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ORIGINAL PAPER

Access to organic and insoluble sources of phosphorus varies among soil Chytridiomycota

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Abstract The sources of minerals accessed by fungi in the Chytridiomycota (chytrid) in soil are largely unknown. The ability of ten species of soil chytrids to use various sources of phosphorus was examined in vitro. While all grew on orthophosphate, fifty per cent of isolates grew on phytic acid, and one isolate grew on DNA as the sole source of phosphorus. All isolates solubilised and utilised $CaHPO_4$. Most isolates utilised hydroxyapatite when NH_4^+ was the nitrogen source. When ammonium was omitted, 50% of isolates solubilised hydroxyapatite. Many soil chytrids may utilise phosphomonoesters as the sole source of phosphorus, and access to DNA appears limited. We suggest that the capacity to use different sources of phosphorus may influence the diversity of chytrids found in Australian soils.

Keywords Chytrids · Chytridiomycota · Phosphorus · Nutrition · Ecophysiology · Solubilisation

Introduction

Soil inhabiting Chytridiomycota (chytrids) are globally distributed. Chytrids are known to grow saprotrophically in bulk soil and leaf litter, and within plant roots (Harder and Uebelmesser 1959; Sparrow 1960; Karling 1977, 1988; Letcher et al. 2004a, b). Despite their ubiquity, the diversity, distribution and nutrition of soil chytrids are poorly understood (Letcher et al. 2004a, b). The nutrition of chytrids has been examined in only a small number of studies on nitrogen (Haskins and Weston 1950; Machlis 1953a, b; Goldstein 1960; Hasija and Miller 1971; Hassan and Catapene 2000) and carbon (Murray and Lovett 1966; Barr 1970, 1984; Hassan and Catapane 2000) substrate utilisation. Furthermore, studies of the utilisation of phosphorus sources by soil chytrids are limited to orthophosphate. This is surprising, as available soil phosphorus may be a determinant of chytrid community composition and diversity (Letcher et al. 2004b).

Phosphorus is not readily available in Australian soils. This low availability often limits plant productivity and is a determinant of plant community structure (Beadle 1962a, b, 1966; Gunary and Sutton 1967; King and Buckney 2002). The limited phosphorus stores in Australian soils are frequently found as insoluble inorganic compounds, recalcitrant organic complexes or a mixture of both (Halstead and McKercher 1975; Cosgrove 1977; Sanyal and DeDatta 1991). In dry sclerophyll forest soils, for example, mean phosphorus was measured at *ca* 90 mg kg^{-1} of soil, of which a significant fraction was correlated with the organic fraction of the soil (King and Buckney 2002). The capacity to access organic and insoluble phosphorus may therefore, be critically important for growth and survival of chytrids in Australian soils.

Many filamentous fungi produce phosphomono and phosphodiesterase enzymes that mobilise phosphorus from organic substrates such as phytate and nucleic acids (Shieh and Ware 1968; Hankin and Anagnostakis 1975; Burt and Cazin 1976; Jennings 1990; Leake and

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Miles 1996). Access to phosphorus present as phosphomono or diesters is not recorded for soil chytrids. It might be predicted that chytrids, like filamentous fungi, will access phosphorus in organic forms because orthophosphate is uncommon in Australian soils.

Recalcitrant phosphorus is also found in a range of inorganic forms whose solubility varies with pH. At lower soil pH, like those found in soils of the Sydney region, phosphorus is frequently found associated with Al, Fe(III) or Mn, or bound to Al or Fe(III) oxide or adsorbed to various aluminosilicate complexes (McLaughlin et al. 1988). At higher soil pH, such as that found in the vertisolic soils of northern NSW, phosphorus typically is found complexed with calcium. Fungal communities release plant-available phosphorus from insoluble sources, indicating the importance of fungi in phosphorus cycling in both agricultural and natural soils (Dighton and Boddy 1989). Some fungi, such as from the family Trichocomaceae, variously solubilise a range of insoluble and poorly soluble phosphorus compounds including; aluminium phosphate (Barthakur 1978; Banik and Dey 1982) calcium monohydrogen phosphate (Rose 1957; Sperber 1958; Molla et al. 1984; Singh et al. 1984; Cunningham and Kuiack 1992; Ilmer and Schinner 1992; Nahas 1996; Whitelaw et al. 1999), β-calcium orthophoshate (Gaur et al. 1973; Barthakur 1978; Banik and Dey 1982, 1983; Whitelaw et al. 1999), fluoroapatite (Agnihotri 1970; Cerezine et al. 1988; Nahas et al. 1990; Vassilev et al. 1995), iron phosphates (Rose 1957; Barthakur 1978; Banik and Dey 1982 Narsian et al. 1994) and hydroxyapatite (Agnihotri 1970; Arora and Gaur 1979). While the phosphorus utilising abilities of some filamentous fungi are relatively well characterised, nothing is known about the abilities of soil chytrids to utilise these recalcitrant stores of phosphorus for growth. Thus, we might predict soil chytrids from these different regions would have the capacity to access the forms of recalcitrant P commonly found in the soil of origin.

This study is part of a program that aims to clarify the determinants of diversity and abundance of chytrids in soil. As phosphorus is crucial for various cell processes and structures, access to phosphate may influence growth and survival of chytrids. The aim of the current study was to test whether soil chytrids can use various organic and inorganic phosphorus sources, in order to determine whether access to phosphorus may influence the ecology of soil chytrids in Australia.

Materials and methods

The ten fungi used in this study were *Catenaria* sp. Dec CC4 10Z, *Chytridiomyces hyalinus* AUS 14, *Cladochytrium* sp. AUS 11, *Powellomyces* sp. AUS 17, two genotypes of *Rhizophlyctis rosea* (AUS 13 and BV80H), *Rhizophlyctis* sp. AUS 16, two unidentified *Rhizophydium* species (AUS 6 and AUS 12) and *Spizellomyces* sp. Mar Ad 2-0. For details of collection (except for *Cladochytrium* sp. AUS 11 and *R. rosea* BV80H) see (Gleason et al. 2004). Isolate AUS 11 was cultured from alpine soil in Tasmania, Australia using onion skin as a bait (P. M. Letcher, unpublished data) while BV80H was isolated using an 80°C heat treatment to suppress other fungal growth in soil from Borry's Vineyard in Orange, NSW, Australia (F. H. Gleason, unpublished data). Dec CC4 10Z and Mar Ad 2-0 were isolated from cultivated alkaline soils of northern NSW, Australia (Commandeur et al. 2005). AUS 6, 11, 12, 14, 16 and 17 are from acid soils. The fungi were selected to represent a range of taxonomic groups and soils of origin.

Prior to the experiment all cultures were maintained on peptone, yeast extract, glucose (PYG) medium that contained L^{-1}: peptone 1.25 g, yeast extract 1.25 g, glucose 3 g (Fuller and Jaworski 1987). For 7 days prior to the experiment all isolates were grown on a low-phosphorus chytrid synthetic medium (CSM) [(L^{-1}): $(NH_4)_2SO_4$ 200 mg, K_2HPO_4 50 mg, $Mg(NO_3)_2.2H_2O$ 200 mg, $CaCl_2.2H_2O$ 50 mg, FeEDTA 500 µg, $MnCl_2.4H_20$ 10 µM, $ZnCl_2$ 10 µM, H_3BO_4 33 µM, $CuSO_4.5H_2O$ 1 µM, $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.2 µM, thiamine 133 µg, alanine 0.9 g, methionine 0.1 g and glucose 4 g l^{-1}] to deplete internal phosphorus stores. In order to obtain an even lawn of fungal material, a single plug of inoculum was placed on the centre of Petri dishes containing solidified low-P CSM and 1 ml of sterile deionised water added. Cultures were then held at 26°C in the dark for 7 days prior to the commencement of the experiment. For all experimental treatments in which biomass was measured two 5.0 mm diameter plugs of mature fungus (with the excess agar excised) were added to five replicate 9 cm diameter Petri dishes each containing 20 ml of liquid CSM medium supplemented with either KH_2PO_4, sodium inositol hexaphosphate (phytic acid), herring sperm DNA (all Sigma) to a starting concentration of 77.5 mg phosphorus L^{-1}. As stationary phase is reached after approximately 10 days in this volume (F.H. Gleason, unpublished data), all fungal material was harvested after 7 days in the dark at 25°C, dried overnight at 80°C, and weighed. For an estimation of the quantity of phosphorus stored within fungal inoculum a phosphorus-free control was included. In the DNA and phytic acid treatments carbon was maintained 2 g L^{-1} by reducing the amount of glucose. In addition, as DNA also contains nitrogen, DNA utilisation was investigated in two treatments. The first included DNA as a sole source of both

nitrogen and phosphorus. In this treatment $MgSO_4$ was added to the same ionic concentrations as present in other treatments. As it is presently unclear whether soil chytrids were able to utilise DNA as a sole source of nitrogen, a second treatment was also included with NH_4^+, NO_3^-, alanine and methionine as sources of nitrogen and DNA as a sole source of phosphorus. Phytic acid was filter sterilised and added to the media post-autoclaving (at 55°C) while DNA was added to the heated media (80°C and stirring) after 48 h immersion in 5 ml 70% ethanol (Leake and Miles 1996).

The use of insoluble phosphorus substrates calcium monohydrogen phosphate and hydroxyapatite treatments was analysed using three replicate double layered agar Petri dishes (Van Leerdam et al. 2001) with the top layer containing 1 g of the finely powdered insoluble phosphorus source and the bottom layer containing phosphorus free CSM. To ensure removal of freely soluble phosphorus, both insoluble sources of phosphorus were washed twice, in sequence, with 1 l of dH_2O, autoclaved and recovered by filtration. In order to ascertain the effect of ammonium uptake on solubility of these phosphorus sources, both calcium monohydrogen phosphate and hydroxyapatite treatments were analysed on media with and without ammonium. Plates were inoculated with 200 µl of a zoospore solution obtained by flooding 10-day-old cultures growing on low phosphorus CSM plates for 1 h with 10 ml dH_20. To ensure the viability of inoculum, 200 µl of the zoospore solution was transferred to three replicate low phosphorus CSM plates as a positive control. Growth on sparingly soluble phosphorus treatments was considered to occur where precipitate cleared and two successive sporangia-zoospore generations were observed microscopically. The degree of clearing was compared to control plates that lacked fungi.

Data analysis

Biomass data were analysed using ANOVA with the post hoc Fishers PLSD procedure or t-tests using SPSS for Windows, Version 14 (SPSS Inc., Chicago, IL, USA).

Results

All chytrids tested produced measurable biomass on orthophosphate as the sole source of phosphorus. Biomass production was broadly similar in all isolates and ranged from 4.5 to 11.6 mg (Table 1). Half of the isolates utilised phytic acid. Only *Cladochytrium* sp. AUS 11, *Rhizophidium* species AUS 6 and AUS 12, *Powellomyces* sp. AUS 17 and *R. rosea* AUS 13 produced measurable biomass (Table 1). For both *Rhizophidium* species, biomass produced on phytic acid did not differ significantly to that produced on orthophosphate (Table 1). In contrast, *Cladochytrium* sp. AUS 11 produced significantly greater biomass on phytic acid than on orthophosphate ($P < 0.05$). Conversely, *R. rosea* AUS 13 produced significantly less biomass on phytic acid compared with that produced on orthophosphate. With the exception of *Cladochytrium* sp. AUS 11, no soil chytrids produced measurable biomass on DNA in either of the DNA treatments (Table 1). *Cladochytrium* sp. AUS 11 produced significantly more biomass on DNA (as a sole source of phosphorus) than orthophosphate, and biomass on DNA and phytic acid were similar (Table 1).

Table 1 Bimass (mg dry weight ± standard error) produced by various soil chytrids on orthophosphate ($PO4^+$), phytic acid and DNA with and without added nitrogen, as sole sources of phosphorus after 7 days in axenic liquied culture in CSM medium

Order	Isolate	Treatment			
		$PO4^+$	Phytic acid	DNA as N and P (without)	DNA as P only (with)
Blastocladiales	*Catenophlyctis* sp. Dec CC4-10Z	11.58(0.8)	–	–	–
Chytridiales	*Chytridiomyces hyalinus* AUS 14	7.74 (0.4)	–	–	–
	Cladochytrium sp. AUS 11	4.50 (0.5)[a]	6.36 (0.4)[b]	5.50 (0.3)[a, b]	6.4 (0.5)[b]
	Rhizophidium sp. AUS 6	5.78 (0.3)[a]	5.46 (0.2)[a]	–	–
	Rhizophidium sp. AUS 12	6.96 (0.5)[a]	6.36 (0.4)[a]	–	–
Spizellomycetales	*Powellomyces* sp. AUS 17	5.66 (1.0)[a]	6.7 (0.5)[a]	–	–
	Rhizophlyctis rosea BV80H	6.5 (0.3)	–	–	–
	Rhizophlyctis rosea A13	6.52 (0.3)[a]	3.48 (0.4)[b]	–	–
	Rhizophlyctis rosea A16	6.18 (0.4)	–	–	–
	Spizellomyces sp. Mar Ad2-0	9.49 (0.5)	–	–	–

Values are mean biomass, from which mean growth for each isolate in the phosphorus was subtracted. Different letters in rows indicate significant differences

"–" = Growth not significantly different to growth in the absence of phosphorus

All chytrids solubilised calcium monohydrogen phosphate. Obvious zones of clearing, particularly under the growing thalii, were observed in all isolates examined. The source of nitrogen did not affect the ability of chytrids to utilise calcium monohydrogen phosphate as a sole source of phosphorus.

All isolates, except *Rhizophidium* sp. AUS 12, solubilised hydroxyapatite when ammonium was the nitrogen source supplied (Table 2). Microscopic examination of *Rhizophidium* sp. AUS 12 suggested that although zoospores were released they did not encyst. In contrast, when nitrogen was supplied as nitrate, only isolates *Catenophlyctis* sp. Dec CC4-10Z, *Cladochytrium* sp. AUS 11, *R. rosea* BV80H, *R. rosea* AUS 13 and *Spizellomyces* sp. Mar Ad2-0 grew and solubilised the phosphorus source (Table 2). Amongst the isolates unable to grow on hydroxyapatite with nitrate two phenomena were observed microscopically. Both *Rhizophidium* isolates and *Chytridiomyces* sp. AUS 14 produced numerous zoospores, which did not encyst. In contrast, zoospores produced by *Powellomyces* sp. AUS 17 and *Rhizophlyctis* sp. AUS 16 encysted to form abnormally shaped proto-sporangia with stunted, abnormal rhizoids. The malformed sporangia did not produce zoospores and the fungi failed to grow beyond initially encysting from the inoculum. All positive control plates inoculated with 200 µl of zoospore suspension grew normally, thus, verifying the viability of the zoospores as a source of inoculum. No change in the insoluble particulate phosphorus sources was observed in the uninoculated control.

Discussion

Like most fungi, all soil chytrids examined in the present study grew on orthophosphate as a sole source of phosphorus. Soluble phosphorus, however, is not readily available in most unfertilised soils. In Australian dry sclerophyll forests, for example, much of the phosphorus is associated with the organic fraction of the soil (King and Buckney 2002). Given the relatively brief residence of other forms of organic phosphorus, such as phospholipids, it is reasonable to assume that much of the organic phosphorus is present as a range of inositol phosphate esters, collectively termed phytate.

In the present study only 50% of soil chytrids examined used phytic acid as the sole source of phosphorus. The ability to grow on phytic acid requires the production of phosphomonoesterases (Sheieh and Ware 1968; Jennings 1990). From the results presented in the current study it may be inferred that phosphomonoesterase activities were expressed by *Cladochytrium* sp. AUS 11, *Rhizophidium* species AUS 6 and AUS 12, *Powellomyces* sp. AUS 17 and *R. rosea* AUS 13. Interestingly, only one of the three *Rhizophlyctis* isolates (AUS 13) grew on phytic acid. If *R. rosea* BV80H is indeed conspecific with *R. rosea* AUS 13 then intraspecific variation with respect to phosphorus nutrition, a phenomena that has previous been observed for numerous filamentous fungi (Cairney 1999), also occurs in chytrids. Interestingly, neither fungus from cultivated alkaline soils accessed phytic acid, a phosphorus source which is presumably less common in agricultural systems.

The persistence of DNA in the environment is dependant on the rate of mineralisation in the soil. In contrast to its short residency time in some agricultural soils (Dalal 1977), DNA may represent a significant fraction of organic phosphorus in slowly mineralising soils (Dyer amd Wrenshall 1941; Zech et al. 1987). Use of DNA as a sole phosphorus and nitrogen source requires both phosphodi and phosphomonoesterase

Table 2 The abilities of various soil chytrids to solubilise calcium monohydrogen phosphate and hydroxyapatite (HA) in standard CSM medium with either the standard mixed nitrogen sources ($NH_4^+ NO_3$, alanine and methionine) or CSM in which $NH_4^+$was omitted

Isolate	Treatment			
	$CaHPO_4$ and mixed N	$CaHPO_4$ and no NH_4^+	HA and mixed N	HA and no NH_4^+
Catenophlyctis sp. Dec CC4-10Z	+	+	+	+
Chytridiomyces hyalinus AUS 14	+	+	+	-
Cladochytrium sp. AUS 11	+	+	+	+
Rhizophidium sp. AUS 6	+	+	+	-
Rhizophidium sp. AUS 12	+	+	-	-
Powellomyces sp. AUS 17	+	+	+	-
Rhizophlyctis rosea BV80H	+	+	+	+
Rhizophlyctis rosea A13	+	+	+	+
Rhizophlyctis sp A16	+	+	+	-
Spizellomyces sp. Mar Ad2-0	+	+	+	+

+ Clearing and normal growth, - No growth and/or incomplete lifecycle

activities along with the action of a nucleotidase. Only *Cladochytrium* sp. AUS 11 grew on DNA as a sole source of nitrogen and phosphorus, implying the release of these enzymes and their activities by this isolate. All other fungi failed to use DNA as either a sole source of nitrogen and phosphorus, or as a sole source of phosphorus in the presence of mixed nitrogen sources. The present study has demonstrated that some chytrids produce phosphomonoesterase activity; the lack of growth on DNA, in the presence of other nitrogen sources, may therefore, reflect the absence of a phosphodiesterase enzyme, or the lack of extracellular activity of this enzyme. Failure to utilise DNA by most chytrids is surprising, as DNA is readily used as a source of phosphorus by various yeasts (Burt and Cazin 1976), some ascomycetes (Hankin and Anagnostakis 1975; Leake and Miles 1996) and some basidiomyceteous taxa (Donly and Day 1984; Sawyer et al. 2003). If the conclusions from the present study can be extended to soil chytrids more broadly, it seems likely that most soil chytrids are unable to access nitrogen and phosphorus from nucleic acids in soil. *Cladochytrium* sp. AUS 11 was the only filamentous chytrid examined in the present study. It may be that filamentous chytrids have differing life strategies to monocentric chytrids. *Cladochytrium* sp. AUS 11 is also the only member of the "Nowakowskiella" clade examined in the present study (Barr 1984; James et al. 2000). Members of this clade have previously been found on decaying plant matter and may be involved in the early stages of plant decomposition (Sparrow 1960). In these environments access to organic forms of phosphorus, found inside plant cells, may be of significant advantage.

All soil chytrids grew on calcium monohydrogen phosphate regardless of whether nitrogen was supplied as ammonium or nitrate. This is perhaps unsurprising as calcium monohydrogen phosphate solubilises sufficiently in neutral media (0.14 g, 100 ml^{-1} soluble at 25°C) to allow normal growth of many fungi (Stumm and Morgan 1995; Whitelaw 2000). A number of studies (reviewed in Whitelaw 2000) have used solubilisation of this substrate to broadly infer solubilisation of inorganic phosphates by fungi. The relatively high solubility of this substrate makes further inferences difficult. However, soil chytrids grew on this phosphorus source in axenic culture and presumably will utilise it in soil.

All chytrids examined, except *Rhizophydium* AUS 12, solubilised hydroxyapatite when ammonium was provided as the nitrogen source. When ammonium was absent, however, only five of ten isolates solubilised hydroxyapatite. Solubilisation was probably partially facilitated by acidification of the growth media via fungal uptake of ammonium. At lower pH values, H^+ interacts with the hydroxyl ion and phosphate groups in hydroxyapatite leading to solubilisation of the substrate (Van Leerdam et al. 2001). Taxonomic position was not correlated with use of hydroxyapatite, as at least one isolate from each of the three orders examined solubilised hydroxyapatite. In other fungi, solubilisation of hydroxyapatite, in the absence of acidification via ammonia uptake, has been demonstrated to be associated with secretion of organic acid. Some members of the chytrid genera *Blastocladia*, *Chytridium, Cladochytridium* and *Cylindrochytrium* (as *Macrochytrium* Crasemann 1954; see Sparrow 1960) have been shown to produce organic acids in axenic culture (Crasemann 1954; Willoughby 1962; Gleason and Gordon 1989). These acids facilitate solubilisation not only via acidification, but also via chelation of precipitating ions such as Ca^{2+} (Jennings 1995). *Catenophlyctis* sp. Dec CC4-10Z, *Cladochytrium* sp. AUS 11, *Rhizophylctis rosea* BV80H, *R. rosea* AUS 13 and *Spizellomyces* sp. Mar Ad 2-0 may thus, solubilise hydroxyapatite following release of organic acids.

In the absence of adequate orthophosphate, soil chytrids appear to be able to access alternative sources of phosphorus in soil. At least some soil chytrids grew on hydroxyapatite and phytic acid, and one fungus grew on DNA. The two fungi from alkaline cultivated soils appear to be able to access the forms of phosphorus probably found in their habitat. Interestingly, *Rhizophydium* spp appear able to grow on phytic acid, possibly placing them at a competitive advantage over other soil chytrids in these acid environments. Overall, however, access to complex phosphate appeared to be reduced among the soil chytrids compared to other soil fungi, and limited sources of availability of phosphorus may influence the soil chytrids found in any habitat.

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