Mycorrhiza (2001) 11:53–56

SHORT NOTE

Ian C. Anderson · Susan M. Chambers
John W.G. Cairney

Variation in nitrogen source utilisation by *Pisolithus* isolates maintained in axenic culture

Accepted: 22 February 2001

Abstract The influence of various inorganic and organic nitrogen sources on biomass production by 17 isolates of Australian *Pisolithus* spp. was investigated before and after a 3-year maintenance period in axenic culture. While some isolates produced similar or higher amounts of biomass on NH_4^+ or certain amino acids after the maintenance period, there was a general trend to reduced biomass production on these substrates. Biomass production by most isolates on bovine serum albumin increased significantly after maintenance. The data are discussed in relation to the use of axenic culture growth experiments for investigations of inter- and intraspecific physiological variation in ectomycorrhizal fungi.

Keywords Ectomycorrhizal fungi · *Pisolithus albus* · *Pisolithus marmoratus* · Organic nitrogen utilisation · Amino acid utilisation

Introduction

It is widely accepted that many ectomycorrhizal (ECM) fungi contribute to the fitness of their hosts by increasing absorption of inorganic nitrogen and/or facilitating access to organic forms of nitrogen in soil (see Smith and Read 1997). However, mycelial absorption and transfer to the host of NH_4^+ and NO_3^- has been demonstrated for only a few fungus–host combinations (Finlay et al. 1988, 1989; Arnebrant et al. 1993; Ek et al. 1994). Similarly, the ability of ECM fungi to effect transfer of nitrogen from simple organic forms to plant hosts has been demonstrated for only a few taxa (Abuzinadah and Read 1986a, b; Abuzinadah et al. 1986; Turnbull et al. 1995). Much of our understanding of the roles played by ECM fungi in the nitrogen nutrition of their hosts relies on observations of nitrogenous substrate utilisation in axenic culture (e.g. Laiho 1970; Lundeberg 1970; Abuzinadah and Read 1988; Finlay et al. 1992; Keller 1996; Anderson et al. 1999; Sarjala 1999). Such studies indicate extensive inter- and intraspecific variation in nitrogen source utilisation in ECM fungi.

Intraspecific variation has been reported in many ECM taxa for a range of physiological parameters, but this may be confounded in some instances by changes arising in mycelia during maintenance in axenic culture (see Cairney 1999). For example, mycelial growth rates of some isolates of certain ECM taxa (Giltrap 1981; Marx 1981; Thomson et al. 1993) and/or their abilities to form ECM with their hosts (Laiho 1970; Marx and Daniel 1976; Marx 1981; Thomson et al. 1993) have been reported to decline during maintenance in axenic culture. Alteration in mycelial growth form, such as a switch from dense to diffuse mycelial growth over a period of a few months (Anderson et al. 1999), may also occur during maintenance in axenic culture. Physiological changes noted during maintenance in axenic culture include a decline in the ability to utilise NH_4^+ as a sole nitrogen source; this was observed over periods of several weeks or months in single *Hebeloma* and *Pisolithus* isolates, respectively (Scheromm et al. 1990; Anderson et al. 1999). While little is known about the basis of such changes, they have been suggested to represent gene modification (e.g. via DNA methylation) or chromosome polymorphisms arising by rearrangement of gene organisation during mitoses or by the action of transposable elements (Di Battista et al. 1996). In order to investigate temporal changes in nitrogen source utilisation by *Pisolithus*, we undertook repeated screening of biomass production on various inorganic and organic nitrogen sources before and after a 3-year period of maintenance in axenic culture.

I.C. Anderson · S.M. Chambers · J.W.G. Cairney (✉)
Mycorrhiza Research Group, School of Science,
Food and Horticulture, University of Western Sydney,
Parramatta Campus, Locked Bag 1797, Penrith South DC,
NSW 1797, Australia
e-mail: j.cairney@uws.edu.au

Materials and methods

The isolates used in this investigation were of three *Pisolithus* species identified by ITS sequence data (see Anderson et al. 1999): 12 isolates of *Pisolithus* species I (putative *P. albus*), four isolates of species II (putative *P. marmoratus*) and one isolate of species III. With the exception of isolates CA01 and NSW1, which were obtained from a culture collection and which had been in culture for >10 years, all isolates were obtained from freshly collected sporocarp tissue during 1996. Where isolates of the same species were collected from the same field location, they were shown by random amplified polymorphic DNA and microsatellite-primed PCR analyses to be genetically distinct individuals (Anderson et al. 1998a, b). The abilities of the isolates to utilise various nitrogen sources for growth were initially investigated in 1997, approximately 1 year after isolation (Anderson et al. 1999). Following this work, all isolates were maintained on modified Melin Norkrans (MMN) agar medium (Marx and Bryan 1975) at 20°C in the dark and subcultured every 6 weeks.

Biomass production on the various nitrogen sources was investigated again during 2000, approximately 3 years after the initial nitrogen utilisation screening, using the methods described by Anderson et al. (1999). Discs of fungal inoculum (5 mm in diameter) were cut from the leading edge of actively growing colonies on MMN agar plates and two discs for each isolate inoculated into 9-cm-diameter Petri dishes containing 25 ml liquid medium. The basal medium contained (per litre) KH_2PO_4 300 mg, $MgSO_4.7H_2O$ 140 mg, $CaCl_2$ 50 mg, NaCl 25 mg, $ZnSO_4$ 3.0 mg, thiamine 0.133 mg, ferric EDTA 12.5 mg. Media were adjusted to pH 5.0–5.5 prior to the addition of the ferric EDTA and autoclaving. The influence of different nitrogen sources on fungal growth was investigated by adding $(NH_4)_2HPO_4$, alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, histidine or bovine serum albumin (BSA) (all from Sigma) to the basal medium to give a starting nitrogen concentration of 106 mg l^{-1}. A control treatment of basal medium with no added nitrogen source was also included. Glucose was added to give final C:N ratios of 5.6:1. Organic nitrogen sources were filter-sterilised into autoclaved basal media using a 0.2-µm membrane filter. In all cases, the same batch of nitrogen substrate was employed in both 1997 and 2000, having been stored according to the manufacturer's instructions. Cultures were incubated at 20°C in the dark for 28 days and each treatment was replicated five times. Mycelial mats were then removed from the liquid media, dried overnight at 80°C and the biomass determined gravimetrically. Data were corrected for growth due to nitrogen contained in inoculum plugs by subtracting the mean value for growth on the control medium with no added nitrogen from the raw data for each treatment. Data for biomass production by each isolate on each nitrogen substrate in 1997 (year 1) (Anderson et al. 1999) and 2000 (year 4) were analysed by ANOVA. The significance of differences between treatments was determined by Fisher's PLSD test using Statview software (Abacus Concepts).

Results and discussion

Significant differences in biomass production were observed between 1997 and 2000 for the majority of isolates on several nitrogen sources (Tables 1, 2). Biomass production on NH_4^+ was significantly higher in year 4 than in year 1 for W34 and there were no significant differences between years 1 and 4 for R01 or W14. In contrast, all other isolates demonstrated a significant reduction in the amount of biomass produced on this substrate in year 4. A similar trend was observed for alanine, aspartic acid, glutamic acid and glutamine, each of which was readily utilised for biomass production by most iso-

Table 1 Mean (±se) biomass increase (µg h^{-1}) for *Pisolithus* isolates in media containing ammonium, alanine, arginine, asparagine or aspartic acid as nitrogen source. Roman numerals in parentheses indicate *Pisolithus* species I–III. The data for year 1 are from Anderson et al. 1999 (*NG* no measurable growth)

Isolate	Ammonium		Alanine		Arginine		Asparagine		Aspartic acid	
	Year 1	Year 4	Year 1	Year 4	Year 1	Year 4	Year 1	Year 4	Year 1	Year 4
R15 (I)	26.79±0.15	19.35±1.49 [b]	1.79±0.60	8.93±1.49 [a]	NG	NG	NG	1.49±0.60 [a]	11.16±0.30	8.93±0.15 [b]
R08 (I)	22.17±1.34	5.95±1.49 [b]	19.35±0.60	5.95±1.49 [b]	NG	NG	0.75±0.15	1.49±0.15	4.91±0.30	NG [b]
W43 (I)	24.40±0.30	22.32±0.07 [b]	14.14±0.45	13.39±0.30	NG	NG	1.79±0.15	1.49±1.49	14.29±0.15	11.90±0.15 [b]
KC05 (I)	26.04±0.30	17.86±1.49 [b]	21.43±1.79	14.88±0.60 [b]	NG	NG	1.04±0.15	NG	14.43±0.15	10.42±0.15 [b]
W34 (II)	1.64±0.45	8.93±0.60 [a]	NG	NG	0.90±0.15	NG [b]	1.34±0.45	NG [b]	NG	NG
W14 (I)	NG	0.45±0.12 [a]	8.18±0.60	1.49±0.15 [b]	2.68±0.75	0.60±0.45 [b]	NG	NG	2.08±0.15	NG [b]
NSW1 (I)	21.43±0.60	10.42±1.49 [b]	19.94±0.90	19.35±0.15	3.27±0.60	13.39±0.45 [a]	7.74±0.75	17.86±0.45 [a]	11.90±0.60	11.90±0.13
W48 (I)	22.92±0.30	20.83±1.49 [b]	21.58±0.15	16.37±0.60 [b]	1.79±0.45	1.49±0.30	3.27±0.60	17.86±1.49 [a]	12.80±0.06	13.39±0.30
W17 (I)	26.79±0.15	2.98±0.45 [b]	13.99±0.60	13.39±0.45	NG	NG	1.34±0.30	NG [b]	10.57±0.60	5.95±0.60 [b]
R01 (I)	NG	NG	6.70±1.49	8.93±1.49 [a]	NG	NG	NG	NG	6.10±0.45	1.49±0.30 [b]
R02 (I)	18.75±1.64	NG [b]	16.22±0.45	1.49±0.15 [b]	3.42±0.15	NG [b]	NG	NG	7.59±0.45	1.49±0.60 [b]
WH01 (I)	4.02±0.30	1.49±0.60 [b]	14.73±0.15	7.44±1.49 [b]	4.46±1.19	2.98±1.49	3.42±0.15	1.49±0.45 [b]	7.89±0.60	4.46±0.75 [b]
CA01 (I)	28.27±0.60	7.44±1.49 [b]	19.64±0.30	16.37±0.30 [b]	1.93±0.15	1.49±0.45	10.42±0.90	16.37±1.49 [a]	15.33±0.15	13.39±0.30 [b]
LJ08 (II)	10.86±1.64	8.93±0.45 [b]	NG	NG	NG	NG	NG	NG	NG	NG
WM02 (II)	16.22±1.04	11.90±1.49 [b]	NG	NG	NG	NG	NG	NG	2.23±0.15	NG [b]
LJ30 (III)	7.74±2.23	2.98±0.60 [b]	NG	NG	NG	NG	NG	NG	NG	0.30±0.15
BW03 (II)	24.55±0.30	19.35±0.60 [b]	7.29±0.45	10.42±0.60 [a]	4.02±0.60	NG [b]	2.23±0.30	1.49±0.45	6.25±0.45	4.46±0.30

[a] Significant increase in biomass between years 1 and 4 ($P<0.05$) [b] Significant decrease in biomass between years 1 and 4 ($P<0.05$)

Table 2 Mean (±se) biomass increase (μg h^{-1}) for *Pisolithus* isolates in media containing glutamic acid, glutamine, histidine or bovine serum albumin (BSA) as nitrogen source. Roman numerals in parentheses indicate *Pisolithus* species I–III. The data for year 1 are from Anderson et al. 1999 (*NG* no measurable growth)

Isolate	Glutamic acid		Glutamine		Histidine		BSA	
	Year 1	Year 4	Year 1	Year 4	Year 1	Year 4	Year 1	Year 4
R15 (I)	9.23±0.15	1.49±0.60 [b]	13.84±0.15	4.46±1.49 [b]	1.19±0.30	NG	1.34±0.15	4.46±0.60 [a]
R08 (I)	16.22±0.30	13.39±0.30 [b]	23.51±0.45	19.35±0.45 [b]	NG	NG	3.13±0.45	1.49±0.45
W43 (I)	14.29±0.30	10.42±0.09 [b]	15.48±0.15	11.90±0.45 [b]	5.65±0.15	NG [b]	3.72±0.60	7.44±0.60 [a]
KC05 (I)	17.86±0.60	10.42±0.45 [b]	18.75±0.15	11.90±0.45 [b]	1.34±0.15	NG	2.38±1.34	10.42±1.49 [a]
W34 (II)	NG	NG	NG	NG	NG	NG	0.75±0.30	4.46±0.45 [a]
W14 (I)	5.95±0.15	NG [b]	9.97±0.90	1.49±0.30 [b]	NG	NG	NG	2.98±0.30 [a]
NSW1 (I)	9.67±0.60	11.90±0.15 [a]	19.20±0.30	17.86±0.12	6.99±0.90	4.46±0.60 [b]	18.45±1.49	32.74±0.15 [a]
W48 (I)	13.84±0.15	11.90±0.30	18.15±0.30	14.88±0.30 [b]	1.79±0.45	1.49±0.30	3.42±0.15	10.42±1.49 [a]
W17 (I)	9.08±0.45	10.42±0.30	12.80±0.15	14.88±0.60 [a]	2.38±0.30	NG [b]	3.57±0.45	5.95±0.45 [a]
R01 (I)	18.30±0.30	16.37±0.30 [b]	23.81±0.60	20.83±0.15 [b]	NG	NG	2.38±0.15	2.98±0.60
R02 (I)	15.03±0.45	8.93±1.49 [b]	16.37±0.60	4.46±0.75 [b]	NG	NG	4.32±0.45	1.49±0.30 [b]
WH01 (I)	12.05±0.15	4.46±0.30 [b]	16.82±0.30	4.46±0.30 [b]	2.23±0.15	NG	2.38±0.15	1.49±0.30
CA01 (I)	15.18±0.30	11.90±0.15 [b]	16.52±0.15	11.90±1.49 [b]	2.53±0.45	NG [b]	4.02±0.45	8.93±0.45 [a]
LJ08 (II)	NG	NG	1.34±0.45	NG [b]	1.34±0.15	NG [b]	0.75±0.30	4.46±1.49 [a]
WM02 (II)	1.93±0.45	NG [b]	NG	NG	NG	NG	NG	NG
LJ30 (III)	NG	NG	1.04±0.45	NG	1.19±0.45	NG	1.79±0.30	1.49±0.15
BW03 (II)	2.83±0.30	4.46±0.30 [a]	9.23±0.75	7.44±1.49	2.23±0.45 [b]	NG [b]	4.02±0.60	7.44±1.49 [a]

[a] Significant increase in biomass between years 1 and 4 ($P<0.05$) [b] Significant decrease in biomass between years 1 and 4 ($P<0.05$)

lates in year 1 (Tables 1 and 2). While there was an increase in biomass production for R01 and R15 on alanine, NSW1 and BW03 on glutamic acid and W17 on glutamine, most other isolates produced significantly less biomass on these amino acids in year 4.

There was no significant change in biomass production for most isolates on asparagine; however, four species I isolates (CA01, NSW1, R15 and W48) produced significantly greater biomass on this amino acid in year 4 (Table 1). Most isolates showed no significant difference in biomass production on arginine or histidine between years 1 and 4; however, several isolates produced significantly less biomass on these amino acids in year 4, while NSW1 produced significantly greater biomass on arginine in year 4 (Tables 1, 2). Most isolates thus showed either a significant decrease or no change in biomass production on most nitrogen sources between years 1 and 4.

These observations are consistent with previous observations of reduced rates of growth of some ECM fungi over time in axenic culture (Giltrap 1981; Marx 1981; Thomson et al. 1993). *Pisolithus* isolates show a similar general trend on a range of simple inorganic and organic nitrogen sources. The obvious exception to this was BSA, on which the majority of isolates (regardless of species) produced significantly more biomass in year 4 than in year 1 (Table 2). In all cases, the isolates for which biomass production increased significantly on BSA in year 4 also produced significantly less biomass on some amino acids and/or NH_4^+ (Tables 1, 2). BSA utilisation by ECM fungi is thought to reflect the action of proteolytic enzymes followed by absorption and metabolism of the resulting amino acids (Read et al. 1989). The fact that those isolates whose biomass increased on BSA in year 4 retained some ability to grow on amino acids, albeit at reduced rates, suggests that increased growth on BSA results from enhanced proteolytic activity in these isolates. While it is conceivable that increased biomass production on BSA resulted from substrate degradation during storage between years 1 and 4, this seems unlikely. All substrates were stored under conditions recommended by the manufacturer and, perhaps more importantly, several isolates grew either equally well or more poorly on BSA in year 4 than in year 1. This implies that the substrate did not become more readily useable following storage.

It is of interest that the general patterns of decreased biomass production on most substrates between years 1 and 4 were shared by the two isolates maintained in axenic culture for >10 years prior to this work (CA01 and NSW1) and the mycelia isolated in 1996. Isolates CA01 and NSW1 were also among those that produced significantly greater biomass on BSA in year 4 (Table 2). Thus it is unlikely in all cases that the observed variation is solely a function of length of time in culture per se. At the conclusion of the 1997 screening, we commenced a standardised subculture regime for all isolates (including CA01 and NSW1). All isolates were subcultured every 6 weeks and maintained under identical conditions. Thus, modification of the conditions under which even relatively old isolates are maintained can, over a 4-year period, result in apparent physiological changes.

The data presented here demonstrate that a general decline in biomass production on most nitrogenous substrates can occur in *Pisolithus* isolates maintained in axenic culture. The extent of this decline can vary between isolates and substrates. In some isolate/substrate combinations, biomass production increased over the same period, suggesting that maintenance of isolates in axenic culture differentially influences individual isolates.

These observations may have important implications for comparative physiological investigation of ECM fungi. The problem of intraspecific variation and the use of single isolates to infer potential functional traits in ECM fungi and in comparative physiological studies have been highlighted previously (Cairney 1999). The present data confirm that absolute comparisons of biomass production by individual isolates of ECM fungi maintained in axenic culture for different time periods are of little value for inferring their relative abilities to utilise nitrogen sources for growth. In only a few cases, however, did *Pisolithus* isolates that produced measurable biomass on a particular nitrogen source in one year produce no detectable biomass in the other year. With the exception of isolate R02 on NH_4^+, all such isolates produced relatively little biomass in the other year, suggesting that their abilities to utilise the substrate(s) in question are extremely limited. Notwithstanding the caveat that activities expressed in axenic culture may not necessarily be expressed during symbiosis, the screening of multiple isolates of different taxa maintained in axenic culture for short time periods thus appears useful for providing a very broad view of their physiological activities. We recommend in future studies of this nature that authors pay due regard to both the length of time and the manner in which isolates have been maintained in axenic culture and that such details are provided in subsequent reports.

Acknowledgements This work was conducted during tenure of an Australian Postgraduate Award to I.C.A. We thank Prof. A.E. Ashford for providing isolates CA01 and NSW1.

References

Abuzinadah RA, Read DJ (1986a) The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. I. Utilisation of peptides and proteins by ectomycorrhizal fungi. New Phytol 103:481–493

Abuzinadah RA, Read DJ (1986b) The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. III. Protein utilisation by *Betula*, *Picea* and *Pinus* in mycorrhizal association with *Hebeloma crustuliniforme*. New Phytol 103:506–514

Abuzinadah RA, Read DJ (1988) Amino acids as nitrogen sources for ectomycorrhizal fungi: utilisation of individual amino acids. Trans Br Mycol Soc 91:473–479

Abuzinadah RA, Finlay RD, Read DJ (1986) The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. II. Utilisation of protein by mycorrhizal plants of *Pinus contorta*. New Phytol 103:495–506

Anderson IC, Chambers SM, Cairney JWG (1998a) Molecular determination of genetic variation in *Pisolithus* isolates from a defined region in New South Wales, Australia. New Phytol 138:151–162

Anderson IC, Chambers SM, Cairney JWG (1998b) Use of molecular methods to estimate the size and distribution of mycelial individuals of the ectomycorrhizal basidiomycete *Pisolithus tinctorius*. Mycol Res 102:295–300

Anderson IC, Chambers SM, Cairney JWG (1999) Intra- and interspecific variation in patterns of organic and inorganic nitrogen utilization by three Australian *Pisolithus* species. Mycol Res 103:1579–1587

Arnebrant K, Ek H, Finlay RD, Söderström B (1993) Nitrogen translocation between *Alnus glutinosa* (L.) Gaertn. seedlings inoculated with *Frankia* sp. and *Pinus contorta* Doug. ex Loud seedlings connected by a common mycelium. New Phytol 124:213–242

Cairney JWG (1999) Intraspecific physiological variation: implications for understanding functional diversity in ectomycorrhizal fungi. Mycorrhiza 9:125–135

Di Battista C, Selosse M-A, Bouchard D, Stenström E, Le Tacon F (1996) Variations in symbiotic efficiency, phenotypic characters and ploidy level among isolates of the ectomycorrhizal basidiomycete *Laccaria bicolor* strain S 238. Mycol Res 100: 1315–1324

Ek H, Andersson S, Arnebrant K, Söderström B (1994) Growth and assimilation of ammonium and nitrate by *Paxillus involutus* in association with *Betula pendula* and *Picea abies* as affected by substrate pH. New Phytol 128:629–637

Finlay RD, Ek H, Odham G, Söderström B (1988) Mycelial uptake, translocation and assimilation of nitrogen from ^{15}N-labelled ammonium by *Pinus sylvestris* plants infected with four different ectomycorrhizal fungi. New Phytol 110:59–66

Finlay RD, Ek H, Odham G, Söderström B (1989) Uptake, translocation and assimilation of nitrogen from ^{15}N-labelled ammonium and nitrate sources by intact ectomycorrhizal systems of *Fagus sylvatica* infected with *Paxillus involutus*. New Phytol 113:47–55

Finlay RD, Frostegård Å, Sonnerfeldt A-M (1992) Utilisation of organic and inorganic nitrogen sources by ectomycorrhizal fungi in pure culture and in symbiosis with *Pinus contorta* Dougal. Ex Loud. New Phytol 120:105–115

Giltrap NJ (1981) Formation of primordia and immature fruiting bodies by ectomycorrhizal fungi in culture. Trans Br Mycol Soc 77:204–205

Keller G (1996) Utilisation of inorganic and organic nitrogen sources by high-subalpine ectomycorrhizal fungi of *Pinus cembra* in pure culture. Mycol Res 100:989–998

Laiho O (1970) *Paxillus involutus* as a mycorrhizal symbiont of forest trees. Acta For Fenn 106:1–57

Lundeberg G (1970) Utilisation of various nitrogen sources, in particular bound soil nitrogen, by mycorrhizal fungi. Stud For Suec 79:1–95

Marx DH (1981) Variability in ectomycorrhizal development and growth among isolates of *Pisolithus tinctorius* as affected by source, age, and reisolation. Can J For Res 11:168–169

Marx DH, Bryan WC (1975) Growth and ectomycorrhizal development of loblolly pine seedlings in fumigated soil infested with the fungal symbiont *Pisolithus tinctorius*. For Sci 21:245–254

Marx DH, Daniel WJ (1976) Maintaining cultures of ectomycorrhizal and plant pathogenic fungi in sterile water cold storage. Can J Microbiol 22:338–341

Read DJ, Leake JR, Langdale AR (1989) The nitrogen nutrition of mycorrhizal fungi and their hosts. In: Boddy L, Marchant R, Read DJ (eds) Nitrogen, phosphorus and sulphur utilisation by fungi. Cambridge University Press, Cambridge, pp 181–204

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

Scheromm P, Plassard C, Salsac L (1990) Effect of nitrate and ammonium nutrition on the metabolism of the ectomycorrhizal basidiomycete, *Hebeloma cylindrosporum* Romagn. New Phytol 114:227–234

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic, London

Thomson BD, Malajczuk N, Grove TS, Hardy GEStJ (1993) Improving the colonization capacity and effectiveness of ectomycorrhizal fungal cultures by association with a host plant and reisolation. Mycol Res 97:839–844

Turnbull MH, Goodall R, Stewart GR (1995) The impact of mycorrhizal colonisation upon nitrogen source utilisation and metabolism in seedlings of *Eucalyptus grandis* Hill ex Maiden and *Eucalyptus maculata* Hook. Plant Cell Environ 18: 1386–1394