

Polyamine levels during the development of zygotic and somatic embryos of *Pinus radiata*

Rakesh Minocha^{a,*}, Dale R. Smith^b, Cathie Reeves^c, Kevin D. Steele^c and Subhash C. Minocha^d

^aUSDA Forest Service, PO Box 640, 271 Mast Road, Durham, NH 03824, USA

^bMetaGenetics, 93SH30 Whakatane Highway, RD4 Rotorua, New Zealand

^cForest Research, Private Bag 3020, Rotorua, New Zealand

^dDept of Plant Biology, Univ. of New Hampshire, Durham, NH 03824, USA

*Corresponding author, e-mail: rminocha@hopper.unh.edu

Changes in the cellular content of three polyamines (putrescine, spermidine and spermine) were compared at different stages of development in zygotic and somatic embryos of *Pinus radiata* D. Don. During embryo development, both the zygotic and the somatic embryos showed a steady increase in spermidine content, with either a small decrease or no significant change in putrescine. This led to a several-fold increase in spermidine/putrescine ratios during development of both types

of embryos. Cell cultures of plant-forming and non-plant-forming lines derived from the same clone and growing on proliferation (maintenance) medium differed significantly in their polyamine levels. Mature, cotyledonary stage somatic embryos capable of germination and formation of plants could be distinguished by their higher spermidine/putrescine ratios from abnormal cotyledonary stage somatic embryos which were incapable of forming plants.

Introduction

Research into somatic embryogenesis of conifers has increased rapidly since the first publication of a reliable protocol for plant regeneration in Norway spruce (Hakman and von Arnold 1985, also see reviews by Tautoris et al. 1991, Gupta and Grob 1995). Pine species have been more recalcitrant, and only a few protocols for reliable plant regeneration through somatic embryogenesis have been published in peer-reviewed journals (Klimaszewska and Smith 1997). However, patents have been granted for protocols on somatic embryogenesis and plant regeneration in commercially important pines, such as *Pinus taeda* (Gupta and Pullman 1993, Smith 1994) and *Pinus radiata* (Smith 1994). Evaluation of the forest management applications of *Pinus radiata* somatic embryogenesis has been under way in New Zealand since 1993 (Aitken-Christie et al. 1994, Smith et al. 1994, Smith et al. 1997).

Unlike the spruces, pine embryogenic tissue tends to lose regeneration capacity within a relatively short period of serial transfers (Smith et al. 1994). Success in the production of embryogenic cell cultures from mature pine tissues has been reported (Smith 1997, Smith et al. 1997), however cultures “reinitiated” from immature somatic embryos tend

to produce plants with abnormal root systems (D. R. Smith, C. L. Hargreaves, L. J. Grace and A. A. Warr, unpublished data). As a consequence, there has been considerable interest in understanding the biochemistry and molecular biology of somatic embryo development in pines as well as other conifers (for reviews see Misra 1994, 1995, Feirer 1995).

It is generally believed that somatic and zygotic embryos show similar morphological and anatomical patterns of development (Goldberg et al. 1989, de Jong et al. 1993, West and Harada 1993). In addition, several reports have been published on molecular and biochemical changes during the development of somatic and/or zygotic embryos (Goldberg et al. 1989, Thomas 1993, Zimmerman 1993, Feirer 1995, Kawahara and Komamine 1995). However, only a few direct comparisons of such changes have been made between the two types of embryos (for review see Misra 1994, 1995). Whereas the zygotic embryos develop inside the protective environment of the surrounding endosperm or megagametophyte (as in gymnosperms), which also provides nutrition, the somatic embryos develop in a defined growth medium in a relatively exposed environment.

Abbreviations – EDM: embryo development medium; EMM1: embryo maturation medium 1; EMM2: embryo maturation medium 2; PUT: putrescine; SPD: spermidine; SPM: spermine.

The observed similarity between the somatic and the zygotic embryos implies that these biochemical and molecular events are probably related to development of the embryo and not to the surrounding environment under which this development is occurring. Thus a basic understanding of the biochemical and molecular events associated with somatic and zygotic embryo development in plants may lead to improved production of somatic embryos on a commercial scale.

There are a number of reports indicating that polyamines play a crucial role in somatic embryo development (for reviews see Minocha and Minocha 1995, Minocha et al. 1995). While the mechanisms of actions of polyamines in plant growth and development are still a matter of controversy (Walden et al. 1997), evidence for their participation in embryogenesis continues to accumulate (Minocha and Minocha 1995, Minocha et al. 1995). Montague et al. (1979) and Robie and Minocha (1989) demonstrated a strong positive correlation between somatic embryogenesis and increased cellular polyamine content in carrot cell cultures. The inhibition of polyamine biosynthesis completely inhibited somatic embryogenesis in this tissue. Bastola and Minocha (1995) later showed that increased putrescine biosynthesis due to the overexpression of a mouse ODC cDNA promoted somatic embryogenesis in transgenic carrot cells. Increased putrescine production in the transgenic cells was found to stimulate the overall metabolic turnover of polyamines (Andersen et al. 1998) and a concurrent decrease in the production of ethylene (Anderson 1995). Transgenic manipulation of the polyamine biosynthetic pathway may thus provide a practical approach to modulate the embryogenic potential of recalcitrant cell lines. This, however, must be based upon knowledge of polyamine and ethylene metabolism in cells undergoing normal embryo development. The present study was undertaken to establish the profile of changes in the metabolism of polyamines during development of zygotic and somatic embryos in *Pinus radiata*. The results presented clearly demonstrate characteristic parallel changes in the metabolism of polyamines during the development of zygotic and somatic embryos in this species.

Materials and methods

Collection of plant material

Two cell lines of a plant-forming embryogenic clone (G95-23) of *Pinus radiata* D. Don. were used in this study. The clone was originated in Rotorua, New Zealand in January 1995 from an immature zygotic embryo within the cultured megagametophyte, using techniques and media described previously (Smith 1994, Smith et al. 1994). Within 12 weeks of establishing the clone in culture, samples were stored in liquid nitrogen (Hargreaves and Smith 1992). The remaining tissue was used to regenerate somatic embryos which were subsequently transferred to soil, while a portion of the embryogenic tissue was serially cultured on maintenance medium for 12 months, during which time it lost the ability to produce mature somatic embryos (non-plant-former). In

February 1996, and again in March 1997, the cryopreserved sub-line (plant-former) was thawed and cultured for the studies described below. It was thus possible to compare two phenotypically different cell lines (plant-forming and non-plant-forming) of the same genotype over a period of 2 years. For the analysis of zygotic embryos, megagametophytes containing zygotic embryos (i.e. complete megagametophytes) were collected at intervals between December 1995 and January 1996, and zygotic embryos isolated from the developing megagametophytes of a single clone were collected between December 1996 and February 1997.

Samples of tissue or developing somatic embryos were taken at various time intervals from the two sub-lines during the progression of tissue from maintenance medium through embryo development medium (EDM), embryo maturation media (EMM1, EMM2), and pre-germination (PGM) and germination media (Smith 1994, D. R. Smith, C. L. Hargreaves, L. J. Grace and A. A. Warr, unpublished data). Embryos were isolated from the tissue mass with fine forceps using a Wild M5 dissecting microscope (12 × objective, 20 × eyepiece) and a Schott 150 W fiber optic cold light source. Before storing in 5% (v/v) perchloric acid (PCA), the embryos were sorted into different developmental stages or quality of embryo within particular developmental stages. For polyamine analysis of somatic embryos, 5 replicate samples containing 10–60 embryos each (depending upon developmental stage and size of embryos at the time of collection) were collected at each time. The “residual tissue” representing the tissue from which all normal and abnormal embryos and suspensors had been removed was also collected. For field samples, 5 or 10 replicate samples, each containing 15 megagametophytes for the first year of collection and 10 megagametophytes for the second year of collection, were analyzed for polyamines. Replicate samples of isolated zygotic embryos contained from 10 to 80 embryos depending upon the developmental stage and size of embryo at the time of collection. All cones were collected from the same trees.

Polyamine analysis

Tissue samples collected in PCA were stored at -20°C for up to 4 months before analysis of polyamines by HPLC. The samples were frozen (-20°C) and thawed (room temperature) three times and centrifuged at 12 000 g for 15 min. Before dansylation, heptanediamine was added to the supernatant as an internal standard. The samples were dansylated for polyamine analysis according to the procedure of Minocha et al. (1990, 1994). Aliquots of 50 or 200 μl of the PCA extract were placed in Reactivials (Kontes, Vineland, NJ, USA) along with 100 μl of saturated Na_2CO_3 and 100 μl of dansyl chloride (10 mg ml^{-1} acetone). The mixture was vortexed and incubated for 1 h at 60°C ; then 50 μl of L-proline (100 mg ml^{-1} H_2O) was added to each vial. The samples were incubated at 60°C for 30 min followed by 3 min of vacuum drying to remove the acetone. Then 400 μl of toluene (Photrex grade, Baker, Phillipsburg, NJ, USA) was added to each vial, the mixtures vortexed and cen-

trifuged for 1 min at 2 000 *g* to separate the two phases. A sample of 200 μ l of the upper toluene layer was removed and placed in a microfuge tube. The samples were evaporated to dryness under vacuum and reconstituted in 1 ml HPLC grade methanol.

The liquid chromatographic system consisted of a HP Series 1050 Quaternary Pump, a Bio-Rad Model AS-105 HRLC autosampler, and a Perkin-Elmer model 204 Fluorescent detector. A Perkin-Elmer Pecosphere-3 $\times$ 3 CR C18, 4.6 $\times$ 33 mm, reversed-phase cartridge column (3 μ m particle size) was used for separation of polyamines using a gradient of heptanesulfonic acid and acetonitrile (Minocha et al. 1990). The excitation and emission wavelengths were set at 340 and 510 nm, respectively. Data collected on a Hewlett-Packard HP3396A integrator were subjected to an internal standard procedure with multi-level calibration of standard response and valley baseline correction of chromatographic peak areas.

Statistical analysis

Results represented in each figure and the data for the three polyamines within each group were analyzed via one-way analysis of variance (ANOVA) to determine whether statistically significant differences occurred among various groups being tested. If *F* values for one-way ANOVA were significant, pairwise comparisons of means were made by Tukey's multiple comparisons test. ANOVA assumes that all the groups being compared have similar variances. This was not true for some of our data sets especially the ones with polyamine ratios. Higher ratios have higher range of variation (see figures). Thus all the statistical analyses were run after logarithmic transformations of the data sets. Data from two types of tissue were compared using *t*-tests. All analyses were performed with Systat Windows, Version 7.01 (Systat, Evanston, IL, USA).

Results

Zygotic embryo development and polyamine content

Developing seeds collected over two different seasons (December 1995–January 1996 and December 1996–February 1997) from three different clones of mature trees were analyzed for their polyamine content. During the first season, the entire megagametophytes containing the embryos were analyzed, for the second season, dissected embryos were analyzed separately from the megagametophytic tissue. The samples collected in December contained either early proembryos or were just beginning to enter a cleavage polyembryony stage of development.

Data presented in Fig. 1A show that at the earliest times of collection for 1995–96 spermidine was the major polyamine in the megagametophytes containing the developing embryos, followed in quantity by putrescine and spermine. The spermidine content increased with the stage of embryo development in both genotypes while putrescine either remained unchanged (clone 539) or decreased (clone 345). Spermine content was always low and increased only slightly over the course of development of the embryo (Fig. 1A,B). The trends in changes of polyamine contents in the megagametophyte containing the developing embryos during the course of embryo development are more revealing when one follows spermidine/putrescine ratios. The gametophytes containing advanced stages of zygotic embryos had significantly higher spermidine/putrescine ratios than those containing early stages of embryos (Fig. 1C,D). The ratios of spermidine/putrescine increased from ~ 1.8 to 5 in clone 539 and from 1 to 2.7 in clone 345. Similar trends in changes were observed for ratios of spermine/putrescine (data not presented).

For 1996–97, separate samples of the isolated developing zygotic embryos and the remaining megagametophytic tissue

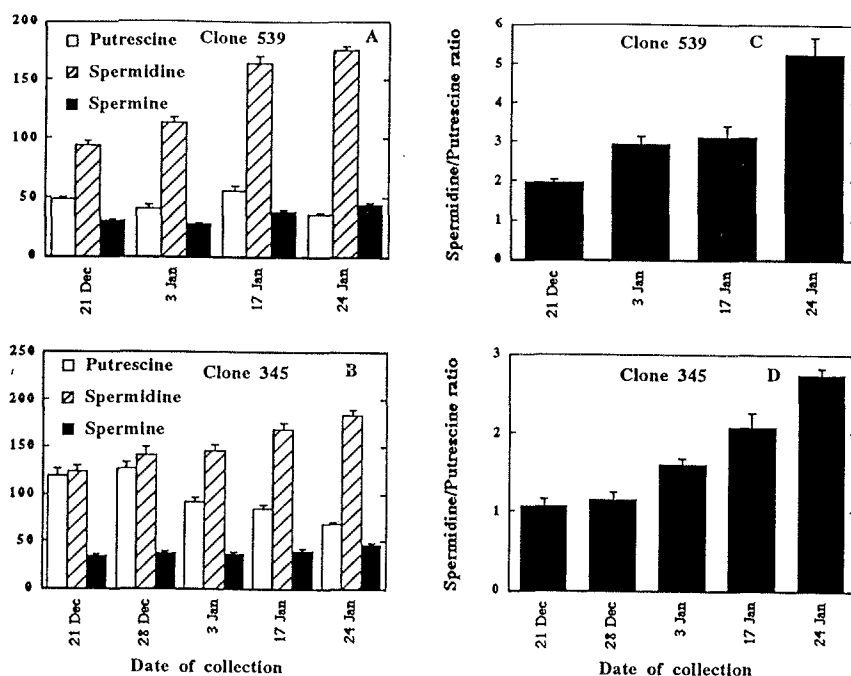


Fig. 1. Time course of changes in cellular polyamines and spermidine/putrescine ratios in the megagametophytes containing developing embryos of clones 345 and 539 of *Pinus radiata*. Samples were collected during the period of December 1995 to January 1996 when the embryos were going through maturation. Data presented are mean $\pm$ SE of 10 replicates, each replicate containing 15 megagametophytes.

Table 1. Clone 268-494 zygotic embryo development (1996–97). ⁽¹⁾ Days from appearance of first proembryo (2 January 1997). ⁽²⁾ 0, No embryo; 1, early proembryo, 2, late proembryo; 3–9, (Figs 1–7 in Spurr, 1949). ⁽³⁾ Embryo development index = sum (developmental stage × frequency). ⁽⁴⁾ Embryo weight = (embryo/embryo + megagametophyte) × 100.

Date	Day ⁽¹⁾	Percent of sample development stage ⁽²⁾										Embryo development index ⁽³⁾	Embryo weight ⁽⁴⁾
		0	1	2	3	4	5	6	7	8	9		
Dec 23		100.0										0.0	0.0
Jan 3	1	0.0	100.0									1.0	0.8
Jan 7	7	0.0	0.0	49.4	36.3	12.2	1.3	0.4	0.4	0.0	0.0	2.7	1.7
Jan 12	12	0.0	6.3	29.4	36.3	16.8	5.7	1.9	1.8	1.8	0.0	3.1	2.2
Jan 27	27	0.0	0.5	2.0	12.0	17.0	15.5	15.5	12.0	25.5	0.0	5.7	3.8
Feb 13	44	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	2.0	93.0	8.9	13.0

were analyzed. In the 23 December 1996 sample, no embryos were observed in any of the megagametophytes, but all of the samples collected on 3 January contained proembryos. Embryo development was subsequently very rapid, although at each collection date a range of developmental stages was observed (Table 1). Embryo development was scored using a system based on the observations of Spurr (1949). Zygotic embryos with a cotyledonary “crown” level with the apical meristem were observed in 25.5% of the megagametophytes collected on 27 January (Table 1), a level of development equivalent to that of somatic embryos at the time of harvest from EMM2 medium. By 13 February, 93% of the zygotic embryos had fully elongated cotyledons, and were capable of germinating directly if placed onto germination medium (D. R. Smith, C. L. Hargreaves, L. J. Grace and A. A. Warr, unpublished data). This degree of maturation was seen in somatic embryos only at the time of transfer from pre-germination medium to germination medium (Smith 1994).

When analyzed separately (collection of 1996–97; clone 268-494), both the developing embryos and the megagametophytes contained spermidine as the major polyamine (Fig. 2). While the embryos showed a steady increase in each of the three polyamines up to the final stages of development, the megagametophytic tissue showed an increase during early stages of embryo development, followed by a decline in all three polyamines during later stages of embryo development (Fig. 2). On a fresh weight basis, the megagametophytic tissue had several-fold higher ($P \leq 0.05$) polyamine levels in the early development stages of the embryo whereas at later stages of development, distribution of total polyamines was significantly higher ($P \leq 0.05$) in the embryo compared to the megagametophytic tissue. The time of increase in putrescine as well as spermidine in the embryo coincided with the decrease in these polyamines in the megagametophytic tissues. The spermidine/putrescine ratios in the two tissues generally followed similar trends – increasing during early development and remaining high during later development (data not presented).

Analysis of polyamines during somatic embryo development

On the maintenance medium, the plant-forming cell line of clone G95-23 produced large numbers of small, compact embryo initials (proembryos) with long suspensors. This stage is referred to as time zero in Fig. 3. At this time the

non-plant-forming cell line was a tangle of suspensor-like cells associated with loose aggregates of small, isodiametric starch-filled cells. The non-plant-forming cell line produced only a few structures with a well-defined embryonic axis when transferred to EDM, instead it showed an abundance of loose aggregates of starch-rich cells with long, suspensor-

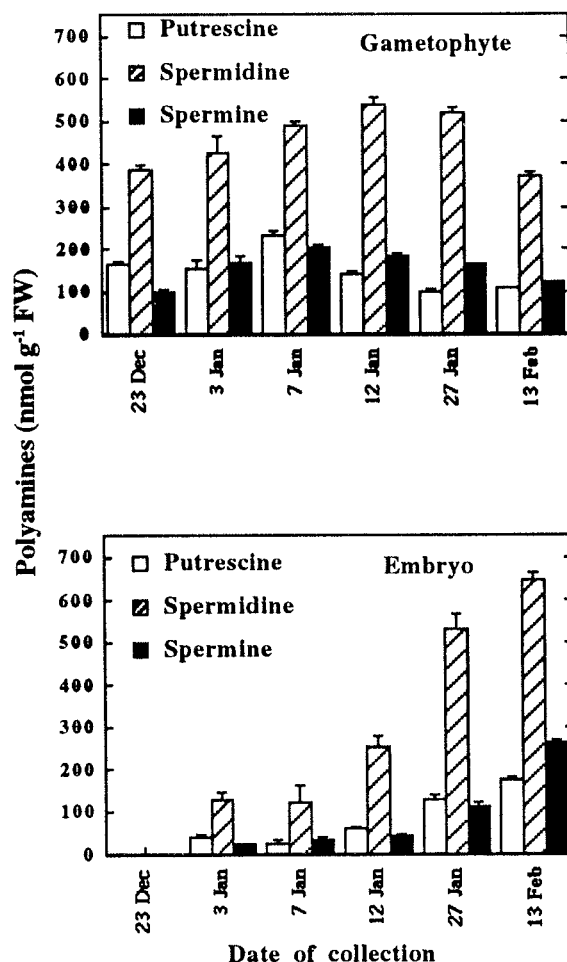


Fig. 2. Time course of changes in cellular polyamines in the megagametophytes and the isolated developing zygotic embryos of clone 494 of *Pinus radiata* during the period of December 1996 to February 1997. Data presented are mean $\pm$ SE of 5–10 replicates, each replicate containing 10 megagametophytes. Number of isolated embryos varied from 10 to 80 per sample depending upon the stage of embryo development.

like cells radiating from them. These were similar in appearance to the “solar” embryo type described in Norway spruce (Jalonon and von Arnold 1991). The plant-forming embryogenic cell line produced numerous structures with a well-defined embryonic axis on EDM. These had bullet-shaped embryo heads above a developing hypocotyl, and well-formed, multi-celled suspensors. There were also structures with a range of abnormal features, including squat embryo heads, frayed suspensors, or totally lacking suspensors. The plant-forming cell line, when grown on EDM, also contained “solar-type” embryos, but these were larger, and the centers more compact than the “solar-type” embryos in the non-plant-forming line. On the maturation media (EMM1, EMM2), bullet-stage embryos in the plant-forming cell line continued to develop until mature, cotyledonary-stage somatic embryos were formed which are regularly capable of germinating to form plants. Representative samples were subsequently put through the germination protocols, and plants were eventually established in soil. There were also “fused” embryos where two or three otherwise acceptable mature somatic embryos were fused together along most of their length. Such embryos were capable of germination, but did so to form “twinning” plants, as sometimes occurs naturally in germinating pine seeds. The plant-forming cell line also produced abnormal cotyledonary-stage embryos. Squat embryos with a whorl of cotyledons, but which lacked a hypocotyl, were the most common type. Embryos with a cotyledonary whorl and hypocotyl, but which lacked a suspensor and root meristem, were also observed. The non-plant-forming cell line produced so few acceptable mature somatic embryos that sampling was not feasible. Grossly distorted structures with no organ development were often observed in this cell line.

Samples from the plant-forming cell line produced 545 cotyledonary stage somatic embryos (embryo development index 8, Tables 1 and 2) on EMM2, of which 154 were judged to be capable of plant formation, and were submitted to the germination protocols. The non-plant-forming line produced a total of 58 cotyledonary stage embryos, of which only six were suitable for transfer to germination media.

For the analysis of polyamines in somatic embryos at different stages of development, the following collections were made: (1) developing embryos were collected on different days from tissue growing on different media, i.e. EDM, EMM1, EMM2, and pre-germination media; and (2) normal and abnormal embryos were collected on different occasions from the same tissue masses growing on the same medium. This allowed us to not only analyze changes in polyamines during embryo development on different media, but also in embryos of different developmental stages growing on the same medium, thus eliminating the medium effect on polyamines that may be unrelated to embryo development. The period of collection, which spanned over 3 months, represents stages of development that are comparable to the ones for zygotic embryos (Table 1). As shown in Fig. 3A, putrescine contents of the embryos at different stages of development and those of the tissue at time zero were generally comparable during the entire period of collections. Spermidine and spermine contents, on the other

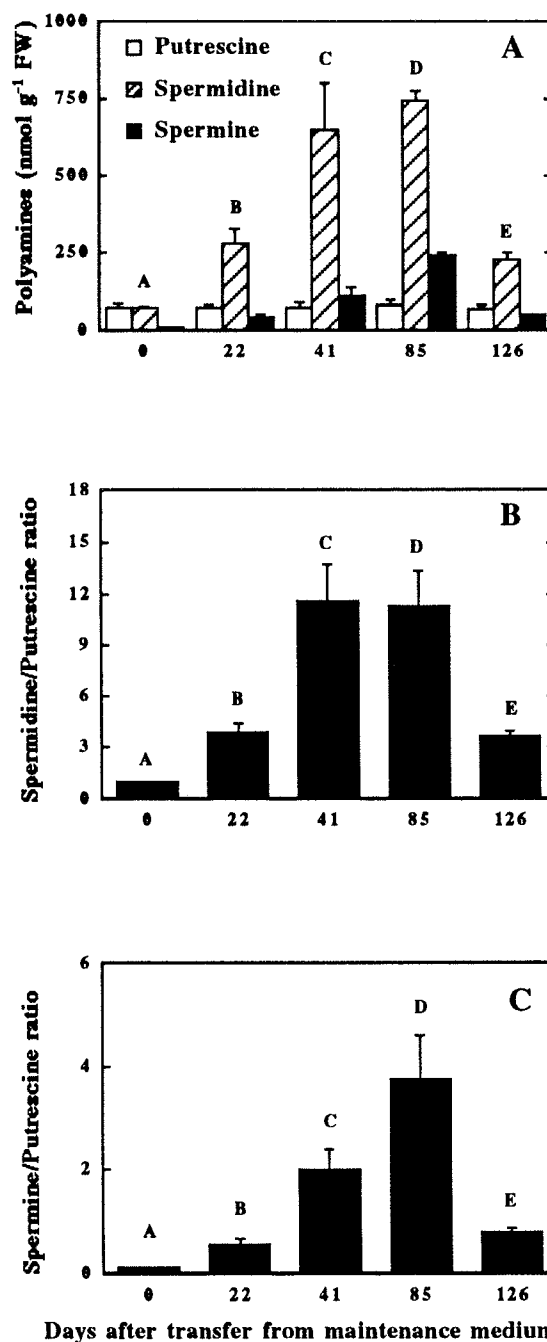
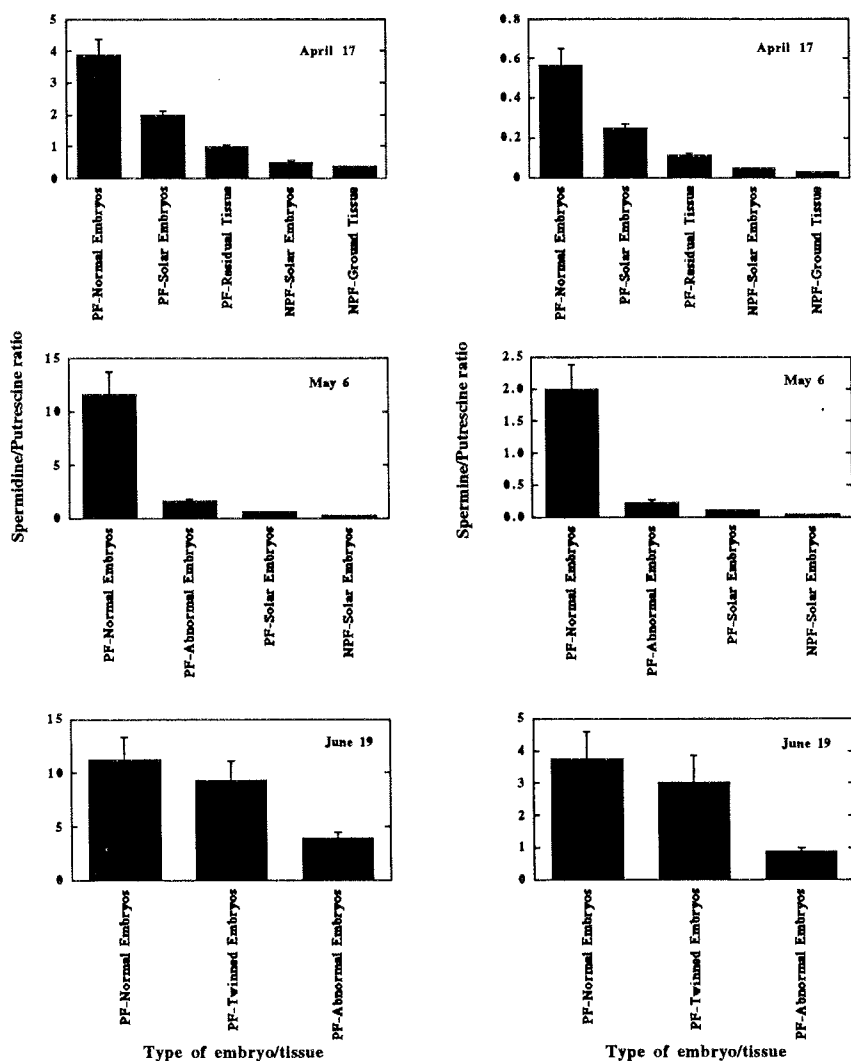


Fig. 3. Changes in cellular polyamines, spermidine/putrescine ratios, and spermine/putrescine ratios in well-formed developing somatic embryos of clone G95-23 of *Pinus radiata*. The following reflect the association of various media with the collection times. A, Zero time (embryonal suspensor mass on maintenance medium EDM); B, 22 days on EDM; C, 41 days (28 days EDM + 13 days EMM1); D, 85 days (28 days EDM + 12 days EMM1 + 45 days EMM2); E, 126 days (28 days EDM + 12 days EMM1 + 76 days EMM2 + 10 days germination medium). Data presented are mean $\pm$ SE of 5 replicates.

hand, showed significant increases with time on transfer to EMM1, spermidine being much higher than either putrescine or spermine. This change is further reflected in the ratios of spermidine/putrescine and spermine/putrescine.

Fig. 4. Comparison of spermidine/putrescine ratios and spermine/putrescine ratios in different forms of somatic embryos and "residual tissue" at different times during maturation in the plant former (PF) and the non-former (NPF) sub-lines of clone G95-23. The NPF sub-line did not produce enough embryos in advanced stages of development for sample collections. The "residual tissue" represents the tissue from which all normal and abnormal embryos including their suspensors had been removed before collection. Data presented are mean $\pm$ SE of 5 replicates.



treoscine ratios than the latter (Fig. 4). However, there was no significant difference in the spermidine/putrescine or spermine/putrescine ratios between the mature twinned and the normal embryos, both of which were capable of forming plants ($P \leq 0.05$).

The profiles of polyamine contents of zygotic and somatic embryos at similar stages of development are presented in Table 2. The zygotic proembryos had a spermidine/putrescine ratio of 3.2, rising to 4.9 as the cotyledons began to appear (refer to Table 1). This ratio remained high, falling subsequently to 3.7 with maturation of the embryos. For somatic embryos, it was not possible to separate proembryos at very early stages of development from the residual tissue, and, therefore, samples were collected beginning with the bullet stage (equivalent to embryo development index of 3 for zygotic embryos). While total polyamine contents of the zygotic and the somatic embryos were similar during the early stages of embryo axis formation, the spermidine/putrescine ratios of somatic embryos rose to a level almost twice as much as those of the zygotic embryos at an embryo development index of around 6–8. At the final stage of

maturation, the total polyamine content of somatic embryos dropped to a level much lower than that in the zygotic embryos (Table 2). However, the spermidine/putrescine ratios in these embryos were similar to those in the zygotic embryos, both of which were by then capable of germination. Spermine/putrescine ratios also showed similar trends, the changes being smaller in the zygotic embryos than in the somatic embryos.

Discussion

The development of somatic and zygotic embryos has been compared in many plants at all levels of observations, e.g. morphological, anatomical, ultrastructural, nutritional, biochemical and molecular (Wann et al. 1987, Goldberg et al. 1989, Hakman et al. 1990, Flinn et al. 1993, Hakman 1993, Krasowski and Owens 1993, Suhasini et al. 1997; also see reviews by Misra 1994, 1995). Among the biochemical and molecular studies, major emphasis has been on the metabolism of lipids and storage proteins, the role of ABA and desiccation, and the differential expression of several

genes, mostly belonging to the Lea (late embryo-specific abundant) group of proteins (Kapik et al. 1995, Dong and Dunstan 1996a,b). It is generally believed that developing somatic and zygotic embryos show similar patterns of biosynthesis of several macromolecules. In some cases, the metabolism of small molecules such as amino acids and polyamines has also been analyzed (Wann et al. 1987, Feirer 1995) and found to be similar in the two types of embryos. Rapid changes in amino acid composition have been observed during the development of both types of embryos. In zygotic embryos of *Pinus strobus*, Feirer (1995) observed that arginine, glutamine and asparagine were the predominant amides that showed major changes during development. Similar research was earlier used to develop the pine embryogenesis medium used for the work described here (Smith 1994).

Changes in cellular polyamine metabolism during somatic embryogenesis have been reported for several plants (see reviews by Minocha and Minocha 1995, Kumar et al. 1997, Walden et al. 1997) including a few conifers (Santanen and Simola 1992, Minocha et al. 1993, Amarasinghe et al. 1996; also see review by Minocha et al. 1995). When proliferating embryogenic tissue of conifers is transferred to maturation medium, an increase in spermidine content is often seen. While Amarasinghe et al. (1996) observed an increase in putrescine preceding the increase in spermidine in interior spruce tissue, most other studies show a sharp decline in putrescine concomitant with an increase or a decrease in spermidine (reviewed in Minocha et al. 1995). It must be emphasized here that all previous studies have analyzed the entire embryogenic tissue or callus mass after transfer to the maturation medium, without a distinction being made between the changes in developing embryos and those in the surrounding residual tissue. This may help explain some of the differences in results obtained from different laboratories. The data presented here are the first to report polyamine contents separately in the developing embryos at different stages of development and the residual tissue which is incapable of regeneration.

The results presented here reveal that an increase in the level of spermidine relative to that of putrescine is a characteristic feature of the developing *Pinus radiata* somatic embryos. As the embryo development proceeds from the bullet stage, when the embryo axis is being determined, to the cotyledon formation stage, spermidine content always increases. On the other hand, trends in the changes of putrescine contents are unpredictable and vary with the genotype. Furthermore, it is seen that the mature cotyledonary stage somatic embryos, which are most likely to germinate and produce viable plants (emblings), can be distinguished by their polyamine ratios from the abnormal embryos that do not germinate to form plants. The changes observed in developing somatic embryos are strikingly similar to those also seen in the zygotic embryos. It was noted that while the spermidine/putrescine ratios of somatic embryos that are ready to germinate (embryo development index of 9, Table 1) are similar to those of zygotic embryos at the same stage (embryo development index of 8.9, Table 2), the total polyamine content of somatic embryos at this stage was only 31% of that of the zygotic embryos. How-

ever, at about the time prior to reaching the final stages of development (embryo development index of 8), the total polyamine contents of somatic embryos were comparable with those in zygotic embryos of development index of 8.9. This apparent difference in polyamine metabolism may be related to the differences in the abilities of the two types of embryos to germinate, i.e. higher germination rates of zygotic embryos than the somatic embryos. Thus this difference may be targeted for genetic manipulation to improve both the frequency of formation of somatic embryos and their conversion into normal plants. The changes in total polyamine contents of the developing seeds in this species are similar to those reported by Feirer (1995) in *Pinus strobus*, and also show similarity with polyamine changes during somatic embryo development in *Picea abies* (Santanen and Simola 1992) and *Picea rubens* (Minocha and Long, unpublished data).

Pine somatic embryogenesis protocol requires that the tissue is transferred to different media (EDM/EMM1/EMM2/PGM) at each stage of development of somatic embryos (Smith 1994). This raises the question of whether the observed changes in polyamine levels in somatic embryos are related to the developmental stage of the embryo or are a consequence of the growth of cells on different media. To resolve this issue, all stages of somatic embryo samples were collected not only from different media, but also from the same medium for comparison. Data in Fig. 4 come from a collection of different types of embryos from the same medium. Note that the spermidine/putrescine ratio of normal embryos in the plant-forming cell line increased significantly between the April 17 and May 6 sampling dates. On the other hand, the spermidine/putrescine ratio of solar embryos in the same cell line remained approximately the same at the two sampling dates. We believe this demonstrates that the polyamines measured in normal somatic embryos reflect the levels appropriate to the stage of development rather than a response to change of medium.

A few detailed accounts of biochemical differences between the embryo and the megagametophytic tissue in conifers have been published (for review see Misra 1994). It is generally believed that the developing zygotic embryo obtains a large proportion of its nutrition from the surrounding megagametophyte. The same also holds true during the process of germination of the seed (Misra 1994, King and Gifford 1997, and references therein). This includes precursors for the biosynthesis of proteins, lipids and, probably, nucleic acids. Kong et al. (1997) have suggested that the megagametophyte in *Picea glauca* may also be a resource for the transport of hormones to the developing embryo. The developing somatic embryos, on the other hand, obtain primarily the inorganic nutrients and sugar from the surrounding medium and must activate their own pathways for the biosynthesis of most of these compounds. Data presented in Fig. 2 are consistent with the possibility of transport of polyamines from the megagametophytic tissue to the developing embryo, since the increase in polyamine content of the embryo coincided with a decline in the megagametophyte. Another possibility is that the changes in the two tissues were due to increased polyamine biosynthesis in the embryos coincident with a decline in the

synthesis and/or an increase in the catabolism of polyamines in the megagametophytes. Analysis of the polyamine biosynthetic enzyme activities in the two tissue types should help resolve this question. Previous reports strongly suggest the possibility of a transport of amino acids and other precursors from the megagametophytic tissue to the embryo during its development (Misra 1995). Whether or not such a transfer of metabolites from the residual tissue to the developing somatic embryo also occurs in the *in vitro* conditions is not known, nor is it clear as to what role the suspensor plays in the development of somatic embryos.

Embryogenic cell masses of all plants consist of a heterogeneous population of cells some of which are competent to form embryos and others that are not (for review see Carman 1990). The embryogenic competence of these cells is often short lived, particularly in woody plants, and it is influenced by the growth conditions as well as genetic factors. A few attempts have been made to distinguish between the competent embryo-forming cells and those that are incompetent to form embryos. Differential expression of genes, DNA methylation levels, isozymes, enzyme activities, and antisera against extracellular proteins have been used to characterize the embryo-forming cells before their development into somatic embryos (see reviews by Carman 1990, Misra 1994, Kawahara and Komamine 1995). The identification of a characteristic extracellular glycoprotein associated with embryogenic cells led to its use in the medium for a substantial improvement of the somatic embryogenic potential of nonembryogenic carrot cells (de Vries et al. 1988, Kreuger and van Holst 1993). Egertsdotter and von Arnold (1995) have also shown that the presence of specific proteins, such as the arabinogalactans, can be correlated with a specific morphology of somatic embryos in Norway spruce.

The low number of normal somatic embryos produced from *Pinus radiata* embryogenic tissue is probably due to an overabundance of the non-embryo-forming, suspensor-like cells compared with the small embryo initials. Increasing the relative proportion of competent proembryos (small compact cell masses) to non-plant-forming vacuolated cells might improve the yield of mature somatic embryos significantly. This may be possible by selective suppression of the growth of non-embryogenic cells, or selective promotion of the proliferation of competent cell types. The data presented here clearly demonstrate that there is a characteristic difference in the cellular polyamine metabolism between the developing somatic embryos and the surrounding residual tissue that does not form somatic embryos. The differences in polyamine metabolism of these two cell types could potentially be the target for biochemical or genetic manipulation to preferentially favor or suppress one group of cells over the other, such as have been done with embryogenic carrot cell suspensions (Robie and Minocha 1989, Minocha and Minocha 1995).

Conclusions

A characteristic pattern of changes in polyamine metabolism during the development of somatic as well as zygotic embryos in *Pinus radiata* is evident. This involves major

changes in putrescine/spermidine ratios during the development of both types of embryos. Furthermore, the ratios of polyamines are significantly different in the developing embryos and the nondifferentiating residual tissue. Thus it may be possible to selectively alter the relative ratios of the two types of cells (embryo forming cells and the suspensor/matrix cells) by modulating polyamine metabolism through the use of inhibitors and/or the approach of genetic manipulation as successfully demonstrated by Bastola and Minocha (1995) for carrot cells. This may result in higher yields of somatic embryos per gram fresh weight of cultured tissue as well as improved maintenance of the embryogenic potential of these cell lines through serial transfers.

Acknowledgements – This research was funded by a New Zealand Government Foundation for Research, Science and Technology grant to the Biotechnology Division of New Zealand Forest Research Institute Ltd. Funding from the New Zealand Lotteries Grant Board towards purchase of cryopreservation equipment is also acknowledged. S. C. Minocha thanks the Biotechnology Division, NZFRI for a grant for sabbatical travel to New Zealand from January to June 1996. The assistance of Cathy Hargreaves for cryopreservation of embryogenic tissue, Chris Neefus for help in statistical analysis of data and Stephanie Long for a portion of polyamine analyses done at UNH is gratefully acknowledged. Scientific contribution number 1991 from the New Hampshire Agricultural Experiment Station.

References

- Aitken-Christie J, Gough K, Maddocks D, Sigley M, Burger F, Carter PCS (1994) Towards commercialisation of conifer embryogenesis. TAPPI Proc. 1994. Biological Sciences Symposium, TAPPI Press, Atlanta, GA: 181–189. ISBN 0-89852-930-1
- Amarasinghe V, Dharmi R, Carlson JE (1996) Polyamine biosynthesis during somatic embryogenesis in interior spruce (*Picea glauca* × *Picea engelmannii* complex). Plant Cell Rep 15: 495–499
- Anderson SI. M.S. Thesis, University of New Hampshire, NH, USA
- Andersen SI, Bastola DR, Minocha SC (1998) Metabolism of polyamines in transgenic cells of carrot expressing a mouse ornithine decarboxylase cDNA. Plant Physiol 116: 299–307
- Bastola DR, Minocha SC (1995) Increased putrescine biosynthesis through transfer of mouse ornithine cDNA in carrot promotes somatic embryogenesis. Plant Physiol 109: 63–71
- Carman JG (1990) Embryogenic cells in plant tissue cultures: occurrence and behaviour. In Vitro Cell Dev Biol 26: 746–753
- de Jong AJ, Schmidt EDL, de Vries SC (1993) Early events in higher-plant embryogenesis. Plant Mol Biol 22: 367–377
- de Vries SC, Booij H, Meyerink P, Huisman G, Wilde DH, Thomas TL, van Kammen A (1988) Acquisition of embryogenic potential in carrot cell-suspension cultures. Planta 176: 196–204
- Dong J, Dunstan DI (1996a) Characterization of three heat shock protein genes and their developmental regulation during somatic embryogenesis in white spruce [*Picea glauca* (Moench) Voss]. Planta 200: 85–91
- Dong J, Dunstan DI (1996b) Expression of abundant mRNAs during somatic embryogenesis of white spruce [*Picea glauca* (Moench) Voss]. Planta 199: 459–466
- Egertsdotter U, von Arnold S (1995) Importance of arabinogalactan proteins for the development of somatic embryos of Norway spruce (*Picea abies*). Physiol Plant 93: 334–345
- Feirer RP (1995) The biochemistry of conifer embryo development: amino acids, polyamines and storage proteins. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic Embryogenesis in Woody Plants, Vol. 1. Kluwer Academic Publishers, Dordrecht, pp 317–336. ISBN 0-7923-3035-8
- Flinn BS, Roberts DR, Newton CH, Cyr DR, Webster FB, Taylor EP (1993) Storage protein gene expression in zygotic and somatic embryos of interior spruce. Physiol Plant 89: 719–730
- Goldberg RB, Barker SJ, Perez-Grau L (1989) Regulation of gene expression during plant embryogenesis. Cell 56: 149–160

- Gupta PK, Pullman GS (1993) Methods for reproducing conifers by somatic embryogenesis using stepwise hormone adjustment. United States Patent no. 5,236,841
- Gupta PK, Grob JA (1995) Somatic embryogenesis in conifers. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic Embryogenesis in Woody Plants, Vol. I. Kluwer Academic Publishers, Dordrecht, pp 81–98. ISBN 0-7923-3035-8
- Hakman I (1993) Embryology in Norway spruce (*Picea abies*). An analysis of the composition of seed storage proteins and deposition of storage reserves during seed development and somatic embryogenesis. *Physiol Plant* 87: 148–159
- Hakman I, von Arnold S (1985) Plantlet regeneration through somatic embryogenesis in *Picea abies* (Norway spruce). *J Plant Physiol* 121: 149–158
- Hakman I, Stabel P, Engstrom P, Eriksson T (1990) Storage protein accumulation during zygotic and somatic embryo development in *Picea abies* (Norway spruce). *Physiol Plant* 80: 441–445
- Hargreaves C, Smith DR (1992) Cryopreservation of *Pinus radiata* embryogenic tissue. *Int Plant Propag Soc Combin Proc* 42: 327–333
- Jalonen P, von Arnold S (1991) Characterization of embryogenic cell lines of *Picea abies* in relation to their competence for maturation. *Plant Cell Rep* 10: 384–387
- Kapik RH, Dinus RJ, Dean JFD (1995) Absciscic acid and zygotic embryogenesis in *Pinus taeda*. *Tree Physiol* 15: 485–490
- Kawahara R, Komamine A (1995) Molecular basis of somatic embryogenesis. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry. Somatic Embryogenesis and Synthetic Seed I, Vol. 30. Springer, Berlin, pp 30–40. ISBN 3-540-57448-4
- King JE, Gifford DJ (1997) Amino acid utilization in seeds of loblolly pine during germination and early seedling growth. *Plant Physiol* 113: 1125–1135
- Klimaszewska K, Smith DR (1997) Maturation of somatic embryos of *Pinus strobus* is promoted by a high concentration of gellan gum. *Physiol Plant* 100: 949–957
- Kong L, Attree SM, Fowke LC (1997) Changes of endogenous hormone levels in developing seeds, zygotic embryos and megagametophytes in *Picea glauca*. *Physiol Plant* 101: 23–30
- Krasowski MJ, Owens JN (1993) The ultrastructural and histochemical post-fertilization megagametophyte and zygotic embryo development of white spruce (*Picea glauca*) emphasizing the deposition of seed storage products. *Can J Bot* 71: 98–112
- Kreuger M, van Holst GJ (1993) Arabinogalactan proteins are essential in somatic embryogenesis of *Daucus carota* L. *Planta* 189: 243–248
- Kumar A, Altabella T, Taylor MA, Tiburcio AF (1997) Recent advances in polyamine research. *Trends Plant Sci* 2: 124–130
- Minocha R, Kvaalen H, Minocha SC, Long S (1993) Polyamines in embryogenic cultures of Norway spruce (*Picea abies*) and red spruce (*Picea rubens*). *Tree Physiol* 13: 365–377
- Minocha R, Minocha SC, Simola LK (1995) Somatic embryogenesis and polyamines in woody plants. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic Embryogenesis in Woody Plants, Vol. I. Kluwer Scientific Publishers, Dordrecht, pp 119–142. ISBN 0-7923-3035-8
- Minocha R, Shortle WC, Long S, Minocha SC (1994) A rapid and reliable procedure for extraction of cellular polyamines and inorganic ions from plant tissues. *J Plant Growth Regul* 13: 187–193
- Minocha SC, Minocha R (1995) Role of polyamines in somatic embryogenesis. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, Somatic Embryogenesis and Synthetic Seed I, Vol. 30. Springer, Berlin, pp 53–70. ISBN 3-540-57448-4
- Minocha SC, Minocha R, Robie CA (1990) High-performance liquid chromatographic method for the determination of dansyl-polyamines. *J Chromatogr* 511: 177–183
- Misra S (1994) Conifer zygotic embryogenesis, somatic embryogenesis, and seed germination: Biochemical and molecular advances. *Seed Sci Res* 4: 357–384
- Misra S (1995) Molecular analysis of zygotic and somatic conifer embryos. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic Embryogenesis in Woody Plants, Vol. I. Kluwer Scientific Publishers, Dordrecht, pp 119–142. ISBN 0-7923-3035-8
- Montague MJ, Koppenbrink JW, Jaworski EG (1979) Polyamine metabolism in embryogenic cells of *Daucus carota*: I. Changes in intracellular content and rates of synthesis. *Plant Physiol* 62: 430–433
- Robie CA, Minocha SC (1989) Polyamines and somatic embryogenesis. I. The effects of difluoromethylornithine and difluoromethylarginine. *Plant Sci* 65: 445–454
- Santanen A, Simola LK (1992) Changes in polyamine metabolism during somatic embryogenesis in *Picea abies*. *J Plant Physiol* 140: 475–480
- Smith DR (1994) Growth medium for plant embryogenic tissue. Australia/Canada Patent # PM5232, USA patent # 5565355 (1996)
- Smith DR (1997) The role of in vitro methods in pine plantation establishment: the lesson from New Zealand. *Plant Tissue Cult Biotechnol* 3: 63–73
- Smith DR., Walter C, Warr A, Hargreaves CL, Grace LJ (1994) Somatic embryogenesis joins the plantation forestry revolution in New Zealand. TAPPI Proc. 1994 Biological Sciences Symposium, TAPPI Press, Atlanta: 19–29. ISBN 0-89852-930-1
- Smith DR, Donaldson LA, McDonald AG (1997) Current research and future applications for cultured pine cells. Proc. IUFRO conference on Genetics of *Pinus radiata*, Rotorua, New Zealand (December 1997) FRI Bulletin No. 203, pp 285–288
- Spurr AR (1949) Histogenesis and organization of the embryo in *Pinus strobus* L. *Am J Bot* 36: 629–664
- Suhasini K, Sagare AP, Sainkar SR, Krishnamurthy KV (1997) Comparative study of the development of zygotic and somatic embryos of chickpea (*Cicer arietinum* L.). *Plant Sci* 128: 207–216
- Tautorus TE, Fowke LC, Dunstan DI (1991) Somatic embryogenesis in conifers. *Can J Bot* 69: 1873–1899
- Thomas TL (1993) Gene expression during plant embryogenesis and germination: an overview. *Plant Cell* 5: 1401–1410
- Walden R, Cordeiro A, Tiburcio AF (1997) Polyamines: small molecules triggering pathways in plant growth and development. *Plant Physiol* 113: 1009–1013
- Wann SR, Johnson MA, Noland TL, Carlson JA (1987) Biochemical differences between embryogenic and nonembryogenic callus of *Picea abies* (L.) Karst. *Plant Cell Rep* 6: 39–42
- West MAL, Harada JJ (1993) Embryogenesis in higher plants: an overview. *Plant Cell* 5: 1361–1369
- Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plants. *Plant Cell* 5: 1411–1423