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Full Length Research Paper

Effects of exogenous polyamines and inhibitors of polyamine biosynthesis on endogenous free polyamine contents and the maturation of white spruce somatic embryos

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The maturation of somatic embryos is a pivotal stage of somatic embryogenesis process. Thus, this study is aimed at investigating the role of polyamines on the maturation of somatic embryos of white spruce (*Picea glauca*). Two inhibitors of the biosynthesis of polyamines methylglyoxal bis-(guanylhydrazone) (*MGBG*) and dicyclohexylamine (*DCHA*) or three exogenous polyamines (putrescine, spermidine and spermine) were added into a modified HLM-1 maturation medium inoculated with embryogenic tissues. Medium responses were assessed with respect to the production of mature somatic embryos, the cellular concentration of free polyamines and the concentration of 1-aminocyclopropane-1-carboxylic acid (*ACC*) and N-malonylaminocyclopropane-1-carboxylic acid (*mACC*), the precursor of ethylene biosynthesis and its conjugate, respectively. Results show that *DCHA* brought about a significant decrease in the concentration of free cellular putrescine during the first two weeks of maturation, whereas *MGBG* induced a significant decrease of both spermidine and spermine. Moreover, both inhibitors (1 mM *DCHA* or 1 mM *MGBG*) reduced the total number (63 and 52%, respectively) and the number of normal (62 and 54%, respectively) somatic embryos. Addition of 5 mM spermidine or 1 mM spermine increased the total number (94 and 113%, respectively) and the number of normal (134 and 143%, respectively) somatic embryos. Finally, the intracellular concentrations of *ACC* and *mACC* significantly increased in the presence of exogenous spermidine while decreasing in the presence of *MGBG*, suggesting that spermidine interacts with ethylene metabolism. These results clearly demonstrate that polyamines play an important quantitative and qualitative role during the maturation of white spruce somatic embryos.

Key words: Conifer, ethylene, maturation stage, polyamines, somatic embryogenesis.

INTRODUCTION

Polyamines (PAs), a group of aliphatic amines, are ubiquitous and play a critical role in various growth and developmental processes in plants as well as in other living organisms such as animals and bacteria. In plants, the major polyamines are putrescine, spermidine, and spermine. The polyamines biosynthetic pathway is relatively well established, and inhibitors are available for several biosynthetic enzymes (Figure 1). Putrescine is formed either by the direct decarboxylation of L-ornithine (L-Orn) through ornithine decarboxylase (ODC; EC 4.1.1.17) or by decarboxylation from L-arginine (L-Arg) to agmatine by arginine decarboxylase (ADC; EC 4.1.1.19) and thereafter to putrescine. Conversion of putrescine to spermidine and spermine requires the addition of one or

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Abbreviations: **ABA**, abscisic acid; **ACC**, 1-aminocyclopropane-1-carboxylic acid; **AOA**, a-aminooxyamino acid; ***DCHA***, dicyclohexylamine; **HLM**, half Litvay's medium; **L-Orn**, L-ornithine; ***mACC***, N malonylaminocyclopropane-1-carboxylic acid; **MGBG**, methylglyoxal bis-(guanylhydrazone); **SAM**, S-adenosylmethionine; **SAMdec**, SAM decarboxylase.

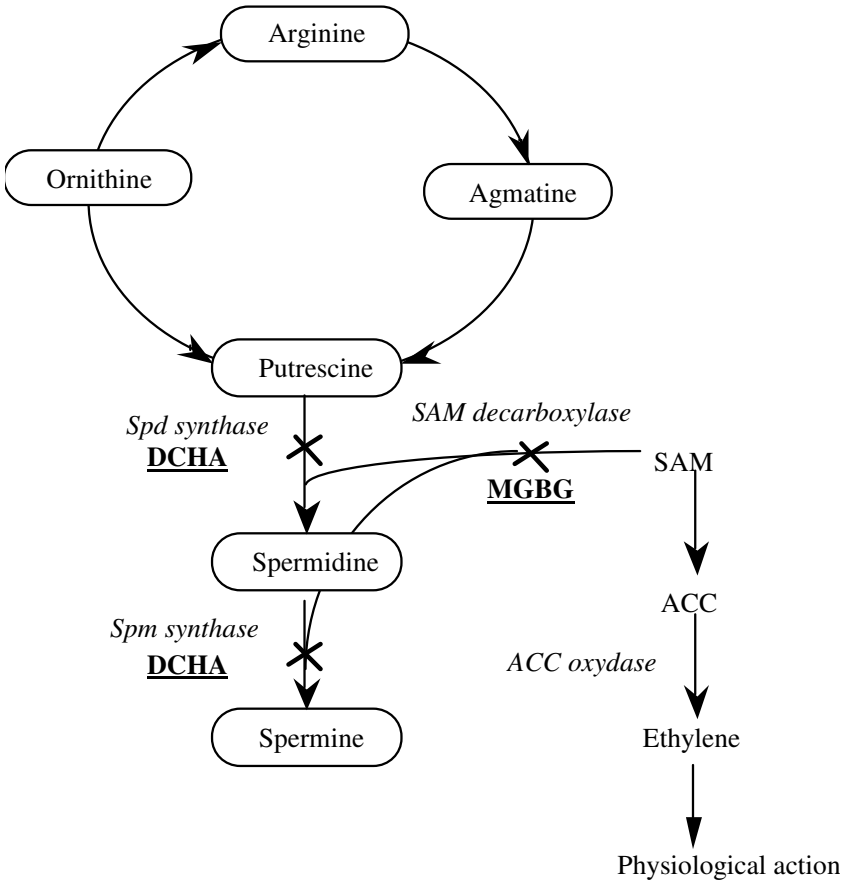

Figure 1. The proposed pathways of ethylene and polyamine synthesis and their key enzymes (Smith, 1985).

two aminopropyl groups from decarboxylated S-adenosylmethionine (dSAM) by spermidine synthase (EC 2.5.1.16) and spermine synthase (EC 2.5.1.22) respectively. The aminopropyl moiety results from the decarboxylation of S-adenosylmethionine (SAM) by SAM decarboxylase (SAMdec; EC 4.1.1.50).

Numerous reports have shown a good correlation between polyamine levels and a variety of fundamental processes such as macromolecular biosynthesis, cell division, cell and tissue differentiation, organogenesis, and embryogenesis (Smith, 1985; Galston and Kaur-Sawhney, 1995; Minocha and Minocha, 1995). Some investigators suggested that PAs are either essential plant growth regulators (PGRs) or secondary messengers of PGRs (Smith, 1985; Galston and Kaur-Sawhney, 1995). Extensive studies of polyamines in suspension cultures of *Daucus carota* and other herbaceous species showed that they might play a critical role in regulating somatic embryogenesis (SE) (Minocha and Minocha, 1995; Galston and Kaur-Sawhney, 1995). In conifers, analysis and quantification of PAs in *Picea abies* (Santanen and Simola, 1992, 1994; Minocha et al., 1993), *Picea glauca* x *Picea engelmannii* complex (Amarasinghe et al., 1996), *Picea rubens (*Minocha et al., 1993), *P. glauca* (Kong et al. 1998), *Pinus sylvestris* (Sarjala et al. 1997), and *Pinus radiata* (Minocha et al., 1999) has suggested their association with processes such as differentiation and development of somatic embryos.

To characterize spruce somatic embryogenesis, effects of *in vitro* gaseous environment and ethylene were investigated during maturation of white spruce and black spruce somatic embryos by El Meskaoui et al. (2000, 2006) and El Meskaoui and Tremblay (1999, 2001). They suggested that the effect of ethylene on maturation of somatic embryos stage could be related to PA metabolism. It is well documented that ethylene and polyamine biosynthetic pathways are linked through SAM. However, although PAs and ethylene are biosynthetically related, their physiological functions are distinct and somewhat antagonistic. This raised the question about the role of polyamines on the maturation of white spruce somatic embryos. To obtain more information regarding a possible competitive interaction

between ethylene and polyamine biosynthetic pathways during the maturation of somatic embryos, levels of endogenous *ACC* and its conjugate N-malonylaminocyclopropane-1-carboxylic acid (*mACC*) were also determined in both *MGBG* and spermidine-treated tissues.

MATERIALS AND METHODS

Plant materials

Embryogenic tissues were induced from mature zygotic embryos of *P. glauca* (Moench Voss.) according to Tremblay (1990). The embryogenic line (G-316) had been maintained on the HLM-1 medium (Tremblay, 1990) through a 14-days subculture cycle. Somatic embryos were allowed to mature according to El Meskaoui et al. (2000).

Maturation experiments

Time course of cellular putrescine (*Put*), spermidine (*Spd*) and spermine (*Spm*) during maturation of white spruce somatic embryos

To study the time course of cellular *Put*, *Spd* and *Spm* during maturation of white spruce somatic embryos, an experiment was conducted using a completely randomized design with 4 repetitions. Intracellular levels of free polyamines were determined at sampling dates (0, 7, 14, 21, 28, and 35 days), each Petri dish constituted an experimental unit in which 5 embryogenic tissue portions (80 ± 5 mg each) were inoculated. Embryogenic tissues were weighted, frozen at -80˚C for 48 h and then being frozen-dried for 48 h at -50˚C, 50 nbar. Dry embryogenic tissues were stored in the dark at -20˚C until analysis by high performance liquid chromatography (HPLC).

Effects of *MGBG*, *DCHA*, *Put*, *Spd* and *Spm* on somatic embryo maturation

The effects of *MGBG*, *DCHA*, *Put*, *Spd* and *Spm* (Sigma, St Louis, Mo, USA) on the maturation of white spruce somatic embryos were evaluated through five independent experiments (1 experiment /compound). The polyamines biosynthetic inhibitors *MGBG* and *DCHA* were individually tested respectively at 0.0, 0.1, 1.0, and 2.0 and 0.0, 0.01, 0.1 and 1.0 mM. While exogenous polyamines *Put, Spd,* and *Spm* were individually tested at 0.0, 0.5, 1.0, 2.0, 5.0 and 10 mM. All these compounds were filter-sterilized before addition to the sterilized maturation medium. Each individual 5 replicates-experiment was completely randomized. Each Petri dish constituted an experimental unit in which 5 embryogenic tissue portions (80 ± 5 mg each) were inoculated. After 5 weeks in maturation, the numbers of normal (embryos with 2 to 8 cotyledons distributed symmetrically around the meristem) and abnormal (fasciated, monocotyledonous or swollen embryos, and embyos with fused cotyledons) embryos was determined.

Effects of *MGBG*, *DCHA*, *Put*, *Spd* and *Spm* on cellular levels of free PAs.

An independent experiment was conducted to assess the effect of exogenous polyamines and polyamine biosynthetic inhibitors on the intracellular levels of free polyamines of embryogenic tissues during the maturation period. Here, *Put* and *Spd* were used at 5 mM, *Spm* at 1 mM, *MGBG* at 1 mM, and *DCHA* at 0.1 mM. This experiment was conducted as a completely randomized design with 4 repetitions. Each Petri dish was inoculated with five embryogenic tissue portions (80 ± 5 mg each). Samplings were carried out 0, 7, 14, 21, 28, and 35 days after the onset of the maturation process. Embryogenic tissues were weighted, placed in a freezer at -80˚C for 48 h and then frozen-dried for 48 h at -50˚C, 50 nbar. Dry embryogenic tissues were stored in the dark at -20˚C until HPLC analysis to assay cellular levels of free PAs.

Effects of *Spd* and *MGBG* on cellular levels of *ACC* and of its conjugate *mACC*

To determine relationship between ethylene and polyamine metabolism, the effects of *Spd* (5 mM) and *MGBG* (1 mM) on *ACC* and *mACC* contents were studied. The five replicate-experiments were completely randomized. Each Petri dish was inoculated with six embryogenic tissue portions (80 ± 5 mg each). Samplings were carried out 0, 5, 10, 15, 20, 25 and 30 days after the onset of the maturation process. *ACC* and *mACC* were extracted with 85% ethanol and quantified as previously described by El Meskaoui et al. (2000).

Extraction and benzoylation of polyamines

Polyamines analysis was carried out according to the procedures described by Flores and Galston (1982). For each treatment and each replicate, fifty mg (dry weight) of embryogenic tissue were homogenized in 4 ml of 5% (v/v) perchloric acid. After extraction for 30 min in an ice bath, samples were centrifuged at 30 000 x g for 20 min at 4 ˚C. Before benzoylation, an artificial amine (heptane-diamine, Sigma) was added to the supernatant as internal standard. Aliquots of each tissue extract were mixed with 1 ml NaOH (2N) and 10 µl benzoyl chloride (Sigma). The mixtures were vortexed for 30 s and incubated for 20 min at room temperature. Benzoylation was stopped by adding 2 ml of saturated NaCl, and polyamines were extracted in 2 ml diethylether. The mixtures were vortexed and centrifuged for 2 min at 2 000 x g to separate two phases. One ml of the upper clear diethylether layer, containing the free polyamines, was removed and placed in a microfuge tube. The samples were evaporated to dryness under vacuum and reconstituted in 100 µl methanols (Mat, Qc, Canada; HPLC grade). Standard curves of *Put, Spd, Spm* and heptanediamine were determined after benzoylation and extraction through same process described above.

HPLC analysis of PAs

Thirty µl of benzoylated samples or polyamine standards were injected into a HPLC system (712 WISP, Waters, Milford, MA, USA) equipped with a Waters 994 UV detector set. The samples were injected onto a reverse phase C_{18} column (4.6 x 250 mm, 5 µm particle diameter) and eluted from the column at room temperature (24˚C). The solvent system run isocratically at 64% methanol, at a flow rate of 1 ml/min. Benzoylated polyamines were detected at 254 nm. Polyamine levels were calculated as the average of the 4 independent replicates.

Germination of somatic embryos

After 35 days in the maturation stage, 30 normal and mature somatic embryos with visible cotyledons produced from *Spd* and *Spm*-treated tissues as well as from controls were germinated in Petri dishes as described by El Meskaoui et al. (2000). The frequencies of embryos developing an epicotyl (calculated as the number of embryos with epicotyl/total number of embryos trans-

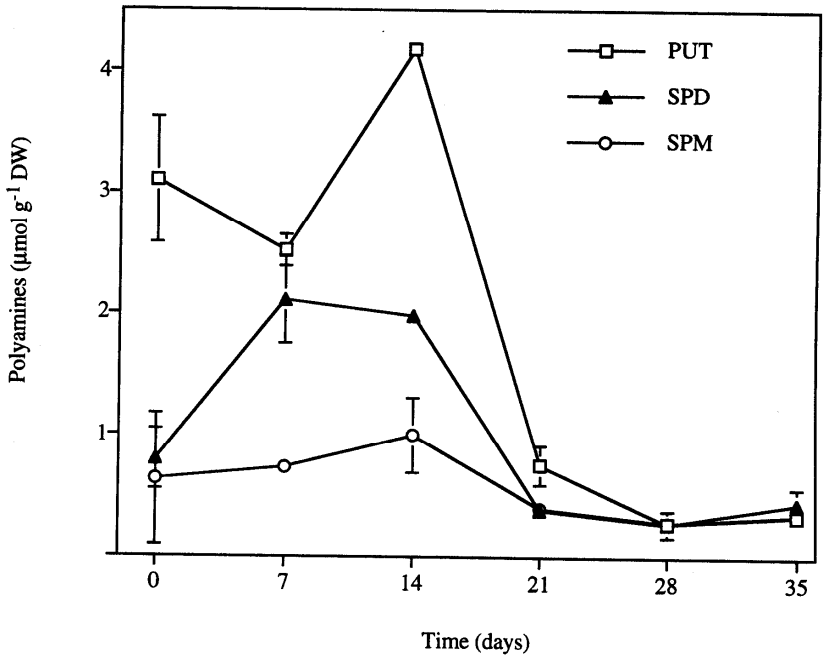

Figure 2. Changes in putrescine, spermidine and spermine concentrations per g DW in the embryogenic tissue of white spruce on maturation medium. The bars represent SE, n=4.

ferred to germination medium) were determined after 9 weeks in germination.

Environmental conditions

Maintenance, maturation and germination followed protocols described by El Meskaoui et al. (2000).

Statistical analysis

Data from the maturation experiments were analyzed using the SAS GLM procedure (SAS Institute Inc., Cary, NC, USA). For each experiment, the homogeneity of variance was verified by Bartlett's test and the means were compared using Bonferroni's multiple range tests (Sokal and Rohlf, 1995) at $P \leq 0.05$. Germination data were analyzed and means were separated by an adjusted G-test (Sokal and Rohlf, 1995) at $P \leq 0.05$.

RESULTS

Temporal changes of free cellular polyamines in embryogenic tissue during maturation

Analysis of putrescine, spermidine and spermine intracellular contents throughout the maturation period revealed putrescine as the predominant polyamine, followed by spermidine and spermine (Figure 2). The highest endogenous content of putrescine was recorded 14 d after the beginning of the maturation process (4.2 µmol g^{-1} dry weight), followed by a 10 fold at 21 d. Endogenous spermidine concentration showed a 2.5-fold increase at day 7 (2.1 µmol g^{-1} dry weight) followed by a consistent decrease until the end of the maturation period. Spermine was the least abundant polyamine; its highest level (0.9 µmol g^{-1} dry weight) was recorded at day 14.

Effects of addition of polyamine biosynthetic inhibitors and of exogenous polyamines on the maturation of white spruce (line G-316) somatic embryos

Addition in the culture medium of 1 and 2 mM *MGBG*, an inhibitor of the biosynthesis of polyamine, respectively decreased the total and normal numbers of embryos by 52; 46% and 78; 85%. Whereas 1 mM *DCHA* brought about a 63 and 62% decrease in the total and normal number of somatic embryos, respectively (Table 1).

Addition in the culture medium of relatively high amount of exogenous spermidine or spermine significantly affected the maturation process. The total and normal numbers of somatic embryos increased by 94 and 134% respectively, when 5 mM of spermidine was added into the culture medium. Similar increments, 113 and 143% respectively, were observed when 1 mM of spermine was added into the culture medium. Beyond this concentration of spermine, the maturation process of somatic embryos was inhibited. Inhibition was complete at the spermine concentration of 5 mM (Table 2).

Table 1. Effect of different concentrations of *MGBG* and *DCHA* on the maturation of white spruce (line G-316) somatic embryos.

Chemical treatment	(mM)	Total number of somatic embryos[1]	Number of normal somatic embryos[1]	Number of abnormal somatic embryos[1]
MGBG	0	45.4^{a}	27.2^{a}	18.2^{a}
	0.1	38.4^{a}	21.4^{a}	17^{ab}
	1	22^{b}	12.6^{b}	9.4^{bc}
	2	10.2^{c}	4.2^{c}	6^{c}
DCHA	0	46^{a}	26.8^{a}	19.2^{a}
	0.01	42.8^{ab}	24.8^{a}	18^{a}
	0.1	29^{bc}	16.6^{ab}	12.4^{ab}
	1	17.2^{c}	10.2^{b}	7^{b}

[1]Within each treatment, data in columns displaying the same letters are not different at a level $p \leq 0.05$ according to Bonferroni's test. Each datum is the mean of 5 replicates. Each replicate was initiated with six pieces of 80 mg each of embryogenic tissue. Statistical analyses were applied separately on each experiment corresponding to each chemical compound.

Table 2. Effect of different concentrations of PAs on the maturation of white spruce (line G-316) somatic embryos.

Chemical treatment	(mM)	Total number of somatic embryos[1]	Number of normal somatic embryos[1]	Number of abnormal somatic embryos[1]	Ratio abnormal/normal[1]
Put	0	35 ^{a}	21.2 ^{a}	13.8 ^{a}	0.71^{a}
	0.5	36.2 ^{a}	21.6 ^{a}	14.6 ^{a}	0.74 ^{a}
	1	43.2 ^{a}	23.4 ^{a}	19.8 ^{a}	0.86 ^{a}
	2	37.4 ^{a}	21.8 ^{a}	15.6 ^{a}	0.71 ^{a}
	5	40 ^{a}	23.4 ^{a}	16.6 ^{a}	0.72 ^{a}
Spd	0	35 ^{b}	21.2 ^{b}	13.8 ^{a}	0.71 ^{a}
	0.5	32.8 ^{b}	17.8 ^{b}	15 ^{a}	0.88 ^{a}
	1	38.6 ^{b}	23.4 ^{b}	15.2 ^{a}	0.66 ^{a}
	2	37 ^{b}	21 ^{b}	15.4 ^{a}	0.76 ^{a}
	5	68 ^{a}	50 ^{a}	17.6 ^{a}	0.35 ^{a}
Spm	0	35 ^{b}	21.2 ^{b}	13.8 ^{b}	0.71 ^{a}
	0.5	34.2 ^{b}	22 ^{b}	12.2 ^{bc}	0.56 ^{b}
	1	74.6^{a}	51.6 ^{a}	23 ^{a}	0.45 ^{b}
	2	20.2 ^{c}	13.8 ^{b}	6.4 ^{c}	0.57 ^{b}
	5	0	0	0	0

[1]Within each treatment, data in columns displaying the same letters are not different at a level $p \leq 0.05$ according to Bonferroni's test. Each datum is the mean of 5 replicates. Each replicate was initiated with six pieces of 80 mg each of embryogenic tissue. Statistical analyses were applied separately on each experiment corresponding to each chemical compound.

Furthermore, the addition of 5 mM *Spd* or 1 mM *Spm* improved the synchronicity of maturation and markedly increased somatic embryo production on all around of embryogenic pieces that occurred normally (controls) at the interface between the medium and the base of embryogenic tissues. Compared to the controls, *Spd* or *Spm* also markedly reduced the browning and senescing of embryogenic tissues). Unlike spermidine and spermine, putrescine did not significantly affect maturation of somatic embryos and remained ineffective in reducing

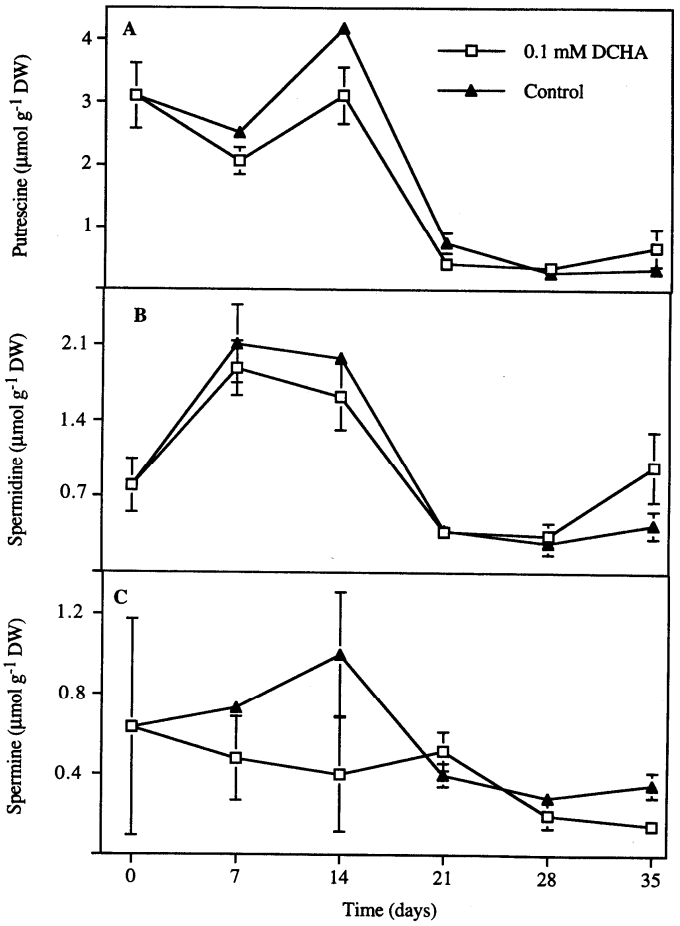

Figure 3. Time course of cellular putrescine, spermidine and spermine in the embryogenic tissue of white spruce grown in the absence or presence of 0.1 mM *DCHA*. The bars represent SE, n=4.

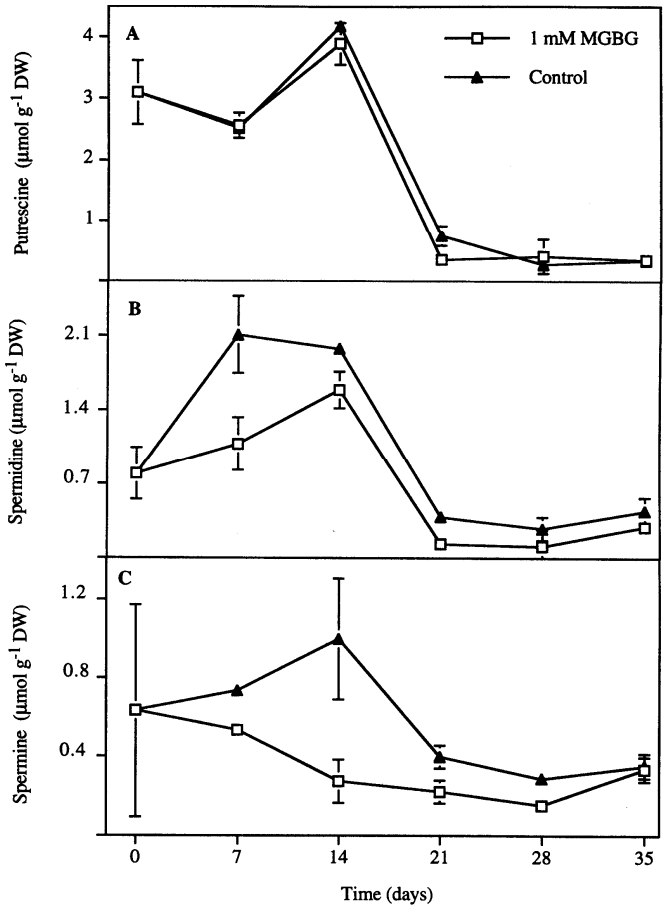

Figure 4. Time course of cellular putrescine, spermidine and spermine in the embryogenic tissue of white spruce grown in the absence or presence of 1 mM MGBG. The bars represent SE, n=4.

browning, for all the concentrations tested.

Effect of *DCHA* and *MGBG* on free cellular polyamine levels

Addition of 0.1 mM *DCHA* to the maturation medium significantly reduced the endogenous *Spm* and *Put* levels after 7 and 14 days of culture, whereas the concentration of spermidine was unaffected (Figure 3). Addition of 1 mM *MGBG* to the culture medium had no effect on putrescine concentration, while it significantly decreased spermidine and spermine concentrations throughout the maturation period (Figure 4).

Effect of exogenous polyamines on free cellular polyamine levels

In the presence of exogenous putrescine, the intracellular concentrations of putrescine, spermidine and spermine markedly increased during the first half of the maturation period followed a rapid decrease to about the control levels. Addition of putrescine caused a 30-fold increase (from 3 to 90 µmol g^{-1} dry weight) in the endogenous putrescine concentration on day 7 (Figure 5A), a 20-fold increase (from 0.8 to 19 µmol g^{-1} dry weight), in the spermidine concentration at day 14 (Figure 5B) and a 5-fold increase (from 0.65 to 3.7 µmol g^{-1} dry weight) in the Spm levels on day 7 (Figure 5C).

Spermidine treatment markedly increased the concentrations of intracellular polyamines during the entire maturation period (Figure 6). In the presence of 5 mM exogenous spermidine, intracellular putrescine and spermidine respectively increased by 10-fold (from 3 to 28 µmol g^{-1} dry weight) (Figure 6A) and 45-fold (from 0.8 to 36 µmol g^{-1} dry weight) during the first 2 weeks of the maturation period, followed by a drastic decrease in both cases at day 21 (Figure 6B). Whereas the intracellular spermine concentration followed a different pattern: it significantly increased from about 0.65 µmol g^{-1} dry weight to reach a maximum value of 1.7 µmol g^{-1} dry weight at day 21, and then slightly decrease to 1.3 µmol

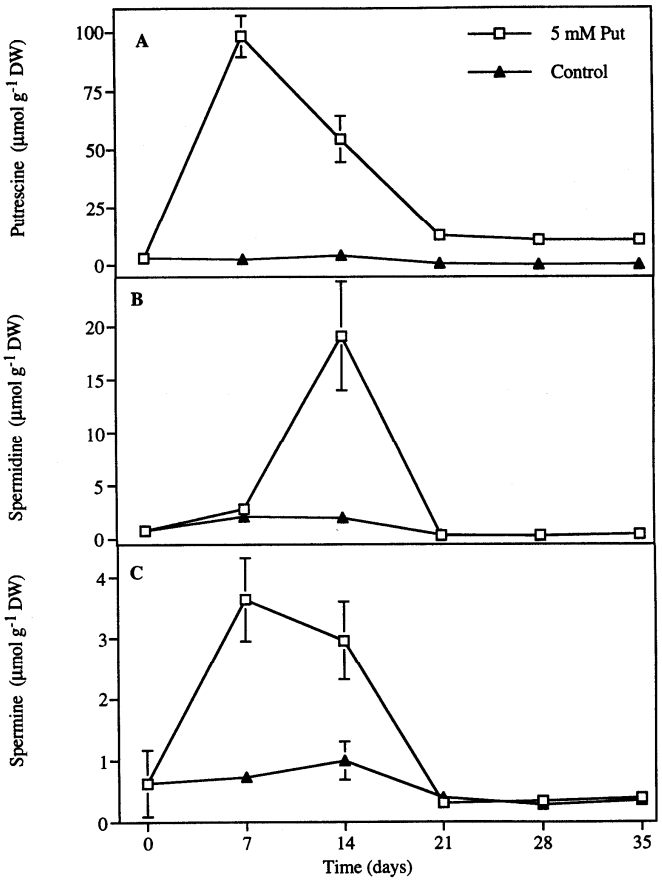

Figure 5. Time course of cellular putrescine, spermidine and spermine in the embryogenic tissue of white spruce grown in the absence or presence of 5 mM putrescine. The bars represent SE, n=4.

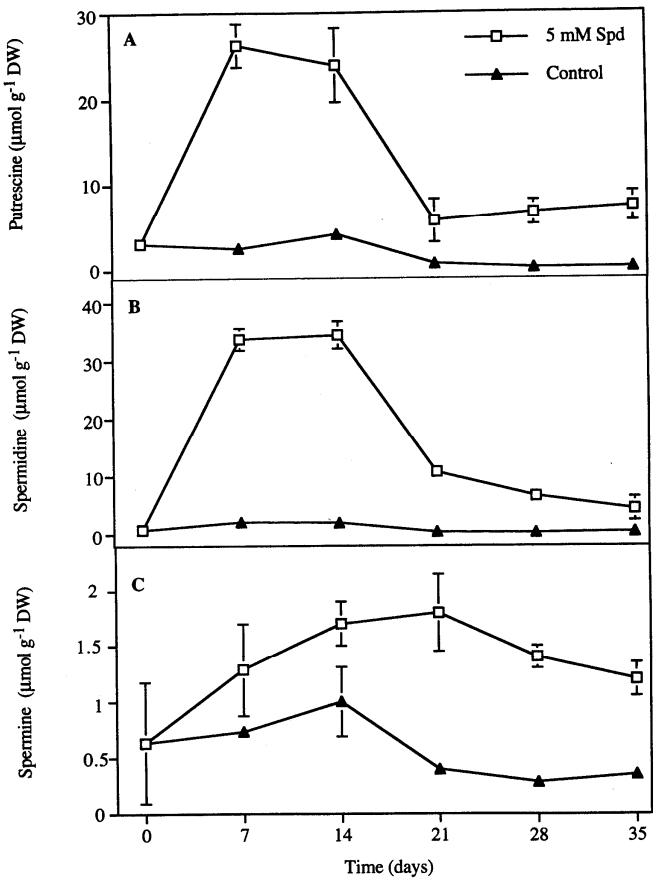

Figure 6. Time course of cellular putrescine, spermidine and spermine in the embryogenic tissue of white spruce grown in the absence or presence of 5 mM spermidine. The bars represent SE, n=4.

g^{-1} dry weight (Figure 6C).

Addition of exogenous spermine also affected the levels of free intracellular polyamines during maturation (Figure 7). In the presence of 1 mM exogenous spermine, the intracellular putrescine concentration increased after 7 days of culture and in the last two weeks of the experiment (Figure 7A). The pattern of the intracellular spermidine concentration was similar to that of the controls, although slightly higher (Figure 7B). Intracellular spermine concentration increased by 15 fold (0.65 to 10 µmol g^{-1} dry weight) at day 14 and remained significantly higher than the controls until the end of experiment (Figure 7C).

Effect of spermidine and *MGBG* on intracellular concentrations of *ACC* and *mACC*

Intracellular concentrations of *ACC* and *mACC* were monitored after addition of either *MGBG* or spermidine to the maturation medium (Figure 8). Addition of 5 mM of exogenous spermidine increased cellular concentration of *ACC*, whereas *MGBG* did not affect the cellular concentration of *ACC* throughout the maturation period (Figure 8A). On the other hand, the cellular concentration of *mACC* was significantly increased by both spermidine and *MGBG* treatments. Spermidine induced the highest *mACC* response throughout the maturation process (Figure 8B).

Germination

No differences were observed in the development of embryos obtained from spermidine- and spermine-treated tissues as compared to the controls. In every case, the frequencies of embryos developing an epicotyl varied from 90 to 100% (data not shown). However, somatic embryos produced from spermidine or spermine-treated tissues germinated more rapidly then those obtained from the controls (data not presented).

DISCUSSION

Current evidence suggests that regulation of the

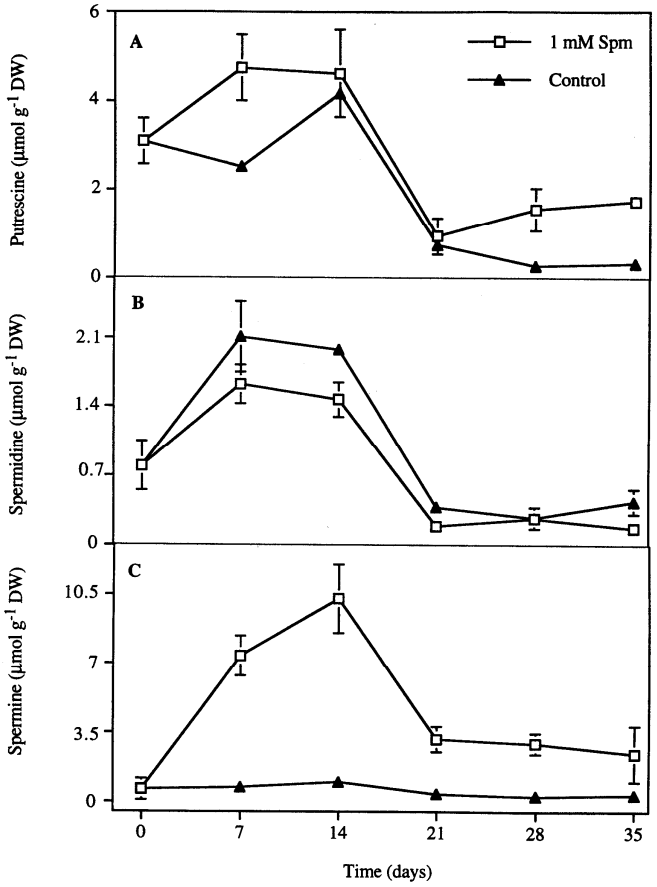

Figure 7. Time course of cellular putrescine, spermidine and spermine in the embryogenic tissue of white spruce grown in the absence or presence of 1 mM spermine. The bars represent SE, n=4.

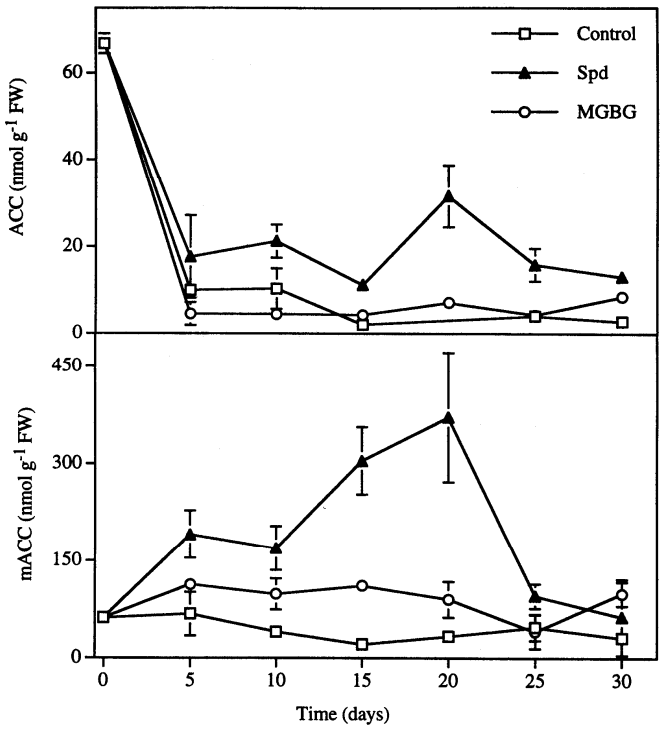

Figure 8. Profile of the cellular pools of ACC and its conjugate *mACC* in the embryogenic tissue of white spruce grown in the absence or in presence of MGBG (1 mM) or spermidine (5 mM).

intracellular polyamine levels play a pivotal role not only in cell proliferation and differentiation but also in the regulation of plant growth and development of nearly all of the species studied. Changes in the levels of polyamines have been reported in several important cellular processes. Elevated levels of polyamines are related to environmental stresses (Flores, 1990) and to various physiological processes including not only cell division and differentiation but also embryo development (Evans and Malmberg, 1989; Kushad and Dumbroff, 1991; Galston and Kaur-Sawhney, 1995). In somatic embryogenesis, temporal changes in cellular polyamines levels have been reported for several plants (Minocha and Minocha, 1995; Kumar et al., 1997; Walden et al., 1997), including a few conifers (Sarjala et al., 1997; Santanen and Simola, 1992; Minocha et al., 1993; Amarasinghe et al., 1996; Kong et al., 1998; Minocha et al., 1999). It has been reported that among common polyamines, putrescine and spermidine are generally most abundant polyamines, while spermine is only present in lower or in trace amount. It has also been shown that free polyamine levels increase during conifer somatic embryo maturation, spermidine being the most abundant (Minocha et al., 1993; Amarasinghe et al., 1996; Kong et al., 1998; Minocha et al., 1999). In contrast, analyses of temporal changes in the current study showed that putrescine was the predominant followed by spermidine and spermine (Figure 2). Consistent with our data, other studies have shown that cellular free putrescine was the most abundant polyamine followed by spermidine and spermine which were less abundant as shown in *P. glauca* x *P. engelmannii* complex. (Amarasinghe et al., 1996), *D. carota* (Feirer et al., 1984; Mengoli et al., 1989), and *Vitis vinifera* (Faure et al., 1991). Therefore, this difference in polyamines predominance cannot be generalized, but might depend on species, the developmental stage, the duration of treatments, the physiological status of cell lines as well as the media examined (Evans and Malmberg, 1989; Egea-Cortines and Mizhari, 1991; Flores and Galston, 1984; Kushad and Dumbroff, 1991; Slocum, 1991; Tiburcio et al., 1997; Chang and Kang, 1999).

The requirements for polyamines during different stages of somatic embryogenesis were shown by application of inhibitors of polyamines biosynthesis (Feirer et al., 1984; Feirer et al., 1998; El Hadrami and D'Auzac, 1992; Meijer and Simmonds, 1988; Minocha and Minocha, 1995). In the present work, the addition of the polyamines biosynthesis inhibitors *DCHA* and *MGBG* reduced polyamines biosynthesis (Figures 4 and 5) but also the production of mature somatic embryos (Tables 1

and 2) supporting the idea that polyamines is important to somatic embryos development. Conversely, treatments of embryogenic tissues with 5 mM spermidine and 1 mM spermine during the maturation process increased intracellular spermidine and spermine concentrations (Figures 6 and 8) while markedly stimulating the production of matured somatic embryos (Table 2). These results support previous studies showing a strong positive correlation between conifer embryo development or its morphological quality and a high spermidine concentration (Minocha et al., 1993; Amarasinghe et al., 1996; Kong et al., 1998; Minocha et al., 1999).

It is interesting to note that most of the biological functions of polyamines can be explained by their polycationic nature, which allows interactions with anionic macromolecules such as DNA, RNA and with negative groups of membranes. It is well documented that polyamines affect the nitrogen pool in plant cells (Walden et al., 1997; Kumar et al., 1997). Interestingly, in our study we noticed a relation between polyamines effects and their amino group amount. Indeed, the effective concentrations which promoted mature somatic embryo production were 1 mM spermine which has four amino groups, and 5 mM spermidine which is triamine suggesting the involvement of aminogroups. Bagani and Serafini-Fracassini (1985) reported that exogenous polyamines serve as a mere nitrogen source for the plants. Also polyamines can act as free radical scavengers and protect senescing membranes against lipid peroxidation. Another possible mechanism is that polyamines might be active, not by themselves, but through their catabolic pathways (Walden et al., 1997; Kumar et al., 1997) or through their interaction with ethylene biosynthesis. In this study, addition of spermidine and spermine delayed the necrotic /senescing status of embryogenic tissues. A body of experimental evidence attests that spermidine and spermine are effective antisenescence agents and that ethylene promotes necrosis/senescence phenomenon (Evans and Malmberg, 1989; El Meskaoui et al., 2000; El Meskaoui and Tremblay, 2001). Spermidine and spermine themselves were shown to interact with ethylene biosynthesis pathway by blocking the conversion of *ACC* to ethylene, possibly because they can directly or indirectly act as free radical scavengers (Smith, 1985; Kumar et al., 1997; Walden et al., 1997; Velikova et al., 1998). Our data show that the addition of spermidine increased polyamines concentration and the *ACC* cellular concentration and its conjugate (Figure 8). This result suggests that increase in polyamine concentration may be inhibited by the activity of *AdoMet dec*, causing a shunt of *AdoMet* to the ethylene pathways. However, it is well recognized that exogenous polyamines inhibit ethylene production by reducing the activity of *ACC* synthase and *ACC oxidase* (Kushad and Dumbroff, 1991). Our results indicate that a rise in both *ACC* level and its conjugate supports that polyamines inhibit *ACC* oxidase. To confirm this, it is necessary to quantify either ethylene production or *ACC* oxidase activity in spermidine-treated embryogenic tissues. Together with previous results on ethylene metabolism in white spruce (El Meskaoui et al., 2000) we could postulate that an effective mechanism possibly exists for modulating somatic embryo maturation in which polyamine and ethylene metabolisms are both involved and may play complementary roles.

Our results support the idea that polyamines are essential to maturation of spruce somatic embryos. Application of spermidine or spermine improved mature somatic embryos production and may be useful to synchronize the maturation stage. Also spermidine and spermine are effective in reducing necrosis/senescence phenomenon. Our data show that polyamines, in particular spermidine and spermine synthesis, are important to the maturation of somatic embryo and could be a target to genetic manipulation as demonstrated in others studies (Hatanaka et al., 1999; Nabha et al., 1999; Primikirios and Roubelakis-Angelakis, 1999; Bastola and Minocha, 1995; Andersen et al., 1998).

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