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POLYAMINES AND THEIR BIOSYNTHETIC ENZYMES DURING SOMATIC EMBRYO DEVELOPMENT IN RED SPRUCE (*PICEA RUBENS* SARG.)

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Summary

The major objective of this study was to determine if the observed changes in polyamines and their biosynthetic enzymes during somatic embryo development were specifically related to either the stage of the embryo development or to the duration of time spent on the maturation medium. Somatic embryos of red spruce (*Picea rubens*) at different developmental stages, grown in the embryo development and maturation media for various lengths of time, were separated from the associated subtending tissue (embryogenic and the suspensor cell masses) and analyzed for their polyamine content as well as for polyamine biosynthetic enzyme activities. Polyamine content was also analyzed in embryos representing different stages of development that were collected from the same culture plate at the same time and the subtending tissue surrounding them. Putrescine was the predominant polyamine in the pro-embryogenic tissue, while spermidine was predominant during embryo development. Significant changes in spermidine/putrescine and spermine/putrescine ratios were observed at all stages of embryo development as compared to the pro-embryogenic cell mass. Changes in the ratios of various polyamines were clearly correlated with the developmental stage of the embryo rather than the period of growth in the maturation medium. Whereas the activities of both ornithine decarboxylase and arginine decarboxylase increased by week 3 or 4 and stayed high during the subsequent 6 wk of growth, the activity of *S*-adenosylmethionine decarboxylase steadily declined during embryo development.

Key words: arginine decarboxylase; ornithine decarboxylase; polyamines; red spruce; *S*-adenosylmethionine decarboxylase; somatic embryos.

Introduction

Somatic embryogenesis is used as a tool for vegetative propagation as well as for biochemical and molecular studies of the regulation of embryo development in plants (Misra et al., 1993; Dong and Dunstan, 2000; Ashihara et al., 2001; von Arnold et al., 2002). While a number of herbaceous and woody plants can be propagated by this method, commercial success with most forest trees is rather limited (Jain et al., 1995). In spite of a few examples of success (Jain et al., 1995; Stasolla and Yeung, 2003), conifers generally pose a unique set of problems for commercial applications of somatic embryogenesis, e.g. the potential of raising embryogenic cultures is limited to only a few embryonic tissues, the yields of somatic embryos are low, and embryogenic masses lose their embryogenic potential with time of culture. Although it is possible to modulate somatic embryogenic potential *in vitro* using different treatments, genotype remains the most important factor affecting the maturation of somatic embryos (El Meskaoui and Tremblay, 2001). In order to increase the efficiency of embryo development, an improved understanding of the biochemical and molecular events that precede and accompany the development of somatic embryos is essential. Such knowledge should permit experimental manipulations of the tissue culture medium or the growth conditions to induce biochemical and molecular changes that normally occur in the developing embryos, thus improving the process.

Changes that accompany somatic embryo development include: (1) the production of soluble signal molecules such as endochitinases, arabinogalactan proteins (AGPs), and lipochito-oligosaccharides (Chapman et al., 2000; von Arnold et al., 2002); (2) changes in small heat shock proteins (Puigderrajols et al., 2002), germins and germinilla proteins (Patnaik and Khurana, 2001); (3) induction of a variety of early and late embryo-specific proteins (Chatthai and Misra, 1998; Cairney et al., 1999; Dong and Dunstan, 1999; Nato et al., 2000); (4) changes in overall gene expression profiles (Cairney et al., 2000; Yasuda et al., 2001); (5) changes in proteins related to anti-oxidant metabolism (Bagnoli et al., 1998); and (6) changes in cellular polyamines (Minocha and Minocha, 1995; Minocha et al., 1995).

The common polyamines (PAs), including putrescine (Put), spermidine (Spd) and spermine (Spm), have been shown to play an important role in basic cellular processes, including organogenesis and somatic embryogenesis (Minocha and Minocha, 1995; Cohen, 1998 and references therein). In the case of the well-characterized somatic embryogenesis system of carrot, it has been shown that somatic embryo development is accompanied by increased PA

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metabolism (see review by Minocha and Minocha, 1995), and that genetic manipulation of Put metabolism can lead to increased somatic embryogenesis (Bastola and Minocha, 1995). Also, the manipulation of tissue PA ratios by adding Spd in the medium resulted in the induction of somatic embryo production in cell cultures of rice that had lost embryogenic potential through repeated subculture (Shoeb et al., 2001). An increase in Spd/Put ratio was also observed during somatic and zygotic embryo development in *Pinus radiata* (Minocha et al., 1999b).

The metabolism of PAs involves: (1) the biosynthesis of Put from arginine and/or ornithine by the enzymes arginine decarboxylase (ADC) and ornithine decarboxylase (ODC), respectively; (2) the conversion of Put into Spd and subsequently into Spm by successive additions of aminopropyl moieties from decarboxylated *S*-adenosylmethionine (SAM), which is the product of SAM decarboxylase (SAMDC) reaction; and (3) the catabolism of Put, Spd and Spm by diamine and PA oxidases (see reviews by Slocum and Flores, 1991; Cohen, 1998). Cellular PA levels in some plants can also be regulated by their conjugation with phenolic acids and covalent binding to macromolecular fractions (Martin-Tanguy, 1997).

Differentiating embryogenic cultures of Norway spruce have significantly higher levels of PAs than non-embryogenic cultures (Minocha et al., 1993; Santanen and Simola, 1994; Amarsinghe et al., 1996). In most biochemical studies of somatic embryos published earlier, either the entire tissue mass on a given day of growth on the embryo induction medium was analyzed and compared with the tissue grown on non-induction medium, or mixtures of developing embryos representing several stages of development were analyzed. Thus, the results represent either the resultant averages of developing embryos and the subtending tissue together or several stages of embryo development. Furthermore, embryos of different stages of development were often collected from tissue masses of different chronological age on the induction/maturation medium. Thus, it has been difficult to discern if the biochemical changes in the tissue are related to the chronological age of the tissue or the stage of embryo development. In the present study, this problem has been addressed by: (1) collecting different stages of developing embryos both from cultures of different ages (i.e., the cells grown in a particular medium for different lengths of time) and also from the collection of different stages of developing embryos from the same age subtending tissue; and (2) collecting the developing embryos and the subtending tissue on the same day from the same medium. The present study is the first to specifically address the issue of whether the changes in PAs at different stages of development of the embryos are related to the developmental stages of the embryos or to the length of time for which the tissue was grown in a particular medium. It is thus possible to decide whether the observed changes in PAs are related to the stage of somatic embryo development or whether they are a coincidental medium effect. The results presented here support the former interpretation.

Materials and Methods

Culture method. Pro-embryogenic suspension cultures of *Picea rubens* Sarg. (genotypes 91.9 and 97.1) were maintained on solid half-strength LP proliferation medium ($\frac{1}{2}$LP) as previously described (Minocha et al., 1993). Liquid cultures were maintained in half-strength LM proliferation medium ($\frac{1}{2}$LM) (Litvay et al., 1981) with modifications that included the addition of 3.42 m*M* glutamine, 9.05 μ*M* 2,4-dichlorophenoxyacetic acid (2,4-D), 4.44 μ*M* 6-benzyladenine (BA), 1.0 g l^{-1} casein hydrolyzate, and 2% (w/v) sucrose (Minocha et al., 1993, 1996). Also, iron-EDTA was replaced by 40 mg l^{-1} of Sequestrine containing 7% (w/w) iron chelate (Plant Products Co. Ltd., Brampton, Canada). The medium was adjusted to pH 5.7 before autoclaving. Cells were subcultured at 7d intervals by transferring 10–12 ml of cell suspension into 45 ml of fresh medium in 250 ml Erlenmeyer flasks. The flasks were incubated in the dark at 25 ± 2°C on a gyratory shaker at 120 rpm. The reason for switching to liquid $\frac{1}{2}$LM medium for suspension cultures was that the suspension cultures would often turn thick and brown when cultured in $\frac{1}{2}$LP medium.

Embryo maturation protocol. The 7-d-old cultures growing in $\frac{1}{2}$LM liquid proliferation medium were subcultured into liquid $\frac{1}{2}$LM maturation medium using a standard subculture ratio of 45 ml of fresh medium to 10–12 ml of 7-d-old cells. These cultures were incubated in the dark at 25°C on a gyratory shaker at 120 rpm for an additional 7 d. The maturation medium contained all ingredients of the proliferation medium except 2,4-D and BA, and also contained 7.5% (w/v) polyethylene glycol (PEG-3350), 30 μ*M* abscisic acid (ABA), 1 μ*M* indole-3-butyric acid (IBA), and 3% sucrose. For embryo development, 1–2 ml of 7-d-old cultures growing in $\frac{1}{2}$LM liquid maturation medium were transferred to solid maturation medium containing 0.4% (w/v) Phytagel (Sigma Chemical Company, St. Louis, MO) on filter papers. The filters were initially transferred to fresh medium at 4 wk and, thereafter, at 2-wk intervals.

Sample collections for polyamine analyses. At weekly intervals, samples consisting of either 100 mg fresh weight (FW) of cell mass (zero time) from suspensions grown in proliferation medium or in maturation medium for 1 wk, or 50 embryos of the predominant stage of development for each of the subsequent weeks, were collected and placed in 500 μl of 5% perchloric acid (PCA). This study was repeated four times in its entirety, three times with the genotype RS 91.9 (derived from a seed from Moosehead Lake, ME) and once with the genotype RS 97.1 (derived from a seed received from the Canadian Forest Service, Fredericton, New Brunswick, Canada).

Samples of specific developmental stages of embryos (stage experiments) were collected on the same day from cultures growing on maturation medium for varying lengths of time, i.e., stages 1–3, 4–7, and 8, from cultures grown on maturation medium for 2, 5–6, and 8–10 wk, respectively. For example, embryo stages 4 and 7 were collected from the same filter paper (maturation medium for 6 wk), although stage 7 embryos were predominant at this time. These samples consisted of three replicates per stage and 25 embryos per replicate in 250 μl of 5% PCA. This part of the study was repeated twice in its entirety, with the genotype RS 91.9. Each replicate embryo sample was collected from a separate Petri plate. All samples were frozen at −20°C immediately after collection until ready for processing for PA analysis.

Analysis of polyamines. The samples were frozen (−20°C) and thawed (room temperature) for a total of three times (Minocha et al., 1994), centrifuged at 13 500 × *g* for 10 min, and the supernatant fraction was used for dansylation according to the procedures of Minocha et al. (1990, 1994). Briefly, 10 μl aliquots of 0.2 m*M* heptanediamine (internal standard) and 100 μl aliquots of sample extract or 100 μl of a mix of three standards (0.02 m*M* each of Put, Spd, and Spm in 5% PCA) were mixed with 100 μl of saturated sodium carbonate solution and 100 μl of dansyl chloride (20 mg ml^{-1} in acetone) in microfuge tubes. The tubes were vortexed and incubated in a water bath at 60°C. After 60 min, 50 μl of proline (100 mg ml^{-1} in water) were added to the reaction mix to react with excess dansyl chloride. Following an additional 30 min incubation, acetone was evaporated from the tubes in a SpeedVac (Savant, Farmingdale, NY) under vacuum, 400 μl of toluene were added to each tube, and the tubes were centrifuged at 13 500 × *g* for 1 min. From the toluene phase, a 200 μl aliquot was transferred to another microfuge tube. Toluene was completely evaporated under vacuum, the residue dissolved in 1 ml of methanol, vortexed, centrifuged briefly at 13 500 × *g*, and analyzed by HPLC.

The liquid chromatographic system consisted of a Perkin Elmer series 400 pump, a Hitachi autosampler (Model AS-4000) fitted with a 20 μl loop (10 μl actual injections), a Perkin Elmer Pecosphere 3 × 3 CR C_{18}, 33 × 4.6 mm i.d. cartridge column (3 μm particle size), and a fluorescence detector (LS-1, Perkin Elmer). The excitation and emission wavelengths were set at 340 and 510 nm, respectively. The separation of PAs was carried out using a gradient of acetonitrile and heptanesulfonic acid (10 m*M*, pH 3.4) according to the gradient profile published earlier (Minocha et al., 1990). Total run time for each sample was about 8.7 min.

Enzyme assays. The samples consisting of either 100 mg of pro-embryogenic masses (zero time) grown in proliferation medium or 100 mg of

embryogenic masses from cell suspensions grown in maturation medium for 1 wk, and 100 embryos of the predominant stage of development for each of the subsequent weeks were collected at weekly intervals (two replicates). Each replicate embryo sample was collected from a separate Petri plate. For the collections at 2 wk, embryos from several plates were mixed to obtain sufficient material. Samples were placed in 250 µl of appropriate buffer in a 15 ml Kimble glass test tube for each enzyme assay as described below and frozen at −20°C immediately after collection until ready to be processed. These samples were analyzed for activity of ADC, ODC, and SAMDC using intact cells or whole embryos according to the procedure of Minocha et al. (1999a). The reaction mixture for ODC and ADC activity consisted of 250 µl of cell suspension and 50 µl of 12 m*M* ornithine containing 1.85 kBq L-[1-^{14}C]ornithine (New England Nuclear, Boston, MA; specific activity 2.15 GBq mmol^{-1}) or 12 m*M* arginine containing 3.7 kBq of DL-[1-^{14}C]arginine (Moravek Biochemicals Inc., Brea, CA; specific activity 2.07 GBq mmol^{-1}), respectively. Reaction was run in a 16 × 100 mm Kimble test tube fitted with a $^{14}CO_2$ trap. Following incubation at 37°C for 60 min, 500 µl of 0.5 *N* H_2SO_4 was injected through the stopper to terminate the reaction. After an additional 30 min incubation, the filter papers were removed and counted for radioactivity. The activity of enzyme was expressed on a fresh weight (FW) basis as nmol $^{14}CO_2$ released per h per g FW. The activity of SAMDC was assayed according to Minocha et al. (1999a) for intact cells. The reaction set up was similar to that of ODC/ADC, but contained 50 µl of 1.2 m*M* SAM along with 3.7 kBq of L-[1-^{14}C]SAM (New England Nuclear; specific activity 2.18 GBq mmol^{-1}). The reaction time in this case was 30 min instead of 60 min. The experiment with enzyme assays was repeated twice in its entirety with the genotype RS 91.9.

Statistical analysis. The data for the three PAs within each group were analyzed as a series of 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, Tukey's multiple comparisons test was used to compare the mean of pro-embryogenic cell masses with means for all other embryo collections for significant differences ($P \leq 0.05$). ANOVA assumes that all the groups being compared have a similar range of variation around them. This was not true for some of our data sets, especially the ones for PA ratios: higher ratios had a higher range of variation. Thus, all statistical analyses for ratios of PAs were run after logarithmic transformations of the data sets. All analyses were performed with Systat Windows, Version 7.01 (Systat Inc., Evanston, IL). It is known that PA levels may vary from week to week in cell and tissue cultures and therefore it is not always easy to pool the data over experiments. However, the trends remained the same not only within repeat experiments with the same genotype but also with another genotype. Therefore, data have been presented here from a single representative experiment belonging to genotype 91.9; where appropriate, the range of variation in results from one experiment to the other is discussed.

Results

The data presented here are representative of data gathered from other experiments for both genotypes and where necessary the range of variation in results from one experiment to the other is also discussed.

Growth and embryo development. The pro-embryogenic cultures maintained in liquid proliferation medium (week 0) contained numerous organized meristematic clusters that were attached to long suspensor-like cells (Fig. 1 – week 0). These clusters have been variously referred to as 'pro-embryogenic clusters,' 'pro-embryos,' 'proembryogenic masses,' and 'embryogenic calluses' in the literature (Stasolla and Yeung, 2003). Within 1 wk of transfer to maturation medium, the meristematic clusters developed into organized embryo heads that were much larger in size, although the attached suspensors had shrunk slightly (Fig. 1 – week 1). On transfer to solid agar-based maturation medium, the embryos developed into globular or torpedo stages within another week (Fig. 1 – week 2). By the end of week 3 on maturation medium, the embryos were in the form of elongated bullet-shaped structures (Fig. 1 – week 3), and by week 4, the embryos were long and cylindrical with visible protuberances for cotyledons (Fig. 1 – week 4). During the next week, the embryo axes had elongated further and the cotyledons were slightly open and well defined (Fig. 1 – week 5). During the 6th and 7th weeks, the embryos were nearly mature with cotyledons not yet fully extended (Fig. 1 – weeks 6 and 7), and finally in the 8th week, the embryos looked mature and cotyledons were fully extended (Fig. 1 – week 8). Additional time on this medium (up to 11 wk) did not further change the morphological appearance of embryos (data not shown).

Changes in cellular polyamines. The pro-embryogenic clusters maintained in liquid proliferation medium (wk 0) had Put as the predominant PA (Fig. 2*A* – week 0). Cellular Put was up to twofold higher than Spd while Spm was present in very low concentrations at week zero. On growth in liquid maturation medium for a week, the total PA levels decreased, Put showing the maximum decrease, reaching a value significantly lower than that of Spd (Fig. 2*A*). This resulted in an increase in the Spd/Put ratio from less than 1 on day 0 to about 3.0 at the end of 1 wk in the maturation medium (Fig. 2*B* – week 1). Although total Spm was still present in relatively small quantities at this time, the ratio of Spm/Put was nevertheless 5–10-fold higher in these cultures as compared to cultures growing on proliferation medium (Fig. 2*C* – week 1).

When the cultures were transferred to solid maturation medium on filter papers, the cellular Put levels declined further within a week to below detection limits (< 1 nmol g^{-1} FW), and Spd and Spm levels increased to mean values of 350–1400 and 50–200 nmol g^{-1} FW, respectively, in different experiments (Fig. 2*A* – week 2). By week 3, Put reappeared in these embryos and varied between mean values of 300 and 840 nmol g^{-1} FW in different experiments. Total PAs were several-fold higher as compared to week 1 and remained high until the embryos were fully mature. Total PAs as well as Put levels peaked around week 4 and stayed high up to week 5 and dropped significantly by week 6 in all experiments. Spermidine content increased up to 4 wk of culture, decreasing for the next 2 wk and remaining steady after that; Put went up once again between 6 and 10 wk of culture. Cellular Spd/Put ratios were in the range of 2–4 during the 11 wk except at the end of week 6, when the Spd/Put ratio was greater than 10.

Cellular Spm ranged between an average of 300 and 400 nmol g^{-1} FW throughout the 3rd to 11th weeks of culture, as compared to 20–40 nmol g^{-1} FW in the pro-embryogenic clusters for various experiments. The Spm/Put ratios were significantly higher ($P \leq 0.05$) at all stages of embryo development as compared to pro-embryogenic cells (0 wk). These ratios peaked around 6–7 wk after transfer of the tissue to maturation medium. Neither the total PAs nor Spd/Put and Spm/Put ratios changed much between the 8th and the 11th week of culture.

Embryos were also collected at specific developmental stages on the same days from cultures growing on maturation media for varying lengths of time, i.e., stages 1–3 were collected from 2-wk-old cultures, stages 4–7 from 5- or 6-wk-old cultures, and stage 8 from cultures on maturation medium for 8 or 10 wk. While on a given day, most developing embryos were at a certain stage of development, there was sufficient asynchrony of development to allow collection of embryos of several other stages from the same plate on the same day. Comparison of PAs in embryos at different stages of development collected from the same filter paper allowed us to distinguish between the effects of a particular developmental stage and the time

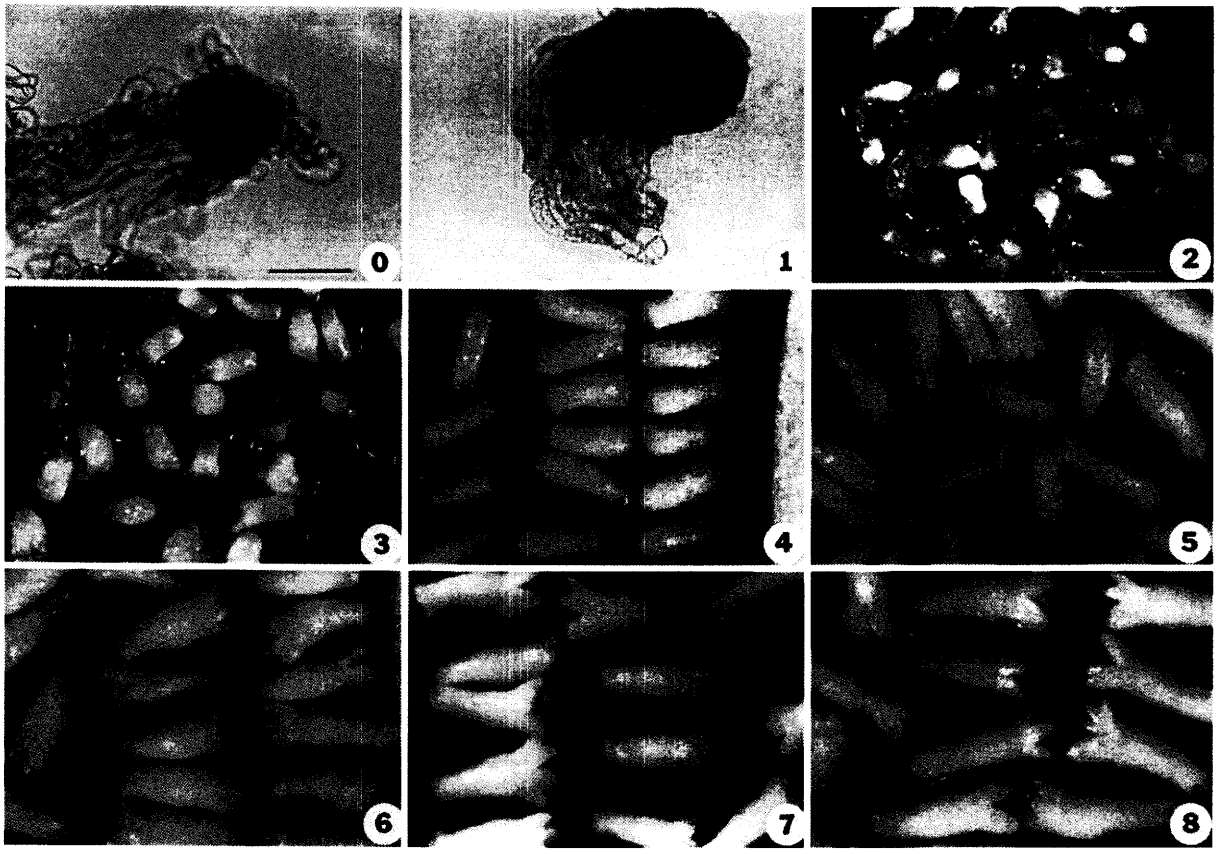

FIG. 1. Stages in the development of somatic embryos of red spruce on the maturation medium containing ABA and IBA. *Numbers* indicate the weeks on maturation medium. For weeks 0 and 1, the cultures were maintained in liquid medium; for weeks 2–8, the cultures were on solid medium. Observations: week 0: early stage pro-embryo showing organized meristematic clusters attached to long suspensor-like cells; week 1: embryos with larger heads and shrunken suspensors as compared to week 0; week 2: globular or torpedo-stage embryos; week 3: elongated bullet-stage structure; week 4: long and cylindrical embryos with visible slits for the cotyledons; week 5: embryos with elongated axis and cotyledons slightly open; weeks 6 and 7: nearly mature embryos with cotyledons not yet fully extended; week 8: mature embryos with fully extended cotyledons. *Bar* shown in week 0 represents 0.67 μm for both weeks 0 and 1. *Bar* shown in week 2 represents 10 μm for weeks 2–8.

for which the cultures were kept on the maturation medium. Figure 3 shows the developmental stages of embryos collected on a given day using a regime described under Materials and Methods. Data presented in Fig. 4 show that the characteristic increase in the Spd/Put and Spm/Put ratios were more strongly correlated with the stage of development than with the number of weeks on a given medium. Stage 1 and 2 embryos together were comparable morphologically to week 2 collections. Similar to the situation of 2-wk-old embryos, stages 1 and 2 also contained little or no Put. Stages 3, 4, 5, 6, and 7 embryos were generally comparable to samples collected on weeks 3, 4, 5, 6, and 7 in morphology as well as in cellular PA content. The Spd/Put and Spm/Put ratios were ≥ 5 and ≥ 1, respectively, in both cases (i.e., weekly collection as well as stage-specific collections) for all weeks. The number of cotyledons on a mature embryo appeared to neither affect the total PA content (Fig. 5) nor the ratios of PAs (data not shown).

For the first few weeks on solid medium, the whole tissue mass was also collected from the plates (this tissue mass included developing embryos as well as the subtending tissue) and compared with separately collected embryos for cellular PA content. The PA content of the collected embryos was always significantly higher as compared to the entire tissue mass that included surrounding subtending tissue and developing embryos (Fig. 6). While the three PAs increased steadily in the developing embryos, the surrounding tissue mass showed only minor changes with time.

Changes in polyamine biosynthetic enzymes. The activities of both ADC and ODC (on a FW basis) declined by 30–50% in the embryogenic cell masses within 1 wk of growth in liquid maturation medium (Fig. 7*A*). The enzyme activities stayed low for another 2 wk and then increased to a level that was higher than that found in the pro-embryogenic cell masses; thereafter, both ADC and ODC remained high throughout the duration of embryo development. The activity of ADC was several-fold higher than ODC on any given day of analysis. When calculated on a per embryo basis, the trends in the changes of ADC and ODC activity were similar to those on a FW basis, i.e., both enzyme activities showed a steady increase between

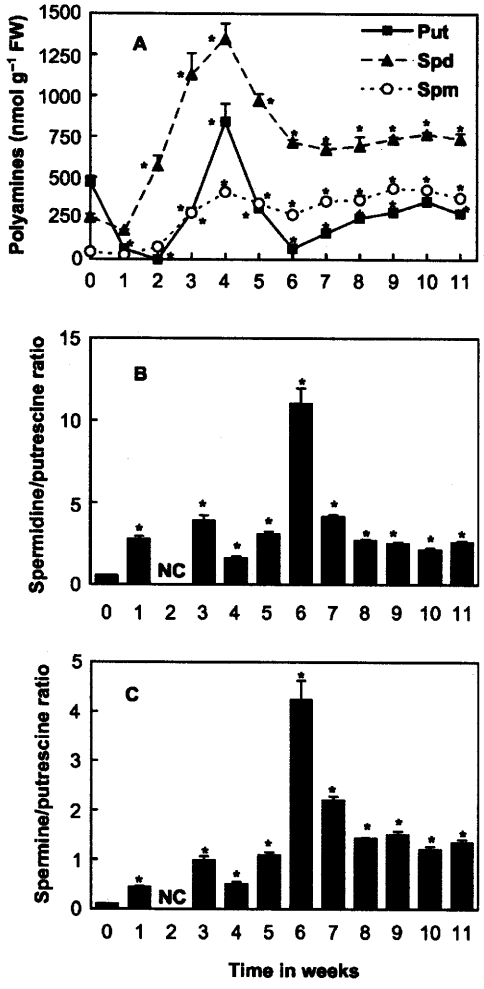

FIG. 2. Changes in (*A*) putrescine, spermidine, and spermine; (*B*) spermidine/putrescine ratio; and (*C*) spermine/putrescine ratio in the developing somatic embryos of red spruce with time of culture. NC, ratio not calculable since putrescine was 0. Data represent mean ± SE, $n = 5$. For the weekly collections and analyses, only the predominant developmental stage was collected.

3 and 6 wk of culture and stayed high thereafter (Fig. 7*B*). No developed embryos were available for 0 and 1 wk times.

On a FW basis, SAMDC activity declined by more than 50% in the embryogenic cell masses within 1 wk of growth in the liquid maturation medium. It declined even further in the next 4 wk, albeit at a slower rate (Fig. 7*C*). After 5 wk of culture, there was little change in SAMDC activity throughout the duration of embryo development. When calculated on a per embryo basis in the developing embryos, SAMDC activity was low during weeks 2 and 3 of embryo development (Fig. 7*D*). Not counting a spike of SAMDC on week 4, it remained low for most of the embryo development period; rising rapidly in mature embryos at 10 wk. This time coincides with the desiccation of mature embryos. Once again, no embryos were available for SAMDC analysis at 0 and 1 wk times.

DISCUSSION

The results presented here show that significant and characteristic changes in the cellular content of PAs and their biosynthetic enzymes occur coincidentally with a developmental shift from proembryogenic masses to developing embryos (weeks 0–2), and again during the period of early development and maturation of the somatic embryos. On transfer to medium promoting embryo development, the cellular contents of Put exhibited a rapid decline (Fig. 2*A*), and a shift occurred in the ratio of Spd/Put from less than 1 to about 3 (Fig. 2*B*). A parallel rise in all three PAs occurred during the next 2–3 wk, but the ratios of Spd/Put and Spm/Put always remained ≥ 1.0, rising to as high as 10 around week 6. In contrast, the subtending tissue surrounding the embryos still exhibited a Spd/Put ratio of ≤ 1.0. Similar results were obtained regardless of whether developing embryos were collected at weekly intervals (e.g., 5-wk-old embryo from tissue that has been on maturation medium for exactly 5 wk) or collected simultaneously from tissue cultured on a maturation medium for several weeks. It can therefore be argued that the observed differences in PAs and polyamine ratios are not due to the medium effect, but are developmental stage-specific. This argument is further strengthened by the results presented in Fig. 4, where again the embryos of different stages of development collected from the same medium on the same days show similar patterns of Spd/Put and Spm/Put ratios as shown in Fig. 2.

Overall, the pattern of changes in total soluble PAs during development and changes in the ratios of Spd/Put are consistent with previous studies on somatic embryogenesis in conifers and angiosperms (Minocha and Minocha, 1995; Minocha et al., 1995). In wild carrot, the addition of Spd alone could restore embryogenic potential in cultures that were treated with PA biosynthesis inhibitors, indicating a direct role of Spd in somatic embryogenesis (El Hadrami and D'Auzac, 1992; Feirer, 1995). Such a role for Spd has also been reported in *Picea abies* (Santanen and Simola, 1992), *Pinus radiata* (Minocha et al., 1999b), *Medicago sativa* (Cvikrová et al., 1999), and *Panax ginseng* (Monteiro et al., 2002). In all these cases, significant increases in Spd levels were associated with the formation of somatic embryos.

Several successful attempts have been made to manipulate somatic embryogenesis in recalcitrant tissues by either supplementation of the growth medium with Spd, Put or their precursors, or by genetic manipulation of PA metabolism. Yadav and Rajam (1998) reported that eggplant leaf discs with higher Put levels and higher ADC activity also had higher embryogenic capacity. This capacity could be easily modulated by either adding exogenous Put or an inhibitor of Put biosynthesis to the cultures. In cell cultures of rice, manipulation of tissue PA ratios by adding Spd to the medium resulted in the induction of somatic embryo production in cell lines that had lost embryogenic potential through repeated subculture (Shoeb et al., 2001). Increased Spd/Put ratio was also observed during somatic and zygotic embryo development of *Pinus radiata* (Minocha et al., 1999b). The data presented here support the idea that PA metabolism, especially manipulation of the ratios of Spd/Put and Spm/Put, may play a significant role in somatic embryo development in red spruce as well.

Kevers et al. (2000) reported a fourfold increase in the number of somatic embryos in *Panax ginseng* when PAs or their precursors, arginine and ornithine, were added to the induction or regeneration

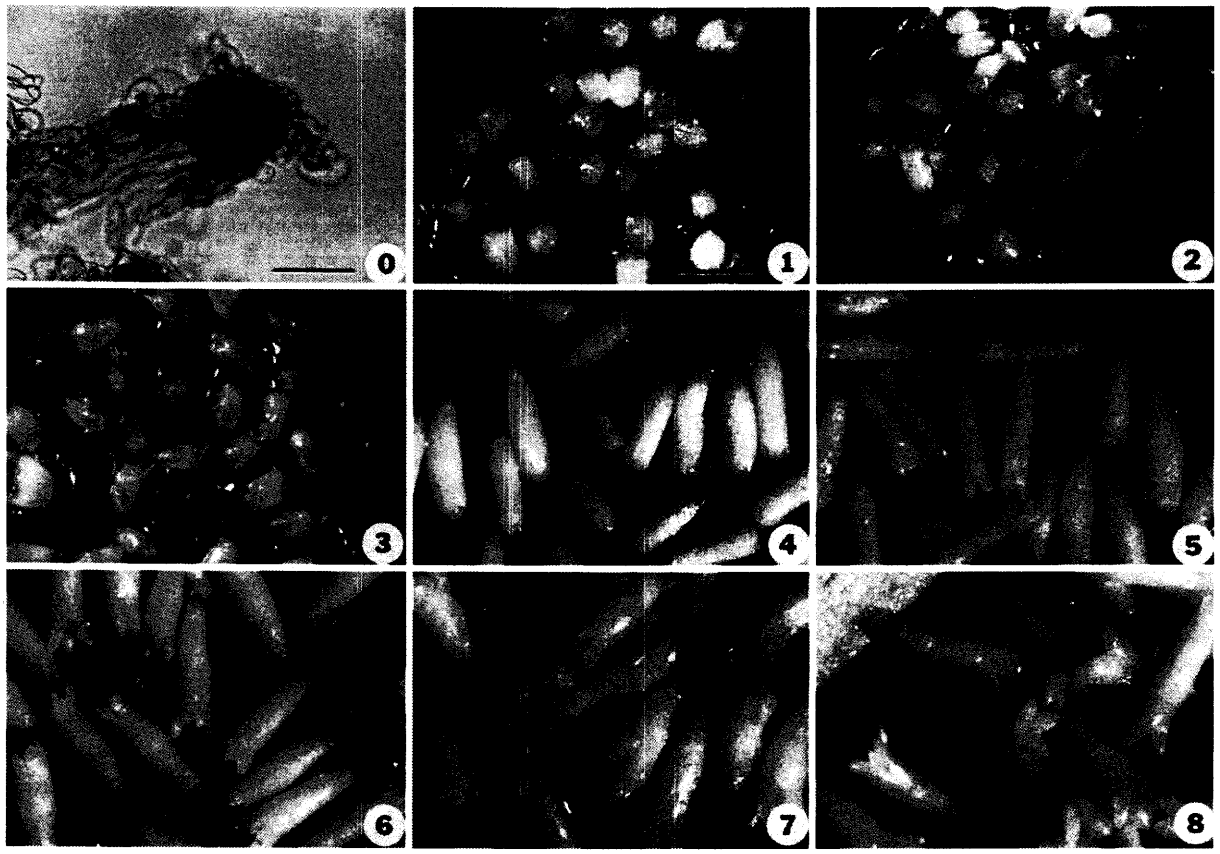

FIG. 3. Stages in the development of somatic embryos of red spruce collected on the same day from cultures grown on maturation medium containing ABA and IBA for varying lengths of time (for details see Materials and Methods). *Numbers* indicate the stages of development in progression towards maturity parallel to those in Fig. 1. *Bar* shown in stage 0 represents 0.67 μm. *Bar* shown in stage 1 represents 10 μm for stages 1–8.

medium. Maximum increase was observed when Spd was added to either medium. Also, the inhibitors of PA biosynthesis reduced the number of embryos produced. The addition of exogenous PAs to root-derived pro-embryogenic callus of this species, however, was deleterious, leading to browning of callus, reduced initiation capacity, and increased tendency to hyper-hydricity (Kevers et al., 2002). This is similar to the adverse effects of exogenous Spd and Spm on embryogenesis of carrot cultures observed by Robie and Minocha (1989). This observation, combined with the promotion of somatic embryogenesis by increased biosynthesis of Put via transgenic expression of an *odc* gene, led Bastola and Minocha (1995) to emphasize the importance of increased PA metabolism in contrast to only an increased cellular PA content in regulating somatic embryogenesis in carrot. The question as to whether changes in PA metabolism could affect somatic embryogenesis by metabolic interactions (competition) with ethylene metabolism has been discussed earlier (Minocha, 1988; Santanen and Simola, 1992, 1994; Minocha and Minocha, 1995; Kong et al., 1998; Minocha et al., 1999b). While several studies appear to support the hypothesis of a competition between the two pathways, others are somewhat contradictory (Quan et al., 2002). It should be pointed out that only a few studies have simultaneously measured the PAs and ethylene levels. El Meskaoui et al. (2000) reported that in white spruce, a reduction in ethylene production was beneficial to somatic embryo production. El Meskaoui and Tremblay (2001) later linked lower ethylene production with a higher capacity of somatic embryo production in a black spruce cell cultures; the low embryogenic capacity cell line had relatively higher levels of ethylene production. The authors suggested that the decrease in ethylene by α-aminooxyacetic acid (AOA) may have led to feedback increase in PA or SAM levels, causing promotion of somatic embryogenesis. Joy et al. (1996) suggested that the physiology of each developmental stage of carrot somatic embryos is distinct. During embryo development, for example, the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway is prominent in non-embryogenic cells and germinating embryo stages. But in pre-embryogenic masses, globular stages, and torpedo-stage embryos, metabolism that utilizes the ornithine cycle is predominant as well as enhanced. Santanen and Simola (1999) reported that in maturing Norway spruce seeds, radioactive labels from arginine and ornithine mostly ended up in Put, suggesting that either Spd and Spm synthesis was slow during embryo maturation or their catabolism was high. Our findings are consistent with the above observation in that the ADC activity was high during the early

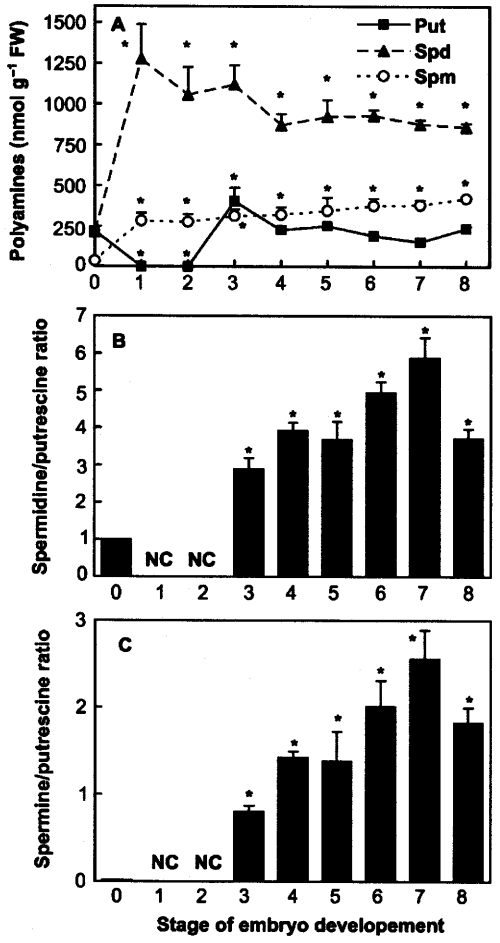

FIG. 4. Changes in (*A*) putrescine, spermidine, and spermine; (*B*) spermidine/putrescine ratio; and (*C*) spermine/putrescine ratio in the developing somatic embryos over time. NC, ratio not calculable since putrescine was 0. Embryos of different stages were collected on the same day from cultures grown on solid medium for varying lengths of time. Data represent mean ± SE, $n = 3$.

stages of somatic embryo development and accompanying SAMDC activity continually decreased, even though Spd levels were quite high at this time, possibly indicating a slow synthesis of Spd in maturing embryos. The increase in SAMDC at week 4 in our study coincided with the peak in cellular Spd and Spm levels. Santanen and Simola (1999) also found tenfold higher levels of Put and ADC activity in embryos as compared to megagametophytes. However, Spd and Spm levels were the same in both types of tissue. In our study, the levels of all these PAs were higher in the isolated embryos as compared to the accompanying non-embryogenic cell mass. One cannot, however, equate megagametophytic tissue with the subtending tissue in cultures.

In conclusion, while there is little doubt that PAs play an important role in somatic embryogenesis, and the process can be modulated by manipulating the cellular PA contents either by media supplementation or genetic engineering, neither the biochemical mechanism of the PA role nor the molecular mechanism of their

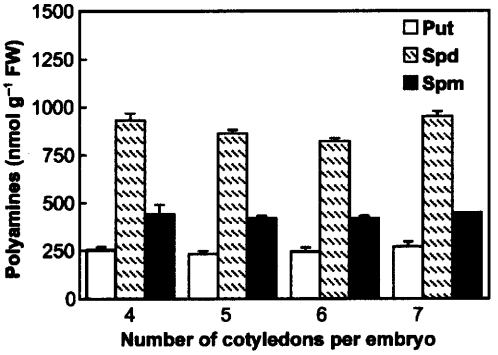

FIG. 5. Comparison of polyamines (putrescine, spermidine, and spermine) in mature embryos with different numbers of cotyledons. Data are mean ± SE, $n = 3$.

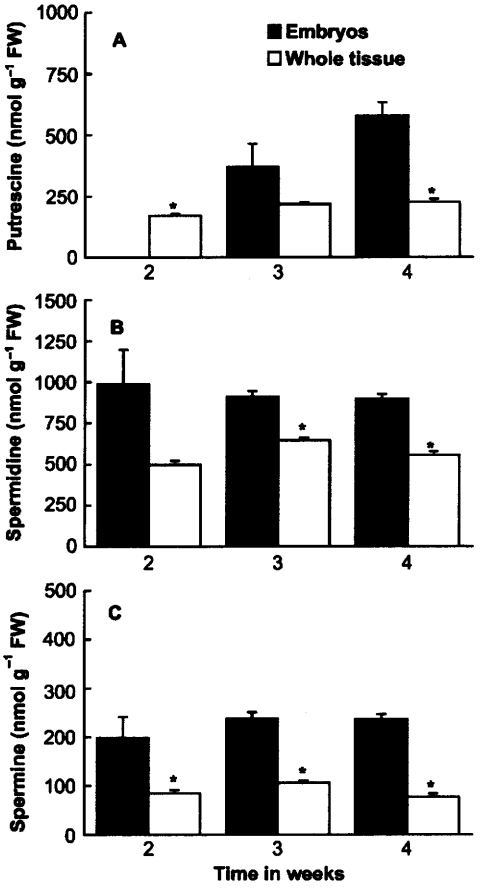

FIG. 6. Comparison of (*A*) putrescine, (*B*) spermidine, and (*C*) spermine in the isolated developing embryos versus entire tissue mass containing developing embryos and subtending tissue (embryogenic tissue plus the suspensor cell mass). Data are mean ± SE, $n = 5$.

action is known as yet. The present study clearly demonstrates that the changes in polyamines at different stages of development of the embryos are related to the developmental stages of the embryos *per se* and not to the length of time for which the tissue was grown

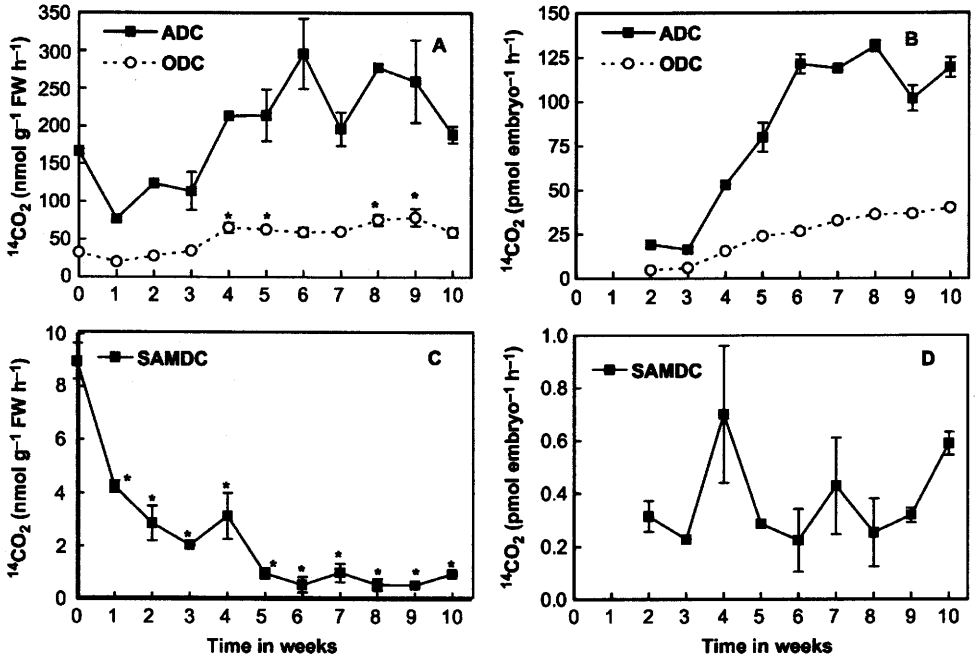

FIG. 7. Activities of ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) represented as nmol per g FW (*A*) and per embryo (*B*); and *S*-adenosylmethionine decarboxylase (SAMDC) represented as nmol per g FW (*C*) and per embryo (*D*). Data are mean ± SE, $n = 2$. For the weekly collections and analyses, the predominant developmental stage was collected.

in a particular medium. It is thus possible to answer the question of whether the observed changes in polyamines are related to the stage of development of the somatic embryos or whether they are related to the medium effect. The results presented here clearly indicate that the former is the case.

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REFERENCES

Amarasinghe, V.; Dhami, R.; Carlson, J. E. Polyamine biosynthesis during somatic embryogenesis in interior spruce (*Picea glauca* × *Picea engelmanii* complex). Plant Cell Rep. 15:495–499; 1996.

Ashihara, H.; Stasolla, C.; Loukanina, N.; Thorpe, T. A. Purine metabolism during white spruce somatic embryo development: salvage of adenine, adenosine and inosine. Plant Sci. 160:647–657; 2001.

Bagnoli, F.; Capuana, M.; Racchi, M. L. Developmental changes of catalase and superoxide dismutase isoenzymes in zygotic and somatic embryos of horse chestnut. Aust. J. Plant Physiol. 25:909–913; 1998.

Bastola, D. R.; Minocha, S. C. Increased putrescine biosynthesis through transfer of mouse ornithine cDNA in carrot promotes somatic embryogenesis. Plant Physiol. 109:63–71; 1995.

Cairney, J.; Xu, N.; Mackay, J.; Pullman, J. Transcript profiling: a tool to assess the development of conifer embryos. In Vitro Cell. Dev. Biol. Plant 36:155–162; 2000.

Cairney, J.; Xu, N.; Pullman, G. S.; Ciavatta, V. T.; Johns, B. Natural and somatic embryo development in loblolly pine: gene expression studies using differential display and DNA arrays. Appl. Biochem. Biotechnol. 77–79:5–17; 1999.

Chapman, A.; Blervacq, A. S.; Vasseur, J.; Hilbert, J. L. Arabinogalactan-proteins in *Cichorium* somatic embryogenesis: effect of beta-glucosyl Yariv reagent and epitope localisation during embryo development. Planta 211:305–314; 2000.

Chatthai, M.; Misra, S. Sequence and expression of embryogenesis-specific cDNAs encoding 2S seed storage proteins in *Pseudotsuga menziesii* [Mirb.] Franco. Planta 206:138–145; 1998.

Cohen, S. S. A guide to the polyamines. New York: Oxford University Press; 1998.

Cvikrova, M.; Binarova, P.; Cenklova, V.; Eder, J.; Machackova, I. Reinitiation of cell division and amino acid and aromatic monoamine levels in alfalfa explants during the induction of somatic embryogenesis. Physiol. Plant. 105:330–337; 1999.

Dong, J.-Z.; Dustan, D. I. Cloning and characterization of six embryogenesis-associated cDNAs from somatic embryos of *Picea glauca* and their comparative expression during zygotic embryogenesis. Plant Mol. Biol. 39:856–864; 1999.

Dong, J.-Z.; Dustan, D. I. Molecular biology of somatic embryogenesis in conifers. In: Jain, S. M.; Minocha, S. C., eds. Molecular biology of woody plants, vol. 1. Dordrecht: Kluwer Academic Publishers; 2000:51–87.

El Hadrami, I.; D'Auzac, J. Effects of polyamine biosynthetic inhibitors on somatic embryogenesis and cellular polyamines in *Hevea brasiliensis*. J. Plant Physiol. 140:33–36; 1992.

El Meskaoui, A. E.; Desjardins, Y.; Tremblay, F. M. Kinetics of ethylene biosynthesis and its effects during maturation of white spruce somatic embryos. Physiol. Plant. 109:333–342; 2000.

El Meskaoui, A. E.; Tremblay, F. M. Involvement of ethylene in the maturation of black spruce embryogenic cell lines with different maturation capacities. J. Exp. Bot. 52:761–769; 2001.

Feirer, R. P. The biochemistry of conifer embryo development: amino acids, polyamines and storage proteins. In: Jain, S. M.; Gupta, P. K.; Newton, R. J., eds. Somatic embryogenesis in woody plants, vol. 1. Dordrecht: Kluwer Academic Publishers; 1995:317–336.

Jain, S. M.; Gupta, P. K.; Newton, R. J., eds. Somatic embryogenesis in woody plants, vol. 1. Dordrecht: Kluwer Academic Publishers; 1995.

Joy, I. V. R. W.; McIntyre, D. D.; Vogel, H. J.; Thorpe, T. A. Stage-specific nitrogen metabolism in developing carrot somatic embryos. Physiol. Plant. 97:149–159; 1996.

Kevers, C.; Gaspar, T.; Dommes, J. The beneficial role of different auxins and amino acids at successive stages of somatic embryo formation and development of *Panax ginseng* in vitro. Plant Cell Tissue Organ Cult. 70:181–188; 2002.

Kevers, C.; Le Gal, N.; Monteiro, M.; Dammes, J.; Gaspar, T. Somatic embryogenesis of *Panax ginseng* in liquid cultures: A role for amino acids and their metabolic pathways. J. Plant Growth Regul. 31:209–214; 2000.

Kong, L.; Stephen, A. M.; Fowke, L. C. Effects of polyethylene glycol and methylglyoxal bis(guanylhydrazone) on endogenous polyamine levels and somatic embryo maturation in white spruce (*Picea glauca*). Plant Sci. 133:211–220; 1998.

Litvay, J. D.; Johnson, M. A.; Verma, D.; Einspahr, D.; Wayrauch, K. Conifer suspension culture medium development using analytical data from developing seeds. Inst. Paper Chem. Tech. Paper Ser. 115:1–17; 1981.

Martin-Tanguy, J. Conjugated polyamines and reproductive development: biochemical, molecular and physiological approaches. Physiol. Plant. 100:675–688; 1997.

Minocha, R.; Kvaalen, H.; Minocha, S. C.; Long, S. Polyamines in embryogenic cultures of Norway spruce (*Picea abies*) and red spruce (*Picea rubens*). Tree Physiol. 13:365–377; 1993.

Minocha, R.; Long, S.; Maki, H.; Minocha, S. C. Assays for the activities of polyamine biosynthetic enzymes using intact tissues. Plant Physiol. Biochem. 37:1–7; 1999a.

Minocha, R.; Minocha, S. C.; Simola, L. K. Somatic embryogenesis and polyamines in woody plants. In: Jain, S. M.; Gupta, P. K.; Newton, R. J., eds. Somatic embryogenesis in woody plants, vol. 1. Dordrecht: Kluwer Academic Publishers; 1995:337–359.

Minocha, R.; Shortle, W. C.; Coughlin, D. J.; Minocha, S. C. Effects of Al on growth, polyamine metabolism, and inorganic ions in suspension cultures of red spruce (*Picea rubens*). Can. J. For. Res. 26:550–559; 1996.

Minocha, R.; Shortle, W. C.; Long, S.; Minocha, S. C. A rapid and reliable procedure for extraction of cellular polyamines and inorganic ions from plant tissues. J. Plant Growth Reg. 13:187–193; 1994.

Minocha, R.; Smith, D.; Reeves, C.; Steele, K. D.; Minocha, S. C. Polyamine levels during the development of zygotic and somatic embryos of *Pinus radiata*. Physiol. Plant. 105:55–164; 1999b.

Minocha, S. C. Relationship between polyamine and ethylene biosynthesis in plants and its significance for morphogenesis in cell cultures. In: Zappia, V.; Pegg, A. E., eds. Progress in polyamine research. New York: Plenum Publishing Corp.; 1988:601–616.

Minocha, S. C.; Minocha, R. Role of polyamines in somatic embryogenesis. In: Bajaj, Y. P. S., ed. Somatic embryogenesis and synthetic seed I. Biotechnology in agriculture and forestry, vol. 30. Berlin: Springer-Verlag; 1995:53–70.

Minocha, S. C.; Minocha, R.; Robie, C. A. High-performance liquid chromatographic method for the determination of dansyl-polyamines. J. Chromatogr. 511:177–183; 1990.

Misra, S.; Attree, S. M.; Leal, I.; Fowke, L. C. Effect of abscisic acid, osmoticum, and desiccation on synthesis of storage proteins during the development of white spruce somatic embryos. Ann. Bot. 71:11–22; 1993.

Monteiro, M.; Kevers, C.; Dommes, J.; Gaspar, T. A specific role for spermidine in the initiation phase of somatic embryogenesis in *Panax ginseng* CA Meyer. Plant Cell Tiss. Organ Cult. 68:225–232; 2002.

Nato, A.; Fresneau, C.; Moursalimova, N.; De Buyser, J.; Lavergne, D.; Henry, Y. Expression of auxin and light-regulated arrestin-like proteins, G proteins and nucleoside diphosphate kinase during induction and development of wheat somatic embryos. Plant Physiol. Biochem. 38:483–490; 2000.

Patnaik, D.; Khurana, P. Germins and germin like proteins: an overview. Indian J. Exp. Biol. 39:191–200; 2001.

Puigderrajols, P.; Jofre, A.; Mir, G.; Pla, M.; Verdaguer, D.; Huguet, G.; Molinas, M. Developmentally and stress-induced small heat shock proteins in cork oak somatic embryos. J. Exp. Bot. 53:1445–1452; 2002.

Quan, Y.; Minocha, R.; Minocha, S. C. Genetic manipulation of polyamine metabolism in poplar II: effects on ethylene biosynthesis. Plant Physiol. Biochem. 40:929–937; 2002.

Robie, C. A.; Minocha, S. C. Polyamines and somatic embryogenesis. I. The effects of difluoromethylornithine and difluoromethylarginine. Plant Sci. 65:45–54; 1989.

Santanen, A.; Simola, L. K. Changes in polyamine metabolism during somatic embryogenesis in *Picea abies*. J. Plant Physiol. 140:475–480; 1992.

Santanen, A.; Simola, L. K. Catabolism of putrescine and spermidine in embryogenic and non-embryogenic callus lines of *Picea abies*. Physiol. Plant. 90:125–129; 1994.

Santanen, A.; Simola, L. K. Metabolism of L[U-^{14}C]-arginine and L[U-^{14}C]-ornithine in maturing and vernalised embryos and megagametophytes of *Picea abies*. Physiol. Plant. 107:433–440; 1999.

Shoeb, F.; Yadav, J. S.; Bajaj, S.; Rajam, M. V. Amino acids as biomarkers for plant regeneration capacity: improvement of regeneration by modulation of amino acid metabolism in different genotypes of indica rice. Plant Sci. 160:1229–1235; 2001.

Slocum, R. D.; Flores, H. E. Biochemistry and physiology of polyamines in plants. Boca Raton, FL: CRC Press; 1991.

Stasolla, C.; Yeung, E. C. Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. Plant Cell Tiss. Organ Cult. 74:15–35; 2003.

von Arnold, S.; Sabala, I.; Bozhkov, P.; Dyachok, J.; Filonova, L. Developmental pathways of somatic embryogenesis. Plant Cell Tiss. Organ Cult. 69:233–249; 2002.

Yadav, J. S.; Rajam, M. V. Temporal regulation of somatic embryogenesis by adjusting cellular polyamine content in eggplant. Plant Physiol. 116:617–625; 1998.

Yasuda, H.; Nakajima, M.; Ito, T.; Ohwada, T.; Masuda, H. Partial characterization of genes whose transcripts accumulate preferentially in cell clusters at the earliest stage of carrot somatic embryogenesis. Plant Mol. Biol. 45:705–712; 2001.