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10

Plant Somatic Embryogenesis: Some Useful Considerations

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1. Introduction

This review chapter discusses basic clues of plant somatic embryogenesis. Firstly, the similarities between zygotic and somatic embryogenesis will be compared, starting from the polarity of the egg cell inside the embryo sac and ending with the mature embryo inside a seed.

The rest of the chapter will review and discuss the most important factors needed for the conversion of a somatic cell into an embryo and finally into a whole plant.

2. Zygotic embryogenesis *versus* somatic embryogenesis

2.1 Zygotic embryogenesis

In order to understand the formation and production of plant somatic embryos it is important to briefly look at the process of zygotic embryogenesis given their high similarity. Double fertilization is one of the main characteristics of angiosperms where one male gamete fertilizes the egg cell and a second male gamete fertilizes the central cell of the embryo sac (Russell, 1993).

Embryogenesis has evolved as a successful reproductive strategy in higher plants. The life cycle of angiosperm plants (flowering plants) is divided into two phases: the diploid sporophytic phase and the haploid gametophytic phase (Fan et al., 2008). The functions of the gametophyte are short lived and less complex than those of the sporophyte and are only devoted to produce haploid male and female gametes (Fan et al., 2008; Reiser & Fischer, 1993; Yadegari & Drews, 2004). Male gametes or microgametophytes (pollen grains) are developed inside the anthers and are formed from a pollen mother cell which undergoes a meiotic process that gives rise to a tetrad of haploid cells called microspores. During the maturation towards the pollen formation, the microspore suffers an asymmetric mitotic division giving rise to a two new cells: the vegetative and the generative cells. The generative cell undergoes a second mitotic division producing two sperms, while the vegetative cell remains undivided and bears the capacity of producing the polen tube which

will grow in the female tissue of the carpel serving as the sperm carrier (Mc Cormick, 2004) (Figure 1).

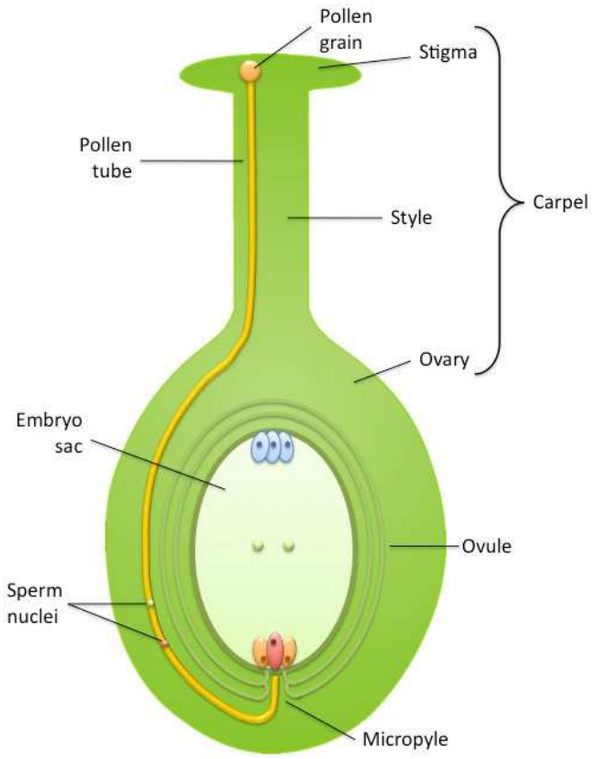

Fig. 1. Ideogram of an angiosperm female apparatus in the flower and the path of the pollen tube toward the embryo sac.

The female gametophyte called **embryo sac** is developed inside the carpel, which consists of three elements: the ovary, the style and the pollen grain receptacle called stigma. The ovary may hold one or several ovules which bear the female gametophyte or embryo sac. An ovule is formed by three layers of cells surrounding the embryo sac, the nucellus and the inner and outer integuments. The integuments do not join at the tip of the ovule leaving an opening called micropyle, which is the "door" for the penetration of the pollen tube into the embryo sac (Figures 1 and 2).

The female gametophyte, megagametophyte or embryo sac is developed inside the ovule. Each ovule contains one megaspore mother cell, which after two rounds of meiotic cell divisions gives rise to a strand of four haploid megaspores. In the majority of angiosperm plant species three of these cells degenerate, but the closest to the chalaza survives as the functional megaspore, enlarges and undergoes three mitotic divisions to form the embryo sac. The embryo sac may follow different patterns of development in different species, however the most common consists of four types of cells, arranged as follows: Three antipodal cells (at the chalazal end), one central cell containing two polar haploid nuclei (that generally migrate towards the center of the embryo sac), two synergid cells flanking the egg cell (all three positioned at the micropylar end) (Maheshwari, 1937; Yang et al., 2010) (Figure 2).

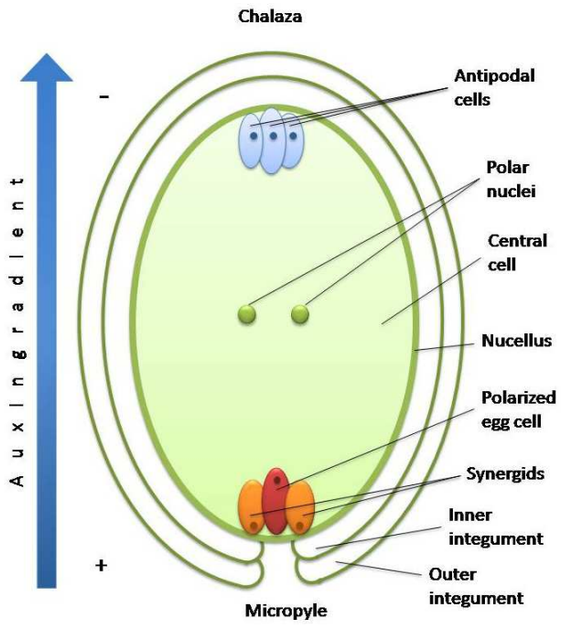

Fig. 2. Ideogram of the female angiosperm gametophyte showing the polarity of the embryo sac and the egg cell given mainly by an endogenous auxin gradient (blue arrow to the left). Modified from Pagnussat et al. (2009).

In the sexual reproduction of angiosperms, the pollen grain is transferred from the anther to the stigma where it germinates and forms the pollen tube (Yadegari & Drews, 2004) which travels long distances directed first by sporophytic signals and then by the female gametophyte (Wetering & Russell, 2004). Afterwards, the pollen tube reaches the micropyle where it is guided by signals generated by the synergid cells through high calcium concentrations (Tian & Russell, 1997). Then the spermatic cells are discharged into one of the synergids through the filiform apparatus (Yadegari & Drews, 2004). Double fertilization takes place when one spermatic nucleus fuses with the egg cell forming the zygote (diploid), while the second sperm fuses with the polar nuclei of the central cell to initiate the endosperm (generally triploid) (Russell, 1993).

The observed polarity of zygotic embryos starts with the formation of the embryo sac. This polarity in the embryo sac is due to a gradient of the natural auxin indole-acetic acid along the micropyle-chalaza axis whose expression starts at the micropylar region outside of the embryo sac. In the same manner and following this pattern, the haploid egg cell which after its fertilization produces the embryo, is also highly polarized with its nucleus located at the chalazal pole (Pagnussat et al., 2009). Pagnussat et al. (2009) reported that it is possible that auxin does not regulate the position of the nuclei during the embryo sac formation, however it participates in the regulation of cell fate at cellularization. After fertilization, the resulting diploid zygote remains highly polarized, while the other male gamete fuses with the central cell of the embryo sac which then develops into the triploid endosperm, acting as a nutritive and protective element for the embryo. In the majority of the plant species, the somatic embryogenesis process follows the above pattern. In the case of somatic embryogenesis being the initial somatic embryogenic cell equivalent to the zygote, and the *in vitro* culture medium being equivalent to the nutritive and protective endosperm (Figure 3).

In angiosperms, the first division of the zygote is highly asymmetric. Actin governs the migration of the premitotic nucleus into the future division plane and the placement of the preprophase band in these asymmetrically dividing cells (Rasmussen et al., 2011). Once fertilized, the zygote elongates and divides asymmetrically, with the smaller apical cell generating most of the embryo and the larger basal cell giving rise mainly to the extra embryonic suspensor. Subsequent divisions of the large basal cell give rise to the suspensor and at its tip the hypophyseal region where the radicle will be formed and finally giving the symmetry to the whole plant (Toonen & de Vries, 1996; Gutiérrez-Mora et al., 2004). The single-celled zygote soon acquires the potential to develop into an embryo undergoing a series of complex cellular and morphological processes that finally produce the sporophyte or plant (Rao, 1996). Further information in these topics can be found in Russell (1992); Rotman et al. (2003); Gutiérrez-Marcos et al. (2006); He et al. (2007) and Capron et al. (2008), among many others.

2.2 Somatic embryogenesis

Somatic embryogenesis is the maximum expression of cell **totipotency** in plant cells. In short, totipotency is the hability of a plant cell to undergo a series of complex metabolic and morphological coordinated steps to produce a complete and normal plant or sporophyte without the participation of the sexual processes. Thus, somatic embryogenesis is the developmental process by which theoretically any somatic cell develops into a zygotic like structure that finally forms a plant (Rao, 1996; Jiménez, 2005). Like their zygotic counterparts, somatic embryos have a single cell origin (Rao, 1996). The single cell origin of somatic embryos has been elegantly reported by several authors, in particular, this unicellular origin of somatic embryos has been reported in *Agave tequilana* (Gutiérrez-Mora et al., 2004; Portillo et al., 2007).

Usually, the somatic embryogenic process consists of two main steps, the induction of the process and the expression of the resultant embryos (Rodríguez-Garay et al., 2000; Gutiérrez-Mora et al., 2004; Jiménez, 2005). The process is initiated with somatic cells theoretically from any part of the plant, however, substantial differences in competence are found in practice. The cells which are more competent for somatic embryogenesis are generally those coming from young tissues, immature zygotic embryos among them. However, stems, roots and leaves may be useful as well. Usually, somatic embryos are induced by simple manipulation of the cultural *in vitro* conditions. One of the main elements in the culture medium are the growth regulator substances (GRS) such as auxins, cytokinins, abscisic acid and gibberellins among other components. Also, it is important to mention that the hormonal endogenous substances play important roles in the somatic embryogenic process. Out of the above mentioned GRS, auxins are the most important components in the induction of the process (Dodeman et al., 1997; Jiménez, 2005; Jiménez & Thomas, 2006; Rao, 1996; Feher, 2006). Somatic cells need the signal for the cell polarization and the asymmetric division given by auxins as it happens in their zygotic counterparts (Gutiérrez-Mora et al., 2004; Pagnussat et al., 2009). The participation of the other GRS is important in the balance of hormonal constituents needed to achieve somatic embryogenesis.

With regard to the initial steps of the development of a somatic embryo, the induction process is generally initiated by the action of a selected auxin (the most used auxin for most species is 2,4-Dichlorophenoxiacetic acid (2,4-D)) (Nomura & Komamine, 1986; Jiménez,

2005). In this revision the cellular process is illustrated by the formation of a somatic embryo of *Agave tequilana* Weber cultivar Azul (Gutiérrez-Mora et al., 2004; Portillo et al., 2007).

The initial induced somatic cell emulates its sexual counterpart "the zygote". This is a highly polarized cell with its nucleus positioned to an extreme of the cell, leaving the other extreme highly vacuolated (Figure 3a and a'). The first transversal cell division is asymmetrical giving rise to a small apical cell and a highly vacuolated basal cell (Figure 3b and b'). A second division of the apical cell gives rise to the embryo proper or two-celled proembryo and the highly vacuolated cell which is putatively the first cell of the suspensor (Figure 3c and c'). A third round of cell division produces a four-celled embryo head, and the first suspensor cell has suffered a second division. Observed subsequent cell divisions of the suspensor cells give rise to the putative hypophyseal region where the plant radicle will be formed. After this round of cell divisions, subsequent and well coordinated divisions will

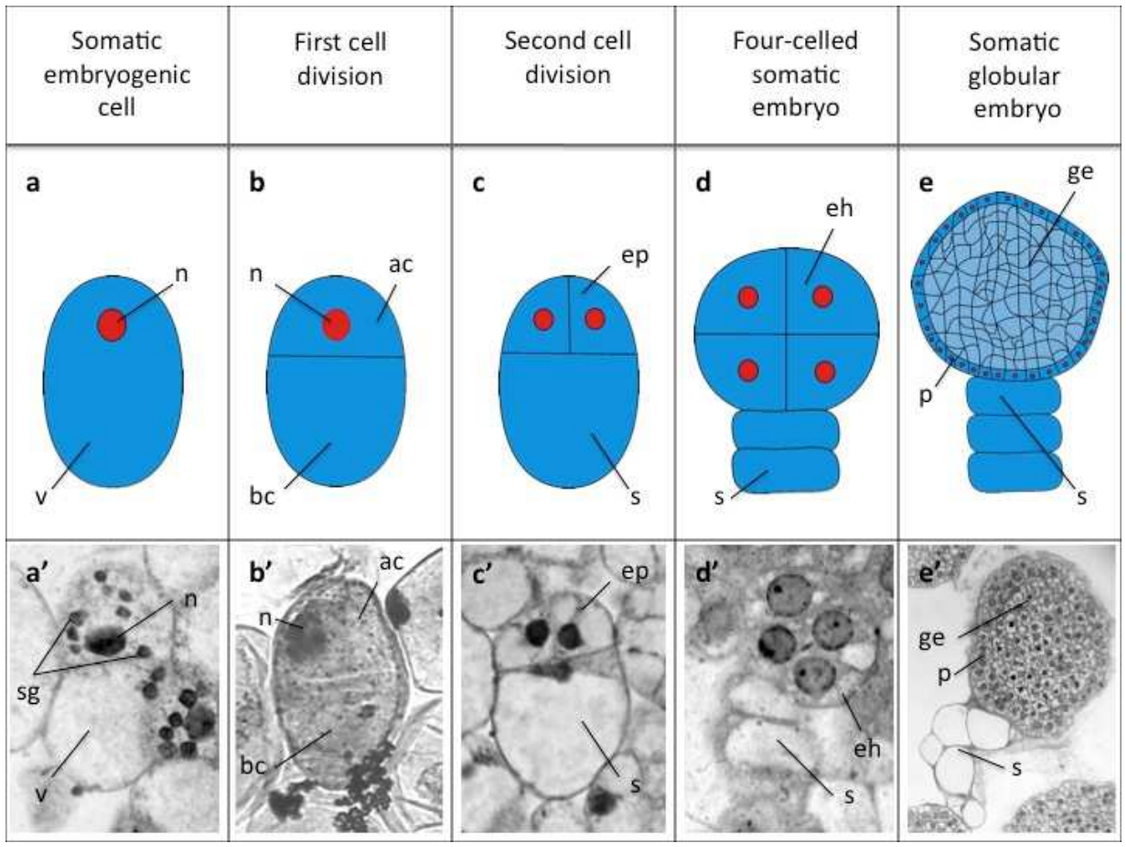

Fig. 3. Early cell divisions in the somatic embryogenesis of the monocotyledonous species *Agave tequilana* Weber var. Azul. **a) - e)**. Ideogram of the somatic embryogenic process, which represents the real somatic embryogenic process **a') - e')**. **n** – nucleus, **v** – vacuole, **sg** – starch granules, **ac** – apical cell, **bc** – basal cell, **ep** – embryo proper, **s** – suspensor, **eh** – embryo head, **p** – protoderm, **ge** – globular embryo. Figures 3a', 3c' and 3d' are from Portillo, L., et al. Somatic embryogenesis in *Agave tequilana* Weber cultivar azul. In Vitro Cellular and Developmental Biology – Plant, 2007, Vol. 43, pp. 569-575. Copyright© 2007 by the Society for In Vitro Biology, formerly the Tissue Culture Association. Reproduced with permission of the copyright owner.

form the globular stage of the somatic embryo (Figure 3e and e'). It is important to mention that at this globular stage the somatic embryos of most of the species are very similar having protoderm. Also, it is important to mention that in many species the suspensor is not observed because it does not remain and does not divide. However, this cellular and morphological point of the somatic embryo is important for the formation of the radicle and the final symmetry of the whole plant (Gutiérrez-Mora et al., 2004; Yeung et al., 1996; Supena et al., 2008).

Moreover, after the globular stage of the somatic embryos, the fate of their morphology follows their genetic lineage: monocotyledonous or dicotyledonous as it can be observed in Figure 4. A distinctive characteristic of the dicotyledonous species is the formation the cotyledon primordium (cp) which gives to the classical heart form to somatic embryos, similar to their zygotic counterparts. On the other hand, somatic embryos of monocotyledonous species show the classical torpedo shape which is disrupted at germination.

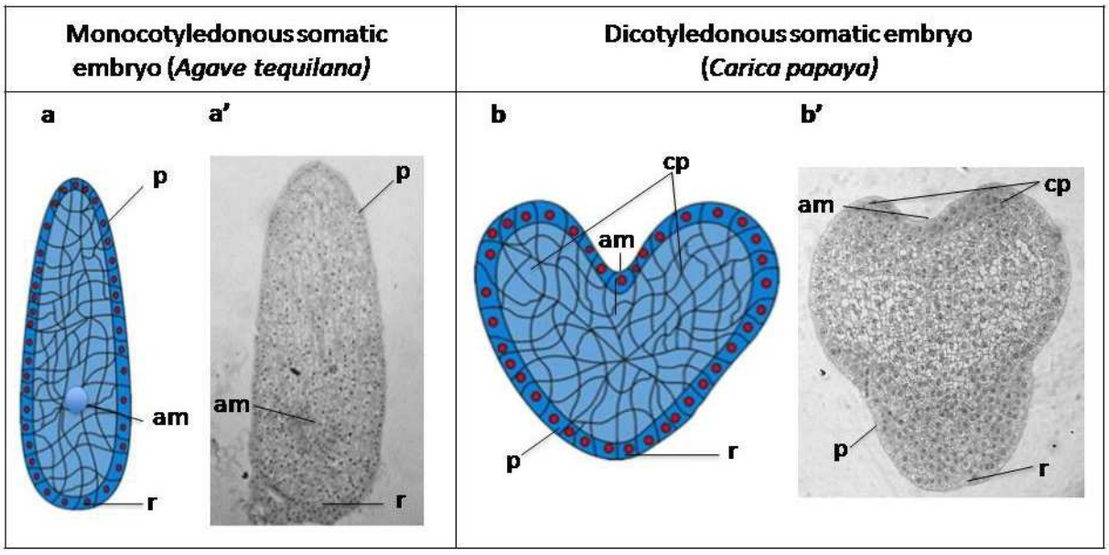

Fig. 4. Differential morphology between monocotyledonous and dicotyledonous somatic embryos after the globular stage. a) and a') Ideogram and real *Agave tequilana* (monocotyledonous) somatic embryo, respectively. b) and b') Ideogram and real *Carica papaya* (dicotyledonous) somatic embryo, respectively. **r** – radicle, **am** – apical meristem, **p** – protoderm, **cp** - cotyledonar primordium.

3. The role of phytohormones

Plants are sessile organisms which have endogenous signals (hormonal compounds) to cope biotic and abiotic challenges (Gilroy & Trewavas, 2001). Phytohormones are chemical cues which are produced at relatively low concentrations and move around the plant triggering diverse responses in tissues and cells. Some of the most important characteristics of endogenous hormonal elements are (Öpik & Rolfe, 2005):

They work at low concentrations, in general between 10^{-6} a 10^{-9} M at the site of action. High concentrations inhibit their action. A medium for hormonal transport is needed if the site of

its synthesis is different from that for its action. Hormones are mainly transported through the vascular system of the plant. This is not always true as is the case for ethylene. It is necessary that the target site has the capacity to respond. Plant hormones are produced endogenously by the plant itself. Plant growth regulators (PGR) are synthetic compounds with hormone-like activity which are given to the plant under *in vitro* or *ex vitro* conditions. There exist several groups of these compounds according to their physiological action:

Auxins.- The original endogenous hormone is the Indole-3-acetic acid (IAA). Some of the most used synthetic PGR with auxin activity are indole-3-butyric acid (IBA), naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), etc.

Cytokinins.- In whole plants the natural cytokinins are zeatin and zeatin riboside. Some of the most used synthetic compounds are kinetin, thidiazuron (TDZ), benzyladenine (BA), among others.

Gibberellins (GA).- In nature, plants produce more than 110 different kinds of GA, however the most used compounds are GA_1, GA_3, GA_4 and GA_7

Abscisic acid(ABA).- This is a natural compound which is used in both *in vitro* and *ex vitro* conditions.

The process of somatic embryogenesis requires different concentrations and combinations at its different stages in order to finally produce an embryo. The two most important stages, the induction and the expression of embryos may require different medium composition with regard to nutrients and growth regulators.

In nature, auxins are produced in apical and root meristems, young leaves, seeds and developing fruits, and their main functions are cell elongation and expansion, suppression of lateral buds, etc. (Öpik & Rolfe, 2005). In somatic embryogenesis this is considered one of the most important elements producing cell polarity and asymmetrical cell division. In general, relatively high auxin concentrations (2,4-D, IAA, etc.) favor callus formation and the induction process (cell polarity). Afterwards, when the induction stage has been achieved, it is necessary to reduce or eliminate the auxins in order to initiate the bilateral symmetry and the expression of the somatic embryos.

On the other hand, in nature, cytokinins are an important factor for cell division, and stimulate the formation and development of lateral or axilar buds, retard senecense and inhibit root formation. In *in vitro* somatic embryogenesis, cytokinins are utilized in combination with auxins and play an important role in cell proliferation. In the production of somatic embryos of some plants such as pea and soybean, the adition of cytokinins to the culture medium inhibits the induction effect of auxins (Lakshmanan & Taji, 2000). However, in other species such as *Zoysia japonica* (Asano et al., 1996), *Begonia gracilis* (Castillo & Smith, 1997) and *Oncidium sp*. (Chen & Chang, 2001), the use of cytokinins favours the induction of somatic embryos.

With regard to gibberellins (GA), in whole plants, these hormonal compounds are mainly produced in the apical zone, fruits and seeds. They stimulate stem growth and are responsible for the distances between nodes by stimulating cell elongation, also, they regulate the transition from the juvenile stage to the adult stage of the plant and promote seed germination by regulating the rupture of the embryo dormancy. In *in vitro*

embryogenic cultures, the addition of GA promotes the regeneration process and the germination of somatic embryos (Li & Qu, 2002).

Finally, another important hormonal factor (but not the last) is the abscisic acid (ABA). This compound is produced mainly in chloroplasts. Its main functions are stomata closure, seed dormancy and the inhibition of axillary buds growth. The addition of ABA to *in vitro* embryogenic cultures inhibits the early embryo germination and stimulates the coordinated maturation of the somatic embryo. However, prolonged exposition to ABA, this element suppresses growth of the formed *in vitro* plants (Bozhkov et al., 2002).

Plant growth regulators are critical in determining the pathway of the plant cells. The effects of growth regulators in somatic embryogenesis have been studied in a variety of plant species. The potential of 2,4 D has been reported as the most efficient growth regulator in *Eleutherococcus sessiliflorus, Gymnema silvester, Holestemma ada-kodien, Paspalum scrobiculatum, Andrographis paniculata* (Choi et al., 2002; Kumar et al., 2002; Martin, 2004). Similarly, Malabadi et al. (2005) developed an effective protocol for inducing somatic embryogenesis in conifers using triacontanol (TRIA) as growth regulator. Moreover, adding Picloram (PIC), 6-benzylaminopurine (BAP) and naphthaleneacetic acid (NAA) was reported to influence plant regeneration via somatic embryogenesis in *Arachis pintoi* and *Arachis glabrata* (Rey & Mroginski, 2006; Vidoz et al., 2004). On the other hand, high-frequency regeneration via somatic embryogenesis of recalcitrant cotton (*Gossypium hirsutum L.*) was possible in medium containing kinentin and 2,4 dichlorophenoxyacetic acid fortified with B5 vitamins and the addition of zeatin (Khan et al., 2010). Carbenicillin, a well-known antibiotic in the culture media showed a growth regulator activity in somatic embryogenic callus induction (Shehata et al., 2010). The effects of 2,4-D, kinetin and 6-benzylaminopurine in the micropropagation of *Anthurium andreanum* 'Tera' through somatic embryogenesis were reported by Beyramizade et al. (2008). Germination of somatic embryos was possible in Pigeonpea *(Cajanus* L. Mills.), *Arachis pintoi and Pennisetum glaucum (L.) R. Br and Phalaenopsis* in MS medium supplemented with BA (Mohan & Krishnamurthy, 2002; Rey & Mroginski, 2006; Jha et al., 2009; Gow et al., 2010). Furthermore, it has been reported that the oxidative stress induced by specific grow regulators is associated with callus regeneration (Szechyńska et al., 2007). Exogenously supplying polyamines during the multiplication stage has been reported as being deleterious at successive stages of somatic embryo formation of *Panax ginseng*. Nevertheless, adding spermidine at the initiation stage enhanced the effect of the synthetic auxin 3-(benzo[b]selenyl) acetic acid (BSAA) (Kevers et al., 2002).

4. *In vitro* environmental factors

Plant regeneration through somatic embryogenesis has been reported in several studies. However, developing regeneration methods that meet the physical and chemical demands of the plant cells is still a largely empirical process. Identifying ideal *in vitro* culture conditions can be extremely difficult due to the wide number of factors that contribute to the induction, development and conversion of the somatic embryo into a plant. With the aim to overcome difficulties and determine optimum conditions for *in vitro* propagation via somatic embryogenesis, the effects of these factors have been studied in a significant number of plant species.

4.1 Culture medium

Culture medium is one of most important factors to be considered for *in vitro* plant cell culture and it can be used in either solid or liquid state. Also, it must supply the essential minerals required for growth and development. The most common medium used in in vitro plant cultures is that developed by Murashige and Skoog (1962) which has been reported to be used in plant regeneration of several species (Ascencio-Cabral et al., 2008; Castillo & Smith, 1997; Fitch et al., 1993; Mohan & Krishnamurthy, 2002; Kevers et al., 2002; Konieczny et al., 2008, among many others). Alternatively, B5 medium has been used for the *in vitro* regeneration of *Arabidopsis thaliana* (Gaj, 2001). Furthermore, Thuzar et al. (2011) revealed that using N6 medium in *in vitro* cultures promoted somatic embryogenesis and plant regeneration of *Elaeis guineensis* Jacq. As well as these, somatic embryogenesis of *Agave tequilana* has been reported when cultured on Schenk and Hildelbrant (SH) medium (Rodríguez-Sahagún et al., 2011).

4.2 Gelling agents

When plant cells or tissues are to be cultured on the surface of the medium, it must be solidified. Even though agar is the most frequent type of gelling agent used in culture media, the water potential of a medium solidified with gel is more negative than that of liquid medium, due to their matric potential (Amador & Stewart, 1987, as cited by George et al., 2008). Nevertheless, Ascencio-Cabral et al. (2008), reported a significant effect on plant regeneration from somatic embryos of *Carica papaya L* when media was solidified with Difco® Bacto Agar. Results showed that the gelling agent not only had a strong effect by itself but also it interacted positively with other factors such as light and the presence of the glucoside phloridzin, producing a high rate of healthy plantlets. Apart from this, the addition of phenolic glycosides into the growth medium have been reported to help reduce the occurrence of hyperhydricity and plant regeneration was improved (Ascencio-Cabral et al., 2008; Witrzens et al., 1988). Another example of the use of a gelling agent is Phytagel or Gellan gum. Complete plants were produced after somatic embryo germination. Cultures were transferred to the same basal medium without growth regulators, and solidified with 5g/l of the agar substitute Phytagel. After 16 weeks, somatic embryos started to germinate and developed typical plantlet morphology (Torres-Muñoz & Rodríguez-Garay, 1996).

4.3 Carbon source

Carbon sources have been reported to have a significant effect in *in vitro* plant regeneration. In general, sucrose is the carbohydrate of choice as carbon source, probably because it is the most common carbohydrate in the plant phloem (Murashige & Skoog, 1962; Thorpe, 1982; Lemos & Baker, 1998; Fuentes et al., 2000, as cited by Ahmad et al., 2007). Fitch et al. (1993) reported the effect on frequency of somatic embryos in cultures of *Carica papaya* L. when the culture medium was supplied with different concentrations of sucrose. Results showed that tissue in medium containing 7% of sucrose was able to enhance somatic embryogenesis (Fitch, 1993). In addition, Rybczyński et al. (2007) reported that supplementing 0.2-0.4% sucrose to culture media boost the efficiency of the photosynthetic apparatus of somatic embryos of *Gentiana kurroo*. However, other carbohydrates can also be suitable and in special conditions may be better than sucrose (Slater et al., 2003). Furthermore, somatic embryogenesis of *Citrus deliciosa* was promoted by supplementing the culture media with

galactose (Cabasson et al., 1995). Selecting the suitable source of carbohydrates and the concentration has been reported to induce high-efficiency somatic embryogenesis in cell cultures of *Phalaenopsis and Prunus incisa* (Tokuhara & Mii, 2003; Cheong & Pooler, 2004).

4.4 Amino acids

Moreover, studies have been conducted to optimize different types and concentrations of amino acids on the induction of somatic embryogenesis in strawberry (*Fragaria x ananassa* Duch.) cultivars. Results revealed that stimulation of embryogenesis and embryo development was strictly dependent on the type and concentration of amino acid in the medium. Proline was much more effective than glutamine and alanine on induction and development of somatic embryogenesis (Gerdakaneh et al., 2011).

4.5 Environmental factors

Moreover, physical factors such as light, photoperiod, temperature, gaseous environment and osmotic pressure have to be controlled when cultured *in vitro*. In order to find the most suitable environmental conditions to produce somatic embryos several works have been conducted.

4.6 Temperature

Applying a heat-shock encouraged somatic embryogenesis in cultures of *Avena sativa* (Kiviharju & Pehu, 1998), *Zea mays*, *Triticum aestivum* L. and rye (Fu et al., 2008). Alternatively, a cold pre-treatment doubled the embryogenic response in *in vitro* maize cultures (Pescitelli et al., 1990). Thermal shock (cold and heat) and incubation in mannitol, cultures of *Dianthus chinensis* (Fu et al., 2008) showed a strong interaction between the genotype and culture conditions for the production of somatic embryos. Aslam et al. (2011) evaluated the effect of freezing and non-freezing temperature on somatic embryogenesis in *Catharanthus roseus* (L.), their results showed that somatic embryo development (production, maturation and germination) was sensitive to temperature variations.

4.7 Light

In addition, light has a significant effect in plant development. The importance of light in plant regeneration of wheat cultures has been reported in several studies (Liang et al., 1987; Jaramillo & Summers, 1991). Somatic embryogenesis in quince was reported as positively regulated by phytochrome (D'Onofrio et al., 1998). Furthermore, research conducted by Torné et al. (2001) demonstrated that somatic embryogenesis in *Araujia sericifera* was promoted by light provided by gro-lux lamps. Alternatively, light or dark treatments have been reported to induce embryogenesis in cultures of *Prunus incisa Thunb*. cv. (Cheong & Pooler, 2004). In contrast, the expression and the maturation of embryos of *Agave tequilana* Weber were successfully achieved when embryos were exposed to red light for 15 days in LOG medium at λ= 630 nm (Rodríguez-Sahagún et al., 2011). Germanà et al. (2005) evaluated the effect of light quality in a culture of *Citrus clementina* Hort. ex Tan., cultivar Nules; as a result embryogenic callus was produced only under photoperiodic conditions of white light. Ascencio-Cabral et al. (2008), reported a significant effect of light quality on germination and plant length from somatic embryos of *Carica papaya* L. In this study,

embryos exposed to gro-lux and wide-spectrum light germinated healthier and developed regular roots. However, Gow et al. (2009) reported negative effects on direct somatic embryogenesis of *Phalaenopsis* orchids in cultures exposed to light; in addition, light induced embryo necrosis and low plantlet regeneration. Furthermore, varying the culture period effectively enhanced somatic embryogenesis when culture conditions were 60 days for induction in darkness and 45 days for subculture in light (Gow et al., 2010).

4.8 Ethylene biosynthesis

Another factor affecting somatic embryogenesis is ethylene biosynthesis which has been reported to inhibit regeneration (Giridhar et al., 2004), it has been reported that by blocking its synthesis plant regeneration increased. Giridhar et al. (2004) reported that by adding silver nitrate at different stages of the plant regeneration through somatic embryogenesis of *Coffea arabica* L. and *Coffea canephora* was good for the production of somatic embryos. Recent research conducted by Kępczyńska & Zielińska (2011) focused on the effects ethylene inhibitors binding to receptors at different phases of somatic embryogenesis in *Medicago sativa* L.; the findings showed that ethylene biosynthesis and its action influenced individual phases of somatic embryogenesis. Moreover, alterations of these processes affected adversely the activity of the production of somatic embryos.

5. A new whole plant

A great number of *in vitro* produced plants do not survive the transfer from the *in vitro* to the *ex vitro* environment under greenhouse or field conditions. Due to their anatomical and physiological characteristics, these kinds of plants need a gradual adaptation or acclimatization to *ex vitro* environments in order to survive and be productive. The greenhouse and field have substantially lower relative humidity, higher light level and septic environment that are stressful to micropropagated plants compared to *in vitro* conditions (Hazarika, 2003, Hazarika & Bora, 2010). Plantlets were developed within the culture vessels under low level of light, aseptic conditions, on a medium containing ample sugar and nutrients to allow for heterotrophic growth and in an atmosphere with a high level of humidity. These contribute a culture-induced phenotype that cannot survive the environmental conditions when directly placed in a greenhouse or field. The physiological and anatomical characteristics of micropropagated plantlets necessitate that they should be gradually acclimatized to the environment of the greenhouse or field (Kozai, 1991).

Abnormalities in morphology, anatomy and physiology of plantlets cultivated *in vitro* can be repaired after transfer to *ex vitro* conditions. However, many plant species need gradual changes in environmental conditions to avoid desiccation and photoinhibition. During acclimatization to *ex vitro* conditions, leaf thickness generally increases, leaf mesophyll progresses in differentiation into palisade and spongy parenchyma, stomatal density decreases and the stomatal shape changes from circular to an elliptical one. The most important changes include development of cuticle, epicuticular waxes, and effective stomatal regulation of transpiration leading to stabilization of water status. For photosynthetic parameters it seems very important at which conditions *in vitro* plantlets have been grown. According to this, transfer can be accompanied with a transient decrease in photosynthetic parameters. Further, an increase in chlorophyll content, maximum photochemical efficiency, actual quantum yield of photosystem II, and net

photosynthetic rate is usually observed in dependence on the environmental conditions during acclimatization. Acclimatization can be speed up by hardening of plantlets *in vitro* or after transplantation by decreasing the transpiration rate by antitranspirants including ABA, or by increasing the photosynthetic rate by elevated CO_2 concentration (Pospíšilová et al., 1999).

Hyperhydricity is a factor which is considered as a physiological disorder that can be induced by diverse stress conditions. Previous research about hyperhydricity, report that the observed anatomical and physiological problems are the result of several altered or disrupted metabolic pathways, such as changes in the synthesis of proteins that negatively affect enzymes involved in the photosynthetic apparatus (Rubisco), also, the disruption in the synthesis of cellulose and lignin (PAL, glucan synthase) and the alteration of processes associated to ethylene synthesis (peroxidases). Changes in the synthesis of proteins affect enzymes which are linked to interconnected metabolic pathways. Low protein levels have been found in hyperhydric leaves as compared to normal leaves and a 30 kD protein has been found only in anomalous leaves (Van Huystee, 1987); and also other proteins (30-32 kD) associated to lignin synthesis (Kevers et al., 1984).

On the other hand, stems exhibit hypertrophy of cortical and pith parenchyma, large intercellular spaces, hypolignification of the vascular system (Kevers et al., 1985), and a reduced and/or abnormal vascular system (Letouzé & Daguin, 1987). Jausoro et al., (2010) reported disorganized cortex, epidermal holes, epidermal discontinuity, collapsed cells, and other structural characteristics were observed in hyperhydric shoots of *Handroanthus impetiginosus*.

Hyperhydricity is the expression of several phases with diverse degrees of abnormalities in affected plants. These plants are not able to survive the stress imposed by the transfer of the *in vitro* to the *ex vitro* environment. In order to have success in this transfer process, it is necessary that the plants to undergo through a gradual change to aquire their normal anatomical stage for a succesful aclimatization process (Debergh et al., 1992).

In order to overcome hyperhydricity, several strategies have been proposed, basically related to environmental issues that help to control relative humidity and water availability through the manipulation of solutes in de growth medium (Maene & Debergh, 1987). Plantlets with well developed leaves under low humidity and high irradiance show a photosynthetic and metabolic normal activity.

In *in vitro* cultures, the photosynthetic activity is scarce, for which the adaptation of the foliar system toward an active photosynthesis is necessary. For the above mentioned adaptation, several strategies have been proposed: elimination of carbon sources, mechanical defoliation of plantlets, induction of storage organs (Ziv & Lilien-Kipnis, 1990), use of growth retardants to inhibit foliar growth in order to improve the proliferation rate, meristems growth, and the enrichment of CO_2 under high luminic intensity (Ziv, 1989). Shoot hyperhydricity, resulting in failure to root and/or survive transplanting is a frequent problem in sunflower (Baker et al., 1999). Hyperhydricity can be controlled in various ways including improved vessel aeration (Rossetto et al., 1992), reducing cytokinin levels (Williams & Taji, 1991), increasing agar concentration (Brand, 1993) and changing the concentration of medium constituents (Ziv, 1989). Losses up to 60% of cultured shoots or explants have been reported due to hyperhydricity in commercial plant micropropagation

(Piqueras et al., 2002). On the other hand stems exhibit hypertrophy of cortical and pith parenchyma, large intercellular spaces, hypolignification of vascular system (Kevers et al., 1985), and a reduced and/or abnormal vascular system (Letouzé & Daguin, 1987).

6. Conclusion

Zygotic embryogenesis is a key process in flowering plants, and it is a well coordinated series of developmental events governed from the very beginning by cell polarity and asymmetric cell division in which male and female cells participate.

Somatic embryogenesis is almost a mirror copy of the above process, but without the participation of sexual organs and cells. Single somatic cells are programmed to follow similar developmental steps by the manipulation of *in vitro* environmental factors such as culture medium which includes several components: a gelling agent (when necessary), a carbon source, several nutrient elements and most importantly hormonal-like factors. Also, the somatic embryogenic processes need special physical environmental factors for the incubation of cell and tissue cultures, such as temperature and light among others.

The practical uses of somatic embryos include the massive propagation of plants of high commercial value and more importantly their use in basic research and in plant breeding programs where biotechnological tools are used.

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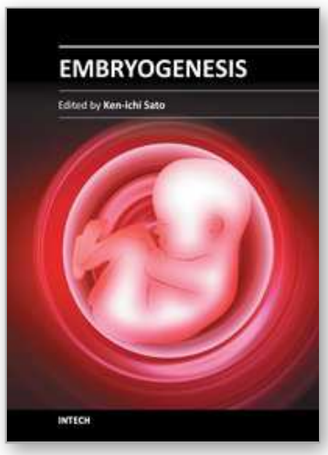

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The book "Embryogenesis" is a compilation of cutting edge views of current trends in modern developmental biology, focusing on gametogenesis, fertilization, early and/or late embryogenesis in animals, plants, and some other small organisms. Each of 27 chapters contributed from the authorships of world-wide 20 countries provides an introduction as well as an in-depth review to classical as well as contemporary problems that challenge to understand how living organisms are born, grow, and reproduce at the levels from molecule and cell to individual.

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