Somatic Embryogenesis - Apical Meristems and Embryo Conversion

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ABSTRACT A large amount of information is currently available for somatic embryogenesis of plants. However, one common problem related to somatic embryos is that the conversion rate can be low for some species. Apical meristems are responsible for post-embryonic growth of the embryo. The low percentage observed is most likely a result of poor apical meristem development or defects in the meristem organization during somatic embryogenesis. In flowering plants, apical meristems are well developed by the late heart stage of zygotic embryo development. In conifers, such as white spruce, apical meristems are formed at the pre-cotyledon stage. Thus, apical meristem development occurs very early, prior to the maturation stage of embryo development. Once formed, meristems are stably determined. In the somatic embryo, as exemplified by white spruce, since embryo development is not synchronous, tissue differentiation including apical meristem formation occurs throughout the "maturation" stage. Different apical meristem organizations can be found among different individuals within a population. In contrast to their zygotic counterparts, the apical meristems appear not to be stably determined as their organization, as the shoot apical meristem especially, can be easily modified or disrupted. Precocious germination seldom results in functional plantlets. All these observations suggest that the conditions for somatic embryo maturation have not been optimized or are not suitable for meristem formation and development. The following strategies could improve meristem development and hence conversion: 1. Simulate *in ovulo* conditions to promote meristem development prior to the "maturation" treatment. 2. Prevent deterioration of apical meristem organization during somatic embryo maturation. 3. Promote further meristem development during embryo germination.

Key words: Apical meristems, conversion, somatic embryo

Introduction

Somatic embryogenesis is an important tool for plant propagation. Successes in somatic embryo formation have been recorded for many species. At present, a large amount of information is currently available for somatic embryogensis in plants. For reviews on this subject, see Thorpe (1995) and Jain et al. (1995-1999).

In somatic embryogenesis, one of the most common problems is that the conversion rate can be low; this is especially so in woody plants. The term conversion is defined as having both root elongation and epicotyl growth with new leaf formation (Hay and Charest 1999).

Conversion is a better way of assessing the quality of the embryo as both apical meristems are functional. The term gemination usually refers only to root elongation. In many somatic embryos, root elongation is not necessarily followed by shoot growth. Thus, one cannot claim successes based on the generation of bipolar embryo-like structures alone, if the conversion rate is low. As apical meristems are responsible for post-embryonic growth of the embryo, the low percentage observed is most likely the result of poor apical meristem development or defects in the meristem organization during somatic embryogenesis. The objective of this review is to draw attention to the importance of apical meristems during embryo development and post embryonic growth of the embryo. A proper understanding of apical meristem development and physiology will greatly enhance our ability to produce somatic embryos

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of improved quality. In this review, the conifer somatic embryo system is used as the main example to illustrate the importance of apical meristems in embryo conversion.

also expressed in the subapical cells of the developing embryo, might be required for preventing differentiation of the meristematic cells, as cell differentiation and fusion of organs was observed in *stm* mutant embryos (Long and Barton 1998). Furthermore, the observation

Formation of apical meristems in the zygotic embryo

The importance of the apical meristems in plant growth and development has long been recognized. The organization of the apical meristems has been a subject of many reviews (see Evans and Barton 1997).

In flowering plants, apical meristem formation occurs at the early globular stage with the differentiation of the epiphysis at the future shoot pole and the hypophysis at the future root pole. These cells are located at opposite ends of the globular embryo and have similar cytological characteristics (Krishnamurthy 1994; Yeung et al. 1996). The epiphysis gives rise to the subapical cells of the shoot meristem. A number of studies indicate the importance of cells in this region. Changes in the subapical cells signal the beginning of shoot apical meristem formation. In *Phlox drummondii*, Miller and Wetmore (1945) considered that the first indication of shoot meristem formation is the formation of a "fan-shaped area composed of smaller isodiametric, dark staining meristem cells". In the canola (*Brassica napus*) pollen embryo, the subapical cells change in morphology prior to the differentiation of the surface layer into a distinct surface of the shoot apical meristem (Yeung et al. 1996, Figure.1A-C).

The importance of the subapical cells during shoot meristem differentiation is also confirmed by moelcular studies. In *Arabidopsis*, initiation of shoot meristem differentiation occurs early during embryogenesis, as indicated by the expression analysis of the shoot meristem gene *WUSCHEL* (*WUS*) (Mayer et al. 1998). The early expression of *WUS*, together with mutant analysis (Laux et al. 1996) sugests that this gene might be required to maintain the subapical cells of the immature embryos in a pluripotent mode (see Lenhard and Laux 1999). The activity of these cells, necessary for the continuation of shoot meristem development, is tightly regulated by the activation of other genes. When the embryo attains a globular shape, expression of *SHOOT MERISTEMLESS* (*STM*) is initiated (Long and Barton 1998). This gene,

Figure 1. Shoot apical meristem development in canola microspore embryos. A, A population of canola pollen embryos. Scale bar = 5 mm; B, Microspore embryo 12-14 days after culture (DAC). The embryo begins to elongate and takes on a torpedo shape. The shoot apical (arrow) is located at the apical notch and the root apical meristem (root apical meristem) is located behind the root cap. Scale bar = 20 μm; C, Early cotyledon stage embryo. The shoot meristem (*) expands laterally in size and takes on a dome shape. Scale bar = 20 μm; D, At the cotyledon stage, the shoot meristem (*) maintains its structural integrity. A highest conversion rate is achieved at this stage. Scale bar = 40 μm; E, Microspore embryo beyond 35 DAC. The shoot apical meristem cells gradually change into parenchyma cells with abundant starch deposits. Scale bar = 40 μm; F, Microspore embryo 50 DAC. Intercellular air spaces (*) begin to appear between cells in the shoot pole (arrowhead). The original meristem cells (arrowhead) become highly vacuolated with prominent starch grains within the cytoplasm. This completely obliterates the shoot meristem organization (for details, see Yeung et al. 1996). Scale bar = 40 μm.

that shoot meristem differentiation is initiated in *stm* mutants (although further development is blocked) (Barton and Poethig 1993), but not in *wus* mutants (Mayer et al. 1998) confirms the later participation of *STM* in shoot meristem formation. Two other important genes expressed during the final stages of shoot meristem development are *ZWILLE* (*ZLL*) and *CLAVATA* (*CLV*) (see Lenhard and Laux 1999). Mutant analysis suggests that *ZLL* might be required for keeping the meristematic cells in an undifferentiated mode, possibly by regulating *STM* expression (Moussian et al. 1998), whereas *CLV* is involved in maintenance of the shoot apical meristem during post-embryonic growth (Clark et al. 1996).

The root meristem is located between the root cap and the procambium. In *Capsella*, the hypophysis gives rise to the root apex including the apical meristem (see Krishnamurthy 1994; Raghavan and Sharma 1995). Cells derived from the hypophysis also give rise to the quiescent centre of the root. Recently, a gene, *HOBBIT*, has been identified in *Arabidopsis* that is essential to root meristem formation (Willemsen et al. 1998). Future studies may provide additional insight into the root-forming process during embryogeny.

Similar to the flowering plants, the structural organization of the vegetative shoot apex in gymnosperms is well documented (Gifford and Corson 1971). In Pinaceae, the shoot apex varies in shape from convex to conical, and the cells at the meristem surface are large and distinct (Johnson 1951). Although a large amount of information is available concerning the vegetative shoot apex, detailed documentation of meristem formation during embryogeny is limited only to a few species, i.e. *Pseudotsuga* (Allen 1947), *Pinus strobus* (Spurr 1949), *Picea abies* (Gregory and Romberger 1972; see also Johnson 1951 and Singh 1978), and *Picea glauca* (Yeung et al. 1998). The organization of the root apical meristem in gymnosperms has received even less attention. Information concerning root meristem ontogeny during embryo development is only available for a few species such as *Pseudotsuga* (Allen 1947) and *Pinus strobus* (Spurr 1949). Recently, we provided a detailed description of apical meristem formation during zygotic embryo development of white spruce (Yeung et al. 1998). This study can serve as a model for meristem initiation and development during somatic embryo development.

In white spruce, apical meristems are initiated at the pre-cotyledon stage of embryo development (Yeung et al.

1998). The first notable sign of shoot and root meristem development in white spruce is the appearance of starch at the respective poles of the embryo. Starch granules gradually accumulate in the subapical cells of the shoot pole and are soon followed by vacuolation in the subapical cells. With the formation of the large vacuolated cells, the surface cell layer at the summit of the shoot pole enlarge and differentiate into the surface initials of the shoot meristem. This observation again indicates that the subapical cells may play an important role in the differentiation of the surface shoot meristem initials similar to flowering plants. As the embryo matures, the large vacuoles within the subapical cells are replaced by small ones with a concomitant increase in the cytoplasmic density of the subapical cells. The subapical cells located next to the surface initials become the central mother cells of the shoot meristem, and those located near the cotyledon junction divide periclinally and give rise to the epicotyl rib meristem. The remaining subapical cells near the procambium become part of the pith. In the root pole, two layers of root meristem initials appear at the junction between the developing procambium and the root cap. The initials are distinct as they are larger in size than surrounding cells. Soon after their formation, mitotic activity stops. Upon germination, mitotic figures can be found initially within these root initials. However, as the root ages, these initials appear to become the quiescent centre of the root. At present, molecular markers similar to those found in flowering plants are not available for the apical meristem cells of gymnosperms. Studies in this area are urgently needed in order to obtain new insights into meristem formation and development during embryogeny. It is interesting to note that a homeobox gene, similar to the *STM* gene, has been found in the vegetative apex of spruce, and this gene appears to be essential for meristem function (Sundas-Larsson et al. 1998).

From the preceding description, it is clear that apical meristem development occurs very early in both the flowering plants and conifers. During zygotic embryo development, the apical meristems follows a specific structural pattern of development. The overall organization reflects subsequent activity that follows during germination.

302

Properties of apical meristems in zygotic embryos

The process of shoot meristem initiation appears to be labile and is easily disrupted by physiological treatments. Once this process is interrupted, shoot meristem formation is aborted. In *Brassica napus*, interference with apical meristem development at the globular stage of embryo development with the use of triiodobenzoic acid (TIBA) resulted in the alteration of the shoot apical meristem leading to low conversion (Ramesar-Fortner 1999). With TIBA treatment, the tunica-corpus organization of the meristem was disrupted. In carrot somatic embryos, conversion failure was a result of an absence of a structurally recognizable shoot apical meristem (Ammirato 1987; Nickle and Yeung 1993). Thus, physiological perturbations at the time of apical meristem formation can lead to the abnormalities observed, especially at the shoot pole. The embryonic environment has to be conducive to meristem differentiation. The "stable" environment surrounding the zygotic embryo ensures the initiation of the apical meristems.

In canola, TIBA exerted its effect only at the globular stage of embryo development. Once the embryo reached the heart stage or later, TIBA could not alter shoot meristem morphology and one hundred percent conversion was observed (Ramesar-Fortner 1999). This observation indicates that the formation of the shoot meristem occurs within a narrow window of time during the course of embryogeny. The failure to stimulate such a process at a precise time during the course of embryogenesis can lead to disruptions in the shoot pole.

In zygotic embryos, apical meristems are "stable" structures once formed (Waring 1978, 1979). From a structural standpoint, the shoot apical meristem is well established by the late heart stage. Since the treatment of the embryo with TIBA at the heart stage and later does not alter shoot meristem organization and does not influence the conversion frequency as compared with the control (Ramesar-Fortner 1999), clearly indicates that the shoot meristem is a "stable" structure once established. Furthermore, the stability of the meristem can be indirectly demonstrated by the fact that immature embryos can precociously germinate as soon as the apical meristems are formed, i.e. after the heart stage of embryo development (Yeung and Sussex 1979). The

subsequent "maturation stage" is not a pre-requisite for precocious germination to occur. Precocious germination has been widely reported for angiosperm species (see Raghavan 1976). In gymnosperm species, in his extensive studies on zygotic embryo culture, LaRue (1936; see also Raghavan 1976; Norstog 1982), successfully cultured immature gymnosperm embryos 2-4 mm in length and plantlets resulted from these cultures. This clearly demonstrates that the meristems once formed are "stable" and can assume their function under appropriate conditions.

A closer look at the apical meristems of somatic embryos

Little is known about the process of meristem formation during somatic embryogenesis. Tissue differentiation, especially meristem formation, varies depending on the cell line. A carrot cell culture line, which was shown to be highly embryogenic, had very low plantlet conversion (Nickle and Yeung 1993, 1994). This was due to the absence of a shoot apical meristem. Treatment with abscisic acid at the globular and torpedo stages greatly increased the conversion percentage. Histological sections revealed that ABA treatment maintained the highly cytoplasmic cells in the shoot apex. In the canola microspore embryo, apical meristem initiation appeared normal during early embryo development (Yeung et al. 1996). However, upon prolonged culture, the tissue pattern began to deteriorate. The procambium and the meristem organization were drastically altered. Large intercellular spaces developed within the shoot pole (Figure. 1D-F) and resulted in a lower conversion frequency. These studies indicate that shoot meristem formation occurs early during the course of embryogeny. However, the process of meristem initiation can be labile and different factors in the culture medium can either enhance or destabilize the shoot apical meristem.

Relatively more information is available for meristem formation during conifer somatic embryogenesis. In white spruce embryogenic tissues, a variety of organizations can be found. In some lines, well organized "embryonal head-like" structures can be present; in other lines, only dense cells are present. Furthermore different cell lines have different embryo-forming capacities. Since development is not synchronous, embryo
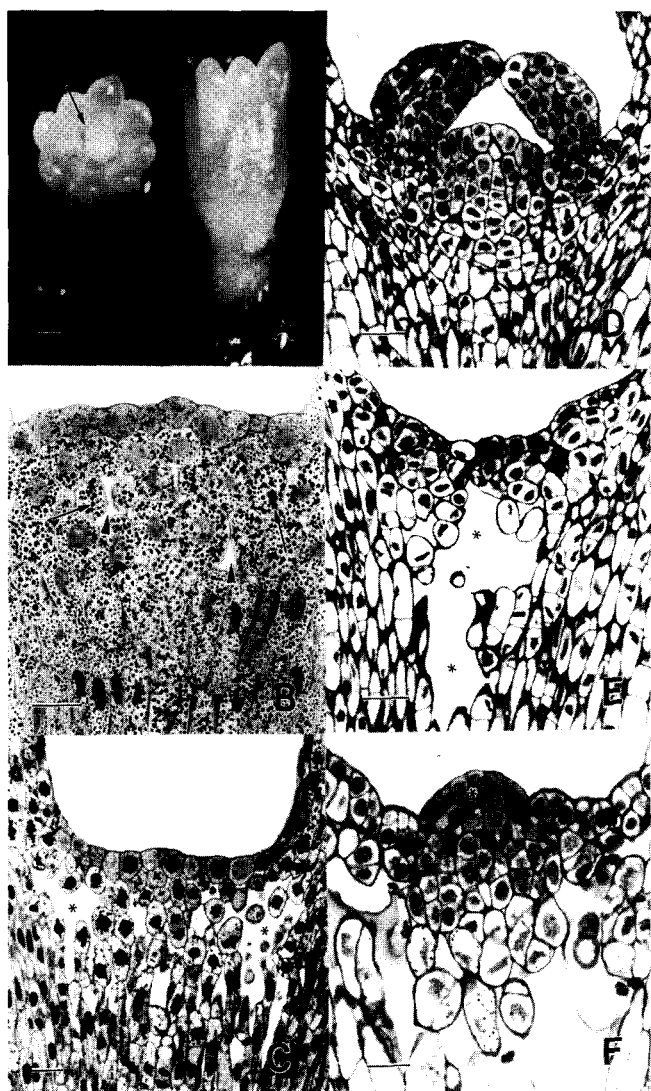
Figure 2. Shoot apical meristem development in white spruce somatic embryos. A, White spruce somatic embryos showing the morphology of the embryo. The shoot apical meristem (arrow) is located in the apical notch. Scale bar = 1 mm; B, The structure of a shoot apex in a mature somatic embryo. The shoot apex is flat. Cells within the first layer of the meristem have dense cytoplasm with prominent nuclei. Relatively little starch accumulates in this layer. Intercellular spaces (arrowheads) exist within the shoot apex. Except for the first layer, other cells are vacuolated and with abundant starch granules (arrows) within the cytoplasm of cells; C, In some somatic embryos, large spaces (*) are present in the shoot pole. A clearly defined shoot meristem is absent; D, After the partial drying treatment, a certain percentage of somatic embryos will convert with the formation of well defined shoots; E, In some somatic embryos, especially those having large spaces in the shoot pole, embryos usually fail to convert primarily due to a total destruction of the shoot meristem organization. Large air spaces (*) continue to develop within the shoot pole upon germination; F; Depending on the cell line, with the ascorbic acid treatment, some shoot meristem cells can divide and organize into a shoot meristem (*) even in the absence of a clearly defined shoot meristem organization in the mature embryo. For details, see Kong and Yeung (1992) and Stasolla and Yeung (1999). Scale bar for B-F = 40 μm.

development and maturation varies between individuals within the same culture. In white spruce, although the general structural pattern of somatic embryo development was similar to its zygotic counterpart (Kong et. al. 1999), a number of differences could be found. Many events, such as starch accumulation, cell vacuolation, differentiation of apical initials, and increase in cytoplasmic density are not tightly coupled, and a well defined pattern similar to the zygotic embryo does not exist. Although shoot apical initials are present (Figure. 2B), they are seldom clearly defined and are not as distinctly large as those in the zygotic embryo. This may be due to the fact that subapical cells are not well developed prior to initial cell differentiation. In the root pole, the number of root apical meristem initials appears to be fewer and not as distinct as their zygotic counterpart. Precocious germination leading to functional plantlet formation has not been reported in conifer somatic embryos suggesting physiological "immaturity" of the apical meristem cells.

One of the most disturbing events that occurs during somatic embryo maturation of white spruce is the formation of intercellular spaces in the shoot pole (Kong and Yeung 1992, Figure. 2B,C). The disruption of the shoot pole is most likely caused by the accumulation of ethylene in the culture vessel (Kong and Yeung 1995). Plant cells, tissue, and organs are able to produce a significant quantity of ethylene in in vitro culture systems. In white spruce somatic embryos, the addition of an ethylene biosynthesis inhibitor, such as aminoethoxyvinylglycine (AVG), greatly reduced the morphological abnormality of the shoot pole (Kong and Yeung 1995). The alteration of the shoot meristem organization again argues for the fact that the shoot apical meristem of somatic embryos is not a stably determined structure as in their zygotic counterparts and is subject to change depending on the culture environment. In the case of the root pole, although intercellular spaces have been observed, large spaces are seldom present. This indicates that there are physiological differences between the two poles of the embryo.

The osmotic embryonic environment / water content

The difference in properties of apical meristems

between zygotic and somatic embryos may be a result of their respective environments. When comparing the zygotic and somatic embryo systems, the main difference between the two is their respective embryonic environment. The zygotic embryo is protected and enclosed by the seedcoat and nutritive tissues, whereas the somatic embryos are either exposed to air or in an artificial liquid environment. Physical and chemical factors from the embryonic environment can play important roles in the regulation of embryo development. These factors may be necessary for the complete expression of the innate genetic capacity of the developing embryo (Steeves and Sussex 1989). The embryonic environment surrounding the somatic embryo may not be conducive to meristem formation and determination, and may account for the low conversion frequency.

One of the most unique features of the zygotic embryonic environment is the low (negative) osmotic value of seed components. It has long been recognized that the liquid endosperm of flowering plants in which young embryos are constantly bathed has a low osmotic value (see Yeung and Brown 1982). The lowering of the water potential of the culture medium is beneficial to the growth of young embryos (see Raghavan 1976, 1997). The inclusion of osmotic agents, such as sucrose, allows for embryo maturation and prevents precocious germination. Thus, the osmotic environment has a morphogenetic role to play during embryo development.

In recent years, polyethylene glycol 4000 has become the compound of choice, especially when liquid suspension is used (see Attree and Fowke 1993). PEG is a non-plasmolysing osmoticum (Carpita et al. 1979) and it can simulate natural water stress conditions at appropriate concentrations (Attree et al. 1991). The addition of osmoticum to *in vitro* embryo culture systems has been shown to promote embryo development (Attree et al. 1991; Xu et al. 1990). One of the most dramatic demonstrations of the beneficial effect of PEG is the work of Ilic-Grubor et al. (1998a, b). In the canola pollen embryo system, the inclusion of 22% PEG and 0.1% sucrose resulted in "normal" somatic embryo development similar to their zygotic counterparts. Germination of these embryos resembled seed embryos. This study proves beyond doubt the usefulness of PEG during *in vitro* embryogenesis.

In conifer somatic embryogenesis, the inclusion of an osmoticum has been found to enhance embryo matura-

tion (Hay and Charest 1999). Sucrose and PEG 4000 are the most common osmotic agents used (see Hay and Charest 1999). However, it is important to note that not all cell lines react favourably to the osmoticum (Find 1997).

Although at present, the mechanism of action of different osmotica is not clear, the inclusion of osmoticum is beneficial to somatic embryo development and maturation. It is essential to re-emphasize the fact that the environment surrounding the zygotic embryo is unique. Zygotic embryos develop slowly, especially during histodifferentiation, under a negative water potential. Thus, the normal process of embryo development is closely coupled to the osmotic environment surrounding the embryo.

Strategies of improving meristem development and hence conversion

From the preceding discussion, although morphologically-developed somatic embryo can be observed, there is no guarantee that the bipolar structure can indeed function as an embryo. The apical meristems can be abnormal, and lead to low conversion rates.

The physical and chemical environments play key roles in embryo development (Steeves and Sussex 1989). A proper understanding of the embryonic environment will provide clues as how to manipulate the culture system in order to obtain the best results. Factors such as osmotic potential, the partial pressure of oxygen, major forms of nutrients, and changing hormonal supplies are important in designing the culture conditions for a particular cell line. In order to enhance somatic embryo conversion, optimization of meristem development is the key. The following are strategies that can enhance meristem development.

1. Manipulation of the culture environment prior to the "maturation" treatment. Knowing that meristem development occurs prior to embryo maturation, efforts should be made to optimize the conditions for the growth of the "embryonal-head" region. The importance of the *in ovulo* condition has been discussed (Yeung 1995). The conditions such as changing the osmotic environment, reducing sucrose, or manipulating oxygen tension may be beneficial to early embryo development and meristem differentiation.

The work of Gupta and Pullman (see Gupta and Grob 1995) clearly illustrates the benefit of treating embryogenic tissues prior to the maturation treatment. They increased the osmolality of the maintenance medium prior to the maturation treatment. Without this treatment, several genotypes of *Pinus taeda* and *Pseudotsuga menziesii* were unable to develop good quality somatic embryos with subsequent ABA treatment (Gupta and Grob 1995). Other manipulations such as using different "natural" growth substances, e.g. IAA and zeatin and changes in oxygen tension might have beneficial effects on meristem development.

2. Prevent meristem deterioration. The second manipulation is at the embryo maturation stage to avoid the effect of ethylene. Ethylene often accumulates within culture vessels. Our work has demonstrated that the use of an ethylene biosynthesis inhibitor, AVG prevented the formation of intercellular spaces in the meristem of white spruce (Kong and Yeung 1994, 1995). However, ethylene continues to increase and accumulates within the culture vessel. Thus, one should try to avoid prolonged culture in the maturation medium. The use of an osmoticum and utilizing a liquid maturation medium may also minimize the action of ethylene.

The partial drying treatment, as devised by Roberts et al. (1990), has proven to be an extremely important protocol for enhancing embryo conversion. The decrease in water content of 10-20 percent greatly enhanced conversion. The partial drying treatment alters some physiological and biochemical process of the somatic embryos. This treatment greatly reduced the sensitivity of the embryo to abscisic acid and abolished ethylene biosynthesis (Kong 1994). Furthermore, the storage product deposition pattern was more similar to the zygotic embryo (Kong and Yeung, unpublished reports). Thus, the partial drying treatment mimicked some physiological process occurring in normal seed development. This treatment also enhanced conversion in some genotypes.

3. Encouraging continual meristem development during germination. Somatic embryos, being bipolar, have both root and shoot poles. It appears that the conditions for root germination are less stringent than that of the shoot growth. Upon germination, the percentage of root growth is usually higher than shoot growth (see Hay and Charest 1999). Thus encouraging shoot development is an important strategy for plantlet develop-

ment. In white spruce, the shoot apical meristem continued to develop during germination leading to epicotyl formation (Kong and Yeung 1992). The failure of shoot development was due mainly to the formation of large intercellular spaces in the shoot pole. In this type of cell line, further shoot growth and new shoot production was possible when the somatic embryos are treated with ascorbic acid (Stasolla and Yeung 1999, Figure. 2D-F). In our preliminary observation, it was observed that for embryos having large intercellular spaces, ascorbic acid could stimulate some shoot apical cells to divide. Meristemoids appeared at the shoot pole. Further division and differentiation followed, and resulted in the formation of a new shoot (Stasolla and Yeung, unpublished results). This pattern of shoot formation is similar to shoot organogenesis, as described in radiata pine cotyledonary explants in culture (Yeung et al. 1981). Once vascular connections are established between the newly formed shoot and the hypocotyl of the germinating embryo, functional plantlets were formed (Stasolla and Yeung, unpublished results).

In any culture system, it may be wise to carry out a detailed structural analysis of the cell line of interest (see Yeung 1999). The structural information can provide background information and pin-point the problem, if any, with a particular cell line. Through the combination of different strategies outlined above, one can improve the quality of the embryo produced and/or the generation of plantlets from somatic embryos of lesser quality.

Conclusion

Plantlet production via the process of somatic embryogenesis is now firmly established as an important method for plant propagation. However, a better theoretical understanding of the process is still necessary. In this review, the importance of the apical meristem is emphasized. In the authors' opinion, the quality of the somatic embryo is determined by the quality of its apical meristem. Whenever possible, the conditions for meristem development should be optimized by the manipulation of the embryogenic culture.

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