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SHORT NOTE

Localisation of phosphomonoesterase activity in ectomycorrhizal fungi grown on different phosphorus sources

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Abstract Phosphorus (P) is a major limiting nutrient for plants in boreal forest ecosystems where a substantial part of the total P is sequestered in organic compounds. Some ectomycorrhizal (ECM) fungi are known to produce phosphomonoesterases, enzymes that degrade organic P sources. Here, we test 16 ECM species for this enzymatic activity by growing them on media containing orthophosphate, phytic acid or apatite. A method with an overlay gel that determined both phosphomonoesterase activity and its spatial distribution was developed. The phosphomonoesterase activity was not significantly higher when growing on organic P; conversely some isolates only produced measurable enzyme activity when grown on apatite. Species-specific variations with respect to phosphomonoesterase activity as well as growth responses to different substrates were found. The production of phosphomonoesterases was found to be widespread in ECM fungi and the enzyme activity did not need induction by organic P. The enzyme activity was highest in the central parts of the mycelia, potentially reflecting breakdown and recycling of phospholipids from old hyphae or potentially higher mycelial density.

Keywords Ectomycorrhizal fungi · Acid phosphatase · Phosphomonoesterase · Organic phosphorus use

Introduction

Phosphorus (P) is an essential element for all cells, being present in both nucleic acids and phospholipids as well as in metabolites. In the boreal forest ecosystem, P is second only to nitrogen as a major limiting nutrient for plant growth (Dalal 1977). Phosphorus is present in soil in three main forms: in solution as orthophosphate (PO_4^{3-}), ionically bound in primary and secondary minerals or bound in organic compounds (Jennings 1995). The major part of soil P, sometimes as much as 90%, is sequestered in organic forms as phosphomonoesters and phosphodiesters (e.g. nucleic acids and phospholipids, Cosgrove 1967). Inositol phosphate is the dominant form of phosphomonoesters and constitutes a minor component of fresh litter, but accumulates during decomposition to make up 20–50% of the total organic P in soil (Richardson 1994; Schachtman et al. 1998).

Ectomycorrhizal (ECM) fungi are known to improve nutrient acquisition by increasing the absorptive surface area and are able to release nutrients from organic substrates by the production of extracellular or wall-bound enzymes (Lindahl et al. 2005). P acquisition by trees has been shown to be improved significantly by ECM fungi and, in laboratory experiments, colonised plants have a higher P status than their non-mycorrhizal counterparts (Bougher et al. 1990; Colpaert and Van Laere 1996).

Acid phosphatases (E.C.3.1.3.2) or phosphomonoesterases are the enzymes primarily responsible for degradation of the organic P resources in soils (Burns and Dick 2002). These enzymes cleave phosphate-ester bonds to release inorganic P from a range of substrates such as inositol phosphate, polyphosphates and phosphorylated sugars (Burns and Dick 2002).

There is some indication that phosphomonoesterase activity is not dependent upon activation by organic P. Joner et al. (2000) and Olsson et al. (2002) found that phosphomonoesterase activity in arbuscular mycorrhizal fungi was not induced by either phosphorus limitation or

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the presence of organic P substrate. Likewise, Antibus et al. (1992) found no general difference in enzyme production after growth on inorganic and organic P sources when growing eight ECM isolates on these two substrates.

Ultimately, all P is derived from mineral sources with apatite ($Ca_{10}(PO_4)_6(OH,F,Cl)_2$) as the predominant, primary mineral in the Earth's crust. P in free form is rapidly immobilised in soil by sorption onto clay minerals or hydrous oxides of Al, Fe and Mn. Many ECM fungi are able to use apatite as a P source (e.g. Wallander et al. 1997) and some (e.g. *Suillus* spp. and *Paxillus involutus*) are known to produce a wide range of low molecular mass organic acids that can act as weathering agents (Machuca et al. 2007; Landeweert et al. 2001).

The methods commonly used to measure cell wall-associated and extracellular phosphatase activity is the *p*-nitrophenyl phosphomonoesterase (*p*NPP) assay (Jennings 1995) and the ELF-97 assay (see Alvarez et al. 2004; Alvarez et al. 2006). The *p*NPP assay allows high throughput of samples and has been widely used to examine a range of organisms (Bartlett and Lewis 1973; Park et al. 1992; Senna et al. 2006). However, this method does not provide any information on where in the mycelia enzyme activity is localised. In addition, Tibbett (2002) states that the large variation between studies in growth temperature, assay temperature, filtration (were *p*NPPase can be lost) and washing steps have important bearings on the results. The ELF-97 method does, on the other hand, provide information on both the position and the intensity of the activity; however, it is very time-consuming as it is based on visual quantification by microscope. Consequently, it is not practical to examine a wider range of isolates with this method.

Lindahl and Finlay (2006) developed a new method to determine activities of chitinolytic enzymes by basidiomycete fungi that allows spatial quantification of enzyme activities on solid surfaces. By overlaying the fungus with a thin gel containing a fluorogenic substrate, they could determine the enzymatic activity on the surface of a fungal mycelium using a fluorescence spectrophotometer. The detector is located above the mycelia and as a consequence of this, activity data are predominantly recorded from the mycelial surface. Here, we use the agar overlay method of Lindahl and Finlay (2006) to assess phosphomonoesterase activities across intact ECM fungal mycelia, growing in pure culture.

Sixteen species of ECM fungi, representing a wide range of taxa, were examined for growth on orthophosphate and on organic phosphorus in the form of phytic acid. To assess intraspecific variation, four isolates of *Amanita muscaria* were included. In addition, nine isolates were also grown on mineral phosphorus in the form of apatite. The phosphomonoesterase activity was examined over the mycelia grown on the different P sources. The primary aims of this study were to determine if the agar overlay method proved to be a suitable method and to assess if phosphomonoesterase activity was dependent on organic P for induction. Another aim was to assess where in the mycelia the activity of this enzyme is localised. In addition, we hypothesise that ECM fungi differ in their preferences for different P sources which would be reflected by differential growth rates.

Materials and methods

Growing ECM fungi on different phosphorus sources

Cultures were obtained from fresh sporocarp material, except for *Cenococcum geophilum*, *Meliniomyces bicolor* and *Piloderma* cf. *fallax* which were isolated from surface sterilised mycorrhizal root tips. Stock cultures were maintained on modified Melin–Norkrans (MMN) media (Marx 1969) in darkness at 25°C.

Nineteen isolates representing 16 species of ECM fungi were tested for phosphomonoesterase activity; one ericoid fungus (*Rhizoscyphus ericae*) and one wood rot fungus (*Serpula lacrymans*) were also included. To confirm the identities of the isolates, the internal transcribed spacer ribosomal RNA gene region (ITS) was sequenced, using the method of Rosling et al. (2003), and sequence similarity was compared with known mycorrhizal taxa in the UNITE (Kõljalg et al. 2005) and GenBank (Benson et al. 2005) databases, using the BLASTN algorithm (Altschul et al. 1990). The GenBank accession numbers and ecological information of the isolates is presented in Table 1.

The fungal isolates were grown on three different media where the P was supplied in simple form as ortophosphate (PO_4^{3-}), in organic form as phytic acid (a variety of inositol phosphate) or in mineral form as apatite powder. To ensure that all fungi would start growing, all treatments had 20% of the phosphorus supplied as simple phosphate (0.15 g L^{-1} $(NH_4)_2HPO_4$). The orthophosphate media was half MMN with 15 g agar L^{-1} (Marx 1969) where additional P was supplied as 0.5 g L^{-1} KH_2PO_4 (total P concentration 4.8 mM). In the organic phosphorus media, the KH_2PO_4 was replaced by phytic acid (0.4 g L^{-1}, P8810 Sigma, total P concentration 4.8 mM) and in the apatite media with 0.1 % *w*/*v* apatite powder (Rosling et al. 2004).

The pH of the media was set to 4.5 before autoclaving and the media were automatically dispensed in Petri dishes with 18 mL in each dish. After autoclaving, the pH was measured to ensure that it remained at 4.5 before fungal inoculation. The three treatments were inoculated with 5 mm circular plugs cut from the actively growing edge of the mycelium in three replicates for each fungus and

Table 1 Nineteen isolates of ectomycorrhizal fungi, one ericoid fungus and one dry rot fungus grown on various phosphorus substrates and tested for acid phosphomonoesterase activity

Species	Collection code	GenBank accession number	P substrate[a]	Host and origin
Amanita muscaria (L.:Fr.) Hook	UP500	DQ658859	C, O	*Pinus sylvestris*, Riddarhyttan, Sweden
A. muscaria	UP501	DQ658860	C, O	Mixed forest, Uppsala, Sweden
A. muscaria	UP607	EU526864	C, O, A	Mixed forest, Uppsala, Sweden
A. muscaria	UP538	EF493267	C, O	Mixed forest, Uppsala, Sweden
A. spissa (Fr.) Kumm	UP502	DQ658858	C, O, A	Mixed forest, Gusum, Sweden
Cenococcum geophilum Fr.	UP219	EF493314	C, O	*P. sylvestris*, *P. abies*, central Lithuania
Cortinarius glaucopus (Schaeff.:Fr.) Fr.	UP21	DQ658854	C, O	*P. abies*, Flakaliden, Sweden
Laccaria bicolor (Maire) Orton	UP506	DQ658853	C, O	*P. sylvestris*, *P. abies*, central Lithuania
Lactarius chrysorrheus Fr.	UP510	DQ658873	C, O, A	*Quercus* forest, Trento, Italy
Meliniomyces bicolor Hambleton & Sigler	UP526	DQ658891	C, O, A	*P. abies*, Uppsala, Sweden
Piloderma aff. *fallax*	UP581	AY884239	C, O	Mixed forest, Nyänget, Sweden
Rhizopogon roseolus (Corda) Th. M. Fr.	UP588	EF493255	C, O	*P. sylvestris*, Kiruna, Sweden
Rhizoscyphus ericae (D.J. Read) W.Y. Zhuang & Korf	UP505	DQ658887	C, O	Highly acidic stagnohumic gley, North York Moors, UK
Russula sanguinea (Bull.) Fr	UP529	DQ658889	C, O, A	Mixed forest, Uppsala, Sweden
Serpula lacrymans (Schumach. ex Fr.) Gray	UP609	AJ245948	C, O	Isolated from timber in Berlin, Germany
Suillus bovinus (L.:Fr.) Roussel	UP591	EF493249	C, O, A	*P. sylvestris*, Kiruna, Sweden
S. luteus (L.:Fr.) Roussel	UP531	DQ658862	C, O	*P. sylvestris*, Abisko, Sweden
S. variegates (Sw.:Fr.) O. Kuntze	UP532	DQ658863	C, O, A	*P. sylvestris*, Kiruna, Sweden
Tricholoma fulvum (DC.:Fr.) Sacc.	UP88	DQ658855	C, O, A	Mixed forest, Uppsala, Sweden
T. scalpturatum (Fr.) Quél.	UP93	DQ658857	C, O, A	*Betula pendula*, Uppsala, Sweden
Xerocomus communis Bull.	UP104	EF493247	C, O	*Quercus* sp., Italy

[a] The media which the fungi were grown on: *C* control plates with orthophosphate, *O* organic P in the form of phytic acid, *A* apatite

medium. All isolates were tested on orthophosphate medium and organic P medium, and nine isolates were also tested on the apatite substrate (Table 1). The plates were incubated in the dark at 25°C for 4 weeks.

Growth measurements and enzyme assay

The mycelial size was determined as the mean of two, perpendicular radial measurements, and the area of the mycelium was calculated. Substrate conditioning by the fungi was roughly determined by estimating the pH of the media close to the mycelia in three replicates, using pH indicator paper (Macherey-Nagel, Düren, Germany) in the sensitivity range of pH 3.5–6.8.

Phosphomonoesterase activity was measured using the agar overlay method of Lindahl and Finlay (2006). The fungal mycelia were covered with 5 mL of 1% low melting point (65°C) agarose solution. The agarose contained 50 μM of the fluorogenic substrate 3,6,8 Tris (dimethylaminosulfanyl)-1-pyrenol phosphate pyridine salt (Sigma) and 0.2% Tween to ensure that the agarose would be in contact with hydrophobic mycelia (e.g. *C. glaucopus*). The mycelia with the agar overlay were then incubated in darkness for 15 min. The reaction was stopped by rinsing the agar surface with 5 mL of 1 M glycine/NaOH buffer at pH 10.6 for 2 min. The glycine buffer was decanted and the outer rim of each Petri dish was cut off level with the agar, to ensure that the detector of the spectrophotometer was close to the agar surface. Fluorescence was measured with a Perkin Elmer LS50F fluorescence spectrophotometer (Perkin Elmer, Wellesley, MA, USA) with the settings; excitation 495 nm, emission 460 nm and slit 2.5 nm. Twelve transects were recorded across each plate. In addition, a few detailed scans with a spatial resolution of 1 mm were performed for imaging purposes.

Mycelial autofluorescence was controlled by measuring the fluorescence of mycelia without fluorogenic substrate in the overlay agar. Fluorescence outside the mycelia was low and was used to correct for background fluorescence. The mean fluorescence (in arbitrary units) per measure point was calculated over each mycelium.

Calibration of the enzyme assay

The arbitrary fluorescent units provided by the spectrophotometer were converted to standardized phosphomonoesterase activity using a commercial phosphomonoesterase (Sigma, P1146). A standard curve was established by

suspending 0, 1, 1.5 or 2 units of the enzyme into 0.2 mL of water. The standards were spread over an agar plate (with three replicates for each concentration), incubated in room temperature for 5 min and then assayed using the agar overlay method in the same way as for the experimental plates. One unit is defined as the amount of enzyme that cleaves 1.0 mg of 3,6,8 Tris (dimethylaminosulfanyl)-1-pyrenol phosphate pyridine salt per minute at pH 4.5 at 25°C. Phosphatase activity per square millimetre was calculated from the total units added, divided by the area of the dish. The relationship between the arbitrary fluorescence units (mean fluorescence per measure point) and the enzyme activity was best described by the linear function $y=56.34x+15.36$ ($R^2=0.98$).

Statistical analysis

The statistical analysis was performed with Minitab version 15 (State College, PA, USA). Both growth areas and enzyme activities were log-transformed, in order to reduce heteroscedasticity. Before log transformation, 1 was added to all enzyme activity data. A general linear model was fitted to the data, using isolate and treatment and organic or orthophosphate, as explaining dummy variables. A separate analysis was performed on the nine isolates also grown on apatite, hence including orthophosphate, organic and the apatite treatments. Tukey's pair-wise comparison was performed to determine significant differences within isolate between different treatments and between isolates within treatments for the four *A. muscaria* isolates.

Results

Mycelial growth

All isolates grew on all media, although the mycelial growth varied between isolates and P sources (Fig. 1a). The average mycelial area (±standard error of the mean) was largest in the orthophosphate treatment (814±77 mm^2) followed by the organic treatment (737±79 mm^2). Overall, the mycelial size was significantly different depending on the isolate ($P<0.0001$), treatment ($P<0.0001$) and the combination ($P<0.0001$). The differences were more dependent on isolate than on treatment. For the nine isolates also grown on apatite, the average mycelial size was 581±70 mm^2. When compared to orthophosphate and organic P treatments, the mycelial areas were significantly different depending on the isolate ($P<0.0001$) and the combination of isolate and substrate ($P<0.0001$) but not depending on substrate. Significant differences in mycelial area between treatments within each isolate are indicated in Fig. 1a.

Fig. 1 Growth (**a**) and phosphomonoesterase activity (**b**) of 19 ectomycorrhizal fungal isolates, one dry rot fungus and one ericoid mycorrhizal fungus on agar plates. The *bars* indicate standard error. The phosphorus in the agar plates was supplied as orthophosphate, in mineral form as apatite or as organic P (phytic acid). All isolates were grown on orthophosphate and organic P and nine isolates were in addition tested on apatite. The phosphomonoesterase activities were measured by overlaying the agar plates with a thin gel containing fluorogenic substrate. The *small letters* indicate when differences between treatments for the same isolates significantly differs ($P<0.05$) as determined by Tukey's pair wise comparisons. For full species names, see Table 1. The spatial phosphomonoesterase activities of *Amanita muscaria* UP500 (**c**) and *Suillus bovinus* (**d**) in arbitrary units after incubation with an overlay gel containing the fluorogenic substrate 3,6,8 Tris (dimethylaminosulfanyl)-1-pyrenol phosphate pyridine salt. The fungi were grown on agar plates with phosphorus supplied as phytic acid. Note that the scales are different between **a** and **b**. No enzymatic activities were found outside the mycelia

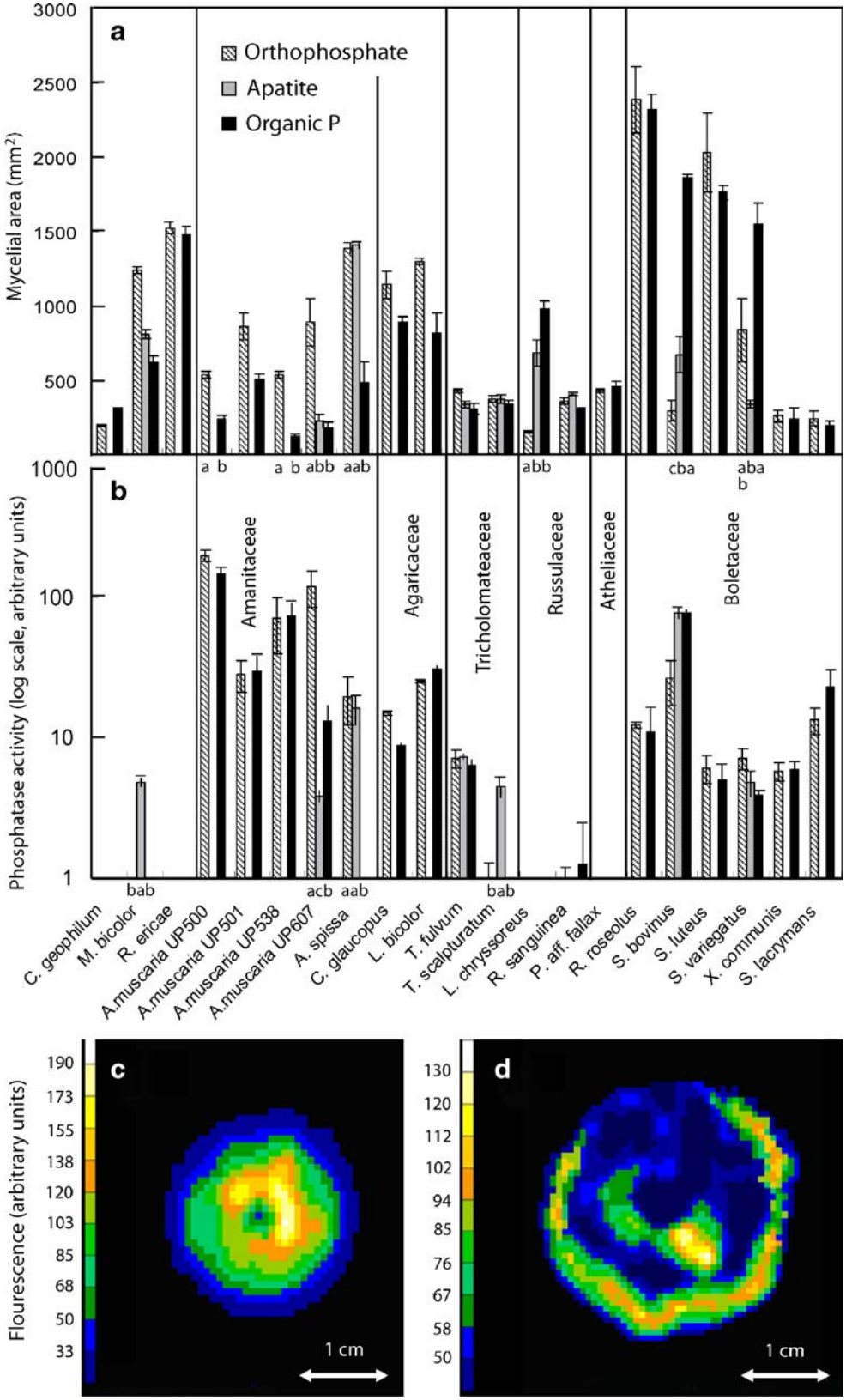
Three isolates of *A. muscaria* (UP500, UP538, UP607), *A. spissa*, *C. glaucopus*, *L. bicolor* and *M. bicolor* grew significantly better on the orthophosphate plates compared to the plates with organic P (Fig. 1a). The opposite was observed in the isolates of *Lactarius chrysorrheus*, *Suillus bovinus* and *Suillus variegatus*, which grew more on organic P (Fig. 1a).

No isolate had significantly higher growth on apatite compared to orthophosphate or organic P. Six species grew slowly and produced equally small mycelia in all treatments (*C. geophilum, Tricholoma fulvum*, *T. scalpturatum*, *R. sanguinea*, *Xerocomus communis* and *S. lacrymans*, see Fig. 1a). No significant differences were found in mycelial area between the treatments for the four *A. muscaria* isolates.

Enzyme activity

Overall, there was no significant correlation between mycelial size and enzymatic activity. The highest enzyme activities of most isolates were localised in the central parts of the mycelia (Fig. 1c). The exceptions were *S. bovinus* and *L. bicolor* that had the highest activity in the actively growing parts at the edge of the colony (Fig. 1d). No phosphomonoesterase activity could be detected in any treatment for the species *C. geophilum*, *L. chrysorrheus*, *P.* cf. *fallax* and *R. ericae* (Fig. 1b).

The enzyme activities were significantly different depending on the isolate ($P<0.0001$), treatment ($P=0.006$) and the combination of the two ($P<0.0001$). The enzyme activity was strongly dependent on isolate. Overall, the phosphatase activity was not significantly different between the orthophosphate treatment and the organic treatment. When analyzing individual isolates, *A. muscaria* (UP607) and *A. spissa* had significantly higher enzyme activity in the orthophosphate treatment compared to the organic treatment.


a
Orthophosphate
Apatite
Organic P
Mycelial area (mm²)
3000
2500
2000
1500
1000
500
a b
a b abb aab
abb
cba
aba
b
b
Phosphatase activity (log scale, arbitrary units)
1000
100
10
1
bab
acb aab
bab
Amanitaceae
Agaricaceae
Tricholomateaceae
Russulaceae
Atheliaceae
Boletaceae
C. geophilum
M. bicolor
R. ericae
A.muscaria UP500
A.muscaria UP501
A.muscaria UP538
A.muscaria UP607
A. spissa
C. glaucopus
L. bicolor
T. fulvum
T. scalpturatum
L. chrysoreus
R. sanguinea
P. aff. fallax
R. roseolus
S. bovinus
S. luteus
S. variegatus
X. communis
S. lacrymans
Flourescence (arbitrary units)
c
190
173
155
138
120
103
85
68
50
33
1 cm
d
130
120
112
102
94
85
76
67
58
50
1 cm

When analyzing the nine isolates which were, in addition, grown on apatite, the phosphatase activity was significantly different depending on the isolate ($P<0.0001$), treatment ($P<0.0001$) and the combination ($P<0.0001$). The isolates of *M. bicolor* and *T. scalpturatum* only produced measurable amounts of enzyme when grown on apatite (Fig. 1b). *A. muscaria* (isolate UP607) expressed the least enzyme activity on apatite in contrast to *A. spissa* that had comparable activities on the orthophosphate and the apatite treatment.

There was intraspecific variation between the four *A. muscaria* isolates with respect to enzyme activity within treatments. The isolates had the same level of enzyme activity on both treatments, except isolate UP607 that had significantly higher enzyme activity on orthophosphate compared to the organic treatment.

No extracellular phosphomonoesterases activity could be detected outside the mycelial front in any of the isolates in this study, indicating that the enzymes were wall-bound.

pH changes

In general, pH changes appeared to be species dependent, but independent of the substrate. The isolates of *A. muscaria*, *C. glaucopus* and *S. luteus* increased the pH to levels between 5 and 6 while *L. chrysorrheus*, *M. bicolor*, *R. ericae* and *S. lacrymans* decreased pH to below 4. The remaining isolates maintained the pH at 4–5. No statistical analysis was performed on the pH values as the method only offered a rough indication of the pH change.

Discussion

The majority of the P in acid, boreal forest soils is present as organic phosphorus, and acid phosphatase production by ectomycorrhizal fungi is therefore significant for obtaining P in these systems (Häussling and Marschner 1989). Here, we were able to demonstrate species-specific variations with respect to level of phosphomonoesterase activity as well as growth responses to different P substrates. In addition, we found that phosphomonoesterase activity did not require organic P for activation and that enzyme activity was generally highest in the central parts of the mycelia.

The agar overlay method used in this study provided simultaneous whole colony analysis of the surface acid phosphatase activity. The enzymatic assay was well suited to screen a large number of isolates and had, in addition, the advantage of showing the spatial distribution of the activity over the mycelial surface. However, the enzyme activity is predominantly measured at the surface since the detector is located above the mycelial surface. Hence, the actual activity might have been higher than detected in this study. *L. chrysorrheus* and *Russula sanguinea*, species with hydrophilic hyphae that grew into the agar, had no or little enzyme activity. However, high enzymatic activity was detected in *L. bicolor*, another hydrophilic species, indicating that the method is suitable to measure surface enzyme activities to some extent for hydrophilic isolates.

ECM fungal community composition has been observed to vary through a vertically stratified soil profile (Rosling et al. 2003). In such profiles, the forms of P vary from complex organic forms in the litter layer, to simpler organic forms and mineral forms further down in the profile (De Brouwere et al. 2003). It is plausible that different P utilisation strategies among ECM fungi are a contributing determinant for the composition of mycorrhizal communities though vertically differentiated soil profiles. The isolates in this study are mostly isolated from fruit bodies and information on where in the soil profile they proliferate is not available. However, *S. variegatus* is frequently found in the upper soil layers (Genney et al. 2006), and in our study, this species grows better on organic P and orthophosphate compared to apatite.

There was some intraspecific variation among the *A. muscaria* isolates in this study. The isolate UP607 differed in enzymatic activity compared to the other isolates although the ecology of this species does not diverge markedly from the other isolates. It is possible that the variation within these species had been higher if the isolates originated from more diverse environments (Table 1).

The majority of the species expressed the highest enzyme activity in the interior parts of the mycelia. Lindahl and Finlay (2006) found a similar pattern when growing wood-rotting fungi on spruce wood and screening for chitinolytic enzymes. They suggested that the fungi utilise endochitinases to degrade older parts of the mycelia in order to recycle the nitrogen and translocate it to newer parts of the mycelia. The same can be suggested for P use, the breakdown of phospholipids in the cell walls of old hyphae may be the cause of the high enzyme activity. Another possibility is that the interior activity simply reflects a higher concentration of cells in the more dense mycelia of the interior parts of the colony. The maximum activity detected in the front of the mycelia of *L. bicolor* and *S. bovinus* could reflect more expansive phosphorus uptake strategies in these fungi. Both species are pioneer species and may be adapted to forage for nutrients more competitively than later colonisers.

In this study, the phosphomonoesterase activities did not require organic P for activation. This is consistent with Antibus et al. (1992) who found no general differences in enzyme production after growth on inorganic and organic P sources. In fact, *A. muscaria* (UP607) and *A. spissa* actually expressed significantly higher enzyme activity in the orthophosphate treatment compared to the organic treat-

ment. In addition, the isolates of *M. bicolor* and *T. scalpturatum* only produced measurable amounts of enzyme when grown on apatite. This could reflect increased enzymatic activity under P-limiting growth conditions (Aleksieva and Micheva-Viteva 2000). It is likely that phosphomonoesterases are always active, to some extent, at the mycelial surface to activate proteins on the outer surface of the plasma membrane for physiological activity (Arnold 1987).

Only a few isolates expressed significantly different enzyme activity between different media. In some species (e.g. *S. variegatus*), the enzyme activity remained at the same level even though the growth was very different (Fig. 1b). It is possible that the phosphomonoesterase is constitutively expressed at the tested conditions for certain fungi included in the study.

When growing on ammonia, the pH of the culture media decreases as protons are released during ammonium uptake (Jennings 1995). In this study, the *A. muscaria* isolates and *C. glaucopus* instead increased the pH. However, the pH can increase when phosphate ions are transported through high affinity proton symporters (Kothe et al. 2002). In addition to P transport by proton symporters, ECM fungi may also possess sodium symporters that would render the pH of the media unchanged (Kothe et al. 2002). Substrate acidification may also be an active mechanism to increase mineral dissolution under P-limiting growth conditions (Rosling et al. 2007). Among the isolates that did not produce any enzymatic activity, *L. chrysorrheus* and *R. ericae* lowered pH to below 4. This may reflect a strategy to obtain P from weathering rather than phosphomonoesterase activity.

The pH optima for most of phosphomonoesterases of the species included in the study is unknown. It is plausible that the pH value of the media was unsuitable for induction of phosphomonoesterase activity in some cases. Previous studies using *C. geophilum* have demonstrated maximum phosphatase activity with no available P and at a pH of approximately 4 as well as phosphorus concentrations around 3.5 mM and a pH around 5 (Alvarez et al. 2004). The individual pH optima of the phosphomonoesterase activity must be known before the maximal enzymatic capacity for different species can be assessed.

In conclusion, the agar overlay method proved suitable for screening fungal cultures for phosphomonoesterase activities. The production of phosphomonoesterases is widespread in ECM fungi, and to assess the maximal enzymatic activity, the individual pH optima has to be considered. Phosphomonoesterase activity does not require induction by organic P and most species in this study expressed higher enzyme activities in the central parts of the mycelium. This may reflect breakdown and recycling of phospholipids from old hyphae or simply higher mycelial density.

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