Plant Cell, Tissue and Organ Culture **42:** 47–55, 1995.

Effects of inhibitors of polyamine biosynthesis and of polyamines on strawberry microcutting growth and development

E. Tarenghi, M. Carré & J. Martin-Tanguy*
INRA, Station de Génétique d'Amélioration des Plantes, BV 1540, 21034 Dijon Cédex, France (requests for offprints)*

Received 22 June 1994; accepted in revised form 7 February 1995

Key words: Arginine decarboxylase, *Fragaria* × *ananassa* Duch, micropropagation *in vitro*, ornithine decarboxylase, polyamines, polyamine inhibitors.

Abstract

The primary free polyamines identified during growth and development of strawberry (*Fragaria* × *ananassa* Duch.) microcuttings cultivated in vitro were putrescine, spermidine and spermine. Polyamine composition differed according to tissue and stages of development; putrescine was predominant in aerial green tissues and roots. α-DL-difluoromethylarginine (DFMA), a specific and irreversible inhibitor of the putrescine-synthesizing enzyme, arginine decarboxylase (ADC), strongly inhibited growth and development. Application of agmatine or putrescine to the inhibited system resulted in a reversal of inhibition, indicating that polyamines are involved in regulating the growth and development of strawberry microcuttings. α-DL-difluoromethylornithine (DFMO), a specific and irreversible inhibitor of putrescine biosynthesis by ornithine decarboxylase, promoted growth and development. We propose that ADC regulates putrescine biosynthesis during microcutting development. The application of exogenous polyamines (agmatine, putrescine, spermidine) stimulated development and growth of microcuttings, suggesting that the endogenous concentrations of these polyamines can be growth limiting.

Abbreviations: ADC – arginine decarboxylase, ODC – ornithine decarboxylase, DFMA – α-difluoromethylarginine, DFMO – α-difluoromethylornithine, Put – putrescine, Spd – spermidine, Sp – spermine, DW – dry weight, PA – polyamine, PPF – photosynthetic photon flux

Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is micropropagated from single node shoot segments excised from shoots grown *in vitro*. Among the different factors regulating proliferation and growth, polyamines could play a major role. Since the application of exogenous polyamines stimulates the development of several higher plants, endogenous concentrations of these amines could be growth limiting (Egea-Cortines & Mizrahi 1991; Galston & Florès 1991; Martin-Tanguy & Carré 1993). Furthermore, the growth of all living organisms requires polyamines and their content is proportional to cellular growth rates (Galston & Flores 1991), raising the possibility that polyamines play a role in cell division in plants. Polyamine inhibition of senescence of oat protoplasts and enhancement of incorporation of radioactive precusors [^{3}H-uridine] and [^{3}H]-leucine into RNA and proteins suggested an antisenescence or growth promoting action for polyamines in plants (Galston et al. 1978).

The role of polyamines in cell division in plants has been studied in a wide range of systems, including morphogenesis *in vitro* (see review: Galston & Flores 1991; Martin-Tanguy & Carré 1993), floral induction (Tiburcio *et al.* 1988), floral differentiation (Galston & Flores 1991), fruit set and development (Egea-Cortines & Mizrahi 1991) and internodal growth (Dumortier *et al.* 1983).

In higher plants the diamine, putrescine, is formed from arginine or ornithine by arginine decarboxylase (ADC) or ornithine decarboxylase (ODC), which are

specific, rate-limiting enzymes. The product of ADC is agmatine. Putrescine is converted to the triamine, spermidine (Spd), and then to the tetramine, spermine (Spm), by successive transfers of aminopropyl groups from decarboxylated S-adenosylmethionine. The irreversible suicide inhibitors DFMA (Kallio *et al.* 1981) and DFMO (Metcalf *et al.* 1978) specifically inhibit plant ADC and ODC activities. They are not metabolized in plants.

The function of polyamines in growth (cell division and cell elongation) or morphogenic processes in plant systems has been studied using the following three approaches:

- seeking correlations between polyamine levels, activities of their biosynthetic enzymes and cell division or morphogenic processes
- studying the effects of inhibitors of polyamine biosynthesis with and without exogenous polyamines on development;
- examining the effects of endogenous polyamines on cell division.

We are presently using these approaches to examine two morphogenic processes in which cell division is involved, root and shoot formation during strawberry microcutting development.

Materials and methods

Plant materials - culture media

Mother plants were produced from *in vitro* microcuttings of 'Darsenga' strawberry. Microcuttings were made from *in vitro* plants (with roots) after two months of culture. Each initial cutting, approximately 12 mm long and bearing an axillary bud and one petiole, was cultured on 25 ml of medium in a 25 × 150 mm glass tube closed by a screw cap and a Teflon washer. The basal medium consisted of the microelements of Murashige & Skoog (1962) and the macroelements of Shah & Dalal (1980). They were (mg l^{-1}): KNO_3 (1000), NH_4NO_3 (500), $CaCl_2 \cdot 2H_2O$ (400), $MgSO_4 \cdot 7H_2O$ (300), KH_2PO_4 (100). The explants were cultured at 25 ± 2 °C, under a 16-h photoperiod (Philips TFL - 100 fluorescent tubes, 100 μmol m^{-2} s^{-1} PPF). The relative humidity in the chamber was 70–80%.

Polyamines (agmatine sulfate, putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride) and inhibitors of polyamine biosynthesis (DFMA and DFMO), were added to the medium to yield final concentrations of 1 mM. All compounds were dissolved in water and filter sterilized through a 0.22 mm Millipore filter before adding to the sterilized media. Polyamines and inhibitors were applied at the beginning of the experiment. Media were solidified with 7 g l^{-1} Difco Bacto-Agar. The pH of culture medium was adjusted to 5.8 with 1N KOH and autoclaved for 30 min at 110 °C. DFMA and DFMO were provided by Merrell Dow Institute (Cincinnati, Ohio, USA). Polyamine standards were purchased from Sigma Chemical Co.

Growth variables measured

The following four developmental variables after application of polyamine biosynthesis inhibitors or exogenous polyamines were determined: root and leaf number, root length, and stem length. Reinitiation of growth or micropropagation rate (development of roots and shoots) was measured after 35 days of culture and expressed as a percentage of control. Forty explants were cultured for each experimental condition. All data were means ± SD of three experiments, each point representing 40 ± 10 explants.

Analysis of polyamine content

Tissue was extracted in 5% (v/v) cold $HClO_4$ at a ratio of about 100 mg ml^{-1} $HClO_4$ according to the methods of Flores & Galston (1982). After extraction for 1 h in an ice bath, samples were pelleted at 48,000 *g* for 20 min, and the supernatant phase, containing the 'free' PA fraction, was stored frozen at -20 °C. $HClO_4$ extracts were stable for polyamine analysis by HPLC for more than 6 months under these conditions, provided excessive refreezing and thawing were avoided.

HPLC in combination with fluorescence spectrophotometry was used to separate and quantitate polyamines (putrescine, spermidine and spermine) (Flores & Galston 1982; Smith & Davies 1985), prepared as their dansyl derivatives from plant tissue. The polyamines were derivatized according to the method of Flores & Galston (1982). Fifty to 100 μl aliquots of the supernatant were added to 200 μl of saturated sodium carbonate and 400 μl of dansyl chloride in acetone (7.5 mg ml^{-1}) in a 5-ml tapered reaction vial. After brief vortexing, the mixture was incubated in darkness at room temperature overnight. Excess dansyl reagent was removed by adding 10 mg of proline and incubating the mixture for 30 min. Dansylated polyamines were extracted in 0.5 ml benzene. The organic phase

was collected and stored in glass vials at 20 °C. Standards were processed in the same way, and 2 to 10 nmol were dansylated for each, alone or in combination.

HPLC was performed according to the method of Smith & Davies (1985). The column used was reversed-phase Bondapak C18 (particle size 9 μm; 3.9 × 300 mm) (Waters Assoc.). Samples were eluted from the column with a programmed methanol:water (v/v) solvent gradient, changing from 60% to 95% in 23 min at a flow rate of 1 ml min^{-1}. Elution was completed by 27 min. The column was washed with 100% methanol for 5 min and reequilibrated at 60% methanol before the next sample was injected.

Polyamines in the eluates from the column were detected with a fluorescence spectrophotometer equipped with an 8-μl flow through cell (model 650-10 LC, Perkin Elmer). For dansylated PAs, an excitation wavelength of 365 nm was used with an emission wavelength of 510 nm. Peaks with their areas and retention times were recorded by an attached integrator (Model 3390 A, Hewlett-Packard).

Standard polyamines were treated similarly and each of them was analysed in amounts between 2–10 nmol in order to permit a quantitative assessment of polyamines in the plant samples. Standard curves, run together with tissue extracts in each experiment, were reproductible within 5%. The limit of detectability was 25 to 50 pmol. Heptanediamine was used as an internal standard

Determination of ADC and ODC activities

ADC and ODC activities were determined after several modifications of the procedures described by Birecka *et al.* (1985). Samples were ground in a chilled mortar at a ratio of 1 g fresh weight ml^{-1} of 100 mM Tris-HCl (pH 7. 5) containing 1 mM EDTA, 10 mM mercaptoethanol, 0.5% (w/v) ascorbic acid and 10% w/w active charcoal to absorb phenolics. The extract was centrifuged 20 min at 32,000 *g*. The supernatant was made 50% satured with $(NH_4)_2SO_4$ for 1 h with gentle stirring. The pellet was collected after 20 min centrifugation at 25,000 *g* and resuspended in a minimum of extraction buffer. This fraction was then dialysed against two changes (1 liter each) of 10 mM Tris-HCl containing 1 mM EDTA and 10 mM mercaptoethanol for 8 h. All the procedures were carried out between 0 and 4 °C. The dialysed extract was used to determine the ADC and ODC activities Burtin 1991).

To assay ODC, 50 μl of extract were mixed with 5 μl ($U^{14}C$) ornithine (7.46 Gbq/mmol, Commissariat à l'Energie Atomique, CEA), 45 μl Tris-HCl (100 mM, pH 8.0 containing 10 mM mercaptoethanol), 0.1 mM pyridoxal phosphate and 5.5 mM cold ornithine (Burtin 1991). To assay ADC, 50 μl of extract were mixed with 3 μl ($U^{14}C$) arginine (11.1 Gbq/mmol, Commissariat à l'Energie Atomique, CEA), 45 μl Tris-HCl (100 mM, pH 8.0 containing 10 mM mercaptoethanol), 0.1 mM pyridoxal phosphate and 2:65 mM cold arginine. Reaction mixtures were incubated for 1 h at 30 °C. The reactions were stopped with 10 μl of 17 M acetic acid. For blanks 10 μl of 17 M acetic acid were added at zero time. Denatured proteins were removed after 5 min centrifugation at 15,000 *g*. Aliquots of 10 μl were analysed by thin layer electrophoresis on cellulose plates (Merck Avicel). Electrophoresis was performed in acetic acid-pyridine-water (5:1:94) for 1 h at 300 V. Unlabelled agmatine and putrescine as standards were also spotted on the plate and developed with ninhydrin (5% w/v in ethanol). The cellulose was scraped off and transferred to scintillation vials and the radioactivity determined in a Beckman LS 1801 scintillation counter (Burtin 1991).

Polyamine levels, ADC and ODC activities were measured in aerial parts and roots at different stages of growth and development (at day 5 and day 10: no visible appearance of leaf; at day 15: leaf formation; at day 20, day 25 and day 35: elongation of leaves; at day 20: root formation; at day 25 and at day 35: elongation of roots).

Results

Changes in polyamine levels and related enzymes during the development of strawberry microcuttings

The concentrations of polyamines (putrescine and spermidine) in initial cuttings were less than 0.30 $\mu mol.g^{-1}$ dry weight. The main free polyamines in aerial tissues were putrescine and spermidine (Fig. 1A). Spermidine remained at a relatively low level throughout the 35-day period of development. A substantial accumulation of putrescine was observed and the maximum level occurred at day 15; then it decreased during leaf elongation. Putrescine, spermidine and spermine were identified in roots (Fig. 1B). Spermine concentrations were always low (less than 0.10 μmol g DW^{-1}) and were not detected after 25 days in culture. The maximum level of putrescine occurred at day 25, and decreased during root elongation. The maximum of

spermidine occurred on day 20. Putrescine was the major polyamine in the roots and represented about 70% of the total amine pool at day 25. Putrescine occurred in higher concentrations in shoots than in roots.

ODC activity was not detected in microcuttings throughout the 35-day period of development. A transitory activation of ADC occurred in aerial parts and in roots (data not shown). In green tissues a peak was reached at day 10 (12 pKat g DW^{-1}). In roots ADC activity reached a peak on day 20 (10 pKat g DW^{-1}).

Effects of polyamine biosynthesis inhibitors on development, growth and polyamine levels in strawberry microcuttings

Treatment with DFMA depressed growth and development of strawberry microcuttings (Table 1). Emergence of the first leaves occurred at day 20 of culture. Roots were formed by day 25 of culture. Toxic effects were not observed. A combination of DFMA and agmatine or putrescine led to a complete reversion of the effects of DFMA alone and enhanced all aspects of growth and development (Table 1). With DFMO micropropagation rate and growth were stimulated (Table 1).

DFMA treatment did not result in an accumulation of putsescine in aerial parts and in roots throughout the 35-day period of development (Table 2). At day 15 the concentration of putrescine in the aerial parts was only 10% of that of the untreated explant. Spermidine was absent in aerial parts and roots of DFMA-treated microcuttings (Table 2). After treatment with DFMA and agmatine or putrescine, putrescine increased in microcuttings during the first 10 days of culture (Table 2). On day 10, the level of putrescine in treated explants was six times greater than the value in untreated microcuttings. Spermidine levels were the same as in untreated cultures throughout the 5-week period of development. With the combination of DFMA and agmatine or putrescine, putrescine (Table 2) increased in the roots. On day 20 the level of putrescine in the DFMA + agmatine or DFMA + putrescine treatment was 5 times greater than the level found in untreated explants. Spermidine levels were not affected after this treatment (Table 2). Addition of DFMO caused an increase in putrescine levels in aerial parts during the first 12 days of culture (Table 2). On day 10, the level of putrescine was 5 times greater than the level found in untreated microcuttings. DFMO did not affect spermidine levels throughout the whole period of development (Table 2). Levels of putrescine were higher in roots of DFMO-treated explants than in untreated explants throughout the whole period of development. On day 20 the level of putrescine was five times greater than the level in untreated microcuttings. DFMO did not affect spermidine levels.

Effects of exogenous polyamines on growth development and polyamine levels in strawberry microcuttings

Root formation and aerial vegetative growth were influenced by the addition of polyamines (Table 3). Micropropagation rate, number of roots per explant, number of leaves, length of roots and stems were greatly enhanced after treatment with agmatine, putrescine and spermidine (Table 3). Maximum development was obtained with putrescine. The application of spermine resulted in a decrease in the percentage of reinitiation of growth and development and a decrease in the four developmental variables measured (Table 3).

The distribution of polyamines in strawberry microcuttings after treatment with exogenous polyamines in aerial parts and in roots are shown in Fig. 2 and 3. After treatment with putrescine, a rapid and substantial accumulation of putrescine was observed in aerial parts during the first 10 days of development. On day 10 compared to untreated microcuttings the level was increased by 80%. Treatment with putrescine did not affect spermidine levels throughout the entire period of development (35 days) (Fig. 2 compared to Fig. 1A). Putsescine and spermidine levels increased in aerial parts after addition of spermidine during the first 15 days of culture, with a maximum at day 15 (Fig. 2 compared to Fig. 1A).

After treatment with putrescine a rapid and substantial accumulation of putrescine was observed in roots during the 35-day period of development (Fig. 3 compared to Fig. 1B). At day 20, the level of putrescine in treated explants was eight times greater than the value in untreated microcuttings. Treatment with putrescine did not increase spermidine levels (Fig. 3 compared to Fig. 1B). After treatment with spermidine, putrescine and spermidine levels increased in roots during the whole period of culture but not for putrescine at day 35 (Fig. 3 compared to Fig. 1 B). On day 20 the level of spermidine in treated explants was 12 times greater than the value in untreated microcuttings. Treatment with spermidine did not affect spermine levels (data not shown).

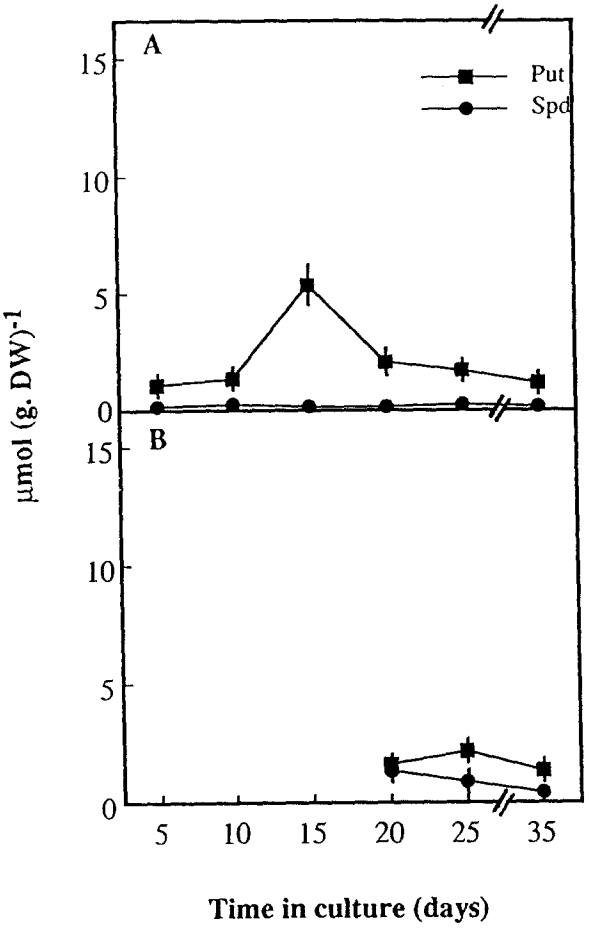

Fig. 1. Changes in free polyamine levels during growth and development (*A*: aerial part; *B*: root system) of *in vitro* strawberry microcuttings. putrescine: ■ spermidine: • Means ± SD of three experiments, each point representing 40 ± 10 explants. Vertical bars show SD if greater than the dimension of the symbol.

Table 1. Effects of the addition of polyamine biosynthesis inhibitors on the growth variables of strawberry microcuttings after 35 days in culture. Numbers represent means ± SD of three experiments, each representing 40 ± 10 explants.

Treatment	Number of roots	Number of leaves	Length of roots (mm)	Length of stems (mm)	Micropropagation rate (% of control)
0	10 ± 2	10 ± 2	25 ± 5	30 ± 5	100
DFMA[a] (1 mM)	3 ± 1	3 ± 1	10 ± 2	10 ± 2	25
DFMA[b] (1 mM) + Agm (1 mM)	30 ± 3	20 ± 2	30 ± 5	60 ± 10	250
DFMO (1 mM)	35 ± 3	42 ± 5	50 ± 10	30 ± 5	300

[a]Root formation at day 25 of culture, shoot formation at day 20 of culture.
[b]Similar results were obtained with DFMA and Put at 1 mM except on the length of roots; with DFMA + Put the length of roots was 50 ± 10. Root formation in both cases occurred at day 20 of culture, shoot formation at day 10 of culture.
[c]Root formation at day 20 of culture, shoot formation at day 10 of culture.

Table 2. Effects of the addition of polyamine biosynthesis inhibitors on polyamine levels in aerial part and root system in strawberry microcuttings, values expressed as μmol g DW^{-1}

Time in culture (days)	No treatment		DFMA (1 mM)		DFMA 1 mM) + Put (1 mM)		DFMO (1 mM)	
	Put	Spd	Put	Spd	Put	Spd	Put	Spd
				Aerial part				
5	0.80 ± 0.04	0.30 ± 0.04	n.d	n.d	3.40 ± 0.50	0.40 ± 0.50	2.80 ± 0.20	0.30± 0.04
10	1.10 ± 0.20	0.40 ± 0.04	0.10 ± 0.04	n.d	6.80 ± 0.50	0.30 ± 0.04	5.60 ± 0.50	0.40 ± 0.04
15	5.60 ± 0.50	0.30 ± 0.04	0.70 ± 0.04	n.d	5.10 ± 0.50	0.30 ± 0.04	5.10 ± 0.50	0.30 ± 0.04
20	2.10 ± 0.20	0.30 ± 0.04	0.20 ± 0.04	n.d.	2.50 ± 0.20	0.30 ± 0.04	1.80 ± 0.20	0.30 ± 0.04
25	1.10 ± 0.20	0.30 ± 0.04	0.30 ± 0.01	n.d	1.20 ± 0.20	0.40 ± 0.04	1.30 ± 0.20	0.40 ± 0.04
35	0.90 ± 0.20	0.20 ± 0.04	0.40 ± 0.04	n.d	1.10 ± 0.20	0.40 ± 0.04	0.90 ± 0.20	0.30 ± 0.04
				Root system				
20	1.50 ± 0.20	1.25 ± 0.20	-	-	7.80 ± 0.50	1.20 ± 0.20	7.10 ± 0.50	1.30 ± 0.20
25	2.0 ± 0.20	0.70 ± 0.04	0.10 ± 0.04	n.d	5.35 ± 0.50	0.50 ± 0.20	4.20 ± 0.50	0.70 ± 0.20

Means ± SD of two experiments, each representing 40 ± 10 explants.
n.d.: not detected; - no morphogenic response

Table 3. Effects of the addition of polyamines on the growth variables of strawberry microcuttings after 35 days in culture.

Treatment	Number of roots	Number of leaves	Length of roots (mm)	Length of stem (mm)	Micropropagation rate (% of control)
O	10 ± 2	10 ± 2	25 ± 5	30 ± 5	100
Agm[a] (1mM)	30 ± 3	25 ± 2	30 ± 5	70 ± 10	250
Put (1 mM)	35 ± 3	30 ± 2	55 ± 10	70 ± 10	250
Spd[a] (1 mM)	25 ± 2	22 ± 2	40 ± 10	70 ± 10	150
Spm (1 mM)	-	4 ± 2	-	20 ± 2	25

Numbers represent means ± SD of three experiments, each representing 40 ± explants.
[a]Root formation occurred at day 20 of culture, shoot formation at day 10.

Treatment with spermine did not affect putrescine and spermidine levels in the aerial parts (data not shown). In aerial parts treatment with spermine resulted in an accumulation of spermine from day 5 to day 25 with a maximum on day 15 (data not shown). At day 15 level of spermine was about 20 μmol g DW^{-1}.

Discussion

Polyamines (agmatine, putrescine, spermidine) have been shown to stimulate the growth of several higher plants, suggesting that the endogenous concentrations of these amines can be growth-limiting (Eilers *et al.* 1988; Bendeck de Cantu & Kandeler 1989; Egea-Cortines & Mizrahi 1991; Galston & Flores 1991; Martin-Tanguy & Carré 1993). It is unlikely that the polyamines in these experiments may act merely as a source of nitrogen when stimulating growth, since they were administered at 1.0 mM in standard medium, and spermine inhibited growth and development.

Application of DFMA, a suicide inhibitor of the putrescine-synthesizing enzyme, arginine decarboxylase, led to an inhibition of microcutting growth. Application of agmatine and putrescine to the inhibited system resulted in a reversal of inhibition, indicating that polyamines are essential compounds in the complex system involved in strawberry microcutting growth. In this investigation, we present evidence that ADC regulates putrescine biosynthesis during micro-

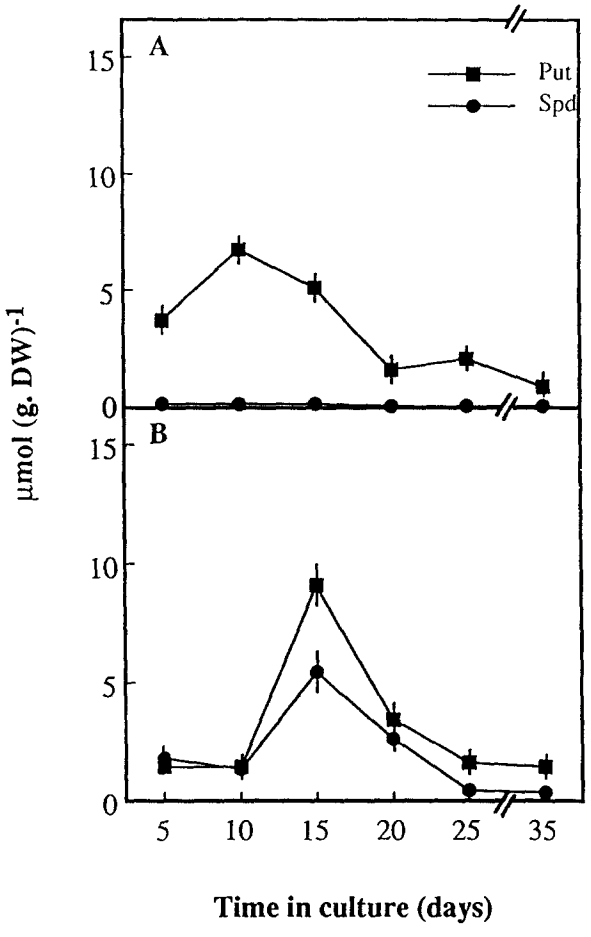

Fig. 2. Effects of addition of polyamines (*A*: Put; *B*: Spd) on free polyamine levels in aerial parts of *in vitro* microcuttings. putrescine: ■, spermidine: •. Vertical bars show SD if greater than the dimension of the symbol.

cutting development. DFMO, a suicide inhibitor of the putrescine- synthesizing enzyme, ornithine decarboxylase, promoted growth and development. This paradoxical effect of DFMO may be related to its unexplained, but consistently observed, slight enhancement of plant ADC activity and accumulation of putrescine (Galston *et al.* 1978; Burtin *et al* 1989; Martin-Tanguy & Carré 1993; Aribaud *et al.* 1994; Bonneau *et al.* 1994a, b; Beranger-Novat *et al.* 1994). Both ODC and ADC are active in plant tissues and their relative contributions to putrescine and polyamine biosynthesis is dependent upon the type of tissue and the developmental process (Tiburcio *et al.* 1988; Galston & Flores 1991; Martin-Tanguy & Carré 1993; Aribaud & Martin-Tanguy 1994; Aribaud *et al.* 1994; Bonneau *et al.* 1994b; Beranger-Novat *et al.* 1994). Vegetative bud differentiation in tobacco tissue culture was inhibited by DFMA, but not by DFMO (Tiburcio *et al.* 1988 ; Burtin et at. 1989). Addition of agmatine or putrescine reversed this inhibition by DFMA. In contrast, DFMO inhibited the *in vitro* and *in vivo* development of floral buds, whereas DFMA did not (Tiburcio *et al.* 1988; Galston & Flores 1991; Burtin *et al.* 1991; Aribaud & Martin-Tanguy 1994). Addition of putrescine reversed this effect. In several cases, somatic embryogenesis was inhibited by DFMA, whereas DFMO was ineffective (Feirer *et al.* 1984; Mengoli *et al.* 1989; Bonneau *et al.* 1990). The inhibition caused by DFMA could be reversed by added agmatine or putrescine. ADC may control the levels of polyamines crucial to embryogenesis (Egea-Cortines & Mizrahi 1991). DFMA, but not DFMO, inhibited *in vitro* growth and development of grapevine microcuttings (Martin-Tanguy & Carré 1993). Application of agmatine or putrescine resulted in a reversal of inhibition. It thus appears that in several plants, ADC may be involved in vegetative

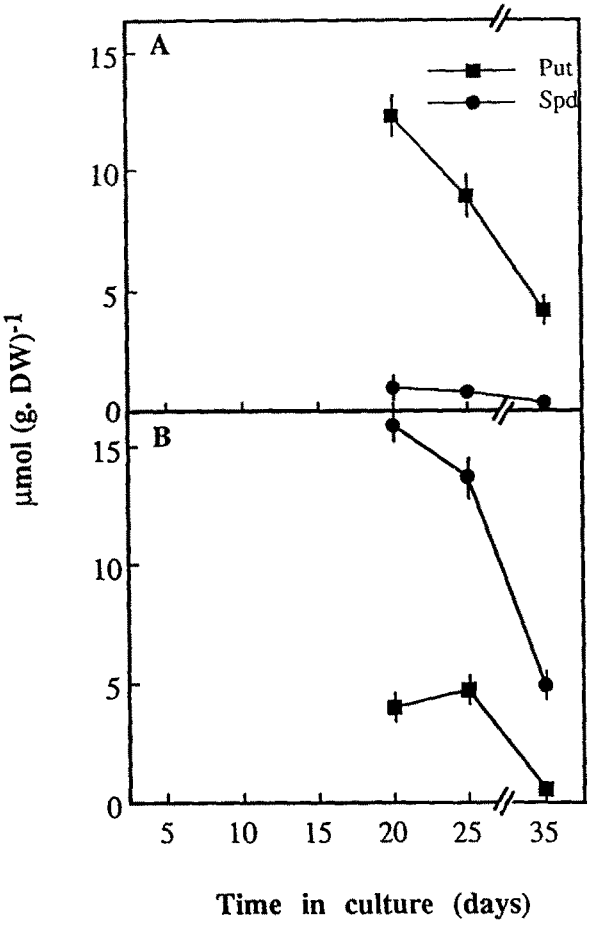

Fig. 3. Effects of addition of polyamines (*A*: Put; *B*: Spd) on free polyamine levels in roots of strawberry microcuttings. putrescine: ■, spermidine: •. Vertical bars show SD if greater than the dimension od the symbol.

development, whereas ODC may be required for floral induction, sexual differentiation, root formation and callus formation (Burtin *et al.* 1990, 1991).

Acknowledgement

This paper represents a part of research submitted by Elisabeth Tarenghi in partial requirements for the doctorate degree at the University of Dijon. This work was supported in part by Grants from the Pernod-Ricard Society (France).

References

Aribaud M & Martin-Tanguy J (1994) Polyamine metabolism, floral initiation and floral development in Chrysanthemum (*Chrysanthemum morifolium* Ramat.). Plant Growth Regul. 15: 23–31

Aribaud M, Carré M & Martin-Tanguy J (1994) Polyamine metabolism and *in vitro* cell multiplication and differentiation of chrysanthemum leaf explants. Plant Growth Regul. 15: 143–155

Bendeck de Cantu L & Kandeler R (1989) Significance of polyamines in flowering in *Spirodela punctata*. Plant Cell Physiol. 30: 455–458

Béranger-Novat N, Monin J & Martin-Tanguy J (1994) Polyamines and their biosynthetic enzymes in dormant embryos of the spindle tree (*Euonymus europaeus* L.) and in dormancy break obtained after treatment with gibberellic acid. Plant Sci. 102: 139–145

Birecka H, Bitonti A & Mc Cann (1985) Assaying ornithine and arginine decarboxylase in some plant species. Plant Physiol, 79: 509–514

Bonneau L, Beranger-Novat N, Martin-Tanguy J & Monin J (1990) Polyamines and somatic embryogenesis in spindle tree (*Euonymus europeaus* L.). The effects of difluoromethylarginine and difluoromethylornithine. In: Flores HT, Arteca RW & Shannon

JC (Eds) Polyamines and Ethylene. Biochemistry, Physiology and Inte ractions (pp 363–364). American Society of Plant Physiologists, Rockville, MD, USA

Bonneau L, Carré M & Martin-Tanguy J (1994a) Polyamines and related enzymes in rice differing in germination potential. Plant Growth Regul. 15: 75–82

Bonneau L, Carré M & Martin-Tanguy J (1994b) Polyamine metabolism during seedling development in rice. Plant Growth Regul. 15: 83–92

Burtin D (1991) Métabolisme des amines libres et conjuguées au cours de la croissance et du développement de *Nicotiana tabacum* var. Xanthi n.c: approches biochimiques et molé culaires. Ph D, Dijon

Burtin D, Martin-Tanguy J, Paynot M & Rossin N (1989) Effects of the suicide inhibitors of arginine and ornithine decarboxylase activities on organogenesis, growth, free polyamine and hydroxycinnamoylputrescine levels in leaf explants of *Nicotiana* × *anthi* n.c. cultivated *in vitro* in a medium producing callus formation. Plant Physiol. 89: 104–110

Burtin D, Martin-Tanguy J, Paynot M, Carré M & Rossin N (1990) Polyamines, hydroxycinnamoylputrescines, and root formation in leaf explants of tobacco cultivated *in vitro*. Effects of the suicide inhibitors of putrescine synthesis. Plant Physiol 93: 1398–1404

Burtin D, Martin-Tanguy J & Tepfer D (1991) α-DL-difluoromethylornithine, a specific, irreversible inhibitor of putrescine biosynthesis, induces a phenotype in tobacco similar to that ascribed to the root-inducing, left-hand transferred DNA of *Agrobacterium rhizogenes*. Plant Physiol, 95: 461-468

Dumortier FM, Flores HE, Shekhawat NS & Galston AW (1983) Gradients of polyamines and their biosynthetic enzymes in coleoptiles and roots of corn. Plant Physiol, 72: 915–918

Egea-Cortines M & Mizrahi Y (1991) Polyamines in cell division, fruit set and development, and seed germination. In: Slocum RD & Flores HE (Eds), Biochemistry and Physiology of Polyamines in Plants (pp 143–154). CRC Press, Boca Raton, FL, USA

Eilers RJ, Sullivan JG & Skirvin RM (1988) Analyzing the effects of exogenous polyamines and growth regulators on planting efficiency of sweet potato protoplasts using a central composite test design. Plant Cell Rep. 7: 216–219

Feirer RP, Mignon G & Litvay JD (1984) Arginine decarboxylase and polyamines required for embryogenesis in wild carrot. Science 223: 1433–1435

Flores H & Galston AW (1982) Analysis of polyamines in higher plants by high performance liquid chromatography. Plant Physiol. 69: 701–706

Galston AW & Flores HE (1991) Polyamines and plant morphogenesis. In: Slocum RD and Flores HE (Eds) Biochemistry and Physiology of Polyamines in Plant (pp 175–186). CRC, Boca Raton, FL, USA

Galston AW, Altman A & Kaur-Sawhney R (1978) Polyamines, ribonucleases, and the improvement of oat leaf protoplasts. Plant Sci. Lett.. 11: 69–79

Kallio A, McCann PP & Bey P (1981) DL-α-difluoromethylarginine: a potent enzyme activated inhibitor of bacterial arginine decarboxylase. Biochemistry 20: 3163–3166

Martin-Tanguy J & Carré M (1993) Polyamines in grapevine microcuttings cultivated *in vitro*. Effects of amines and inhibitors of polyamine biosynthesis on polyamine levels and microcutting growth and development. Plant Growth Regul. 13: 269–280

Mengoli M, Bagni N, Luccarini G, Ronchi VN & Serafini-Fracassini D (1989) *Daucus carota* cell cultures: polyamines and effect of polyamine biosynthesis inhibitors in the preembryogenic phase and different embryo stages. J. Plant Physiol, 134: 389–394

Metcalf BW, Bey P, Danzin C, Jung MJ, Casara P & Vevert JP (1978) Catalytic irreversible inhibition of mammalian ornithine decarboxylase by substrate and product analogues. J. Amer. Chem. Soc. 100: 2251–2253

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Shah RR & Dalal KC (1980) *In vitro* multiplication of *Glycyrrhiza*. Current. Sci. 49: 69–71

Smith TA & Davies PJ (1985) Separation and quantification of polyamines in plant tissue by high performance liquid chromatography of their dansyl derivates. Plant Physiol. 78: 89–91

Tiburcio A, Kaur-Sawhney R & Galston AW (1988) Polyamine biosynthesis during vegetative and floral bud differentiation in thin layer tobacco tissue cultures. Plant Cell Physiol. 7: 1241–1249