as previously mentioned, when embryogenic callus cultures lose their embryogenic competence, it is accompanied by a reduction in their endogenous free IAA levels, down to the levels found in the non-embryogenic callus lines. In view of the results obtained, it might be postulated that these high free IAA levels in the embryogenic cultures are more likely a consequence of the embryogenic characteristics of the tissues, than a requirement of plant tissues to be able to form embryogenic callus. It means that these high free IAA levels reflect the ability of these cultures for further embryogenic development when the adequate conditionsare supplied, probably by allowing the explants to establish an endogenous auxin gradient as soon as the adequate stimulus is given (usually a reduction in the 2,4-D content of the culture medium).

Although endogenous hormone levelsdo certainly play a role in induction and development of somatic embryogenesis, the importance of sensitivity of the cells to changes in the hormone concentration, both endogenous and exogenous, may be also a key feature, and has to be further studied. There are some other factors, mentioned at the beginning of this paper, which could also be determinant in induction, development and expression of somatic embryogenesis, since this process seems to lack a unique induction

mechanism. Among those factors, the role that differences in the gene expression patterns could play in conferring embryogenic capacities to a particular explant or explant section, is to be considered. In this sense, certain mutant seedlings of *Arabidopsis* can bring some light to the subject. An example of such mutation is *pickle* (*pkl*), which is abnormal in primary root development. When this abnormal root is excised and cultured *in vitro* on medium lacking plant growth regulators, somatic embryos are produced spontaneously, an effect counteracted by administration of GA (Ogas *et al.*, 1997). Mordhorst *et al.* (1998) found that the rate of somatic embryo formation is higher in the cultures of the *primordia timing*, *clavata1* and *clavata3* mutants when compared to the wild-type. These mutants are characterized by an enlarged shoot apical meristem, which could be more responsive to auxin signaling (Mordhorst *et al.*, 1998). Genetic approaches, which have not been extensively exploited in this research field, would contribute to a better understanding of this plant developmental course (Sugiyama, 2000).

ACKNOWLEDGEMENTS

The support of the German Academic Exchange Service (DAAD) to conduct part of the literature review is gratefully acknowledged.

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