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Effects of Polyamines on Shoot and Root Development in *Arabidopsis* Seedlings and Carnation Cultures

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Polyamines, a class of aliphatic amines, are active in the growth and development of bacteria, animals, and plants. To better understand their physiological role in plants, we used spermidine and the inhibitor of SAMDC to analyze the developmental patterns of roots and shoots from *Arabidopsis* seedlings and in-vitro carnation shoot cultures. We also monitored mRNA levels of the polyamine biosynthetic gene after adding various phytohormones to the growing media. Treating wild-type *Arabidopsis* seedlings with polyamine resulted in greater growth after four weeks; 1 mM spermidine increased root lengths by 39% and seedling weights by 44%. Spermidine was more effective in enhancing root growth in the *det2* mutants than in the wild-type plants. In contrast, MGBG, an irreversible inhibitor of SAMDC activity, resulted in root lengths that were 29% of the controls, as well as seedling weights that were only 77% of normal. In our carnation cultures, shoot-growth rates were severely reduced by both spermidine and MGBG; root growth was also markedly inhibited by treatment with spermidine. Transcripts of *ADC* and *SAMDC* were significantly greater in cultures treated with kinetin and IAA, with levels being higher with the former. Previous research has shown that sensitivity and the appropriate cellular content for polyamines vary among plant species, and may also be organ- or tissue-specific. Polyamines may play an important developmental role because of their potentially stimulatory effects on phytohormonal signaling and/or synergism.

Keywords: *Arabidopsis*, arginine decarboxylase (ADC), carnation (*Dianthus*), methyl glyoxal *bis*-guanylhydrazone (MGBG), phytohormone, polyamine biosynthetic gene, root growth, S-adenosylmethionine decarboxylase (SAMDC), shoot development, spermidine

Polyamines, a class of aliphatic amines, have been implicated in a wide range of growth and developmental processes in bacteria, animals, and plants. Although they are known to be involved in cell proliferation in both prokaryotes and eukaryotes, their exact physiological role has not been firmly established. Auvinen et al. (1992) have suggested that the gene in animals that encodes ornithine decarboxylase, by which ornithine is converted to putrescine, may be a proto-oncogene. In addition to research that describes the involvement of polyamines in DNA, RNA, and protein synthesis, Ficker et al. (1994) and Fakler et al. (1995) have reported specific interactions with certain potassium channels and glutamate receptors. In animal systems, the movement of polyamines is via specific transporters, and is energy-dependent (Seiler and Dezeure, 1990). Recent biochemical and physiological research data also suggest that they could be transported through the xylem and phloem (Antognoni et al., 1998). Regardless of whether polyamines can be considered as phytohormones, the identification of such a transport system should provide new insights into their actions in plant cells (Hanzawa et al., 2000).

Watson et al. (1998) have observed greater polyamine biosynthetic activities in a wide range of developmental processes, including embryogenesis, root development, flowering, and leaf senescence. In addition, Li and Chen (2000) have reported that polyamine contents in higher plants can increase in response to various environmental factors, such as acid-, osmotic-, or salinity stresses.

The most common polyamines are the diamines (putrescine and cadaverine) and higher polyamines (spermidine and spermine), all of which are synthesized from basic amino acids. Putrescine is formed from either ornithine decarboxylase or arginine, via arginine decarboxylase. Decarboxylated S-adenosylmethionine (dcSAM), synthesized from S-adenosylmethionine (SAM) by SAM decarboxylase (SAMDC), is used as an aminopropyl-group donor, first by spermidine synthase to form spermidine, and then by spermine to produce spermine. Because the level of dcSAM in living organisms is very low, and because SAMDC has a relatively short half-life (about 1 to 2 h; Li and Chen, 2000), SAMDC is a rate-limiting enzyme in polyamine biosynthesis (Mehta et al., 2002).

Lee et al. (1997b) have suggested that polyamines act as anti-senescence regulators by inhibiting in-vitro ethylene biosynthesis in flowers. SAMDC mRNA accumulates to greater amounts under high rather than low irradiance (Park et al., 2001). In addition, regulated

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expression of antisense SAMDC in the potato changes polyamine levels and results in abnormal phenotypes (Kumar et al., 1996). When antisense expression of ethylene biosynthetic genes induces higher accumulations of polyamines, abiotic stress tolerance is also enhanced (Wi and Park, 2002).

These results are now beginning to reveal direct evidence of a physiological role for polyamines in plant metabolism. Therefore, the objective of our study was to better understand their function in plants. To do this, we analyzed the developmental patterns of roots and shoots in *Arabidopsis* seedlings and carnation cultures by monitoring the effects of spermidine and an inhibitor of SAMDC. We also determined mRNA levels for the SAMDC gene after various phytohormones were added to the growth media.

MATERIALS AND METHODS

Plant Material

Seeds of both the wild-type *Arabidopsis thaliana* Columbia ecotype and the *det2* mutant were surface sterilized for 5 min in 70% EtOH and for 10 min in 5% bleach, then rinsed at least seven times. After being incubated at 4°C for 2 d, they were placed on Petri dishes containing a solid growth medium supplemented with half-strength MS salts, 0.8% agar, and 0.5% sucrose. All seedlings were then grown for four weeks at 25°C under a 16-h photoperiod, which was provided by cool-white fluorescent lamps with an intensity of 150 μmol m^{-2} s^{-1}. Media for the treated seedlings were supplemented with 1 mM of spermidine or 1 mM methyl glyoxal *bis*-guanylhydrazone (MGBG), an irreversible inhibitor of SAMDC activity (Lee et al., 1997a). Control seedlings were grown without those additional chemicals.

For our second plant type, shoot cultures of wild carnation (*Dianthus caryophyllus* L. cv. White Sim) were grown in a Murashige and Skoog (MS) medium supplemented with 3% sucrose, 0.5 mg L^{-1} IAA (indole-3-acetic acid), and 2 mg L^{-1} kinetin, and were transferred every four weeks. To assess shoot and root development, we moved 10-d-old cultures either into a shoot induction medium supplemented with 0.5 mg L^{-1} IAA and 2 mg L^{-1} kinetin for five weeks, or into a rooting medium supplemented with 0.5 mg L^{-1} NAA (naphthalene acetic acid) for four weeks. To determine the specific effect of these phytohormones (auxin, cytokinin and gibberellin (GA)) on shoot or root growth, we precultured the tissues on a hormone-free MS medium for 4 d.

Analyzing Expression of Polyamine Biosynthetic Genes

We extracted total RNA from carnation leaves with guanidine HCl, following the method of Sambrook et al. (1989). Reverse transcription was performed with 1 μg of total RNA, an oligo(dT) primer, and *Bca*PLUS Rtase, according to the manufacturer's protocol (TaKaRa, Japan). The amounts of cDNAs were standardized with PCR for their actin contents, using gene-specific primers from the database entry for tobacco actin (GenBank Accession No. X63603). We also designed gene-specific primers of *SAM synthetase*, *ADC* (*arginine decarboxylase*), and *SAMDC* with conserved regions, to compare with gene entries in GenBank. For quantitative PCR, we added an accurate amount of the reverse transcriptase reaction mixture to 100 μL of the PCR mixture that contained 10 pmol of each primer. Templates were amplified by incubating the reactions for 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The products were separated electrophoretically on 1% agarose gels and transferred to a positively charged nylon membrane (Hybond-N+, Amersham, UK). Southern hybridization was then carried out for 18 h in a prehybridization solution after adding 5×10^5 cpm mL^{-1} of a denatured ^{32}P-labeled cDNA probe. For our probe, cDNA fragments of carnation *SAM synthetase*, *ADC*, and *SAMDC* were separately labeled using a Random Priming Labeling kit (Boehringer Mannheim, Germany) with [α-^{32}P] dCTP (3000 Ci $mmol^{-1}$). Following hybridization, the membranes were washed twice for 15 min with 2× SSC containing 0.1% SDS, and twice for 15 min with a solution of 25 mM $NaHPO_4$, 1 mM EDTA (pH 8.0), and 0.1% SDS. All washes were carried out at 52°C. Finally, the membranes were exposed to Fuji X-ray film with an intensifying screen at −70°C for appropriate exposure.

Determining SAMDC Activity

SAMDC activity was measured in our carnation shoot cultures according to the procedure described by Park and Lee (1994). Both crude tissue extracts and translated products served as enzyme sources, and were reacted with 0.1 μL of [carboxyl-^{14}C]SAM.

RESULTS

Effects of Spermidine and MGBG on Seedling Growth of *A. thaliana*

After the *Arabidopsis* wild-type seedlings had been treated for four weeks with 1 mM spermidine, root

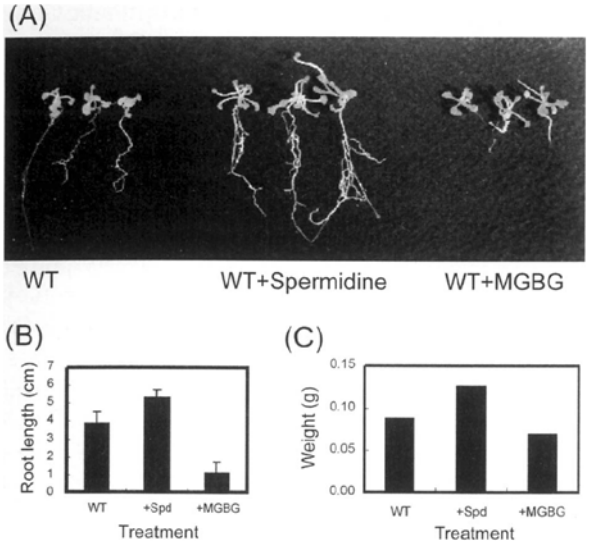

Figure 1. Effects of spermidine and MGBG on *A. thaliana* seedling development and organogenesis. **A.** Wild-type seedlings (WT, Columbia ecotype) were grown for 4 weeks on ½-MS salts media containing 1mM spermidine or 1 mM MGBG. For the control, WT seedlings were grown on media without supplemental chemicals. **B.** Mean root length and fresh weight per seedling when grown in the absence (control) or in the presence of spermidine or MGBG. Error bars represent standard deviation.

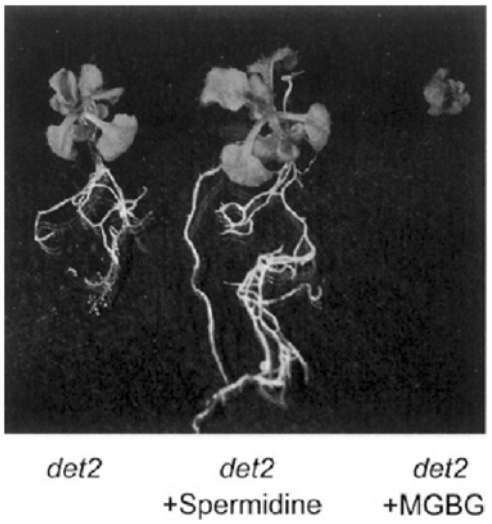

Figure 2. Effects of spermidine and MGBG *A. thaliana det2* seedling development and organosis. Mutant seedlings were grown for 4 weeks on ½-MS salts media containing either 1 mM spermidine or 1 mM MGBG. For the control, the seedlings were grown on media without supplemental chemicals.

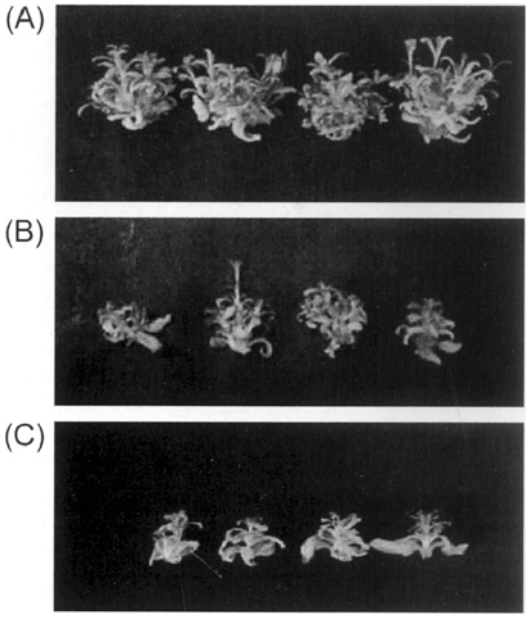

Figure 3. Effects of spermidine and MGBG on shoot Carnation cultures were grown for 5 weeks on SIM supplemented with 0.5 mg L^{-1} IAA and 2 mg L^{-1} kinetin. **A.** For the control, cultures were grown on media without supplemental chemicals. **B.** Cultures grown in the presence of 1 mM spermidine. **C.** Cultures grown in the presence of 1 mM MGBG.

lengths were increased by 39% over the control; seedling weights, by 44% (Fig. 1B). In contrast, treatment with 1 mM of the inhibitor MGBG resulted in root lengths that were only 29% of the control, and seedling weights that were only 77% of normal. Nevertheless, germination of the *Arabidopsis* seeds was not significantly affected by treatment with either spermidine or MGBG (data not shown). Therefore, we suggest that polyamine is required for optimal seedling growth, but not for germination. We also found that MGBG severely inhibited the growth of shoots and completely halted root development in four-week-old *det 2* mutants, a process that was effectively blocked during the second step of brassinosteroid biosynthesis (Fujioka et al., 1997) (Fig. 2). However, seedlings treated with spermidine showed significantly increased root growth and development of lateral roots.

Effects of Spermidine and MGBG on Organogenesis of Carnation Cultures

Organogenesis of carnation shoot cultures was determined after five weeks of incubation in media supplemented with 1 mM spermidine (Fig. 3). When cultures that had been grown on a shoot-induction medium (SIM) were transferred onto the same medium type now supplemented with the polyamine, the formation of multiple shoots was significantly decreased,

Table 1. Effects on morphogenesis of carnation cultures in shoot-induction media supplemented with 0.5 mg L^{-1} IAA and 2 mg L^{-1} kinetin, where tissues were held for four weeks in the presence of 1 mM spermidine or 1 mM MGBG. For the control, cultures were grown on media without supplemental chemicals. Number of shoots is the mean number of emerging shoots from each explant. Values are mean ± SD.

Treatment	Number of shoots	Length (cm)	Weight (g)
Control	26.25±5.59	5.05±0.73	9.24±2.00
Spermidine	13.33±3.23	3.32±0.58	3.30±0.94
MGBG	9.13±2.25	2.59±0.76	2.35±0.67

to 61% of that in the control (Table 1). Likewise, the addition of 1 mM MGBG blocked polyamine biosynthesis in the SIM and disrupted shoot formation (Fig. 3), with the number of new shoots amounting to only 44% of normal production (Table 1). Shoot growth rates were decreased in both supplemental treatments, as reflected in the inhibition of fresh weights to 35% of that measured in the control because of the spermidine, and to 52% as a result of the MGBG (Table 1).

In the root-induction medium (RIM), shoots were not multiple, but they were elongated. Nevertheless, treatment with 1 mM spermidine decreased the shoot elongation rate by almost 50% after 5 weeks compared with untreated controls (Fig. 4). In addition, when 16 shoot cultures were treated with spermidine, 12 showed decreased root growth compared with the control (Fig. 4), while the root development that was induced in the other 4 was equivalent to that found with the control (data not shown). In contrast, treatment with the MGBG supplement in the RIM almost completely interrupted both shoot elongation and root formation.

Effects of Phytohormones on Gene Expression of Polyamine Biosynthesis and SAMDC Activity

After treating the carnation shoot cultures with, GA, kinetin, or IAA, we used reverse transcriptase polymerase chain reactions (RT-PCR) to determine the steady-state levels of genes encode for enzymes in polyamine biosynthesis. Transcripts of *SAM synthetase*, *ADC*, and *SAMDC* were detected in all the control cultures. Moreover, although phytohormone treatment did not affect the expression of *SAM synthetase*, transcript levels of ADC and SAMDC were significantly increased in those cultures treated with kinetin and IAA, with the greater amount being measured with the former, and an intermediate level being detected in IAA-treated cultures. SAMDC activity was significantly higher in both kinetin- and IAA-treated cultures, but was only moderately increased for those grown in the presence of GA.

DISCUSSION

Germination and seedling development are complex processes in which endogenous plant growth regulators play multiple, important roles (Bewley, 1997). One such regulator class, the polyamines, is produced in all organisms, and can have profound effects on plant physiology (Graser and Hartman, 2000).

Several *bis*-guanylhydrazones, of which the methyl glyoxal derivative (MGBG) has been most thoroughly studied, interfere with the metabolism of natural polyamines. That property makes them essential tools for research on the physiological functions of polyamines (Antognoni et al., 1999). The most important aspect

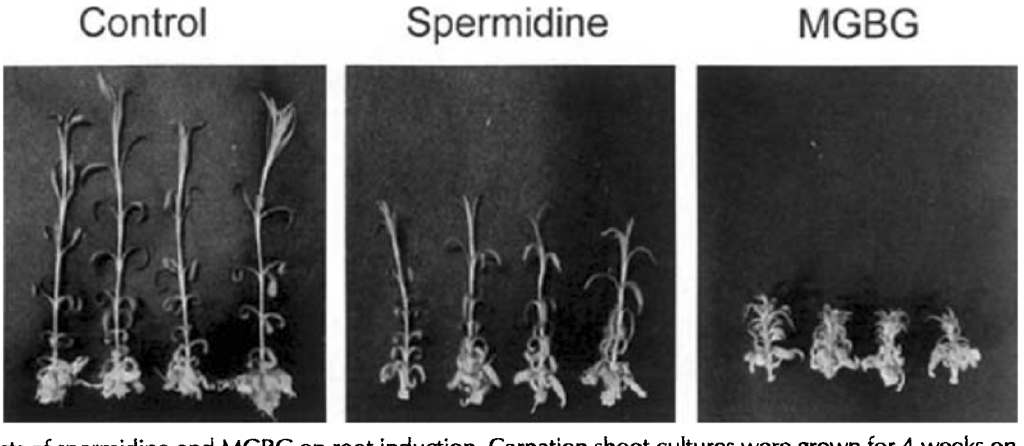

Figure 4. Effects of spermidine and MGBG on root induction. Carnation shoot cultures were grown for 4 weeks on RIM supplemented with 0.5 mg L^{-1} NAA, in the presence of 1 mM spermidine or 1 mM MGBG. For the control, cultures were grown or media without supplemental chemicals.

of MGBG is its ability to inhibit SAMDC. In our experiments, spermidine levels were depleted in explants treated with MGBG. At levels of 0.02 to 0.15 mM MGBG, however, growth was still not significantly inhibited. Nevertheless, Antognoni et al. (1999) have reported a more serious impact at levels of at least 0.2 mM. In fact, their explants treated with the highest concentration (5 mM) displayed symptoms of browning, probably because of toxic side effects from the synthesis of polyphenols. Because of those previous results, therefore, we selected a treatment level of 1 mM MGBG for our tests.

The inhibitory influence of MGBG was more severe in roots than in shoots, which suggests that polyamines, especially spermidine, play a more significant role in controlling root development. However, because putrescine biosynthesis is not affected by MGBG, completely inhibited development would not have been expected (Locke et al., 2000). The effect of MGBG on polyamine content in our *Arabidopsis* seedlings and carnation cultures was, in fact, indicative of one of the most potent growth inhibitors. This result clearly demonstrated the physiological significance of polyamines in plant development.

Although treatment with 1 mM spermidine did not affect germination success (data not shown), it did stimulate *Arabidopsis* seedling growth (Fig. 1). In contrast, spermidine supplements resulted in decreased shoot and root growth in our carnation cultures (Fig. 4), and caused fewer multiple shoots to be induced on the SIM (Table 1). These conflicting results between *Arabidopsis* seedlings and carnation cultures may have resulted from different sensitivities of the polyamine response in those tissue types. Nonetheless, the ubiquitous expression of those polyamines demonstrates their importance in plant cells (Galston and Flores, 1991).

Vegetative tissues, e.g., stems, roots, and leaves, all present transcripts of the *SAMDC* gene, although their levels vary among tissue types (Lee et al., 1997a), as well as in their responses to developmental, hormonal, and environmental conditions (Marco and Carrasco, 2002). Changes in expression may also be affected by the age of the plant, being more important in younger stock. A role in developmental regulation has been suggested based on observations that *SAMDC* mRNA levels change during fruit development and ovary senescence (Alabadi and Carbonell, 1999; Marco and Carrasco, 2002). Therefore, the physiological functions of polyamines may be organ-dependent.

Changes are transient in polyamine contents and gene expression during polyamine biosynthesis, reaching a maximum, then returning to a basal level. For example, research in *Ipomoea nil* by Park et al. (2001) has shown that brassinosteroids induce the maximum level of *SAMDC* transcript after 1 h of treatment, and those transcripts are then reduced to their basal level after 6 h. Lee et al. (1997a) have also reported that activation of the polyamine biosynthetic pathway, which leads to high levels of endogenous polyamines, is toxic to vegetative growth.

Attempts have been unsuccessful in producing sense transgenic plants with 35S *SAMDC* or *ADC* constructs (Kumar et al., 1996; Hanfrey et al., 2002). Those studies, however, have indicated that constitutive increases in polyamine contents by overexpression of polyamine biosynthetic genes are lethal to plants. Therefore, it is generally accepted that the polyamine pathway is tightly regulated at the end-product level (Noury et al., 2000). In our study, spermidine treatment resulted in inhibited shoot and root growth in the carnation cultures. Based on those results, we believe that *Arabidopsis* seedlings, rather than carnation cultures, are the preferred organ for accumulating polyamines. In fact, cellular polyamine contents after spermidine treatment harmed carnation plant growth. This also implies that the sensitivity and appropriate cellular content for polyamines may be species- or organ/tissue-specific.

Martínez-Téllez et al. (2002) have reported that although 0.5 mM spermidine reduces electrolyte leakage and chilling injury in zucchini squash, high polyamine concentrations (i.e., 2.0 mM) are associated with the appearance of chilling-injury symptoms. Our study also showed that carnation cultures could not tolerate treatment with 1 mM spermidine. A comparison of developmental effects and physiological concentrations suggests that this particular polyamine might play a significant role in controlling shoot and root development. These contrasting effects of spermidine may also be related to differences in the endogenous levels of plant growth substances, especially polyamines.

In analyzing the amounts of transcripts for polyamine biosynthetic genes after treatment for 12 h, we found that expression was hormone-dependent in our carnation cultures (Fig. 5). For example, genes for *SAMDC* and *ADC* were induced by treatment with IAA and, more importantly, by kinetin. In contrast, GA treatments did not effectively change the expression of *ADC* or *SAMDC* genes. Kaur-Sawhney et al. (1986) have reported that part of the GA-induced increase in internodal growth might be a result of enhanced polyamine biosynthesis via the ADC pathway.

Marco and Carrasco (2002) have shown differential

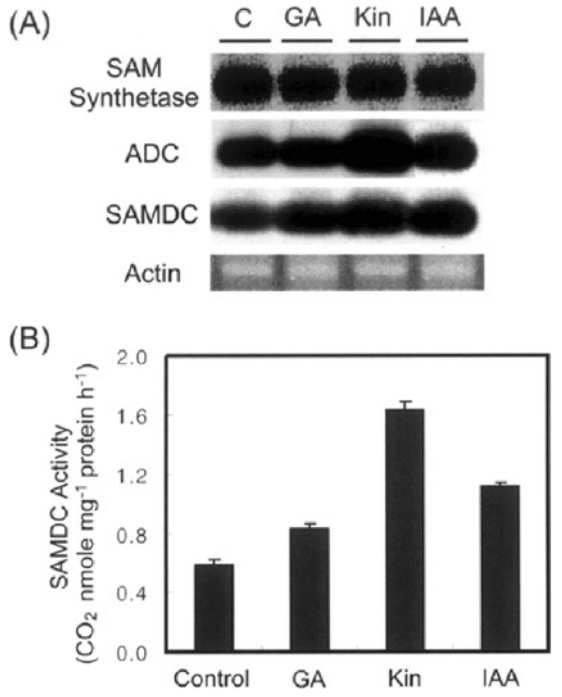

Figure 5. Gene expression of the polyamine biosynthetic pathway and the activity of SAMDC for control plants or phytohormone-treated carnation shoot cultures. **A.** Transcripts were detected by Southern-blot hybridization with each probe of carnation SAM synthetase, ADC, and SAMDC, after RT-PCR with gene-specific primers. Equal amounts of first-strand cDNA in each reaction are shown by RT-PCR with the gene-specific primers for tobacco actin. **B.** SAMDC activity was determined in cultures grown on media in the absence or presence of 1 mM spermidine or 1 mM MGBG. Error bars represent standard deviation.

expression of the pea *SAMDC* gene in vegetative and reproductive tissues, with the highest *SAMDC* mRNA levels being found in undifferentiated calli and tissues experiencing high rates of cell division, as well as at the onset of fruit development. Gene expression for SAM synthetase also differs during parthenocarpic development of pea ovaries when one compares gibberellin- and cytokinin-treated organs with those that are auxin-induced (Gómez-Gómez and Carrasco, 1996). In our carnation study, SAM synthetase-gene expression did not change after hormonal treatment (Fig. 5), which implies constitutive expression. However, Gómez-Gómez and Carrasco (1996) have reported that SAM synthetase especially is regulated by developmental and/or environmental factors that are strictly controlled according to the particular requirements for SAM. Therefore, we suggest that polyamines play a physiological role in organogenesis from carnation shoot cultures, operating through *ADC* and *SAMDC* gene expression with cytokinins and auxins.

Park et al. (2001) have reported that BR-deficient *Arabidopsis* mutants, such as *det2* and *cpd*, show strong dwarfism and dark green, curly leaves. In their study, 6-d-old morning glory seedlings were treated with white light; the amount of *SAMDC* transcript was dramatically increased after 1 h of light exposure, but then returned to a basal level. In addition, treatment with brassinosteroids in the dark caused the *SAMDC* transcript to accumulate to the same level as that found with the light treatment. Therefore, it was suggested that *SAMDC* gene expression was restored by the BR treatment. Even though spermidine treatments in the current study did not completely reverse the inhibition of growth in *det2* mutant seedlings (Fig. 2), root production functioned more effectively in the mutants than in the wild types as a result of the spermidine. Therefore, we propose that polyamines can partially substitute for BR during plant growth.

Although information regarding the effects of GA, cytokinin, and auxin on polyamine biosynthesis is still contradictory, researchers have no doubt that, in the few cases examined, one or more of the enzymes in this pathway was activated by these phytohormones. ADC and SAMDC are particularly active in organogenic tissues, such as shoot-forming tobacco calli, where they are activated within 24 h (Biondi et al., 2001). ADC and SAMDC are also required during the processes of elongation and differentiation. Therefore, we believe that polyamines might play an important role in plant development because of their stimulatory effects on phytohormonal signaling and/or synergism.

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