

Growth of Ectomycorrhizal Fungi on Inorganic and Organic Nitrogen Sources

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Abstract

In forest soils, nitrogen (N) is present in inorganic and organic forms. The organic forms include monomeric amino acids, but also polymers such as chitin. Ectomycorrhizal fungi are known to take up both inorganic and organic N forms, and to depolymerize large organic compounds; however, it is unknown if the compounds are used for growth. The aim of this investigation was to determine the growth of a range of ectomycorrhizal fungi on inorganic and organic N sources. Seven ectomycorrhizal fungi and one endophyte originating from mountain regions either in Austria, Mongolia, or Slovenia were grown in *in-vitro* cultures containing ammonium, nitrate, or chitin. Four ectomycorrhizal fungi were used to investigate growth on amino acids. All fungi, except *Paxillus involutus*, utilized nitrate as a N source. All fungi also grew on both chitin and N-acetylglucosamine, the amino sugar precursor of chitin. *Paxillus involutus* and *Melanogaster broomeanus* showed enhanced growth on chitin-containing media. *Amanita muscaria*, *Rhizopogon roseolus*, and *Suillus granulatus*, but not *Paxillus involutus*, were able to utilize the amino acids glycine and glutamate, as well as the tripeptide triglycine. The ability to utilize the different N sources was independent of the origin of the fungi.

Keywords: ectomycorrhizal fungi; nitrate; ammonium; amino acids; chitin

1. Introduction

Nitrogen is the limiting nutrient in many ecosystems [1]. Forest soils contain soluble forms of both inorganic N as ammonium (NH₄) and nitrate (NO₃), but also organic N primarily in the forms of amino acids [2]. The relative importance of organic forms is higher under N limited conditions [3]. The availability of the different N forms varies with season, but also with soil pH [4]. Whereas more acid, organic matter-rich, boreal forest soils tend to be rich in NH₄ and amino acids [5], neutral and higher pH soils tend to have higher contents of NO₃ [6]. Thus, ectomycorrhizal trees are exposed to a range of soluble N forms depending on the soil pH and the soil N levels. The soluble organic N components are primarily the cleavage products of polymeric N forms [5]. Ectomycorrhizal fungi, which are symbiotic primarily with woody plants, have been shown to absorb many forms of N, both inorganic forms as NH₄ and nitrate NO₃, but also organic forms such as monomeric amino acids and polymeric proteins [7,8]. The N acquired is both retained by the fungus



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but also passed on the host plant in exchange for carbon (C) in the form of sugars and lipids [9].

Polymeric substances such as proteins and chitin form a large N pool, particularly in more acid soils. Cleavage of proteins and utilization of amino acids has been shown for many ectomycorrhizal and ericoid mycorrhizal fungi [10]. The cleavage of proteins is carried out by the secretion of proteases and peptidases [11] to yield amino acids, which are then absorbed by plant roots and mycorrhizal fungi. However, both the uptake of amino acids [12] and the use of amino acids for growth has been shown to vary greatly between different species and strains [13] of ectomycorrhizal fungi.

Chitin is an important component of soil organic matter (SOM) [14] and represents a significant organic N pool [15]. Chitin is derived from the exoskeleton of insects, but also as necromass from fungal cell walls [16,17]. Like proteins, chitin must first be cleaved into its monomeric precursor N-acetylglucosamine [18]. Fungi degrade chitin by hydrolysis using endochitinases and β -N-acetylglucosaminidases (NAGases, or exochitinases) [19]. However, SOM may also be degraded non-enzymatically through Fenton reactions [20].

Although there is increasing awareness of the importance of chitin as a N pool in forest soils [15–17], and the decomposition of fungal necromass as a potential N source [16], little is known about the role of chitin in N nutrition [18]. However, in earlier work [21], it was shown that ericoid mycorrhizas could utilize chitin as a N source, but not two ectomycorrhizal fungi, *Paxillus involutus* and *Rhizopogon roseolus*. It has been shown that ectomycorrhizal fungi can cleave complex substrates, particularly proteins in the form of bovine serum albumen [11], yet few studies have investigated whether these substrates can be utilized for fungal growth.

There is increasing evidence that ectomycorrhizal root tips may be composed of several co-existing fungal partners [22,23]. This co-existence is often an ectomycorrhizal fungus and fungal endophytes [23,24]. The fungal endophytes have been shown to be physiologically active [25] and involved in N and P uptake processes [26], but despite the ecological importance of fungal root endophytes, little is known about their ability to utilize organic N.

In this work, we investigated the ability of seven ectomycorrhizal fungi and one root-associated fungal endophyte to utilize chitin and its derivative N-acetylglucosamine for growth. This was compared to the ability to utilize other N sources: nitrate, amino acids, and a peptide. In addition, as the availability of N forms is pH-dependent, we investigated growth at a number of pH ranges, relevant for forest soils.

2. Materials and Methods

2.1. Sources of the Fungal Isolates

The ectomycorrhizal fungi assessed originated from a number of sources (Table 1). All of the ectomycorrhizal isolates were from mountain regions either in Austria, Mongolia, or Slovenia. All of the fungi were isolated from sporocarps except *Absidia* cf. *psychrophilia*, which was isolated from an ectomycorrhizal root tip of *Pinus sibirica* collected in Mongolia [27]. The fungal isolates originated from different soil types developed from both acid and calcareous parent materials.

Table 1. Sources of fungi. Shown are the country of origin, the dominant tree species at the site of origin, and the soil type.

Taxa	Source Country	Associated Tree Species	Soil Type
<i>Absidia</i> cf. <i>psychrophilia</i>	Mongolia	<i>Pinus sibirica</i>	Neutral
<i>Amanita muscaria</i>	Scotland	<i>Pinus sylvestris</i>	Unknown

Table 1. Cont.

Taxa	Source Country	Associated Tree Species	Soil Type
<i>Amanita rubescens</i>	Slovenia	<i>Quercus petraea</i> , <i>Pinus sylvestris</i> , <i>Fagus sylvatica</i>	Acid
<i>Boletus subtomentosus</i>	Slovenia	<i>Quercus petraea</i> , <i>Carpinus betulus</i> ,	Calcareous
<i>Melanogaster broomeanus</i>	Slovenia	<i>Fagus sylvatica</i>	Calcareous
<i>Paxillus involutus</i> 533	Germany	<i>Picea abies</i>	Acid
<i>Rhizopogon roseolus</i>	Slovenia	<i>Picea abies</i>	Acid
<i>Suillus granulatus</i>	Slovenia	Unknown	Unknown

2.2. Culture of the Fungal Isolates

The fungi were maintained on Modified Melin-Norkrans medium (MMN), and grown at 22 °C. The MMN medium contained 10 g L⁻¹ glucose, 3 g L⁻¹ malt extract, 1.9 mM (NH₄)₂SO₄, 3.7 mM KH₂PO₄, 0.6 mM MgSO₄, 0.5 mM CaCl₂, 0.4 mM NaCl, 6.0 mM FeCl₃, and 10 µg L⁻¹ thiamine [28]. The medium was set using 2% agar and was adjusted to pH 5.6 before sterilization. The medium was sterilized at 121 °C for 15 min. FeCl₃ and thiamine were sterile filtered and added to the medium after it cooled to 70 °C.

pH. To obtain MMN agar with a range of pH values, MMN medium was adjusted to pH 8, 7, 6, and 5 before sterilization. For pH 4 and 3, to prevent degradation of the agar at the low pH during sterilization, the agar and medium were sterilized separately. The double strength medium without agar was adjusted to pH 4 and 3, and aqueous 4% agar was adjusted to pH 5 before sterilization. After sterilization, the medium and agar were mixed, resulting in pH 4 and 3 in the final medium.

Nitrogen sources. The agar plates used MMN medium without malt extract and contained inorganic (NO₃) and organic N sources (amino acids, peptides, amines, and polyamines). The standard MMN medium contains 3.8 mM NH₄. To obtain the different N sources, the NH₄ was substituted by the addition of KNO₃, glycine, triglycine, L-glutamate, N-acetyl-D-glucosamine, or chitin (from shrimp shells), to give a total N concentration of 3.7–3.8 mM N in the MMN medium. The powdered chitin had a N concentration of 6.9%. The stock solutions of glycine, triglycine, glutamate, and glucosamine were sterile filtered and added to the medium after it cooled to 70 °C. Chitin was added from a stock suspension directly to the MMN and autoclaved at 121 °C for 15 min. The agar plates with chitin were slightly turbid. The addition of KNO₃ increased the K concentration to 7.5 mM in the MMN medium, and the different N source MMN medium contained 0.6 mM SO₄ compared to 2.5 mM SO₄ in the basic NH₄ containing medium.

To estimate the effect of pH and N source on the growth of the fungi, a 6 mm diameter plug was taken from the growing edge of a fungal culture and placed in the centre of a new 8.0 cm diameter agar plate. The cultures were maintained at 22 °C and grown for between 21 and 28 days. Every 2 days, the radius of the growing hyphal mat was estimated in 4 cardinal directions and used to calculate the total surface area of the hyphal mat, as a perfect circle. For comparison of the treatments, values were used at a time point when the fastest growing treatment had reached a diameter of 2.0 to 2.6 cm. This point was between 9 and 11 days after beginning the experiment and was dependent upon the fungal taxa used. This point also corresponded to the highest growth rate.

2.3. Statistical Analysis

To normalize growth rates, the growth of all fungi was calculated as the percentage growth of the ammonium containing standard MMN medium. For the pH study, the growth rates were normalized against the growth at pH 5. Data were tested for normality using the Shapiro–Wilk test and equal variance using the Brown–Forsythe test. Significant

differences between treatments were determined by one-way analysis of variance using the Holm–Sidek test, where $p < 0.05$.

3. Results

3.1. Effect of pH on Radial Growth

The growth of all the fungi was relatively unaffected by pH within the range 4 to 7 (Figure 1). *Melanogaster broomeanus* showed a strong reduction in radial growth at pH 8, as did *Paxillus involutus*, which also showed reduced growth at pH 3. *Rhizopogon roseolus* showed no clear pattern of response to pH but showed a lower rate of growth at pH 3 and 4, but this was not significantly different. *Suillus granulatus* had a significantly higher radial growth rate at pH 6 compared to all other pH values.

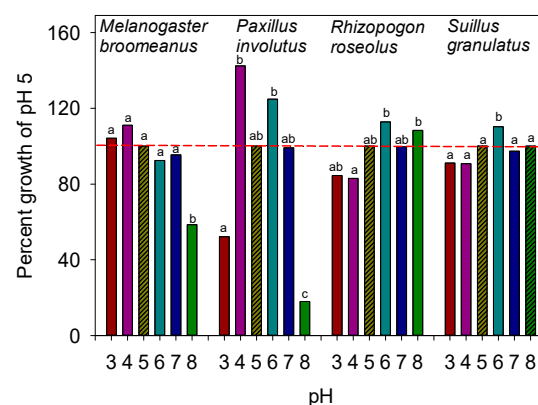


Figure 1. The effect of pH on the radial growth of four ectomycorrhizal fungi. Growth is shown as percentage growth compared to growth at pH 5 (100%). The red broken line shows 100%. Bars not followed by the same letters within a fungal taxa are significantly different. $p < 0.05$.

3.2. Effect of N Source on Radial Growth

Two fungi *Melanogaster broomeanus* and *Rhizopogon roseolus* showed a nearly 4-fold greater radial growth rate on MMN medium containing NO_3 compared to NH_4 (Figure 2a). A significantly higher growth on medium containing NO_3 was also shown for *Absidia* cf. *psychrophilia*, *Amanita muscaria*, and *Suillus granulatus*. In contrast the growth of *Paxillus involutus* on MMN medium containing NO_3 was reduced to 20% of that on medium containing NH_4 . *Amanita rubescens* and *Boletus subtomentosus* showed a slightly lower radial growth rate on NO_3 compared to NH_4 .

All of the fungi were able to use chitin as a N source (Figure 2b). The radial growth of *Melanogaster broomeanus* and *Paxillus involutus* was significantly greater when N was supplied as chitin compared to growth on NH_4 . In contrast, *Amanita muscaria* and *Amanita rubescens* had lower growth rates on chitin compared to NH_4 .

The growth of *Amanita muscaria*, *Paxillus involutus*, *Rhizopogon roseolus*, and *Suillus granulatus* on a number of N sources is shown in Figure 3. The growth of *Amanita muscaria* was lower on glutamate but was greater on glycine and triglycine compared to growth on NH_4 . The growth of *Paxillus involutus* was lower on all of the amino acids, as was the growth of *Suillus granulatus* on glycine and triglycine. However, in *Suillus granulatus*, growth on glutamate was greater than the NH_4 control. *Rhizopogon roseolus* grew better on all amino acids compared to the NH_4 control. All of the fungi were able to utilize glucosamine as a N source. *Amanita muscaria* and *Paxillus involutus* showed greater growth on glucosamine compared to growth on NH_4 .

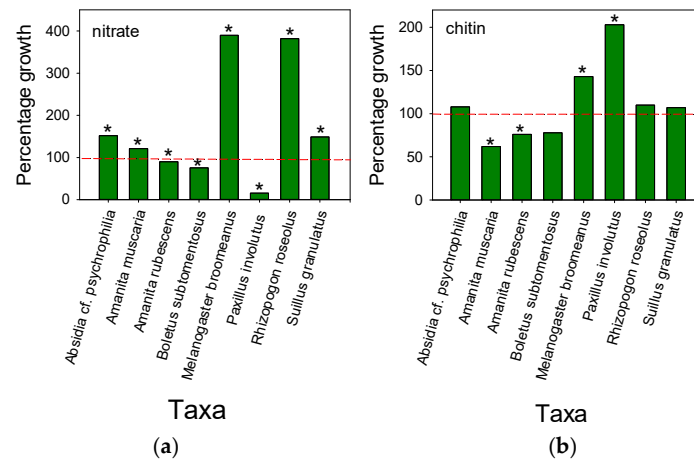


Figure 2. The effect of nitrate (a) and chitin (b) on the radial growth of ectomycorrhizal fungi and *Absidia cf. psychrophilia*. Growth of nitrate and chitin is shown as percentage growth compared to growth on ammonium (100%). The red broken line shows 100%. The values of bars marked with an asterisk (*) are significantly different when comparing growth on nitrate or chitin with growth on ammonium. $p < 0.05$.

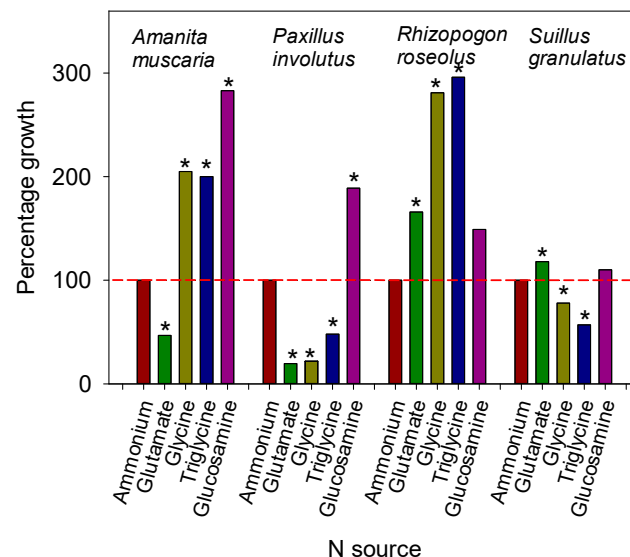


Figure 3. The radial growth of four ectomycorrhizal fungi on various N sources. Growth is shown as percentage growth compared to growth in ammonium (100%). The red broken line shows 100%. The values of bars marked with an asterisk (*) are significantly different when comparing growth on glutamate, glycine, triglycine, or glucosamine with growth on ammonium, $p < 0.05$.

4. Discussion

The degree to which the different ectomycorrhizal fungi were able to utilize the different N sources varied between the different taxa. Most ectomycorrhizal fungi are able to utilize ammonium [4,12] and thus NH_4 is commonly used in culture media. In our investigation, most of the fungi were also able to utilize NO_3 and only *Paxillus involutus* did not grow on the NO_3 containing medium. In all cases, we expressed the growth relative on different N sources in relation to the growth on the standard NH_4 containing MMN medium [28]. Two fungi, *Melanogaster broomeanus* and *Rhizopogon roseolus*, grew 3–4 times better on NO_3 compared to NH_4 . Two other EM fungi, *Amanita muscaria* and *Suillus granulatus*, and the endophyte *Absidia cf. psychrophilia*, also grew significantly better on NO_3 compared to NH_4 . Greater growth on NO_3 compared to NH_4 has been shown in a number of other studies but was species-dependent. Lazarevic et al. [29] showed that

Lactarius deciduous also grew better on NO_3 compared to NH_4 , but that the growth of *Suillus granulatus* was unaffected by N source. In contrast, Francis and Reid [30] also showed a better growth of *Suillus granulatus* on NO_3 . Finlay et al. [10] showed that *Suillus variegatus*, *Piloderma croceum*, *Paxillus involutus*, and *Hebeloma crustuliniforme* grew less well on NO_3 compared to NH_4 . In ectomycorrhizal root tips of *Fagus sylvatica* from a temperate forest, Khokon et al. [12] showed that uptake of NH_4 generally exceeded that of NO_3 for a range of EM taxa, matching the expected availability of these N forms at such sites. However, in a study of over 68 species [31], from both boreal and temperate forests, the majority of the species were also able to utilize NO_3 irrespective of their isolation source. Thus, even fungi isolated from boreal forests, where soil NO_3 levels tend to be low [1], were able to utilize NO_3 . In addition, in 35 out of 43 tested strains partial sequences of the *nar* gene were determined. This gene codes the enzyme nitrate reductase, which is considered to be essential for the assimilation of NO_3 [32]. In our study, only *Paxillus involutus* was unable to utilize NO_3 , and was isolated from an acidic spruce forest in Northern Germany. In forests, sporocarp production of *Paxillus involutus* has been shown to increase under N addition [33]. Ek et al. [34] found that *Paxillus involutus* was able to transfer N as nitrate through the extramatrical mycelium to the host plant, leading Nygren et al. [31] to suggest that this may be a mechanism to avoiding potential toxic effects of NO_3 . Clearly this would not be possible in *in-vitro* cultures, which may explain the lack of growth of *Paxillus involutus* on NO_3 . At the species level, no clear pattern emerges in the relative ability to utilize NO_3 for growth, both in this study and in comparison to previous work. While the ability to assimilate NO_3 seems common amongst ectomycorrhizal fungi, the use of NO_3 for growth is species- and also strain-dependent [35].

Similarly, there were differences between the ectomycorrhizal fungi in the ability to use both monomeric amino acids and the peptide triglycine. *Paxillus involutus*, which was unable to utilize NO_3 , also only poorly utilized the amino acids and the peptide. In contrast, *Rhizopogon roseolus* showed increased growth on media containing all amino acids including glutamate, and also on the NO_3 containing medium. Both *Paxillus involutus* and *Rhizopogon roseolus* originated from acid soil types, showing that there is no clear link between the N source preference and origin. In previous studies, *Hebeloma* isolates were also shown to use alanine and glutamic acid better than NH_4 (Tibbett et al., 1998) [36].

In our study, all fungi were able to utilize and grow on the polymeric substance chitin, and those tested also on the amino sugar glucosamine. Chitin was utilized by both the ectomycorrhizal fungi and the endophyte. Chitin and glucosamine are important organic N pools in boreal forest soils [15,37]. The ability to degrade protein, commonly in the form of bovine serum albumin, is widespread [11,20], but often growth on BSA is poor compared to growth on NH_4 [10,35]. Thus, there appears to be a dichotomy between the ability to degrade a complex substrate and actual utilization for growth. *Paxillus involutus* and *Melanogaster broomeanus* grew better on chitin than on NH_4 , whereas both taxa of *Amanita* grew less well on chitin. In contrast, all three of these fungi were able to utilize the chitin precursor glucosamine. Although *Paxillus involutus* is considered to be an efficient decomposer of soil organic matter [38] and has been shown in other studies to degrade hyphal necromass [17], it was shown that it was not able to utilize N from chitin [18]. In a study of 16 EM fungal taxa [18], only 4 had a significant capacity for chitin degradation, leading to the suggestion that the ability to degrade chitin is not widespread. However, in our study, all of the fungi tested grew with chitin as a N source, and also on the N containing glucosamine, some (*Amanita muscaria*, *Paxillus involutus*) better than on NH_4 . In the method we used, all other sources of N including malt extract were not included in the MMN medium. Chitin was primarily present as fine particles in the agar, suggesting that the fungi degraded the polymer. However, we cannot rule out that during sterilization,

some degradation of the chitin occurred. Similarly, we cannot rule out that there was not some residual N in the agar used to gel the plates and also in the 6 mm inoculation plug. As the MMN contained 10 g L^{-1} glucose, it is unlikely that the glucose contribution from chitin or glucosamine stimulated growth, but rather both act as a source of N.

All of the fungi tested were relatively insensitive to pH except at very low (pH 3) or very high (pH 8) values. Similar results have also been found for other ectomycorrhizal fungi [29,39].

5. Conclusions

Ectomycorrhizal fungi can utilize a number of N sources, both inorganic and organic. Our work shows evidence that ectomycorrhizal fungi and also a root-associated endophyte can actively use chitin as a N source. The ability to utilize the different N sources was independent of the origin of the fungi.

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