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Research in Microbiology 156 (2005) 184–190
Research in Microbiology
www.elsevier.com/locate/resmic

Nitrogen nutrition and isotopic discrimination in tropical ectomycorrhizal fungi

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Received 2 June 2004; accepted 6 September 2004

Available online 30 October 2004

Abstract

It is known that many ectomycorrhizal (EcM) fungi are able to utilise complex organic sources of nitrogen. Two hypotheses were tested using isolates of tropical EcM fungi grown in vitro: (i) EcM fungi isolated from mineral soils of tropical rain forests are less able to utilise organic sources of nitrogen than mineral sources; and (ii) nitrogen isotope discrimination patterns follow those of the nitrogen source utilised. *Pisolithus albus* and *Tomentella* sp. represented tropical EcM fungi and they were grown along with *Thelephora terrestris*. All three species were able to utilise bovine serum albumen as a nitrogen source and *P. albus* produced the greatest biomass on this source of nitrogen. Nitrate was generally less well utilised than ammonium although all three species were able to grow on this nitrogen source. The nitrogen source which led to the greatest biomass also led to the highest fungal nitrogen concentration in *P. albus* and *Tomentella* sp., but not *T. terrestris*. All three species discriminated against ^{15}N when grown on BSA and NO_3 but there were interspecific differences in isotope discrimination when grown on NH_4. From a limited number of isolates, it was found that EcM fungi from tropical soils utilise protein nitrogen as well as mineral nitrogen and there can be considerable nitrogen isotope discrimination during the utilisation of all these nitrogen sources.

Keywords: Bovine serum albumen; $\delta^{15}N$; In vitro growth; Nitrogen isotope discrimination; *Pisolithus*; *Thelephora*; *Tomentella*

1. Introduction

Mycorrhizas are an intimate symbiotic association between plant roots and fungi. They are beneficial to the plant and can improve nutrient uptake, water relations and pathogen resistance. Mycorrhizal colonisation can improve access to poorly mobile mineral nutrients such as phosphorus, and many ectomycorrhizas (EcMs) can also make organic sources of nitrogen available to the host plant ([35] and references therein). Nitrogen is the main limiting nutrient in boreo-temperate forest soils and, as early as 1894, Frank (in [30]) proposed that colonisation by EcM fungi might allow access to organic forms of nitrogen which make up the largest proportion of nitrogen in these soils. Many subsequent studies have shown in vitro utilisation of organic nitrogen by EcM fungi: both simple organic substances such as amino acids, amides and peptides, and more complex organic nitrogen sources such as the proteins gliadin and bovine serum albumen (BSA) [1,2,4,5,10,12,24,29,33, 39,41,42]. More recent studies have shown transfer of assimilated organic nitrogen from EcM fungi to their host plants when they were grown in association, leading to improved mineral nutrition and increased growth of the hosts [12,27,40,42]. EcM fungi can produce a wide range of nutrient mobilising enzymes to obtain organic nutrients [19,31, 32,35], but different fungi have differing abilities to utilise organic nitrogen sources (see above references). This led Abuzinadah and Read [1] to propose a simple classification of EcM fungal species as 'protein' or 'non-protein' fungi based on their ability to grow on the organic nitrogen source, BSA.

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0923-2508/$ – see front matter
doi:10.1016/j.resmic.2004.09.003

The nitrogen isotope signatures of fungi, a potential indicator of nitrogen nutritional patterns, are poorly known, but they are affected by a number of processes and pathways including the nitrogen source and concentration, and fungal uptake, transport and allocation [10,24,47]. Studies have shown some EcM fungi to be more enriched in ^{15}N relative to their substrate compared to other fungi, and it was hypothesised that this was because they had access to ^{15}N-enriched organic matter pools within the substrate [13,14]. An alternative hypothesis relates to discrimination patterns during transfer of organic nitrogen to the host plant, with ^{14}N-enriched amino acids being preferentially transferred whilst ^{14}N-depleted amino acids remain in the fungal tissue increasing its $\delta^{15}N$ value [16,38]. However, Emmerton et al. [10] showed that fungal nitrogen isotope signatures are unlikely to directly reflect that of the source and that discrimination patterns of fungi grown in vitro are not easily predicted. It is therefore instructive to further examine isotopic signatures of EcM fungi as they may help shed some light on the physiology of nitrogen nutrition of these organisms.

EcM fungi are most common in temperate and boreal forests composed of predominantly EcM tree families such as Betulaceae, Fagaceae and Pinaceae. In these forests, nitrogen mineralisation rates are low [6] and there is a deep layer of organic matter [31,46]. However, EcM trees are also found in mixed arbuscular mycorrhizal/EcM communities in the tropical rain forests of Southeast Asia [20–22,26,36,37] where members of the Dipterocarpaceae and Caesalpiniaceae form EcM associations. In these rain forest soils, nitrogen mineralisation rates are higher than in temperate and boreal forests [6,44] and a greater proportion of soil nitrogen is available in the inorganic forms of ammonium and nitrate (e.g., 90% in an Australian rain forest soil [33]). We therefore hypothesised that fungal isolates from tropical forests, where the soil has a lower proportion of its available nitrogen in an organic form, may be less able to utilise these organic nitrogen sources.

In this paper, we examine the utilisation of various nitrogen sources by fungi isolated from tropical forest communities and test, firstly, the hypothesis that they will grow better on inorganic nitrogen sources when compared with organic nitrogen sources. Secondly, we examine change in nitrogen isotope discrimination following growth on these different nitrogen sources.

2. Materials and methods

2.1. Study species

Three EcM isolates were chosen: two tropical isolates (*Pisolithus albus* (Cooke & Mass.) Priest) and one temperate isolate for comparative purposes (*Thelephora terrestris* Ehrh. *ex* Fr.). *P. albus* (Pisolithaceae) was isolated from a fruiting body under *Acacia mangium* Willd. (Mimosaceae) in Gemas, Negeri Sembilan, Malaysia and was included in Martin et al. [25] on the phylogeny of *Pisolithus* species. *Tomentella* sp. (Thelephoraceae) (J. Díez pers. comm.) was isolated from the roots of *Shorea parvifolia* Dyer (Dipterocarpaceae) in a nursery in Lentang, Pahang, Malaysia. *T. terrestris* (Thelephoraceae) was obtained from R. Jackson (via the culture collection of D.J. Read) who isolated it from a nursery in Reading, Berkshire, UK. All three cultures are maintained in the collection of D.J. Read at the University of Sheffield.

2.2. Culture solution

The effect of different nitrogen sources on fungal growth was examined by using a basal, nitrogen-free, Modified Melin Norkrans (MMN) solution (500 mg l^{-1} KH_2PO_4; 155 mg l^{-1} $MgSO_4 \cdot 7H_2O$; 50 mg l^{-1} $CaCl_2 \cdot 2H_2O$; 25 mg l^{-1} NaCl and 7.2 mg l^{-1} $FeCl_3 \cdot 6H_2O$) modified with either ammonium sulphate [$(NH_4)_2SO_4$], calcium nitrate [$Ca(NO_3)_2 \cdot 4H_2O$] or bovine serum albumen (Sigma–Aldrich Company Ltd., Poole, Dorset, UK) to provide 100 mg N l^{-1}. For ammonium and nitrate, the chemicals were added to the solution and the pH was adjusted to 4.7 (with HCl or NaOH) prior to sterilisation by autoclaving. For BSA, the solution was autoclaved, BSA was dissolved in distilled water and added by filter sterilisation through a 0.2 μm Millipore filter; the pH was then adjusted to 4.7 with sterilised HCl and NaOH. Carbon was added to all solutions prior to sterilisation as D-glucose (5 g l^{-1}) to create a C:N ratio of at least 20:1.

2.3. Growth conditions and harvesting

Prior to initiation of the study, the fungal cultures were maintained on solid MMN in the dark. To start the study, a 6 mm disc of inoculum was cut from the edges of actively growing (c. 1 month old) cultures with a sterile No. 3 cork borer. The disc was transferred aseptically into a 250 ml Erlenmeyer flask containing 25 ml of sterilised MMN with 100 mg l^{-1} of the required N source (471 mg l^{-1} of $(NH_4)_2SO_4$, 843 mg l^{-1} of $Ca(NO_3)_2 \cdot 4H_2O$ or 641 mg l^{-1} of BSA). The flasks were sealed with cotton wool and aluminum foil, transferred into one of two replicate incubators and maintained at 25 °C in the dark. 25 °C was found to be the optimum temperature for the growth of *Tomentella* sp. in prior experiments (M. Patahayah, F.Q. Brearley and S.S. Lee, unpubl. data). Fungal mycelium was harvested after two, four and six weeks with four replicates of *P. albus*, eight replicates of *Tomentella* sp. and six replicates of *T. terrestris* from each N source at each harvest. The mycelium was filtered through pre-dried, pre-weighed filter papers and then oven dried at 80 °C for 48 h. Total fungal biomass was calculated as the dried fungal biomass minus a mean of the initial weight of fungal inoculum plus agar added to the flasks at the beginning of the study.

2.4. Fungal tissue analyses

Fungal mycelium (and initial nitrogen sources, as powdered chemicals) were analysed for nitrogen concentration and δ^{15}N by bulking the samples for each species and nitrogen source at each of the three harvest dates, and grinding in liquid nitrogen. They were subsequently analysed using a PDZ Europa ANCA-GSL preparation module connected to a 20-20 isotope ratio mass spectrometer (PDZ Europa Ltd., Northwich, Cheshire, UK). The isotope ratio was calculated as:

$$\delta^{15}\mathrm{N}\ (‰) = (R_{\mathrm{sample}}/R_{\mathrm{standard}} - 1) \times 1000,$$

where R is the isotope ratio of ^{15}N/^{14}N of either the sample or the standard (atmospheric nitrogen).

2.5. Statistical analyses

Fungal biomass and nitrogen concentration were analysed using one-way ANOVAs for each time point; if the results were significant ($P < 0.05$), the means were compared using Turkey's test. Box-Cox transformations were carried out as required to normalise treatment distributions or to equalise heterogeneous variances. Nitrogen isotope ratios were analysed by a one-sample t-test to determine differences from the nitrogen isotope ratio of the nitrogen source within the MMN solution. Two flasks became contaminated and were therefore not included in the analyses. Minitab 12.2 was used for all analyses (Minitab Inc., State College, Pennsylvania, USA).

3. Results

3.1. Biomass accumulation

All three species were able to grow on all three nitrogen sources (Fig. 1). *P. albus* had a rapid growth rate and the biomass of fungi grown on BSA was at least 50%, and often more than 100%, greater than the biomass of fungi grown on either ammonium or nitrate at all three harvests (Fig. 1a). *Tomentella* sp. and *T. terrestris* had slower growth rates and increased little in biomass between the 4- and 6-week harvests (Figs. 1b and 1c). The growth on all three nitrogen sources was similar for both species although the biomass of *Tomentella* sp. on nitrate was lower at the 6-week harvest than at the 4-week harvest (Fig. 1b) and growth of *T. terrestris* only began after 2 weeks, thereafter increasing in biomass comparable to that on BSA and ammonium (Fig. 1c).

3.2. Nitrogen concentration

The nitrogen concentration of fungal mycelium had similar patterns to the biomass, with faster growing fungi having a greater nitrogen concentration. *P. albus* had a very high nitrogen concentration when grown on BSA; when grown on NH_4 or NO_3 the nitrogen concentration was less than half of that on BSA (Table 1). *Tomentella* sp. had the highest nitrogen concentration when grown on BSA and NH_4; the concentration when grown on NO_3 was only around a third of these two values (Table 1). *T. terrestris* also had its highest nitrogen concentration when grown on BSA, with very low concentration when grown on NH_4 or NO_3 (Table 1).

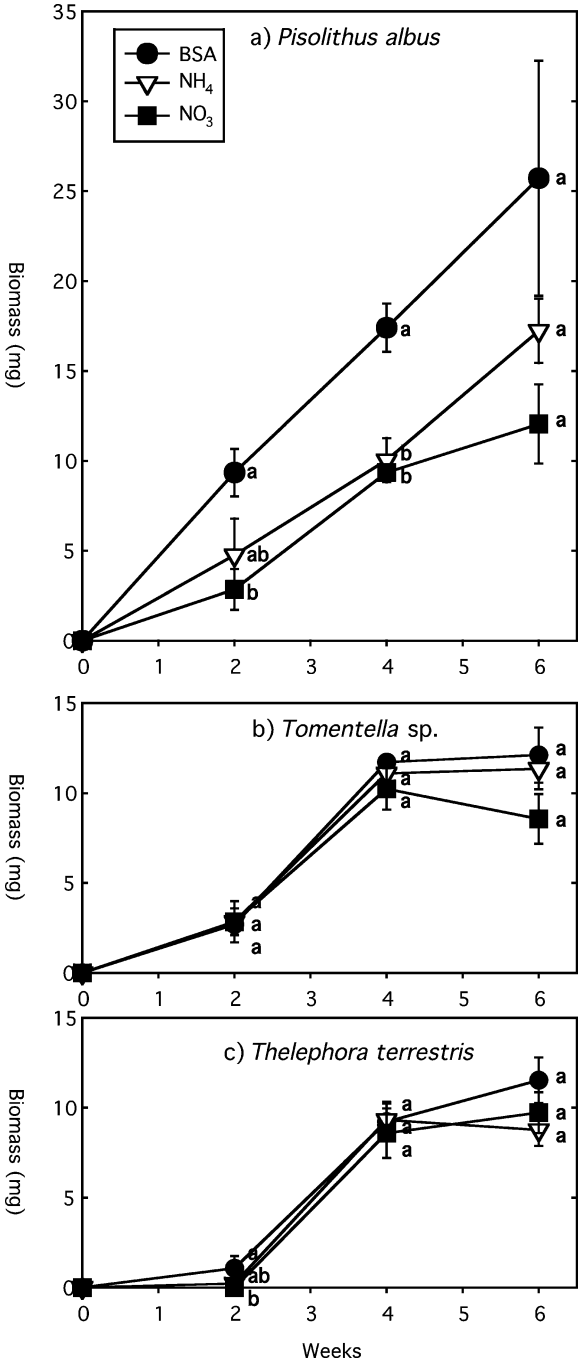

Fig. 1. Biomass of three ectomycorrhizal fungal species grown on three different nitrogen sources for six weeks. All values are means (±SE). Letters at the same harvest for each species indicate differences according to Turkey's test at $P < 0.05$.

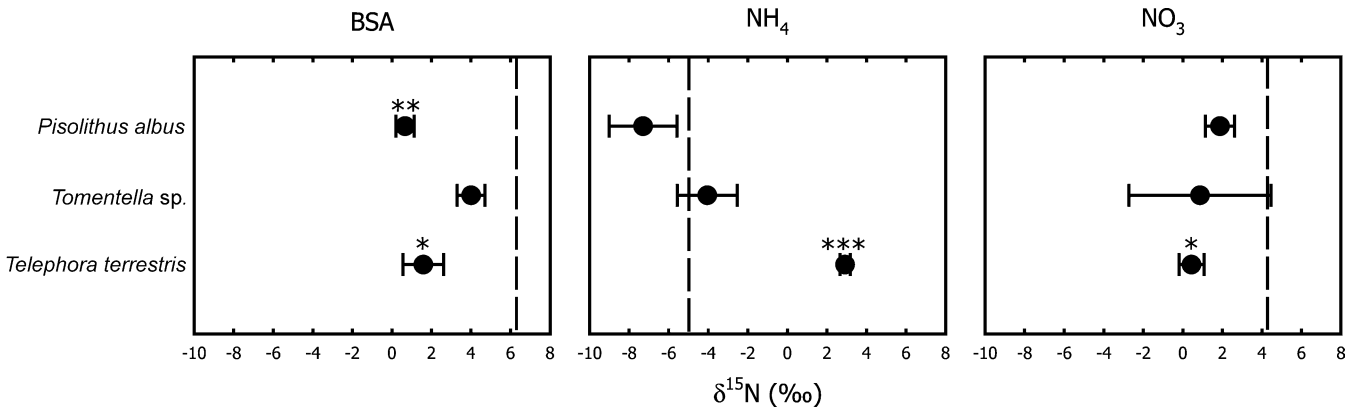

Fig. 2. Nitrogen isotope discrimination patterns of three ectomycorrhizal fungal species grown on three different nitrogen sources for six weeks (initial nitrogen source δ^{15}N values shown as a dashed line). All values are the mean (±SE) of three bulked samples taken at each of three harvests after the start of the experiment. Significant differences in isotope signature from the initial source are indicated as: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 1
Nitrogen concentration (%) of fungal mycelium of three ectomycorrhizal fungal species grown on three different nitrogen sources for six weeks

Nitrogen source	*Pisolithus albus*	*Tomentella* sp.	*Thelephora terrestris*
BSA	9.68 ± 0.72 a	2.64 ± 0.01 a	6.92 ± 1.57 a
NH_4	4.79 ± 0.61 b	3.02 ± 0.38 a	0.83 ± 0.17 b
NO_3	3.26 ± 0.71 b	1.00 ± 0.09 b	0.74 ± 0.22 b

All values are the means (±SE) of three bulked samples taken at each of three harvests. Letters within the same column indicate differences according to Turkey's test at $P < 0.05$.

3.3. Nitrogen isotope discrimination

All three species discriminated against ^{15}N when grown on BSA and on NO_3 although this was only statistically significant for *P. albus* on BSA and *T. terrestris* on BSA and NO_3 (Fig. 2). There were no consistent patterns of discrimination when grown on NH_4, with *P. albus* showing discrimination against ^{15}N and *Tomentella* sp. and *T. terrestris* showing discrimination towards ^{15}N, highly significantly in the case of *T. terrestris* (Fig. 2).

4. Discussion

4.1. Growth of the ectomycorrhizal isolates

This study has shown that all three species of fungi examined utilised all three nitrogen sources to varying degrees. Ammonium is usually the most readily utilised nitrogen source by EcM fungi [2,4,5,17,29,33,35,42] and this may be especially true in mineral soils with rapid nitrogen mineralisation rates. The results for *P. albus* were therefore somewhat unexpected in that the greatest biomass was produced on BSA. All three study species were able to utilise nitrate to a certain degree, but a number of studies [4,5,12,17,33,35,39] have shown a limited ability of certain species to grow on this nitrogen source. Within the cells, nitrate is converted to ammonium by the action of nitrate reductase before the ammonium is then assimilated into amino acids. It has been suggested that it is therefore energetically less expensive to preferentially take up ammonium for direct assimilation into amino acids.

The isolate of *P. albus* used in this study showed the greatest growth on BSA and appeared to take up almost all of the 2.5 mg of nitrogen supplied (25.7 mg × 0.0968 (% N) = 2.49 mg), demonstrating very efficient use of this protein nitrogen. Abuzinadah and Read [1] categorised *Pisolithus tinctorius* as intermediate between 'protein' and 'non-protein' fungi due to varying protein usage depending on culture conditions. Subsequent experiments by Turnbull et al. [42] and Anderson et al. [4,5] have shown *Pisolithus* species to usually grow best on ammonium, but increasing protein utilisation ability was shown following an increased time since isolation, and also with decreased glucose concentrations in the culture solution.

Although there are probably over 60 species of *Tomentella* (Kõljalg in [11]), there appears to be only one report of in vitro growth of any members of this genus. This is the report of Sharples and Cairney [34] who found that the growth rate on BSA was approximately double that on both ammonium and nitrate. When their study was carried out, the fungal species was unidentified; it has now been shown to have close affinities with *Tomentella* [7]. This is in agreement with our results presented here, indicating good protein-using ability of this genus. The association of fruiting bodies of *Tomentella* species with decaying plant material suggests some ability to utilise these organic materials [18]. Further evidence for the protein-using ability of *Tomentella* species comes from Lilleskov et al. [23] who showed a decline in a number of unidentified *Tomentella* species when they were subjected to additions of inorganic atmospherically deposited nitrogen.

Although it could be suggested that *T. terrestris*, as a pioneer species [8], should have little access to organic sources, this does not appear to be the case here. Our results are consistent with those of Finlay et al. [12] who showed the ability of some isolates of *T. terrestris* to grow on nitrogen obtained from a protein source.

4.2. Fungal nitrogen concentration

A number of studies have reported the nitrogen concentration of EcM fungi to be around 1.5–6.5% [1,14,38,45], with fruiting body caps often having a notably higher nitrogen concentration (c. 2% higher in Taylor et al. [38]). Some of the fungi grown on BSA in this experiment had very high nitrogen concentrations although they were within the range of concentrations reported for non-EcM fungi [45]. It is interesting that *T. terrestris* was able to take up nitrogen from BSA but was not able to use this nitrogen to produce greater biomass than on the other nitrogen sources. In the other two species, an increase in nitrogen concentration was associated with an increase in biomass accumulation. Eaton and Ayres [9] suggested that EcM fungi preferentially allocate carbon towards obtaining nitrogen over allocating carbon towards growth. This strategy may allow the fungus to maintain independent growth in the event of sudden cessation of plant-derived carbon [9].

4.3. Nitrogen isotope discrimination

Differences in isotope discrimination patterns between EcM fungi can be due to: (a) selective uptake of a given isotope; (b) consistent differences in discrimination patterns during uptake, assimilation and allocation; (c) differences in the proportion, and isotopic signature, of nitrogen returned to the growing medium and (d) preferential transfer of ^{14}N over ^{15}N from the fungus to the plant [10,24].

All three study species discriminated against ^{15}N when grown on BSA and nitrate but there were no consistent patterns of discrimination when grown on ammonium. Our results are therefore in contradiction with those of Gebauer and Taylor [14] who found most fungi to be enriched in ^{15}N relative to their source; however one obvious difference between in vitro and in vivo studies is that there can be no transfer of nitrogen from the fungus to the plant so, when the fungus is grown in vitro, researchers are only able to examine internal physiological processes. These results support the hypothesis of Emmerton et al. [10] that ^{15}N abundance of fungal tissues is unlikely to directly reflect the isotopic signature of the original source as there is isotope discrimination during both nitrogen uptake and assimilation. This is further confounded by the fact that discrimination patterns are source- and fungal-specific.

The discrimination against ^{15}N in BSA-grown fungi may be due to a release of extracellular protein-degrading enzymes which are generally enriched in ^{15}N [47]. Consistent discrimination against ^{15}N with nitrate uptake, also found by Emmerton et al. [10], is probably related to discrimination during nitrate reduction in the cell, but may also be due to excretion of ^{15}N-enriched, non-reduced nitrate [47]. The most likely explanation, given current knowledge, for inconsistent discrimination patterns during ammonium assimilation are the potential differences in discrimination patterns (as yet unmeasured) between the two main nitrogen assimilation pathways, GS-GOGAT and GDH-GS [10,15,47]. The operation of these two pathways is regulated by both fungal species and the nitrogen source and concentration [3,28,43] and may have considerable implications for isotope discrimination patterns.

4.4. Comparison with related studies

These results contrast with those of Sangtiean and Schmidt [33] who found EcM fungal growth on BSA to be poor when compared with growth on ammonium for fungal species isolated from an Australian rain forest ($< 25\%$), although fungal species from a *Eucalyptus* (Myrtaceae) forest were more able to utilise BSA when compared with ammonium ($< 38\%$). Other subtropical fungi from *Eucalyptus* forests in Turnbull and colleagues' [42] study also showed a very limited ability to grow on BSA, with the exception of an *Amanita* species. Lilleskov et al. [23,24] found a decrease in the numbers of protein using fungi with increasing soil inorganic nitrogen and a decrease in the protein utilisation ability of fungi isolated from these soils. Taylor et al. [39] also found similar patterns over a European nitrogen deposition gradient.

4.5. Conclusions

Our original hypothesis, that fungi isolated from tropical forest communities will grow better on inorganic nitrogen sources when compared with organic nitrogen sources, was not upheld as BSA was utilised equally well as ammonium, and better by *P. albus*. It appears that these tropical EcM fungi are able to effectively utilise a range of nitrogen sources and this 'generalist' nitrogen use may help to explain their host species dominance in certain tropical rain forest soils as they can make a wide range of nitrogen sources immediately available to their host plant. Clearly more work, with a much wider range of species, is needed before this hypothesis can be fully supported.

Acknowledgements

We thank the British Ecological Society for financial support through their Overseas Research Programme and Rick Dunn, Irene Johnson and Judith Shaw for technical assistance.

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