Involvement of polyamines in root development at low temperature in the subantarctic cruciferous species *Pringlea antiscorbutica*

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Abstract

Polyamine involvement in root development at low temperature was studied in seedlings of *Pringlea antiscorbutica* R. Br. This unique endemic cruciferous species from the subantarctic zone is subjected to strong environmental constraints and shows high polyamine contents. In the present study, free polyamine levels were modified by inhibitors of polyamine biosynthesis (d-arginine, difluoromethylornithine, cyclohexylammonium, and methylglyoxal-bis-guanylhydrazone) and variations of the endogenous pools were compared to changes in root growth. The arginine decarboxylase pathway, rather than that of ornithine decarboxylase, seemed to play a major role in polyamine synthesis in *Pringlea antiscorbutica* seedlings. Root, but not shoot, phenotypes were greatly affected by these treatments, which modified polyamine endogenous levels according to their expected effects. A positive correlation was found between agmatine level and growth rate of the primary root. Spermidine and spermine contents also showed positive correlations with primary root growth whereas the putrescine level showed neutral or negative effects on this trait. Free polyamines were therefore found to be differentially involved in the phenotypic plasticity of root architecture. A comparison of developmental effects and physiological concentrations suggested that agmatine and spermine in particular may play a significant role in the control of root development.

Key words: Agmatine, low temperature, polyamines, *Pringlea antiscorbutica*, root development.

Introduction

*Pringlea antiscorbutica* R. Br. (Cruciferae), Kerguelen cabbage, is the sole endemic cruciferous species from the Kerguelen phytogeographic province in the subantarctic zone and possesses such particular vegetative and floral characters that a monospecific genus was created for it (Hooker, 1847, in Chapuis et al., 2000). Given the amount of knowledge on the genetics and physiology of cruciferous species, especially *Arabidopsis thaliana*, *P. antiscorbutica* provides a useful model for understanding adaptive mechanisms of plants to extreme environmental conditions including low temperature. The subantarctic region is characterized by a permanent cold temperature with an annual mean around +4 °C, strong and permanent wind (around 10 m s⁻¹) and very high precipitation (Hennion and Martin-Tanguy, 2000). This extreme environment imposes strong environmental constraints likely to constitute a pressure of selection of populations leading to species adaptation. In Kerguelen, *P. antiscorbutica* shows a wide ecological amplitude, being distributed in large populations from the shore to 1000 m altitude. This means that this species can tolerate a variety of adverse conditions such as salt spray and flood, high diurnal temperature amplitude and frequent freeze-thaw cycles (Hennion and Bouchereau, 1998).

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Abbreviations: ADC, arginine decarboxylase; Agm, Agmatine; CHA, cyclohexylammonium; DFMA, difluoromethylarginine; DFMO, difluoromethylornithine; MGBG, methylglyoxal-bis-guanylhydrazone; ODC, ornithine decarboxylase; Put, putrescine; SAMDC, S-adenosylmethionine decarboxylase; Spd, spermidine; Spm, spermine.

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These environmental conditions result, for sessile organisms and ectotherms, in a number of stresses which may particularly endanger early development and elicit developmental, biochemical and molecular responses. Thus, *P. antiscorbutica* exhibits a high flexibility of polyamine metabolism depending on culture conditions and high contents in polyamines in comparison with other crucifers. Roots and shoots of *P. antiscorbutica* accumulate a massive amount of Agm (Hennion and Martin-Tanguy, 2000). This could be a specific answer to extreme environmental conditions as accumulation of Agm has been correlated with frost resistance (Racz et al., 1996). Moreover, polyamines have been implicated in a range of developmental processes, including embryogenesis, root development, flowering and leaf senescence, although their precise role in these processes remains unclear (Evans and Malmberg, 1989). Polyamines could play a major role in proliferation and growth, since the application of exogenous polyamines stimulates development in several higher plants, suggesting that endogenous concentrations of these amines could be growth-limiting (Martin-Tanguy and Carré, 1993). Moreover, polyamines have been shown to modulate gene expression (Hiraga et al., 2000). Finally, as polyamine metabolism is responsive to external conditions (reviewed in Bouchereau et al., 1999), polyamines may play an important role in interactions between environment and development, especially in roots, whose development is a crucial part for seedling establishment. In *P. antiscorbutica*, seed germination and early seedling development was characterized by short-term changes in amine composition (J Martin-Tanguy, F Hennion, unpublished results). In the present work, polyamine metabolism was modulated in vivo during seedling development by the use of inhibitors of different steps of polyamine synthesis. In plants, Put is generally synthesized by two pathways. One pathway starts with ornithine to give Put via ornithine decarboxylase (ODC), the other produces Agm via arginine decarboxylase (ADC) followed by additional steps to produce Put (Smith, 1980). Difluoromethylornithine, DFMO, and d-arg were used to inhibit, respectively, the ODC- and ADC-catalysed steps of Put formation. Put conversion to Spd and then to Spm by successive transfers of aminopropyl groups from decarboxylated S-adenosylmethionine (Martin-Tanguy and Carré, 1993) was inhibited by two different methods. Methylglyoxal-bis-guanylhydrazine, MGBG, was used to inhibit Spd and Spm synthesis by inhibiting the production of decarboxylated S-adenosylmethionine (Bagni et al., 1981). Cyclohexylammonium, CHA, an inhibitor of Spd synthase, was used to inhibit Spd biosynthesis (Martin-Tanguy and Carré, 1993). Most treatments resulted in significant modifications of root development. A positive correlation was found between Agm, Spd and Spm content and primary root growth. In addition, Put biosynthesis in *P. antiscorbutica* seedlings was found to depend on the ADC pathway, rather than on the ODC pathway. These results are discussed in relation to the plasticity of root development and the responses of root systems to environmental stimuli.

**Materials and methods**

**Plant material**

Seeds were collected from a population of *Pringlea antiscorbutica* R. Br. (Cruciferae) in Mayes Island in the Kerguelen Archipelago (68–70° E, 48–50’ S) during the austral summer in February, 1999. The site was a sheltered coastal area. After collection until use, seeds were kept dry at 4 °C in the dark. Seeds were surface-sterilized for 1 min in 95% ethanol, then soaked for 2 h in calcium hypochlorite and rinsed in distilled water (Hennion and Walton, 1997). Surface-sterilized seeds were plated on square (14 × 14 cm) Petri dishes for germination and growth under axenic conditions. Growth medium consisted of 0.8% (w/v) agar in 1 × Murashige and Skoog basal salt mix (M 5519, Sigma), pH 5.7. Petri dishes were sealed with Parafilm and stored vertically in a growth chamber at 22 °C for 4 d (16 h light regime) for germination (Hennion and Walton, 1997). Petri dishes were then transferred to a controlled growth chamber under conditions close to subantarctic summer conditions (510 °C night/day, 14 h light period, 1.3 MJ m⁻² d⁻¹) (Hennion and Bouchereau, 1998).

**Treatment with inhibitors of polyamine metabolism**

Amines (Agm, Put, Spd) and inhibitors of polyamine biosynthesis (d-arg, DFMO, CHA, MGBG) were dissolved in water and sterilized by microfiltration through 0.2 μm cellulose acetate filters (Polylabo, Strasbourg, France). Treatments were performed by adding either inhibitors (2 mM) or a mixture of inhibitor (2 mM) and polyamine (0.5 mM) to the control medium under axenic conditions.

**Measurement of growth and development parameters**

Seedling growth was studied during a 15 d period. Seeds of *P. antiscorbutica* are known to undergo ageing processes upon conservation, which may result in sensitivity to surface-sterilization. In order to limit this cause of variation of physiological response, comparison of treatments had to be made on experiments that were carried out in strict parallel. Primary root length was measured every 3 d. Growth rate after greening of cotyledons was calculated from the linear part of the curve by linear regression. Lateral root density was given by the number of emerged lateral roots per mm of primary roots at day 15. Morphological parameters were calculated as mean (±SEM) of the measurements from at least 17 seedlings.

**HPLC analysis of polyamines**

Polyamines were analysed in order to verify the potential effects of treatments. At the end of the 15 d growth period, seedlings were frozen in liquid nitrogen, then lyophilized. Shoots and roots were separated after lyophilization. For each treatment, shoots or roots were pooled for amine analysis. It was previously found that, in untreated *P. antiscorbutica* plantlets, polyamine levels showed small, never exceeding 10%, inter-individual variations (Hennion and Martin-Tanguy, 2000). In
in the present study, repetition of analysis of free polyamines in different batches of effector-treated plantlets, within the same set of experiments, gave relative errors never exceeding 20\%, whereas modifications of free polyamines as a result of effector treatment were in the 2–10-fold range. In order to extract free amines, aerial parts and roots were ground respectively in 1 ml or in 0.5 ml of 1 M HCl (Hennion and Martin-Tanguy, 2000). After extraction for 1 h, samples were pelleted by centrifugation at 18 000 g for 30 min, and the supernatant, containing free amines, was stored frozen at −20 °C. HPLC and spectrofluorimetry were used to separate and quantify amines prepared as their dansyl derivatives. The methods of Smith and Davies modified by Hennion and Martin-Tanguy were used with some modifications (Smith and Davies, 1985; Hennion and Martin-Tanguy, 1999). Aliquots (100 µl or 200 µl of samples, depending on sample dry weight) of the supernatant were added to 200 µl of saturated sodium carbonate and 600 µl of 27.8 mM dansyl chloride in acetone in a 5 ml tapered vial. After brief vortexing, the mixture was incubated in darkness at room temperature for 16 h. Excess dansyl reagent was removed by reaction with 300 µl of 1.3 M proline and incubation for 30 min in the dark. Dansylated amines were extracted in 1 ml ethyl acetate. The organic phase was collected and then evaporated to dryness. The residue, containing the free amine fraction, was dissolved in methanol and stored in glass vials at −20 °C. The amine fraction was analysed by HPLC using an LKB 2152 plus LKB 2150 chromatography system with an HPLC column packed with reverse phase spherisorb ODS-2 (particle size 5 µm; 4.6×250 mm; Waters, Milford, USA) as previously described (Hennion and Martin-Tanguy, 1999). Portions (20 µl) of the amine fractions were applied to the column and eluted with a programmed methanol:water solvent gradient, changing from 60% to 95% over 23 min at a flow rate of 0.8 ml min⁻¹. Elution was complete after 7 min. For detection of dansyl amines, an excitation wavelength of 365 nm was used with an emission wavelength of 510 nm (Hennion and Martin-Tanguy, 1999). Results were standardized with equimolar (0.1 nmol) mixtures of dansylated amines. All amine standards were purchased from Sigma.

Results

Patterns of germination and seedling development in P. antiscorbutica

Germination was observed within 4 d at 22 °C or, at the latest, 24 h after transfer to the growth chamber at low temperature. At least 24 seeds were plated for germination. Non-germinated seeds and seedlings showing early growth arrest prior to greening were not taken into account for growth measurements. Figure 1A shows root growth under low-temperature conditions. Greening of cotyledons and hypocotyl began on day 4 and went on until day 6. Primary root growth was biphasic (Fig. 1A). The first stage, which was characterized by low growth rate, corresponded to heterotrophic development. After greening and probable transition from heterotrophy to autotrophy, root growth rate was enhanced and growth followed a linear pattern. Development of root hairs started on day 2 and lateral roots emerged after day 10. The first true leaves emerged on day 14. In accordance with previous results (Hennion and Martin-Tanguy, 2000), inter-individual variations within the same set of experiments were very low. However, variations of values between sets of experiments implied that comparison between untreated and treated plantlets had to be carried out within the same set of experiments and that trends had to be validated on repeated sets of experiments.

The results are presented in Table B. The data indicate that DFMO and DFMO plus Put treatments significantly inhibited root growth. The effect was more pronounced at the lower concentration of DFMO. The inhibitory effect of DFMO on root growth was also observed in previous studies (Hennion and Martin-Tanguy, 2000). However, the effect of DFMO on root growth was not always consistent. The results of the present study suggest that DFMO has a specific effect on root growth, which is not observed with other compounds, such as Put. The results also indicate that DFMO and DFMO plus Put treatments significantly inhibited root growth. The effect was more pronounced at the lower concentration of DFMO. The inhibitory effect of DFMO on root growth was also observed in previous studies (Hennion and Martin-Tanguy, 2000). However, the effect of DFMO on root growth was not always consistent. The results of the present study suggest that DFMO has a specific effect on root growth, which is not observed with other compounds, such as Put.
Effects of inhibitors of Put synthesis

DFMO treatment had no effect on germination sensu stricto, but deeply modified post-germinative development, especially root development (Fig. 1A). Thus, DFMO treatment increased primary root length as a result of stimulation of growth rate after greening of cotyledons. This also indicated that the 2 mM concentration of DFMO was sufficient to elicit a biological effect, in accordance with previous studies on other dicot species (Ben-Hayyim et al., 1994). The small impact of DFMO plus Put treatment on the growth rate of primary roots seemed to highlight the efficiency of reversion. Moreover, neither DFMO nor DFMO plus Put modified lateral root density (Fig. 1B). Seedlings treated with DFMO or DFMO plus Put exhibited significant modifications of polyamine levels (Fig. 2). DFMO and DFMO plus Put treatments had very little effect on the content of Spd and Spm in leaves in accordance with the preferential uptake of effectors by roots, and with limited transfer from root to shoot for these amines. In addition, DFMO plus Put treatment led to Put accumulation in roots not in shoots (Fig. 2A), thus suggesting efficient uptake in roots. Total polyamine content in roots was enhanced by DFMO treatment (8.9 μmol g⁻¹ DW) in comparison with control (6.4 μmol g⁻¹ DW). The major effect of DFMO treatment was a 10-fold higher Spm level in roots (Fig. 2B). Roots from seedlings treated with DFMO plus Put also exhibited an overall increase in polyamines, but the Spm pool only increased 3-fold (Fig. 2B). Given the absence of effects of DFMO on Put levels, inhibition by D-arg was attempted in a distinct set of experiments, where control values showed discrepancies with those of Fig. 2 for the reasons given in the Materials and methods. D-arg treatment led to depletion of Agm and Put pools in seedlings as a whole (Fig. 3A). Moreover, Agm distribution in organs was modified in treated seedlings relative to the control. In D-arg-treated roots, Agm content was increased whereas this diamine showed a 4-fold decrease in shoots as compared to the control (Fig. 3A). Put level was almost 4-fold lower in treated roots relative to the control (Fig. 3A). Spd and Spm contents were almost doubled in D-arg-treated roots (Fig. 3A, B). D-arg treatment thus led to a change of balance in the polyamine pool characterized by a depletion of Put, which may be ascribed to a decrease of Put biosynthesis and also to higher metabolism of Put into Spd. The D-arg plus Agm treatment only partially reversed the effects of D-arg on polyamine pools (Fig. 3). Seedlings treated with the reversion treatment did

Fig. 2. Effects of DFMO and DFMO plus Put treatments on free polyamine contents in roots and shoots. Levels of Agm, Put, Spd (A), and Spm (B) were determined after 15 d of growth in different organs of the P. antiscorbutica seedlings described in Fig. 1. Treatments consisted of 2 mM DFMO or 2 mM DFMO + 0.5 mM Put. Relative error on polyamine content was less than 20% as described in Materials and methods.

Fig. 3. Effects of D-arg and D-arg plus Agm treatments on free polyamine contents in roots and shoots. Levels of Agm, Put, Spd (A), and Spm (B) were determined after 15 d of growth in different organs of P. antiscorbutica seedlings in a distinct set of experiments from that of Fig. 2. Treatments consisted of 2 mM D-arg or 2 mM D-arg + 0.5 mM Agm. Relative error on polyamine content was less than 20% as described in Materials and methods.
not exhibit an Agm pool increase in comparison with d-arg-treated seedlings, but a completely recovery of the Put pool in seedlings as a whole (Fig. 3A). The Put pool was almost 2-fold higher in d-arg plus Agm-treated roots in relation to root from controls (Fig. 3A). This seemed to indicate that conversion of Agm to Put could readily occur in roots after uptake. This treatment also led to the accumulation of Spd and Spm in roots in comparison with control (Fig. 3A, B). These inhibitory and reversion treatments modified the polyamine contents and balance, and resulted in phenotypic alteration. Thus, during DFMO plus Put treatment, Put accumulation occurred in parallel with a decrease of root growth rate (Fig. 1). In the same way, d-arg treatment, which resulted in Put depletion in root, enhanced growth rate from 2.4 mm d\(^{-1}\) (±0.1, SEM, n = 19, control) to 2.8 mm d\(^{-1}\) (±0.2, SEM, n = 20, d-arg treatment). However, this stimulatory effect of the d-arg treatment could be associated with an accumulation of Spd and Spm in roots in a similar way to the DFMO treatment, which led to Spm accumulation and a stimulation of root growth rate. Put level therefore seemed to be negatively linked with root growth rate whereas the levels of Spd and Spm appeared positively linked with this trait. Besides, d-arg plus Agm treatment, whilst enhancing the total polyamine pool, inhibited root growth rate from 2.8 mm d\(^{-1}\) (±0.2, SEM, n = 20, d-arg treatment) to 2.2 mm d\(^{-1}\) (±0.1, SEM, n = 21, d-arg plus Agm treatment). Thus, the balance between polyamines, especially Put, Spd and Spm levels, seemed to play an important role in the control of root development.

**Effects of inhibitors of Spd synthesis**

Germination and heterotrophic development were hardly influenced by CHA and CHA plus Spd treatments (Fig. 4). By contrast, during autotrophic development, CHA treatment drastically depressed primary root length, and CHA plus Spd stimulated growth rate of primary roots (Fig. 4A). Moreover, CHA-treated seedlings seemed to show a trend of decrease of lateral root density, whereas this was reversed during CHA plus Spd treatment (Fig. 4B). The effects of MGBG treatment on seedling phenotype and development were drastic (Fig. 5). Germination was inhibited. Moreover, growth rate was deeply reduced during heterotrophic and autotrophic development (Fig. 5). Thus, MGBG treatment led to a significant decrease in root length compared to the control. MGBG-treated seedlings exhibited alterations of primary root, but not shoot, phenotypes. Moreover, MGBG treatment induced necrosis of some root apical meristems. This necrosis was the probable cause of increase in inter-individual variability for the measured parameters. Seedlings treated with CHA or CHA plus Spd exhibited a modification of polyamine levels (Fig. 6).

The most striking result was the 24-fold depletion of the Spd pool in CHA-treated roots (Fig. 6A). Spm, which is produced by Spd metabolism, was also decreased 5-fold (Fig. 6B). The CHA plus Spd treatment led to a much higher accumulation of Spd and Spm than in the control, thus demonstrating Spd absorption and reversion efficiency. Not only the Spd pool was recovered (Fig. 6A), but also the Spm pool with a 8.5-fold increase compared to CHA-treated roots (Fig. 6B). Put content could not be assessed, because the analytical method did not
discriminate Put from CHA. CHA treatment did not influence the Agm pool in seedlings as a whole, but seemed to alter Agm distribution with a depression in roots (Fig. 6A). MGBG treatment led to an overall decrease in polyamines, mainly in roots (Fig. 7). Thus, Spd content in MGBG-treated roots was 2.3-fold lower than in controls (Fig. 7A). Moreover, MGBG also led to a depression of Agm and Put pools in roots (Fig. 7A).

The decrease in growth of primary roots under CHA treatment and its increase under the reversion treatment could be linked to a decrease or an increase, respectively, of Spd and Spm contents. This result was consistent with the results of DFMO treatment about the positive influence of Spd and Spm levels on root growth. In addition, MGBG treatment, whilst inducing smaller modifications of polyamine content than CHA treatment, led to more drastic phenotypic alterations than CHA.

**Correlations between polyamine levels and root development**

The effects of treatments on both polyamine content and root phenotype demonstrated that modifications of polyamine level exerted a strong action on seedling development. Figure 8 thus illustrates the effects of MGBG or DFMO treatments on the phenotype of 15-d-old seedlings. No effect was observed on shoots, but roots exhibited drastic phenotypic alterations. As discussed above, inhibitor and reversion treatments gave coherent results. Moreover, treatments with the different types of inhibitor in independent experiments gave consistent results in terms of response of root growth to free polyamine levels, which were expressed as a percentage of control values (Fig. 9A–D). A positive correlation existed...
Spd and Spm contents in roots also appeared to be positively linked with root growth rate (Fig. 9B, C) as DFMO treatment enhanced both Spd and Spm contents and rate of root growth whereas CHA treatment depleted Spd and Spm contents and inhibited root growth. Moreover, the hypothesis of Agm, Spd and Spm having a role in root development was supported by the results of MGBG treatment (Fig. 7). MGBG treatment increased Spd and Spm levels in roots and shoots, while DFMO treatment decreased them.

Fig. 7. Effects of MGBG treatment on free polyamine contents in roots and shoots. Levels of Agm, Put, Spd (A), and Spm (B) were determined after 15 d of growth in different organs of the P. antiscorbutica seedlings described in Fig. 5. Treatment consisted of 2 mM MGBG. Relative error on polyamine content was less than 20% as described in Materials and methods.

Fig. 8. Effects of MGBG or DFMO treatment on phenotype of 15-d-old P. antiscorbutica seedlings. Seedlings were grown on Murashige and Skoog medium without treatment (control) or treated with 2 mM MGBG or 2 mM DFMO. Aerial parts were not modified by treatments.

Fig. 9. Relationships between growth rate of primary root and relative free polyamine contents in roots of 15-d-old P. antiscorbutica seedlings. Values of growth rate and relative free polyamine contents were taken from independent sets of experiments. Free polyamine contents are expressed as percentage values of the controls. Relationships of root growth rate with Agm (A), Spd (B), Spm (C), and Put (D) levels in roots are shown. Standard errors for root growth rate and polyamine level are given.

between Agm level in root and root growth rate (Fig. 9A). Spd and Spm contents in roots also appeared to be positively linked with root growth rate (Fig. 9B, C) as DFMO treatment enhanced both Spd and Spm contents and rate of root growth whereas CHA treatment depleted Spd and Spm contents and inhibited root growth. Moreover, the hypothesis of Agm, Spd and Spm having...
stimulatory effects on the growth rate of primary roots was underlined by the phenotypic effects of the CHA plus Spd treatment. Whereas inhibition of Spd synthesis led to inhibition of primary root development, reversion treatment stimulated the contents of Agm, Spd and Spm (Fig. 6) and root growth rate (Fig. 4). By contrast, the low range of Put content in roots tended to be negatively linked with the growth rate of primary roots (Fig. 9D), whereas higher Put accumulation led to little growth decrease during DFMO plus Put treatment. MGBG treatment resulted in necrosis of root apices and thus probably induced changes of balance between primary and secondary root development. This induction and interference between S-adenosylmethionine metabolism and ethylene production (Locke et al., 2000) could explain the specific effects of the MGBG. However, MGBG treatment had no visible effect on ethylene-sensitive processes such as hypocotyl elongation. Moreover, in Fig. 9A, B, C, the values of the MGBG experiments did not specifically differ from the overall trend. By contrast, the MGBG experiment significantly differed from the overall trend in Fig. 9D.

Discussion

Polyamine synthesis in Pringlea antiscorbutica

The effectiveness of all inhibitory treatments in modulating polyamine contents and of reversion by polyamine treatment highlighted that polyamine biosynthetic pathways, in P. antiscorbutica, involved the main enzymatic steps already described in other plant species. CHA treatment led to Spd and Spm pool depletion in roots (Fig. 6A, B), which was partially reversed during CHA plus Spd treatment. This result, in accordance with previous studies on other plant species (Martin-Tanguy and Carré, 1993), obviously indicated Spd synthase involvement in P. antiscorbutica. Similarly, the depletion of Spd and Spm pools, consequent on MGBG treatment, was consistent with recent data showing that the production of Spd is regulated mainly at the SAMDC level (Shen et al., 2000). DFMO treatment was expected to lead to a decrease of Put synthesis as a result of ODC activity inhibition. In Pisum sativum, 2 mM DFMO treatment significantly inhibited Put, Spd and Spm biosynthesis, thus suggesting the involvement of ODC (El Ghachi et al., 1996). The maintenance of Put content during DFMO treatment (Fig. 2A) indicated that the ODC-catalysed pathway may not be implicated during this developmental step in P. antiscorbutica. As D-Arg is a competitive inhibitor of ADC (Rosenfeld and Roberts, 1976; Sung et al., 1994), the 2-fold and 4-fold depletion of Put pools, respectively, in shoots and roots, as a result of D-Arg treatment (Fig. 3A), suggested that Put production depended on ADC activity. The synthesis pathway of free polyamines, Put-Spd-Spm, thus seemed to depend on ADC rather than on ODC. Nevertheless, DFMO and DFMO plus Put treatments led to overall polyamine accumulation in roots. A similar enhancement of Agm and Put levels after DFMO treatment was observed in P. antiscorbutica seedlings during germination and the first days of early development (J Martin-Tanguy, F Hennion, unpublished results). All of these results indicated that DFMO could act on a protein target in P. antiscorbutica. Moreover, Burtin et al. have suggested that ODC activity inhibition by DFMO could activate the ADC pathway, and cause an overall increase of polyamine pool as observed in the present study (Burtin et al., 1989). MGBG treatment led to a depletion of the Agm pool, which could be regarded as ADC inhibition (in agreement with the results of Hiatt and Malmberg, 1986). ADC therefore appeared to be the main enzyme involved in Put biosynthesis in P. antiscorbutica, at least in roots. Further work is needed to verify this hypothesis at the enzyme level.

Free polyamines and root development

Little is known about root development under low temperature. When cultivated at 10 °C, soybean (Glycine max cv. Aldana) primary root growth is slowed down to 1 mm d⁻¹, which corresponds to a 10-fold decrease relative to growth at 25 °C (Janas et al., 2000). The rate of primary root growth of P. antiscorbutica seedlings was 2.4 mm d⁻¹ under 5/10 °C night/day temperature regime (Fig. 1). By contrast, this rate of root growth in P. antiscorbutica showed a 2-fold decrease when plants were cultivated at 25 °C (Hennion and Martin-Tanguy, 2000). These characteristics of P. antiscorbutica are probably related to adaptations to the natural conditions of this species and make it a useful model for studying root development under cold conditions. Treatments modulating the levels of free polyamines strongly modified root phenotypes (Figs 8, 9). In a number of plant species, studies have shown that the overall depletion of polyamine pools was linked to root growth inhibition. Thus, in Phaseolus vulgaris, depletion of Put, Spd, Spm titres, due to a decrease of ADC activity, led to decrease of root length (Palavan-Ünsal, 1987). Nevertheless, in terms of development, a decrease in polyamine level is often associated with ageing and senescence whereas accumulation, more precisely Spd and Spm accumulation, is associated with growing tissue activity and organogenesis (Perez-Amador et al., 1995). In P. antiscorbutica, the correlation between intracellular polyamine content and root growth rate depended on polyamine species. Thus, Agm, Spd and Spm were positively correlated with root growth rate, whereas Put content had neutral or negative effects on this trait, depending on the concentration range (Fig. 9D). Agm
content was modulated positively with DFMO or negatively with MGBG treatments with very important consequences on phenotype. An overall positive correlation was found between Agm content in roots and the growth rate of primary roots (Fig. 9A). The involvement of ADC in root development has been found in *A. thaliana* (Watson et al., 1998). The stimulation of root growth due to the Agm pool increase in *P. antiscorbutica* was thus partially consistent with studies on *A. thaliana* mutants (Watson et al., 1998), since ADC double mutants exhibited a compact root pattern. Similarly, DFMA, an inhibitor of ADC, has been shown to decrease root elongation in lateral roots in hairy root cultures (Biondi et al., 1993). Martin-Tanguy and Carré also showed that DFMA inhibited root growth of grapevine microcuttings (Martin-Tanguy and Carré, 1993). Thus, these results would suggest that a direct link between ADC activity and root development might be the size of the Agm pool. Figure 9 (B, C) shows a positive correlation between Spd or Spm content and primary root growth. Moreover, the decrease of Spd and Spm levels by CHA treatment resulted in a decrease of lateral root development (Fig. 4). Since it is well established that primary root growth and lateral root formation involve intensive mitotic activity and are dependent on an array of endogenous physiological factors (Burtin et al., 1990), the positive connection that is observed in this study between Spd and Spm contents and root development, could be related to Spd and Spm involvement in cell cycle activity, growth and differentiation (Fuller et al., 1977; Rupniak and Paul, 1978). The endogenous Spm content in roots of untreated *P. antiscorbutica* plantlets was in the range of large variations of root growth rate in response to Spm level. Spm was thus a good candidate for growth regulation of root development. By contrast, root growth inhibition was found in *A. thaliana*, when 1 mM Spd was added to culture media (Tassoni et al., 2000). Meanwhile, those treatments led to Put accumulation in seedling roots. In agreement with these data, this study, except for the case of MGBG treatment, suggested a negative connection, within a certain concentration range, between Put level in roots and growth rate of primary roots (Fig. 9D). Similarly, in roots excised from plants of *Nicotiana tabacum*, DFMO treatment was found to cause an increase in root system length in parallel with Put pool depletion, Agm accumulation and no variation of Spd pool (Ben-Hayyim et al., 1996). In these excised roots, DFMO caused a fundamental change in root architecture with an increase in primary root length. These changes were cancelled when 1 mM Put was included in the DFMO treatment, thus indicating that the effects of DFMO on development were indeed due to Put limitation. DFMO decreased free Put in excised roots by a factor of 3 and exogenous Put prevented this effect whereas the Spd titre remained unchanged (Ben-Hayyim et al., 1994). In *P. antiscorbutica*, large variations of the Put pool, up to 8-fold the control value, did not show any effect on root growth, but at lower concentrations a decrease of the Put pool seemed to enhance primary root growth, in accordance with the above studies. However, Lee has shown that exogenously-added Put in concentrations varying from 0.01 to 1 mM enhanced root elongation in excised root of *Oryza sativa* L. *in vitro* at 25 °C (Lee, 1997). In the same study, DFMO was found to inhibit root elongation and polyamine levels in roots and these effects were reversed by DFMO plus Put co-treatment, or 1 mM Put exogenous treatment. Moreover, Spd inhibited root elongation at concentrations greater than 0.1 mM and Spm, at all concentrations used, inhibited the elongation of roots grown at 25 °C (Lee, 1997). Similarly, Tarenghi et al. showed that 1 mM Put exogenous treatment led to an increase of Put level in roots and also to an increase of root length in strawberry microcuttings (Tarenghi et al., 1995). Taken together, all of these results emphasize important inter-species differences, which might also depend on culture conditions. In spite of these discrepancies, a number of unrelated dicot species, such as *Nicotiana*, *Phaseolus*, *Vitis*, *Arabidopsis*, and *P. antiscorbutica* seem to share common features of polyamine involvement in root development. Differential impacts of free polyamines on root development may be inferred from studies of these different plant species. However, the overall balance of free polyamines may be more important than a specific role of each polyamine. Thus, reduced root growth in the presence of CHA corresponded to a joint decrease of Agm, Spd and Spm (Fig. 6).

The phenotypic effects of treatments on roots, not shoots, reflected the striking plasticity of root growth and development (Doerner et al., 1996). The range of root architectures that were obtained in *P. antiscorbutica*, through artificial modifications of polyamine pools, suggested the involvement of polyamines in root phenotypic plasticity in agreement with previous studies (Ben-Hayyim et al., 1994). Thus, treatments such as DFMO (Fig. 8) led to longer primary roots. Environmental stress can also result in an increase or a decrease of polyamine levels, depending on type of stress, plant species and time of stress application (Ali, 2000; Hennion and Martin-Tanguy, 2000; Shen et al., 2000). Thus, artificial modulations of polyamine contents can mimic external stimuli (Hennion and Martin-Tanguy, 2000). In heterogeneous soil, the plasticity of root architecture could confer an advantage depending on resource distribution and edaphic constraints (Lynch, 1995). Ben-Hayyim et al. showed that, in *Nicotiana tabacum*, modifying the polyamine contents could convert a superficial root system to one having a tap root with restricted
elongation of the laterals (Ben-Hayyim et al., 1994). Such a change in root architecture, with deeper foraging of water resource, may be an adaptive response to drought stress. This potential link between response to stress, polyamine levels and root development in P. antiscorbutica is currently being investigated in its natural habitat where plants are subjected to heterogeneous soil conditions and summer drought linked with climate change in the subantarctic.

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