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Use of different nitrogen sources by the edible ectomycorrhizal mushroom *Cantharellus cibarius*

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Abstract The growth of three strains of *Cantharellus cibarius* on liquid media containing ammonium, nitrate and bovine serum albumin (BSA) in different combinations was determined. The most readily utilisable source of N was ammonium. BSA utilisation was limited compared with media containing ammonium. Growth on nitrate was also poor, suggesting a limited capacity of *C. cibarius* to metabolise this nitrogen source. There was some indication of considerable intraspecific variation within *C. cibarius* in the utilisation of nitrogen sources. Possible links between atmospheric nitrogen deposition and the observed decrease of sporocarp formation by *C. cibarius* in Europe are discussed. We highlight the potential ecological significance of bacteria associated with *C. cibarius* which may circumvent the need for fungal extracellular enzymes to access complex nitrogen sources.

Keywords *Cantharellus cibarius* · Organic nitrogen utilisation · Minerotrophic fungi · Sporocarp production

Introduction

Ectomycorrhizal trees predominate in boreal and temperate forests, where nitrogen is generally considered to be the main growth-limiting factor (Read 1991; Tamm 1991). Levels of inorganic soil nitrogen, ammonium and nitrate in these systems are generally very low (Cole 1981), while low-molecular-weight organic N compounds, particularly amino acids, are often present at much higher concentrations (Tamm 1991). It has been shown that some ectomycorrhizal (ECM) fungi associated with boreal tree species can readily utilise a wide range of amino acids (Finlay et al. 1992; Keller 1996; Dickie et al. 1998). Some ECM species can also grow on more complex organic N sources, including proteins (Lundeberg 1970; Abuzinadah and Read 1986a, b; Leake and Read 1989; Finlay et al. 1992; Keller 1996; Anderson et al. 1999) and chitin (Leake and Read 1990). The ability of ECM fungi to utilise protein as an N source has received special attention and the classification of fungi according to their ability to grow on a protein (bovine serum albumin, BSA) compared with ammonium has been proposed (Abuzinadah and Read 1986c). If the competitive ability of fungi to utilise organic and mineral N forms is influenced by their relative availability in soil, then changes in availability should lead to changes in species composition of the ECM community.

In western Europe, nitrogen eutrophication of forest ecosystems via atmospheric N deposition is considered an important factor in the observed decline in ECM sporocarp production and species richness (Arnolds 1991; Cairney and Meharg 1999; Treseder and Allen 2000). Recently, Taylor et al. (2000) examined spruce forests along a European N-S transect and found a clear negative relationship between soil inorganic N availability and ECM community diversity. In addition, fungal species characteristic of northern sites that were able to utilise organic N decreased in number as the availability of soil mineral N increased towards central Europe. It was suggested that the more minerotrophic fungal species out-competed fungi more specialised in using organic N sources. The species considered in most of the above studies are, however, primarily those that readily grow in culture (Leake and Read 1997) and very limited information is available on the physiological capabilities of more recalcitrant species.

The golden chanterelle, *Cantharellus cibarius* Fr., is a highly prized edible ECM fungus with a worldwide distribution (Watling 1997; Danell 1999). The annual world market is estimated to be 150–200 K metric tonnes, with an estimated value of £1 billion (Watling 1997). Given its importance, it is surprising that the eco-physiology of *C. cibarius* has been little studied. Straatsma and coworkers were amongst the first to examine the growth requirements of *C. cibarius* mycelia in culture (Straatsma

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1986; Straatsma and Bruinsma 1986; Straatsma and van Griensven 1986). The work centred on the role of carbon dioxide and carboxylated metabolic intermediates as nutritional factors in vegetative growth. More recently, this work has been expanded by Danell (Danell et al. 1993; Danell 1994a, b; Danell and Camacho 1997), who has studied factors influencing the fruiting of *C. cibarius*.

In Europe, a marked reduction in sporocarp production by *C. cibarius* has been observed over the last 40 years (Jansen and van Dobben 1987; Arnolds 1995). As for other ECM fungi, it has been suggested that this reduction is linked to increased N deposition (Van der Eerden et al. 1998). Here we investigated the ability of *C. cibarius* to utilise ammonium, nitrate and BSA as N sources to test the hypothesis that *C. cibarius* can grow on a protein source. Assuming that increasing mineral N changes the competitive balance between fungi with different N source requirements (Taylor et al. 2000), then better growth of *C. cibarius* on a protein than on ammonium would offer a possible reason for the observed reduction in sporocarp production.

Materials and methods

Fungal strains and culture conditions

The three Swedish strains of *C. cibarius* (SNGT2-A, LBCT6, and SVBT5) used were originally isolated from fruit bodies collected in mixed coniferous forests and maintained on Modified Fries Medium (MFM) (Danell 1994a) at 20°C in complete darkness. Cultures have been maintained in culture for 8–10 years. Inoculum was prepared by transferring 5-mm-diameter plugs cut from the growing front of actively growing mycelia onto fresh MFM agar

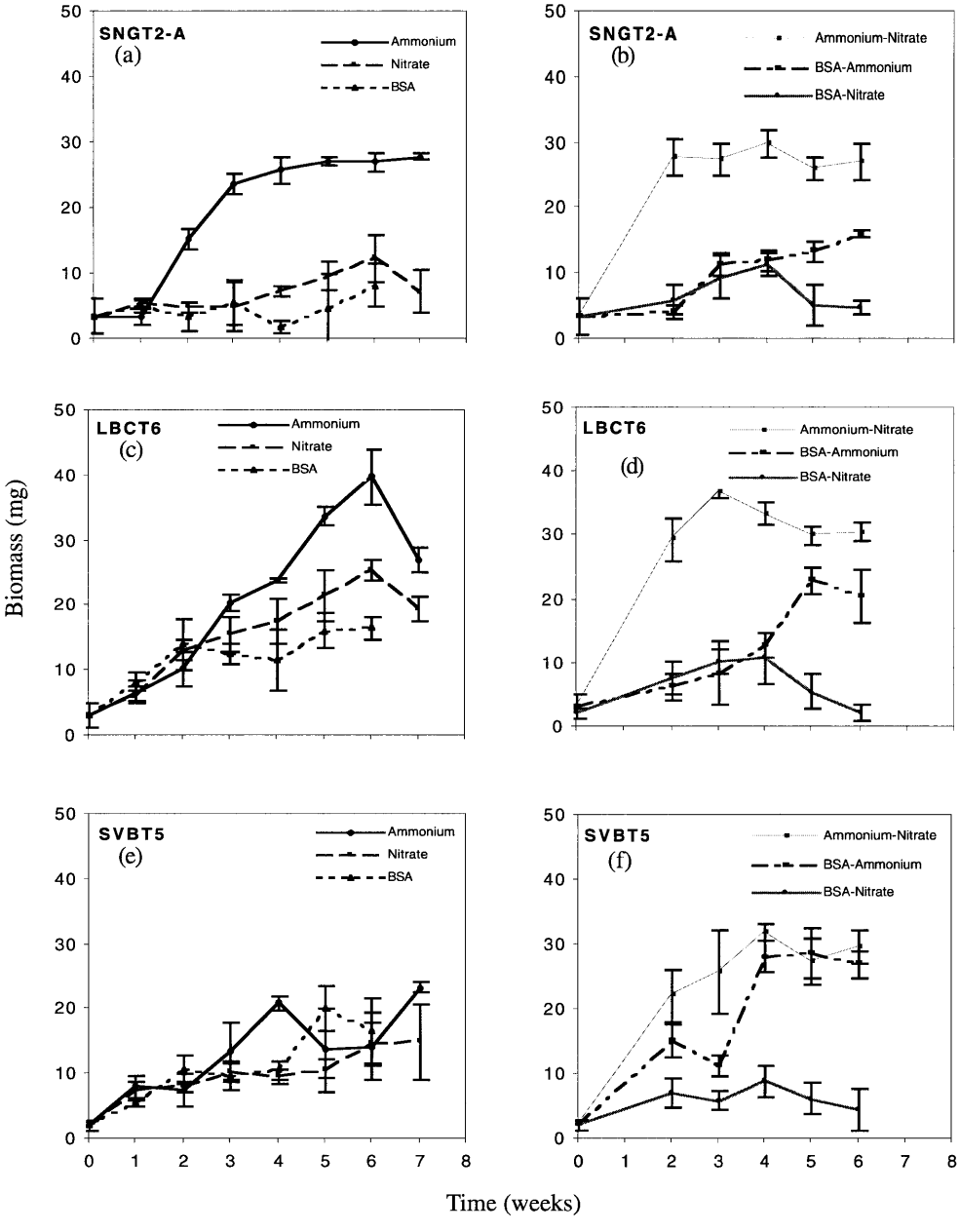

Fig. 1 Dry weight (mg) production of three strains of *Cantharellus cibarius* grown on medium containing ammonium, nitrate, and bovine serum albumin (BSA), singly or in combination. *Vertical bars* indicate 95% confidence limits, *n*=5

covered with a film of cellophane. After incubation for ca. 7 days, plugs with obvious regrowth were transferred to 9-cm Petri dishes. Each dish contained 20 ml of sterile, liquid MFM supplemented with a single or combined nitrogen source. Inoculated dishes were kept at 20°C in complete darkness.

Nitrogen sources

The effect of different N sources on biomass production was examined using MFM as a basal medium, as *C. cibarius* is known to produce greater biomass on this medium than on other commonly used media (Straatsma 1986). Growth was examined with six different N treatments (Fig. 1); mineral N was supplied in the form of NH_4Cl and/or KNO_3, and organic N as the protein BSA (Sigma Chemical Co.). Individually or combined, the nitrogen sources had a final concentration of 152 mg N l^{-1}. This amounted to 3.04 mg N per Petri dish. Carbon was supplied as glucose (2.2 g l^{-1}) and fructose (2 g l^{-1}), to give a final C/N ratio of 11. The pH of the culture solution was adjusted to 4.5. BSA was first dissolved in deionised water and then filter sterilised (0.2-µm filter). The concentration of BSA in the filtrate was determined prior to its addition to the filter-sterilised basal medium. Mycelia were harvested 2, 3, 4, 5, and 6 weeks after inoculation, and at 1 and 7 weeks, respectively, where N uptake was particularly rapid or growth was very slow. Five replicates per strain and treatment were harvested each time. The mycelia were transferred to pre-weighed filter papers, oven dried at 105°C overnight and weighed to the nearest 0.1 mg. Evaporation from dishes was determined by measuring the volume of culture solution remaining in the Petri dishes at each sampling date. The concentrations of the N sources in the culture solution at each sampling date were then adjusted to the original volume of 20 ml. The pH of the liquid media was recorded immediately after harvesting.

BSA remaining in the culture solution was determined immediately after harvesting of the mycelium using the Coomassie Plus Protein Assay Reagent (Pierce Chemical Co.) calibrated with BSA. Measured concentrations of BSA were not corrected for the possible release of extracellular proteins during fungal growth. It is likely that these proteins constitute a very small proportion of total protein in solution (cf. Leake and Read 1989).

Treatments with inorganic N (ammonium and nitrate) were stored at –20°C until analysed using a flow injection analyser (FIAstar Analyzer, Foss-Tecator, Sweden).

Statistical analyses

Differences in dry weight production, pH and N remaining in the culture solution were analysed by one-way analysis of variance. Differences between means were then further analysed using a multiple range test (Tukey, P=0.05). All analyses were made with Minitab Statistical Software (Minitab Inc.).

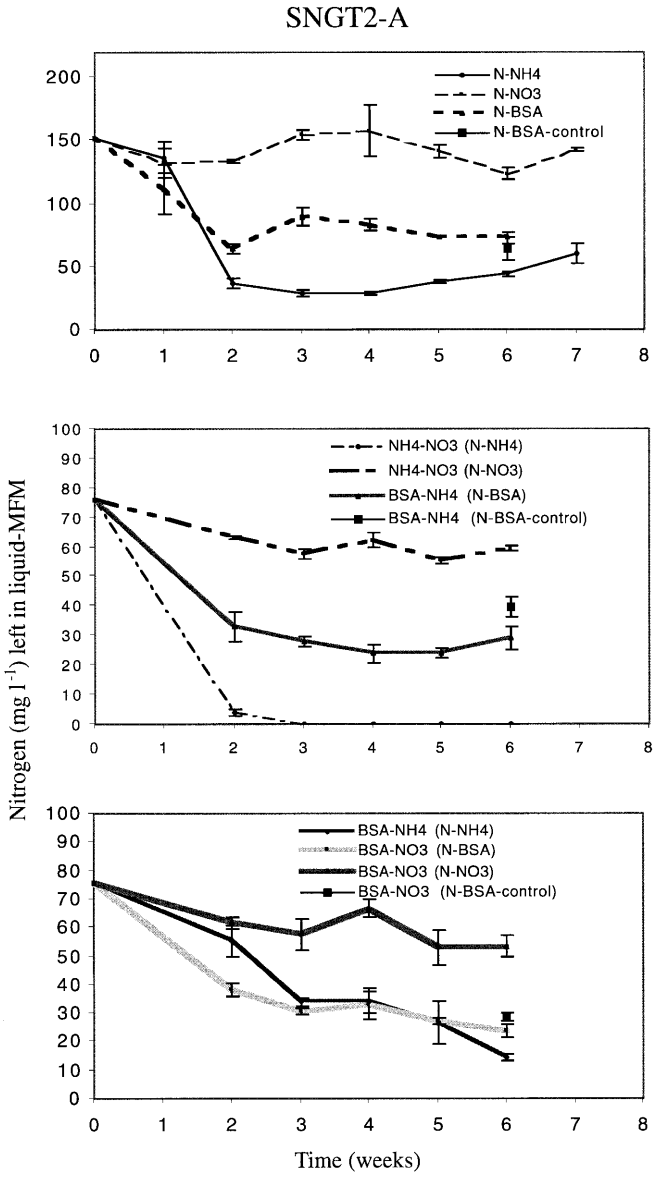

Fig. 2 Nitrogen (mg l^{-1}) remaining in liquid medium (MFM) during 7 weeks of the growth of *Cantharellus cibarius* strain SNGT2-A. The nitrogen concentration in the single treatment was 152 mg l^{-1}. In combined treatments, each N source accounted for 76 mg l^{-1}. *Vertical bars* indicate 95% confidence limits, n=5

Results

Biomass production on different N sources

The *C. cibarius* isolates had rather different patterns of growth on the N sources provided (Fig. 1). Isolate SNGT2-A grew well on media containing ammonium supplied singly or in combination with BSA or nitrate (Fig. 1a, b; Table 1). There was no evidence that this isolate utilised BSA as an N source. Although biomass increased somewhat on the nitrate treatment, there was no indication from the concentration of residual N in the culture solution that this involved utilisation of the nitrate (Fig. 2). In addition, there was no increase in the pH of the culture solution, which also suggests limited assimilation of nitrate (Fig. 3a). SNGT2-A showed some growth on the BSA–nitrate treatment, but this was accompanied by an increase in pH (Fig. 3b) and a decrease in N in the culture medium (Fig. 2). This suggests that some growth involving nitrate utilisation occurred on this medium.

Isolate LBCT6 showed appreciable growth and N utilisation on all N sources provided (Fig. 1c, d; Fig. 4; Table 1) although, as with isolate SNGT2-A, growth was generally better on media containing ammonium. The changes in culture solution pH reflected very closely the uptake of the different N sources (Fig. 3c, d).

Table 1 Maximal biomass (mg dry wt.) of three strains of *Cantharellus cibarius* grown on different nitrogen sources. The initial N concentration was 152 mg l^{-1}. Numbers in parentheses indicate standard errors. Different letters indicate differences, according to Tukey's test, I: among treatments in the same strain, II: among strains in the same treatment

	SNGT2-A				LBCT6				SVBT5			
			I	II			I	II			I	II
Ammonium	27.06	(0.52)	a	b	39.79	(2.16)	a	a	20.92	(0.49)	b	b
Nitrate	12.36	(1.87)	bc	b	25.39	(0.84)	b	a	14.68	(1.64)	bc	b
Ammonium–nitrate	29.90	(1.11)	a	b	36.84	(0.40)	a	a	31.85	(0.50)	a	b
BSA	8.18	(1.69)	c	b	16.56	(0.89)	b	a	20.03	(1.34)	b	a
BSA–ammonium	16.14	(0.34)	b	c	22.92	(1.05)	b	b	28.81	(1.53)	a	a
BSA–nitrate	11.36	(0.86)	bc		10.76	(1.56)	c		8.97	(1.21)	c	

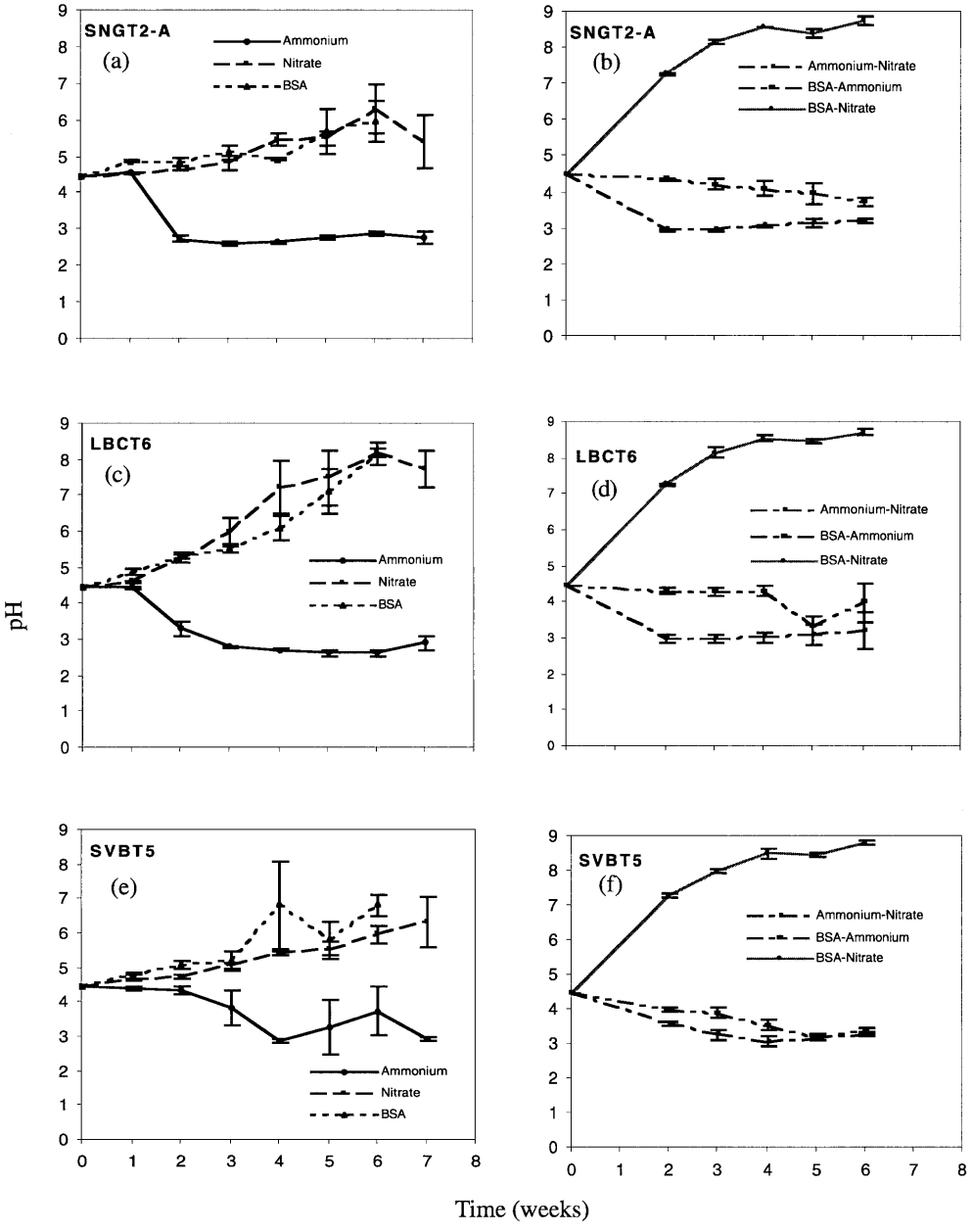

Fig. 3 Change of medium pH during the growth of three strains of *Cantharellus cibarius* on media containing ammonium, nitrate and BSA, singly or in combination. *Vertical bars* indicate 95% confidence limits, *n*=5

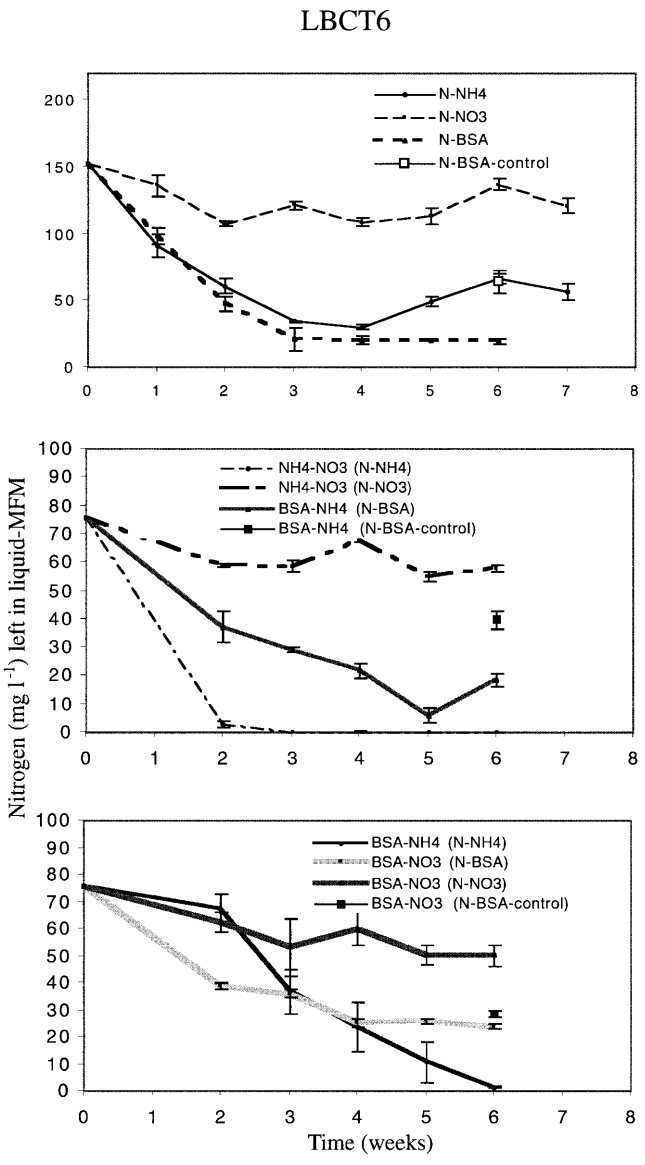

Fig. 4 Nitrogen (mg l^{-1}) remaining in media during 7 weeks growth of *Cantharellus cibarius* strain LBCT6. The nitrogen concentration in the single treatment was 152 mg l^{-1}. In combined treatments, each N source accounted for 76 mg l^{-1}. *Vertical bars* indicate 95% confidence limits, *n*=5

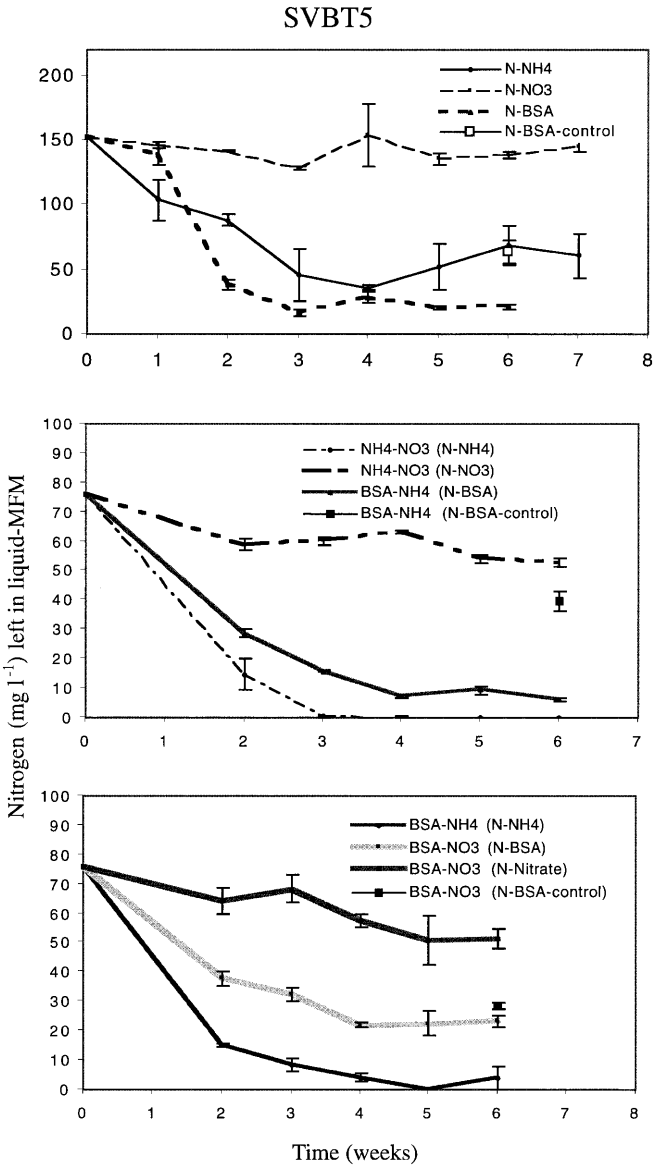

Fig. 5 Nitrogen (mg l^{-1}) remaining in media during 7 weeks of growth of *Cantharellus cibarius* strain SVBT5. The nitrogen concentration in the single treatment was 152 mg l^{-1}. In combined treatments, each N source accounted for 76 mg l^{-1}. *Vertical bars* indicate 95% confidence limits, *n*=5

The third isolate, SVBT5, showed rather slow and erratic growth on single N sources (Fig. 1e). There were no significant differences between the three N sources in biomass produced (Table 1). The erratic growth was also reflected in fluctuations of culture solution pH (Fig. 3e) and residual N (Fig. 5). Growth of SVBT5 on combined N sources was rather more stable than on single N sources and the highest growth was on the ammonium–nitrate and BSA–ammonium treatments (Fig. 1f; Table 1).

Loss of BSA from culture solution in control dishes

There was a clear decrease in BSA concentration in the uninoculated control treatments. The final concentrations of N-BSA in controls for treatments BSA, BSA–ammonium, and BSA–nitrate were 63.90 mg l^{-1} (8.94), 39.62 mg l^{-1} (3.44), and 28.58 mg l^{-1} (1.37), respectively (Figs. 2, 4, 5). The reduction in BSA concentration was accompanied by changes in solution pH: 5.44 (0.63), 4.44 (0.00), and 5.55 (0.03), respectively. The reduction in BSA was probably due to precipitation of the protein rather than abiotic hydrolysis; a very thin layer of finely granular material appeared on the bottom of the Petri dishes.

Discussion

Ammonium is generally recognised as the most readily utilisable source of N for most ECM fungi (Smith and Read 1997) and the results from the present study of *C. cibarius* support this assertion. The results also confirm the work of Straatsma and van Griensven (1986), who reported that ammonium is a suitable source of N for *C. cibarius*. Fungal biomass production was similar when ammonium was supplied singly or in combination with nitrate, with the exception of strain SVBT5, which grew better on the combined treatment. The mycelia grew faster on the combined treatment than on ammonium alone and took up all the ammonium N. This may indicate that ammonium uptake was more efficient when supplied at the lower concentration (76 mg l^{-1} N) than at the higher concentration (152 mg l^{-1} N). Nevertheless, the concentration of ammonium supplied alone was the same as the optimal concentration reported by Straatsma and van Griensven (1986) for growth of *C. cibarius* mycelia in liquid medium. These authors also reported that doubling the ammonium concentration in the medium did not inhibit biomass production; there was no difference in biomass production compared with yields at the standard N concentration. Therefore, inhibition of growth by a high concentration of ammonium was not considered in our study.

The results do not support our original hypothesis that *C. cibarius* can use protein better than ammonium as an N source. In spite of the growth of the *C. cibarius* strains LBCT6 and SVBT5 on BSA, biomass production was lower than on ammonium alone or in combination. The change in pH observed in the treatment BSA–nitrate suggests a symport transport of $2H^+$:NO_3^-, reflected in a large influx of protons (McClure et al. 1990; Hawkins and Lewis 1993) and an alkalisation of the medium. The marked increase in pH may have inhibited also the activity of (mainly acidic) proteases produced to degrade BSA (Leake and Read 1989).

Despite the lack of support for our hypothesis, it is likely that the observed reduction in sporocarp production by *C. cibarius* is linked to increases in soil mineral N availability. Nohrstedt (1994) reported a 30% decrease in sporocarp production by *C. cibarius* in a central Swedish pine forest after application of 150 kg N ha^{-1} ammonium nitrate. In addition, it has been shown that removal of the upper organic layers of the forest floor can improve partially sporocarp production by *C. cibarius* (de Vries et al. 1995) and other ECM fungi (Baar and ter Braak 1996). This was attributed to high concentrations of mineral N, primarily ammonium, within the organic layers that are detrimental to fungal growth. However, the concentrations of mineral N per se may not be as important as the predominant form of the available N (i.e. N-NO_3^- or N-NH_4^+).

The presence of nitrate ion has been shown both in vitro and in soil to have negative effects on the development of some ECM fungi (Richards 1965; Harley and Smith 1983). Only one of the *C. cibarius* isolates used in the present study (LBCT6) showed measurable growth when nitrate was supplied as an N source. This suggests that *C. cibarius* has a limited capacity to metabolise nitrate. Thus, increases in nitrate concentration in soil, either through nitrification of ammonium or directly through atmospheric deposition, may be detrimental to *C. cibarius*. We are not aware of any specific studies of the effects of the nitrate ion upon *C. cibarius* and this requires further investigation.

The different growth patterns of the isolates suggest considerable intraspecific variation within *C. cibarius* in utilisation of N substrates. The three isolates used originated in forests with very similar edaphic conditions and N deposition loads. The variation may, therefore, represent inherent rather than site-specific variation. Cairney (1999) reviewed the occurrence of intraspecific physiological variation in ECM and pointed out that the great majority of studies on ECM fungus physiology involved single isolates of rapidly growing fungi. Where several isolates were included, intraspecific physiological variation was common. Cairney (1999) also pointed out that intraspecific variation may be underestimated when only a small number of isolates or strains of a given fungus are included. Future work with more isolates of *C. cibarius* from a range of ecological conditions should further elucidate the physiological diversity within this fungus. In addition, utilisation of different N sources by *C. cibarius* in the symbiotic state will also be determined in order to assess the significance of N form on host nutrition.

It has been demonstrated on a number of occasions that bacteria are commonly associated with the hyphae of EMC fungi in the soil (Garbaye 1994; Frey et al. 1997; Timonen et al. 1998). In addition, bacteria have been observed within sporocarps of ECM fungi (Garbaye et al. 1990; Pacioni 1990; Varese at al. 1996; Sbrana et al. 2000). Sporocarps of *C. cibarius* are unusual in this respect in that they contain very high numbers of bacteria, in particular fluorescent *Pseudomonas* (Danell et al. 1993; Rangel et al. 2000). If the vegetative mycelia of *C. cibarius* also associate with a wide range of bacteria, the need for fungus extracellular enzymes (e.g. proteases) to utilise organic N may be circumvented by the enzymatic capabilities of the associated bacteria. This also applies to the many other ECM fungi with bacterial flora associated with the hyphae.

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