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RESEARCH PAPER

Changes in polyamine content and localization of *Pinus sylvestris ADC* and *Suillus variegatus ODC* mRNA transcripts during the formation of mycorrhizal interaction in an *in vitro* cultivation system

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Abstract

The involvement of polyamines (PAs) in the interaction between *Pinus sylvestris* L. seedlings and an ectomycorrhizal fungus *Suillus variegatus* (Swatz: Fr.) O. Kunze was studied in an *in vitro* cultivation system. PA concentrations in seedlings were analysed after 1, 3, and 5 weeks in dual culture with *S. variegatus*, and changes in PA pools were compared with the growth of the seedlings. *Pinus sylvestris* arginine decarboxylase (*ADC*) and *S. variegatus* ornithine decarboxylase (*ODC*) mRNA transcripts were localized during the formation of mycorrhizas. During mycorrhiza formation, *Suillus variegatus ODC* transcripts were found in developing hyphal mantle and Hartig net, and *P. sylvestris ADC* transcripts in specific root parenchyma cells adjacent to tracheids and in mitotic cells of the root apical meristem. However, no unambiguous difference in *ADC* transcript localization between inoculated and non-inoculated roots was observed. Regardless of the unchanged distribution of *ADC* transcripts, inoculation with *S. variegatus* increased free putrescine, spermidine, and spermine concentrations in roots within the first week in dual culture. The concentration of free and conjugated putrescine and conjugated spermidine also increased in the needles due to the fungus. The fungus-induced lateral root formation and main root elongation were greatest between the first and third week in dual culture, coinciding with retarded accumulation or a decrease of free PAs. These results show that accumulation of PAs in the host plant is one of the first indicators of the establishment of ectomycorrhizal interaction between *P. sylvestris* and *S. variegatus* in the *in vitro* system.

Key words: Arginine decarboxylase, ectomycorrhizas, ornithine decarboxylase, *Pinus sylvestris*, polyamines, *Suillus variegatus*.

Introduction

Mycorrhizal symbiosis is a mutualistic association between plants and certain fungi that colonize roots. *Pinus sylvestris* L., like other *Pinus* species, form a symbiosis with ectomycorrhizal (ECM) fungi that entirely cover short roots with a hyphal mantle. Moreover, ECM fungi form a highly branched structure called a Hartig net between epidermal and cortical cells. In ECM association, the host plant supplies simple sugars to the fungal partner, whereas the fungus improves plant nutrition by increasing the surface that absorbs nutrients and also by enabling the use of organic forms of nutrients (Smith and Read, 1997;

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Abbreviations: ADC, arginine decarboxylase; ECM, ectomycorrhizal; ODC, ornithine decarboxylase; PA, polyamine; PCA, perchloric acid; Put, putrescine; Spd, spermidine; Spm, spermine.

Lindahl, *et al*., 2002). In addition to nutritional benefit, the host plant may gain improved tolerance against root pathogens in the presence of the mycorrhizal fungus (Smith and Read, 1997).

Polyamines (PAs) are low molecular weight organic cations that are essential for the development and growth of all living organisms (Tabor and Tabor, 1985; Walters, 1995; Bais and Ravishankar, 2002). PAs exist in cells as free bases or perchloric acid (PCA)-soluble or PCA-insoluble conjugated forms. Free bases may in certain situations act like inorganic cations affecting basic cell chemistry, including pH and ion balance. However, the major part of the physiological functions of PAs has been attributed to their ability to bind to important anionic cell constituents, including nucleic acids, phospholipids, proteins, and phenolic compounds (Del Duca and Serafini-Fracassini, 1993; Bagni and Tassoni, 2001; Facchini *et al*., 2002).

Putrescine (Put), spermidine (Spd), and spermine (Spm) are the most common PAs in plants and they have been reported to be involved in cell proliferation, embryo, floral, and fruit development (Bais and Ravishankar, 2002), as well as rhizogenesis (Couée *et al*., 2004) and responses to biotic stress (Walters, 2003). There is also increasing evidence of the role of PAs in mycorrhizal interactions. ECM fungi have been observed to produce and release different PAs *in vitro* depending on the species and strain (Zarb and Walters, 1994; Fornalé *et al*., 1999; Sarjala, 1999; Niemi *et al*., 2002*a*, 2003) and, in studies on mycorrhiza formation, ECM roots of *P. sylvestris* were found to contain higher concentrations of PAs than non-inoculated roots (Kytöviita and Sarjala, 1997; Sarjala and Taulavuori, 2004).

In plants, Put can be synthesized directly from ornithine in a reaction catalysed by ornithine decarboxylase (ODC) (EC 4.1.1.17) or from arginine after decarboxylation to agmatine by arginine decarboxylase (ADC) (EC 4.1.1.19) and subsequent conversion of agmatine into Put by agmatine iminohydrolase (EC 3.5.3.12) and *N*-carbamoylputrescine aminohydrolase (EC 3.5.1.53). Put is converted into Spd and subsequently into Spm by the addition of aminopropyl moieties from decarboxylated *S*-adenosylmethionine (SAM) in reactions catalysed by Spd synthase (EC 2.5.1.16) and Spm synthase (EC 2.5.1.22), respectively (Bais and Ravishankar, 2002).

Genes encoding ADC have been successfully cloned from several plant species (Bell and Malmberg, 1990; Watson and Malmberg, 1996; Nam *et al*., 1997; Mo and Pua, 2002; Hao *et al*., 2005). In developing zygotic embryos of *P. sylvestris*, Put biosynthesis has also been found to occur preferentially through an ADC pathway and to be involved in mitosis (J Vuosku *et al*., unpublished results). However, *Arabidopsis* is currently the only plant species studied that does not have an *ODC* gene (Hanfrey *et al*., 2001; Hummel *et al*., 2004). In plants possessing both ADC and ODC systems, the enzymes have been proposed to have different physiological roles (Flemetakis *et al.*, 2004; Acosta *et al.*, 2005; Delis *et al.*, 2005; Paschalidis *et al.*, 2005). *ADC* mRNA has been found to accumulate under various stress conditions (Chattopadhyay *et al*., 1997; Urano *et al*., 2003; Hao *et al*., 2005), but ADC has been reported to be regulated both transcriptionally and post-translationally (Malmberg and Cellino, 1994; Watson and Malmberg 1996; J Vuosku *et al*., unpublished results). This indicates that different regulatory mechanisms may be involved in ADC expression.

In fungi, ODC seems to be the sole pathway for production of Put, although there is also evidence of ADC activity in certain fungal species. In the mycelium of an ECM fungus *Paxillus involutus* (Batsch) Fr., Put synthesis proceeded predominantly via ODC, but ADC was also present in the mycelium (Fornalé *et al*., 1999). Furthermore, Sannazzaro *et al.* (2004) observed both ODC and ADC activity in the spores of an arbuscular mycorrhizal fungus *Gigaspora rosea*. However, to date, only genes encoding ODC have been identified in fungi (Blasco *et al*., 2002; Niňo-Vega *et al.* 2004; Morel *et al*., 2005) and, depending on the species, ODC has been regulated at the transcriptional (Blasco *et al*., 2002) or post-transcriptional level (Niňo-Vega *et al*., 2004).

An *in vitro* method has been developed to induce formation and growth of *P. sylvestris* roots by means of inoculation with specific ECM fungi (Niemi *et al*., 2002*a*, *b*). A previous study using this *in vitro* cultivation system showed that ECM fungi and specific PAs have a synergistic effect on adventitious root formation of *P. sylvestris* (Niemi *et al.*, 2002*a*). In the present work, this *in vitro* method was used to study changes in growth and PA metabolism in *P. sylvestris* seedlings as they form mycorrhizal interaction with an ECM fungus *Suillus variegatus* (Swatz: Fr.) O. Kunze. Changes in concentrations of free and PCA-soluble and -insoluble PAs in different parts of the seedlings were analysed, and *P. sylvestris ADC* and *S. variegatus ODC* mRNA transcripts were localized in developing mycorrhizal root system.

Materials and methods

Biological material

The ECM fungus, *S. variegatus* was originally isolated from a basidiocarp under a *P. sylvestris* stand in western Finland and was maintained by cultivating on modified Hagem agar medium (Modess, 1941). For inoculations, the mycelia were cultivated for 3 weeks on strips of moist filter paper lying on modified Melin–Norkrans (MMN) agar medium (Marx, 1969) containing 3.7 mM KH_2PO_4, 1.9 mM $(NH_4)_2HPO_4$, 0.45 mM $CaCl_2$, 0.43 mM NaCl, 0.61 mM $MgSO_4.7H_2O$, 0.2 μM thiamine-HCl, 30.8 μM $FeCl_3.6H_2O$, and 55.5 mM glucose, pH 5.8. For RNA isolations, the mycelia grew for 4 weeks on the moist cellophane membrane (P 400, Visella Oy, Valkeakoski, Finland) lying on modified Hagem agar medium.

Seeds from the open-pollinated elite *P. sylvestris* clone 884, originating from the Punkaharju clone collection in Finland (61°48′ N; 29°17′ E), were surface-sterilized with 2% calcium hypochlorite for 20 min, rinsed in sterile water and germinated on

0.7% water agar in glass jars. The germinating seeds were incubated for 25 d in a growth chamber at 25±1 °C under a 16 h photoperiod (140–150 μmol m^{-2} s^{-1}, Osram L36W/23 and Osram L36W/77).

Growth of Pinus sylvestris *seedlings in the presence of* Suillus variegatus

Petri dishes (14 cm diameter) were filled with MMN medium containing 1.1 mM glucose. Two 25-d-old seedlings were transferred from the germination medium and laid horizontally on sterile moist filter paper covering the agar surface. Individual seedlings were inoculated by placing two filter paper strips covered by a 3-week-old mycelium of *S. variegatus* on the main root. In non-inoculated cultures, mycelium-covered filter papers were substituted by sterile moist filter papers under which two small pieces of fresh agar were placed. A semi-circle of brown paper was placed on the lower part of the lid of the Petri dish to protect the fungus and the root system from direct illumination while leaving the shoot unshaded. Petri dishes were slanted at 70° and incubated in the growth chamber under the same conditions as described for germination.

Seedlings were cultivated in the presence of the fungus for 1, 3, and 5 weeks. At harvest, shoot fresh mass, the length of the main root, fresh mass of the roots, and the number of lateral roots were determined on 15 replicates (two seedlings in a Petri dish represented one replicate) per treatment. The number of root tips with a hyphal mantle was evaluated using a dissecting microscope.

The remaining eight seedlings per treatment were harvested for examination of mycorrhizal structures by light microscopy according to the method described by Niemi and Häggman (2002). After fixation, the root samples were infiltrated and embedded using a Spurr resin kit (Agar Scientific Ltd., UK). The sections were cut in a LKB IV Ultratome and stained with toluidine blue (Merck, Germany).

Analysis of polyamines from Pinus sylvestris *seedlings*

After the growth parameters were determined, needles, stems, and roots of non-inoculated and inoculated seedlings were analysed for PAs. For each harvest, 10 seedlings were pooled to form one sample for PA analyses. PAs were determined for three samples of needles, stems, and roots per treatment at each harvest. Free PCA-soluble and PCA-insoluble conjugated PAs in the needles, stems, and roots were extracted in 5% (w/v) PCA according to Sarjala and Kaunisto (1993) and Fornalé *et al*. (1999). PAs in the crude and hydrolysed extracts were dansylated and then separated by high-performance liquid chromatography (HPLC) (Merck, Hitachi, Japan) as described by Sarjala and Kaunisto (1993). The concentrations of PAs are expressed as nmol g^{-1} fresh weight of plant material.

Localization of Pinus sylvestris ADC *and* Suillus variegatus ODC *mRNA transcripts during the development of mycorrhizas*

The *P. sylvestris* sense and antisense *ADC* probes were designed based on *P. sylvestris* sequence data (AF306451) (J Vuosku *et al*., unpublished results). The partial cloning of *S. variegatus ODC* and the generation of sense and antisense probes were performed as described below. Total RNA was isolated from the 4-week-old mycelium of *S. variegatus* cultivated on a cellophane membrane lying on modified Hagem agar medium. RNA was extracted using a slightly modified version of the nucleic acid extraction protocol of Vainio *et al*. (1998). After two repeated extractions with phenol:chloroform:isoamyl alcohol (25:24:1, by vol.), the aqueous phase was extracted twice with hot phenol (65 °C) and phenol:chloroform:isoamyl alcohol (25:24:1, by vol.). This was followed by chloroform:isoamyl alcohol (24:1, v:v) extraction and treatment with DNase I (Invitrogen, Carlsbad, CA, USA). *Suillus variegatus* partial *ODC* cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen).

The PCR amplification of *S. variegatus* putative *ODC* sequence was performed using primers that were designed based on the regions of high sequence conservation with 5′-CTACGCCGTCAAGTGCAAC-3′ as a forward primer and 5′-CGAAACCACCACCGACAT-3′ as a reverse primer. Products with appropriate length were subcloned using a Qiagen PCR Cloning Kit (Germantown, MD, USA) following the manufacturer's instructions. The nucleotide sequences were determined using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and with an ABI PRISM 377 DNA sequencer (Perkin-Elmer, Wellesley, MA, USA).

Digoxigenin-UTP-labelled 389 bp long antisense and sense RNA probes for *in situ* hybridization were generated using a polymerase chain reaction (PCR)-based technique in which a T7 polymerase promoter sequence (5′-TAATACGACTCACTATAGGG-3′) was introduced at the 5′ end of the gene-specific primers (Young *et al*., 1991; David and Wedlich, 2001). The forward primer (5′-GTTGGGTCATAGCAGCCACT-3′) contained the T7 promoter at its 5′ end, which enabled the synthesis of the antisense probe (Fig. 1). The reverse primer (5′-AAGGCTTCTCGCTGCTTTA-3′) with the T7 promoter at its 5′ end was used to generate the sense probe (Fig. 1). PCR products were gel-purified using a Montage DNA Gel Extraction Kit (Millipore Corporation, Billerica, MA, USA). Transcription was carried out at 37 °C for 2 h in a 20 μl mixture containing ~250 ng of purified PCR product as a template, 5 mM dithiothreitol (DTT), Dig RNA labeling mix (Roche, Basel, Switzerland), 20 U of RNaseOUT™ recombinant RNase inhibitor (Invitrogen), and 100 U of T7 RNA polymerase in appropriate buffer

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ccggatccatacgttataaggcttctcgctgctttaggggctggctttgactgcgcatcc  60
P  D  P  Y  V  I  R  L  L  A  A  L  G  A  G  F  D  C  A  S   20
aacggggaaatctcccaggtcctcagcattggcggtgtcgatccatcgaggatcatcttt  120
N  G  E  I  S  Q  V  L  S  I  G  G  V  D  P  S  R  I  I  F   40
gccaacccttgtaaagccacgtctttcgtgcggagcgctgcaagagccggtgtcaacact  180
A  N  P  C  K  A  T  S  F  V  R  S  A  A  R  A  G  V  N  T   60
atgacgttcgacaatgaagatgaattgtacaaaattgcacgcgcccatccgggtgccaaa  240
M  T  F  D  N  E  D  E  L  Y  K  I  A  R  A  H  P  G  A  K   80
cttatcgttcgtattctcgcggatgattccaaaagtatctgtcggttcggtatcaaattc  300
L  I  V  R  I  L  A  D  D  S  K  S  I  C  R  F  G  I  K  F   100
ggagcaccccttgagggtgtccccggcctcttgtcgaaggctaaggagctcgggctagac  360
G  A  P  L  E  G  V  P  G  L  L  S  K  A  K  E  L  G  L  D   120
gtgattggggtcagtttccacgttggaagtggctgctatgacccaaccacctacggggat  420
V  I  G  V  S  F  H  V  G  S  G  C  Y  D  P  T  T  Y  G  D   140
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Fig. 1. Nucleotide and deduced amino acid sequence of the cDNA encoding the putative ornithine decarboxylase (ODC) of *Suillus variegatus*. Boxes indicate the positions of primers used to generate antisense and sense RNA probe for *in situ* hybridization.

(Invitrogen). The sense and antisense RNA probes were treated with DNAase I Amp Grade (Invitrogen).

Non-inoculated and inoculated seedlings of 25-d-old *P. sylvestris* were grown as described above. Root samples for *in situ* hybridization were collected at the beginning of the dual culture and 1, 3, and 5 weeks later by excising a 0.5 cm long sample from the main root tip. Lateral roots colonized by *S. variegatus* were also collected. For *in situ* hybridization, root samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and dehydrated in a graded ethanol series. Before being embedded with paraffin (Merck, Whitehouse Station, NJ, USA), the samples were treated with 2-methyl-2-propanol. Tissue sections (5 μm thick) were mounted on Super Frost Ultra Plus slides (Menzel-Gläzer, Braunschweig, Germany). Section pretreatment and post-hybridization washes were performed according to Lincoln *et al.* (1994). The amount of *ODC* and *ADC* probes used was 200 ng per slide. Root sections were hybridized in a solution containing 9.6 U ml^{-1} RNaseOUT™ recombinant RNase inhibitor (Invitrogen), 200 μg ml^{-1} tRNA, 50% (v/v) formamide, 10× Denhardt's solution, 0.6× salts for *ODC*, 1× salts for *ADC*, and 10% dextran sulphate for 18 h at 55 °C in a H_2O atmosphere. After hybridization, the slides were washed as described by Lincoln *et al.* (1994), and the blocking procedure and the detection were done according to Coen *et al.* (1990). The hybridized transcripts were detected with anti-digoxigenin antibodies conjugated to alkaline phosphatase using a DIG Nucleic Acid Detection Kit (Roche). An antibody conjugate was diluted 1:1000 for detection.

Growth parameters and free PAs were determined for the seedlings used in the *in situ* hybridization experiment. Growth parameters did not differ significantly (P >0.05) from those of the first inoculation experiment. One week after inoculation, the free Put concentration in the needles of the seedlings used in the *in situ* hybridization was significantly (P <0.05) higher than in the first inoculation experiment, whereas for the concentration of free Spd in the roots the situation was the opposite. However, the responses of the seedlings to the fungus were similar in both experiments and, therefore, only the growth and PA data of the first experiment are shown and discussed.

Statistical analyses

Growth and PA results between non-inoculated and inoculated seedlings were compared using *t*-test or non-parametric Mann–Whitney *U*-test. All statistical analyses were conducted with SPSS/PC version 12.0 (SPSS Inc., Chicago, IL, USA).

Results

Growth and mycorrhiza formation of Pinus sylvestris *seedlings*

Inoculation with *S. variegatus* significantly (P <0.05) increased the main root length (Fig. 2A), number of lateral roots (Fig. 2B), and root/shoot ratio (Fig. 2C) within the first week in dual culture. Root growth induction due to the fungus was most pronounced between weeks 1 and 3 (Fig. 2A–C). During this time, there was a 3.0-fold increase in root and 1.4-fold increase in shoot fresh weight of the inoculated seedlings. Comparable values for non-inoculated seedlings were 1.2 and 1.3, respectively. Mycorrhiza formation started soon after inoculation, and after 1 week the hyphae covered 73% of the lateral root tips. At the end of the experiment, 5 weeks after inoculation, 65% of the lateral roots were covered with fungal hyphae and the Hartig net reached the cortex.

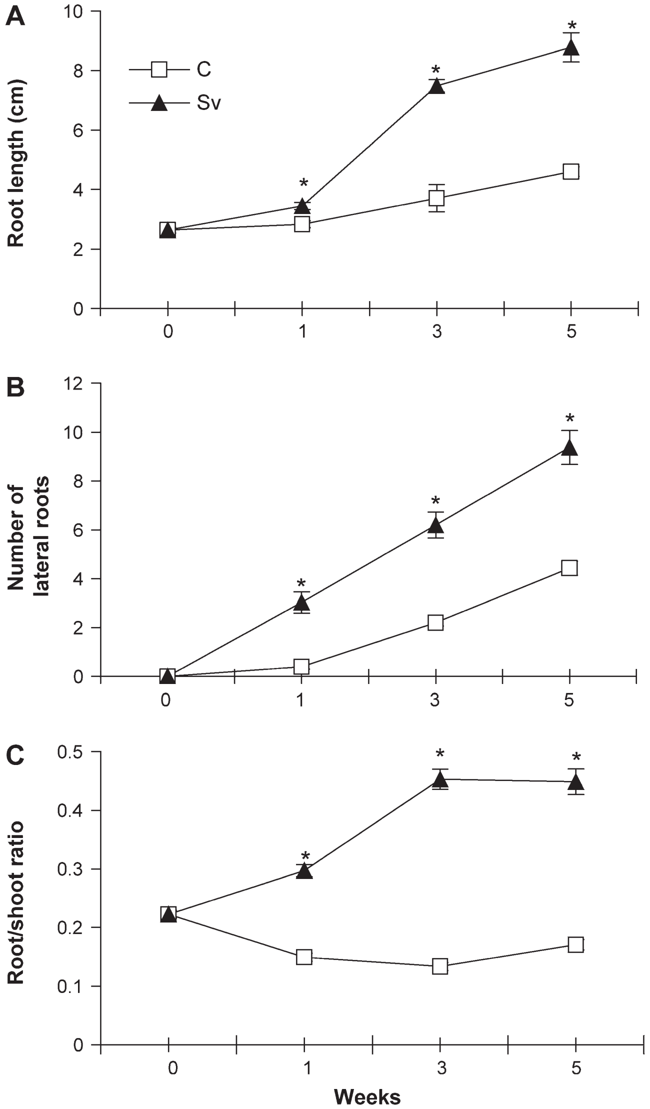

Fig. 2. Effects of inoculation with *Suillus variegatus* on the growth of *Pinus sylvestris* seedlings during 5 weeks of dual culture. Values are means (±SE) of 15 replicates. An asterisk above the data points represents significant (P <0.05) differences between non-inoculated (C) and inoculated (Sv) seedlings according to an independent samples *t*-test.

Polyamine concentrations in Pinus sylvestris *seedlings*

Dual culture for 1 week caused a drastic increase in the concentrations of free Put in needles, stems, and roots of the seedlings (Fig. 3A, D, G). Inoculation also increased the concentrations of free Spd and Spm in stems (Fig. 3E, F) and roots (Fig. 3H, I). In contrast, the concentration of free Spm in the needles was significantly (P <0.05) higher in non-inoculated than in inoculated seedlings (Fig. 3C). During the last weeks of the experiment, there was a general decrease in the concentrations of free Put, Spd, and Spm in the inoculated seedlings (Fig. 3A–I).

Inoculation significantly (P <0.05) increased the concentration of PCA-soluble conjugated Put in needles after 1 week in dual culture (Fig. 4A). In contrast to free Spd,

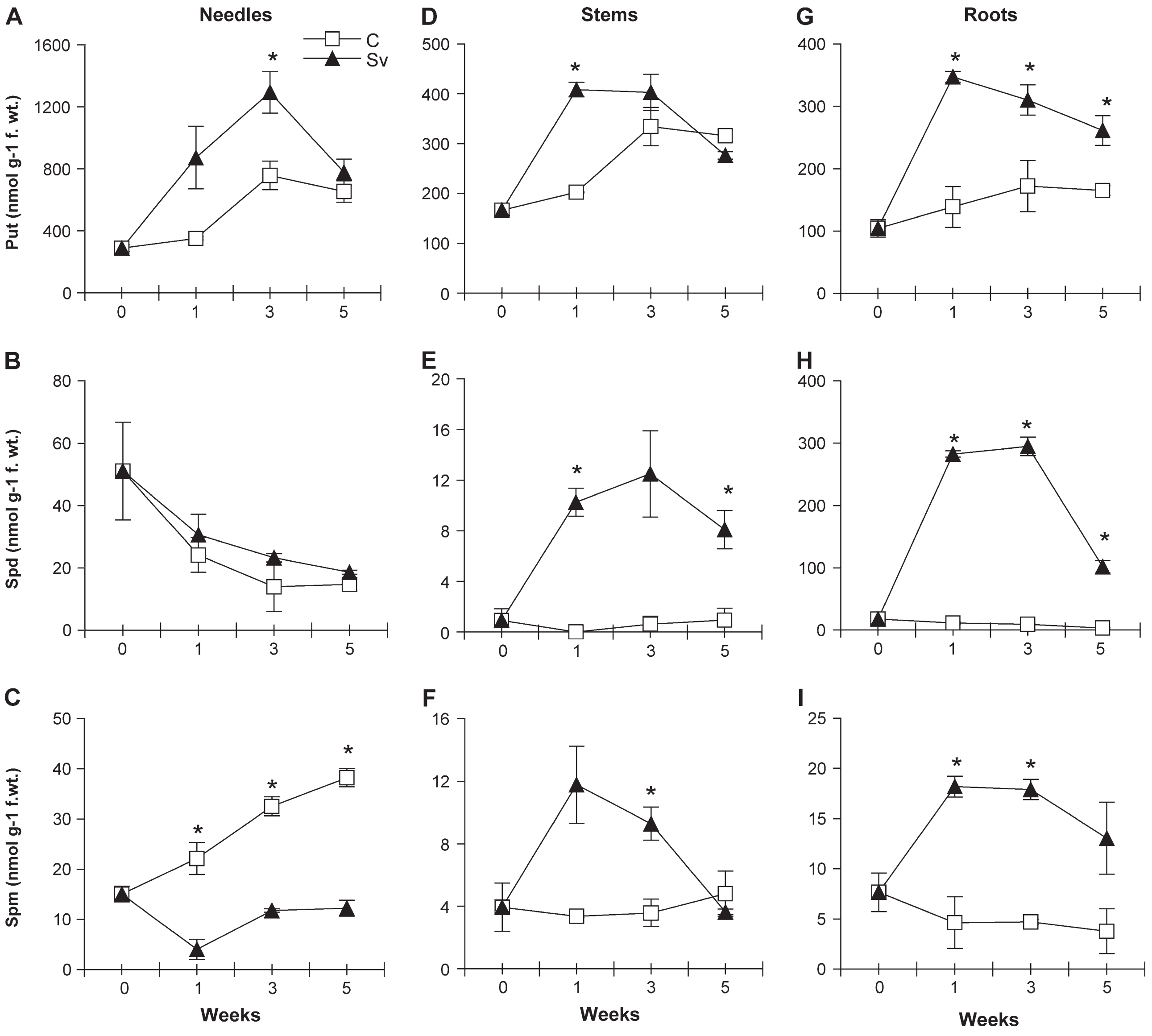

Fig. 3. Effects of inoculation with *Suillus variegatus* on the concentrations (nmol g^{-1} fresh weight) of free polyamines in needles (A–C), stems (D–F), and roots (G–I) of *Pinus sylvestris* seedlings during 5 weeks of dual culture. Values are means (±SE) of three replicates. An asterisk above the data points represents a significant (P <0.05) difference between non-inoculated (C) and inoculated (Sv) seedlings according to an independent samples *t*-test or a non-parametric Mann–Whitney *U*-test. Note the different scales in the figures.

the amount of soluble conjugated Spd in the needles was significantly (P <0.05) higher in inoculated than in non-inoculated seedlings (Fig. 4B). After 1 week in dual culture, the concentrations of soluble conjugated Put and Spd (Fig. 4A, B) in the needles of the inoculated seedlings were higher than those of the comparable free forms (Fig. 3A, B). The amount of soluble conjugated Spm decreased in the needles of the non-inoculated seedlings, whereas in the inoculated seedlings the concentration stayed relatively constant throughout the experiment (Fig. 4C). After 3 weeks in dual culture, the concentrations of soluble conjugated Spd and Spm in stems were significantly (P <0.05) higher in the inoculated than in the non-inoculated seedlings (Fig. 4E, F). In the roots, fungal inoculation caused no significant changes in soluble conjugated PAs (Fig. 4G–I). PCA-insoluble PAs were found in small concentrations or not at all in the seedlings, and there was no significant (P >0.05) difference in insoluble PAs between non-inoculated and inoculated seedlings (data not shown).

Localization of Pinus sylvestris ADC *and* Suillus variegatus ODC *mRNA transcripts during the development of mycorrhizal interaction*

ADC mRNA expression was detected throughout the 5 week experiment. In the main root tips of both non-inoculated and inoculated seedlings, *P. sylvestris ADC* mRNA transcripts accumulated in specific parenchyma cells adjacent to developing or mature tracheids (Fig. 5A).

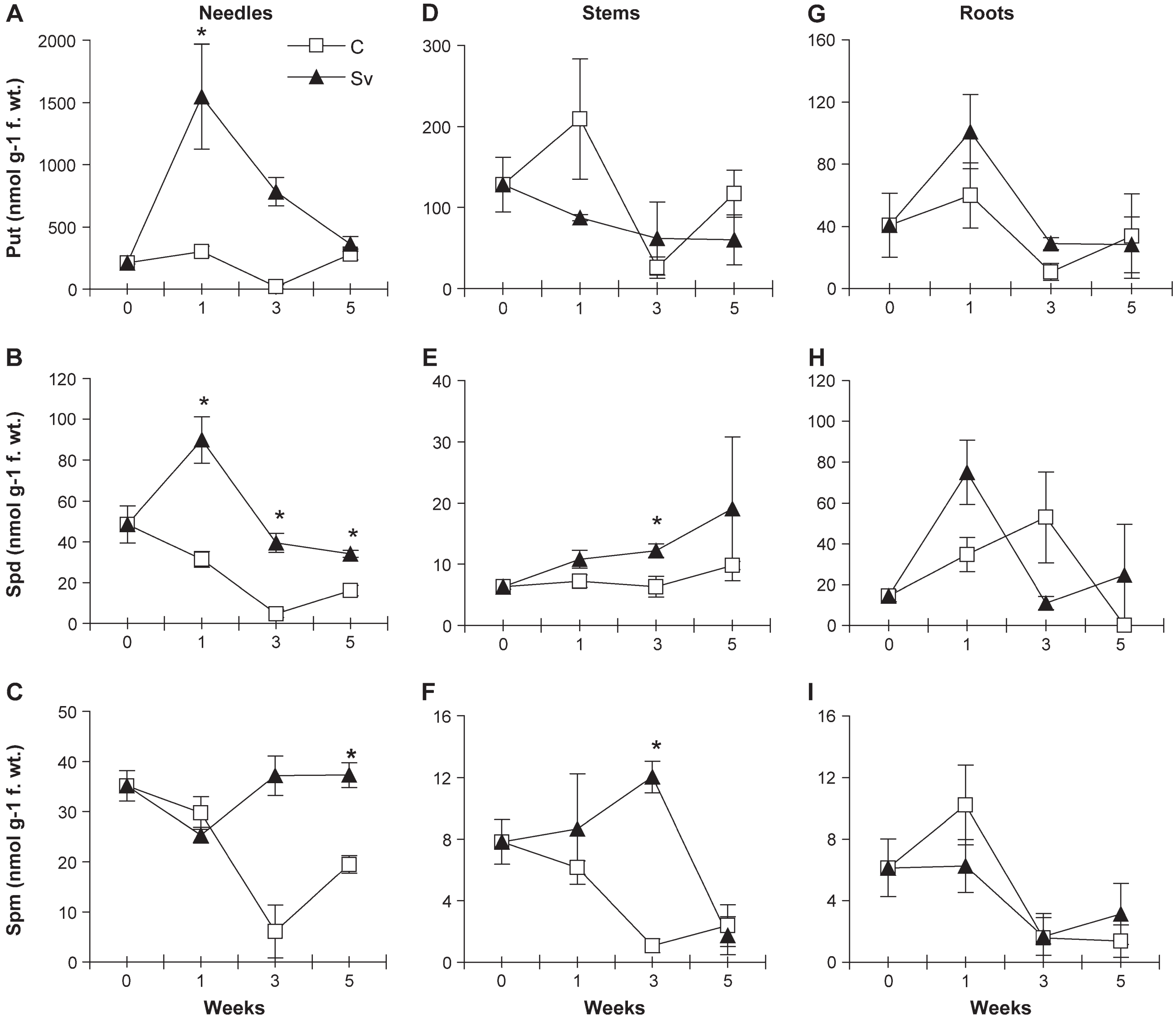

Fig. 4. Effects of inoculation with *Suillus variegatus* on the concentrations (nmol g^{-1} fresh weight) of PCA-soluble polyamines in needles (A–C), stems (D–F), and roots (G–I) of *Pinus sylvestris* seedlings during 5 weeks of dual culture. Values are means (±SE) of three replicates. An asterisk above the data points represents a significant (P <0.05) difference between non-inoculated (C) and inoculated (Sv) seedlings according to a non-parametric Mann–Whitney U-test. Statistical comparisons were performed only when the polyamine was found in all three replicates. Note the different scales in the figures.

The *ADC* expression was also detected more widely throughout the developing vascular tissue (Fig. 5B), especially in inoculated root tips. However, no clear difference in *ADC* expression was found between inoculated and non-inoculated main root tips or between inoculated lateral and main roots. *ADC* transcripts were also localized in mitotic cells of root apical meristem (Fig. 5C, D). *Suillus variegatus ODC* transcripts were found both in fungal hyphae covering lateral roots (Fig. 6A, B) and in the developing Hartig net between epidermal and cortical cells (Fig. 6B). The expression of the *ODC* mRNA transcript was detectable in mycorrhizal hyphae throughout the 5 weeks of dual culture. The specificity of both *ADC* (Fig. 5E) and *ODC* (Fig. 6C) probes was confirmed by the absence of signal in sections hybridized with the sense probes.

Discussion

PAs have been proposed to play an important role in the development of root architecture (Couée *et al.*, 2004). The previous study with *P. sylvestris* hypocotyl cuttings showed that specific exogenous PAs together with ECM fungi have positive effects on adventitious root formation and, moreover, that exogenous PAs have the potential to induce mycorrhiza formation in the *in vitro* cultivation system (Niemi *et al.*, 2002*a*). In the present study, the *in vitro* system was modified to study the effects of the ECM

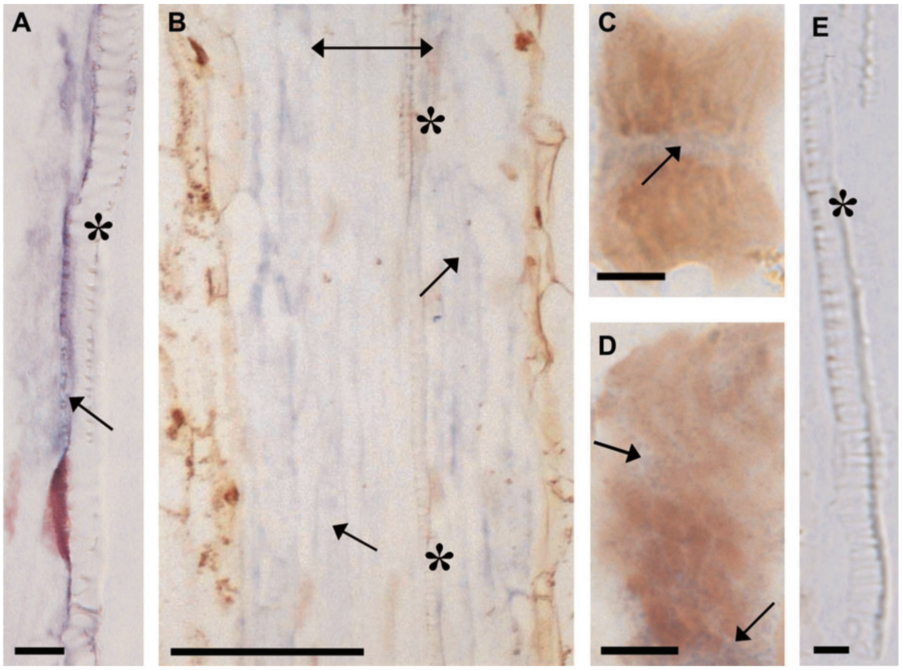

Fig. 5. *In situ* localization of *ADC* mRNA transcripts in the main and lateral roots of *Pinus sylvestris* seedlings during mycorrhiza formation. Hybridization signal was visualized using an alkaline phosphate reaction product (blue-purple colour). (A, C–E) Expression results after 1 week in dual culture and (B) after 5 weeks in dual culture. (A) Part of the vascular tissue of the non-inoculated main root with *ADC* expression adjacent to the tracheid. (B) An inoculated lateral root with *ADC* expression in the vascular tissue. (C) A mitotic cell of an inoculated lateral root in late anaphase. *ADC* expression is seen in the area of the mitotic spindle. (D) A mitotic cell of an inoculated lateral root in prophase. *ADC* expression is seen in the area of the mitotic spindle. (E) Part of the vascular tissue of the non-inoculated main root hybridized with the *ADC* sense probe (negative control). Arrow, transcripts of *ADC*; asterisk, developing or mature tracheid; double-headed arrow, vascular tissue. Bars=10 µm (A, E), 50 µm (B), and 5 µm (C, D).

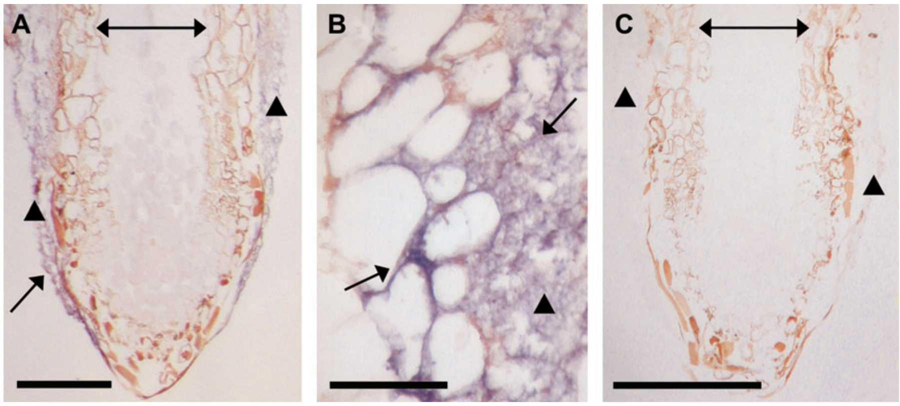

Fig. 6. *In situ* localization of *ODC* mRNA transcripts in the mycelium of *Suillus variegatus* during mycorrhiza formation. The hybridization signal was visualized using an alkaline phosphate reaction product (blue-purple colour). (A) Developing hyphal mantle with *ODC* expression. (B) *ODC* expression in the hyphal mantle and developing Hartig net. (C) An inoculated lateral root hybridized with the *ODC* sense probe (negative control). Arrow, transcripts of *ODC*; triangle, mycelium of *S. variegatus*; double-headed arrow, vascular tissue of *P. sylvestris*. Bars = 200 µm (A, B) and 20 µm (B).

fungus *S. variegatus* on the early growth and PA metabolism of *P. sylvestris* seedlings. Inoculation with *S. variegatus* increased the concentration of free Put in needles, stems, and roots within the first week in dual culture. An increase also in the concentrations of free Spd and Spm in stems and roots suggests that the positive effect of *S. variegatus* was not specific to Put synthesis but that the fungus enhanced PA synthesis in general. The fungus-induced root growth and mycorrhiza formation were greatest at the time of retarded accumulation or decrease of free PAs. These results indicate that accumulation of free PAs in the host plant is predominantly involved in the establishment of the positive ECM interaction between *P. sylvestris* and *S. variegatus* in the *in vitro* system.

ADC and ODC are the key enzymes in the biosynthesis of Put in plants (Bais and Ravishankar, 2002). In

P. sylvestris, Put synthesis seems to occur predominantly via the ADC pathway (J Vuosku *et al.*, unpublished results). In the present study, *P. sylvestris ADC* mRNA transcripts were found in mitotic cells in the apical meristematic regions and in specific parenchyma cells adjacent to tracheids of both inoculated and non-inoculated roots. This shows that increased biosynthesis of Put in the roots due to the fungus was not related to the distribution of *ADC* transcripts. At the same time as *ADC* expression was seen in *P. sylvestris* roots, *S. variegatus ODC* mRNA transcripts were found throughout the developing hyphal mantle and Hartig net. This supports the recent study of Morel *et al.* (2005), in which, using cDNA array, the *Paxillus involutus* ODC-encoding gene was observed to be expressed in ECMs and also in external mycelium. In the present study, concomitant expression of *P. sylvestris ADC* transcripts in specific root cells and *S. variegatus ODC* transcripts in the developing ECM hyphae indicates that the PA biosynthesis of both symbiotic partners was essential for the establishment of mycorrhizas. Since the root and ECM mycelium cannot be separated, PAs of the mantle and Hartig net affected the PA contents of the root samples. However, the concentration of PAs in roots increased drastically within the first week in dual culture, whereas lateral root and mycorrhiza formation was most intensive between the first and third weeks. This indicates that the high amount of PAs in the inoculated roots was due to fungus-induced biosynthesis in the roots.

The expression of *P. sylvestris ADC* mRNA transcripts in the root meristematic region supports the results of the previous study with *P. sylvestris* zygotic embryos, in which *ADC* mRNA transcripts were found in the mitotic spindle in dividing cells (J Vuosku *et al.*, unpublished results). However, to our knowledge, this is the first report of the localization of *ADC* transcripts near tracheids in root tips. The expression of *ADC* close to tracheids indicates that free Put was either related to the development of tracheids or was transported via tracheids. It has been shown that free PAs can be translocated in both phloem and xylem and that free Put is the main PA for long-distance translocation (Antognoni *et al.*, 1998; Shevyakova *et al.*, 2001; Duhazé *et al.*, 2002; Sood and Nagar, 2005). The present finding, i.e fungus-induced increase in free Put, but not in free Spd and Spm of the needles, may indicate that the fungus not only induced Put synthesis in the needles but also induced Put translocation from the roots to shoots.

PAs in the PCA-soluble fraction are conjugated mainly to hydroxycinnamic acids (HCAs) to form HCA amides (HCAAs). Accumulation of HCAAs has been shown to be involved in plant defence reactions against fungal pathogens (Cowley and Walters, 2002; Walters *et al.*, 2002). Peipp *et al.* (1997) observed transient accumulation of four HCAAs in the roots of *Hordeum vulgare* L. during the early stages of AM fungal colonization, and concluded that this accumulation was related to the initiation of defence responses. In the present study, inoculation with *S. variegatus* increased the concentration of PCA-soluble conjugated PAs in *P. sylvestris* seedlings. However, the increase in PA conjugates occurred predominantly in the needles, whereas in the roots no clear difference in the accumulation of conjugates was observed. The present results indicate that the accumulation of PA conjugates is not related to the initiation of a defence reaction in *P. sylvestris*. Conjugated PAs have not been shown to act as amino storage compounds (Facchini *et al.*, 2002) and, therefore, in the present study, the drastic and transient accumulation of conjugated Put and Spd in the needles due to inoculation is not a direct effect of the fungus on plant nutrition. Instead the results suggest that, together with free PAs, conjugated Put and Spd play an important role in early interaction or recognition events between *P. sylvestris* and *S. variegatus*, and are involved in improved growth of the shoots.

In conclusion, inoculation with *S. variegatus* caused a transient increase in the PA concentrations in *P. sylvestris* seedlings that was followed by a significant increase in root growth. This indicates that accumulation of PAs is involved in the establishment of the positive interaction between *P. sylvestris* and *S. variegates* in the *in vitro* cultivation system used here. Localization of *ADC* mRNA transcripts was not changed by the fungus, suggesting that the increase in Put synthesis in the roots due to the fungus was not related to the distribution of *ADC* transcripts. The expression of *S. variegatus ODC* transcripts in developing hyphal mantle and developing Hartig net concomitantly with *P. sylvestris ADC* transcripts shows that PA biosynthesis of both roots and fungal mycelium was essential for the establishment of mycorrhizas.

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References

Acosta C, Pérez-Amador MA, Carbonell J, Granell A. 2005. The two ways to produce putrescine in tomato are cell-specific during normal development. *Plant Science* **168,** 1053–1057.

Antognoni F, Fornale S, Grimmer C, Komor E, Bagni N. 1998. Long-distance translocation of polyamines in phloem and xylem of *Ricinus communis* L. plants. *Planta* **204,** 520–527.

Bagni N, Tassoni A. 2001. Biosynthesis, oxidation and conjugation of aliphatic polyamines in higher plant. *Amino Acids* **20,** 301–317.

Bais HP, Ravishankar GA. 2002. Role of polyamines in the ontogeny of plants and their biotechnological applications. *Plant Cell Tissue and Organ Culture* **69,** 1–34.

Bell E, Malmberg RL. 1990. Analysis of a cDNA encoding arginine decarboxylase from oat reveals similarity to the *Escherichia coli* arginine decarboxylase and evidence of protein processing. *Molecular Genomics and Genetics* **224,** 431–436.

Blasco JL, García-Sánchez MA, Ruiz-Herrera J, Eslava AP, Iturriaga EA. 2002. A gene coding for ornithine decarboxylase (*odcA*) is differentially expressed during *Mucor circinelloides* yeast-to-hyphae transition. *Research in Microbiology* **153,** 155–164.

Chattopadhyay MK, Gupta S, Sengupta DN, Ghosh B. 1997. Expression of arginine decarboxylase in seedlings of indica rice (*Oryza sativa* L.) cultivars as affected by salinity stress. *Plant Molecular Biology* **34,** 477–483.

Coen ES, Romero JM, Doyle S, Elliot R, Murphy G, Carpenter R. 1990. *Floricaula*: a homeotic gene required for flower development in *Antirrhinum majus. Cell* **63,** 1311–1322.

Couée I, Hummel I, Sulmon C, Gouesbet G, Amrani AE. 2004. Involvement of polyamines in root development. *Plant Tissue and Organ Culture* **76,** 1–10.

Cowley T, Walters DR. 2002. Polyamine metabolism in an incompatible interaction between barley and the powdery mildew fungus, *Blumeria graminis* f.sp. *hordei*. *Journal of Phytopathology* **150,** 581–586.

David R, Wedlich D. 2001. PCR-based RNA probes: a quick and sensitive method to improve whole mount embryo *in situ* hybridizations. *BioTechniques* **30,** 769–774.

Del Duca S, Serafini-Fracassini D. 1993. Bound polyamines in plants. *Current Topics in Plant Physiology* **1,** 83–102.

Delis C, Dimou M, Efrose RC, Flemetakis E, Aivalakis G, Katinakis P. 2005. Ornithine decarboxylase and arginine decarboxylase gene transcripts are co-localized in developing tissues of *Glycine max* etiolated seedlings. *Plant Physiology and Biochemistry* **43,** 19–25.

Duhazé C, Gouzerh G, Gagneul D, Larher F, Bouchereau A. 2002. The conversion of spermidine to putrescine and 1,3-diaminopropane in the roots of *Limonium tataricum. Plant Science* **163,** 639–646.

Facchini PJ, Hagel J, Zulak KG. 2002. Hydroxycinnamic acid amide metabolism: physiology and biochemistry. *Canadian Journal of Botany* **80,** 577–589.

Flemetakis E, Efrose R-C, Desbrosses G, Dimou M, Delis C, Aivalakis G, Udvardi M-K, Katinakis P. 2004. Induction and spatial organization of polyamine biosynthesis during nodule development in *Lotus japonicus. Molecular Plant–Microbe Interaction* **17,** 1283–1293.

Fornalé S, Sarjala T, Bagni N. 1999. Endogenous polyamine content and metabolism in the ectomycorrhizal fungus *Paxillus involutus. New Phytologist* **143,** 581–587.

Hanfrey C, Sommer S, Mayer MJ, Burtin D, Michael AJ. 2001. *Arabidopsis* polyamine biosynthesis: absence of ornithine decarboxylase and the mechanism of arginine decarboxylase activity. *The Plant Journal* **27,** 551–560.

Hao Y-J, Kitashiba H, Honda C, Nada K, Moriguchi T. 2005. Expression of arginine decarboxylase and ornithine decarboxylase genes in apple cells and stressed shoots. *Journal of Experimental Botany* **56,** 1105–1115.

Hummel I, Bourdais G, Gouesbet G, Couée I, Malmberg R, El Amrani A. 2004. Differential gene expression of arginine decarboxylase *ADC1* and *ADC2* in *Arabidopsis thaliana*: characterization of transcriptional regulation during seed germination and seedling development. *New Phytologist* **163,** 519–531.

Kytöviita M-M, Sarjala T. 1997. Effects of defoliation and symbiosis on polyamine levels in pine and birch. *Mycorrhiza* **7,** 107–111.

Lincoln C, Long J, Yamaguchi J, Serikawa K, Hake S. 1994. A knotted 1-like homeobox gene in *Arabidopsis* is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. *The Plant Cell* **6,** 1859–1876.

Lindahl B, Taylor AFS, Finlay R. 2002. Defining nutritional constraints on carbon cycling in boreal forests—towards a less phytocentric perspective. *Plant and Soil* **242,** 123–135.

Malmberg RL, Cellino ML. 1994. Arginine decarboxylase of oats is activated by enzymatic cleavage into two polypeptides. *Journal of Biological Chemistry* **28,** 2703–2706.

Marx DH. 1969. The influence of ectotrophic fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. *Phytopathology* **59,** 153–163.

Mo H, Pua E-C. 2002. Up-regulation of arginine decarboxylase gene expression and accumulation of polyamines in mustard (*Brassica juncea*) in response to stress. *Physiologia Plantarum* **114,** 439–449.

Modess O. 1941. Zur Kentniss der Mycorrhizabildner von Kiefer und Fichte. *Symbolae Botanicae Upsaliensis* **5,** 1–147.

Morel M, Jacob C, Kohler A, Johansson T, Martin F, Chalot M, Brun A. 2005. Identification of genes differentially expressed in extraradical mycelium and ectomycorrhizal roots during *Paxillus involutus–Betula pendula* ectomycorrhizal symbiosis. *Applied and Environmental Microbiology* **71,** 382–391.

Nam KH, Lee SH, Lee JH. 1997. Differential expression of *ADC* mRNA during development and upon acid stress in soybean (*Glycine max*) hypocotyls. *Plant Cell Physiology* **38,** 1156–1166.

Niemi K, Häggman H. 2002. *Pisolithus tinctorius* promotes germination and forms mycorrhizal structures in Scots pine somatic embryos. *Mycorrhiza* **12,** 263–267.

Niemi K, Häggman H, Sarjala T. 2003. Ectomycorrhizal fungal species and strains differ in their ability to produce free and conjugated polyamines. *Mycorrhiza* **13,** 283–287.

Niemi K, Sarjala T, Häggman H. 2002a. Effects of diamines on the interaction between ectomycorrhizal fungi and adventitious root formation on Scots pine *in vitro. Tree Physiology* **22,** 373–381.

Niemi K, Vuorinen T, Ernstsen A, Häggman H. 2002*b*. Ectomycorrhizal fungi and exogenous auxins affect root and mycorrhiza formation on *in vitro* Scots pine hypocotyl cuttings. *Tree Physiology* **22,** 1231–1239.

Niño-Vega GA, Sorais F, Calcagno A-M, Ruiz-Herrera J. 2004. Cloning and expression analysis of the ornithine decarboxylase gene (*PbrODC*) of the pathogenic fungus *Paracoccidioides brasiliensis. Yeast* **21,** 211–218.

Paschalidis KA, Roubelakis-Angelakis KA. 2005. Spatial and temporal distribution of polyamine levels and polyamine anabolism in different organs/tissues of the tobacco plant. Correlations with age, cell division/expansion, and differentiation. *Plant Physiology* **138,** 142–152.

Peipp H, Maier W, Schmidt J, Wray V, Strack D. 1997. Arbuscular mycorrhizal fungus-induced changes in the accumulation of secondary compounds in barley roots. *Phytochemistry* **44,** 581–588.

Sannazzaro A, Álvarez C, Menéndez A, Pieckenstains F, Albertó E, Ruiz OA. 2004. Ornithine and arginine decarboxylase activities and effect of some polyamine biosynthesis inhibitors on *Gigaspora rosea* germinating spores. *FEMS Microbiology Letters* **230,** 115–121.

Sarjala T. 1999. Effect of organic and inorganic nitrogen sources on endogenous polyamines and growth of ectomycorrhizal fungi in pure culture. *Mycorrhiza* **8,** 277–281.

Sarjala T, Kaunisto S. 1993. Needle polyamine concentrations and potassium nutrition in Scots pine. *Tree Physiology* **13,** 87–96.

Sarjala T, Taulavuori K. 2004. Fluctuation in free and conjugated polyamines in Scots pine seedlings after changes in temperature and daylength. *Acta Physiologiae Plantarum* **26,** 271–279.

Shevyakova NI, Rakitin VY, Doung DB, Sadomov NG, Kuznetsov VK. 2001. Heat shock-induced cadaverine accumulation and translocation throughout the plant. *Plant Science* **161,** 1125–1133.

Smith S, Read D. 1997. *Mycorrhizal symbiosis*, 2nd edn. Cambridge, UK: Academic Press.

Sood S, Nagar PK. 2005. Xylem and phloem derived polyamines during flowering in two diverse rose species. *Journal of Plant Growth Regulation* **24,** 36–40.

Tabor CW, Tabor H. 1985. Polyamines in micro-organisms. *Microbiological Reviews* **49,** 81–99.

Urano K, Yoshiba Y, Nanjo T, Igarashi Y, Seki M, Sekiguchi F, Yamaguchi-Shinozaki K, Shinozaki K. 2003. Characterization of *Arabidopsis* genes involved in biosynthesis of polyamines in abiotic stress responses and developmental stages. *Plant, Cell and Environment* **26,** 1917–1926.

Vainio EJ, Korhonen K, Hantula J. 1998. Genetic variation in *Phlebiopsis gigantea* as detected with random amplified microsatellite (RAMS) markers. *Mycological Research* **102,** 187–192.

Walters DR. 1995. Inhibition of polyamine synthesis in fungi. *Mycological Research* **99,** 129–139.

Walters DR. 2003. Polyamines in plant disease. *Phytochemistry* **64,** 97–107.

Walters DL, Cowley T, Mitchell A. 2002. Methyl jasmonate alters polyamine metabolism and induces systemic protection against powdery mildew infection in barley seedlings. *Journal of Experimental Botany* **53,** 747–756.

Watson MB, Malmberg RL. 1996. Regulation of *Arabidopsis thaliana* (L.) Heynh arginine decarboxylase by potassium deficiency stress. *Plant Physiology* **111,** 1077–1083.

Young IY, Ailles L, Deugau K, Kisilevsky R. 1991. Transcription of cRNA for *in situ* hybridization from polymerase chain reaction-amplified DNA. *Laboratory Investigation* **64,** 709–712.

Zarb J, Walters DR. 1994. The effects of polyamine biosynthesis inhibitors on growth, enzyme activities and polyamine concentrations in the mycorrhizal fungus *Laccaria proxima. New Phytologist* **126,** 99–104.