Effects of putrescine and inhibitors of putrescine biosynthesis on organogenesis in *Euphorbia esula* L.[1]

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Summary:

Exogenous putrescine (≤ 5 mM) had little effect on root or shoot formation in aseptically isolated hypocotyl segments of leafy spurge (*Euphorbia esula* L.) grown on full-strength B5 medium. Unexpectedly, putrescine inhibited root and shoot formation in hypocotyl segments grown on B5 medium diluted 10-fold. In the full-strength medium, root and shoot formation were inhibited by 0.5 mM concentrations of DL-α-difluoromethylornithine (DFMO) and DL-α-difluoromethylarginine (DFMA). DFMO and DFMA are inhibitors of the ornithine decarboxylase and arginine decarboxylase pathways, respectively, of putrescine biosynthesis in plants. Exogenous putrescine (0.5 to 5 mM) did not reverse either the DFMO or DFMA-induced inhibition of shoot formation. However, the DFMA-induced inhibition of root formation was partially reversed by exogenous putrescine. The auxin, indole-3-acetic acid (IAA), reduced the inhibitory effects of DFMO+DFMA (applied together) on both roots and shoots. In the first few days of culture, the endogenous levels of putrescine and spermidine, but not of spermine, increased in the presence of IAA. The levels of putrescine and spermidine in the tissues did not correlate well with either root or shoot production in the later stages of organ formation; especially in tissues treated with IAA. These results show that there were no obvious correlations between polyamine levels and organogenesis in leafy spurge hypocotyl segments, although residual putrescine or spermidine or both in the tissues at the time of excision may be indirectly involved in the early stages of root formation.

Introduction

Leafy spurge (*Euphorbia esula* L.), a perennial plant introduced from Europe, is of economic importance because it infests pastures, rangelands, road rights of way, and recreational areas. It continues to spread into new non-crop areas (Watson, 1985). Leafy spurge spreads from dehiscing capsules, although its primary means of effective invasion is by root buds formed on an extensive root system (Raju, 1985). Therefore, the process of vegetative reproduction (organogenesis) is of major concern in programs to control this weed, as well as other perennial weeds. Although biocontrol methods look promising and a few herbicides are used for leafy spurge control (Watson, 1985), the weed persists and continues to expand. Eradication seems unlikely and adequate control will undoubtedly require a combination of herbicides and biocontrol methods, which will require many years to become established. Growth regulator-herbicide combinations have been tested for control of leafy spurge (Ferrell *et al.*, 1989). Currently a combination of picloram and 2,4-dichlorophenoxyacetic acid (2,4-D) is the recommended method of control (Lym and Messersmith, 1987). New herbicides that are economical, environmentally safe, and prevent the development of either roots or shoots are required.

Putrescine (1,4-diaminobutane) and the polyamines, spermidine [*N*-(3-aminopropyl)-1,4-butanediamine] and spermine (*N'*,*N'*-bis(3-aminopropyl)-1,4-butanediamine], may be involved in cell division and plant development (Bagni, 1986; Evans and Malmberg, 1989; Galston and Kaur-Sawhney, 1990). Putrescine is metabolized in plants to spermidine and spermine, and then to other compounds or conjugated polyamines or both (Slocum, 1991). The role of polyamines in root development is unclear and often contradictory. Exogenous L-arginine and L-ornithine (both precursors of putrescine, spermidine and spermine) promoted adventitious rooting in mung bean hypocotyls (Shyr and Kao, 1985). Leshem *et al.* (1991) found that changes in polyamine titers were similar in organogenic and non-organogenic tissues of melon (*Cucumis melo* L.). Exogenous polyamines did not promote root formation in mung bean hypocotyls (Friedman *et al.*, 1982) but Friedman *et al.* (1985) reported that the induction of root formation by the synthetic auxin indolebutyric acid (IBA) may require polyamine biosynthesis.

The relationship of the polyamines with auxins and other growth regulators is not understood. Chriqui *et al.* (1986) reported on a close relationship between rhizogenesis and increased levels of putrescine and spermidine. These authors also reported synergistic effects between ornithine and the natural auxin, indole-3-acetic acid (IAA), on rhizogenesis in *Datura innoxia* Mill. leaf explants. IBA stimulated rooting in stem sections (that included the hypocotyl) of mung bean seedlings, and further stimulation was induced by exogenous spermine; spermidine, on the other hand, inhibited rooting (Jarvis *et al.*, 1983). No mention was made of the effects of putrescine, although free putrescine and

spermidine were the predominant polyamines in tissue. The mung bean studies were conducted with hypocotyls from light-grown seedlings, with apices and some leaves still attached, rather than with etiolated tissues as used in the present work. In tobacco callus (Tiburcio *et al*., 1987), root formation seems to be inversely related to the concentration of putrescine.

No clear-cut pattern emerges as to the role of the polyamines in root formation or in their interaction with synthetic or natural auxins. In this paper, we report on the effects of exogenously applied putrescine and two inhibitors of enzymes involved in putrescine biosynthesis, ornithine decarboxylase (ODC) and arginine decarboxylase (ADC). DL-α-difluoromethylarginine (DFMO) and DL-α-difluoromethylornithine (DFMA) are suicide inhibitors of ODC and ADC, respectively (Bagni, 1986; Galston and Kaur-Sawhney, 1990; Minocha, 1988), although their specificity in plant tissues is not understood (Slocum, 1991).

The formation of roots in leafy spurge cell suspension cultures is a function of the age of the culture and the nitrogen status of the medium (Evenson *et al*., 1988). Isolated hypocotyl segments of leafy spurge (Davis and Olson, 1993) gave more consistent results than cell suspension cultures for determining the effects of plant growth regulators or herbicides on organogenesis (Davis *et al*., 1988). Because of the relatively consistent responses of the hypocotyl segments to auxin (Davis and Olson, 1993), root formation was a primary interest. Shoot formation was also determined because untreated hypocotyl segments usually formed more shoots than roots.

The influence of auxin (IAA) was determined because, as described above, auxin type herbicides (2,4-D and picloram) have been the most successful in the control of this perennial weed. The interactions of the polyamines with auxin may be weak links in the physiology of leafy spurge that could be exploited.

Materials and methods

Seeds collected from fields and pastures in southeastern North Dakota were surface sterilized with 70% ethanol followed by 20 minutes in 30 to 60% commercial bleach and three rinses in sterile water. The high concentration of bleach was required to eliminate a seed-borne contaminant. The seeds were germinated on sterile agar-water (0.7% wt/vol), in darkness at 30/20º C (12/12 hour) according to Beasley (1964). After 2 weeks, the etiolated hypocotyls were cut into 1-cm lengths and placed onto 0.7% agar medium with B5 salts and vitamins and 2% (wt/vol) sucrose (Gamborg *et al*., 1968). In some experiments the salts and vitamins were diluted, but the sucrose was 2% throughout. The undiluted B5 medium is designated as 100%, and the diluted media as 30 or 10%, as appropriate. Compounds that were tested for their effects and interactions on organogenesis were: putrescine dihydrochloride, agmatine [4-(aminobutyl)guanidine] sulfate, DFMO, DFMA, and IAA. Filter sterilized aqueous solutions of test compounds were added to autoclaved media. IAA was added in acetone at non-phytotoxic concentrations of acetone (< 0.1% vol/vol; Davis *et al*., 1978). Plastic petri dishes (6 cm diameter) were wrapped in Parafilm and aluminum foil, and incubated at 26º C. Roots and shoots were observed at 7

and (usually) 28 days under a dissecting microscope and they were counted only when they were clearly recognizable as distinct organs (1 to 2 mm long).

Endogenous free polyamines were quantified as dansylated derivatives using high performance liquid chromatography (HPLC). Hypocotyl segments were weighed and frozen at $-20°$ C, and were analyzed as described by Minocha *et al*. (1990). Briefly, analysis was done on samples thawed and frozen 3 times in 5% perchloric acid. Similar results were obtained with samples frozen and thawed 3 times and those ground for 2 minutes in a glass homogenizer, so the freeze-thaw method was used for all samples.

The HPLC system consisted of a Perkin-Elmer Series 400 pump, fitted with a 10-μl injection loop, a C_{18} reversed-phase column (Perkin-Elmer CRC 18, particle size 3 μM), and a Perkin-Elmer fluorescence detector (excitation at 340 nm, emission at 515 nm). The polyamines were eluted using a linear gradient of acetonitrile and heptanesulfonate (10 m*M* in water, pH 3.4) at a flow rate of 2.5 ml/min.

The uptake of [1,4-^{14}C]-putrescine•2HCl (New England Nuclear, Boston, MA, specific activity 90.4 mCi/mmol) into leafy spurge hypocotyl segments was determined by incorporating the radiolabeled compound into B5 media containing 1 m*M* putrescine and following the radioactivity in the hypocotyls with time. The hypocotyls were combusted and the radiolabeled CO_2 evolved was trapped and counted in a liquid scintillation counter.

Nutrients for the B5 media were from Sigma Chemical Co., St. Louis, MO, and were plant tissue culture tested. Polyamines were from Sigma, or Aldrich Chemical Co., Inc., Milwaukee, WI. DFMO and DFMA were from Marion-Merrill-Dow, Cincinnati, OH.

Experiments were repeated 2 or more times. Treatments were compared using a two-sample t test of the computer program Statistix (NH Analytical Software, St. Paul, MN). In most experiments, eight hypocotyl segments were used per petri dish, with 10 dishes per treatment. Triplicate samples were used to determine polyamine concentrations.

Results and discussion

Effects of putrescine, DFMO and DFMA on organogenesis. Both roots and shoots were formed on hypocotyl segments on B5 agar medium containing the normal (100%) salt and vitamin concentrations. The addition of putrescine at 1 or 5 m*M* had no significant effect on either shoot or root formation (Exp. A, Table 1), but putrescine at 0.5 m*M* stimulated root formation (Exp. B, Table 1). DFMO at 0.5 m*M* partially inhibited shoot formation and completely prevented root formation. In other plant species, putrescine has been shown to reverse DFMO-induced inhibition of cell division (Galston and Kaur-Sawhney, 1990). In leafy spurge hypocotyl segments, exogenous putrescine at 0.5, 1, or 5 m*M* added simultaneously with DFMO, did not reverse the DFMO-induced inhibition of root formation.

DFMA at 0.5 m*M* inhibited both root and shoot formation (Table 1). When 0.5 m*M* putrescine was added simultaneously with 0.5 m*M* DFMA, root numbers did not differ significantly from those of the controls but increased significantly over DFMA alone. Pu-

trescine (0.5 to 5 m*M*) had no significant effect on shoot formation or on the DFMA-induced inhibition of shoot formation (Table 1).

Although DFMO or DFMA (0.5 m*M* each) reduced shoot formation nearly equally (~40 to 50%), DFMO by itself was a more effective inhibitor of root formation than was DFMA. This indicates that the ornithine pathway of putrescine biosynthesis may be more important than the arginine pathway in any involvement in rhizogenesis in leafy spurge hypocotyl segments.

Table 1. Shoot and root formation in leafy spurge hypocotyl segments grown 29 days (experiment A) or 28 days (experiment B) in B5 media with putrescine ± DFMO or DFMA)[a].

Concentration, m*M*				
DFMO	DFMA	PUT	Shoots	Roots
A.				
0	0	0	1.5 ± 0.2	0.4 ± 0.1
0	0	1	1.8 ± 0.2	0.5 ± 0.1
0	0	5	1.2 ± 0.2	0.4 ± 0.1
0.5	0	0	0.9 ± 0.2^{b}	0^{b}
0.5	0	1	0.7 ± 0.1^{b}	0^{b}
0.5	0	5	0.8 ± 0.2^{b}	0^{b}
B.				
0	0	0	2.2 ± 0.3	0.4 ± 0.1
0	0	0.5	2.6 ± 0.3	0.7 ± 0.1^{b}
0.5	0	0	1.1 ± 0.2^{b}	0^{b}
0.5	0	0.5	1.6 ± 0.2	0.05 ± 0.03^{b}
0	0.5	0	1.2 ± 0.1^{b}	0.2 ± 0.1^{c}
0	0.5	0.5	1.5 ± 0.2^{b}	0.6 ± 0.1^{c}

[a]Values are number per hypocotyl segment (means ± standard errors).
[b] Differs from controls ($P < 0.05$).
[c]Differs from DFMA alone ($P < 0.01$).

Interaction with IAA. Exogenous IAA (0.23 *µM*) dramatically increased root formation in leafy spurge hypocotyl segments (Fig. 1A). IAA at 0.23 *µM* is the lower threshold for the stimulation of root formation, and concentrations of IAA up to 2.2 *µM* increased root formation (Davis and Olson 1993). In other (unpublished) experiments, 5.7 *µM* IAA also increased root formation. Therefore, the auxin, IAA, has a fairly wide concentration range for the stimulation of root formation in leafy spurge hypocotyl segments.

Root formation was inhibited strongly by DFMO+DFMA (0.5 m*M* each, applied together) (Fig. 1A). Treatment with IAA+DFMO+DFMA consistently increased root formation compared to the controls or to DFMO+DFMA-treated tissues. However, this increase was not equal to that of IAA alone, except at the very early times (7 days in Fig. 1A, and 5 days in similar experiments). Root formation was more sensitive than shoot

formation to DFMO alone (Table 1) or to DFMO+DFMA applied together (Fig. 1), and the effects of treatment on root formation was more consistent than for shoot formation.

Shoot formation was inhibited by DFMO+DFMA or IAA (0.23 μM) applied alone up to 28 days (Fig. 1B). Shoot formation was also inhibited by simultaneous application of all three compounds up to 21 days, but by 28 days the hypocotyl segments treated with IAA+DFMO+DFMA had shoot numbers similar to the controls. It is unclear why IAA should reverse the inhibition of shoot formation by DFMO+DFMA, inasmuch as IAA by itself usually inhibited shoot formation. Perhaps metabolism of all three compounds in the tissues lowered the cellular concentrations below phytotoxic levels, so the tissues were then capable of full shoot expression.

Fig. 1. IAA (0.23 μM) modification of the effects of DFMO + DFMA (0.5 mM each) on organogenesis as a function of time. Values are means ± SE.

Relationship of endogenous polyamine levels to root formation

Putrescine. Putrescine concentrations in leafy spurge control hypocotyl segments decreased (Fig. 2) from 355 to 166 nmol/g fresh weight between Day 2, when organs were not yet visible, and Day 19, when most of the roots were formed (Fig. 1A) and shoots numbers were still increasing (Fig. 1 B). IAA increased putrescine levels over the controls, with the greatest increases occurring at 2 and 5 days (Fig. 2). IAA has also been shown to increase the levels of free putrescine in other plant species (Chriqui *et al.*, 1986; Cocucci and Bagni, 1968). In the IAA-treated leafy spurge tissues, root numbers continued to increase up to 28 days (Fig. 1), with putrescine levels declining to almost the same level as the untreated tissues at 0 days (Fig. 2).

In DFMO+DFMA-treated tissues, putrescine levels declined by Day 2, and were undetectable by Day 5, in the presence or absence of IAA (Fig. 2). However, at 7 days, root numbers in IAA+DFMO+DFMA-treated tissues reached a maximum and were similar to tissues treated with IAA alone (Fig. 1A). Therefore, either putrescine is not required for root formation or the amount of putrescine in the tissues from 0 to 5 days was sufficient for the initiation of root primordia, but not for the roots to penetrate the epidermis. This supports the concept that putrescine may be involved in the cell division stage of plant development, and not cell enlargement. Galston and Kaur Sawhney (1990) discuss the possibility that polyamines produced in plants via the ornithine pathway may be essential
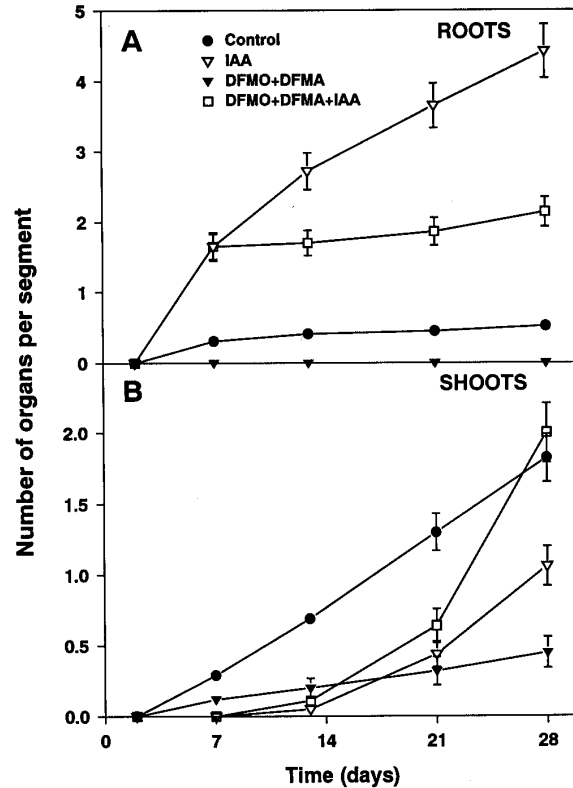
for DNA replication and cell division. Egea-Cortines and Mizrah (1991) summarized much of the work on the relationship of polyamines to cell division.

Spermidine. Spermidine levels increased in control tissues up to 5 days. IAA induced a further, significant ($P < 0.02$) increase over the controls up to Day 8 (Fig. 2). DFMO+DFMA reduced spermidine levels markedly in both controls and IAA-treated tissues. IAA+DFMO+DFMA increased spermidine levels significantly ($P < 0.05$), at least up to 8 days, compared to the DFMO+DFMA-treated tissues. Although both spermidine levels and root formation increased in controls, the changes were not closely related, and were even less so in the presence of IAA (Figs. 1A and 2). Therefore, there does not seem to be a close correlation between spermidine levels and root formation. The putrescine:spermidine ratio was suggested to be an important factor for somatic embryos of grape (Faure *et al.*, 1991), but does not seem to influence root formation in leafy spurge hypocotyl segments.

Spermine. Spermine was not detected in controls at Day 0, but increased slowly to 44 nmol/g fresh weight by 19 days (Fig. 2). Root formation seems to parallel the change in spermine levels in control tissues, but not in IAA-treated tissues (Fig. 2). IAA treatment had little effect on spermine levels, either in controls or when applied with DFMO+DFMA. Since IAA increased root formation without altering spermine levels, there seems to be no obvious direct relationship between spermine levels and root formation.

Unlike putrescine or spermidine, spermine levels were increased by treatment with DFMO+DFMA (Fig. 2). Possibly, much of the residual spermidine (which was never zero in any of the treatments) in the DFMO+DFMA-treated tissues was converted to spermine with no further metabolism, resulting in a net accumulation of spermine.
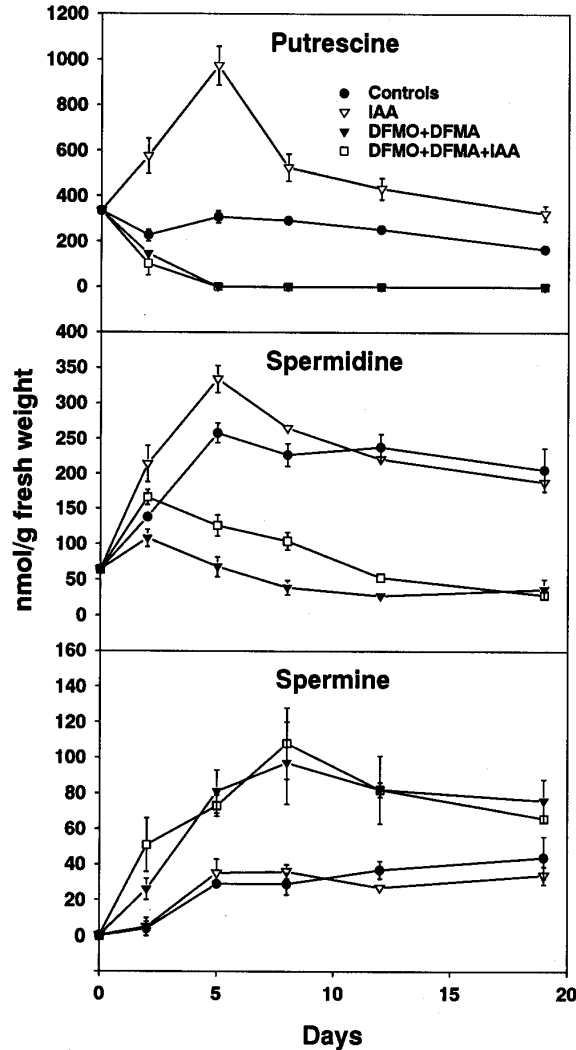

Fig. 2. Cellular polyamine levels in leafy spurge hypocotyl segments treated with DFMO + DFMA (0.5 m*M* each) ± 1.1 μM IAA. Values are means (nmol/g fresh weight) of triplicate samples ± standard errors.

Relationship of endogenous polyamine levels to shoot formation

Putrescine. Putrescine levels in control tissues declined slowly with time (Fig. 2), whereas shoot formation increased linearly with time after 2 days (Fig. 1B). IAA stimulated putrescine accumulation (Fig. 2), but inhibited shoot formation (Fig. 1B). DFMO+DFMA was even more inhibitory to shoot formation by 28 days than was IAA (Fig. 1B). A few shoots were formed in the DFMO+DFMA-treated tissues at 28 days, even though putrescine was depleted by Day 5 (Fig. 2). However, IAA+DFMO+DFMA resulted in shoot numbers similar to those of IAA-treated tissues up to 21 days, increasing to approximately those of the control tissues by 28 days (Fig. 1B). Putrescine was not detected in the IAA+DFMO+DFMA-treated tissues by Day 5 (Fig. 2) and yet shoot formation continued to increase up to 28 days. Thus, there seems to be no direct relationship between putrescine levels and shoot formation.

Spermidine. Spermidine levels in control tissues increased rapidly for 5 days and leveled off with time (Fig. 2). Shoot formation continued to increase linearly up to 28 days (Fig. 1B). IAA increased spermidine levels significantly ($P < 0.02$) by Day 5 (Fig. 2). IAA delayed shoot formation, resulting in only about 60% of the number of shoots as control tissues at 28 days. DFMO+DFMA reduced spermidine levels after 5 days (Fig. 2) but did not result in total depletion of spermidine in DFMO+DMFA-treated tissues. Presumably, the endogenous putrescine present in the hypocotyl segments at the time of excision (0 days) was converted to spermidine in the presence of DFMO+DFMA, and part of that was not metabolized further to spermine, resulting in a low level of residual spermidine.

Indole-3-acetic acid significantly ($P < 0.05$) increased the spermidine levels only for the first 8 days in the DFMO+DFMA-treated tissues (Fig. 2). For the first 21 days, IAA+DFMO+DFMA-treated tissues formed approximately the same numbers of shoots as IAAor DFMO+DFMA-treated tissues. At Day 28 the numbers of shoots were similar to the controls, and were much greater than the tissues treated with either IAA or DFMO+DFMA (Fig. 1B). Therefore, there seems to be no direct or consistent relationship between spermidine levels and shoot formation.

Spermine. Spermine levels were not correlated closely with shoot formation (Figs. 2 and 1B, respectively). Spermine was undetectable at time 0, but increased slowly up to 19 days in control tissues. Shoot formation was also 0 at Day 0 and at 2 days, but increased quite rapidly from Day 7 on. IAA inhibited shoot formation (Fig. 1), without increasing the spermine levels above that of the controls. There were no differences in spermine levels in tissues treated with DFMO+DFMA or IAA+DFMO+DFMA (Fig. 2).

In contrast to the results with putrescine and spermidine, DFMO+DFMA treatment increased the levels of spermine in the presence or absence of IAA (Fig. 2). Apparently there was no interference with the conversion of spermidine to spermine, and the endogenous levels of spermidine in the tissues were high enough for the reaction to occur. Because spermine levels were not affected by IAA but shoot numbers decreased in the tissues treated with IAA alone and increased in DFMO+DFMA-treated tissues, it seems that spermine is not directly involved in shoot formation of leafy spurge.

Organogenesis in dilute media

Because the polyamines were first brought to the attention of plant physiologists by the work of Richards and Coleman (1952) concerning potassium deficiency and putrescine accumulation in plants, it was of interest to investigate the interrelationships of nutrient deprivation, polyamines, and auxin. As a first step, the B5 medium was simply diluted (with the sucrose remaining constant at 2%), and the leafy spurge hypocotyl segments were treated in a fashion similar to those in the full-strength (100%) B5 medium.

Dilution of the salt and vitamin concentrations of the B5 medium to 30% of the normal concentrations (with sucrose constant at 2% wt/vol) resulted in slightly fewer shoots with no effect on roots (Table 2). Further dilution of the salts and vitamins to 10% of the normal concentrations inhibited shoots and roots, although both organs were formed. Five millimolar putrescine in full strength medium had no significant effect on root or shoot formation, but in 30 or 10% media the formation of both organs was inhibited totally. In other experiments (not shown), 3 mM putrescine also prevented root formation in the 10% medium. One millimolar putrescine reduced the number of roots by about 75%, but 0.3 mM putrescine did not inhibit root formation (Fig. 3). IAA stimulated root formation in the 10% medium in the presence or absence of putrescine.

Table 2. Effects of putrescine on organogenesis in hypocotyls grown on B5 medium with varying salt and vitamin concentrations.

Percentage of Salts and Vitamins in B5 Medium	Put	Number of Organs per Segment, mean ± SE	
		Shoots	Roots
100		2.6 ± 0.2	0.6 ± 0.1
30		2.0 ± 0.2[a]	0.6 ± 0.1
10		0.8 ± 0.1[a]	0.3 ± 0.1[a]
100	5 mM	2.2 ± 0.2	0.5 ± 0.1
30	5 mM	0[a]	0.01 ± 0.01[a]
10	5 mM	0[a]	0[a]

[a] Differs from controls (100% B5) ($P<0.05$).

Shoot formation was inhibited in the 10% medium by both IAA (0.2 μM) and putrescine (0.3 or 1 mM (Fig. 3). Three millimolar putrescine completely inhibited shoot formation in the 10% medium in the presence or absence of IAA (data not shown). This contrasts with the results in the 100% medium, where putrescine up to 5 mM had no effect (Table 1).

Difluoromethylornithine+DFMA (0.5 mM each, applied simultaneously) strongly inhibited both shoot and root formation in the 10% medium (Fig. 4). Attempts to reverse this inhibition with exogenous 0.25 mM putrescine (non-inhibitory to root formation in the 10% medium) did not succeed. Putrescine, by itself, at 0.5 mM or greater was inhibitory in the 10% medium.

DL-α-Difluoromethyarginine (applied alone at 0.5 mM) inhibited both shoot and root formation (Fig. 5). Agmatine is an intermediate in the biosynthetic pathway between arginine and putrescine (Slocum, 1991), so its addition should by-pass the block in the biosynthetic pathway due to DFMA inhibition of ODC activity. However, the inhibition due to DFMA in the 10% medium was not overcome by adding agmatine simultaneously with DFMA at either 0.5 or 1 mM. Instead, agmatine by itself at 0.5 or 1 mM inhibited shoot formation. Root formation was stimulated by 0.5 mM agmatine, but inhibited by 1 mM agmatine. Agmatine has been shown to be an inhibitor to ADC activity in plants, but at very high concentrations (Slocum, 1991).
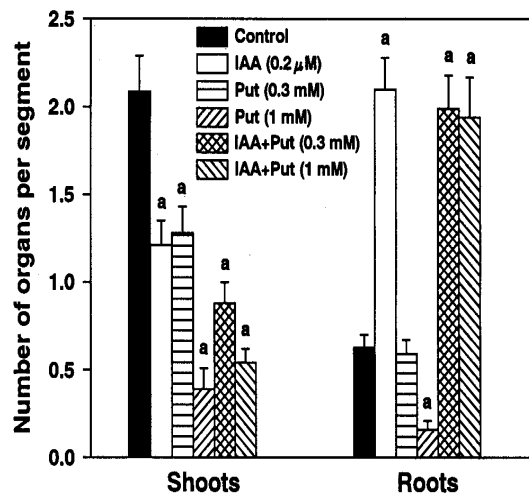

Fig. 3. Putrescine and IAA effects on organogenesis in leafy spurge hypocotyl segments grown in dilute (10%) B5 medium for 28 days. Three-millimolar putrescine (± IAA) was also tested, but was not included in this figure because no organs were formed. Values are means ± SE. α = differs from controls ($P < 0.05$).
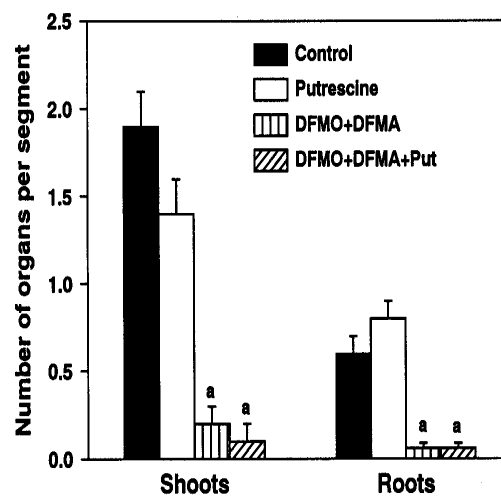

Fig. 4. Effects of 0.25 mM putrescine and DFMO + DFMA (0.5 mM each) on organogenesis in leafy spurge hypocotyl segments cultured in 10% B5 for 28 days. Values are means ± SE. α = differs from controls ($P < 0.05$).
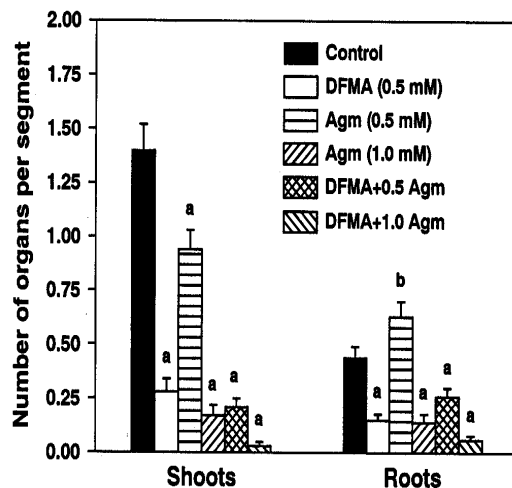
Fig. 5. Effects of 0.5 m*M* DFMA ± agmatine (0.5 or 1 m*M*) on organogenesis in leafy spurge hypocotyl segments. 10% B5, 28 days. Combined data from two experiments. Values are means ± SE. *a* = differs from control (*P* < 0.01). *b* = differs from control (*P* < 0.03).

Uptake of exogenous putrescine

Putrescine is reported to be taken up rapidly by plant tissues (Caffaro *et al.*, 1993; Christ *et al.*, 1989). [14C]-Putrescine was taken up by isolated leafy spurge hypocotyl segments (Table 3) more readily from the 10% B5 medium than from the normal (100%) B5 medium. The recoveries of radioactivity were 2.3-fold and 3.4-fold greater at Day 1 and Day 7, respectively, in the 10% medium than in the 100% medium. The 14C-activity recovered in the control hypocotyl segments grown on the 100% medium (Table 3) is equivalent to approximately 1100 nmol/g fresh weight at 1 day and 2300 nmol/g fresh weight at 7 days, compared to endogenous putrescine levels in controls of approximately 200 to 350 nmol/g fresh weight (Fig. 2). However, because the metabolic fate of the [14C]-putrescine in the leafy spurge hypocotyl segments was not determined, the portion of the [14C]-activity that remained as unaltered putrescine is unknown.

Table 3. Uptake of [14C]-putrescine from isolated hypocotyl segments of leafy spurge cultured on media containing 1 m*M* putrescine[a]

Concentration of B5 Salts and Vitamins	14C Activity, dpm per mg Fresh Weight	
	1 Day	7 Days
100%	17.5 ± 2.7	37.3 ± 5.7
10%	40.6 ± 2.2	127.3 ± 4.4

[a] Values are means ± SE.

Putrescine has been shown to accumulate in plants that are deficient in potassium (Smith, 1991). Dilution of the B5 medium still resulted in a significant amount of potassium in the diluted medium (decreasing from 25 to 2.5 mM). Perhaps increased uptake of exogenous putrescine and decreased metabolism of endogenous putrescine or both resulted in the accumulation of putrescine to a phytotoxic level.

Competition may occur between putrescine, spermine, or spermidine, and one or more of the cations in the B5 medium, which may be in limited supply. Spermidine or especially spermine replaced the Mg^{++} requirement for the biosynthesis of polyphenylalanine in cell-free ribosome preparations from barley embryos (Cohen and Zalik, 1978). In their experiments, other divalent cations could also replace Mg^{++}, indicating that the cation requirement for synthesis of proteins may not be specific and the polyamines may be viable substitutes. DiTomaso *et al*. (1992) indicated that divalent or polyvalent inorganic cations do not compete for the same sites as putrescine for transport through membranes, although competition for sites on nucleic acids or non-membrane proteins was not determined. DiTomaso *et al*. (1989) indicated that putrescine fails to stabilize membranes of corn seedlings if the tissues are depleted of Ca^{++} by the use of a chelating agent. Although the leafy spurge hypocotyl segments grown in 10% B5 medium were not depleted of Ca^{++}, the concentration of Ca^{++} (0.1 mM) or other specific cations may be below the threshold level for optimum membrane stability.

In addition to free polyamines, conjugated polyamines are present in leafy spurge hypocotyl segments (Davis and Olson, unpublished results), but were not determined in this study. Conjugated polyamines have been implicated in the development of reproductive and vegetative organs of tobacco (Torrigiani *et al*., 1987) and may serve as a potential source of free polyamines. In tobacco, DFMA and D-arginine were more effective in reducing the endogenous levels of putrescine than was DFMO, implying that the arginine pathway may be more important than the ornithine pathway for putrescine biosynthesis in tobacco callus.

The results of the present study with leafy spurge hypocotyl segments do not support the direct involvement of free putrescine, spermidine, or spermine in organogenesis. A requirement for these compounds during the early stages of the initiation of roots and shoots is not ruled out completely. DFMO+DFMA inhibited the biosynthesis of putrescine and also root formation. Addition of auxin restored root formation within the first few days of culture. The mechanisms of action and interactions of the polyamines and auxin remain unknown.

Acknowledgements

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