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Tissue density and growth response of ectomycorrhizal fungi to nitrogen source and concentration

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Abstract *Amanita rubescens* Pers., *Lactarius affinis* Pk., *Leccinum aurantiacum* (Fr.) S.F. Gray, *Tylopilus felleus* (Bull. ex Fe.) Karsten, and two isolates of *Suillus intermedius* (Smith & Thiers) Smith & Thiers collected from an approximately 55-year-old *Pinus resinosa* Ait. plantation, and *Pisolithus tinctorius* (Pers.) Coker & Couch obtained from another source, were tested for their abilities to grow with protein as the primary source of nitrogen. Protein plates contained 63 mg l^{-1} N as bovine serum albumen and 7 mg l^{-1} N as arginine. Control plates contained only 7 mg l^{-1} N as arginine. All isolates except *Leccinum aurantiacum* and one isolate of *S. intermedius* attained greater dry weight with protein as the primary source of N. *Lactarius affinis, Leccinum aurantiacum, P. tinctorius*, and both isolates of *S. intermedius* had higher tissue densities on protein medium. *Amanita rubescens* had lower tissue density. To determine if increase in tissue density was an effect of total N concentration or an effect of N source (protein versus arginine), we performed a second experiment in which arginine concentration was increased (7 mg l^{-1} N versus 70 mg l^{-1} N). The second experiment also included *Cenococcum geophilum* Fr. but excluded *T. felleus*. Higher tissue densities with increased nutrients were found in *C. geophilum*, *Lactarius affinis, Leccinum aurantiacum*, and both isolates of *S. intermedius*. Only *A. rubescens* and *P. tinctorius* did not have increased densities. The results suggest that these ectomycorrhizal fungi alter their growth forms according to N concentration. At low N concentrations, a growth form likely to promote exploitation of a large volume of medium for a given biomass is produced. At high concentrations, a growth form likely to promote exploitation of a rich source of N is produced. Whether ectomycorrhizal fungi growing in association with roots would act in a similar fashion is not known.

Key words Ectomycorrhiza · Protein use · Hypha foraging · Arginine

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Introduction

The ability of mycorrhizal fungi to directly obtain nitrogen from protein has been shown by several authors (Abuzinadah and Read 1986; Abuzinadah et al. 1986; Finlay et al. 1992; Turnbull et al. 1995; Sharples and Cairney 1997), although others have questioned whether this occurs in natural substrates (Lundeberg 1970; Bending and Read 1996). Because N in complex organic forms is normally unavailable to plants, acquisition of N from protein by ectomycorrhizal fungi has major implications for forest ecosystems (Read et al. 1989).

Our initial experiment investigated potential for protein use among six ectomycorrhizal fungi isolated from a 10-acre *Pinus resinosa* Ait. plantation. Two additional species were included: *Cenococcum geophilum* Fr., and *Pisolithus tinctorius* (Pers.) Coker & Couch. *Cenococcum*–type mycorrhizas were observed in the plantation, but never successfully isolated. *Pisolithus tinctorius* was included because it is a widely studied species (Cairney and Chambers 1997), and hence useful for comparison with other research. Isolates were grown in axenic culture on a gel-based medium with a small amount of "starter nitrogen" (sensu Abuzinadah et al. 1986) in both control and protein plates. Gel-based media are probably more similar to the natural matrix encountered by ectomycorrhizal fungi than liquid cultures (Bending and Read 1996) and also permit measurement of diameter as well as weight.

During the first experiment, we observed increased hyphal density in response to increased nutrient concentrations. This is consistent with Finlay and Read's (1986a,b) observation of dense patches of mycelium forming in response to localized sites of nutrient enrichment. Read (1991) found that these patches could be induced by organic N enrichment. Bending and Read (1995) showed that these patches had increased translocation of nitrogen, phosphorus, and potassium. Similar patterns of proliferation in response to high nutrient concentrations have been reported for both roots and vesicular-arbuscular mycorrhizal hyphae (St. John et al. 1983; Cui and Caldwell 1996a,b). We are unaware of any previous study of in vitro ectomycorrhizal hyphal density change in homogeneous environments.

In our second experiment, we investigated whether the increased density observed in the first experiment was a response to protein per se or if it could be induced by increased N concentration alone.

Materials and methods

Six of the eight isolates we studied were isolated from a single *P. resinosa* plantation. *Amanita rubescens* Pers., *Lactarius affinis* Pk., *Leccinum aurantiacum* (Fr.) S.F. Gray, *Suillus intermedius* (Smith & Thiers) Smith & Thiers (*S. intermedius* 96), and *Tylopilus felleus* (Bull. ex Fe.) Karsten were isolated in the summer of 1996 from sporocarps collected under a 10-acre, approximately 55-year-old *P. resinosa* plantation near State College, Pa. *Suillus intermedius* was also cultured from a 1994 isolate from the same location (*S. intermedius* 94). *Cenococcum geophilum* was collected by Jim Trappe from *Tsuga mertensiana* (Bong.) Carr. in AK (isolate A175) and provided by the Forestry Sciences Laboratory (USDA Forest Service, Corvallis, Ore.). *Pisolithus tinctorius* was obtained from Plant Health Care, Inc., Pittsburgh, Pa.

We used three growth media for these experiments, all based on a modified gelled Melin-Norkrans solution to which we added various levels of arginine and/or protein (see below). The base solution consisted of 0.5 g l^{-1} $CaCl_2$, 0.5 g l^{-1} KH_2PO_4, 0.25 g l^{-1} $MgSO_4 \cdot 7H_2O$, 6.05 mg l^{-1} $FeSO_4 \cdot 7H_2O$, 0.75 mg l^{-1} KI, 6 mg l^{-1} $MnCl_2 \cdot 4H_2O$, 2.65 mg l^{-1} $ZnSO_4 \cdot 7H_2O$, 1.5 mg l^{-1} H_3BO_3, 0.13 mg l^{-1} $CuSO_4 \cdot 5H_2O$, 0.0024 mg l^{-1} $Na_2MoO_4 \cdot 2H_2O$, 10 g l^{-1} sucrose, 6.25 g l^{-1} gellan gum (Phytagel, Sigma Chemical P-8169) and 0.5 mg l^{-1} nicotinic acid, 0.1 mg l^{-1} thiamine HCl, 0.1 mg l^{-1} pyridoxine HCl and 3.0 mg l^{-1} glycine in 1 ml l^{-1} ethanol. After autoclaving for 15 min at 121 °C, nitrogen sources were added to partially cooled medium by 0.2-μm millipore filtration (see below). Before cooling completely, the media were poured in ca. 26-ml portions into 100-mm × 15-mm petri dishes.

Mother cultures were maintained on high arginine medium (70 mg l^{-1} N as arginine) for use in the following two experiments except for *T. felleus* and *S. intermedius* 96, which were maintained on a slightly different medium (same as base medium with the addition of 0.25 g l^{-1} NH_4Cl, 8 mg l^{-1} NaFeEDTA, 2.5 g l^{-1} malt extract (Difco Lab 0186-17-7), and 0.125 g l^{-1} yeast extract (Difco Lab 0127-17-9) and the omission of $FeSO_4 \cdot 7H_2O$). All cultures were sealed with parafilm, inverted and incubated in the dark at 21 °C.

Experiment 1

For the first experiment, 10 replicates of each isolate were plated onto either protein (treatment) or low arginine medium (control). Protein medium had 7 mg l^{-1} N as arginine and 63 mg l^{-1} N as bovine serum albumin; low arginine medium had 7 mg l^{-1} N as arginine. Twenty plugs were cut with a 3 cork borer (7 mm diameter) from one or more mother culture plates and placed alternately on the two media. Cultures were incubated as above. Cultures which produced more than one growth center per plate or that became contaminated were discarded (final *n* values given in Table 1). *Cenococcum geophilum* completely failed to grow on either medium for unknown reasons and was discarded from the first experiment.

When at least one culture of an isolate neared the edge of its plate or after 56 days, all cultures of that isolate were measured for diameter and prepared for dry weight determination (harvest times given in Table 1). Gel was removed from mycelium by placing mycelium and attached gel in 200 ml of citrate buffer (1.22 g l^{-1} sodium citrate and 0.7968 g l^{-1} citric acid, pH 6) for 24–72 h at room temperature (Doner and Bécard 1991). To facilitate gel removal, cultures of *S. intermedius* 94 were cut into several pieces after 48 h of soaking and allowed to soak an additional 24 h. Mycelia were then filtered from solution and dried for 24 h at 65 °C before weighing.

Experiment 2

We had insufficient mother cultures at the time of the second experiment to include *T. felleus*. *Cenococcum geophilum*, which

Table 1 Harvest ages, weights, diameters, and densities for experiment 1. Significance levels determined by Student's *T*-test (*n.s.* not significant at $P < 0.05$, * significant at $P < 0.05$)

Isolate	Growth medium	Age (days)	*n*	Dry wt. (mg)	Diameter (cm)	Density (mg cm^{-2})
Amanita rubescens	Protein	56	8	4.5*	3.7*	0.43*
	Low arginine		10	2.6	2.1	0.87
Lactarius affinis	Protein	20	10	80.9*	6.7 n.s.	2.27*
	Low arginine		8	19.1	6.8	0.53
Leccinum aurantiacum	Protein	56	6	9.2 n.s.	2.8 n.s.	1.50*
	Low arginine		7	5.4	2.4	1.16
Pisolithus tinctorius	Protein	14	10	2.2*	4.3*	0.15*
	Low arginine		10	1.7	5.0	0.09
Suillus intermedius 94	Protein	28	9	87.2*	4.3 n.s.	6.06*
	Low arginine		10	39.6	4.2	2.89
Suillus intermedius 96	Protein	42	4	1.4*	1.3*	0.91*
	Low arginine		8	5.1	4.1	0.43
Tilopilus felleus	Protein	56	9	8.3*	2.9*	1.26 n.s.
	Low arginine		10	6.4	2.6	1.22

Table 2 Harvest weights, diameters, and densities for experiment 2. All ages are as in Table 1 except *C. geophilum* which was harvested at 98 days. Significance levels determined by Student's *T*-test (*n.s.* not significant at $P<0.05$, * significant at $P<0.05$)

Isolate	Growth medium	*n*	Dry wt. (mg)	Diameter (cm)	Density (mg cm^{-2})
Amanita rubescens	High arginine	9	2.0 n.s.	2.8 n.s.	0.33 n.s.
	Low arginine	10	2.0	2.6	0.39
Cenococcum geophilum	High arginine	10	17.9*	1.3 n.s.	13.16*
	Low arginine	10	12.9	1.5	7.39
Lactarius affinis	High arginine	9	72.9*	4.4*	4.78*
	Low arginine	10	20.7	5.6	0.85
Leccinum aurantiacum	High arginine	9	7.2 n.s.	1.4*	2.83*
	Low arginine	8	5.4	2.3	1.44
Pisolithus tinctorius	High arginine	10	2.3 n.s.	4.1 n.s.	0.18 n.s.
	Low arginine	10	2.7	3.9	0.22
Suillus intermedius 94	High arginine	10	93.3*	3.7 n.s.	8.84*
	Low arginine	10	50.3	3.8	4.45
Suillus intermedius 96	High arginine	9	89.5*	5.5*	3.74*
	Low arginine	9	21.6	5.9	0.80

failed to grow in the first experiment, had adequate growth in the second experiment after 98 days and was retained.

Ten replicates of the seven isolates were plated onto high arginine and low arginine plates (70 mg l^{-1} N and 7 mg l^{-1} N as arginine, respectively). All culturing and harvesting procedures were the same as for the first experiment.

Statistics

Differences between protein and low arginine, and high and low arginine media were tested for significance with Student's *t*–test at $P<0.05$.

Results

Experiment 1

Five isolates (*A. rubescens*, *Lactarius affinis*, *P. tinctorius, S. intermedius* 94, and *T. felleus*) had significantly higher weights on protein medium than on low arginine medium (Table 1). Weights of *Leccinum aurantiacum* were not significantly different between treatments. *Suillus intermedius* 96 was the only isolate with significantly higher weight on low arginine medium.

Final diameters of three isolates were not significantly different on protein and low arginine media (*Lactarius affinis*, *Leccinum aurantiacum*, and *S. intermedius* 94). *Suillus intermedius* 96 and *P. tinctorius* had significantly higher diameters on low arginine than on protein medium. *Amanita rubescens* and *T. felleus* had significantly higher diameter on protein medium.

Densities (weight:area ratios) of five isolates (*Lactarius affinis*, *Leccinum aurantiacum, P. tinctorius,* and *S. intermedius* 94 and 96) were greater on protein medium. Density differences between treatments were visually apparent as darker, thicker growth. No other differences in growth form were observed. *Amanita rubescens* grew more densely on low arginine medium. The density of *T. felleus* was not significantly affected by treatment.

Experiment 2

Four isolates (*C. geophilum*, *Lactarius affinis*, *S. intermedius* 94, and *S. intermedius* 96) were significantly heavier and denser on high arginine medium (Table 2). *Leccinum aurantiacum* was also significantly denser on high arginine medium. *Leccinum aurantiacum, Lactarius affinis*, and *S. intermedius* 96 had significantly higher diameters on low arginine medium. No other differences between treatments were significant.

Discussion

Five of the seven isolates tested were able to use protein as a source of nitrogen as evidenced by increased weight, with *Leccinum aurantiacum* and one isolate of *S. intermedius* being the exceptions. *Leccinum aurantiacum* did show a trend toward increased weight on protein medium and was one of the slower growing of the isolates (average diameter of 2.8 cm at 56 days). It seems likely that *Leccinum aurantiacum* would show significant increased growth on protein medium given a longer incubation time. *Suillus intermedius* 96 was the only isolate apparently inhibited by the presence of protein, although *S. intermedius* 94 was capable of using protein. Variation in ability to grow on protein between isolates of the same species has also been reported by Finlay et al. (1992). *Suillus intermedius* 96 was plated from medium containing NH_4Cl, residues of which may have inhibited protease activity (Abuzinadah et al. 1986), but *T. felleus* plated from the same medium was able to grow on protein medium.

Our results are generally consistent with those of other researchers, where comparisons can be made with the same species or genus. Ramstedt and Söderhäll (1983) detected protease activity in *P. tinctorius*, *S. variegates, S. bovinus* and *A. muscaria.* Abuzinadah and Read (1986) describe *Lactarius rufus* as a non-protein

fungus, *P. tinctorius* as intermediate, and *S. bovinus*, and *A. muscaria* as protein fungi. Niini et al. (1988) detected proteolytic enzymes on plates of *S. bovinus*, and *A. muscaria*. Finlay et al. (1992) found that *S. variegatus* was capable of growing on bovine serum albumin, while *Lactarius rufus* had a variable response. Turnbull et al. (1995) found that *Amanita* sp. grew well on protein but that *Pisolithus* sp. had poor growth on protein. Cairney and Chambers (1997) point out that there may be considerable intraspecific variation in protein use within *P. tinctorius.*

The potential for protein use in this experiment does not necessarily imply that these species are capable of obtaining nitrogen directly from protein in natural systems where proteins may be bound in polyphenol complexes (Bending and Read 1996). Our results do show that these fungi have the potential to utilize free protein if exposed to it, perhaps through the action of saprotrophic fungi or bacteria.

Five of seven isolates showed increased density in the presence of protein in the first experiment, but similar responses to high concentrations of arginine in five out of seven isolates were observed in the second experiment, suggesting that this response was due to increased nitrogen concentration rather than protein per se. This is consistent with the observations of Finlay and Read (1986a,b) of dense patches of mycelium forming in response to localized sites of nutrient enrichment. Our findings show that the increased density can occur in a homogeneous substrate with free-living fungi and is not dependent on a heterogeneous environment or any influence of the plant symbiont.

Increased density in response to nutrients can be seen as a simple foraging strategy consistent with previous findings for roots and VA mycorrhizal hyphae (St. John et al. 1983; Cui and Caldwell 1996a,b). Mycorrhizal fungi in low nutrient areas may grow less densely, exploring a large volume with minimal investment in tissue biomass. When mycorrhizal fungi encounter high concentrations of nutrients, increased density, possibly because of increased branching as found by St. John et al. (1983), may maximize ability to exploit the resource. Whether or not ectomycorrhizal fungi growing from roots would exhibit similar foraging strategies is not known.

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