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SHORT NOTE

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Ectomycorrhizal fungal species and strains differ in their ability to produce free and conjugated polyamines

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Abstract Production of free and conjugated polyamines by one strain of *Laccaria proxima* (Boud.) Maire, three strains (H, O, K) of *Paxillus involutus* (Batsch) Fr., and one strain of *Pisolithus tinctorius* was studied in vitro. Spermidine (Spd) was the main polyamine in the 4-week-old mycelium of all the fungi. It was mainly present in the free form, but it also occurred in conjugated forms. *Paxillus involutus* strain H released large amounts of free putrescine (Put), and the *Pisolithus tinctorius* released a compound probably related to cadaverine (Cad). On the other hand, these two fungi contained less conjugated polyamines than the other fungi. In addition to the amounts, the forms (perchloric acid soluble and insoluble) of conjugated polyamines in the mycelium varied between species and strains. *L. proxima* contained nearly as much insoluble conjugated Spd as free Spd, whereas *Paxillus involutus* strains O and K contained relatively large amounts of soluble conjugated Spd. The results suggest that ectomycorrhizal fungal species and strains differ in their ability and need to produce conjugated polyamines. The small amounts of soluble conjugated polyamines found in the culture filtrates indicate that some specific conjugated polyamines may be involved in polyamine translocation across the plasma membrane.

Keywords Conjugated polyamines · Free polyamines · *Laccaria proxima* · *Paxillus involutus* · *Pisolithus tinctorius*

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Introduction

Polyamines are low molecular weight molecules with positive charges, which are essential for the development and growth of all living organisms. Their cationic nature at physiological pH allows polyamines to interact with negatively charged nucleic acids, phospholipids and pectic acids (reviewed by Tabor and Tabor 1985; Walters 1995; Tiburcio et al. 1997; Bais and Ravishankaar 2002). The most common polyamines, putrescine (Put), spermidine (Spd) and spermine (Spm), exist in cells in both free and conjugated forms. Growth responses have been postulated to be dependent on the amount of free polyamines, and conjugate formation is one way to regulate the free polyamine pool in the cell. However, binding of polyamines in a variety of different cellular constituents indicates that conjugation may play an important role in, for example, membrane stabilization, polyamine translocation, detoxification of phenolic compounds and defence reactions against biotic and abiotic stress (reviewed by Del Duca and Serafini-Fracassini 1993; Martin-Tanguy 1997; Tassoni et al. 1998).

In polyamine conjugates, a polyamine is covalently bound to a partner molecule, and it can only be released by strong acidic hydrolysis. In perchloric acid (PCA), conjugates occur either as soluble or insoluble forms. For example, polyamines linked to monomers of hydroxycinnamic acids belong to the PCA-soluble fraction, whereas polyamines conjugated with dimers of the same acids are insoluble. However, most of the polyamines in the PCA-insoluble fraction seem to be bound to high molecular weight compounds such as nucleic acids and proteins (reviewed by Del Duca and Serafini-Fracassini 1993; Bagni and Tassoni 2001).

A mycorrhiza is a symbiotic association between a fungus and the roots of a plant, which results in the effective exchange of water and nutrients taken up by the fungus with photosynthates produced by the host plant (Smith and Read 1997). Information has recently started to accumulate on the polyamine metabolism of mycorrhizal fungi (Zarb and Walters 1994a, 1994b; El

Ghachtouli et al. 1996; Fornalé et al. 1999; Sarjala 1999; Niemi et al. 2002), and also on the involvement of polyamines in the establishment of mycorrhizas. Inoculation with ectomycorrhizal (ECM) fungi can change the concentration of free polyamines in roots (Kytöviita and Sarjala 1997) and, furthermore, a supply of specific polyamines can enhance the formation of both ECM (Niemi et al. 2002) and arbuscular mycorrhizal (El Ghachtouli et al. 1995) symbiosis. In the present study, our aim was to compare the ability of different ECM fungal species and strains to synthesize and release free and conjugated polyamines in vitro. Of the conjugated polyamines, both PCA-soluble and PCA-insoluble forms were analysed.

Materials and methods

Fungal material

The ECM fungi were one strain of *Laccaria proxima* (Boud.) Maire, three strains (H, O, K) of *Paxillus involutus* (Batsch) Fr., and one strain of *Pisolithus tinctorius.* They were all originally isolated from basidiocarps under Scots pine (*Pinus sylvestris* L.). The *L. proxima* strain was obtained from the culture collection of the University of Lund, Sweden (strain 90003). *Paxillus involutus* strain H provided by Prof. V. Hintikka was isolated from Helsinki in southern Finland, strain O from Oulu in central Finland, and strain K from Kolari in northern Finland. The *Pisolithus tinctorius* strain was originally isolated in Sweden (Strandberg-Arveby 1980) and was deposited in the culture collection of the Swedish University of Agricultural Sciences, Uppsala, Sweden (strain 1984a). Identification of the *Pisolithus tinctorius* strain at DNA level has been done by Sims et al. (1999). For the present study, all the fungal strains were maintained on Melin-Norkrans (MMN) medium (Marx 1969) modified by Heinonen-Tanski and Holopainen (1991).

Polyamine analyses

For polyamine analyses, the fungi were cultivated in 40 ml MMN liquid medium containing 3.7 mM KH_2PO_4, 4.7 mM NH_4Cl, 0.45 mM $CaCl_2$, 0.43 mM NaCl, 0.61 mM $MgSO_4.7H_2O$, 0.2 μM thiamine HCl, 30.8 μM $FeCl_3.6H_20$ and 41.6 mM glucose, pH 5.8 (Marx 1969; Heinonen-Tanski and Holopainen 1991). Two mycelial plugs, each 5 mm in diameter and cut from the margin of a 1-month-old fungal colony, were placed in the medium and cultivated in the dark at 21±1°C. Four weeks later, the mycelium and culture medium were separated by filtration. The mycelium was washed with distilled water, carefully dried between a filter paper at room temperature and then weighed, whereas the culture filtrate was freeze-dried. Both the mycelia and culture filtrates were stored at – 80°C until analysed.

Free and conjugated polyamines in the mycelium and culture filtrate were extracted in 5% (v/v) PCA according to Sarjala and Kaunisto (1993) and Fornalé et al. (1999), respectively. Both PCA-soluble and PCA-insoluble conjugated polyamines were determined from the mycelia, but only soluble conjugated forms from the culture filtrates. Polyamines in the crude and hydrolysed extracts were dansylated and then separated by HPLC (Merck Hitachi) as described by Sarjala and Kaunisto (1993). The concentrations of polyamines in the mycelia and culture filtrates were expressed as nmol g^{-1} fresh weight fungal mycelium.

Statistical analyses

Differences in the production of free and conjugated polyamines by the ECM fungal strains were compared with a non-parametric Kruskal-Wallis test. Statistical comparisons were made only when a certain polyamine was found in more than two replicates.

Results

Spd was the most abundant polyamine in the 4-week-old mycelia of all the fungi (Table 1). It was predominantly in

Table 1 Concentrations of free and conjugated [perchloric acid (PCA)-soluble and PCA-insoluble] polyamines (nmol g^{-1} fresh weight mycelium) in the mycelium of a 4-week-old culture of ectomycorrhizal fungi in liquid Melin-Norkrans (MMN) medium. Values are means (±SE) of four replicates. *P*values denote probabilities for differences among fungal strains according to a non-parametric Kruskal Wallis test. Statistical comparisons were performed only when a certain polyamine was found in more than two replicates

Polyamine	*Pisolithus tinctorius*	*Laccaria proxima*	*Paxillus involutus* H	*Paxillus involutus* O	*Paxillus involutus* K	*P*
Putrescine						
Free	10.8±2.1	19.9±6.2	71.9±15.0	68.8±22.0	129.4±24.9	0.0001
Soluble conjugated	0	0	2.7±2.7 [a]	1.06±1.0 [a]	19.4±7.1	
Insoluble conjugated	0	0	0	0	0	
Spermidine						
Free	145.4±23.0	280.0±16.8	244.3±46.1	354.4±39.4	451.4±90.7	0.006
Soluble conjugated	0	3.4±3.4 [a]	16.8±16.8 [a]	100.5±63.4	94.3±43.5	
Insoluble conjugated	7.7±2.1	188.7±53.2	9.2±3.0	14.0±3.74	37.0±5.3	0.001
Spermine						
Free	4.7±1.1	2.3±0.3	1.0±0.2	1.6±0.8	0	0.004
Soluble conjugated	0.62±0.37 [a]	0.9±0.5 [a]	0.9±0.5 [a]	1.6±1.3	0.9±0.2	
Insoluble conjugated	1.1±0.2	2.6±1.0	1.7±0.9	1.9±1.2	1.3±0.6	0.307
Cadaverine						
Free	6.2±1.0	0	0	0	0	
Soluble conjugated	0	0	0	0	0	
Insoluble conjugated	0	0	0	0	0	

[a] Polyamines were found in only one or two replicates

Table 2 Concentrations of free and conjugated (PCA-soluble and PCA-insoluble) polyamines (nmol g^{-1} fresh weight mycelium) in the 4-week-old culture filtrate of ectomycorrhizal fungi in liquid MMN medium. Values are means (±SE) of four to six replicates. *P*values denote probabilities for differences among fungal strains according to a non-parametric Kruskal Wallis test. Statistical comparisons were performed only when a certain polyamine was found in more than two replicates

Polyamine	*Pisolithus tinctorius*	*Laccaria proxima*	*Paxillus involutus* H	*Paxillus involutus* O	*Paxillus involutus* K	*P*
Putrescine						
Free	0	5.7±1.6	503.7±168.0	29.3±5.0	25.9±8.2	0.0001
Soluble conjugated	0.1±0.1 [a]	0.1±0.1 [a]	0	0	1.0±1.0 [a]	
Spermidine						
Free	53.2±19.2	36.7±5.2	7.0±2.6	2.9±0.8	8.6±2.5	0.001
Soluble conjugated	0	3.3±3.3 [a]	0	2.5±0.8	0.2±0.2 [a]	
Spermine						
Free	0.7±0.3	0.9±0.1	7.1±1.6	2.4±0.7	1.0±0.3	0.001
Soluble conjugated	0	0	0	0.1±0.1 [a]	0.1±0.1 [a]	
Cadaverine-like compound						
Free	[b]	0	0	0	0	
Soluble conjugated	0	0	0	0	0	

[a] Polyamines were found in only one or two replicates
[b] Concentration of the unknown polyamine was not determined

free form, but was also found in soluble and/or insoluble conjugated forms. *Paxillus involutus* strains O and K contained large amounts of soluble conjugated Spd, whereas in the mycelium of *Paxillus involutus* strain H, *Pisolithus tinctorius*, and *L. proxima* it occurred only in trace amounts. On the other hand, *L. proxima* contained nearly as much insoluble conjugated Spd as free Spd. The amount of Put was highest in the three *Paxillus involutus* strains. It was predominantly in the free form, but was also found as traces in the soluble conjugated form. *Pisolithus tinctorius* was the only fungus with cadaverine (Cad) production. Only very small amounts of Spm were found in the mycelia of the fungal species and strains studied (Table 1).

Pisolithus tinctorius was the only fungus to release an unknown compound with a retention time (14.65 min) between that of Cad (14.09 min) and *N*-methylPut (15.40 min) (Table 2). The compound is probably related to Cad or *N*-methylPut, because these two compounds have the same molecular weight and the same number of carbon atoms and amino groups. *Paxillus involutus* strain H released a much larger amount of free Put than *Paxillus involutus* strains O and K. Spm was present only in trace amounts in the culture filtrates. The amount of soluble conjugated polyamines was very small in the culture media or there were no conjugated polyamines at all, as was the case with *Paxillus involutus* strain H.

Discussion

Spd was the main polyamine in the mycelia of all five fungal strains studied, and Spm was found only in trace amounts, which in general agrees with earlier studies on ECM fungi (Fornalé et al. 1999; Sarjala 1999; Niemi et al. 2002). Polyamines were found both in free and conjugated forms in the mycelium. When studying the polyamine metabolism of one *Paxillus involutus* strain, Fornalé et al. (1999) found no soluble conjugated forms in the mycelium, but the amount of free polyamines compared to the amount of insoluble conjugated polyamines increased during a 4-week culture. In the present study too, 4-week-old mycelia of all three *Paxillus involutus* strains contained hardly any Put in conjugated form. However, a relatively large amount of soluble conjugated Spd was still present. On the other hand, in the mycelium of *L. proxima* the amount of free and insoluble conjugated Spd was nearly equal. These differences in the amount of free and conjugated polyamines may indicate differences between ECM fungal species and strains in their growth rate and rhythm, but they also suggest that species and strains may have different abilities and needs to form conjugated polyamines.

An increasing number of reports on plants have shown that conjugated polyamines do not act only as storage forms for free polyamines but are also involved in a wide variety of physiological processes in cells (reviewed by Del Duca and Serafini-Fracassini 1993; Martin-Tanguy 1997; Bais and Ravishankaar 2002). Recently, Tassoni et al. (1998) reported on a specific interaction between Spd and plasma membrane proteins, and suggested that binding proteins were either Spd receptors or plasma membrane carriers for Spd. In plants, polyamines are also known to bind to a large number of phenolic compounds, and it has been proposed that conjugation may reduce the toxicity of certain phenolics (reviewed by Martin-Tanguy 1997). *Paxillus involutus* produces large amounts of phenolic compounds, but their effects on growth and interaction with polyamines are still unknown. Trace amounts of soluble conjugated polyamines were also found in some culture filtrate samples, and in the case of *Paxillus involutus* strain O in all the replicates. This result is in contrast to earlier reports concerning ECM fungi (Fornalé et al. 1999; Niemi et al. 2002), and indicates that certain conjugated polyamines may be translocated across the plasma membrane. Also in plants, the results on the involvement of conjugated forms in polyamine translocation have been ambiguous (Havelange et al. 1996; Antognoni et al. 1998).

In the study of Fornalé et al. (1999), the release of free Put and Spd showed a considerable increase with increasing age of the mycelium of *Paxillus involutus*. In the present study with 4-week-old mycelia, *P. involutus* strain H was the only one to release a large amount of Put into the culture medium. Effective release of Put probably resulted in a much smaller amount of free Spd and its conjugates in the mycelium of strain H compared to strains O and K. Put released from the mycelium may play an important role in a specific interaction between *P. involutus* strain H and roots. Our previous study showed that the strain H induced adventitious rooting of Scots pine hypocotyl cuttings in vitro, and the fungus and exogenous Put had a synergistic effect on root formation (Niemi et al. 2002). When studying the interaction between *Suillus variegatus* (Swartz: Fr.) O. Kunze and Scots pine roots, Kytöviita and Sarjala (1997) found a larger amount of Put in mycorrhizas than in non-mycorrhizal short roots. However, the *P. involutus* strain H studied here was not able to form mycorrhizal structures with Scots pine (Niemi et al. 2002) and, therefore, the possible role of Put in ECM formation still remains unknown.

In the present study, *Pisolithus tinctorius* contained Cad and released a Cad-like compound into the culture filtrate, which supports our earlier report on the polyamine metabolism of this fungus (Niemi et al. 2002). Zarb and Walter (1994b) reported on the routine production of Cad and its higher derivatives in some ECM fungi, but Cad synthesis has also been associated with polyamine starvation (Alhonen-Hongisto and Jänne 1980; Paulus et al. 1982). In the present study, the mycelium of *P. tinctorius* contained less free and conjugated polyamines than other fungi. However, Cad and a Cad-like compound may be essential for the specific interaction between this fungal strain and Scots pine roots. In our previous study, *P. tinctorius* enhanced adventitious rooting of Scots pine hypocotyl cuttings, and addition of Cad into the medium further increased the rooting frequency and subsequent root growth, as well as mycorrhiza formation (Niemi et al. 2002).

In addition to the importance of different polyamines in the root-fungus interaction, polyamines released by the fungus may be involved in fungus-microbe interactions. While growing in the soil and colonizing a root system, an ECM fungus interacts with a large number of rhizosphere microbes. Certain microbes have been shown to stimulate the growth of ECM fungi and to induce formation of ECM symbiosis (reviewed by Garbaye 1994). As is the case with plants and fungi, polyamines are essential to the growth and development of microbes (Tabor and Tabor 1985) and, therefore, one can speculate on the possible role played by polyamines released by ECM fungi in the growth and activity of beneficial fungi in the rhizosphere.

In conclusion, this study showed that conjugated polyamines are essential for fungal polyamine metabolism, and that fungal species and strains may have different needs to produce soluble and insoluble conjugated polyamines. The role of conjugated forms is still largely under evaluation, but this study indicates that at least some of them may be translocated across the fungal plasma membrane. Release of free Put by the *Paxillus involutus* strain H and a Cad-like compound by *Pisolithus tinctorius* suggests that specific polyamines may be strain-specific compounds for interactions with roots or rhizosphere microbes.

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References

Alhonen-Hongisto L, Jänne J (1980) Polyamine depletion induces enhanced synthesis and accumulation of cadaverine in cultured *Ehrlich ascites* carcinoma cells. Biochem Biophys Res Commun 93:1005–1013

Antognoni F, Fornalé 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, Ravishankaar GA (2002) Role of polyamines in the ontogeny of plants and their biotechnological applications. Plant Cell Tissue Organ Cult 69:1-34

Del Duca S, Serafini-Fracassini D (1993) Bound polyamines in plants. Curr Topics Plant Physiol 1:83–102

El Ghachtouli N, Paynot M, Morandi D, Martin-Tanguy J, Gianinazzi S (1995) The effect of polyamines on endomycorrhizal infection of wild-type *Pisum sativum*, cv. Frisson (nod^+ myc^+) and two mutants (nod^- myc^+ and nod^- myc^-). Mycorrhiza 5:189–192

El Ghachtouli N, Paynot M, Martin-Tanguy J, Morandi D, Gianinazzi S (1996) Effect of polyamines and polyamine biosynthesis inhibitors on spore germination and hyphal growth of *Glomus mosseae*. Mycol Res 100:597–600

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

Garbaye J (1994) Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol 128:197–210

Havelange A, Lejeune P, Bernier G, Kaur-Sawhney R, Galston AW (1996) Putrescine export from leaves in relation to floral transition in *Sinapis alba*. Physiol Plant 96:59–65

Heinonen-Tanski H, Holopainen T (1991) Maintenance of ectomycorrhizal fungi. In: Norris JR, Read DJ, Varna AK (eds) Techniques for the study of mycorrhizas. (Methods in Microbiology, vol 23) Academic Press, London, pp 413–422

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

Martin-Tanguy J (1997) Conjugated polyamines and reproductive development: biochemical, molecular and physiological approaches. Physiol Plant 100:675–688

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

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

Paulus TJ, Kiyonu P, Davis RH (1982) Polyamine deficient *Neurospora crassa* mutants and synthesis of cadaverine. J Bacteriol 152:291–297

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 Physiol 13:87–96

Sims K, Sen R, Watling R, Jeffries P (1999) Species and population structures of *Pisolithus* and *Scleroderma* identified by combined phenotypic and genomic marker analysis. Mycol Res 103:449–458

Smith S, Read D (1997) Mycorrhizal symbiosis, 2nd edn. Academic Press, San Diego, Calif.

Strandberg-Arveby A (1980) Ärttryffel—en sällsynt, ful svamp (English summary). Sven Bot Tidskr 74:329–333

Tabor CW, Tabor H (1985) Polyamines in micro-organisms. Microbiol Rev 49:81–99

Tassoni A, Antognoni F, Battistina ML, Sanvido O, Bagni N (1998) Characterization of spermidine binding to solubilized plasma membrane proteins from zucchini hypocotyls. Plant Physiol 117:971–977

Tiburcio AF, Altabella T, Borrell A, Masgrau C (1997) Polyamine metabolism and its regulation. Physiol Plant 100:664–674

Walters DR (1995) Inhibition of polyamine synthesis in fungi. Mycol Res 99:129–139

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

Zarb J, Walters DR (1994b) The formation of cadaverine, aminopropylcadaverine and *N*,*N*-bis (3-aminopropyl)cadaverine in mycorrhizal and phytopathogenic fungi. Lett Appl Microbiol 19:277–280