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# Effect of organic and inorganic nitrogen sources on endogenous polyamines and growth of ectomycorrhizal fungi in pure culture

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**Abstract** The abilities of three ectomycorrhizal fungi, *Paxillus involutus, Suillus variegatus* and *Lactarius rufus*, to utilize organic and inorganic nitrogen sources were determined by measuring the growth and endogenous free polyamines (putrescine, spermidine and spermine) of pure culture mycelium. Differences were found in the utilization of the nitrogen sources and in the polyamine concentrations between the fungal species and between isolates of *L. rufus*. All the fungi grew well on ammonium and on several amino acids. Endogenous polyamine levels varied with the nitrogen source. Spermidine was commonly the most abundant polyamine; however, more putrescine than spermidine was found in *P. involutus* growing on inorganic nitrogen or arginine. Low amounts of spermine were found in *S. variegatus* and some samples of *L. rufus*. None or only a trace of spermine was found in *P. involutus* mycelium. In all fungi, putrescine concentrations were higher with ammonium than with the nitrate treatment. The total nitrogen content of peat did not determine the ability of *L. rufus* strains isolated from peatland forest sites to utilize organic nitrogen.

**Key words** Ectomycorrhiza · Nitrogen · Putrescine · Spermidine · Spermine

## Introduction

Over 5 million hectars of peatland are used in forestry in Finland and balanced mineral nutrition in those areas is a prerequisite for growing forests (Kaunisto 1997). The importance of nitrogen to forest productivity is well established and its role is also pronounced in drained peatland forests. In forest soils, where the mineralization rate is often low, soluble nitrogen in the soil is largely in the form of organic compounds. Ectomycorrhizal fungi are thought to contribute to the nitrogen nutrition of their host plants in both a quantitative and a qualitative manner. Finlay et al. (1992) demonstrated the utilization of amino acids and proteins by several ectomycorrhizal fungi in pure culture and in symbiosis with *Pinus contorta* Dougl. ex Loud. Potential nitrogen sources include simple organic forms of nitrogen such as soluble amino acids and peptides as well as soluble proteins (Abuzinadah and Read 1986, 1988; Abuzinadah et al. 1986; Keller 1996). Näsholm et al. (1998) demonstrated the uptake of organic nitrogen in the field by the boreal forest plants *Pinus sylvestris* L., *Picea abies* (L.) Karst., *Vaccinium myrtillus* L. and *Deschampsia flexuosa* (L.) Trin.

Keller (1996), studying the ability of several mycorrhizal species and fungus isolates associated with *Pinus cembra* L. in subalpine areas to utilize inorganic and organic nitrogen sources, found considerable intraspecific variation in ability to use proteins. After classifying several species and a large number of isolates from the subalpine zone, Keller (1996) suggested that differences in nitrogen utilization are important ecophysiological markers of ectomycorrhizal fungi.

Polyamines are part of the overall metabolism of nitrogenous compounds, although they do not seem to function in normal nitrogen nutrition (Altman and Levin 1993). Among them putrescine, spermidine and spermine are essential for normal growth and development of plants, animals and micro-organisms (Galston and Kaur-Sawhney 1990). A homeostatic effect of polyamines, i.e. the maintenance of cellular pH and cation/anion balance, is only one of several proposed mechanisms of action of polyamines in living organisms (Smith 1985; Altman and Levin 1993). Studies on interactions between polyamines and ammonium/nitrate nutrition have revealed that the nitrogen source directly affects polyamine biosynthetic pathways (Altman and Levin 1993). According to El Ghachtouli et al. (1995), polyamines in arbuscular mycorrhizal fungi may act on

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mycelial growth to increase contact events with host roots, thus affecting establishment of mycorrhizae. In earlier work by Kytöviita and Sarjala (1997), ectomycorrhizal symbiosis was found to affect the polyamine levels of Scots pine seedlings by increasing putrescine levels in mycorrhizal roots. At the same time, putrescine decreased and spermidine increased in Scots pine needles. Activities of arginine and ornithine decarboxylase, biosynthetic enzymes of polyamines, together with ergosterol content have been used to indicate metabolic activity and hyphal growth of *Hebeloma crustuliniforme* (Bull. ex St. Amans.) Quél in pure culture (Johnson and McGill 1990).

The impact of mycorrhizal symbiosis on the nutrition of trees on drained peatlands has not been widely studied. However, because organic forms of nitrogen predominate in the inorganic fraction in soil solutions (Williams and Edwards 1993), mycorrhiza can presumably play an important role in nitrogen uptake, especially on peatland forests where the mineralization rate is low. In this present study, mycorrhizal fungi were isolated from a drained peatland Scots pine forest from experimental plots with varied total nitrogen content, in order to test their ability to utilize different inorganic and organic nitrogen compounds. The endogenous free polyamine levels in mycelium were also determined both to characterize the fungi and to elucidate the role of polyamines in the maintenance of cell homeostasis under varying nitrogen conditions. Three ectomycorrhizal species common to drained peatland forests, *Paxillus involutus* (Fr.) Fr, *Suillus variegatus* (Fr.) O. Kuntze and *Lactarius rufus* (Scop. ex Fr.) Fr., were studied.

## Materials and methods

### Growth of fungi

The growth of fungi on different nitrogen sources was examined in pure culture using modified Melin-Norkrans liquid medium (Marx 1969) as the basal medium. Ammonium and malt extract were omitted and different nitrogen sources added individually to provide a final concentration of 60 mg $l^{-1}$ N. The nitrogen sources were ammonium sulphate (0.284 g $l^{-1}$), calcium nitrate (0.506 g $l^{-1}$), bovine serum albumen (BSA) (0.375 g $l^{-1}$), gliadine (0.429 g $l^{-1}$), glutamic acid (0.723 g $l^{-1}$), glutamine (0.313 g $l^{-1}$), alanine (0.382 g $l^{-1}$), arginine (0.187 g $l^{-1}$), asparagine (0.283 g $l^{-1}$), and ornithine (0.223 g $l^{-1}$). Glucose was added as the carbon source to give a final carbon to nitrogen ratio of 20:1. The media were adjusted to pH 4.5 by the addition of $H_2SO_4$ or NaOH and autoclaved at 120 °C for 20 min. The organic nitrogen was added to the basal medium through a 0.2-μm sterile filter.

One isolate each of *P. involutus* and *S. variegatus* and four isolates (K8/32, K19/33, A109/15, A109/4) of *L. rufus* were used. Cultures were obtained from fruiting bodies collected from drained peatland under stands of *Pinus sylvestris* (L.) in experimental areas of the Finnish Forest Research Institute in western Finland. The field sites were chosen according to total nitrogen concentrations in peat as determined by the Kjeldahl method in 1995 in plots of two of the experiments presented in detail by Kaunisto (1982). For *L. rufus,* the total nitrogen concentration in peat varied: 0.8% dry wt. in the plot of *L. rufus* K8/32, 1.6% in the plot of *L. rufus* K19/33, 2.7% in the plot of *L. rufus* A109/15 and 2.3% in the plot of *L. rufus* A109/4. The total nitrogen content of peat in the plot of *P. involutus* was 1.5–1.6% dry wt. and 0.8% in the plot of *S. variegatus*. Discs of fungal inoculum were cut from the edge of actively growing colonies on agar plates and transferred to sterile Petri dishes containing 10 ml of culture medium. There were 5 replicate dishes for each combination of species and nitrogen source. Ornithine was used as a nitrogen source only with *P. involutus, S. variegatus* and one isolate of *L. rufus*, whereas all other nitrogen sources were tested with *P. involutus, S. variegatus* and the four isolates of *L. rufus*. The growth of *P. involutus* and *S. variegatus* was followed for 2 weeks and that of *L. rufus* for 4 weeks by measuring increase in diameter of the fungal mat and fresh weight of the fungal mycelium, before polyamine analysis.

### Polyamine analyses

Frozen samples of the fungal mycelium were ground in liquid nitrogen and 5% (v/v) $HClO_4$. The extract was centrifuged at 37 000 *g* for 15 min. Analysis of free polyamines in the supernatant was performed after dansylation (Sarjala and Kaunisto 1993) by HPLC (Merck Hitachi) using a Lichrospher 100 RP-18 column 5 μm (Merck) with a methanol-water gradient. Significant differences among the nitrogen sources were tested by one-way ANOVA.

## Results

The fungi showed clear differences in growth on different nitrogen sources when measured as mat diameters. All isolates of *L. rufus* showed a low growth rate, with usually the highest growth on ammonium (Fig. 1). Only one of the isolates (A109/4) grew better on other organic nitrogen sources (ornithine, glutamine, alanine). All isolates of *L. rufus* grew on nitrate, glutamine and arginine but differed in their utilization of BSA, gliadine, glutamic acid, alanine and asparagine (Fig. 1). *P. involutus* grew as well or better, on nitrate and arginine than on ammonium, whereas the other organic nitrogen sources were not so well utilized. Negligible growth of *P. involutus* was measured on asparagine and no growth at all on gliadine or ornithine (Fig. 1). *S. variegatus* grew better on glutamine and arginine than on ammonium (Fig. 1). Glutamic acid and alanine were almost as good a source of nitrogen as ammonium. Slow growth of *S. variegatus* was observed on BSA and gliadine and no growth was found on nitrate, asparagine or ornithine (Fig. 1).

The correlation coefficients for colony diameter and fresh weight for the *L. rufus* isolates were: K8/32 $r=0.942$ ($P<0.001$), K19/33 $r=0.887$ ($P<0.01$), A109/15 $r=0.811$ ($P<0.01$) and A109/4 $r=0.895$ ($P<0.01$), for *P. involutus* $r=0.641$ ($P<0.07$) and for *S. variegatus* $r=0.915$ ($P<0.001$).

The results for endogenous free polyamine concentration showed large differences between the fungi (Table 1). Spermidine was the most abundant. All the isolates of *L. rufus* had very low or no free putrescine or spermine. Spermidine levels in *L. rufus* were lower than those in *P. involutus* and *S. variegatus*. Spermidine concentration in *L. rufus* depended on the nitrogen source (Table 1), with the highest values on ammon-

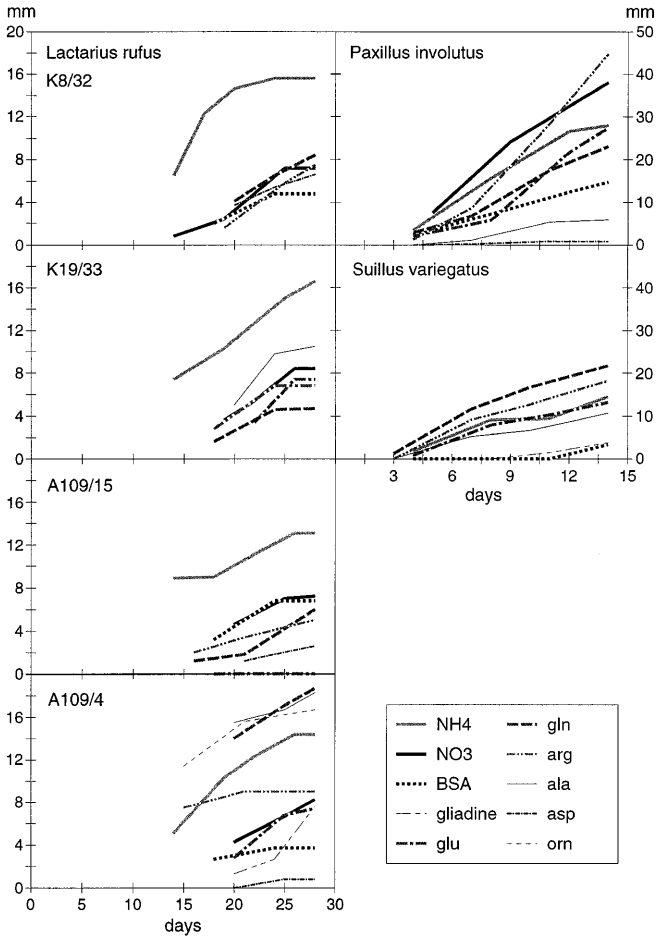


**Fig. 1** Growth responses as diameter of colonies (mm) of four isolates of *Lactarius rufus* (K8/32, K19/33, A109/15, A109/4), *Paxillus involutus* and *Suillus variegatus* on different nitrogen sources (*$NH_4$* ammonium, *$NO_3$* nitrate, *BSA* bovine serum albumen, *glu* glutamic acid, *gln* glutamine, *arg* arginine, *ala* alanine, *asp* asparagine, *orn* ornithine)

ium, glutamine, alanine and ornithine in isolates A109/4, K19/33 and K8/32. Isolate A109/15 had relatively low polyamine concentrations in all treatments.

*P. involutus* had more putrescine in the mycelium than the other species, whereas only traces of spermine were found (Table 1). Putrescine concentration of *P. involutus* was highest on ammonium, glutamic acid, arginine and asparagine. Spermidine concentration varied with the nitrogen source, being highest on ammonium, glutamic acid, arginine and asparagine.

*S. variegatus* mycelium contained less putrescine than spermidine and also only low amounts of free spermine. Most putrescine was found on ammonium and alanine. Spermidine levels were highest on ammonium, glutamic acid, glutamine and alanine.

## Discussion

The three fungal species in this study, *P. involutus, S. variegatus* and *L. rufus*, showed clear differences between each other in ability to utilize organic nitrogen and in endogenous free polyamine profiles. *L. rufus* had a slow growth rate in general and clearly preferred ammonium as a nitrogen source, except for isolate A109/4. *P. involutus* utilized inorganic forms of nitrogen, i.e. ammonium and nitrate, and grew on several organic forms. *S. variegatus* did not grow on nitrate and grew faster on some amino acids than on ammonium. The large differences in nitrogen utilization between the species and the fungal strains reported in other studies (e.g. Finlay et al. 1992; Keller 1996) were also found here.

Piispanen and Lähdesmäki (1983) demonstrated that the concentrations of 21 amino acids increased after drainage of peat, although total nitrogen content decreased. According to these authors, soluble nitrogenous compounds were fourfold higher in dry than in wet peat. Among the amino acids which increased manyfold in peat were glutamic acid, glutamine, alanine and arginine. These were utilized by *P. involutus* and *S. variegatus*, and partly by *L. rufus*, in the present study.

There was no correlation between polyamine concentration and growth of mycelium, measured as either colony diameter or fresh weight, for the three ectomycorrhizal fungi.

Two of the fungi, *S. variegatus* and *L. rufus* K8/32, were isolated from a very low nitrogen site (0.8% dry wt. peat). *S. variegatus* utilized quite well organic nitrogen sources but not nitrate. The *L. rufus* isolate used ammonium better than any of the organic nitrogen sources. *L. rufus* A109/4, which was isolated from a plot with quite a high total nitrogen content (2.3% dry wt.), had the highest ability among *L. rufus* isolates to utilize organic nitrogen sources, showing better growth on glutamine, alanine and ornithine than on ammonium. The *L. rufus* isolates from the plots with the lowest (0.8% dry wt.) (*L. rufus* K8/32) and the highest nitrogen contents (2.7% dry wt.) (*L. rufus* A109/33) utilized the same organic nitrogen compounds and showed no differences in growth in pure culture.

Näsholm et al. (1998) concluded that organic nitrogen is important for plant species in boreal forests, irrespective of their type of root-fungal association and that they can bypass nitrogen mineralization, as earlier suggested by Chapin et al. (1993) and Northup at al. (1995). The variation between mycorrhizal fungal strains and species in ability to utilize different nitrogen sources no doubt influences the adaptation of forest trees to varying nutritional conditions and ability to compete for nitrogen with other plant species. In the present study, the total nitrogen content of peat did not determine the ability of the *L. rufus* strains to utilize different forms of nitrogen. However, the number of strains tested here was quite low for drawing firm conclusions. *L. rufus* is probably one of the most common symbionts of *Pinus sylvestris* on drained peatland forests, but in this study it was not as efficient as the other fungal species in utilizing organic nitrogen in pure cul-

**Table 1** Endogenous free polyamine concentrations (mean nmol per g fresh wt. ± SE) in fungal mycelium grown on different sources of nitrogen. F values in the one-way ANOVA for differences among all treatments and their probabilities are given, except for treatments in which polyamines were found only in one or two of the three replicate samples (means in parentheses) (*n* no growth, – not determined, *Lr Lactarius rufus*)

| Nitrogen source | Lr K8/32 | Lr K19/33 | Lr A109/15 | Lr A109/4 | *Paxillus involutus* | *Suillus variegatus* |
|---|---|---|---|---|---|---|
| Putrescine | | | | | | |
| NH4 | 2.2 ± 0.1 | 0.9 ± 0.5 | 1.9 ± 0.1 | (0.3) | 572.9 ± 130.7 | 132.1 ± 20.6 |
| NO3 | 0 | 0 | 0 | 0 | 108.8 ± 9.1 | n |
| albumen | 0 | n | 0 | 0 | 16.4 ± 6.3 | 10.1 ± 4.8 |
| gliadine | n | n | n | (3.3) | n | 30.2 ± 6.3 |
| Glu | n | (0.6) | n | 0 | 173.2 ± 41.4 | 20.6 ± 6.8 |
| Gln | 0 | (2.3) | 0 | 0.6 ± 0.4 | 80.4 ± 27.6 | 18.7 ± 4.5 |
| Ala | n | (0.7) | n | 6.9 ± 1.0 | 110.6 ± 36.9 | 71.8 ± 16.3 |
| Arg | (1.3) | (11.5) | (1.0) | 0 | 672.3 ± 25.7 | 10.8 ± 5.3 |
| Asp | 0 | n | (4.1) | (2.2) | 396.4 ± 58.0 | n |
| Orn | – | – | – | 0 | n | n |
| F | | | | | 22.113 | 15.943 |
| *P* | | | | | 0.0001 | 0.0001 |
| Spermidine | | | | | | |
| NH4 | 79.6 ± 14.3 | 68.6 ± 2.8 | 36.2 ± 0.5 | 96.1 ± 5.0 | 279.7 ± 67.2 | 709.3 ± 104.6 |
| NO3 | 3.9 ± 0.7 | 4.8 ± 0.4 | 13.7 ± 1.3 | 6.8 ± 0.4 | 27.2 ± 5.2 | n |
| albumen | 27.5 ± 0.2 | n | 69.8 ± 58.3 | 22.2 ± 11.9 | 24.4 ± 7.9 | 32.0 ± 19.6 |
| gliadine | n | n | n | 10.3 ± 1.8 | n | 65.5 ± 19.3 |
| Glu | n | 7.9 ± 0.7 | n | 32.1 ± 5.8 | 352.9 ± 99.4 | 124.5 ± 41.3 |
| Gln | 64.2 ± 5.2 | 95.4 ± 10.9 | 59.6 ± 7.4 | 161.2 ± 21.4 | 201.3 ± 47.8 | 162.3 ± 18.8 |
| Ala | n | 52.6 ± 9.8 | n | 239.8 ± 58.9 | 108.2 ± 37.0 | 256.6 ± 38.1 |
| Arg | 9.3 ± 0.2 | 33.5 ± 9.2 | 59.0 ± 9.8 | 93.3 ± 27.3 | 259.1 ± 22.8 | 88.8 ± 17.7 |
| Asp | 30.6 ± 7.3 | n | 91.3 ± 25.1 | 65.7 ± 19.1 | 839.0 ± 20.3 | n |
| Orn | – | – | – | 104.3 ± 31.8 | n | n |
| F | 18.827 | 24.543 | 0.926 | 8.226 | 6.202 | 22.785 |
| *P* | 0.0001 | 0.0001 | 0.500 | 0.0001 | 0.002 | 0.0001 |
| Spermine | | | | | | |
| NH4 | 1.5 ± 0.4 | 0.9 ± 0.2 | 0.6 ± 0.03 | 1.6 ± 0.04 | 0 | 5.6 ± 0.8 |
| NO3 | 0.6 ± 0.5 | 0.6 ± 0.3 | 0 | (1.5) | 0 | n |
| albumen | 2.1 ± 0.4 | n | 0 | 0 | 0 | 1.5 ± 0.8 |
| gliadine | n | n | n | (0.9) | n | 4.2 ± 4.2 |
| Glu | n | 2.3 ± 1.2 | n | (1.8) | (1.0) | 2.5 ± 0.7 |
| Gln | 0 | 4.7 ± 1.7 | 0 | 2.2 ± 0.9 | 0 | 2.9 ± 0.3 |
| Ala | n | (0.6) | n | 2.5 ± 0.6 | 0 | 5.4 ± 1.6 |
| Arg | (0.9) | 8.6 ± 5.3 | 9.4 ± 6.1 | 0 | (1.0) | 1.9 ± 0.4 |
| Asp | 0 | n | (2.4) | 10.7 ± 2.2 | 0 | n |
| Orn | – | – | – | 0 | n | n |
| F | | | | | | 1.719 |
| *P* | | | | | | 0.194 |

ture. This indicates that other factors make it a successful symbiont. Furthermore, the ability to utilize different nutrients in pure culture may not reflect the situation in a mycorrhizal association (Finlay et al. 1992). This emphasizes the importance of also studying intact mycorrhizal plants.

The endogenous polyamine levels of the fungal mycelium varied with the nitrogen source in the medium. These comparisons between *P. involutus*, *S. variegatus* and *L. rufus* show that large differences in polyamine metabolism are possible between fungal species.

Arginine and ornithine are the precursors for putrescine synthesis in fungi (Biondi et al. 1993; Walters 1995). Although all the fungi in this study utilized arginine as a nitrogen source, only *P. involutus* showed an increased putrescine level when grown on arginine. Ornithine was not utilized by *P. involutus* or *S. variegatus*, whereas the only *L. rufus* isolate (A109/4) tested on ornithine grew well and showed an increased spermidine content. Although *P. involutus* and *S. variegatus* did not utilize ornithine, this does not exclude use of endogenously generated ornithine for polyamine synthesis. Zarb and Walters (1994a) showed that *Laccaria proxima* (Boud.) Maire possesses arginine decarboxylase but *P. involutus* does not (Zarb and Walters 1994b).

Polyamines act as a metabolic buffer and maintain cellular pH in conditions where ammonium assimilation produces excess protons (Smith 1985; Altman and Levin 1993). Furthermore, putrescine has been shown

to accumulate with ammonium nutrition (Young and Galston 1983; Smith 1984). This may explain why both *P. involutus* and *S. variegatus* contained higher concentrations of putrescine on ammonium than on other nitrogen sources. This effect was not clearly seen with *L. rufus*, which contained very low or no putrescine. The cause of the high polyamine concentration found in *P. involutus* grown on asparagine is not known; growth was very poor on this nitrogen source. This variation between ectomycorrhizal fungi may reflect the effect of polyamines on the homeostatic mechanism of their cells and pH regulation and emphasizes the regulatory role of the fungal partner in the utilization of organic and inorganic nitrogen.

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