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Intraspecific variation in use of different organic nitrogen sources by the ectomycorrhizal fungus *Hebeloma cylindrosporum*

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Abstract The ectomycorrhizal (ECM) fungus *Hebeloma cylindrosporum* is an appropriate model to study the intraspecific functional diversity of ECM fungi in forest ecosystems. Numerous metabolic genes, specifically genes related to nitrogen assimilation, have been characterised for this species and the spatial and temporal structures of its natural populations have been extensively worked out. In this paper, we reveal the extent to which intraspecific variation exists within this fungus for the ability to use organic nitrogen, an important functional characteristic of ECM fungi. In addition to ammonium and nitrate, *H. cylindrosporum* can use at least 13 different amino acids out of 21 tested as sole nitrogen source, as well as urea and proteins. By screening 22 genetically different wild type haploid strains we identified obvious differences in use of six nitrogen sources: alanine, glycine, phenylalanine, serine, bovine serum albumin and gelatine. Of the 22 haploid strains, 11 could not use at least one of these six nitrogen sources. The inability of some haploid strains to use a nitrogen source was found to be a recessive character. Nevertheless, obvious differences in use of the four amino acids tested were also measured between wild type dikaryons colonising a common *Pinus pinaster* root system. This study constitutes the basis for future experiments that will address the consequences of the functional diversity of an ECM fungus on the functioning of the ECM symbiosis under natural conditions.

Keywords Amino acid and protein utilisation · Ectomycorrhizal fungi · *Hebeloma cylindrosporum* · Intraspecific variation · Nitrogen nutrition

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Introduction

It is now well established that a single root system of an individual ectomycorrhizal (ECM) plant is simultaneously associated with many different ECM fungal species as well as with different individuals of the same species. Furthermore, although a host plant is permanently associated with ECM fungi, the composition, species richness and diversity of the associated ECM fungal community change during the course of forest ageing (Smith et al. 2002) as well as in response to various disturbances of the ecosystem such as nitrogen deposition (Peter et al. 2001; Lilleskov et al. 2002a; Avis et al. 2003). To this taxonomic and genetic diversity should correspond a functional diversity that needs to be evaluated to appreciate the functioning of the ECM symbiosis under field conditions.

The ability of ECM fungi to use simple (e.g. amino acids) or complex (e.g. proteins) organic nitrogen sources has been the focus of many studies. This ability contrasts with the almost exclusive preference of plants for inorganic nitrogen sources. Abuzinadah and Read (1986b) demonstrated for the first time that mycorrhizal plants of *Pinus contorta* associated with the ECM fungi *Paxillus involutus*, *Rhizopogon roseolus* and *Suillus bovinus* could grow on a substrate supplemented with proteins as sole nitrogen source. Since then, it has been shown that mycorrhizal fungi can access organic nitrogen at very early stages of organic matter decomposition and could act as primary decomposers of, for example, pollen grains (Perez-Moreno and Read 2001a) or of the microfauna (Klironomos and Hart 2001; Perez-Moreno and Read 2001b). The use of organic nitrogen forms to the benefit of the host plant is thought to be highly relevant in boreal forest ecosystems where environmental conditions prevent, or considerably slow down, mineralisation of organic matter (reviewed in Read and Perez-Moreno 2003).

Quantitative differences in use of inorganic and organic N sources have been documented both between ECM fungal species (Abuzinadah and Read 1986a; Dickie et al. 1998; Lilleskov et al. 2002b) and also between strains belonging to the same species (Anderson et al. 1999; Rangel-Castro et al.

2002; Sawyer et al. 2003a,b). At the interspecific level, Abuzinadah and Read (1986a) distinguished three classes of ECM fungal species: 'protein', 'non-protein' and 'intermediate' according to their ability to use protein as sole nitrogen source. More recently, Lilleskov et al. (2002b) suggested that fungal species that predominate in forest habitats characterised by low concentrations of inorganic nitrogen could on average use protein and amino acid-N more efficiently than species predominating in habitats characterised by high concentrations of inorganic nitrogen. Similar ranges of variation in use of organic N sources have been reported at the intraspecific level. For example, it was reported that some individuals of *Amanita muscaria* could use proteins as efficiently as ammonium, whereas others were found unable to use proteins at all (Sawyer et al. 2003a). Whether variation in use of organic nitrogen sources, as revealed in pure-culture experiments, could be related to the ability of individual ECM species to respond and adapt to variations in N availability in the field has yet to be demonstrated.

Both the origins (i.e. the genetic and molecular basis) of intraspecific variation in use of organic N and its consequences (such as the ability to adapt to changing environmental conditions) can now be addressed using model ECM fungal species that can easily be grown in pure culture and for which molecular data are accumulating. This is the case for the agaric *Hebeloma cylindrosporum* Romagnesi, which has been extensively used as a model to study inorganic (both ammonium and nitrate) assimilation at the biochemical, genetic and molecular levels (Wagner et al. 1988, 1989; Marmeisse et al. 1998; Jargeat et al. 2000, 2003; Javelle et al. 2001, 2003). More recently, a general amino acid permease has been cloned from this fungus (Wipf et al. 2002) and two programmes of random sequencing of cDNAs [generating expressed sequence tags (EST)] have led to the characterisation of several genes involved in protein and amino acid utilisation (Wipf et al. 2003; R. Lambilliotte, S. Zimmermann and H. Sentenac, personal communication).

Furthermore, the spatial and temporal structures of natural populations of *H. cylindrosporum* have been extensively worked out (Gryta et al. 1997; 2000; Guidot et al. 2002, 2004). One of the most significant observations is that this species occurs in two different habitats of the planted *Pinus pinaster* sand dune forest ecosystems along the south-western Atlantic coast of France. In these two habitats, which differ by e.g. their level of disturbance and the quantity of organic matter (and potential available nitrogen), the dynamics of *H. cylindrosporum* populations are strikingly different and illustrate the two extreme population dynamics described for other ECM fungal species. In the habitat most affected by human-induced disturbance, populations of *H. cylindrosporum* are exclusively composed of several small, annual individuals that are eliminated from the root systems within a year following fruit body formation (Gryta et al. 1997; Guidot et al. 2002, 2004). This situation contrasts with the almost exclusive occurrence of a few large, perennial individuals that survive for several years (up to 10–15 or more) in the second habitat, which is affected by wind erosion and/or sand accretion but to a far lesser extent by human activities (Gryta et al. 1997, 2000; Guidot et al. 2002). Knowledge of such differences in populations of *H. cylindrosporum* opens the possibility to study the relationship between genetic diversity and functional diversity of individual ECM fungal species in forest ecosystems.

As a prerequisite to investigating intraspecific functional diversity of *H. cylindrosporum* in its natural environment, the aim of the present paper was to reveal, in pure culture, the extent of intraspecific variation in use of different organic N sources by *H. cylindrosporum*. We initially studied haploid homokaryotic strains in order to unravel variations that could be masked in heterozygous dikaryotic mycelia. Dikaryotic strains collected in the two contrasting habitats where *H. cylindrosporum* occurs were then studied.

Materials and methods

Fungal strains

The basidiomycete agaric *H. cylindrosporum* Romagnesi is a pioneer species commonly found associated with *P. pinaster* trees in the European Atlantic sand dune forest ecosystem. In south-west France, this species occurs in two distinct habitats. The first (referred to as the 'dune habitat' in Guidot et al. 2002) is the extreme western fringe of the forest that makes the transition with the bare dune. In this habitat, populations of *H. cylindrosporum* are dominated by large perennial individuals, each producing numerous fruit bodies. The second (the 'forest habitat' in Guidot et al. 2002) is more inland, within the forest, and is characterised by a high level of human-induced disturbance. In this latter habitat, populations are characterised by annual individuals, each producing on average less than two fruit bodies. The soil is sandy (98–99% non calcareous sand), slightly acidic (pH in water about 5.7) without any obvious horizon. The percent of organic matter is low, ranging from less than 0.1–0.5% in the 'dune habitat' to about 3% in the 'forest habitat'.

Variability in the use of different nitrogen sources was studied among (1) a collection of 22 homokaryotic haploid mycelia originating from in vitro germination of basidiospores collected from 22 genetically distinct fruit bodies, (2) 6 dikaryotic mycelia resulting from in vitro crosses between 5 of the 22 homokaryons and, (3) 7 wild-type dikaryotic mycelia originating from 7 genetically distinct fruit bodies. Two of the latter were neighbouring (1 m apart) fruit bodies collected in the 'dune habitat' [strains GCD-N and GCD-S collected in 1993 in the 'Grand Crohot' (GCD) site; Gryta et al. 1997, 2000] (Fig. 5A) and the five others were very close (3–5 cm apart) fruit bodies collected in the 'forest habitat' [strains LPF97X-1 to -5, collected in 1997 in the 'Le Porge' (LPF) site; Guidot et al. 2001] (Fig. 6A). Two or three of these latter five strains were frequently found associated on 5-cm-long root segments collected in the field (unpublished results). Of the 22 fruit bodies used to isolate the haploid mycelia, 21

were collected in 1995 in 12 *P. pinaster* forest stands along a transect of 160 km on the Atlantic coast of 'Les Landes' in south-west France, two stands on the coast of Brittany in western France (TSM and TUR strains) (Guidot et al. 1999) plus one stand in The Netherlands (the two NL strains). Strains originating from the same site received the same letter code (see Fig. 2). The final strain was the homokaryon h1 isolated from in vitro fruiting of the HC1 dikaryon (Debaud and Gay, 1987). The habitat type ('dune' or 'forest') where these 22 fruit bodies had been collected has not been identified.

Media and culture conditions

All strains were maintained on YMG agar medium (Rao and Niederpruem 1969) supplemented with 30 mg l^{-1} chloramphenicol. The basal N2P2 medium (Gay 1990), either liquid or solid (10 g l^{-1} of agar), used to study nitrogen source utilisation contains (l^{-1}): $CaCl_2$, 50 mg; $MgSO_4 \cdot 7H_2O$, 150 mg; KH_2PO_4, 245.7 mg; NaH_2PO_4 $2H_2O$, 4.5 mg; Na_2HPO_4 $2H_2O$, 158 mg; glucose, 2.5 g; thiamine, 40 ng; biotin, 0.4 ng; Fe(III) citrate, 10 mg and Heller's micronutrients (Heller 1953). After autoclaving, single nitrogen sources (ammonium, nitrate, L-amino acids, urea, proteins) were added to the medium as filter-sterilised stock solutions to give a final concentration of 3.78 mM nitrogen (53 mg N l^{-1}). These nitrogen sources came from different manufacturers and their degree of purity was >99%. The pH of the medium was then adjusted to 6.0 (or 4.0 in the case of protein utilisation) by adding 25 mM (final concentration) filter-sterilised MES buffer. The medium was also supplemented with 30 mg l^{-1} chloramphenicol.

All cultures were performed in 90 mm Petri dishes filled with 25 ml medium. Solid media were overlaid with a cellophane membrane to allow recovery of the mycelium. Each plate was inoculated with a single 5 mm inoculum cut from the margin of an actively growing thallus. Three replicates were prepared for each nitrogen source. Each experiment included a control medium without added nitrogen.

All cultures were performed at 22°C in the dark. At the end of the culture period of either 6, 12 or 20 days, mycelia were recovered, dried overnight at 80°C and the biomass determined gravimetrically. Data were corrected by subtracting the mean value for growth in basal medium with no added nitrogen in order to take into account the weight of the inoculum and the contribution of its nitrogen content to the final dry weight of the mycelium. In order to compare relative growth rates between strains on a particular N source, data were also expressed as mean percentage of their biomass production relative to their biomass production on NH_4^+.

Statistical analyses

Data were analysed by one-way ANOVA, and significant differences between treatments were determined by Fisher's PLSD test. Correlations between treatments were established using Spearman's rank coefficient. A principal component analysis (PCA) was performed on the matrix of homokaryotic strain growth-N treatment interaction values. Groups of homokaryotic strains were determined visually from the two-principal-coordinates scattergram of PCA. All statistical analyses were performed using Statview software.

Results

Growth kinetics and differences in use of nitrogen sources

In a first experiment we tested the ability of the haploid laboratory strain h1 to use ammonium, nitrate, the 20 proteogenic amino acids (except tyrosine), L-citrulline, L-ornithine and urea as sole nitrogen source. Mycelia were recovered 6, 12 or 20 days after inoculation of solid medium. As expected, the different N sources tested were not used with the same efficiency (Fig. 1). Among the best nitrogen sources were the two key amino acids of N primary metabolism (Gln, Glu), N-rich amino acids (Arg, Asn), amino acids that frequently accumulate in fungal cells (Ala, Asp) as well as the inorganic forms of N (ammonium and nitrate) and urea, which are commonly found in soils. Glycine and serine were also efficiently used by strain h1. Aromatic (Phe, Trp), chemically complex (His, Pro) and sulfur-containing (Cys, Met) amino acids predominated among the N sources that were not used or which resulted in very poor yield (not significantly different at 20 days from growth on N-free medium; Fig. 1). This preliminary experiment also revealed that the growth kinetics of the mycelium differed between N sources; utilisation of some N sources (e.g. valine) required a long lag period and therefore the ranking of nitrogen sources according to mycelial biomass differed between 12 and 20 days of growth (Fig. 1); 20 days corresponded to the time where, in most cases, plates became entirely covered by mycelium; therefore comparisons performed at later times could have been biased.

Variability in use of organic nitrogen sources among haploid strains

In a second experiment, we screened 21 genetically different haploid strains for variability in use of the 24 nitrogen sources previously tested on strain h1, in addition to the two proteins bovine serum albumin (BSA) and gelatine. This was a crude visual screen on solid medium, without dry weight measurements, intended to reveal major differences between strains. As for strain h1, none of the 21 homokaryons seemed to use proline, lysine, histidine, cysteine, threonine, tryptophan, methionine, citrulline or ornithine. Noticeable growth was observed for at least some of the strains on all other N sources, including the two proteins tested. Ammonium, nitrate, aspartic acid, glutamic acid, asparagine, glutamine, arginine and urea were always the

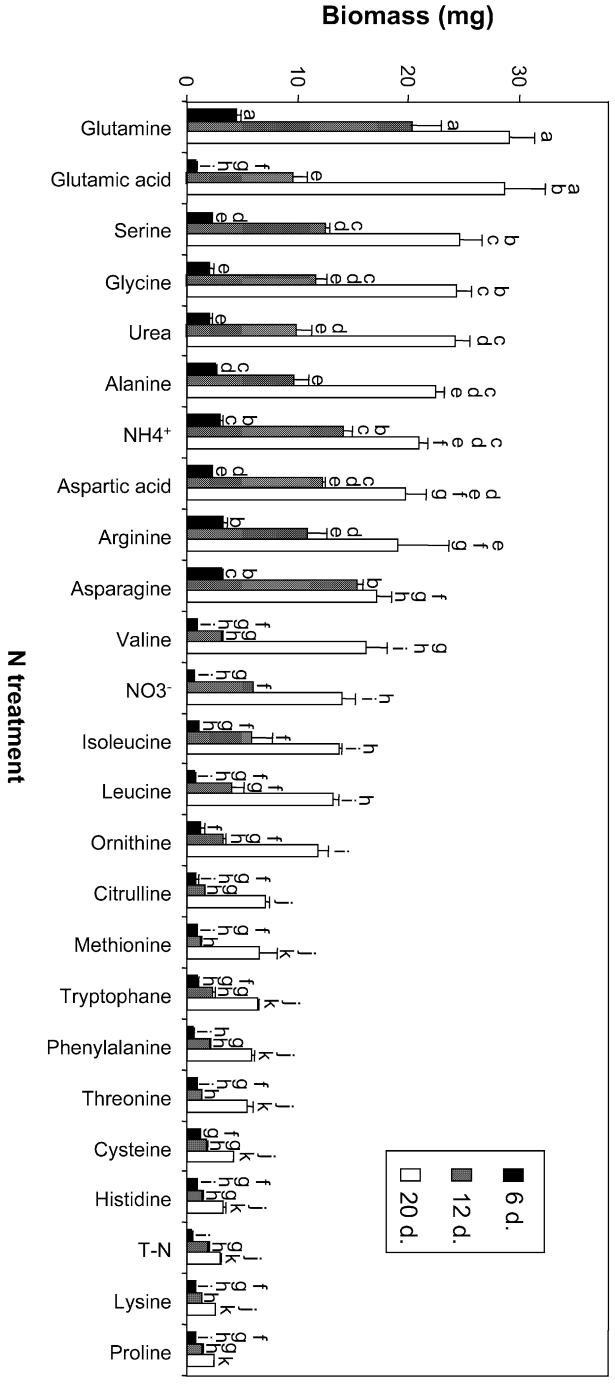

Fig. 1 Mycelial biomass production of the homokaryotic strain h1 of *Hebeloma cylindrosporum* on 24 different nitrogen sources. Mycelia were grown on solid medium covered by a cellophane membrane and collected after 6, 12 or 20 days. The final concentration of the N sources added to the medium was 3.78 mM nitrogen. Biomass (mg dry weight of mycelium per plate) is expressed as the mean of three replicates ±SE. Different letters above the columns indicate significant differences ($P<0.05$) between biomass production (as determined by Fisher's PLSD test). Only compare letters above bars of similar colour. *T-N* Control medium without added nitrogen

preferred N sources for all strains. Major differences were observed between strains in the use of alanine, glycine, phenylalanine, serine, gelatine and BSA. At least one of the strains could not use one of these latter six organic N sources at all. Growth on these six sources was then quantified in a subsequent experiment.

In this subsequent experiment, biomass production was measured for the 22 haploid strains after 20 days of growth in liquid medium to suppress the potential contribution of traces of N-compounds present in the agar. To take into account the different abilities of the strains to grow in vitro, growth of a strain on each organic source was expressed as a percentage of its value on ammonium, which is a standard N source for all strains of *H. cylindrosporum*. As illustrated in Fig. 2, huge differences in use of the six N sources tested were detected between strains, except to some extent phenylalanine, which was poorly utilised by most strains. For each given source, while some strains completely failed to use it (−100% values, e.g. strain LP5 for serine), others performed at the same level as on ammonium (zero values, e.g. strains GUR, LP1, LPD or NL2 for serine) or far better, with biomass at least 50% greater than that achieved on ammonium (e.g. strains MOB and LAC for serine). Patterns of utilisation of the proteins gelatine and BSA at pH 6.0 were quite similar. Only a few strains had different growth rates on these two proteins, e.g. TUR, which preferred gelatine to BSA at pH 6.0 (Fig. 2B).

Since protein degradation and utilisation are known sometimes to be strongly pH-dependent processes (Abuzinadah and Read 1986a), we compared the use of BSA at pH 4.0 and at pH 6.0. Acidification led either to no statistically significant effect (e.g. strains GUR and LPS could not use BSA at either pH while strain MIM grew equally well at pH 4.0 and 6.0; Fig. 2B) or to significant growth improvement in the case of eight strains (e.g. strains LAC (+11,700%), TUR (+377%) and NL2 (+357%); Fig. 2B).

We used the non-parametric Spearman rank coefficient of correlation (ρ) on the values presented in Fig. 2 to test if the patterns of utilisation of the different organic N sources were correlated. Interestingly, the patterns of utilisation of the four amino acids showing the greatest variation in utilisation (alanine, glycine, phenylalanine and serine) were all significantly correlated (smallest ρ value of 0.636 and *P* values always below 0.01; Table 1) but none of these were correlated to patterns of protein utilisation. The patterns of utilisation of the two proteins were also correlated, but this correlation was less significant than between the four amino acids tested (Table 1).

A principal component analysis (PCA) suggested a significant homokaryotic strain growth-N treatment interaction (Fig. 3A). The PCA principal coordinate F1, which represents 47% of the matrix variance, ranked strains according to their growth on the four amino acids tested (alanine, phenylalanine, serine and glycine) (Fig. 3A). On this axis, we arbitrarily distinguished four sets of strains according to their pattern of utilisation of these amino acids (Fig. 3B). The group on the left side of the axis encompasses strains LP5 and LIX, which do not use or grow

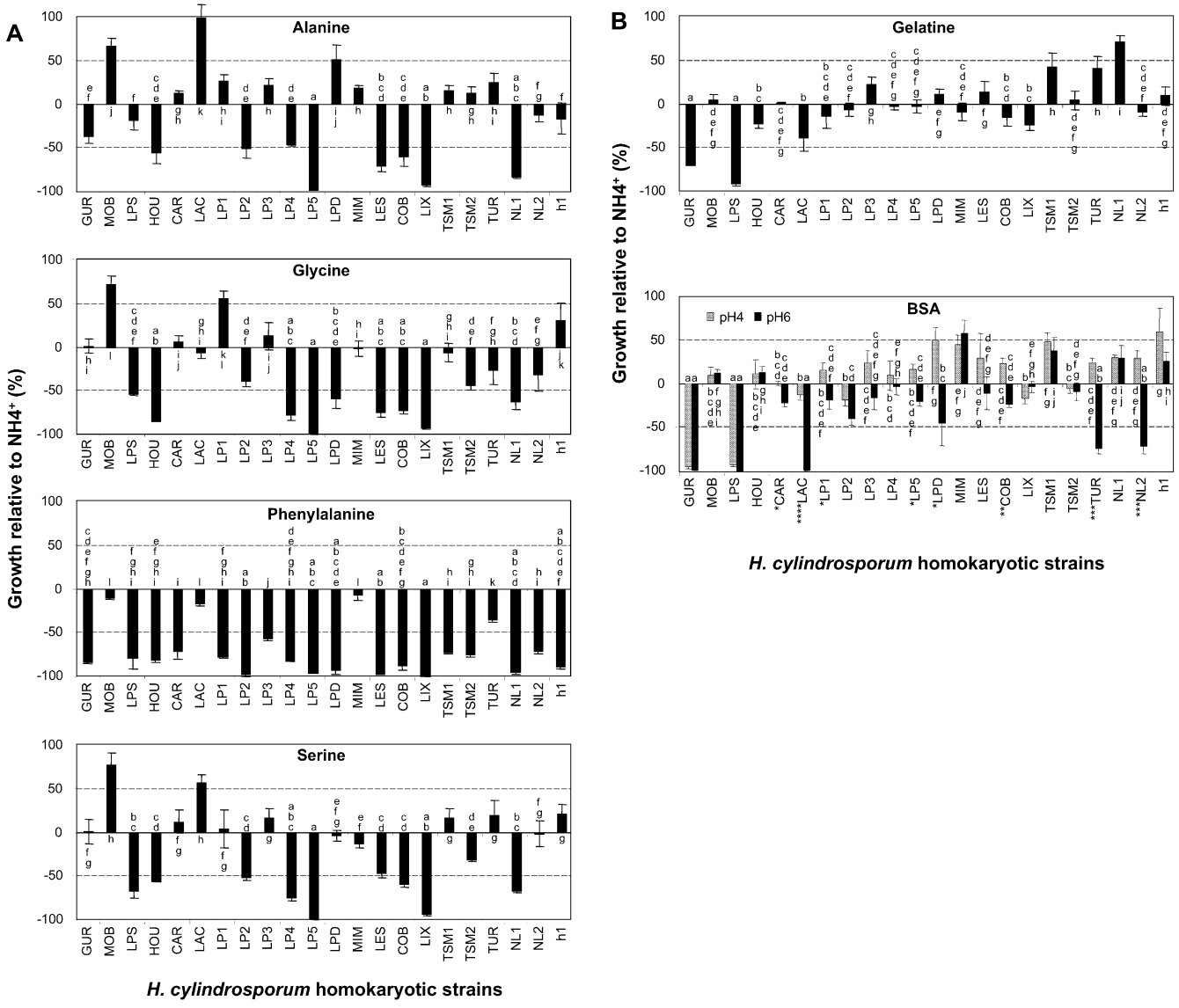

Fig. 2A, B Mycelial growth of 22 homokaryotic strains of *H. cylindrosporum* on six different organic nitrogen sources. Growth is expressed as mean percentage of the biomass production relative to the biomass production on NH_4^+ according to the ratio: $\left(\frac{\text{Biomass on N source}-\text{Biomass on medium without N}}{\text{Biomass on } NH_4^+ - \text{Biomass on medium without N}} \times 100\right) - 100$ (e.g. a zero value means that identical biomasses were measured on ammonium and on the corresponding N source, a −100 value means that the N source was not used at all, while a +100 value means that biomass was twice as much on the corresponding N source than on ammonium). Biomasses were measured after 20 days growth in liquid medium containing different organic nitrogen sources at a final concentration of 3.78 mM nitrogen. The pH of all media was adjusted to 6.0 after autoclaving using 25 mM MES buffer. Mean values of three replicates ±SE are given. Different letters above columns indicate significant differences ($P<0.05$) between growth (as determined by Fisher's PLSD test). **A** Growth on amino acids. **B** Growth on proteins. For the BSA-containing medium, cultures were performed at pH 6.0 and 4.0. Only compare letters above bars of similar colour. * Significant difference between growths at pH 6.0 and at pH 4.0 ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$), **** ($P<0.0001$)

very poorly on all four amino acids, while the group on the right side encompasses strains MOB and LAC, which are characterised by the opposite phenotype. The second coordinate F2, which represented 33% of the matrix variance, ranked strains according to their growth on proteins (Fig. 3A). On this axis we defined two sets of strains (Fig. 3B), the set on the lowest part of the graph encompassing the three strains LPS, GUR and LAC, which on average produced the lowest biomass on proteins.

Variability in use of organic nitrogen sources among controlled dikaryotic strains

Homokaryons are never observed and isolated from field samples, they either die soon after spore germination or quickly fuse with compatible mycelia to produce heterozygous dikaryons. As a result, nutritional defects identified in haploid mycelia could potentially be masked in heterozygous dikaryons. In order to determine if the inability to use some organic N sources is a recessive or dominant character, we analysed the nutritional patterns of dikaryons

Table 1 Correlation between the patterns of utilisation of six different organic nitrogen sources by 22 homokaryotic strains of *Hebeloma cylindrosporum* according to the non parametric Spearman rank coefficient of correlation (ρ). *BSA* Bovine serum albumin

N treatment	Glycine	Phenylalanine	Serine	Gelatine	BSA pH 6	BSA pH 4
Alanine	0.733***	0.779***	0.824***	0.143 ns[a]	−0.164 ns	0.146 ns
Glycine		0.636**	0.860****	0.144 ns	0.010 ns	0.098 ns
Phenylalanine			0.667**	0.024 ns	−0.020 ns	0.048 ns
Serine				0.259 ns	−0.084 ns	0.209 ns
Gelatine					0.429*	0.665**
BSA pH 6						0.455*

*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$
[a]Not significant

resulting from crosses between homokaryons with distinct abilities to use (or not) amino acids or proteins. Only homokaryons with the most extreme phenotypes were selected according to their position in the PCA scattergram (Fig. 3B).

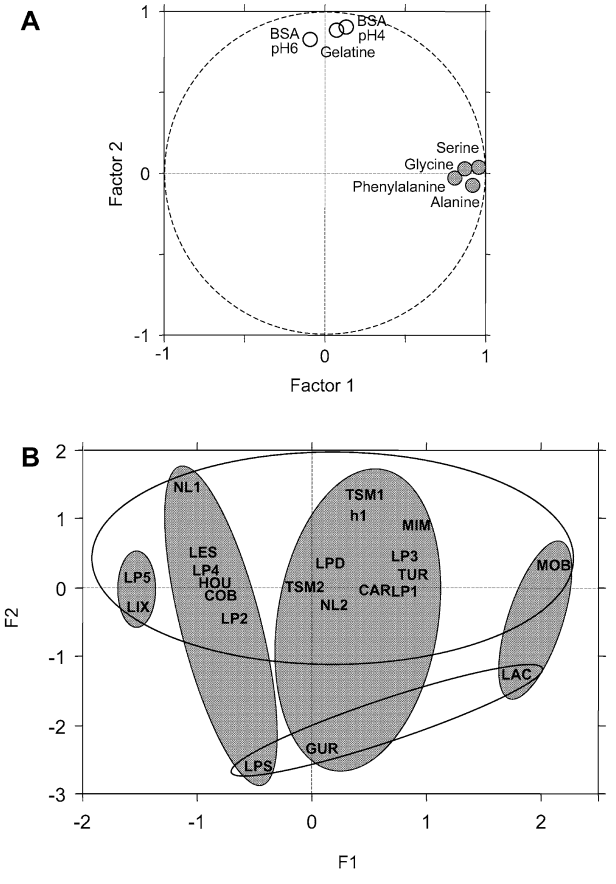

Fig. 3A, B Interaction between growth of 22 homokaryotic strains of *H. cylindrosporum* and the organic nitrogen source present in the culture medium according to a principal component analysis (PCA). **A** Distribution of the variables on the correlation circle. **B** Distribution of the strains on the two principal coordinates (F1, F2). Groups of strains were determined visually. The four grey groups ranked strains according to their growth on the four amino acids tested (alanine, phenylalanine, serine and glycine) and the two white groups ranked strains according to their growth on the two proteins tested [bovine serum albumin (BSA) and gelatine]

For amino acid utilisation, we selected the LP5 and LIX homokaryons, which failed to use the four amino acids tested. They were crossed with the laboratory strain h1, which does not grow on phenylalanine only, and with the MOB homokaryon, which uses phenylalanine to some extent and grows better on the other three amino acids than on ammonium. In this experiment, although growth of the MOB homokaryon was not as high as in the experiment presented in Fig. 2A, this strain remained a good user of the four amino acids and two proteins tested. The phenotypes of the resulting dikaryons were not clear cut. Residual (on alanine and glycine) or significant (on serine) growth on amino acids was recorded for the LP5 X LIX dikaryon (Fig. 4A). In the case of crosses between a homokaryon unable to use the four amino acids and a homokaryon able to use them, the resulting dikaryons had the ability to grow on alanine, serine and glycine but with a lower efficiency compared to the second 'parental' homokaryotic strain. These results thus suggested that the inability to use alanine, serine or glycine is a recessive character. Use of phenylalanine was obvious only in the MOB X LP5 cross (Fig. 4A). For protein utilisation, we selected the LPS strain that used neither gelatine nor BSA at pH 4 or 6.0, and we crossed it with the MOB strain that produced a similar biomass on proteins and ammonium. Growth of the resulting dikaryon on both gelatine and BSA at pH 6.0 (Fig. 4B) suggested that the inability of strain LPS to use these proteins is a recessive character.

Variability in use of organic nitrogen sources among dikaryotic field isolates

This analysis was conducted on two sets of genetically distinct strains that coexist on *P. pinaster* root systems. The six organic N sources studied were those whose utilisation was variable among homokaryotic mycelia. Biomass was measured 20 days after inoculation.

The two strains GCD-N and GCD-S collected in the dune habitat both utilised proteins efficiently and were characterised by poor or no growth on alanine, glycine and serine, but differed significantly in their ability to use phenylalanine (Fig. 5B). The GCD-S strain was characterised by a pattern of nitrogen source utilisation already observed among haploid strains (e.g. strains LP5 and LIX, Fig. 2).

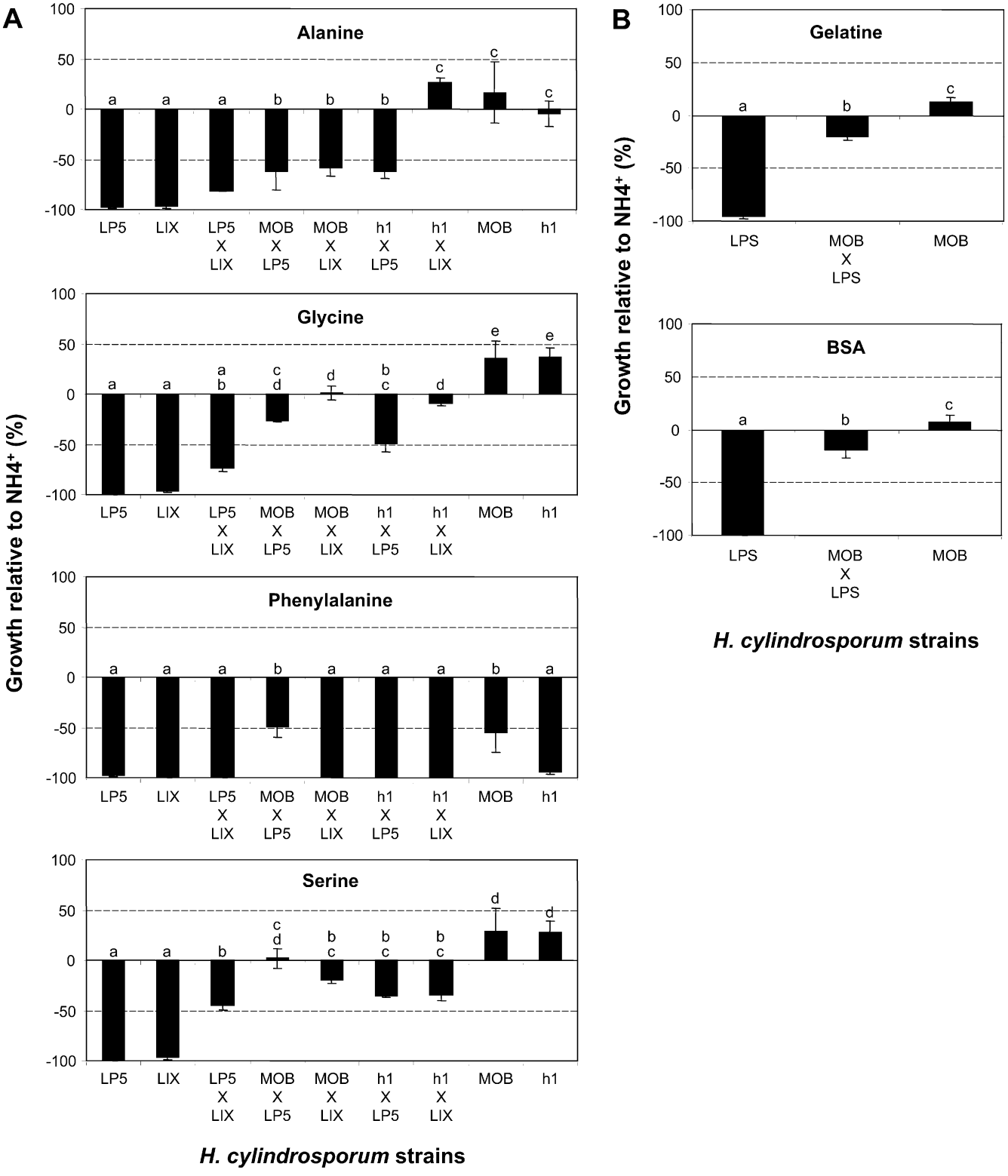

Fig. 4A, B Mycelial growth of controlled dikaryotic strains of *H. cylindrosporum* compared to their 'parental' homokaryotic strains on six different organic nitrogen sources. See Fig. 2 for legend. **A** Growth on amino acids. **B** Growth on proteins at pH 6.0

All five LPF97X dikaryons originating from five genetically different fruit bodies collected within a 15×15 cm square in the forest habitat utilised gelatine efficiently and phenylalanine very poorly but differed significantly in their ability to use alanine, glycine, serine and BSA (Fig. 6B).

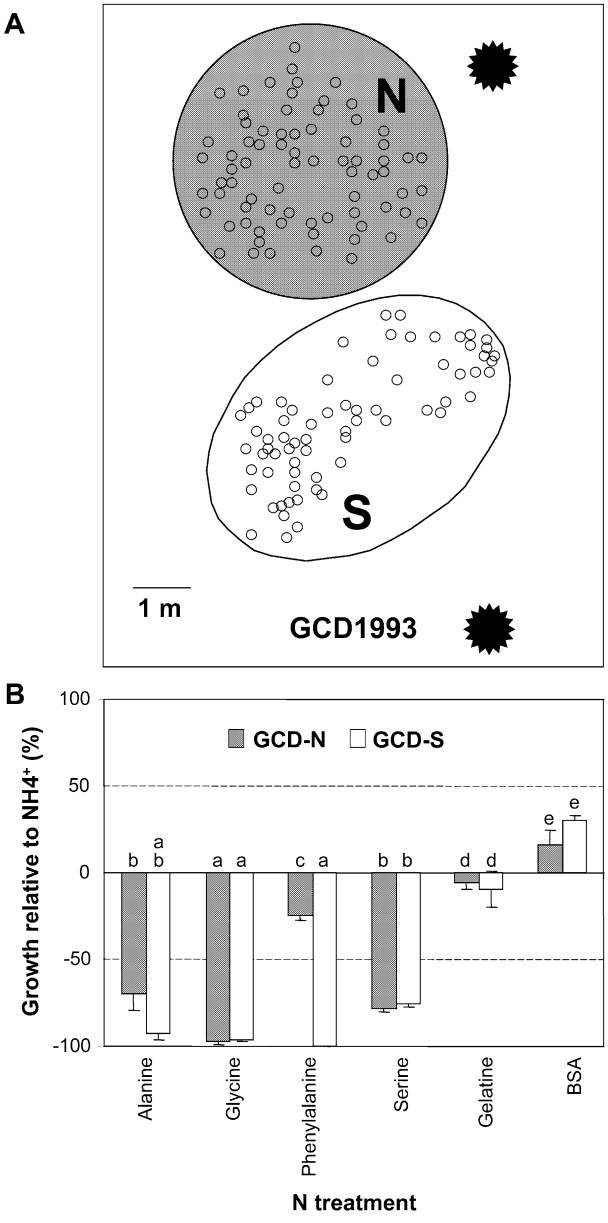

Fig. 5A, B Intraspecific variation in use of different organic nitrogen sources by two wild dikaryotic strains of *H. cylindrosporum* from the dune habitat. **A** Distribution of fruit bodies of *H. cylindrosporum* strains GCD-N and GCD-S in the GCD site of the dune habitat in 1993 (Gryta et al. 1997; 2000). *Small circles* Fruit bodies, *large stars Pinus pinaster* trees. **B** Growth of the strains GCD-N and GCD-S on six different organic nitrogen sources. See Fig. 2 for legend

Discussion

H. cylindrosporum can utilise a wide range of amino acids and other simple (e.g. urea) or complex (e.g. proteins) compounds as nitrogen source. The N utilisation profile of strain h1 (Fig. 1) is similar to that already reported for this strain by Wipf et al. (2002). Strain h1 and all other strains tested very efficiently used most of the amino acids that usually predominate in the soil solution (Abuarghub and Read 1988). Indeed, many of these amino acids frequently accumulate in living cells of micro- and macroorganisms either because they are central to N primary metabolism (e.g. glutamate, glutamine, alanine, aspartate) and/or because of their low C:N ratio (e.g. asparagine, arginine). Glutamine and glutamic acid, the two amino acids most readily used by *H. cylindrosporum*, have also been reported, together with arginine and asparagine, to be readily used by most of ECM fungal species examined so far, e.g. *Suillus bovinus* and *Amanita muscaria* (Chalot and Brun 1998). Interestingly, most 'minor' amino acids (cysteine, methionine, proline, threonine, tryptophan and tyrosine), which usually do not accumulate in soil, cannot be used as sole nitrogen source by any of the 22 homokaryotic strains tested in this study and have also been reported not to be utilised by any ECM fungal species tested so far (Abuzinadah and Read 1988; Chalot and Brun 1998; Anderson et al. 1999; Sawyer et al. 2003a,b). Some of these amino acids have complex structures and their utilisation may require specific catabolic pathways, as exemplified by the proline utilisation pathway whose genes cluster in the genome of the ascomycete mould *Aspergillus nidulans* (Arst and MacDonald 1975). Such gene clusters may have been lost during the course of fungal evolution at the transition from a saprophytic to a symbiotic life style.

We chose to study in detail the use of nitrogen compounds for which the most obvious differences were observed (i.e. nitrogen compounds that were not utilised at all by some of the 22 haploid strains tested while other strains utilised these same compounds readily). One-third of the 18 N sources efficiently assimilated by at least one strain of *H. cylindrosporum* (from glutamine to ornithine in Fig. 1 plus phenylalanine, BSA and gelatine) were thus selected. This selection comprised the four amino acids alanine, glycine, phenylalanine and serine and the two proteins tested. In this study, as in many others dealing with protein assimilation, we chose to use the proteins BSA and gelatine. It is likely that a screening made with proteins more representative of those seen in nature might slightly modify the present results. Indeed, thalli of *Amanita rubescens* have a protease activity 6-fold higher when grown on proteins extracted from beech litter, as compared to the activity measured on a BSA- or gelatine-containing medium (Botton and Chalot 1995). However, compared to BSA and gelatine, such field proteins are not easy to produce.

Both the calculation of Spearman's rank coefficients (Table 1) and PCA (Fig. 3) clearly indicated that not all physiological defects are linked to each other. This implies that the ability to use organic N compounds by an ECM fungus should not be considered as a simple and unique character. Although some of these characters seem to be, to some extent, associated (e.g. the capacity to use the four different amino acids tested here), we have no evidence to conclude that a single genetic determinant controls utilisation of these molecules. Indeed, for a character as simple in appearance as protein utilisation, differences in the response to a shift in the pH of the growth medium suggest that the inability to use a protein at pH 6 could result from causes as diverse as mutations in the structural gene of the corresponding protease, differences in pH sensing or dif-

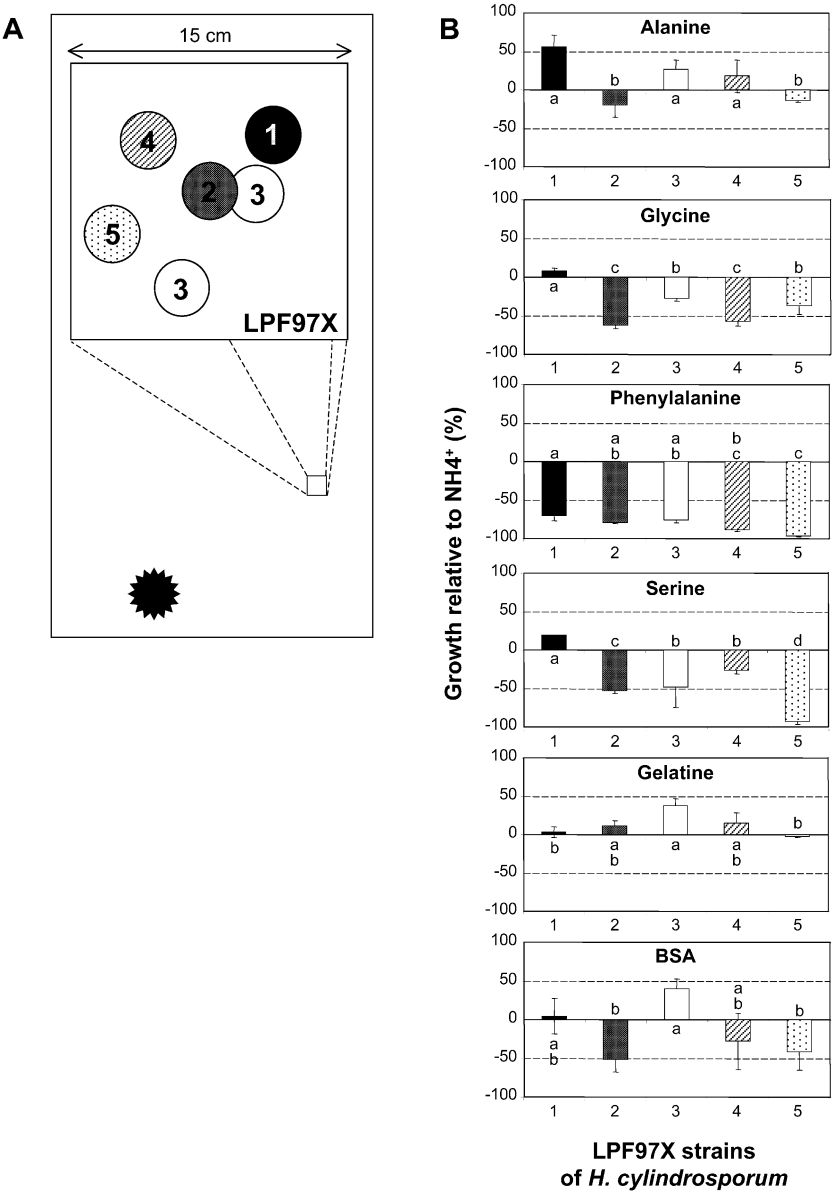

Fig. 6A, B Intraspecific variation in use of different organic nitrogen sources by five wild dikaryotic strains of *H. cylindrosporum* from the forest habitat. **A** Distribution of fruit bodies of *H. cylindrosporum* strains LPF97X-1 to -5 in the LPF site of the forest habitat in 1997. *Circles/numbers* Fruit bodies, *large star P. pinaster* tree. **B** Growth of strains LPF97X-1 to -5 on six different organic nitrogen sources. See Fig. 2 for legend

ferences in the regulatory networks that control the levels of transcription of several structural genes whose expression is necessary for the assimilation of simple molecules. The use of microarray analysis, which allows simultaneous monitoring of the transcription levels of a large set of genes participating in different cellular functions, is an experimental approach that can be used to pin down the molecular basis of polymorphisms affecting complex physiological traits (Townsend et al. 2003).

A major finding of the present study was that the inability of some haploid strains to use a particular nitrogen source was a recessive character. The frequencies at which growth defects on the six selected N sources were detected in our haploid strain collection may therefore not correspond to the frequencies at which they occur in natural populations composed of heterozygous dikaryotic individuals. The inability to use a nutrient source present in the natural environment of the species can be regarded as detrimental. It would be of interest to see whether or not this character is counter selected within natural populations of *H. cylindrosporum*. This could be tested experimentally by comparing the frequency of the phenotype within a sample of dikaryotic isolates to its frequency within a sample of homokaryotic strains isolated from the same population. Absence of counter selection of the phenotype could mean that acquisition of the corresponding nutrient source may not confer a selective advantage to the species.

Another major finding of this study was that it brought to the fore the functional diversity in use of N sources between wild dikaryotic strains isolated from local populations in each of two habitat types where *H. cylindrosporum* occurs, even between strains colonising a common short root segment in a natural ecosystem (case of the LPF97X-1 to -5 strains of the ‘forest habitat’; Fig. 6; unpublished result). In the present study, the number of wild

dikaryotic strains analysed was not sufficient to draw any definitive conclusion about a possible relationship between the N utilisation profiles of the strains and the characteristics of their habitat of origin. For example, we know that the two habitat types where *H. cylindrosporum* occurs differ in the quantity of organic matter and thus in the quality, and potentially the quantity, of nitrogen sources. Results obtained in this study represent an opportunity to establish a potential link between functional diversity within ECM fungal populations and habitat characteristics. Functional differences between strains can be determined by combining phenotypic screens as in the present study and by comparing the diversity and the expression levels of genes participating in N assimilation directly in field samples (mycelia or mycorrhizas). Several genes participating in either inorganic or organic N metabolism have been cloned in the case of *H. cylindrosporum* (Jargeat et al. 2000, 2003; Javelle et al. 2001, 2003; Wipf et al. 2002, 2003).

In conclusion, the results of the present study constitute the basis for future experiments that will investigate the functional diversity of *H. cylindrosporum* in its natural environment. This will contribute to a better appreciation of the role of ECM fungal diversity in the functioning of the symbiosis under natural conditions.

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