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SHORT NOTE

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Variation in nitrogen source utilisation by nine *Amanita muscaria* genotypes from Australian *Pinus radiata* plantations

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Abstract The abilities of nine genotypes of *Amanita muscaria* (L.:Fr) Pers. to utilise a range of inorganic and organic nitrogen sources for growth was examined in axenic liquid cultures. Considerable intraspecific variation was observed in biomass yields on all substrates; however biomass yield was highest on glutamine and/or NH_4^+ for all genotypes. Yields on aspartic acid, glutamic acid and histidine were generally low relative to NH_4^+, while utilisation of arginine and glycine showed marked variation between genotypes. Eight genotypes produced significantly less biomass on bovine serum albumin than on NH_4^+, raising questions regarding classification of *A. muscaria* as a 'protein fungus'.

Keywords Nitrogen nutrition · Amino acid utilisation · Protein utilisation · Protein fungi · Intraspecific variation

Introduction

Ectomycorrhizal (ECM) associations are regarded as important in nutrient cycling in temperate and boreal forests worldwide (Smith and Read 1997). Emphasis has been placed upon the roles of ECM fungi in nitrogen cycling processes, in particular their importance in enhancing nitrogen acquisition by their tree hosts in forest soils where nitrogen mineralisation is frequently limited (Buscot et al. 2000). It is now well established that ECM fungi can enhance host nitrogen status via increased acquisition of inorganic nitrogen and/or facilitating access to organic nitrogen substrates (Chalot and Brun 1998). Many ECM fungi can, to varying degrees, access nitrogen in simple organic forms such as amino acids, amides, peptides and protein (e.g. Abuzinadah and Read 1986; Finlay et al. 1992; Keller 1996; Dickie et al. 1998).

Abuzinadah and Read (1986) investigated the ability of a range of ECM fungi to utilise bovine serum albumin (BSA) in axenic culture. They reported that biomass production for certain taxa on BSA was poor relative to that on NH_4^+, whereas for other taxa biomass production on BSA was similar to or greater than that on NH_4^+. The latter were designated as 'protein fungi' and the former as 'non-protein fungi', with an intermediate category also proposed between the two extremes (Abuzinadah and Read 1986). The data, however, were derived using only single isolates of each taxon, and there is considerable evidence for intraspecific physiological variation in ECM fungi (reviewed by Cairney 1999). In the context of nitrogen utilisation, where multiple isolates of particular taxa have been screened, considerable intraspecific variation in nitrogen source utilisation has been demonstrated (e.g. Keller 1996; Anderson et al. 1999; Rangel-Castro et al. 2002). Furthermore, storage of ECM fungi in axenic culture can influence utilisation of some nitrogenous substrates (Anderson et al. 2001). Such observations suggest that categorisation of ECM fungi into functional groups based on activities of single isolates may be rather misleading, particularly when inferences are made regarding ecological functioning of the taxa concerned (e.g. Abuzinadah and Read 1986; Taylor et al. 2000).

Amanita muscaria (L.:Fr)Pers. is a widely distributed taxon that forms ECM with a range of gymnosperm and angiosperm tree hosts (Yang et al. 1999). It has further been introduced via soil inoculum with exotic pines to some parts of the southern hemisphere and is regarded as potentially invasive of native ECM communities in native forest systems (Bougher 1996). Based on growth characteristics of a single isolate, *A. muscaria* was classified as a protein fungus by Abuzinadah and Read (1986). Subsequently, however, Keller (1996) reported that biomass production by four *A. muscaria* isolates was considerably poorer on BSA than on NH_4^+, casting doubt on the previous classification of the taxon. In order to address

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this, we investigated utilisation of a range of inorganic and organic nitrogen sources by nine genetically different isolates of *A. muscaria*.

Materials and methods

Nine isolates of *A. muscaria* were obtained from basidiome stipe material collected from two *Pinus radiata* plantation forest sites in New South Wales (NSW), Australia during the period April–June 1999 (Table 1). Isolates MM3, MM13, MM19 and MM20 were collected from Mount Macquarie State Forest (33° 39′ S, 149° 11′ E) and isolates FL16, FL19, FL23, FL25 and FL28 from Forest Lodge plantation (33° 50′ S, 149° 58′ E). All isolates were maintained on modified Melin Norkrans (MMN) agar medium (Marx and Bryan 1975) with subculturing every 4–8 weeks. Inter-simple sequence repeat PCR has previously shown that these isolates represent nine genetically distinct mycelial individuals (Sawyer et al. 2001).

Preliminary growth experiments indicated that *A. muscaria* batch cultures in liquid MMN displayed active growth for ca. 2–25 days following inoculation (data not shown). Therefore, all nitrogen-utilisation treatments were harvested at 23 days after inoculation. The influence of nitrogen source on biomass production was determined by inoculating two discs (5.0 mm diameter) of each isolate, cut from the leading edge of actively growing colonies on MMN agar, into 9.0-cm Petri dishes containing 25 ml liquid medium. The basal medium for all nitrogen treatments contained (l^{-1}): KH_2PO_4, 300 mg; $MgSO_4.7H_2O$, 140 mg; $CaCl_2$, 50 mg; NaCl, 25 mg; $ZnSO_4$, 3 mg; thiamine, 0.133 mg; ferric EDTA, 12.5 mg. Media were adjusted to pH 5–5.5 prior to the addition of the ferric EDTA and autoclaving. The influence of nitrogen source on biomass yield was determined by adding either $(NH_4)HPO_4$, $Ca(NO_3)_2$, arginine, histidine, aspartic acid, glutamic acid, glutamine, glycine or bovine serum albumin (BSA) to give a starting nitrogen concentration of 106 mg l^{-1}. Glucose was added to each treatment to give a standard C:N ratio of 39:1. Organic nitrogen sources were added by filter sterilisation (0.2-μm membrane filter) into the autoclaved basal media. Organic nitrogen sources were dissolved in basal medium and then adjusted to pH 5–5.5 before filter sterilisation to ensure that the starting pH of each substrate was constant. A control treatment containing basal medium with no added nitrogen was also included.

All treatments were replicated five times for each isolate and cultures incubated at 20°C in the dark. Mycelial mats were then removed from the liquid media, dried overnight at 80°C and the biomass determined gravimetrically. Data were corrected for growth in the presence of the nitrogen contained in the inoculum discs by subtracting the mean value for growth in basal medium with no added nitrogen from the raw data for each treatment. Data were analysed by one-way ANOVA and significant differences between treatments determined by Fisher's pairwise comparisons test using Minitab software (Minitab Inc., 1997). In order to compare relative growth rates between isolates on a particular substrate, data were also expressed as mean percentage of biomass production relative to NH_4^+.

Results and discussion

All genotypes produced measurable biomass on all inorganic and organic nitrogen sources, with the exception of genotype FL23 on NO_3^- and four genotypes (MM19, MM20, MM3 and FL23) on histidine, which produced no measurable biomass (Table 1). This confirms that *A. muscaria* can utilise a broad range of inorganic and organic substrates as nitrogen sources. For all genotypes, biomass production was highest on glutamine; however,

Table 1 Mean biomass yield (*μg* dry wt.) of nine *Amanita muscaria* isolates after 23 days growth in axenic liquid culture containing different nitrogen sources. Figures in parentheses indicate standard errors. Yields followed by different letters within rows are significantly different ($P<0.05$) as determined by Fisher's pairwise comparisons (*BSA* bovine serum albumin, *ng* no growth)

Isolate	NH_4^+	NO_3^-	Arginine	Histidine	Aspartic acid	Glutamic acid	Glutamine	Glycine	BSA
MM19	39.38 (3.31)a	3.63 (0.59)b,c	5.43 (1.47)b	ng	1.24 (0.39)c	3.73 (0.59)b,c	40.96 (6.37)a	38.20 (3.49)a	6.12 (1.06)b,c
MM20	18.99 (1.17)a	2.89 (0.74)b	31.60 (6.78)c	ng	ng	2.71 (0.78)b	41.65 (2.78)d	44.28 (2.35)d	10.03 (1.45)b
MM13	12.19 (1.59)a	7.68 (0.74)b	9.34 (1.19)a,b	0.28 (0.115)d	1.79 (0.12)d	8.60 (1.04)a,b	22.68 (1.40)e	26.27 (2.21)e	12.28 (2.58)a,d
MM3	21.98 (3.98)a	2.16 (1.36)b	36.80 (5.84)c	ng	1.79 (0.66)b	3.22 (0.81)b	31.39 (1.52)c,d	25.30 (3.01)d	5.70 (1.86)b
FL19	37.49 (2.19)a	0.37 (0.16)b	33.88 (2.69)a	0.37 (0.18)b	16.28 (0.71)c	20.98 (2.74)c	46.92 (0.34)d	45.40 (1.73)d	14.08 (0.44)c
FL23	24.84 (1.24)a	ng	23.48 (2.62)a	ng	3.78 (1.98)b	5.79 (1.70)b	69.48 (4.67)c	49.04 (8.09)d	10.03 (1.24)b
FL25	4.60 (0.14)a	0.83 (0.16)b	1.38 (0.07)b	1.66 (0.21)b	2.12 (0.41)b	1.19 (0.28)b	11.91 (1.04)c	0.14 (0.02)d	0.12 (0.02)d
FL28	25.30 (1.75)a	0.23 (0.12)b	24.88 (2.67)a	0.46 (0.12)b	5.75 (1.45)c	6.12 (0.76)c	34.09 (7.96)d	23.74 (1.04)a	5.01 (0.64)c
FL16	7.96 (1.63)a	0.05 (0.02)b	1.24 (0.97)c	0.92 (0.12)c	0.74 (0.46)c	2.39 (2.16)d	15.64 (0.69)e	4.05 (1.61)d	1.98 (0.59)d

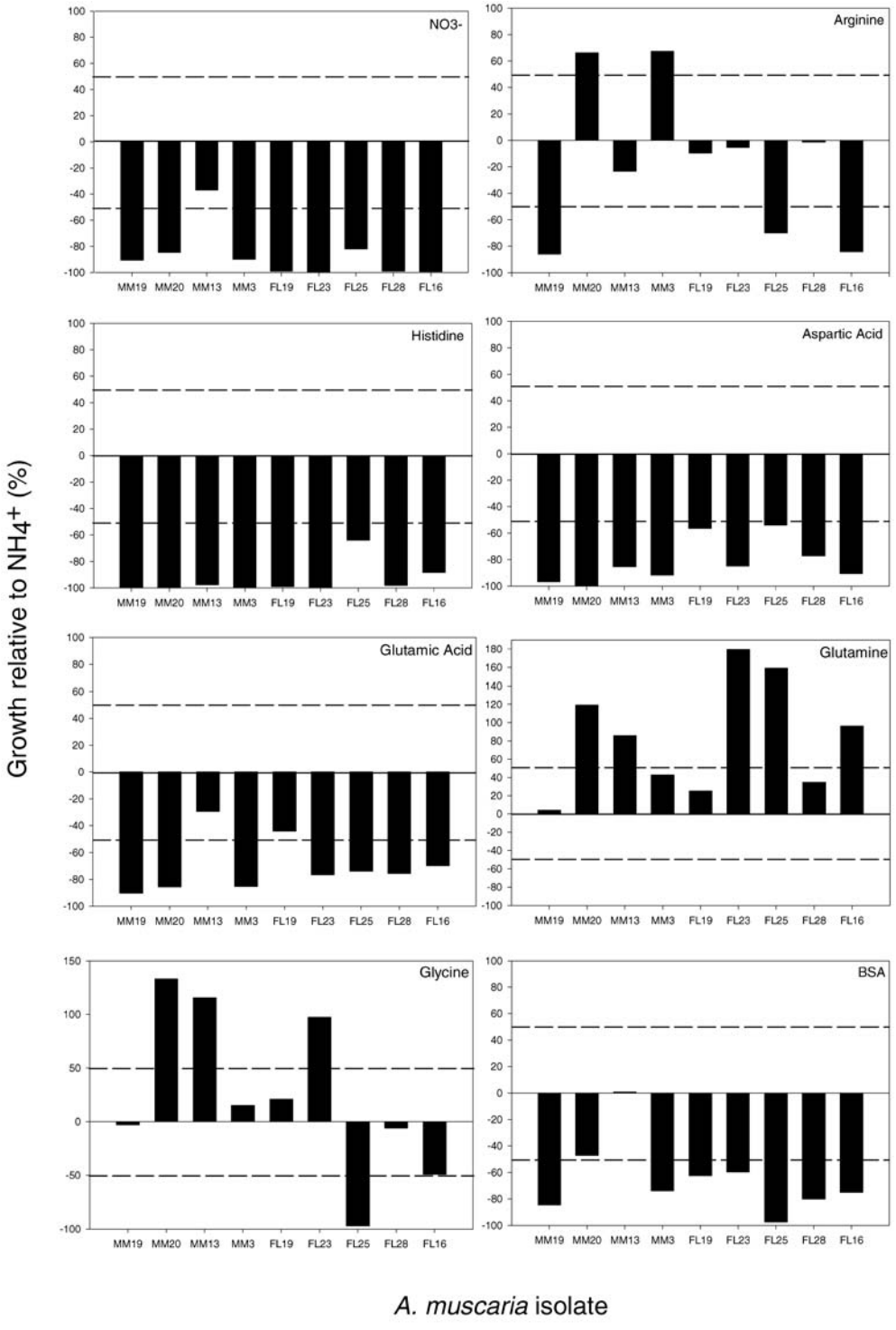

Fig. 1 Mean growth (%) relative to NH_4^+ of nine *Amanita muscaria* genotypes on a range of inorganic and organic nitrogen sources

for some this was not significantly different to NH_4^+ (genotype MM19), arginine (genotype MM3) or glycine (genotypes MM19, MM20, MM13 and FL19) (Table 1). For most genotypes, biomass production on NH_4^+ was greater than on the remaining substrates but not significantly different to or significantly less than for some genotypes on arginine (genotypes MM3, MM13, FL19 and FL28), glutamic acid (genotype MM3) and glycine (genotypes MM20, MM13, MM3, FL28, FL19 and FL23) (Table 1). Biomass production on NO_3^- was generally poor for all genotypes, being either significantly less than or in some cases not significantly different to any other amino acid. Exceptions were histidine (genotype MM13), aspartic acid (genotype MM13) and glycine (genotype FL25), for which biomass production was significantly higher than on NO_3^- (Table 1).

Nitrogen mineralisation in eastern Australian *P. radiata* plantation soils is slow. Thus NH_4^+ is the major inorganic from of nitrogen (Connell et al. 1995). In common with most other ECM fungi and previous observations on *A. muscaria*, all genotypes in the present study produced significantly more biomass on NH_4^+ than NO_3^- (France and Read 1984; Littke et al. 1984; Abuzinadah and Read 1988; Keller 1996; Anderson et al. 1999; Sangtiean and Schmidt 2002), indicating that they may effectively exploit the major inorganic nitrogen pool.

Biomass yields on histidine, aspartic acid and glutamic acid were significantly lower than those on NH_4^+, with the exception of genotypes MM19 and MM13 on glutamic acid, for which biomass production was not significantly different to biomass production on NH_4^+ (Table 1, Fig. 1). Biomass yields on glycine and arginine were variable, with some genotypes producing significantly more or less biomass on at least one of these substrates relative to NH_4^+ (Table 1). Genotypes MM20 and MM3 produced >50% more biomass on arginine than on NH_4^+, while the other genotypes produced 10–85% less biomass on arginine than on NH_4^+ (Fig. 1).

The considerable intraspecific variation observed among the *A. muscaria* genotypes with respect to their abilities to utilise amino acids confirms previous observations with other *A. muscaria* isolates and other ECM fungal taxa (Keller 1996; Anderson et al. 1999). All genotypes were isolated into axenic culture during the 1999 fruiting season (Sawyer et al. 2001) and have since been subjected to identical subculturing regimes. We can, therefore, discount the possibility that the observed variation arose as a result of variable length of time in culture or subculturing protocol (see Anderson et al. 2001). With the exception of two genotypes on glycine, all genotypes in the present study utilised the neutral amino acids glycine and histidine well. This is consistent with the conclusions of Abuzinadah and Read (1988) and Keller (1996) for other *A. muscaria* isolates. Our observations for the acidic and basic amino acids, however, contrast with those of Abuzinadah and Read (1988). Biomass yield of an *A. muscaria* isolate from an Austrian pine forest on either glutamic or aspartic acids was found by these authors to be higher than on NH_4^+. However, in the present study, biomass yields on these substrates were poor relative to NH_4^+ and significantly lower than on glutamine. While this might reflect a deficiency in their relative abilities to assimilate glutamic acid, the fact that growth of most isolates was also poor on aspartic acid seems more likely to indicate a relative inability to absorb acidic amino acids, at least under the experimental conditions we adopted. Abuzinadah and Read (1988) also reported that biomass yield of the *A. muscaria* isolate on arginine was higher than on NH_4^+, and that yields on histidine or lysine were ca. 50% of the yield on NH_4^+. Only two genotypes in the present study grew better on arginine than on NH_4^+, while most produced little or no growth on histidine, emphasising the importance of screening multiple genotypes of a taxon before drawing conclusions regarding its physiological potential or making comparisons with other taxa. Interestingly, Nehls et al. (1999) found that a general amino acid permease from an isolate of *A. muscaria* was an efficient transporter of basic amino acids, including histidine. The relative inability of some *A. muscaria* genotypes to utilise histidine may, thus, reflect variation in transporter activity or at the intracellular metabolic level. Careful tracer experiments with labelled substrates would be required to resolve this.

Biomass production on BSA was significantly lower than on NH_4^+ for all genotypes except MM13, for which there was no significant difference. Yields on BSA were either significantly lower than or not significantly different to those on the amino acids, except for histidine (all genotypes) and aspartic acid (genotypes MM19, MM20, MM13 and FL16), for which biomass production was higher on BSA (Table 1, Fig. 1). Notwithstanding that BSA may not induce maximal protease activity in some ECM fungi (Chalot and Brun 1998), Abuzinadah and Read (1986) classified *A. muscaria* as a 'protein fungus' based on the its ability to utilise BSA as well as or better than NH_4^+ in axenic culture, and it is frequently cited as such in the literature (e.g. Smith and Read 1997; Taylor et al. 2000). Implicit in this is that *A. muscaria* has a greater ability to utilise simple protein as a nitrogen source than non-protein or intermediate taxa. It must be stressed, however, that the classification was based on the activity of a single *A. muscaria* isolate.

Only genotype MM13 from the present study, which produced similar biomass on both BSA and NH_4^+, would fit the description of a protein fungus. The remaining eight genotypes produced only a fraction of their NH_4^+ biomass on BSA. According to the classification of Abuzinadah and Read (1986), these genotypes would be regarded as non-protein fungi, as would the four isolates screened by Keller (1996). Other recent work has indicated that *A. muscaria* excretes aspartic proteases that can hydrolyse extracellular protein (Nehls et al. 2001) and possesses a broad-spectrum amino acid transporter that could effect absorption of the products of protein degradation (Nehls et al. 1999). There is, thus, no doubt that *A. muscaria* can utilise simple proteins and peptides as nitrogen sources. It is also clear from the combined data of the present and previous studies that *A. muscaria* displays considerable intraspecific variation in this regard. With this in mind, we suggest that the term protein fungus is an inappropriate description of *A. muscaria*. In a broader context, our data emphasise the need to consider multiple genotypes when investigating physiological activities of ECM fungal taxa. If multiple genotypes are screened and frequency distributions of nitrogen utilisation characteristics within taxa determined (sensu Cairney et al. 2000), then meaningful interspecific comparisons may be possible. In the absence of such data, partitioning taxa into putative functional groups such as protein fungi or non-protein fungi is likely to be misleading and may result in erroneous inferences about ecological function (Cairney 1999).

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