

Polyamines in embryogenic cultures of Norway spruce (*Picea abies*) and red spruce (*Picea rubens*)

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Summary

Embryogenic cultures of red spruce (*Picea rubens* Sarg.) and Norway spruce (*Picea abies* (L.) Karst.) were initiated from dissected mature zygotic embryos. The tissues were grown on either proliferation medium or maturation medium. On proliferation medium, the embryogenic tissue continued to produce early stage somatic embryos (organized meristems attached to elongated, suspensor-like cells), whereas on maturation medium fully mature embryos developed from the embryonic tissue. Analysis of polyamines in tissues grown on these two media showed that: (1) both putrescine and spermidine concentrations were always higher in cultures grown on proliferation medium than in cultures grown on maturation medium; (2) in both species, spermidine concentrations declined with time in the tissues grown on maturation medium; and (3) spermine was present in only minute quantities and showed only a small change with time. The presence of difluoromethylornithine in the culture medium had little effect on polyamine concentration, whereas the presence of difluoromethylarginine caused a decrease in putrescine concentrations in both red spruce and Norway spruce tissues grown on proliferation medium or maturation medium.

Keywords: *abscisic acid, difluoromethylarginine, difluoromethylornithine, maturation, putrescine, somatic embryogenesis, spermidine, spermine.*

Introduction

The differentiation of somatic embryos from tissue cultures has been reported for several economically important conifers (Bonga and Durzan 1987). The development of somatic embryos is very sensitive to changes in the constituents of the medium and the physical environment of culture (Bhojwani and Razdan 1983, Goldberg et al. 1989, Carman 1990). Unlike the embryogenic cultures of angiosperms (e.g., carrot) where it is difficult to recognize a mass of embryogenic cells, embryogenic tissues of conifers are characterized by the presence of long suspensor-like proliferative cell masses. These cell masses continue to produce early stage somatic embryos, and maturation and plantlet formation can only occur when the cell mass is exposed to a particular combination of chemical and physical conditions (Hakman and Fowke 1987a, 1987b, Nagmani et al. 1987, Hakman and von Arnold 1988, Boulay et al. 1988, Harry and Thorpe 1991).

Most of our knowledge of the biochemical and molecular aspects of somatic embryogenesis is based on studies with herbaceous angiosperms. Relatively little

information is available on the physiological and biochemical changes associated with the proliferation and maturation stages of somatic embryogenesis in gymnosperms. A few laboratories have attempted to characterize the metabolic status of conifer cell and tissue cultures undergoing somatic embryogenesis or adventitious shoot regeneration (Grey et al. 1987, Durzan 1987, Wann et al. 1987, Hakman and von Arnold 1988, Kumar and Thorpe 1989). These studies have focused on the metabolism of carbohydrates and nitrogen. Changes in the cytoplasmic proteins and mRNAs during somatic and zygotic embryogenesis in *Picea glauca* (Moench) Voss have also been investigated (Misra and Green 1990, 1991).

Several reports have implicated the aliphatic polyamines (spermidine and spermine) and their precursor, putrescine, in the process of somatic embryogenesis in tissue cultures of angiosperms (Minocha 1988, Galston and Flores 1991). Not much is known about polyamine metabolism in gymnosperms (Slocum and Flores 1991), although some recent studies point to a role of polyamines in stress responses and *in vitro* growth and development of *Pinus radiata* D. Don and *Picea abies* (L.) Karst. (Königshofer 1989, 1990, 1991, Tenter and Wild 1991, Daoudi et al. 1991, Kumar and Thorpe 1989, Santanen and Simola 1992).

The present study was undertaken to develop a procedure for producing fully developed somatic embryos of red spruce (*Picea rubens* Sarg.) and to characterize the polyamines in embryogenic cultures of Norway spruce (*Picea abies*) and red spruce grown under two different conditions that support either proliferation of embryogenic tissue or maturation of somatic embryos. Specific inhibitors were used to examine the putrescine biosynthetic pathway and to determine its role in the maturation of somatic embryos.

Materials and methods

Seeds of red spruce were collected from Moosehead Lake, Maine, USA, and stored at -20°C for up to one year. The seeds were soaked in running tap water for 2 days, surface sterilized with 2.5% sodium hypochlorite solution for 10 min and rinsed with sterile distilled water 3–4 times. Embryos were dissected out aseptically and transferred to growth medium.

Initiation and maintenance of embryogenic cultures

Embryogenic cultures of red spruce were initiated on modified LP medium (von Arnold and Eriksson 1981) supplemented with $9.05\text{ }\mu\text{M}$ 2,4-dichlorophenoxyacetic acid (2,4-D), $4.44\text{ }\mu\text{M}$ benzyladenine (BA) and 1% sucrose. The medium, designated the proliferation medium (PM), was solidified with 0.7% Bacto agar and autoclaved at 15 psi for 20 min. Each petri dish contained 25 ml of proliferation medium and 10–12 embryos. In the case of Norway spruce, two different embryogenic tissue lines, denoted Nos. 36 and 57, were used. These lines had been initiated in 1986 in the laboratory of Professor Sara von Arnold, Department of Forest Genetics, the Swedish University of Agricultural Sciences and maintained at the Agricultural University of Norway, Ås, Norway since 1990. For maintenance, embryogenic

tissues of both red spruce and Norway spruce were subcultured at 2-week intervals on proliferation medium. For maturation of somatic embryos, LP medium supplemented with 3% sucrose, 30 μM abscisic acid (ABA), and 1 μM indolebutyric acid (IBA) was used. This is called the maturation medium (MM). Tissue pieces (80–100 mg fresh weight per piece, seven to eight pieces per plate) were subcultured from proliferation medium to maturation medium; thereafter, they were transferred to fresh maturation medium at 2-week intervals until mature embryos developed. Concentrations of polyamine biosynthesis inhibitors used were based on studies with several other tissues.

All cultures were maintained at 25 ± 1 °C in the dark. For experiments on maturation medium, Day zero was the day on which the tissue was transferred from proliferation medium to maturation medium. Each experiment, except the exogenous putrescine feeding experiment (Table 1), was performed at least three times. In most cases, the results for the two Norway spruce tissue lines were similar; therefore, only the results for tissue line No. 57 are presented.

The inhibitors, DL- α -difluoromethylarginine (DFMA) and DL- α -difluoromethylornithine (DFMO), and putrescine were filter sterilized and added to partially cooled (60 °C), autoclaved media. Tissue samples were taken from the cultures 2, 5, 8, and 12 days after subculture. The tissue at this stage contained numerous embryos at early stages of maturation. These embryos were too small to be isolated in sufficient quantities for polyamine analysis.

Quantification of polyamines

Samples of tissue (150–170 mg fresh weight) were transferred to 5% perchloric acid (PCA, 200 mg fresh weight per ml of PCA) and stored at -20 °C for up to a month. Before dansylation, the samples were frozen and thawed three times and then centrifuged at 13,500 g for 15 min. One hundred μl of supernatant was dansylated

Table 1. The effect of exogenous putrescine (2.0 mM) on the cellular concentrations of putrescine and spermidine (nmol gFW^{-1}) in Norway spruce and red spruce embryogenic tissues grown on proliferation medium. This experiment was conducted twice. Each number is the mean of three replicates, an asterisk denotes a single observation.

Days	Norway spruce		Red spruce	
	Control	Putrescine	Control	Putrescine
<i>Putrescine</i>				
2	198 $\pm$ 19	3100*	400 $\pm$ 19	2133 $\pm$ 21
5	225 $\pm$ 7	2731 $\pm$ 76	363 $\pm$ 11	2880 $\pm$ 233
8	211 $\pm$ 14	2958 $\pm$ 60	334 $\pm$ 13	2197 $\pm$ 196
12	306 $\pm$ 18	3808 $\pm$ 46	451 $\pm$ 34	4122 $\pm$ 119
<i>Spermidine</i>				
2	55 $\pm$ 3	53*	72 $\pm$ 3	53 $\pm$ 3
5	62 $\pm$ 3	68 $\pm$ 14	95 $\pm$ 3	83 $\pm$ 10
8	61 $\pm$ 2	54 $\pm$ 2	103 $\pm$ 4	75 $\pm$ 1
12	92 $\pm$ 4	88 $\pm$ 4	109 $\pm$ 3	117 $\pm$ 9

according to the procedure described by Minocha et al. (1990). Dansylated polyamines were dissolved in 1 ml of methanol. Polyamines were separated by reversed phase HPLC in a gradient of acetonitrile/heptanesulfonate and quantified by fluorometry (Minocha et al. 1990). Heptanediamine was used as an internal standard for polyamine analysis. The elution times for putrescine, heptanediamine, spermidine, and spermine were 2.24, 2.71, 3.22 and 4.05 min, respectively (Figure 1). In addition to the three common polyamines, the HPLC method can also detect agmatine, cadaverine, acetylspermidine and acetylspermine, but none were detected in these tissues.

Results

Within 3–4 weeks of culture on proliferation medium, excised embryos of red spruce started to proliferate to form either a white, solid, nonembryogenic tissue or a white, translucent, embryogenic tissue, or both. The two types of tissues were either produced from different embryos or were mixed and originated from the same embryo. Because only the translucent type of tissue was embryogenic, it was cultured separately and used for further experiments.

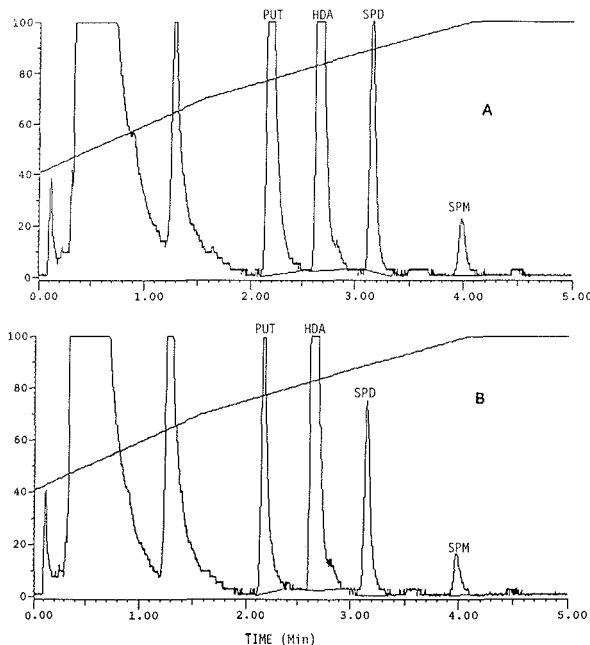


Figure 1. Representative chromatograms from HPLC separation of dansyl-polyamines in (A) Norway spruce and (B) red spruce callus grown on proliferation medium. PUT = putrescine, HDA = heptanediamine, SPD = spermidine, SPM = spermine. The gradient profile used for separation is described in Minocha et al. (1990).

Growth and somatic embryogenesis

Two different embryogenic tissue lines (Nos. 57 and 36) of Norway spruce, which had been initiated five years ago, were assayed for polyamines and their response to polyamine biosynthesis inhibitors. Tissue line No. 57 produced more embryos than line No. 36 when the two lines were cultured under identical conditions on proliferation medium. On transfer to maturation medium, 30–50 distinct embryos could be seen on each tissue clump (about 200 mg fresh weight), of which 15–20 turned green within 8–10 weeks. During the two-week subculture period on maturation medium, there was a relatively small increase in the fresh weight of the tissue compared to a two- to three-fold increase in fresh weight of tissue on the proliferation medium indicating that while the somatic embryos were undergoing maturation, tissue growth was retarded. Neither DFMO nor DFMA caused an apparent change in the growth of embryogenic tissues on proliferation medium or maturation medium during the 12-day test period. Studies are underway to quantify the long-term effects of DFMO and DFMA on the growth rate of these cultures. Our observations on somatic embryogenesis in Norway spruce (data not shown) confirm previously published findings by several authors (von Arnold and Eriksson 1985, Nagmani et al. 1987, Wann et al. 1987).

All experiments on somatic embryogenesis and polyamine analysis of red spruce were done with tissue that had been initiated less than 20 months ago. The physical appearance of the embryogenic tissue of red spruce was identical to that of Norway spruce tissue cultured on the same medium. The callus has maintained its characteristic appearance on proliferation medium through more than 2 years of subculture. Microscopically, numerous organized meristematic clusters attached to long suspensor-like cells were visible in this tissue (Figure 2A). Multiple embryos attached to the same group of loosely organized suspensor cells were commonly observed. Each tissue clump (150–200 mg fresh weight) produced 40–60 somatic embryos within 6–8 weeks of transfer to the maturation medium (Figure 2B). Usually most of these embryos turned green on maturity and germinated into seedlings on transfer to germination medium (LP medium without ABA) (Figure 2C).

Polyamines

Putrescine was the predominant polyamine in both species, its concentration was three- to fourfold higher than the concentration of spermidine. The concentration of spermine was low in tissues of both species at all times. On proliferation medium, the cellular concentration of putrescine in Norway spruce tissue lines Nos. 36 and 57 increased with time between 2 and 12 days of culture (Figure 3). In contrast, there was generally a decrease in the concentration of putrescine with time in tissue grown on the maturation medium. As a result, after 8 and 12 days of culture, putrescine concentrations were approximately four- to eightfold higher in tissue cultured on proliferation medium than in tissue cultured on maturation medium. In red spruce cultured on maturation medium, significant declines in putrescine concentrations were observed with time, whereas changes in putrescine concentrations were less pronounced in tissue cultured on proliferation medium (Figure 3). In some experi-

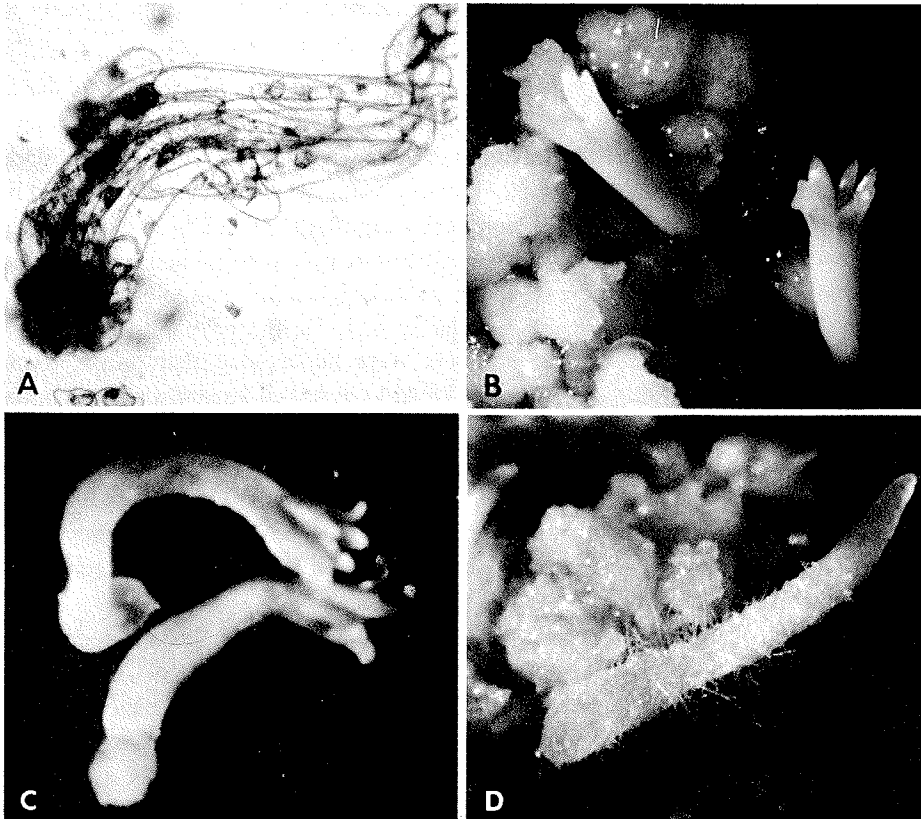


Figure 2. Stages of development of somatic embryos in red spruce. (A) A developing somatic embryo attached to suspensor-like cells grown on proliferation medium. (B) Somatic embryos after 8 weeks of growth on maturation medium. (C) Eight-week-old plantlets grown on germination medium. Embryos were transferred to the germination medium after 8 weeks of growth on maturation medium. (D) Root development on maturation medium supplemented with 2.0 mM DFMO.

ments, on Day 12, there was an increase in putrescine concentration in red spruce tissue grown on proliferation medium. On any given day, the putrescine concentration was always higher in tissue cultured on proliferation medium than in tissue cultured on maturation medium.

In Norway spruce, cellular concentrations of spermidine did not change appreciably with duration of culture in proliferation medium; however, on Day 8, a sharp decline in spermidine concentration was seen in tissue cultured on maturation medium (Figure 4). By Day 12, spermidine was almost absent from the Norway spruce tissue. Red spruce tissue showed an approximately 30% increase in spermidine concentration on Day 5 of culture in proliferation medium, whereas there was a substantial decrease in spermidine concentration with time in tissue cultured on maturation medium (Figure 4). In both species on any given day, spermidine concentrations were generally higher in tissues grown on proliferation medium than

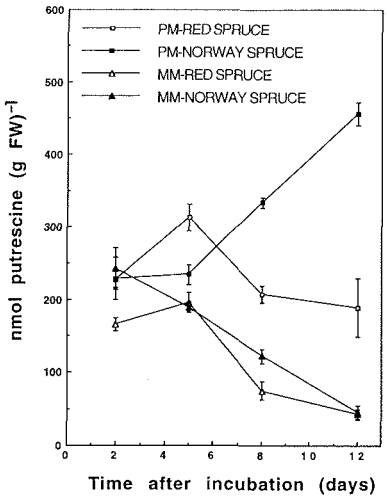


Figure 3. Changes in cellular concentrations of putrescine in Norway spruce and red spruce tissues grown on proliferation (PM) or maturation (MM) medium for different lengths of time. Values are mean $\pm$ SE of four replicates for Norway spruce and three replicates for red spruce.

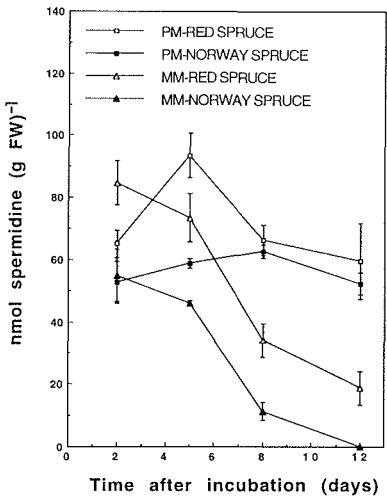


Figure 4. Changes in cellular concentrations of spermidine in Norway spruce and red spruce tissues grown on proliferation (PM) or maturation (MM) medium for different lengths of time. Values are mean $\pm$ SE of four replicates for Norway spruce and three replicates for red spruce.

in tissues grown on maturation medium.

Spermine concentrations, which were always very low independently of the culture medium, ranged between 0 to 18 nmol g_{FW}^{-1} (data not shown) and did not show a consistent trend with duration of culture.

Effects of inhibitors on cellular polyamines

Up to Day 8 with few exceptions, the inclusion of 2.0 mM DFMO in either medium had no significant effect on cellular putrescine concentrations of red spruce tissue or the two tissue lines of Norway spruce (Figure 5). By Day 12, however, there was a significant decrease in cellular putrescine concentrations in tissues of both species cultured on proliferation medium. In contrast, the presence of DFMA in the culture medium resulted in significant decreases in the cellular putrescine concentrations of both species at all times of analysis. In most cases, the presence of DFMO in the culture medium did not significantly alter cellular spermidine concentrations in either of the Norway spruce cell lines or in red spruce (Figure 6). Cellular spermidine concentrations were not significantly affected by the inclusion of DFMA in the culture medium, except for red spruce tissue grown on maturation medium where a decrease in spermidine concentration was observed. Exposure of red spruce and

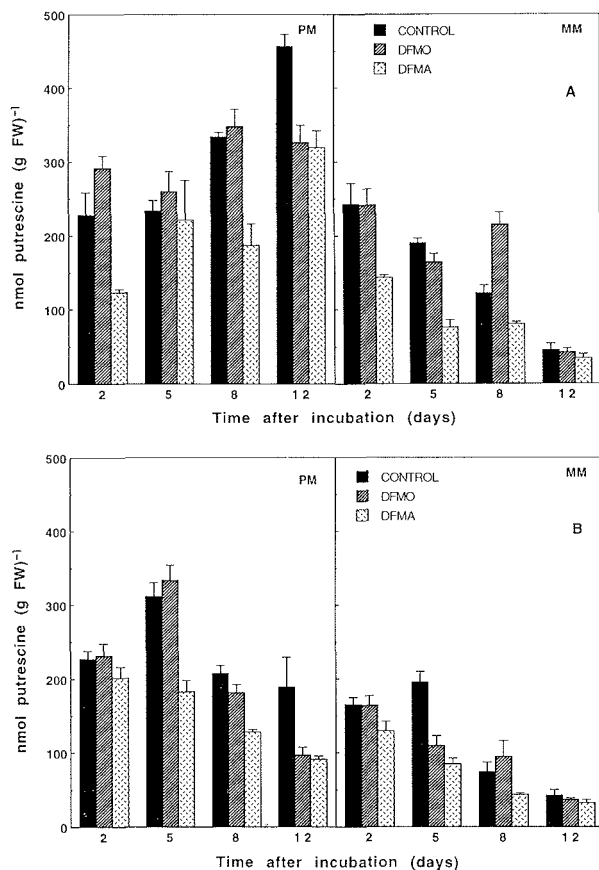


Figure 5. The effects of 2.0 mM DFMO and 0.1 mM DFMA on cellular concentrations of putrescine in (A) Norway spruce and (B) red spruce tissues grown on proliferation (PM) or maturation (MM) medium for different lengths of time. Values are mean $\pm$ SE of four replicates for Norway spruce and three replicates for red spruce.

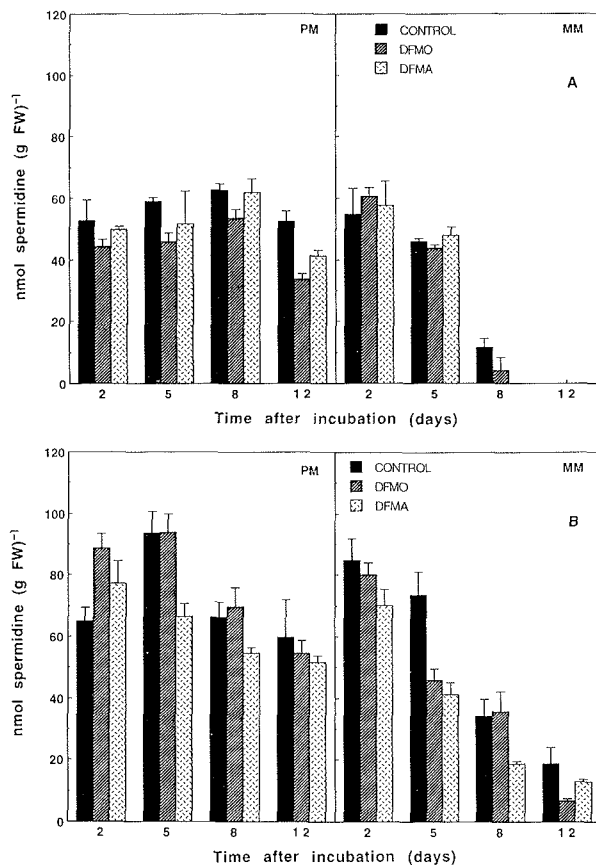


Figure 6. The effects of 2.0 mM DFMO and 0.1 mM DFMA on cellular concentrations of spermidine in (A) Norway spruce and (B) red spruce tissues grown on proliferation (PM) or maturation (MM) medium for different lengths of time. Values are mean $\pm$ SE of four replicates for Norway spruce and three replicates for red spruce.

Norway spruce tissues to either DFMO or DFMA had no effect on cellular spermine concentrations (data not shown).

When the tissues were grown on proliferation medium supplemented with 2.0 mM putrescine, cellular putrescine concentrations increased eight- to tenfold and remained high throughout the culture period. However, there was no change in the cellular concentrations of spermidine in Norway spruce tissue and there was a slight decrease in spermidine concentration in red spruce tissue (Table 1).

Effect of inhibitors on somatic embryogenesis

The addition of 2.0 mM DFMO or 0.1 mM DFMA had no visible effect on maturation of somatic embryos on maturation medium and did not cause appreciable changes in growth rates of tissues cultured on proliferation medium. In some cases, long roots with dense root hairs were seen on tissues cultured in the presence of

DFMO or DFMA (Figure 2D). Although no quantitative data were collected, the somatic embryos were somewhat smaller in the presence of DFMA than in the control and DFMO-treated tissues.

Discussion

The development and morphology of embryogenic tissue, the stages of maturation of somatic embryos, and the nutritional requirements for somatic embryogenesis were similar for Norway spruce and red spruce. There was also much similarity in the biochemical changes during somatic embryogenesis in Norway spruce and red spruce cultures.

In contrast to animals, which utilize ornithine decarboxylase (ODC) as the sole enzyme for putrescine biosynthesis, plant cells have an additional pathway that is catalyzed by arginine decarboxylase (ADC) (Pegg 1986, Smith 1990, Slocum 1991). Either one or both of these pathways may be active in different plant tissues. Our preliminary results indicate that ADC is the predominant pathway for putrescine biosynthesis in embryogenic tissue of both Norway spruce and red spruce, because the activity of ODC is barely detectable (data not presented). This is in line with the observed inhibitory effects of DFMA on cellular putrescine concentrations in both the proliferation and the maturation media.

In many plant tissues, spermidine is the predominant polyamine and is generally followed in quantity by putrescine and spermine (Kumar and Thorpe 1989, Robie and Minocha 1989). In addition, significant quantities of agmatine, cadaverine, or polyamine conjugates may be present in some tissues (Slocum 1991). In contrast, putrescine is the major polyamine in several different tissues of Norway spruce trees (Königshofer 1990, 1991, Tenter and Wild 1991, Santanen and Simola 1992). We found that, in both Norway spruce and red spruce tissues, putrescine concentrations were consistently higher than spermidine concentrations, and the concentration of spermine was always very low.

Our results confirm several other studies indicating that exogenously supplied putrescine is not converted to spermidine in most plant tissues (Rudulier and Goas 1977, Kumar and Thorpe 1989). Kumar and Thorpe (1989) showed that, in excised cotyledons of *Pinus radiata*, less than 10% of the ^{14}C -putrescine appeared as spermidine. Tobacco and carrot cell cultures also show either no effect or a slight decrease in cellular concentrations of spermidine in response to exogenously supplied putrescine (S.C. Minocha, unpublished observations).

The role of polyamines in morphogenesis and somatic embryogenesis in cell cultures of herbaceous angiosperms has been extensively studied (Minocha 1988, Galston and Flores 1991), but little is known about the changes in polyamine concentrations during the development of either zygotic or somatic embryos in woody plant species. Generally, cellular polyamine concentrations increase in differentiating cultures of herbaceous angiosperms before the appearance of somatic embryos (Montague et al. 1978, 1979, Feirer et al. 1984, Fienberg et al. 1984, Robie and Minocha 1989, Galston and Flores 1991). Inhibition of polyamine biosynthesis

results in an inhibition of somatic embryogenesis or organogenesis, or both (Feirer et al. 1984, Robie and Minocha 1989). In contrast to the work on herbaceous angiosperms, our results on gymnosperms indicate a reverse trend, i.e., we observed a decrease rather than an increase in the cellular concentrations of putrescine and spermidine during the development of somatic embryos of both Norway spruce and red spruce grown on maturation medium. However, the proembryogenic tissues of both species grown on proliferation medium had high concentrations of cellular putrescine and spermidine. Santanen and Simola (1992) also observed higher concentrations of polyamines in tissues of Norway spruce grown on proembryogenic culture medium than in tissues cultured on medium containing ABA, and they observed a decline in cellular putrescine concentrations during the maturation of somatic embryos. However, they report an initial increase in spermidine concentration for the first 5 or 10 days followed by a decline both in free and conjugated spermidine (Santanen and Simola 1992). It should be pointed out that somatic embryogenesis in most conifers is quite different from morphogenesis in angiosperms. In most angiosperms, somatic embryogenesis and organogenesis involve a rapid growth phase for several days during which time embryos and plantlets are formed that continue to grow without a period of apparent dormancy (maturation). In most conifers, on the other hand, tissue on the proliferation medium is fast growing and continues to produce early stage embryos, whereas maturation of these somatic embryos parallels the development of zygotic embryos. Also, in contrast to media used for somatic embryogenesis and organogenesis of angiosperms, the maturation medium used for conifers contains ABA, which is known to promote maturation of somatic as well as zygotic embryos (Hakman and Fowke 1987a, 1987b, Boulay et al. 1988, Hakman and von Arnold 1988, Misra and Green 1990, 1991, Santanen and Simola 1992). The mature embryos must be transferred to a fresh medium lacking ABA before they can develop into plantlets. This contrasting pattern of somatic embryogenesis in herbaceous angiosperms and conifers may explain why the cellular polyamine concentration is lower in the maturing somatic embryos of conifers than in tissue growing on proliferation medium. Alternatively, the decline in polyamine concentrations in tissue cultured on maturation medium could be a direct response to ABA. We are currently comparing polyamine concentrations in the developing somatic embryos with those of the non-differentiated tissue cultured on maturation medium.

Acknowledgments

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References

- Bhojwani, S.S. and M.K. Razdan. 1983. Plant tissue culture: theory and practice. Elsevier, New York, pp 91–112 and 199–236.
- Bonga, J.M. and D.J. Durzan. 1987. Cell and tissue culture in forestry. Vol. 3. Martinus Nijhoff, Publ., Dordrecht, pp 1–462.
- Boulay, M.P., P.K. Gupta, P. Krogstrup and D.J. Durzan. 1988. Development of somatic embryos from cell suspension cultures of Norway spruce (*Picea abies* Karst.). Plant Cell Rep. 7:134–137.
- Carman, J.G. 1990. Embryogenic cells in plant tissue cultures: occurrence and behavior. *In Vitro Cell Dev. Biol.* 26:746–753.
- Daoudi, E.H., M. Bonnet-Masimbert, and J. Martin-Tanguy. 1991. Evolution des polyamines dans les bourgeons et les rameaux de *Pseudotsuga menziesii* (Mirb.) Franco à l'état floral. *Ann. Sci. For.* 48:367–376.
- Durzan, D.J. 1987. Physiological states and metabolic phenotypes in embryonic development. *In Cell and Tissue Culture in Forestry*, Vol. 2. Eds. J.M. Bonga and D.J. Durzan. Martinus Nijhoff Publ., Dordrecht, pp 405–439.
- Feirer, R.P., G. Mignon and J.D. Litvay. 1984. Arginine decarboxylase and polyamines required for embryogenesis in the wild carrot. *Science* 223:1433–1435.
- Fienberg, A.A., J.H. Choi, W.P. Lubich and Z.R. Sung. 1984. Developmental regulation of polyamine metabolism in growth and differentiation of carrot culture. *Planta* 162:532–539.
- Galston, A.W. and H.E. Flores. 1991. Polyamines and plant morphogenesis. *In Biochemistry and Physiology of Polyamines in Plants*. Eds. R.D. Slocum and H.E. Flores. CRC Press, Boca Raton, FL, pp 175–186.
- Goldberg, R.B., S.J. Barker and L. Perez-Grau. 1989. Regulation of gene expression during plant embryogenesis. *Cell* 56:149–160.
- Grey, D., G. Stepan-Sarkissian and M.W. Fowler. 1987. *In Cell and Tissue Culture in Forestry*, Vol. 2. Eds. J.M. Bonga and D.J. Durzan. Martinus Nijhoff Publ. Dordrecht, pp 31–60.
- Hakman, I. and L.C. Fowke. 1987a. An embryogenic cell suspension culture of *Picea glauca* (white spruce). *Plant Cell Rep.* 6:20–22.
- Hakman, I. and L.C. Fowke. 1987b. Somatic embryogenesis in *Picea glauca* (white spruce) and *Picea mariana* (black spruce). *Can. J. Bot.* 65:656–659.
- Hakman, I. and S. von Arnold. 1988. Somatic embryogenesis and plant regeneration from suspension cultures of *Picea glauca* (white spruce). *Physiol. Plant.* 72:579–587.
- Harry, I.S. and T.A. Thorpe. 1991. Somatic embryogenesis and plant regeneration from mature zygotic embryos of red spruce. *Bot. Gaz.* 152:446–452.
- Königshofer, H. 1989. Seasonal changes in polyamine content in different parts of juvenile spruce trees (*Picea abies* (L.) Karst.). *J. Plant Physiol.* 134:736–740.
- Königshofer, H. 1990. Polyamine content in needles, shoot-axes, buds and xylem exudates of mature spruce trees (*Picea abies* (L.) Karst.). *J. Plant Physiol.* 136:377–380.
- Königshofer, H. 1991. Distribution and seasonal variation of polyamines in shoot-axes of spruce (*Picea abies* (L.) Karst.). *J. Plant Physiol.* 137:607–612.
- Kumar, P.P. and T.A. Thorpe. 1989. Putrescine metabolism in excised cotyledons of *Pinus radiata* cultured *in vitro*. *Physiol. Plant.* 76:521–526.
- Minocha, S.C. 1988. Relationship between polyamine and ethylene biosynthesis in plants and its significance for morphogenesis in cell cultures. *In Progress in Polyamine Research*. Eds. V. Zappia and A.E. Pegg. Plenum Publishing Corp., New York, pp 601–616.
- Minocha, S.C., R. Minocha and C.A. Robie. 1990. High-performance liquid chromatographic method for the determination of dansyl-polyamines. *J. Chromatogr.* 511:177–183.
- Misra, S. and M.J. Green. 1990. Developmental gene expression in conifer embryogenesis and germination. I. Seed proteins and protein body composition of mature embryo and the megagametophyte of white spruce (*Picea glauca* (Moench) Voss.). *Plant Sci.* 68:163–173.
- Misra, S. and M.J. Green. 1991. Developmental gene expressions in conifer embryogenesis and germination II. Storage protein synthesis in developing embryo and the gametophyte in white spruce (*Picea glauca*). *Plant Sci.* 78:61–71.

- Montague, M.J., T.A. Armstrong and E.G. Jaworski. 1979. Polyamine metabolism in embryogenic cells of *Daucus carota* L. I. Changes in intracellular content and rates of synthesis. *Plant Physiol.* 63:341–345.
- Montague, M.J., J.W. Koppenbrink and E.G. Jaworski. 1978. Polyamine metabolism in embryogenic cells of *Daucus carota*. II. Changes in arginine decarboxylase activity. *Plant Physiol.* 62:430–433.
- Nagmani, R., M.R. Becwar and S.R. Wann. 1987. Single-cell origin and development of somatic embryos in *Picea abies* (L.) Karst. (Norway spruce) and *P. glauca* (Moench) Voss (white spruce). *Plant Cell Rep.* 6:157–159.
- Pegg, A.E. 1986. Recent advances in the biochemistry of polyamines in eukaryotes. *Biochem. J.* 234:249–262.
- Robie, C.A. and S.C. Minocha. 1989. Polyamines and somatic embryogenesis. I. The effects of difluoromethylornithine and difluoromethylarginine. *Plant Sci.* 65:45–54.
- Rudulier, D.L. and G. Goas. 1977. Devenir de la putrescine-1,4-¹⁴C chez *Glycine max*. *Physiol. Plant.* 40:87–90.
- Santanen, A. and L. K. Simola. 1992. Changes in polyamine metabolism during somatic embryogenesis in *Picea abies*. *J. Plant Physiol.* 140:475–480.
- Slocum, R.D. 1991. Polyamine biosynthesis in plants. In *Biochemistry and Physiology of Polyamines in Plants*. Eds. R.D. Slocum and H.E. Flores. CRC Press, Boca Raton, FL, pp 23–40.
- Slocum, R.D. and H.E. Flores. 1991. Biochemistry and physiology of polyamines in plants. CRC Press, Boca Raton, FL, pp 1–264.
- Smith, T.A. 1990. Plant polyamines—metabolism and function. In *Polyamine and Ethylene: Biochemistry, Physiology and Interaction*. Eds. H.E. Flores, R.N. Arteca and J.C. Shannon. Amer. Soc. Plant Physiol., Bethesda, MD, pp 1–17.
- Tenter, M. and A. Wild. 1991. Investigations on the polyamine content of spruce needles relative to the occurrence of novel forest decline. *J. Plant Physiol.* 137:647–654.
- von Arnold, S. and T. Eriksson. 1981. *In vitro* studies of adventitious shoot formation in *Pinus contorta*. *Can. J. Bot.* 59:870–874.
- von Arnold, S. and T. Eriksson. 1985. Initial stages in the course of adventitious bud formation on embryos of *Picea abies*. *Physiol. Plant.* 64:41–47.
- Wann, S.R., M.A. Johnson, T.L. Noland and J.A. Carlson. 1987. Biochemical differences between embryogenic and nonembryogenic callus of *Picea abies* (L.) Karst. *Plant Cell Rep.* 6:39–42.