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Review paper

Involvement of plant hormones and plant growth regulators on *in vitro* somatic embryogenesis

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Abstract

In spite of the importance attained by somatic embryogenesis and of the many studies that have been conducted on this developmental process, there are still many aspects that are not fully understood. Among those features, the involvement of plant hormones and plant growth regulators on determining the conversion of somatic onto embryogenic tissues, and on allowing progression and maturation of somatic embryos, are far away from being completely comprehended. Part of these difficulties relies on the frequent appearance of contradictory results when studying the effect of a particular stimulus over a specific stage in somatic embryogenesis. Recent progress achieved on understanding the interaction between exogenously added plant growth regulators over the concentration of endogenous hormones, together with the involvement of sensitivity of the tissues to particular hormone groups, might help clarifying the occurrence of divergent patterns in somatic embryogenesis, and in tissue culture in general. The aspects described above, emphasizing on the effect of the concentration of plant hormones and of the addition of plant growth regulators during the different phases of somatic embryogenesis, will be reviewed in this paper. Citations will be limited to review articles as much as possible and to individual articles only in those cases in which very specific or recent information is presented.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid; ABA – abscisic acid; CK – cytokinin; E – embryogenic; (GA_3) – gibberellic acid; GA – gibberellin; IAA – indole-3-acetic acid; BAP – N^6-benzylaminopurine; iP – N^6(Δ^2-isopentenyl) adenine; iPA – N^6(Δ^2-isopentenyl) adenosine; NAA – naphthalene acetic acid; NE – non-embryogenic; PGR – plant growth regulator; SE – somatic embryogenesis

Introduction

Somatic embryogenesis (SE) is the developmental pathway by which somatic cells develop into structures that resemble zygotic embryos (i.e., bipolar and without vascular connection to the parental tissue) through an orderly series of characteristic embryological stages without fusion of gametes (Jiménez 2001). From the time SE was described for the first time, almost 50 years ago (Steward et al. 1958; Reinert 1959), and due to the importance shown, it has been the subject of many

studies. The connotation SE has acquired is the consequence of its usefulness as a tool to investigate zygotic embryogenesis, as well as an adequate system for mass-propagation of plants (Jiménez 2001).

SE has been traditionally divided in two main stages, namely induction and expression. In the former one, somatic cells acquire embryogenic (E) characteristics by means of a complete reorganization of the cellular state, including physiology, metabolism and gene expression (Fehér et al. 2002). It is usually after a change in one or more culture conditions [e.g., culture medium, composition of plant growth regulators (PGRs), carbohydrate source, osmotic potential, etc.] that the induced tissues or cells reach the expression stage, in which cells display their E competence and differentiate into somatic embryos.

The importance of plant hormones and PGRs in both stages of SE has been widely documented during the last decades. In this review, 'plant hormones' will designate the endogenous compounds produced naturally by the tissues and cells, while 'PGRs' will account for those synthetic compounds added exogenously. Most studies on the participation of these compounds in SE has been conducted with the 'classical' groups [i.e., auxins, cytokinins (CKs), gibberellins (GAs), abscisic acid (ABA) and ethylene], as the following pages make obvious. From the other substances that share some characteristics with the 'classical' hormones, and that have been included more recently into this group (i.e., jasmonates, salicylic acid, brassinosteroids, and polyamines), there is only evidence for polyamines to participate in SE. However, they will not be considered in this review. Further information on the participation of polyamines in SE can be found elsewhere (e.g., Minocha and Minocha 1995; Kakkar et al. 2000; Bais and Ravishankar 2002).

Most success achieved so far in understanding the mechanisms involved in determination and progress of SE in plants has been accomplished with model plant species, such as carrot, alfalfa and white spruce, and, more recently, hormone signaling pathways have been validated with the use of *Arabidopsis* mutants (Gazzarrini and McCourt 2003). Very few reviews have been published dealing specifically with the role of hormones on initiation and development of SE in higher plants (e.g., Jiménez 2001). In most other reviews on SE, morphological and biochemical, and more recently, molecular aspects of this developmental process, are also included (Komamine et al. 1992; Nomura and Komamine 1995; Thorpe 2000; Stasolla et al. 2002).

Two main approaches have been followed to try to understand hormone regulation during SE. In the first and most widely used, PGRs have been added to the culture medium (evaluating different substances, concentrations and moments of application) to induce the desired developmental pattern, frequently in trial and error experiments. This approach is still being used and several new articles appear every year that propose the proper conditions, dose and combination of PGRs that permits efficient induction and progression of SE in recalcitrant or less-studied species, genotypes or explant types (e.g., Arunyanart and Chaitrayagun 2005; Park et al. 2005; Stefanello et al. 2005). Later on and with the development of enough sensible techniques to analyze small molecules present in minute amounts in tissues, such as plant hormones are, the role that these compounds might play in governing SE was investigated by means of determining and relating their endogenous concentrations to morphogenesis and development. In addition to the former two aspects, this review will also include other features that developed more recently, such as the interaction between added PGRs and endogenous hormones and the role played by sensitivity of the explants during SE.

The aim of this review is to summarize the vast amount of information published so far on the role of plant hormones and PGRs on the different phases of SE development. Those works that involve molecular and genetic studies of hormonal regulation will not be discussed here (further information on this particular topic can be found in Dudits et al. 1995; Dodeman et al. 1997; Dong and Dunstan 2000; Thorpe 2000; Stasolla et al. 2002; Phillips 2004; von Arnold et al. 2005, among other sources). Whenever possible, review articles will be employed to avoid citing huge amounts of literature that could be found elsewhere. Individual articles will only be cited when they present very recent or specific information that will help to explain or illustrate a particular aspect.

Role played by endogenous hormones on SE

Generalities

To study the role that endogenous hormone contents play on SE, different situations have been evaluated. In a first one, the endogenous hormones in responsive and non-responsive explants were compared to verify their involvement in initiating this developmental process. Also, the same aspect was evaluated in explants showing differences in E competence. Moreover, the evolution of the endogenous hormone levels along progression of SE was also the subject of intensive research. Finally, the use of inhibitors/antagonists of hormones that might give some light on the role of endogenous hormones was also analyzed. In this section, the evidence of the participation of endogenous hormones belonging to distinct groups on SE will be reviewed.

It is important to mention that initial explants, as well as E cultures derived from them, are composed of different kinds of cells, with varying degrees of competence. Analysis of such tissues and cell conglomerates would allow the global quantification of endogenous hormones in the tissues, but specific hormone levels in responsive or E cells could not be accounted, since they average with the levels in non-responsive or non-embryogenic (NE) cells. It is only with the use of synchronized systems, such as the ones developed for carrot (Osuga et al. 1999, and references therein) and alfalfa (reviewed by Fehér et al. 2002), that more exact contents have been measured.

Relationship between endogenous hormone contents in explant tissues and their E competence

Level of endogenous hormones is considered to be one of the crucial factors determining E potential of explants (Fehér et al. 2003; Gaj 2004). Explant tissues as source material to induce SE are very diverse, depending mainly on the species in study. There are very responsive plants, such as carrot, in which almost any part of the plant can be used to establish E cultures (Jiménez et al. 2005 and references therein), and others more recalcitrant, in which only very specific, usually juvenile, explants are responsive. The latter is the case of most cereals and conifers (Bhaskaran and Smith 1990; Stasolla et al. 2002).

In some of the works, in which immature zygotic embryos were used as explants (mainly in cereals), the endogenous hormone contents were measured in the isolated embryos (Kopertekh and Butenko 1995; Jiménez and Bangerth 2001b, 2001c,d), while in some other works, those contents were analyzed in the whole grains (Carnes and Wright 1988; Hess and Carman 1998). The results in the latter studies may not reflect the situation very exactly, because it has been observed that the whole kernel hormone level poorly reveals the levels in the immature embryos. This is simply because the endosperm constitutes the majority of kernel dry matter, and the hormone levels in the endosperm might vary greatly to those of the immature embryos, as observed in wheat and maize (Jiménez and Bangerth 2001b,c; Hess et al. 2002).

In the least responsive species, it is common to find genotypes that react more readily than others to a particular set of inductive conditions. One approach employed to study relationships between endogenous hormone levels and E competence has been the comparison of those contents in genotypes with differential E potential. There are several works in which such differences have been reported. For example, higher indole-3-acetic acid (IAA) and ABA and lower CK levels were found in zygotic embryos of the most competent wheat genotype evaluated by Kopertekh and Butenko (1995). In a similar experiment, but using another set of wheat genotypes, also differing in their E competence, Jiménez and Bangerth (2001b) found higher ABA levels in the competent one as the unique difference among them. This result was related to differences in the rate of precocious germination in the individual genotypes, which indirectly affects E callus formation, as reported by Qureshi et al. (1989). Furthermore, very recently, Tran Thi and Pleschka (2005) reported a positive relationship between the endogenous contents of ABA in petioles in some *Daucus* species and their capacity for SE.

Additionally, Centeno et al. (1997) found higher endogenous levels of $N^6(\Delta^2$-isopentenyl) adenine (iP) and lower of zeatin in E genotypes of *Corylus avellana*, even if the total CK contents did not vary among evaluated genotypes. Concerning a different hormone group, Hutchinson et al. (1997) reported that high endogenous GA contents play a negative role on induction of SE in geranium

(*Pelargonium*×*hortorum*) hypocotyl explants. On the other hand, Limanton-Grevet et al. (2000) and Jiménez and Bangerth (2001c) practically did not find any significant variation in the levels of several hormones between genotypes of asparagus and maize, differing in their E potential, pointing against a determinant role of endogenous hormone levels in the initial explants on their E competence.

Instead of comparing genotypes varying in their E competence, in some cases tissues or organ parts of the same genotype, but with different E ability, were evaluated. In several works, in which leaf sections from different parts of the leaves were analyzed, higher IAA levels in E than in NE explants were found, e.g., Rajasekaran et al. (1987a) in *Pennisetum purpureum*. Wenck et al. (1988) in *Dactylis glomerata*. Ivanova et al. (1994) in *Medicago falcata*. The same behavior was found for ABA in *Pennisetum purpureum* (Rajasekaran et al. 1987b) but the opposite was found for this hormone in *Medicago falcata* (Ivanova et al. 1994). Wenck et al. (1988) and Rajasekaran et al. (1987a) reported lower endogenous levels of CKs in *Dactylis glomerata* leaves and in leaf regions of *Pennisetum purpureum*, respectively, with high E capacity.

Even if in early works in this field it was postulated that endogenous hormone levels were the main difference between genotypes with various grades of competence (reviewed by Bhaskaran and Smith 1990), the contrasting results cited above show that this is not always the case. Supporting this latter asseveration, Jiménez and Bangerth (2001d) found that two barley genotypes, largely known for having broad differences in the contents of IAA and GAs ($GA_{1,3,20}$) (Mounla et al. 1980), did not differ evidently in their E potential.

Involvement of endogenous hormones during induction of SE

Auxin is considered to be the most important hormone in regulating SE in vitro (Cooke et al. 1993). This regulation probably occurs through the establishment of an auxin gradient during the induction phase, which is essential for initiating bilateral symmetry during embryogenesis in somatic and zygotic embryos in dicotyledonous and monocotyledonous (Schiavone and Cooke 1987; Liu et al. 1993; Fischer and Neuhaus 1996). For this gradient to be established, relatively high levels of IAA in the competent tissues may be necessary. It has been reported that the culture of explants in medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), the classic induction treatment for many species, increases the endogenous auxin levels in the responsive explants (Michalczuk et al. 1992a; Pasternak et al. 2002), being this synthesis one of the crucial signals determining E fate of cultured cells (Thomas et al. 2002).

Most studies on hormone contents during induction of SE evaluate only one single timepoint during the process, either estimating the hormone levels on the explants prior to their culture (discussed in the previous section), or when E or NE cultures have already developed (at the end of the induction process). In the few works, in which endogenous hormone contents were followed-up during induction of SE, a transient increase in the endogenous IAA levels seems to be a common feature. Charrière et al. (1999) observed it 24 h after increasing the concentration of sucrose from 3 to 12%, one of the inducer treatments in immature zygotic embryos of sunflower (*Helianthus annuus*), while Thomas et al. (2002) detected it 14 h after including a CK into the culture medium, another inductor for the same model system. The latter pattern was shown to correlate in time with the reactivation of the cell division in the region of the explants where the morphogenic reaction took place. In another work, Grieb et al. (1997) evaluated changes in the levels of IAA, ABA and six CKs during the first 18 d of culture of carrot petiole explants with a supplement of auxin. They found a peak of IAA at day six, but also a continual decrease of ABA during the period evaluated, and an increase in the CKs up to day 10.

As stated previously in this review, explant tissues consist of cells with distinct capacity to respond to an induction treatment and therefore to become E. For that reason, after treating competent explants with an adequate induction treatment, many cells, sometimes the majority of them, do not acquire E capacity, resulting in a mixed population of cells that have to be selected. More than 10 years ago, Kiyosue et al. (1993) postulated that characterizing the differences between E and NE cells would help to elucidate the mechanisms involved in the induction and maintenance of E

competence of somatic cells. However, even if characterization of endogenous hormone levels in E and NE cultures has been accomplished in several model systems during the past years, progress in understanding the mechanisms involved in SE has come primarily from other type of studies (i.e., analysis of mutants in model plants and surveys on gene expression) (Gazzarrini and McCourt 2003).

Examples of E cultures that have higher contents of endogenous auxins than their NE counterparts can be found in carrot (Li and Neumann 1985; Sasaki et al. 1994; Jiménez and Bangerth 2001a), *Pennisetum purpureum* (Rajasekaran et al. 1987b), *Medicago falcata* (Ivanova et al. 1994), sugarcane (Guiderdoni et al. 1995), *Prunus* spp. (Michalczuk and Druart 1999), wheat (Jiménez and Bangerth 2001b) and maize (Jiménez and Bangerth 2001c). However, there is a couple of works in which no differences could be found between E and NE cultures (Besse et al. 1992 in oil palm and Michalczuk et al. 1992a in carrot). Further evidence that correlate high levels of endogenous auxins with E competence comes from works in which a reduction in the E capacity, observed after prolonged culture in inductive conditions, coincides with a reduction in the endogenous IAA, practically to the levels present in the NE lines, e.g., Rajasekaran et al. (1987b) in *P. purpureum*, Kopertekh and Butenko (1995), Jiménez and Bangerth (2001b) in wheat, Jiménez and Bangerth (2001c) in maize.

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, an aspect that will be described in detail below), the endogenous IAA levels must be reduced to allow the establishment of the mentioned polar auxin gradient. Continuous growth in medium containing 2,4-D does not allow reduction in endogenous auxin levels (Nissen and Minocha 1993) resulting in inhibition of E development. Fischer-Iglesias et al. (2001) evidenced a bidirectional transport of auxins in wheat embryos growing on medium without auxin, and an altered distribution pattern of this hormone when external auxin is supplied.

In very efficient and highly controlled E systems, such as the one described by Pasternak et al. (2002) in alfalfa, it has been even possible to compare evolution of endogenous hormone contents in E and NE cultures. These authors identified a peak in IAA synthesis in NE cultures, which showed a delay of a few days in appearance, when compared to the peak observed in E cells. In sunflower, tissues grown under the E conditions described earlier in this review (by modifying the sucrose content in the medium) showed a 4-fold increase in their IAA content, as compared to those tissues that followed the caulogenic pathway (Thomas et al. 2002). Evidence from these and other experiments suggests that temporal and spatial changes in endogenous auxin levels may be one of the first signals leading to SE (reviewed by Fehér et al. 2003).

Endogenous levels of ABA appear to be significant for initiation of E cultures, especially in some monocots (reviewed by Bhaskaran and Smith 1990), but also in carrot (Kiyosue et al. 1992). Favoring this hypothesis, Rajasekaran et al. (1987b) in *Pennisetum purpureum*, Kiyosue et al. (1992) and Jiménez and Bangerth (2001a) in carrot, Guiderdoni et al. (1995) in sugarcane, Jiménez and Bangerth (2000) in grapevine and Nakagawa et al. (2001) in melon found higher ABA levels in E callus lines, when compared to NE ones. However, the opposite was found in *Hevea brasiliensis* (Etienne et al. 1993) and in alfalfa (Ivanova et al. 1994), whose E callus cultures accumulated lower levels of ABA than their NE counterparts did.

Concerning GAs, the situation is less clear, because the few works in which endogenous contents of these hormones in E and NE cultures have been analyzed, show ambiguous data. For example, Jiménez and Bangerth (2001c) found higher GA ($GA_{1,3,20}$) levels in E maize lines, but Noma et al. (1982) found the contrary for polar GAs (probably GA_1) in carrot and anise. On the other side, Jiménez and Bangerth (2001a) in carrot, Jiménez and Bangerth (2001b) in wheat and Jiménez and Bangerth (2000) in grapevine did not find any difference in GA levels among cultures showing different E characteristics.

Results in anise (Ernst and Oesterhelt 1985 and references therein) and in grapevine (Jiménez and Bangerth 2000) indicate that CK levels seem to be more related to the growth of the callus cultures than to their E competence. However, several reports indicate a relationship between E capacity and endogenous CK contents. Rajasekaran et al. (1987a) and Pintos et al. (2002) found higher levels of CKs in NE than in E callus in *Pennisetum purpureum* and in *Medicago arborea*, respectively.

Guiderdoni et al. (1995) reported higher levels of iP and $N^6(\Delta^2$-isopentenyl) adenosine (iPA) in E calluses than in the NE calluses of sugarcane, the opposite for zeatin, and no differences in the zeatin riboside levels. The individual role that the iP- and zeatin-type CKs might play has been discussed elsewhere (Jiménez 2001). Even if the relationship between auxin and CK levels in determining E properties has been postulated to be important in *Pennisetum* (Rajasekaran et al. 1987a), this could not to be confirmed in other species (Jiménez and Bangerth 2001a–c).

Concerning ethylene, higher levels of this hormone have been found in NE than in E cultures of white spruce and carrot (Kumar et al. 1989; Feirer and Simon 1991). A further difference between E and NE cultures can be related to the uptake rates of exogenous PGRs, as reported in winter wheat cultures (Filek et al. 2004).

Changes in endogenous hormone contents during expression of SE

The expression stage of SE can be divided in two substages, the first one elapsed from the time a stimulus to induce progression in embryo development is applied (e.g., eliminating 2,4-D from the culture medium), to the moment in which the first visible changes are observed. In the second phase embryos pass through the typical stages of zygotic embryogenesis, i.e., globular, heart-shaped and torpedo-shaped stages in dicots, globular, scutellar (transition), and coleoptilar stages in monocots, or globular, early cotyledonary and late cotyledonary embryos in conifers (Gray et al. 1995; Toonen and de Vries 1996; Dong and Dunstan 2000).

Few works concentrate in the earliest of these substages, which usually comprises the first 7 days after the induction treatment. In one of these works, Michalczuk et al. (1992a) observed a rapid decline in both free and conjugated 2,4-D metabolites while IAA levels expressed remained relatively steady within seven days after transfer from the auxin-containing medium. In a very early report also related to auxins, Fujimura and Komamine (1979a) did not find significant changes in IAA levels during the first 2 weeks after eliminating 2,4-D in carrot suspension cultures.

Analyzing progression of ABA concentrations in carrot SE, Kamada and Harada (1981) found that the amount of endogenous ABA in cell clusters and embryos did not change and remained low during the first 7 days of culture. In a more recent work, Jiménez et al. (2005) analyzed evolution of endogenous IAA, ABA, GAs ($GA_{1,3,20}$) and several CKs, during the first 7 days after 2,4-D was removed from the culture medium. They only found minor changes in concentration of the evaluated hormones along time, i.e., a peak of IAA in callus 48 h after 2,4-D removal, and some fluctuations in the CKs, making high levels of zeatin/zeatin riboside to coincide with low concentrations of iP/iPA.

There are several plant model systems in which expression of SE is induced by stimuli different than a reduction in the content of 2,4-D in the culture medium. For example, in E callus cultures of nucellar origin in several *Citrus* species, proembryos are induced to develop into embryos by a change in the energy source of the culture medium from sucrose to glycerol. Using this system, Jiménez et al. (2001) found that the treatment that stimulated the further development of the formed somatic embryos also stimulated auxin and CK accumulation within the first 5 days, maintaining the levels of ABA and GAs steady.

An advantage of analyzing endogenous hormones after the first morphological changes in SE had occurred (the second substage described above), is the fact that the cultures can be purified and synchronized by different procedures (reviewed by Osuga et al. 1999; Sharma 1999). This allows analysis of stage-specific embryo populations, and avoids the dilution effect observed when using the heterogeneous cell populations described above. Using this approach, Michalczuk et al. (1992a) reported that auxin levels decline steadily after the globular stage in all subsequent stages of embryo development. In another work, it has been observed that, after remaining low during the first 7 days of culture in absence of 2,4-D (see above), the concentrations of ABA increased during further development of carrot somatic embryos until day 10, and then decreased (Kamada and Harada 1981). Similarly, Rajasekaran et al. (1982) found that ABA levels in hybrid grapevine somatic embryos decreased from the globular to the mature stage.

Application of a maturation treatment, such as chilling at 4 °C, induced partial desiccation of well-developed somatic embryos in some species.

Desiccation considerably improves the germination frequency of somatic embryos by either reducing endogenous ABA content (Kermode et al. 1989), such as chilling do (Rajasekaran et al. 1982; Jiménez and Bangerth 2000), or by changing the sensitivity to ABA (Finkelstein et al. 1985). However, there is at least one example in which endogenous ABA levels in chilled somatic embryos of carrot were similar to those of non-chilled embryos (Spencer and Kitto 1988). When evaluating evolution of endogenous ABA contents in *Quercus robur*, Prewein et al. (2004) found a reduction along germination of somatic embryos.

Studies on development of endogenous levels of GAs during latter progression of SE are limited to, to the best of our knowledge, two works conducted early in the 1980s. In the earliest one, Noma et al. (1982) found carrot and anise developing somatic embryos to contain lower levels of polar GAs (GA_1 like), but 13–22 times higher levels of less polar GAs ($GA_{4/7}$ like) than embryos staggered in their development. One year later, Takeno et al. (1983) reported that free and highly water-soluble GA-like substances in a hybrid grape decreased on a dry weight basis during embryo development, but increased on an embryo basis.

Considering ethylene, Kong and Yeung (1994) studied evolution of endogenous levels of this hormone after transferring somatic embryos of white spruce into maturation medium. They found an initial rise at day one, followed by a decline and by a gradual rise in the latter half of the culture period (day 22nd). In a more recent investigation, El Meskaoui and Tremblay (2001) related supraoptimal production of ethylene to a low maturation capacity of somatic embryos of a particular cell line of black spruce. They related the adequate maturation capacity in a different cell line in this species to an adequate ethylene production.

Use of inhibitors/antagonists as tool to determine the role of endogenous hormones

An indirect approach to evaluate the effect of endogenous hormone concentrations on different morphogenetic processes in plants, SE among them, has been the use of inhibitors of or antagonists to the different hormone groups.

The necessity for auxins on SE has been established in several plants by using polar auxin transport inhibitors and substances with antiauxin properties. When included in induction media, it has been observed that polar auxin transport inhibitors hampered embryo development in carrot (Schiavone and Cooke 1987; Tokuji and Kuriyama 2003), changed the morphogenic pathway from E to organogenic in sunflower (Charrière and Hahne 1998) and inhibited somatic embryo formation in ginseng (Choi et al. 1997) and *Eleutherococcus senticosus* (Choi et al. 2001). Experiments with the antiauxins (substances that inhibit biosynthesis of auxins), 2,4,6-trichlorophenoxyacetic acid and *p*-chlorophenoxyisobutyric acid, supported the findings described above for polar auxin transport inhibitors, since addition of these compounds inhibited embryogenesis in carrot (Fujimura and Komamine 1979b, Tokuji and Kuriyama 2003).

Regarding CKs, it has recently been observed that purine riboside, an anti-CK, also inhibited direct SE in carrot (Tokuji and Kuriyama 2003). Additionally, the use of triazine- and carbamate-type of anti-CKs influenced SE in *Dactylis glomerata* by reducing the number of somatic embryos produced when high concentrations of the former were employed, while the opposite was found for the latter compound (Somleva et al. 1995).

As previously stated, several works (Rajasekaran et al. 1987b; Kiyosue et al. 1992; Guiderdoni et al. 1995; Jiménez and Bangerth 2000, 2001a) pointed out to a significant role of endogenous ABA during the induction phase of SE. Further experimental evidence for the contribution of this hormone was provided by Senger et al. (2001), by reducing endogenous ABA contents in *Nicotiana plumbaginifolia* by diverse means (i.e., by producing a transgenic line constitutively expressing an anti-ABA single chain variable fragment antibody, by treating wild-type cultures with the ABA-synthesis inhibitor flouridone, and by using two ABA-synthesis mutants). As a result, they observed disturbed morphogenesis at preglobular formation of somatic embryos, which could be reverted by exogenous ABA application. Similarly, the lost in E capacity, caused by the application of flouridone in *Pennisetum purpureum*, was partially overcome by the addition of ABA (Rajasekaran et al. 1982). Also, inclusion of

flouridone inhibited secondary embryogenesis from E cell clusters originated from carrot seed coats cultured in absence of PGRs, while this inhibition was counteracted by including ABA into the culture medium (Ogata et al. 2005).

Concerning GAs, there are contrasting reports about their involvement in SE, according to the results from experiments employing inhibitors of their biosynthesis. Rajasekaran et al. (1987a) observed a neutral effect, since neither paclobutrazol nor the reduced levels of GAs, which may have resulted from its application, altered E character of the *P. purpureum* explants evaluated. A negative effect was reported by Mitsuhashi et al. (2003), who found that uniconazole, another inhibitor of GA biosynthesis, induced shrunken embryos when applied during expression of SE in carrot. Similarly, the use of paclobutrazol in alfalfa significantly decreased the number of somatic embryos formed (Ruduś et al. 2002). Another effect of uniconazole is the aforementioned promotion of secondary SE in carrot (Tokuji and Kuriyama 2003). Finally, Pullman et al. (2005b) recently found an improvement in initiation of SE in several conifers using paclobutrazol.

It has been observed that ethylene plays a negative role on induction of SE (reviewed by Buddendorf-Joosten and Woltering 1994; Litz and Yurgalevitch 1997). Supporting this statement, it has been reported that application of inhibitors of ethylene biosynthesis increased induction rate of SE, as observed in maize, white spruce and soybean with aminoethoxyvinyl-glycine (Vain et al. 1989; Kong and Yeung 1994; Santos et al. 1997), and in carrot with cobalt, nickel and salicylic acid (Roustan et al. 1989, 1990a). A similar effect was observed using inhibitors of ethylene action, such as silver nitrate in carrot, soybean and date palm (Roustan et al. 1990b; Santos et al. 1997; Al-Khayri and Al-Bahrany 2001) and by using silver nitrate and silver thiosulfate in maize (Vain et al. 1989). Several works reported an influence of the genotype in the response to inhibitors of ethylene biosynthesis and action (Litz and Yurgalevitch 1997; El Meskaoui and Tremblay 2001; Huang et al. 2001; Al-Khayri and Al-Bahrany 2004). Nissen (1994) worked with a carrot line in which addition of low concentrations of aminocyclopropane carboxylic acid, an ethylene precursor, or ethephon, a compound metabolized to ethylene by plant tissues, stimulated SE. In this line, inhibitors of ethylene biosynthesis caused a slight inhibition in SE. A similar effect was observed during direct SE in *Oncidium* (Orchidaceae) leaf cultures (Chen and Chang 2003). On the other side, there are some reports in which modulation of endogenous levels of ethylene by the use of inhibitors or modulation of the action of this compound by antagonists did not have an effect on maturation and conversion of somatic embryos into plants (e.g., *Picea sitchensis* Selby et al. 1996 and *P. mariana* El Meskaoui and Tremblay 1999).

Effect of PGRs on SE

On induction of SE

Addition of PGRs into the culture medium is the preferred way to induce morphogenetic responses in *vitro* in most plant tissue culture systems evaluated, being SE no exception. It has even been observed that, depending on the PGR composition of a particular culture medium, either SE, organogenesis or axillary bud development can be induced, e.g., in seedlings of *Arachis hypogaea* (Victor et al. 1999) and in embryonic axes developed from mature seeds of *Juglans regia* (Fernández et al. 2000).

In only less than 7% of the protocols surveyed by Gaj (2004), SE was induced in culture media devoid of PGRs, being several new examples reported constantly, e.g., *Eleutherococcus koreanum* (Park et al. 2005). Lakshmanan and Taji (2000) pointed out that detailed study of those model systems in which addition of PGRs are not necessary to induce SE will be very valuable to elucidate early regulatory events in embryo development.

In the majority of the species studied, in which addition of PGRs is necessary to induce SE, auxins and CKs are key factors in the determination of E response, probably because they strongly participate in cell cycle regulation and cell division (Francis and Sorrell 2001; Fehér et al. 2003; Gaj 2004). However, ABA, ethylene, GAs and other hormones have regulatory roles which must not be ignored in culture systems. Moreover, a new generation of PGRs, such as thidiazuron, a CK that belongs to the phenylureas, is emerging as successful alternative for high-frequency direct

regeneration of somatic embryos, even from well differentiated explant tissues (Gairi and Rashid 2004a,b; Panaia et al. 2004; Zhang et al. 2005).

Raemakers et al. (1995) and Gaj (2004) presented statistics about the number of species that respond to and of protocols that use different PGR groups and combinations to induce SE. While Raemakers et al. (1995) informed that 45% of the species reported in the publications evaluated by them, responded to an auxin treatment for induction of SE, Gaj (2004) mentioned that in more than 80% of the protocols studied, SE was induced in the presence of auxins alone, or in combination with CKs. Raemakers et al. (1995) also reported that about 48% of the dicot species evaluated reacted to a combination of auxins and CKs for induction of SE. These latter authors also stated that, among auxins, the most frequently used was 2,4-D (49%) followed by naphthalene acetic acid (NAA, 27%), IAA (6%), indole-3-butyric acid (6%), Picloram (5%) and Dicamba (5%). Gaj (2004) pointed out the important role of 2,4-D, by mentioning that in more than 65% of the recent protocols, this compound was applied alone or in combination with other PGRs.

Lakshmanan and Taji (2000), on their side, reviewed the response of legumes to different auxin sources during induction of SE. They pointed out that, even if most species respond favorably to auxins, especially to 2,4-D, this PGR was much less efficient than IAA in inducing somatic embryos in suspension cultures of *Chamaecytisus austriacus* (Greinwald and Czygan 1991) and it completely inhibited the production of E callus in *Hardwickia binata* (Das et al. 1995). They also mentioned that, for many legume species, use of 2,4-D resulted in a high frequency of morphologically abnormal embryos, which failed to convert into plantlets later on. In an earlier review, Nomura and Komamine (1995) summarized the results of several works in carrot, in which the effect of different exogenous auxin sources on induction of SE were evaluated. 2,4-D appears to act as an effective stressor, being one of the triggers of E development in cultured plant cells (reviewed by Fehér et al. 2003). This mode of action should be also considered in those systems in which very high concentrations of exogenous auxins are necessary for induction of SE in some plant systems (e.g., *Pisum sativum* Özcan et al. 1993 and *Serenoa repens* Gallo-Meagher and Green 2002). NAA, being the second most frequently used auxin to induce SE, as reported by Raemakers et al. (1995, see above), has shown this outcome alone or in combination with CKs mainly in woody dicots (e.g., Cuenca et al. 1999; Pinto et al. 2002; Hernández et al. 2003; Toribio et al. 2004, and references therein).

Concerning the role that another group of PGRs, the CKs, has played on plant SE, Gaj (2004) reported that induction of SE by members of this group occurred in less than 14% of the publications evaluated by her. For several species in the genus *Medicago*, in which SE occurs indirectly, the effect of CKs appears to be mostly on extensive cell proliferation prior to embryo dedifferentiation (reviewed by Lakshmanan and Taji 2000). Even in some cases, addition of CKs inhibited the induction of SE promoted by auxins, e.g., direct SE in pea, soybean and *Coronilla varia*, (reviewed by Lakshmanan and Taji 2000). Reports of species that respond to CKs as the sole source of PGRs include *Zoysia japonica* (Asano et al. 1996), *Begonia gracilis* (Castillo and Smith 1997), six citrus species (Carimi et al. 1999) and *Oncidium* sp. (Chen and Chang 2001). Among the protocols in which CKs were used as the sole PGR for induction of SE, Raemakers et al. (1995) mentioned that N^6-benzylaminopurine (BAP) was the most frequently employed (57%), followed by kinetin (37%), zeatin (3%) and thidiazuron (3%). Concerning this last product, it has been observed that it induces SE in *Cajanus cajan* and in peanut more efficiently than auxins, resulting in the development of one of the most competent genotype-independent peanut SE systems described to date (Saxena et al. 1992; Murthy et al. 1995). Several review works, with detailed information and examples of plant systems in which exogenous CKs act alone inducing SE, are available (e.g., Komamine et al. 1992; Nomura and Komamine 1995; Raemakers et al. 1995; Lakshmanan and Taji 2000; Gaj 2004). In some plant systems, the couple of CKs with auxins has been more effective to induce SE than CKs alone (reviewed by Merkle et al. 1995). For example, in conifers a low percentage of sucrose, in combination with auxins and CKs, is generally necessary for induction of this process (reviewed by Dong and Dunstan 2000; Stasolla et al. 2002).

In addition to auxins and CKs, supplement of other PGRs has been found to be necessary for induction of somatic embryos in some cases. There are several examples that evidence stimulation of SE by means of ABA. In one of them, an increase in the number of somatic embryos formed in explants of E genotypes of *Dactylis glomerata* was observed by inclusion of this PGR (Bell et al. 1993). Also, seedlings of carrot cultured on medium containing ABA formed somatic embryos directly from the epidermal cells, being the number of embryos formed dependant of the concentration of this PGR (Nishiwaki et al. 2000). Moreover, inclusion of ABA into a culture medium that normally induces organogenesis in sunflower immature zygotic embryos, produced somatic embryos instead (Charrière and Hahne 1998). Induction of SE in hybrid bermudagrass also benefited from ABA supplement (Li and Qu 2002).

The effect of exogenously applied GAs on SE is highly variable from one to another species or tissues. For example, when GAs were added to the culture medium, mainly as gibberellic acid (GA_3), they inhibited SE in carrot (Fujimura and Komamine 1975; Tokuji and Kuriyama 2003), citrus (Kochba et al. 1978) and geranium (Hutchinson et al. 1997). However, there are also some examples, in which exogenous GA_3 stimulated embryogenesis, such as in chickpea immature cotyledon cultures (Hita et al. 1997) and in *Medicago sativa* petiole-derived tissue cultures (Ruduś et al. 2002).

A similar effect (i.e., inhibition of SE) was observed, in general, for ethylene (applied as ethephon or ethrel) (reviewed by Minocha and Minocha 1995; Nomura and Komamine 1995; Thorpe 2000). However, in certain carrot cell lines, addition of low concentrations of etephon or aminocyclopropane carboxylic acid stimulated SE (Minocha and Minocha 1995).

Sometimes, a multi-step protocol is necessary to induce SE in certain woody species. For example, Fernández-Guijarro et al. (1995) could only induce SE in *Quercus suber* by reducing the high concentrations of BAP and NAA present in the first step (medium) to lower levels in the second one. A similar methodology was successfully employed by Hernández et al. (2003) with the same species, but adding a preconditioning phase that consisted in placing the explants on medium devoid of PGRs.

On secondary SE (proliferation)

In some E systems (e.g., the carrot system), SE is a recurrent process (i.e., new somatic embryos are initiated from existing somatic embryos). The proliferative process has been termed secondary, recurrent or repetitive embryogenesis (Raemakers et al. 1995). In some species, this proliferation may occur indefinitely (Merkle et al. 1995; Thorpe 2000). Usually, E callus is maintained and proliferated on a medium similar to that used for initiation, being the use of liquid cultures preferred for large-scale propagation (von Arnold et al. 2002).

For most species studied, auxin is the main factor associated with proliferation but, at the same time, with inhibition of development of proembryogenic masses into somatic embryos, probably by inhibiting electric cellular polarity (Thorpe 2000; von Arnold et al. 2002; Fehér 2003) or by impairing establishment of an auxin gradient (discussed above). For example, repetitive SE in peanut requires the presence of 2,4-D and the secondary embryos produced appeared to be arrested between the late globular and early torpedo stages of development (Durham and Parrott 1992). Secondary SE is induced in cassava by another synthetic auxin, picloram, while further development of the somatic embryos required removal of this compound (Groll et al. 2001).

However, there are some examples of species that deviate from the model described above. Primary somatic embryos of alfalfa induced on medium containing IAA, NAA and kinetin produced new somatic embryos directly, when transferred onto culture medium devoid of PGRs. Repetitive somatic E capacity of these cultures remained stable for 2 years (Parrott and Bailey 1993). Similarly, the combination of a different auxin (2,4-D) with the CK BAP, induced secondary SE in *Morus alba* (Agarwal et al. 2004). Also, in some species, secondary SE occurs in absence of PGRs (e.g., das Neves et al. 1999; Koh and Loh 2000; Puigderrajols et al. 2000; Ćalić et al. 2005). Additionally, in the case of the banana cultivar Dwarf Brazilian, addition of coconut milk induced secondary SE (Khalil et al. 2002). Coconut milk has been known for some decades now to be a natural source of CKs (Amasino 2005). Synthetic CKs have also stimulated this phenomenon in several woody species, such as *Abies numidica*, cherry, coffee and mango (Vooková et al. 2003;

Fernández-Da Silva and Menéndez-Yuffá 2003; Gutièrrez and Rugini 2004; Xiao et al. 2004). In another example, Tokuji and Kuriyama (2003) observed that inhibition of GA synthesis promoted secondary embryogenesis from the primary embryo. Moreover, Mondal et al. (2001) reported that BAP, indole-3-butyric acid and glutamine are necessary to produce secondary somatic embryos in a synchronous manner.

In conifers, maintenance of E tissue occurs in a liquid or on solid medium of composition similar to the induction medium, but with a lower concentration of auxin and CK, and often a reduced amount of sucrose (Stasolla et al. 2002). It has also been observed that ABA reduces secondary embryogenesis in this plant group and in *Quercus ilex* (reviewed by von Arnold et al. 2002; Mauri and Manzanera 2004). Secondary SE, although rare in monocots, has also been reported in bermudagrass and was induced a few weeks after removal of ABA from the culture medium (Li and Qu 2002). On the contrary, secondary, and even 'tertiary', SE has been recently described to be induced on E cultures of carrot when ABA was applied (Ogata et al. 2005). Induction of secondary SE was also stimulated in *Rosa hybrida* cv. Carefree Beauty and in hybrid larch by this PGR (Li et al. 2002; Saly et al. 2002). Additionally, in the latter work, it was observed that enrichment of the vessel atmosphere with ethylene, or addition of ethephon or aminocyclopropane carboxylic acid reduced induction of this process.

On expression of SE

As practically every developmental process in plants, expression of SE might be triggered by different factors, depending on species, cultivar, and physiological conditions of the donor plant and so on. However, in those cases in which the exogenous application of auxins has proved to be the most efficient treatment to induce SE, further development of the existing somatic embryos has commonly been reached by reducing or removing auxin from the culture media, as mentioned previously.

However, some cases that deviate from this general behavior have been recorded in the literature. For example, there are few examples in which somatic embryos continue their development in the same medium in which they formed, e.g., *Arachis hypogaea* (Hazra et al. 1989; Wetzstein and Baker 1993), *Eleutherococcus koreanum* (Park et al. 2005). In addition, a change in the auxin type, together with a reduction in its concentration induced formation of somatic embryos in *Psophocarpus tetragonolobus* (Ahmed et al. 1996). In several instances, it was the addition of low levels of CK (e.g., zeatin), together with a reduction in auxin levels, what was beneficial, such as in carrot (Fujimura and Komamine 1975). Also, a positive effect of CKs on embryo progression was observed in *Corydalis yanhusuo* (Sagare et al. 2000).

There are several examples of other PGRs exerting an effect on expression of SE in a number of model systems, sometimes with contradictory results. For example, ABA did not affect the number of carrot embryos in globular and early heart stages, but caused a decrease in the amount of embryos in heart and torpedo stages (Fujimura and Komamine 1975). It has been also documented that this PGR caused a decrease in the total number of somatic embryos in carrot (Kamada and Harada 1981). In most conifer species evaluated so far, somatic embryo development usually has to be stimulated by exogenous ABA, a treatment that concomitantly reduces cell proliferation, probably by affecting nucleotide biosynthesis (reviewed by Dong and Dunstan 2000; Stasolla et al. 2002). Very recently, a positive interaction of ABA with activated carbon in development and yield of somatic embryos was pointed out by Pullman et al. (2005a) for Norway spruce. Probably the main effect of exogenous ABA on progression of SE has been an improvement in embryo morphology, e.g., in caraway, as described by Ammirato (1977), an event probably related to the effect of this PGR on maturation of the embryos, as will be described below.

Exogenous application of GA_3 inhibited development of somatic embryos in most species evaluated (Takeno et al. 1983; Hutchinson et al. 1997 and references therein). However, it has also been observed that the combination of L-glutamic acid and GA_3 in cultures of *Hardwickia binata*, greatly improved the frequency of normal embryo differentiation (Das et al. 1995), and that GA_3 strongly stimulated somatic embryo production in *Medicago sativa* (Ruduś et al. 2002). Concerning ethylene, Roustan et al. (1994) observed an inhibitory effect on embryo formation in carrot

when this compound was applied at the beginning of the embryo developmental phase.

On maturation of somatic embryos and conversion of somatic embryos into plants

Another important phase in zygotic, but also in somatic, embryo development is the process of maturation. During this phase embryos undergo various morphological and biochemical changes, which are evident by deposition of storage materials, repression of germination and acquisition of desiccation tolerance (the latter aspect mainly in species with orthodox seeds) (Thomas 1993; McKersie and Brown 1996). Nevertheless, there are several examples in the literature in which somatic cultured embryos do not develop normally, germinate, nor convert into normal plantlets. In other cases, embryo development and maturation are interrupted by precocious germination, leading to the occurrence of poorly developed plantlets. Great efforts have been devoted to circumvent these problems, especially by supplementing culture media with certain PGRs that allow latter phases of SE to progress similarly to those in zygotic embryogenesis.

Inclusion of ABA into the culture medium during the final phases of somatic embryo development, resembling, in certain way, the natural increase in endogenous hormones observed in several zygotic embryos, is necessary to stimulate maturation and, at the same time, to prevent precocious germination, especially, but not only in conifers (Mauri and Manzanera 2004; Sharma et al. 2004; García-Martín et al. 2005). Bozhkov et al. (2002) found that the yield of mature somatic embryos of Norway spruce on ABA-containing medium was increased up to 10-fold when a pretreatment of 1–9 days with this PGR was applied. In legumes, a similar effect to the one observed in conifers was also noted. Moreover, partial desiccation or exposure to cold, heat, water and osmotic stresses have shown to enhance somatic embryo germination and conversion in many members of this family (reviewed by Lakshmanan and Taji 2000). Very recently, Blöchl et al. (2005) related the effect of ABA on maturation of alfalfa somatic embryos to an accumulation of raffinose oligosaccharides, such as it occurs during late seed development in orthodox seeds. In spite of the previous results, in peanut, application of ABA failed to improve somatic embryo maturation or conversion (Mhaske et al. 1998).

Concerning individual effect of other PGR groups, addition of GA_3 to the regeneration medium of bermudagrass, which usually contains BAP, accelerated germination/regeneration of the somatic embryos present (Li and Qu 2002). With reference to ethylene, its application in form of ethephon during maturation has been related to an increase in morphological abnormalities in white spruce (Kong and Yeung 1994), but did not show any apparent effect on *Picea sitchensis* (Selby et al. 1996).

Again in legumes, maturation has also been stimulated by inclusion of a CK alone, or in combination with an auxin, into the culture medium, being relevant the particular substance added (reviewed by Lakshmanan and Taji 2000). Nevertheless, there are some examples in this family, in which maturation and further development of somatic embryos occur only on growth regulator-free medium (Buchheim et al. 1989; Durham and Parrott 1992). Sreenivasu et al. (1998) explained such an event by suggesting that differentiated somatic embryos possibly acquire the ability to endogenously synthesize the hormones required to continue their development.

Even if obtaining high quantities of somatic embryos has not constituted a problem in several plant systems, a bottleneck encountered for massive propagation of certain species is the conversion of the somatic embryos into plants (Gaj 2004). In some cases, somatic embryos develop into small plants on culture medium without PGRs, whereas there are several experiments in which addition of different PGRs, together with the use of a different or altered basal medium, were necessary (reviewed by von Arnold et al. 2002). Among PGRs studied, CKs and auxins appear to have certain regulatory functions during somatic embryo germination and conversion, as demonstrated by the positive effect of the use of these PGRs separately or in combination (reviewed by Lakshmanan and Taji 2000).

The positive effect of ABA on inhibition of precocious germination and stimulation of maturation stimulation (see above) extends well into conversion of somatic embryos into 'normal-shaped' plants. This fact is well documented in grapevine (Rajasekaran et al. 1982; Goebel-Tourand et al.

1993) and *Brassica oleraceae* (Hansen 2000). In *Medicago falcata* this was only evident when the treatment was performed at the torpedo stage (Kuklin et al. 1994). Despite the stimulatory effect of ABA, prolonged exposure to this compound was reported by Bozhkov et al. (2002) to suppress the growth of the formed plants.

Dormancy of zygotic embryos in several species is counteracted by chilling. This treatment has been related to an increase in endogenous GAs (Takeno et al. 1983) and to a reduction in ABA endogenous contents (Rajasekaran et al. 1982; Jiménez and Bangerth 2000). Similarly, in somatic embryos of several species, especially in those which undergo dormancy, germination and conversion of somatic embryos into plants were stimulated by inclusion of GA_3 into the culture medium (reviewed by Gaj 2004).

There are also some cases in which particular treatments have a carry-on effect on later development of the explants. That is the case for the detrimental effect of the 2,4-D used during induction of SE observed afterwards on the regeneration ability of the somatic embryos obtained (Özcan et al. 1993; Rodríguez and Wetzstein 1998). The morphological abnormalities observed, such as multi-cotyledon or 'fan-shaped' embryos, have been related to disruption in polar auxin transport (Liu et al. 1993).

Interaction between PGRs and endogenous hormones during SE

Several observations support the premise that PGRs added exogenously exert part of their effect by modifying the concentrations of endogenous hormones (Gaspar et al. 1996, 2003). This mechanism has also been postulated to explain partially the regulation of SE by supplied PGRs (Neumann 1988; Carman 1990; Ribnicky et al. 1996; Thorpe 2000). Especially, the effect of added auxins and CKs has been related to an interaction with other endogenous plant hormones, such as ABA, ethylene, and GAs, producing, at the end, the conspicuous changes in development (Gaspar et al. 1996; Lakshmanan and Taji 2000). Modulation of endogenous hormones by exogenous PGRs may occur either directly (through enzyme synthesis) or indirectly (through effectors), as it was postulated for auxins by Gaspar et al. (1996).

There is evidence for PGRs modifying levels of endogenous hormones belonging, both to the same and to a different group, during SE. Examples of the former include the increase in the contents of IAA as a result of 2,4-D supply into the culture medium in carrot E cultures (Michalczuk et al. 1992a,b) and alfalfa leaf protoplasts (Pasternak et al. 2002). Moreover, two variant carrot cell lines able to grow at very high concentrations of 2,4-D (92 μM) increased their levels of endogenous IAA in response to this situation (Ceccarelli et al. 2002). Furthermore, Liu et al. (1998) reported that NAA and indole-3-butyric acid treatments promote an increase in the endogenous IAA levels in soybean hypocotyl explants.

The other alternative, modulation of endogenous hormone levels by exogenous PGRs belonging to a different group, is exemplified in a very early work, in which Noma et al. (1982) observed that 2,4-D regulated the relationship among polar and less polar GAs in carrot and anise. More recently, Charrière et al. (1999) reported an increase in the endogenous contents of IAA in immature zygotic embryos of sunflower as a consequence of ABA application. This PGR, added in high quantities, also reduced ethylene contents during maturation of somatic embryos of white spruce (Stasolla et al. 2002). In another report involving modulation of ethylene levels, an increase in the synthesis of this gaseous hormone was observed in response to application of high amounts of 2,4-D, which impaired embryo development (Minocha and Minocha 1995). A further example of the interaction among PGRs and endogenous plant hormones has been proposed to be the mechanism by which thidiazuron induces SE in peanut. This CK apparently modulates endogenous levels of auxins and CKs, which caused the observed effect (Murthy et al. 1995). This is supported by the reduction in the endogenous contents of IAA and BAP caused by thidiazuron in callus cultures of *Scutellaria baicalensis* (Zhang et al. 2005)

Sensitivity as a factor regulating SE

Trewavas (1981) raised, more than twenty years ago, the point that sensitivity to plant hormones has an important role in the way hormones modulate several processes in plants. He postulated that the sensitivity of the tissues to a change in the

hormone concentration (probably perceived by particular receptors) is more important than the change in the concentration itself.

Involvement of sensitivity during induction of SE could be evidenced by the fact that only responsive tissues react to the PGR contents in culture media (Bell et al. 1993; Somleva et al. 1995). Sensitivity to auxins might explain, at least partially, differences in response between plant species, genotypes or cells in the same explants or in explants with different origin, in their capability to become E (Dudits et al. 1995). Divergences in sensitivity are probably the consequence of variation in the ability of certain explants to produce the proper receptors, and thus continuing with the E developmental pattern (Guzzo et al. 1994). There is evidence that E lines are more sensitive than their NE counterparts to particular PGRs. This was observed by Bögre et al. (1990), in protoplast-derived cells or root explants from alfalfa. It has also been detected that 2,4-D can modulate the level of auxin-binding proteins in the membranes of carrot cell suspension cultures (reviewed by Lo Schiavo, 1995), being this a mechanism by which sensitivity might be affected.

Additionally, loss in E competence in sweet potato after prolonged time in culture was related, by Padmanabhan et al. (2001), to a decrease in auxin-responsiveness. Also, variation in CK requirements for optimal expression of SE in different species of *Medicago* and *Trifolium* were explained by Lakshmanan and Taji (2000), to be the consequence, in addition to genetic variability, of differential sensitivity to CKs. Moreover, since auxin- and CK-autonomy of habituated tissues could not be explained simply by an overproduction of these hormones (Jiménez 2001), it is high probable that sensitivity plays a role in development of this phenomenon (Gaspar et al. 2003).

Concluding remarks

In spite of the large amount of research conducted during the last years, knowledge is still vague in regards to the mechanisms by which plant hormones are involved in regulation of SE. There is a pattern well defined for highly responsive species and genotypes, in which the auxin 2,4-D plays a positive role for induction, while withdrawal of this compound triggers expression, allowing development of somatic embryos. However, this scenery is not so clear for the more recalcitrant genotypes, in which the requirements can vary greatly.

Evaluating endogenous hormones in explants varying in their degree of competence, as well as along development of SE, was proposed by the mid-1990s to be an approach that would improve induction and expression of this developmental process in recalcitrant genotypes (Merkle et al. 1995). However, use of custom-designed culture media that counteracts the deficiency of a particular hormone in an explant, by supplying the corresponding PGR, has not been employed frequently. The former has occurred despite the relatively large number of studies in which endogenous hormones were analyzed in the explants and tissue cultures. Moreover, to date the usual strategy to develop adequate 'recipes' to culture, multiply and regenerate plant through SE still involves addition of PGRs in a trial and error basis.

Findings summarized in this review give a clear indication on the absence of a unifying mechanism for induction, development and expression of SE in the different species and genotypes in which studies have been conducted. It has been postulated that the use of distinct methodologies to purify plant extracts and to quantify plant hormones is, at least, partially, responsible for the differences reported in various works (Jiménez 2001). However, nowadays the methodologies employed are highly reliable and very large differences among their results are not to be expected (reviewed by Ljung et al. 2004). It is more feasible that the encountered differences are the result of genotypic diversity among cultivars and species or of physiological determination of the explants.

The absence of tight relationships between endogenous hormones and E competence, as well as the large variability in the requirements of PGRs to promote and govern SE, as described in this review, point out to the participation of additional factors. Interaction between PGRs and endogenous hormones seems to be one of the mechanisms involved that might explain the absence of a common pattern of hormonal regulation in this process, and has to be studied in more detail. An aspect that is gaining importance is the differential sensitivity of particular tissues/genotypes to specific factors (i.e., PGR and endogenous hormones). Recent advances in identifying the molecular receptors for some hormonal groups (reviewed by

Napier 2004), together with comprehending the responses that plant hormones and PGRs induce at the level of gene expression might bring new insights to the subject (Dong and Dunstan 1997; Shakirova et al. 2002) and might help to gain a better understanding of the actors involved.

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