

16. Somatic Embryogenesis and Polyamines in Woody Plants

Rakesh Minocha, Subhash C. Minocha and Liisa Kaarina Simola

Contents

1. Introduction	337	6. Spermine	350
2. Polyamines	338	7. ODC, ADC, and SAMDC	
3. Polyamines in Conifers and Hardwoods:		Activities	350
General Comments	341	8. Polyamine Degradation and	
4. Polyamines and Somatic Embryogenesis:		Interconversions	353
Background	343	9. Conclusions	355
5. Polyamines and Somatic Embryogenesis		References	355
in Woody Plants	344		
5.1. Putrescine	345		
5.2. Spermidine	349		

1. Introduction

The formation of whole plants from cultured cells is interesting not only because of its applications for mass propagation but also as a prime example of the process of controlled development and differentiation in plants. Cultures capable of producing somatic embryos with high frequency provide ideal experimental systems to study and understand the biochemical basis of hormonal regulation of the developmental process. This knowledge should lead to the planning of media and various treatments that allow us to obtain whole plants from single cells in recalcitrant plant species.

In contrast to zygotic embryos which mature under the restrictive environment of endospermic tissue and show a high degree of commitment at very early stages of development (Bhojwani and Razdan, 1983; Goldberg *et al.*, 1989), somatic embryos are highly plastic and much less committed to maturity (Carman, 1990). Their development is easily disturbed by changes in medium constituents, physical environment, density of cultures, and other exogenously applied chemical factors.

Whereas a large amount of literature has accumulated on the morphogenetic and biochemical events underlying embryogenic development in cell cultures of herbaceous dicotyledons as exemplified by carrot (Street, 1977; Sharp *et al.*, 1980; Ammirato, 1984; Raghavan, 1986; Minocha, 1988; Carman, 1990; De Jong *et al.*, 1993), only limited information is available for the development of zygotic or somatic embryos in woody plants. A few laboratories have attempted to characterize the metabolic status of conifer cell and tissue cultures undergoing somatic embryogenesis or adventitious

shoot regeneration (Simola and Honkanen, 1983; Grey *et al.*, 1987; Durzan, 1987; Wann *et al.*, 1987; Hakman and von Arnold, 1988; Kumar and Thorpe, 1989; Simola and Santanen, 1990; Tautorius *et al.*, 1991). These studies have focused mostly on the metabolism of carbohydrates and nitrogen. Somatic embryos of *Picea abies* cultured under appropriate conditions demonstrate ultrastructural and chemical similarities (Hakman, 1993) to zygotic conifer embryos (Simola, 1974a,b, 1975). Changes in proteins and mRNA during somatic and zygotic embryogenesis in *Picea glauca* have recently been reported by Misra and Green (1990, 1991).

2. Polyamines

Polyamines are naturally occurring aliphatic amines that carry a net positive charge at physiological pH. The most prevalent polyamines in higher plants are spermidine, spermine, and their precursor putrescine. Other less frequently observed polyamines include cadaverine, norspermidine, norspermine, homospermidine and homospermine. Their ubiquitous occurrence in all living organisms and in all cell types has led to the speculation that polyamines are essential for cellular growth and differentiation (Smith, 1985; Evans and Malmberg, 1989; Slocum and Flores, 1991). Polyamines have been found to exist in free, conjugated, and bound forms.

Polyamine biosynthesis and cellular concentrations can be modulated by various plant growth regulators (Evans and Malmberg, 1989; Rastogi and Davies, 1991, and references therein). Likewise, the modulation of cellular polyamines by exogenous polyamines or polyamine biosynthetic inhibitors can alter the endogenous levels of plant growth regulators (Roberts *et al.*, 1984). A possible function of these compounds as growth regulators or as secondary messengers in plant cells has been discussed (Slocum *et al.*, 1984; Smith, 1985; Galston and Kaur-Sawhney, 1987; Phillips *et al.*, 1987; Evans and Malmberg, 1989).

The variety of physiological responses in which a role for polyamines has been suggested is indeed large. Obviously, a single mode of action cannot be envisioned. Undoubtedly, the polybasic nature of these compounds is essential to the many metabolic activities they help to regulate, but their mechanism of action remains in question. It is believed that polyamines accomplish many of their functions by binding with negatively charged sites on various macromolecules (see Bachrach and Heimer, 1989a,b; Slocum and Flores, 1991, and references therein).

In plants, polyamines have been implicated in the regulation of light-induced growth responses, embryogenesis, organogenesis, pollen formation, flower development, fertilization, fruit development and senescence (Slocum *et al.*, 1984; Minocha, 1988; Evans and Malmberg, 1989; Slocum and Flores, 1991). In addition, a role for polyamines has also been proposed in the response of plants to stress from both biotic and abiotic factors (Young and

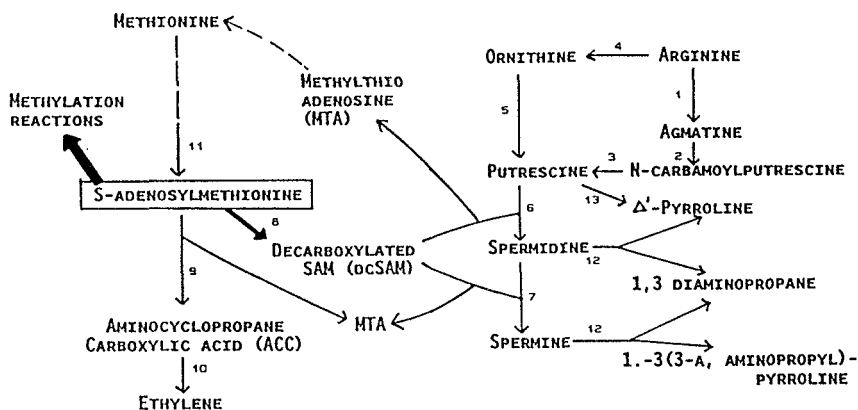


Figure 1. Combined pathway for biosynthesis of polyamines and ethylene in plants. The enzymes involved are: (1) arginine decarboxylase (ADC); (2) agmatine iminohydrolase; (3) N-carbamoylputrescine amidohydrolase; (4) arginase; (5) ornithine decarboxylase (ODC); (6) spermidine synthase; (7) spermine synthase; (8) SAM decarboxylase (SAMDC); (9) ACC synthase; (10) ethylene forming enzyme; (11) S-adenosylmethionine synthase; (12) polyamine oxidase (PAO); (13) diamine oxidase (DAO).

Galston, 1983; Flores and Galston, 1984; Villanueva *et al.*, 1987; Krishnamurthy and Bhagwat, 1989; Wang and Zug, 1989; Slocum and Flores, 1991; Tenter and Wild, 1991; Minocha *et al.*, 1992; Prediari *et al.*, 1993).

Ornithine decarboxylase (ODC) (EC 4.1.1.17) is a ubiquitous enzyme that catalyzes the conversion of L-ornithine to the diamine putrescine (Fig. 1). Ornithine decarboxylation is the only *de novo* pathway to putrescine synthesis in animals. In higher plants as well as prokaryotes and some fungi, there is a second pathway for putrescine synthesis (Slocum *et al.*, 1984; Smith, 1985; Pegg, 1986; Khan and Minocha, 1989a,b). In this pathway, the decarboxylation of L-arginine by arginine decarboxylase (ADC; EC 4.1.1.19) leads to the formation of putrescine, via agmatine and N-carbamoylputrescine intermediates. The distribution of these enzymes in different tissues and in different plants is regulated in a developmental and tissue-specific manner. For example, proliferating suspension cultures of carrot possess only ADC while developing green mature somatic embryos contain both ODC and ADC, the former being predominant (Robie and Minocha, 1989).

Spermidine and spermine are synthesized by the sequential addition of an aminopropyl group to putrescine (Fig. 1). The aminopropyl moiety is donated to putrescine by decarboxylated S-adenosylmethionine (dcSAM) (Torget *et al.*, 1979; Cohen *et al.*, 1981; Pegg, 1986). In addition to its role as the major biological methylating agent and in the biosynthesis of polyamines, SAM is also the precursor for ethylene biosynthesis in plants. Decarboxylation of SAM by SAM decarboxylase is nonreversible, committing SAM to the polyamine biosynthetic pathway. Spermidine and spermine synthases are assumed

to be separate enzymes, though the mechanisms of their action may be identical (Smith, 1985; Pegg, 1986).

Many other polyamines have been reported in plants whose synthesis and potential functions were reviewed by Phillips and Kuehn (1991). Polyamines can also serve as precursors for secondary metabolites including anabasine, nicotine, atropine and unusual cinnamic acid amide conjugates. Some of the conjugates appear to be involved in resistance to pathogen infections, while others apparently are involved in plant reproduction (Smith *et al.*, 1983; Flores and Martin-Tanguy, 1991). Total concentrations of cellular polyamines are obviously influenced by the concentration of these secondary metabolites. Techniques are now available for isolation and quantitation of both perchloric acid (PCA) soluble and insoluble (i.e., free vs. conjugated or covalently bound) polyamines to help in the accurate determination of endogenous polyamine levels (Minocha *et al.*, 1990; Smith, 1991a). This is particularly important in view of the possibility that polyamine titers are regulated in part by interconversion between free polyamines and their conjugates.

A number of compounds have been synthesized that act as potent inhibitors of the polyamine biosynthetic enzymes (Pegg, 1986; McCann *et al.*, 1987). These compounds have proven useful in elucidating the importance of polyamines in cellular activities, and in understanding the regulation of polyamine biosynthesis. The suicide inhibitors, DL- α -difluoromethylornithine (DFMO) and DL- α -difluoromethylarginine (DFMA) specifically inhibit ODC and ADC, respectively. In addition, D-ornithine and monofluoromethylornithine have also been used to inhibit ODC activity.

Inhibition of cell growth with these compounds seems to be cytostatic rather than cytotoxic as the exogenous application of polyamines generally reverses the effect of the ODC and ADC inhibitors. Despite a specific and almost universal inhibition of ODC activity by DFMO in animals, this compound does not function as an effective inhibitor for all prokaryotic and plant ODCs. For example, DFMO does not inhibit ODC activity of certain cultivars of *Solanum tuberosum*, *Avena sativa*, *Pisum sativum*, *Amaranthus* sp., *Daucus carota*, *Catharanthus roseus*, and a few cultivars of *Nicotiana tabacum* (Galston, 1983; Flores and Galston, 1984; Slocum and Galston, 1985; Robie and Minocha, 1989; Minocha *et al.*, 1991a) when tested *in vitro*. In general, DFMA has been highly effective as a suicide inhibitor of ADC in plants.

Another widely used inhibitor of polyamine biosynthesis is MGBG (methylglyoxal bis(guanylhydrazone)), a competitive and reversible inhibitor of SAMDC that generally inhibits spermidine and spermine biosynthesis. In some instances, MGBG is not specific for SAMDC inhibition (Pegg, 1986) and may also cause stabilization of this enzyme (Hiatt *et al.*, 1986; Malmberg and Hiatt, 1989).

Polyamines are oxidatively deaminated by the enzymes diamine oxidases (DAOs) and polyamine oxidases (PAOs) (Federico and Angelini, 1991). Plant diamine oxidases are most active with putrescine and cadaverine,

though enzymes with broad specificities also occur in some species. Polyamine oxidases are known to occur mostly in monocots, especially cereals (Smith, 1991b). Only recently have these enzymes been detected in dicots (Bagga *et al.*, 1991) and conifers (Santanen and Simola, 1994). Unlike the diamine oxidases, the PAOs are specific for aliphatic polyamines. It is unclear why di- and polyamine oxidases occur so erratically throughout the plant kingdom. Some plants, such as those in the Solanaceae, apparently do not contain amine oxidase activity. The ultimate fate of polyamines in plants is thus quite complicated and not fully understood.

3. Polyamines in Conifers and Hardwoods: General Comments

Cellular levels of polyamines in different tissues of a number of woody plant species have been reported (Table 1). It is obvious from the published work that the major polyamines (putrescine, spermidine, and spermine) found in woody plants are the same as in the herbaceous plants. There are no published reports containing any information on the occurrence of rare polyamines, possibly because only a few species of woody plants have been studied. Like the situation with herbaceous plants, different species and different tissues of the same plant differ widely in having either putrescine or spermidine as the predominant polyamine. Both ODC and ADC have been observed in woody plants, their distribution also being tissue and species dependent as in herbaceous plants (R. Minocha and S.C. Minocha, unpublished data). While the information is scant, both conjugated and bound forms of polyamines have been reported in woody plants.

Some polyamines and their combinations have been shown to have favorable effects on protoplast and callus cultures of woody plants. Ornithine (25 μ M) and putrescine (50 and 100 μ M) were found to stimulate cell division and colony formation in mesophyll protoplasts of *Alnus glutinosa* and *A. incana* (Huhtinen *et al.*, 1982/83). When the effect of polyamines was tested on initiation of megagametophyte callus of *Picea abies*, it was found that these compounds could not replace casein hydrolysate and glutamine in most cases, but a combination of three polyamines (putrescine 0.25, spermidine 0.1, and spermine 0.025 mM) favored the development of roots (Simola and Honkanen, 1983). The microspore callus cultures of this species were able to grow in the dark if the nutrient medium contained putrescine (0.1 mM). The effects of spermidine and spermine on growth were similar but root differentiation was stimulated in blue, red, and fluorescent light by spermine only (Simola and Huhtinen, 1986).

Table 1. Amount of polyamines in different woody plants.

Species	Putrescine	Spermidine	Spermine	Reference
<i>Larix decidua</i> callus	52.8	48.5	14.6	Minocha <i>et al.</i> , unpublished data
<i>Picea abies</i> needles, clone 14*	50	27	16	Dohmen <i>et al.</i> , 1990
needles, clone 11*	65	38	11	
callus	228.7	52.7	0.0	Minocha <i>et al.</i> , 1993
zygotie embryos	209.4	728.1	221.9	Santanen and Simola, unpublished data
megagametophyte	25.6	131.0	21.3	Santanen and Simola, unpublished data
<i>Picea rubens</i> needles	112.6	173.2	7.0	Minocha <i>et al.</i> , unpublished data
callus	227.3	65.0	10.3	Minocha <i>et al.</i> , 1993
roots	109.4	57.6	0.0	Minocha <i>et al.</i> , unpublished data
suspension	139.7	354.3	78.5	Minocha <i>et al.</i> , unpublished data
<i>Pinus radiata</i> cotyledon (ng/cot)	130 (0d) 40 (3d)	105 (0d) 80 (3d)	105 (0d) 70 (3d)	Biondi <i>et al.</i> , 1986
cotyledon ($\mu\text{mol/gFW}$)*	1.05 (0d) 0.4 (3d)	2.9 (0d) 1.1 (3d)	0.2 (0d) 0.25 (3d)	Kumar and Thorpe, 1989
<i>Citrus aurantium</i> leaves	41	90*	75	Kushad and Yelenosky, 1987
<i>Citrus jambhiri</i>	28	90*	14	
<i>Citrus sinensis</i>	29	70*	210	
<i>Hevea brasiliensis</i> callus (40 d)	4060	322	270	El Hadrami and D'Auzac, 1992
<i>Mangifera indica</i> ($\mu\text{mol/gFW}$) nucellus- monoembryonic callus	9.3 382.3	0.6 2.2	0.0 0.4	Litz and Schaffer, 1987
somatic embryos	8.6	0.0	0.0	
zygotie embryos	0.5	1.4	0.2	
<i>Populus</i> <i>nigra</i> $\times$ <i>maximowiczii</i> suspension	2039	386	45	Sun and Minocha, unpublished data
<i>Populus tremuloides</i> callus	5.3	40.0	6.3	Minocha <i>et al.</i> , unpublished data
<i>Prunus avium</i> leaf*	110	45	—	Biondi <i>et al.</i> , 1990
shoot 63	37	traces		
<i>Pyrus communis</i> * shoot cultures (2 wk)	460	120	19	Predari <i>et al.</i> , 1993

The polyamine levels are expressed as nmol/gFW unless otherwise stated (d = days).

* Indicates estimated amount from published figures.

4. Polyamines and Somatic Embryogenesis: Background

In addition to a multitude of potential functions of polyamines in plants, it has been suggested that polyamines play a critical role in morphogenesis in plant cell cultures (Minocha, 1988; Galston and Flores, 1991). With respect to somatic embryogenesis, the role of polyamines has been studied extensively in carrot (Montague *et al.*, 1978, 1979; Feirer *et al.*, 1984; Minocha, 1988; Mengoli *et al.*, 1989; Robie and Minocha, 1989; Minocha *et al.*, 1991b,c). The following statements summarize the relationship between polyamines and somatic embryogenesis in this tissue: 1) presence of auxin in the medium completely suppresses somatic embryogenesis; 2) increased biosynthesis of polyamines precedes the development of somatic embryos upon removal of auxin; 3) inhibitors of polyamine biosynthesis (e.g., DFMA and MGBG) strongly inhibit the development of somatic embryos. Studies from our laboratory (Robie and Minocha, 1989; Khan and Minocha 1991; Minocha *et al.*, 1991b,c; Nissen and Minocha, 1993; D.R. Bastola and S.C. Minocha, unpublished data) further show that: 1) cells grown in the presence of auxin contain less polyamines than those grown in an auxin-free medium; 2) DFMO, in a manner unrelated to its effects on ODC, promotes the biosynthesis of polyamines, inhibits ethylene production and promotes somatic embryogenesis in the absence of auxin, and, most importantly, allows the development of somatic embryos even in the presence of auxin; 3) the promotory effects of DFMO cannot be mimicked by exogenous addition of polyamines or other analogs; 4) transgenic cell lines of carrot that overexpress a mouse ODC cDNA and thus produce considerably higher levels of putrescine (as compared to control cells), also show a stimulation of somatic embryogenesis in the auxin-free medium, and produce somatic embryos even in the presence of otherwise inhibitory concentrations of 2,4-D. Thus, stimulation of putrescine biosynthesis achieved either through treatment with DFMO (Robie and Minocha, 1989) or through the overexpression of ODC (D.R. Bastola and S.C. Minocha, unpublished data) have similar consequences, i.e., a partial reversal of the inhibitory effect of auxin. Additional research is needed on mechanism(s) by which increased cellular levels of putrescine induce the development of somatic embryos.

Parallel studies with several other herbaceous angiosperms also have demonstrated the inhibition of somatic embryogenesis by inhibitors of polyamine biosynthesis (Meijer and Simmonds, 1988; Minocha, 1988; Galston and Flores, 1991). These results indicate that a continued biosynthesis of polyamines is an essential aspect of the metabolism during the differentiation and development of somatic embryos in plants.

In contrast to the herbaceous angiosperms, however, there have been only a few studies on polyamines in relation to the process of embryogenesis in woody plants, especially trees. Litz and Schaffer (1987) were the first to study changes in cellular polyamine content during the development of somatic and adventitious embryos in a tree species (*Mangifera indica*). Biondi *et al.* (1986,

1988) have reported changes in polyamine metabolism in the cotyledons of *Pinus radiata* cultured on shoot-forming and non shoot-forming media. Santanen and Simola (1992) compared the free, bound, and conjugated polyamines in non-embryogenic callus cultures with the differentiating embryogenic callus cultures of *Picea abies* grown on ABA-containing maturation medium. Minocha *et al.* (1993) have studied the metabolism of free polyamines in *Picea abies* and *Picea rubens* pro-embryogenic callus cultures grown on the proliferation medium containing auxin and cytokinin, and on the maturation medium containing ABA and IBA. The effects of exogenous addition of polyamines and their biosynthetic inhibitors on free polyamine levels and growth rate in the differentiating embryogenic cultures of rubber tree (*Hevea brasiliensis*) were reported by El Hadrami and D'Auzac (1992). All of these studies, like those on carrot, were done with tissues that were producing somatic embryos but also contained a large proportion of non-differentiating cells. However, a detailed analysis of polyamines in different stages of the developing somatic or zygotic embryos is not currently available, though, as discussed by Feirer, Chapter 15 of this book, preliminary studies have been undertaken in *Pinus strobus*. Santanen and Simola (1994) also reported on the putrescine and spermidine catabolism in non-embryogenic and embryogenic callus lines and late stages of developing embryos of *Picea abies*. The following is a summary of the available literature on polyamines and somatic embryogenesis and morphogenesis in tissue cultures of woody plants, and how these results compare with the reported data on herbaceous angiosperms.

5. Polyamines and Somatic Embryogenesis in Woody Plants

Somatic embryogenesis in conifers is known to be quite different from that in carrot and other angiosperm species. In most angiosperms, somatic embryogenesis entails a rapid growth phase for several days during which embryos and plantlets are formed that continue to grow or "germinate" without an intervening period of dormancy (maturation). In most conifers, on the other hand, tissue on the proliferation medium grows fast and produces only early stage embryos (Tautorius *et al.*, 1991). Maturation of these embryos is similar to the maturation of zygotic embryos. Maturation medium for conifers commonly contains ABA in contrast to the medium used for somatic embryogenesis in angiosperms (Hakman and Fowke, 1987a,b; Boulay *et al.*, 1988; Hakman and von Arnold, 1988; Misra and Green, 1990, 1991; Simola and Santanen, 1990). These mature embryos must be transferred to a fresh medium lacking ABA and other growth hormones in order to initiate germination.

As described elsewhere in this book, in most conifers, the pro-embryogenic tissue maintained on the proliferation medium consists of numerous organized meristematic clusters attached to long suspensor-like cells. Pro-

embryogenic callus produces visible globular embryos on maturation medium within 10 to 15 days in both *Picea abies* and *Picea rubens*. Embryos with greenish or yellowish green cotyledons are visible in large quantities within 4 to 8 weeks. Multiple embryos attached to the same group of loosely organized suspensor-like cells are commonly observed. Since embryogenesis is not synchronous in these cultures, embryos at different stages of development can be found at any one time on the same piece of tissue. Embryogenic cultures maintained on maturation medium increase in dry as well as wet weight mostly due to the developing embryos. However, the increase in weight of these cultures always is less than that of the pro-embryogenic tissue maintained on the proliferation medium.

Differentiating embryogenic cultures of *Picea abies* contained significantly higher levels of polyamines than non-embryogenic cultures (Fig. 3). These observations are similar to those reported for carrot and egg plant (*Solanum melongena*) tissues (Montague *et al.*, 1978; Fobert and Webb, 1987; Robie and Minocha, 1989). Differentiating embryogenic tissues of *Picea abies* and *Picea rubens*, maintained on the maturation medium, had lower levels of polyamines compared to the same tissues grown on the proliferation medium (Fig. 2). The decline of polyamines on the maturation medium may partly be related to the effects of ABA itself on this tissue and not to embryogenesis since non-embryogenic tissue grown on ABA containing medium also showed this decline, though at a slower rate (Santanen and Simola, 1992). Either putrescine or spermidine was the predominant polyamine in embryogenic tissue depending on how long the tissue had been on a particular medium, the developmental stage of the tissue, and whether the cultures were grown in suspension or on solid media. Spermine was present in minute quantities at all times tested. In general, embryogenic cultures had a higher concentration of free putrescine than spermidine both in *Picea abies* and *Hevea brasiliensis* (El Hadrami and D'Auzac, 1992; Minocha *et al.*, 1993). By contrast, Litz and Schaffer (1987) had found a much lower concentration of polyamines in the somatic as well as zygotic embryos compared to nucellus tissue and callus of *Mangifera indica* (Table 1).

5.1. Putrescine

The cellular content of free putrescine increased between 2 to 12 days after subculture of the pro-embryogenic tissue of two different tissue lines (genotypes) of *Picea abies* on the proliferation medium (Fig. 2). In contrast, there was a reverse trend for changes in free putrescine content during the same period in embryogenic cultures grown on the maturation medium. A similar profile of changes also was seen in *Picea rubens* cultures grown on maturation medium (Fig. 2). Free putrescine was always higher in tissues grown on the proliferation medium compared to those grown on the maturation medium (for details see Minocha *et al.*, 1993). In *Picea abies* the level of conjugated putrescine was found to be high in the ABA-containing medium at the

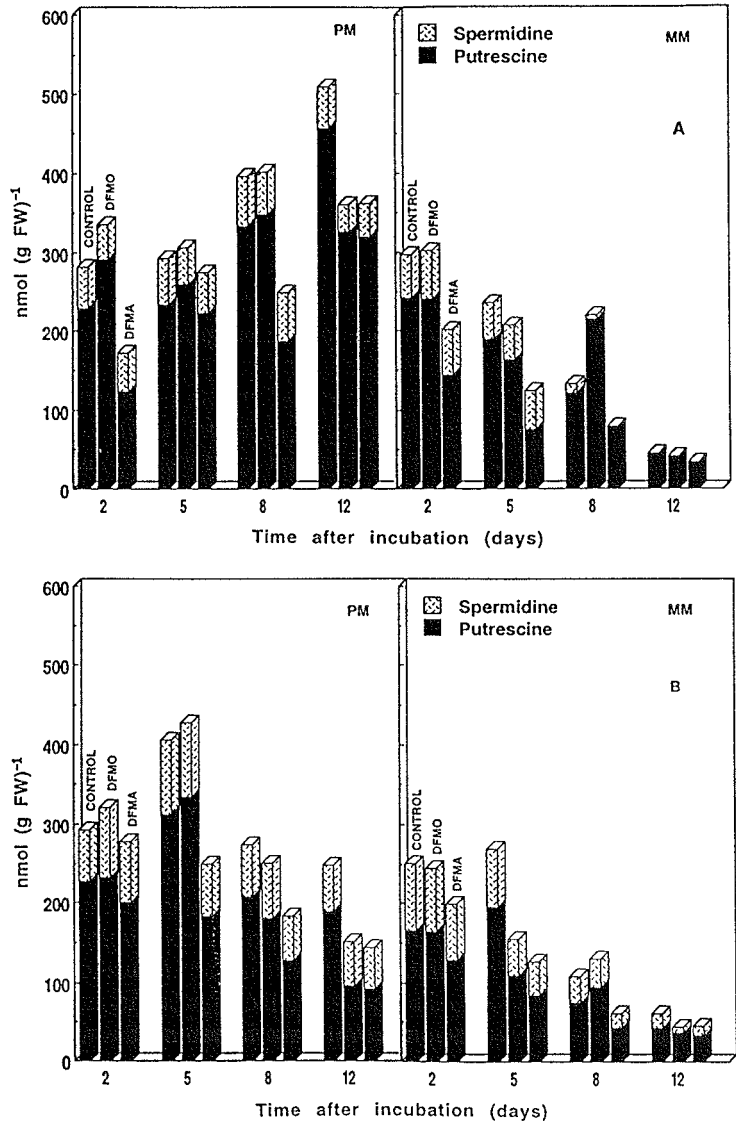


Figure 2. Effect of 2.0 mM DFMO and 0.1 mM DFMA on cellular levels of putrescine and spermidine in (A) *Picea abies* and (B) *Picea rubens* tissues grown on proliferation (PM) or maturation (MM) medium for different lengths of time. Values are mean $\pm$ SE of 4 replicates for *Picea abies* and 3 replicates for *Picea rubens*. (Modified from Minocha *et al.*, 1993.)

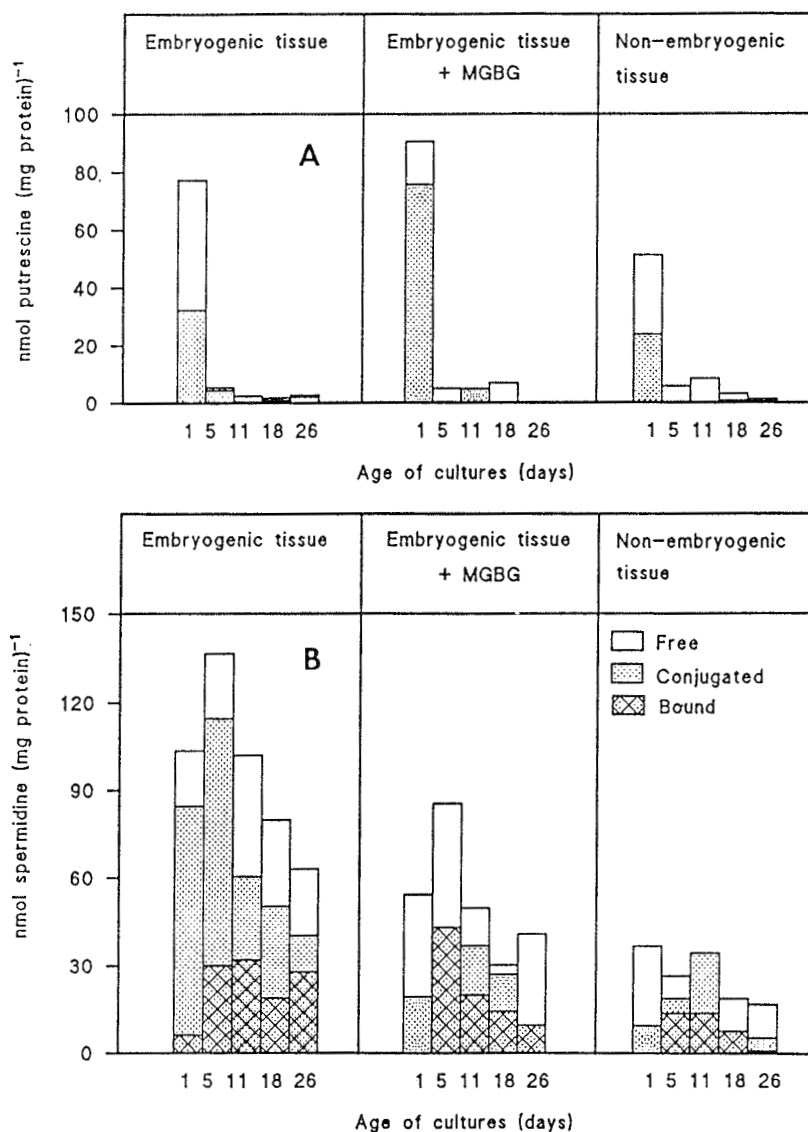


Figure 3. Changes in (A) putrescine and (B) spermidine content in embryonic and non-embryonic tissues of *P. abies* on ABA (+MgBg) containing medium. (Modified from Santanen and Simola, 1994.)

beginning of experiment in embryonic as well as non-embryonic cultures (Fig. 3).

Covalently bound putrescine was not found in *Picea abies* embryonic tissue (Santanen and Simola, 1992). With few exceptions, DFMO (2.0 mM) had little effect on free putrescine level for the first 8 days of subculture in

any experiment (Fig. 2). Occasionally, free putrescine level was higher in DFMO treated cells than control cells. This effect of DFMO is similar to the situation with carrot (Robie and Minocha, 1989; Nissen and Minocha, 1993). By contrast, DFMA was a strong inhibitor of free putrescine levels at all times tested (Fig. 2). DFMO has been shown to have stimulatory effects on embryogenesis in the case of carrot and maize (*Zea mays*) cell cultures (Mengoli *et al.* 1989; Robie and Minocha, 1989; Nissen and Minocha, 1993; Torné *et al.* 1993). However, in *Hevea brasiliensis* and in *Medicago sativa*, DFMO inhibited the embryogenesis process (Meijer and Simmonds, 1988; El Hadrami and D'Auzac, 1992). This may be due to the different pathways for putrescine biosynthesis in different tissues. Unfortunately, the distribution of ODC and ADC in these tissues is not known.

In a study of the changes in cellular polyamines during development of shoot forming (+BA) and non shoot-forming (−BA) callus from *Pinus radiata* cotyledons, Kumar and Thorpe (1989) observed a decline in putrescine levels in both types of cultures during the 21-day culture period; the sharpest decline occurred during the first 3 days. There was a somewhat faster decline in the non shoot-forming tissue than in the shoot-forming tissue. These results are similar to those of Biondi *et al.* (1986) with the same tissue. In their studies, however, an earlier decline (1 to 3 days) was followed by a steady increase for up to 10 days.

Marked differences in polyamine levels were observed in embryos and calli originating from different tissues of different varieties of *Mangifera indica* with either monoembryonic or polyembryonic adventitious embryos (Litz and Schaffer, 1987). The nucellar calli produced from monoembryonic varieties contained significantly more polyamines than those from polyembryonic varieties. Also, in contrast to the observations with *Picea abies* (Santanen and Simola, 1992), the non-embryogenic calli contained higher levels of polyamines than the embryogenic calli. Putrescine was the predominant polyamine in all varieties and in all tissues except in the zygotic embryos, where low levels of all polyamines were detected. Cellular polyamine levels in somatic embryos were comparable to those in the nucellus and were much lower (10 to 50 fold) than those in the callus. Exogenous supply of polyamines generally had no effect on the initiation of callus or somatic embryos.

In *Hevea brasiliensis*, the levels of putrescine increased with the time of culture up to 40 days, thereafter showing a decline up to 70 days (El Hadrami and D'Auzac, 1992). The addition of putrescine or arginine into the medium increased somatic embryogenesis potential of the calli while both DFMO and DFMA caused a reduction in somatic embryogenesis. While DFMA inhibited cellular putrescine in this tissue, there was a significant increase in putrescine in the presence of DFMO. Surprisingly, MGBG, which inhibited both spermidine and spermine synthesis, also caused a reduction in cellular putrescine. On the other hand, spermidine as well as spermine levels were higher in the presence of DFMA. No direct analysis of the enzyme activities for ODC, ADC, or SAMDC was reported.

Although MGBG (0.1 mM) inhibited free putrescine levels in differentiating embryogenic cultures of *Picea abies*, it showed a simultaneous increase in conjugated putrescine (day 1, Fig. 3). El Hadrami and D'Auzac (1992) also observed a similar decrease in free putrescine in embryogenic cultures of *Hevea brasiliensis*. However, free putrescine levels more than doubled within 4 days of subcultures on auxin-free medium containing MGBG in differentiating carrot cultures (Minocha *et al.*, 1991c).

5.2. Spermidine

In *Picea abies*, free spermidine levels did not change appreciably in the pro-embryogenic tissue during 12 days of culture on the proliferation medium (Fig. 2). In *Picea rubens* tissue, on the other hand, there was a slight increase in spermidine on day 5 under similar conditions. However, a decline in free spermidine levels was observed with time in cultures of both the species grown on the maturation medium (Fig. 2). These results are apparently different from the findings of Santanen and Simola (1992) who observed an increase in free spermidine levels at day 11, concomitant with a decrease in conjugated spermidine (Fig. 3). It should be pointed out, however, that the two laboratories have reported polyamine levels differently: whereas Minocha *et al.* (1993) expressed polyamine levels as $\text{nmol g}^{-1}\text{FW}$, Santanen and Simola (1992) expressed these as nmol mg^{-1} protein.

Similar to its effects on putrescine, DFMO usually had no effect on free spermidine levels in either *Picea abies* or *Picea rubens* tissues. DFMA inhibited spermidine only in differentiating embryogenic tissue on the maturation medium after 5–8 days of culture (Fig. 2). MGBG strongly decreased conjugated spermidine level in differentiating embryogenic tissues (Fig. 3).

Pinus radiata cotyledons grown on media with or without BA showed a sharp decline in cellular spermidine during the first 3 days of culture, after which only slight changes (mostly an increase) were seen during the next 18 days (Kumar and Thorpe, 1989). There were no significant differences in response to the presence of BA in the medium. These results are in apparent contrast to the data of Biondi *et al.* (1986), who observed a sharp rise in spermidine in the cotyledons by day 3 following a decline during the first 2 days. Again, it should be noted that the data reported by Kumar and Thorpe (1989) and Biondi *et al.* (1986) are in different units, i.e., $\mu\text{mol/gFW}$ in the former and ng/cotyledon in the latter. Dicyclohexylamine (DCHA, 1.0 mM) inhibited the growth rate of cotyledons and caused a severe decline in cellular spermidine levels both in the presence and the absence of BA. The formation of adventitious buds in the presence of BA was only partially inhibited by DCHA.

Spermidine levels increased within 20 days of culture in *Hevea brasiliensis*, remaining high for the next 70 days (El Hadrami and D'Auzac, 1992). Both DFMA and DFMA + DFMO treatments caused an increase in spermidine on day 40 while DFMO alone caused a decrease of spermidine at this time.

No effects of inhibitors were seen on days 60 and 70. Exogenous supply of spermidine enhanced the embryogenic potential of this tissue when used alone and in combination with other polyamines. Spermidine was also able to reverse the inhibitory effect of MGBG on somatic embryogenesis.

Cellular spermidine levels were higher in the non-embryogenic callus obtained from nucellus tissue of a monoembryonic mango as compared to the embryogenic callus as well as the somatic and zygotic embryos (Litz and Schaffer, 1987). However, in embryogenic callus raised from the nucellus of a polyembryonic variety, the cellular spermidine was higher than that in the non-embryogenic callus, somatic embryos and adventitious embryos.

6. Spermine

Spermine levels always were low in both *Picea rubens* and *Picea abies* tissues (Minocha *et al.*, 1993). Neither DFMO nor DFMA had an effect on the production of cellular spermine. In contrast to putrescine and spermidine, both of which decreased by 50 to 60 percent during the first 3 days of culture of *Pinus radiata* cotyledons, spermine levels increased by 60 percent during this period in the presence of BA (Kumar and Thorpe, 1989). No such increase in spermine was seen in the absence of BA. In earlier studies with the same tissue, Biondi *et al.* (1986) observed a decline in spermine during the first 2 days. This was followed by a steady rise between days 3 and 10. This increase in spermine was completely prevented by DCHA, presumably through its inhibition of spermidine biosynthesis.

In *Hevea brasiliensis*, however, spermine levels were comparable to those of spermidine and showed a peak at day 40, the same time as for putrescine and spermidine (El Hadrami and D'Auzac, 1992). DFMA caused an increase in spermine, while DFMO caused a slight decrease. MGBG inhibited cellular spermine as expected.

7. ODC, ADC, and SAMDC Activities

As is the case with several plants like *Daucus carota*, *Catharanthus roseus*, and *Nicotiana* sp. (Tiburcio *et al.*, 1987; Robie and Minocha, 1989; Minocha *et al.*, 1991a), ADC seems to be the dominant pathway for putrescine production in embryogenic tissues of both *Picea abies* and *Picea rubens* (Minocha *et al.*, 1993). In both these species, DFMA inhibits putrescine production presumably through the inhibition of ADC activity (Fig. 4). Whereas ADC activity did not show specific trends during the 12 days of growth of the pro-embryogenic tissue kept on the proliferation medium, it increased with time in tissues grown on the maturation medium for both species. In line with its lack of effect on putrescine production, DFMO either slightly promoted or had no effect on ADC activity. While no enzyme assays were reported for

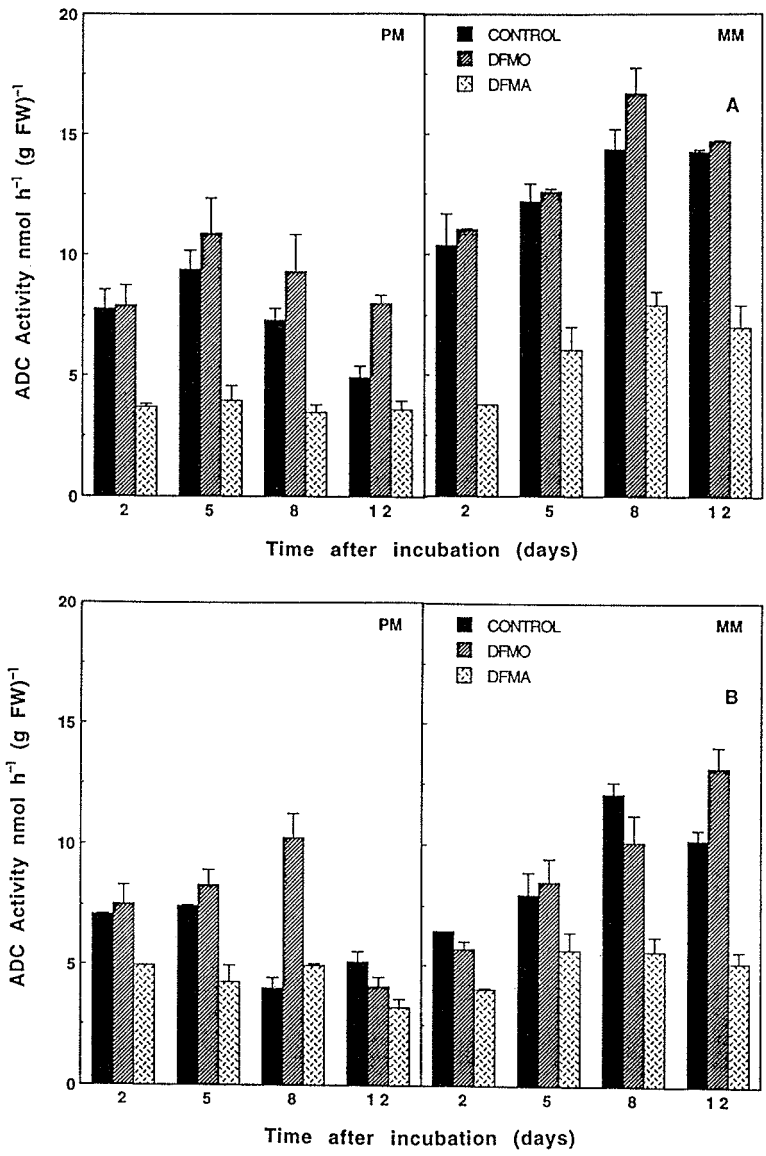


Figure 4. Effect of 2.0 mM DFMO and 0.1 mM DFMA on cellular levels of arginine decarboxylase (ADC) activity in (A) *Picea abies* and (B) *Picea rubens* tissues grown on proliferation (PM) or maturation (MM) medium for different lengths of time. Values are mean $\pm$ SE of 4 replicates for *Picea abies* and 3 replicates for *Picea rubens*. (Minocha *et al.*, unpublished data.)

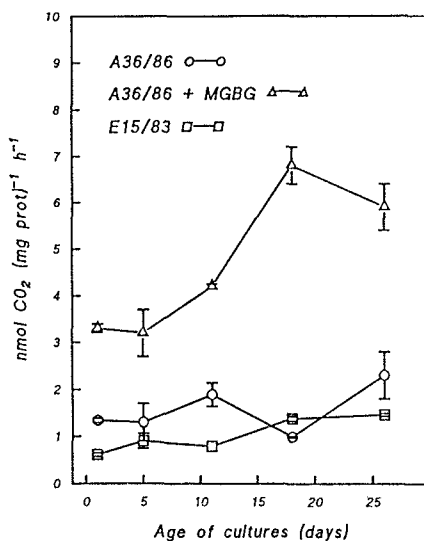


Figure 5. SAMDC activities in callus cultures of *P. abies* during 26 days of culture on ABA-containing medium A36/38 embryogenic callus, E15/83 non-embryogenic callus. Values are mean $\pm$ SE of three replicates. (Santanen and Simola, 1992.)

Hevea brasiliensis tissue by El Hadrami and D'Auzac (1992), their results on the effects of DFMO and DFMA indicate that ADC also is the main enzyme responsible for putrescine biosynthesis in this tissue. In contrast, in *Solanum melongena* and other solanaceous species, ODC seems to play a major role in putrescine biosynthesis (Slocum and Galston, 1985; Fobert and Webb, 1987). In the case of carrot embryogenic suspensions, ADC was the predominant enzyme during the first 10 days after culture and its inhibition by DFMA inhibited both putrescine levels and somatic embryogenesis. However, by day 12 when mature green embryos had appeared in these cultures, ODC was detected and showed a steady increase thereafter, accompanying the growth of these embryos (Robie and Minocha, 1989).

The activity of SAMDC also increased with time in embryogenic cultures of *Picea abies* grown on the maturation medium (Fig. 5). The specific activity of SAMDC was slightly higher in embryogenic cultures than in non-embryogenic cultures except for day 18. It is difficult to explain the increase in ADC and SAMDC activities in tissue grown on the maturation medium where overall polyamine levels as well as growth rate were lower than in the pro-embryogenic callus cultures on the proliferation medium. A further complication arises from the fact that somatic embryos constitute only a small proportion of the total tissue mass. While the somatic embryos are showing active growth, the remainder of the tissue mass is not. Since the enzyme and polyamine analysis were done on total tissue, the observed results are probably representative of the slow-growing tissue. Studies are

underway to separately analyze enzyme activities in the somatic embryos and the surrounding tissue.

In the cotyledons of *Pinus radiata*, the activity of SAMDC increased sharply within the first 2 days of culture. This was followed by a steady decline during the next 8 days (Biondi *et al.*, 1988). This change coincided with change in the rate of conversion of ^{14}C -putrescine into spermidine and spermine. However, dicyclohexylamine, which inhibited the production of spermidine in this tissue, had little effect on measurable SAMDC activity during the entire 10-day culture period. No enzyme activities have been reported for *Hevea* or *Mangifera*.

8. Polyamine Degradation and Interconversions

The uptake and metabolism of putrescine has been studied in *Pinus radiata* cotyledons grown on shoot forming and non shoot-forming media (Kumar and Thorpe, 1989). Most of the ^{14}C -putrescine was metabolized into γ -aminobutyric acid (GABA), aspartic acid and glutamic acid. A similar fast metabolic conversion of ^{14}C -putrescine into GABA was reported in other higher plants (Flores and Filner, 1985). Only a small proportion (2 to 9 percent) of ^{14}C appeared in spermidine and spermine. The conversion of ^{14}C -putrescine into spermidine and spermine increased by 4 to 5 fold in the non shoot-forming cotyledons on days 3 and 10. However, overall catabolism of putrescine was highest in the shoot forming cotyledons where only 17 percent of the label was still in putrescine after 3 days as compared to 33 percent in the non shoot-forming cotyledons at the same time. A similar slow conversion of putrescine into spermidine and spermine in the same tissue was also reported earlier by Biondi *et al.* (1988). In their studies, a peak of incorporation of ^{14}C -putrescine into spermidine and spermine was observed on day 2 in the presence of BA and on day 5 in the absence of BA. Dicyclohexylamine inhibited the conversion of putrescine into both spermidine and spermine and also inhibited the uptake of putrescine. However, the activity of SAMDC in the tissue was not significantly affected by the presence of DCHA in the medium. Biondi *et al.* (1986) had earlier shown that treatment of cotyledons with DCHA prevented the accumulation of spermidine and spermine in the tissue during the 10-day culture period.

Catabolism of putrescine and spermidine in embryogenic and non-embryogenic tissues of *Picea abies* was studied by Santanen and Simola (1994) using (1,4- ^{14}C)-putrescine and (1,4- ^{14}C)-spermidine as substrates. Except for day 1, both putrescine oxidation and spermidine oxidation rates increased with time of subculture. Activity was highest toward the end of growth period and also in later stages of embryo development (Fig. 6). Because both putrescine and aminopropylpyrrolidine were formed by the degradation of spermidine in non-embryogenic tissue (Santanen and Simola, 1994), it was suggested that there might be two separate routes for spermidine oxidation

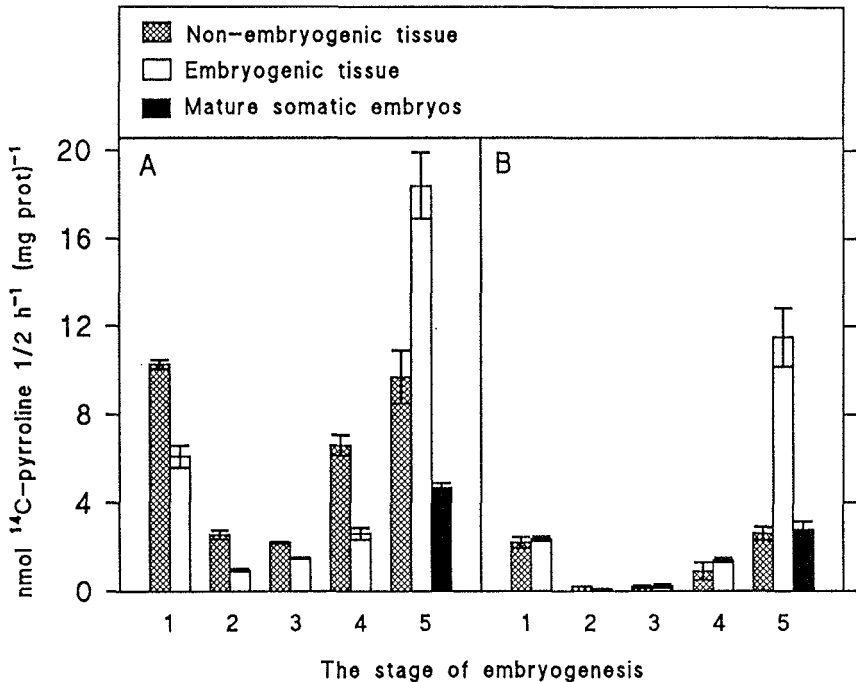


Figure 6. Activities of putrescine and spermidine oxidative enzymes in embryogenic and non-embryogenic tissues of *P. abies* (pH 8) during 5 stages of somatic embryogenesis in embryogenic tissue. At developmental stage 5, enzyme activity in somatic embryos and non-differentiated tissue were assayed separately. (A) DAO activity; (B) PAO activity. Values are mean $\pm$ SE of three replicates. (Modified from Santanen and Simola, 1994.)

in spruce tissue; one to aminopropylpyrroline by DAO and a second to putrescine and 3-aminopropanal by PAO, as reported earlier in fungal cells (Kobayashi and Horikoshi, 1982; Bagni and Pistocchi, 1992). In general, putrescine showed about a 5 times higher rate of degradation as compared to spermidine in both types of cultures. Maturing embryos showed much lower rates of putrescine and spermidine oxidation as compared to the non-differentiating embryogenic tissue growing on the same maturation medium (Fig. 6). In nonembryogenic tissue, oxidation of putrescine and spermidine by DAO and PAO proceeded via the formation of pyrroline intermediate, since pyrroline dehydrogenase activity also was observed in these cultures. Both DAO and PAO activities were inhibited in *Picea abies* by 1.0 mM aminoguanidine. Whereas the highest level of spermidine oxidation activity was located in cell wall fraction, the location of putrescine oxidation activity varied between cell wall, supernatant fraction and the homogenates (Santanen and Simola, 1994). The rather high polyamine degradation activity in aging embryogenic tissue (non-differentiated cells growing on maturation

medium, Fig. 6) is not consistent with observations on some other tissues. The dissimilar nitrogen metabolism, developmental stage and physiological state of the tissue may explain why some tissues have low activity (ageing leaves, Kaur-Sawhney *et al.*, 1981) while others have high activity (intensively dividing tuber cells of *Helianthus tuberosus*, Torrigiani *et al.*, 1989).

9. Conclusions

The paucity of information on the metabolism of polyamines in cell cultures of woody plants makes it difficult to draw conclusions on their role in somatic embryogenesis. Further, the fact that different laboratories have reported results in different units (see Table 1) makes it nearly impossible to compare the results of these studies. However, analysis of results of the published studies with herbaceous plants leaves no doubt as to the importance of polyamine metabolism in the development of somatic embryos. In-depth studies are needed with well-established embryogenic cultures of conifers and hardwoods to establish the role of polyamines in the process of somatic embryogenesis in these species. Nevertheless, manipulation of polyamine biosynthetic pathways by use of inhibitors and transgenic techniques has demonstrated that somatic embryogenesis can be affected through polyamine metabolism in a number of unrelated species. This should lead to greater success in obtaining somatic embryogenesis in recalcitrant woody species, and enhancing it in those that show somatic embryogenesis at a low frequency.

References

- Ammirato, P.V., 1984. Induction, maintenance, and manipulation of development in embryogenic cell suspension cultures. In: I.K. Vasil (Ed.), *Cell Culture and Somatic Cell Genetics of Plants*, Vol. 1, pp. 139–151. Academic Press, New York.
- Bachrach, U. and Y.M. Heimer (Eds.), 1989a. *The Physiology of Polyamines*, Vol. 1. CRC Press, Boca Raton, FL.
- Bachrach, U. and Y.M. Heimer (Eds.), 1989b. *The Physiology of Polyamines*, Vol. 2. CRC Press, Boca Raton, FL.
- Bagga, S., A. Dharma, G.C. Phillips and G.D. Kuehn, 1991. Evidence for the occurrence of polyamine oxidase in the dicotyledonous plant *Medicago sativa* L. (alfalfa). *Plant Cell Rep.* 10: 550–554.
- Bagni, N. and R. Pistocchi, 1992. Polyamine metabolism and compartmentation in plant cells. In: K. Mengel and J. Bilbeam (Eds.), *Nitrogen Metabolism of Plants*, pp. 229–248. Oxford Science Publications, Oxford, UK.
- Bhojwani, S.S. and M.K. Razdan, 1983. *Plant Tissue Culture: Theory and Practice*, pp. 91–112; 199–236. Elsevier, New York.
- Biondi, S., N. Bagni and A. Sansovini, 1986. Dicyclohexylamine uptake and effects on polyamine content in cultured cotyledons of radiata pine. *Physiol. Plant.* 66: 41–45.
- Biondi, S., T. Diaz, I. Iglesias, G. Gamberini and N. Bagni, 1990. Polyamines and ethylene in relation to adventitious root formation in *Prunus avium* shoot cultures. *Physiol. Plant.* 78: 475–483.

- Biondi, S., P. Torrigiani, A. Sansovini and N. Bagni, 1988. Inhibition of polyamine biosynthesis by dicyclohexylamine in cultured cotyledons of *Pinus radiata*. *Physiol. Plant.* 72: 471–476.
- 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.
- Cohen, S.S., R. Ballint and R.K. Sindhu, 1981. The synthesis of polyamines from methionine in intact and disrupted leaf protoplasts of virus-infected chinese cabbage. *Plant Physiol.* 68: 1150–1155.
- De Jong, A.J., E.D.L. Schmidt and S.C. DeVries, 1993. Early events in higher plant embryogenesis. *Plant Mol. Biol.* 22: 367–377.
- Dohmen, G.P., A. Koppers and C. Langebartels, 1990. Biochemical response of Norway spruce (*Picea abies* (L.) Karst.) towards 14 month exposure to ozone and acid mist: Effects on amino acid, glutathione and polyamine titers. *Environ. Pollut.* 64: 375–383.
- Durzan, D.J., 1987. Physiological states and metabolic phenotypes in embryonic development. In: J.M. Bonga and D.J. Durzan (Eds.), *Cell and Tissue Culture in Forestry*, Vol. 2, pp. 405–439. Martinus Nijhoff Publishers, Dordrecht.
- El Hadrami, I. and J. D'Auzac, 1992. Effects of polyamine biosynthetic inhibitors on somatic embryogenesis and cellular polyamines in *Hevea brasiliensis*. *J. Plant Physiol.* 140: 33–36.
- Evans, P.T. and R.L. Malmberg, 1989. Do polyamines have roles in plant development? *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40: 235–269.
- Federico, R. and R. Angelini, 1991. Polyamine catabolism in plants. In: R.D. Slocum and H.E. Flores (Eds.), *Biochemistry and Physiology of Polyamines in Plants*, pp. 41–56. CRC Press, Boca Raton, FL.
- 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.
- Flores, H.E. and P. Filner, 1985. Polyamine catabolism in higher plants: characterization of pyrroline dehydrogenase. *Plant Growth Reg.* 3: 277–291.
- Flores, H.E. and A.W. Galston, 1984. Osmotic stress-induced polyamine accumulation in cereal leaves. *Plant Physiol.* 75: 102–109.
- Flores, H.E. and J. Martin-Tanguy, 1991. Polyamines and plant secondary metabolites. In: R.D. Slocum and H.E. Flores (Eds.), *Biochemistry and Physiology of Polyamines in Plants*, pp. 57–76. CRC Press, Boca Raton, FL.
- Fobert, P.R. and D.T. Webb, 1987. Effects of polyamines, polyamine precursors, and polyamine biosynthetic inhibitors on somatic embryogenesis from eggplant (*Solanum melongena*) cotyledons. *Can. J. Bot.* 66: 1734–1742.
- Galston, A.W., 1983. Polyamines as modulators of plant development. *Bioscience* 33: 382–388.
- Galston, A.W. and H.E. Flores, 1991. Polyamines and plant morphogenesis. In: R.D. Slocum and H.E. Flores (Eds.), *Biochemistry and Physiology of Polyamines in Plants*, pp. 175–186. CRC Press, Boca Raton, FL.
- Galston, A.W. and R. Kaur-Sawhney, 1987. Polyamines as endogenous growth regulators. In: P.J. Davies (Ed.), *Plant Hormones and Their Role in Plant Growth and Development*, pp. 280–295. Kluwer Academic Publishers, Dordrecht.
- Goldberg, R.B., S.J. Baker and L. Perez-Grau, 1989. Regulation of gene expression during plant embryogenesis. *Cell* 56: 149–160.
- Grey, D., G. Stepan-Sarkisian and M.W. Fowler, 1987. Biochemistry of forest tree species in culture. In: J.M. Bonga and D.J. Durzan (Eds.), *Cell and Tissue Culture in Forestry*, Vol. 2, pp. 31–60. Martinus Nijhoff Publishers, Dordrecht.
- Hakman, I., 1993. Embryology in Norway spruce (*Picea abies*): An analysis of the composition of seed storage proteins and deposition of storage reserves during seed development and somatic embryogenesis. *Physiol. Plant.* 87: 148–159.
- Hakman, I. 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.
- Hiatt, A.C., J. McIndoo and R.L. Malmberg, 1986. Regulation of polyamine biosynthesis in tobacco. *J. Biol. Chem.* 261: 1293–1298.
- Huhtinen, O., J. Honkanen and L.K. Simola, 1982/83. Ornithine- and putrescine-supported divisions and cell colony formation in leaf protoplasts of alders (*Alnus glutinosa* and *A. incana*). *Plant Sci. Lett.* 28: 3–9.
- Kaur-Sawhney, R., H.E. Flores and A.W. Galston, 1981. Polyamine oxidase in oat leaves: A cell wall-localized enzyme. *Plant Physiol.* 68: 494–498.
- Khan, A.J. and S.C. Minocha, 1989a. Biosynthetic arginine decarboxylase in phytopathogenic fungi. *Life Sci.* 44: 1215–1222.
- Khan, A.J. and S.C. Minocha, 1989b. Polyamine biosynthetic enzymes and the effect of their inhibition on the growth of some phytopathogenic fungi. *Plant Cell Physiol.* 30: 655–663.
- Khan, A.J. and S.C. Minocha, 1991. Polyamines and somatic embryogenesis in carrot. II. The effects of cyclohexylammonium phosphate. *J. Plant Physiol.* 137: 446–452.
- Kobayashi, Y. and K. Horikoshi, 1982. Purification and characterization of extracellular polyamine oxidase produced by *Penicillium* sp. *Biochim. Biophys. Acta.* 705: 133–138.
- Krishnamurthy, R. and K.A. Bhagwat. 1989. Polyamines as modulators of salt tolerance in rice cultivars. *Plant Physiol.* 91: 500–504.
- Kumar, P.P. and T.A. Thorpe, 1989. Putrescine metabolism in excised cotyledons of *Pinus radiata* cultured *in vitro*. *Physiol. Plant.* 76: 521–526.
- Kushad, M.M. and G. Yelenosky, 1987. Evaluation of polyamine and proline levels during low temperature acclimation of citrus. *Plant Physiol.* 84: 692–695.
- Litz, R.E. and B. Schaffer, 1987. Polyamines in adventitious and somatic embryogenesis in mango (*Mangifera indica* L.). *J. Plant Physiol.* 128: 251–258.
- Malmberg, R.L. and A.C. Hiatt, 1989. Polyamines in plant mutants. In: U. Bachrach and Y.M. Heimer (Eds.), *The Physiology of Polyamines*, Vol. 2, pp. 148–159. CRC Press, Boca Raton, FL.
- McCann, P.P., A.E. Pegg and A. Sjoerdsma (Eds.), 1987. *Inhibition of Polyamine Metabolism*, pp. 1–371. Academic Press, New York.
- Meijer, E.G.M. and J. Simmonds, 1988. Polyamine levels in relation to growth and somatic embryogenesis in tissue cultures of *Medicago sativa* L. (alfalfa). *J. Exp. Bot.* 39: 787–794.
- Mengoli, M., N. Bagni, G. Luccarini, V. Nuti Ronchi and D. Serafini-Fracassini, 1989. *Daucus carota* cell cultures: Polyamines and effect of polyamine biosynthesis inhibitors in the pre-embryogenic phase and different embryo stages. *J. Plant Physiol.* 134: 389–394.
- Minocha, R., H. Kvaalen, S.C. Minocha and S. Long, 1993. Polyamines in embryogenic cultures of Norway spruce (*Picea abies*) and red spruce (*Picea rubens*). *Tree Physiol.* 13: 365–377.
- Minocha, R., S.C. Minocha, A. Komamine and W.C. Shortle, 1991a. Regulation of DNA synthesis and cell division by polyamines in *Catharanthus roseus* suspension cultures. *Plant Cell Rep.* 10: 126–130.
- Minocha, R., S.C. Minocha, S. Long and W.C. Shortle, 1992. Effects of aluminum on DNA synthesis, cellular polyamines, polyamine biosynthetic enzymes, and inorganic ions in cell suspension cultures of a woody plant, *Catharanthus roseus*. *Physiol. Plant.* 85: 417–424.
- Minocha, S.C., 1988. Relationship between polyamine and ethylene biosynthesis in plants and its significance for morphogenesis in cell cultures. In: V. Zappia and A.E. Pegg (Eds.), *Progress in Polyamine Research*, pp. 601–606. Plenum Publishing Corporation, New York.
- Minocha, S.C., R. Minocha and A. Komamine, 1991b. Effects of polyamine biosynthesis inhibitors on s-adenosylmethionine synthetase and s-adenosylmethionine decarboxylase activities in carrot cell cultures. *Plant Physiol. Biochem.* 29: 231–237.
- 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.
- Minocha, S.C., N.S. Papa, A.J. Khan and A.I. Samuelsen, 1991c. Polyamines and somatic

- embryogenesis in carrot III. Effects of methylglyoxal bis(guanylhydrazone). *Plant Cell Physiol.* 32: 395–402.
- 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 expression in conifer embryogenesis and germination II. Crystalloid protein synthesis in the developing embryo and megagametophyte of white spruce (*Picea glauca* (Moench) Voss). *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. II. Changes in arginine decarboxylase activity. *Plant Physiol.* 63: 341–345.
- Montague, M.J., J.W. Koppenbrink and E.G. Jaworski, 1978. Polyamine metabolism in embryogenic cells of *Daucus carota*. I. Changes in intracellular content and rates of synthesis. *Plant Physiol.* 62: 430–433.
- Nissen, P. and S.C. Minocha, 1993. Inhibition by 2,4-D of somatic embryogenesis in carrot as explored by its reversal by difluoromethylornithine. *Physiol. Plant.* 89: 673–680.
- Pegg, A.E., 1986. Recent advances in the biochemistry of polyamines in eukaryotes. *Biochem. J.* 234: 249–262.
- Phillips, G. and G. Kuehn, 1991. Uncommon polyamines in plants and other natural sources. In: R.D. Slocum and H.E. Flores (Eds.), *Biochemistry and Physiology of Polyamines in Plants*, pp. 121–136. CRC Press, Boca Raton, FL.
- Phillips, R., M.C. Press and A. Eason, 1987. Polyamines in relation to cell division and xylogenesis in cultured explants of *Helianthus tuberosus*: Lack of evidence for growth regulatory action. *J. Expt. Bot.* 38: 164–172.
- Predieri, S., D.T. Krizek, C.Y. Wang, R.M. Mirecki and R.H. Zimmerman, 1993. Influence of UV-B radiation on developmental changes, ethylene, CO₂ flux and polyamines in cv. Doyenne d'Hiver pear shoots grown *in vitro*. *Physiol. Plant.* 87: 109–117.
- Raghavan, V., 1986. Somatic embryogenesis. *Embryogenesis in Angiosperms*, pp. 115–151. Cambridge Univ. Press, Cambridge, UK.
- Rastogi, R. and P.J. Davies, 1991. The effects of light and plant growth regulators on polyamine metabolism in higher plants. In: R.D. Slocum and H.E. Flores (Eds.), *Biochemistry and Physiology of Polyamines in Plants*, pp. 187–199. CRC Press, Boca Raton, FL.
- Roberts, D.R., M.A. Walker, J.E. Thompson and E.B. Dumbroff, 1984. The effects of inhibitors of polyamine and ethylene biosynthesis on senescence, ethylene production and polyamine levels in cut carnation flowers. *Plant Cell Physiol.* 25: 315–322.
- Robie, C.A. and S.C. Minocha, 1989. Polyamines and somatic embryogenesis in carrot. I. The effects of difluoromethylornithine and difluoromethylarginine. *Plant Sci.* 65: 45–54.
- Santanen, A. and L.K. Simola, 1992. Changes in polyamine metabolism during somatic embryogenesis in *Picea abies*. *J. Plant Physiol.* 140: 475–480.
- Santanen, A. and L.K. Simola, 1994. Catabolism of putrescine and spermidine in embryogenic and non-embryogenic callus lines of *Picea abies*. *Physiol. Plant.* 90: 125–129.
- Sharp, W.R., M.R. Sondhal, L.S. Caldas and S.B. Maraffa, 1980. The physiology of *in vitro* asexual embryogenesis. *Hort. Rev.* 2: 268–310.
- Simola, L.K., 1974a. The ultrastructure of dry and germinating seeds of *Pinus sylvestris* L. *Acta Bot. Fenn.* 103: 1–31.
- Simola, L.K., 1974b. Subcellular organization of cotyledons of *Picea abies* during germination. *Port. Acta Biol., Ser. A*, 14: 413–428.
- Simola, L.K., 1975. Changes in the subcellular organization of endosperm and radicle cells of *Picea abies* during germination. *Z. Pflanzenphysiol.* 78: 41–51.
- Simola, L.K. and J. Honkanen, 1983. Organogenesis and fine structure in megagametophyte callus lines of *Picea abies*. *Physiol. Plant.* 59: 551–561.
- Simola, L.K. and O. Huhtinen, 1986. Growth, differentiation, and ultrastructure of microspore callus of *Picea abies* as affected by nitrogenous supplements and light. *N.Z. J. For. Sci.* 16: 357–368.

- Simola, L.K. and A. Santanen, 1990. Improvement of nutrient medium for growth and embryogenesis of megagametophyte and embryo callus lines of *Picea abies*. *Physiol. Plant.* 80: 27–35.
- Slocum, R.D. and H.E. Flores, 1991. *Biochemistry and Physiology of Polyamines in Plants*, pp. 1–264. CRC Press, Boca Raton, FL.
- Slocum, R.D. and A.W. Galston, 1985. *In vivo* inhibition of polyamine biosynthesis and growth in tobacco ovary tissue. *Plant Cell Physiol.* 26: 1519–1526.
- Slocum, R.D., R. Kaur-Sawhney and A.W. Galston, 1984. The physiology and biochemistry of polyamines in plants. *Arch. Biochem. Biophys.* 235: 283–303.
- Smith, M.A., 1991a. Chromatographic methods for the identification and quantitation of polyamines. In: R.D. Slocum and H.E. Flores (Eds.), *Biochemistry and Physiology of Polyamines in Plants*, pp. 229–242. CRC Press, Boca Raton, FL.
- Smith, T.A., 1985. Polyamines. *Ann. Rev. Plant Physiol.* 36: 117–143.
- Smith, T.A., 1991b. A historical perspective on research in plant polyamine biology. In: R.D. Slocum and H.E. Flores (Eds.), *Biochemistry and Physiology of Polyamines in Plants*, pp. 1–22. CRC Press, Boca Raton, FL.
- Smith, T.A., J. Negrel and C. Bird, 1983. The cinnamic acid amides of the di- and polyamines. *Adv. Polyamine Res.* 4: 347–369.
- Street, H.E., 1977. Embryogenesis and chemically induced organogenesis. In: W.R. Sharp, P.O. Larsen, E.F. Paddock and V. Raghavan (Eds.), *Plant Cell and Tissue Culture Principles and Applications*, pp. 123–153. Ohio State University Press, Columbus, OH.
- Tautorus, T.E., L.C. Fowke and D.I. Dunstan, 1991. Somatic embryogenesis in conifers. *Can. J. Bot.* 69: 1873–1899.
- 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.
- Tiburcio, A.F., R. Kaur-Sawhney and A.W. Galston, 1987. Effect of polyamine biosynthetic inhibitors on alkaloids and organogenesis in tobacco callus cultures. *Plant Cell Tiss. Org. Cult.* 9: 111–120.
- Torget, R., L. Lapi and S.S. Cohen, 1979. Synthesis and accumulation of polyamines and S-adenosylmethionine in chinese cabbage infected by turnip yellow mosaic virus. *Biochem. Biophys. Res. Commun.* 87(4): 1132–1139.
- Torné, J.M., I. Claparols, X. Figueras and M. Santos, 1993. Effect of DL- α -difluoromethyl-ornithine pretreatments in maize callus differentiation. *Plant Cell Physiol.* 34: 371–374.
- Torrigiani, P., D. Serafini-Fracassini and A. Fara, 1989. Diamine oxidase activity in different physiological stages of *Helianthus tuberosus* tuber. *Plant Physiol.* 89: 69–73.
- Villanueva, V.R., M. Mardon, Th.M. Le Goff and F. Moncelon, 1987. Development of a multi-component analysis system. Application and preliminary results of a comparative study of cellular metabolism in healthy and damaged *Picea* trees from polluted areas. *J. Chromatogr.* 393: 97–105.
- Wang, C.Y. and L.J. Zug, 1989. Effect of low-oxygen storage on chilling injury and polyamines in zucchini squash. *Sci. Hort.* 39: 1–7.
- 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.
- Young, N.D. and A.W. Galston, 1983. Putrescine and acid stress: Induction of arginine decarboxylase activity and putrescine accumulation by low pH. *Plant Physiol.* 71: 767–771.