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Diversity in phosphorus mobilisation and uptake in ectomycorrhizal fungi

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Abstract

• **Introduction** Phosphorus (P) is often the first or second element limiting aboveground net primary productivity of forests. Besides low available inorganic orthophosphate (Pi) concentrations, soil may contain high total P contents, as insoluble mineral P or as organic P. Most plants form mycorrhizal associations that improve their P nutrition. Three main hypotheses have been proposed to explain this positive effect through an increase of (1) P mobilisation from mineral P, (2) P mobilisation from organic P and (3) soil exploration and P uptake. However, the positive effect of mycorrhizal symbiosis may be variable with the fungal species forming the association. This could be due to the different abilities of mycorrhizal fungi to mobilise P and/or to take up Pi from the soil.

• **Objectives** The aim of this review was to examine our current knowledge about the capacity of ectomycorrhizal fungi to release organic compounds as low-molecular-weight organic anions and phosphatases thought to have a role for mineral and organic P mobilisation, respectively. The diversity of Pi transporters among mycorrhizal species is also examined.

• **Results** The main conclusion is that the study of the functional diversity of ectomycorrhizal fungi in situ is still a

challenging question and could be addressed by combining different tools now available to make large-scale studies.

Keywords Low-molecular-weight organic acids · Oxalate · Phosphatase · Phytase · Fungal P transporters · Phosphate · Gene expression · Organic and mineral phosphorus

1 Introduction

Phosphorus (P) is an essential element for plant nutrition and can only be taken up as inorganic orthophosphate (Pi), either as H_2PO_4^- or HPO_4^{2-} , depending on the pH of soil solution. It has critical functions in many processes, such as energy metabolism, synthesis of nucleic acids and membranes, as well as in photosynthesis (Raghothama 1999; Vance et al. 2003). In soil, free concentrations of Pi are thought to range from 1 to 10 μM (Bieleski 1973; Hinsinger 2001; Vance et al. 2003), and this low availability limits the productivity of plants in many terrestrial ecosystems. It is often the first or second element limiting aboveground net primary productivity of forests. Besides these low concentrations in free Pi, soil may contain high levels of phosphorus that are not directly available to plant roots or microorganisms as P is combined either to cations to form mineral P or to carbon-containing compounds to form organic P. Given the Pi supply constraints in many soils, it is not surprising that plants have evolved strategies to acquire and/or efficiently use P. Plant adaptations that enhance the acquisition of inorganic Pi have been reviewed many times (e.g. Bucher 2007; Lambers et al. 2008; Lynch and

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Brown 2008; Raghothama 1999; Richardson et al. 2009; Vance et al. 2003). Such adaptations include modifications to root structure and morphology, as well as biochemical (e.g. root exudates). However, in natural conditions, approximately 80% of land plants live in association with specialised soil fungi to form mycorrhizal roots. The most widespread mycorrhizal association exists between herbaceous plants and arbuscular mycorrhizal (AM) fungi forming AM symbiosis (Parniske 2008; Smith and Read 2008). Woody plants from the gymnosperms and several angiosperms growing in boreal and temperate regions also live in symbiotic association with mycorrhizal fungi that form ectomycorrhizal (ECM) roots (Marmeisse et al. 2004). Whatever the mycorrhizal type (AM or ECM), mycorrhizal fungi produce extraradical hyphae which are able to explore the soil away from the mycorrhizal roots and form a tight association with the plant root where exchanges between fungal and root cells are occurring.

The formation of mycorrhizal roots is considered as the most widespread response to increase phosphate acquisition by plants (Burleigh et al. 2002; Smith et al. 2000; Tibbett and Sanders 2002). This is mainly due to the repeated observation that mycorrhizal plants show growth improvement and increased plant P content than non-mycorrhizal plants (Chalot et al. 2002; Smith and Read 2008). Three main hypotheses have been proposed to explain this improved P nutrition of mycorrhizal plants: an increase of (1) P mobilisation from mineral P, (2) P mobilisation from organic P and (3) soil exploration and P uptake. In particular, the role of extraradical hyphae is thought to be decisive for the functioning of the symbiosis (Finlay 2009). However, the positive effect of mycorrhizal symbiosis may be variable with the fungal species forming the association. This could be due to the different abilities of mycorrhizal fungi to mobilise P and/or to take up Pi from the soil. The aim of this review was to examine published data that may explain such differences between mycorrhizal fungal species, especially those forming ectomycorrhizae.

2 Diversity of fungal species to mobilise mineral P

Soils contain a large part of phosphate that can be precipitated with calcium, iron or aluminium to form many different potential phosphate minerals under crystalline or amorphous form (Barber 1984). Some of the more common minerals are listed in Table 1. In soils above pH 7, calcium phosphates should be dominant, whilst in acid soils, iron and aluminium phosphates are the dominant forms (Barber 1984). Solubility isotherms of phosphate minerals have been calculated as a function

of pH and concentration of Pi. Figure 1 shows some examples of common soil mineral phosphate isotherms calculated using a calcium activity arbitrarily set at $pCa=2.5$, a value consistent with those found in non-calcareous soil solutions. The activities of Al^{3+} and Fe^{3+} were controlled by the solubility of their oxides representing an average value of calcium and are given in Fig. 1. From these isotherms, it is possible to predict the solubility of each mineral when the pH and $[H_2PO_4]$ in the soil solution are measured. If a point is above a line, the solution will be supersaturated relative to that mineral. If a point is under the line, the solution will be undersaturated (Barber 1984). In other words, Fig. 1 shows that crystalline aluminium and iron phosphate minerals will form at very low $[H_2PO_4]$ at acidic pH (even at pH 5), whereas calcium phosphate minerals could be dissolved easily by a pH decrease from 7 to 5. In addition to precipitated forms, phosphate ions can be specifically adsorbed by soluble metal hydrous oxides or by clays (Hunt et al. 2007). Several organic compounds bearing carboxylic groups, either of high molecular weight such as humic (HA) and fulvic acids (FA) or low-molecular-weight organic anions (LMWOAs) such as oxalate, may interact with Pi in soils to influence the amount of available Pi in solution. As indicated by Hunt et al. (2007), the interactions between organic compounds and mineral surfaces in soil may result in positive effects on Pi availability due to three processes—(1) competition between carboxylates and Pi for mineral adsorption sites; (2) complexation of surface metals and release of these metals into solution, thereby removing adsorption sites; and (3) increased repulsion of phosphate anions by sorption of organic compounds to positive sorption sites—and in negative effects by enhancing the formation of cation bridges, leading to an increase in P sorption sites and a decrease in Pi availability. When assessed in solution, HA or FA purified from soils (Borggaard et al. 2005) or HA of commercial origin (Hunt et al. 2007) displayed a low capacity to modify the adsorption of phosphate by metal oxides, leading to the conclusion that humic substances have limited influence on phosphate adsorption by aluminium and iron oxides (Borggaard et al. 2005; Guppy et al. 2005). In contrast to HA, oxalate supplied in the solution at low concentration (≈ 2 or 8 mM) together with goethite and gibbsite displayed a significant ability to decrease Pi adsorption on metal oxides (Hunt et al. 2007). In this context, the production of LMWOAs by ectomycorrhizal fungi may play a decisive role for Pi release from phosphate minerals.

Ectomycorrhizal fungal species have been shown to be able to produce a range of LMWOAs (Plassard and Fransson 2009). The first studies on the capacity of

Table 1 Some common soil phosphate minerals (from Barber 1984)

Calcium phosphates	Aluminium phosphates	Iron phosphates
$\text{Ca}_5(\text{PO}_4)_3\text{F}$ Fluoroapatite	$\text{AlPO}_4 \cdot 2\text{H}_2\text{O}$ Variscite	$\text{FePO}_4 \cdot 2\text{H}_2\text{O}$ Strengite
$\text{Ca}_5(\text{PO}_4)_3\text{OH}$ Hydroxyapatite	$\text{H}_6\text{K}_3\text{Al}_5(\text{PO}_4)_8 \cdot 18\text{H}_2\text{O}$ Potassium Taranakite	
$\text{Ca}_3(\text{PO}_4)_2$ Tricalcium phosphate	AlPO_4 Berlinite	
$\text{Ca}_4\text{H}(\text{PO}_4)_3 \cdot 2.5\text{H}_2\text{O}$ Octacalcium phosphate		
CaHPO_4 Dicalcium phosphate		
$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ Dicalcium phosphate dihydrate, brushite		

ectomycorrhizal fungi to produce LMWOAs were carried out by Lapeyrie and colleagues (Lapeyrie 1988; Lapeyrie et al. 1987, 1991). Lapeyrie et al. (1991) used 11 different ectomycorrhizal strains and found that oxalate production ranged from 27 to 120 $\mu\text{g mg}^{-1}$ fungal dry weight, whereas other LMWOAs could not be detected. These results were confirmed by later experiments (Arviu et al. 2003). In particular, *Paxillus involutus*, *Suillus* sp. and *Rhizopogon roseolus* were strong oxalate producers (Arviu et al. 2003; Lapeyrie et al. 1987). Now, approximately 30 species of ECM fungi belonging to the genera *Cortinarius*, *Lactarius*, *Paxillus*, *Piloderma*, *Pisolithus* and *Suillus* were found to be able to release substantial amounts of LMWOAs, with oxalate being the predominant form (Courty et al. 2010).

Nevertheless, a huge intraspecific variation in the capacity to release organic acids exists among these LMWOAs-producing fungal genera, as shown for *P. involutus* by Lapeyrie et al. (1991). On the other hand, almost no production of organic acids was detected in some ECM species belonging to the genera *Amanita*, *Cenococcum*, *Hebeloma*, *Thelephora* and *Tylospora* (Courty et al. 2010) or in the species *Laccaria bicolor* (Lapeyrie et al. 1991) or *Hebeloma cylindrosporum* (Arviu et al. 2003). Taken together, these data indicate that a huge diversity of the ability to produce oxalate occurs among ectomycorrhizal species and even among isolates of the same species. The ecological meaning of the diversity has not yet been established. In non-oxalate-producing species or isolates, whether this is due to a low production rate (through a lack or a low level of enzymes responsible for oxalate biosynthesis) or to the degradation of produced oxalate (by enzyme decarboxylation of oxalate) remains to be determined.

Besides the specific diversity displayed by ectomycorrhizal fungal species, several environmental factors may increase or decrease organic anion production by ectomycorrhizal fungi. Exposure of ectomycorrhizal fungi to different metals (including Al, Fe, Pb, Cd, Cu and Ni) induced variable effects ranging from negative ones to positive ones (see Table 1 in Plassard and Fransson 2009), with an exudation of oxalate increased by 15–45% after Pb and Cd exposure. However, exposure to Al and Fe generally did not stimulate oxalate production by the fungal species used. In contrast to metal exposure, nitrogen source appears to be a major factor as in vitro studies carried out in pure culture show a consistent stimulation of oxalic acid production in the presence of nitrate and inhibition by ammonium for *P. involutus* (Gharieb and Gadd 1999; Lapeyrie et al. 1987; 1991). We can hypothesise that it is

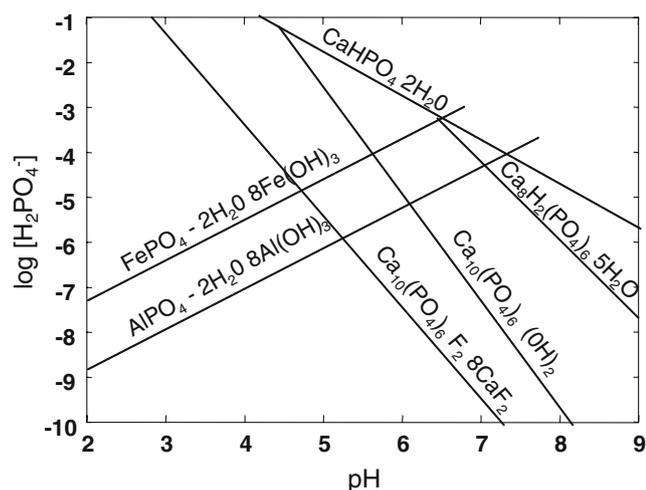


Fig. 1 Solubility isotherms for indicated crystalline phases. Activity of calcium was arbitrarily set at $\text{pCa}=2.5$. The activity of Al^{3+} and Fe^{3+} were controlled by the solubility of their oxides (redrawn from Fig. 9.3 in Barber 1984)

the NH_4^+ ion per se which has an inhibitory effect on oxalate synthesis as organic nitrogen did not inhibit organic acid release in *R. roseolus*, contrary to ammonium (Plassard, unpublished data). When nitrate is supplied as the sole nitrogen source in the medium, the presence of calcium and bicarbonate ions was shown also to promote oxalate production in the fungus grown in pure culture (Lapeyrie et al. 1987) or in association with the plant (Casarin et al. 2003, 2004).

From a physiological point of view, the efflux of oxalate through fungal cell membranes occurs as anion transport because the cytosolic pH (around 7) is higher than the pK of oxalic acid (pK for oxalate⁻/oxalate²⁻ is 4.19; Jones 1998), meaning that the organic acid is actually present as organic anion in the cytosol. Therefore, when carboxylates are exuded as anions, their charge could be balanced by a cation efflux that can be either K^+ or H^+ (Roelofs et al. 2001) or alternatively by anion influx (Arvieu et al. 2003). Oxalate released with K^+ or against anion influx will have only complexing effects, whereas oxalate released with H^+ will have both complexing and acidifying effects. The production of oxalate only should be a better strategy to mobilise Pi combined to metals in acidic soils, as observed in Proteaceae developing in highly weathered, acidic Australian soils and releasing citrate and K^+ (Roelofs et al. 2001; Lambers et al. 2008). Conversely, the production of oxalate together with protons should be a better strategy when Pi is combined with calcium (see Fig. 1). Interestingly, inter- and intraspecific differences in acidifying properties of oxalate-producing ectomycorrhizal species have been reported in vitro (Arvieu et al. 2003). However, in the study of Casarin et al. (2003, 2004), only the acidifying ECM species *R. roseolus* and the poor oxalate producer ECM species *H. cylindrosporum* were used in association with *Pinus pinaster* plants grown in rhizoboxes containing a Mediterranean soil layer with a low level of easily available P. In these conditions, only *R. roseolus* hyphae were able to release oxalate into the soil that combined with calcium to form calcium oxalate (Casarin et al. 2003). Oxalate release was accompanied by acidification and increase of Pi availability of rhizosphere soil (Casarin et al. 2004). Finally, this fungal association significantly increased the P nutrition of the host plant, indicating its ability to enhance Pi bioavailability from this soil. It would be of great interest to use two contrasting ectomycorrhizal species, able to produce high amounts of oxalate with or without acidifying effect, to study the effects of complexing/acidifying properties due to organic anion release on P bioavailability in tropical acidic soils. Indeed, these soils are characterised by a high level of metal oxides where acidification could decrease P availability.

So far, most of our knowledge about the capacities of ECM species to produce organic anions comes from in vitro studies (Rosling 2009). Despite the fact that in vitro experiments are valuable tools to study processes, systematic in situ measurement of oxalate release by ectomycorrhizal tips could bring more relevant data on the ecological point of view. Progress in this field was hampered by the lack of an easy method to measure LMWOAs in field-sampled ectomycorrhizal tips. Recently, Rineau et al. (2008) reported a microplate assay making it possible to measure the release of oxalate at the level of individual ectomycorrhizal tips. The use of this method could be a very valuable tool to assess at least the capacities of oxalate released by ectomycorrhizal species even if they are not grown in vitro. In addition, the comparison of oxalate release capacity by these ectomycorrhizal tips with the actual concentration of oxalate in soil sample surrounding these tips could help quantify the fate of LMWOAs released into the mycorrhizosphere. However, determining if oxalate—or other LMWOAs—are released with or without protons in situ remains a challenging issue.

3 Diversity of fungal species to mobilise organic P

Remarkably, besides the low level of available free Pi, soils contain a high amount of P that is linked to C-containing compounds to form organic P (Po). The majority of the organic phosphorus is present as phosphate esters (C–O–P bonds) either in the form of phosphate monoesters, including inositol phosphates, or phosphate diesters, such as nucleic acids and phospholipids, together with small quantities of phosphonates (C–P bonds; Condron et al. 2005; Magid et al. 1996). To be used by plants and soil microorganisms, the phosphate group of Po compounds must be released from the ester bond linking it to carbon by enzymes that are phosphatases. Depending on their substrate, the enzymes can be phosphomonoesterases or phosphodiesterases. Most of the studies addressing the release of phosphatases use artificial phosphomonoesters such as *p*-nitrophenyl phosphate (*p*NPP) based on the procedure described in Tabatabai and Bremner (1969) or the fluorescent assay based on the release of 4-methylumbelliferone from 4-methylumbelliferone-phosphate (MUP; Courty et al. 2005, 2006; Pritsch et al. 2004; Pritsch and Garbaye 2011) to estimate phosphomonoesterase activity.

Depending on the pH of the incubation medium, one can distinguish acid phosphomonoesterase activity (ACP) from alkaline phosphomonoesterase activity (ALP), measured respectively at pH around 5 (ACP)

and 8 or more (ALP; Bae and Barton 1989; van Aarle and Plassard 2010). However, so far, most of the ECM fungi showed maximal activities of released or surface-bound phosphomonoesterase at acidic pH when assayed with *p*NPP (Tibbett 2002) or with MUP (Courty et al. 2005). ALP was measured in cell extracts of *Cenococcum graniforme* (renamed *Cenococcum geophilum*), and although the enzyme showed a high association with cell wall material, it was not released into the external medium from young fungal cultures (Bae and Barton 1989). Indeed, Antibus et al. (1986) did not measure a significant surface ALP in the same species but rather an ACP. It can be hypothesised that intracellular ALP will be involved in internal recycling of fungal organic P, whereas surface-bound or released ACP will be involved in the mineralization of soil organic P. Indeed, as most forest soils are not alkaline, the production of acid phosphatases by ECM fungi seems to be relevant of the field conditions encountered by ECM fungi.

Different abilities to release ACP were reported among ectomycorrhizal species and strains when grown in pure culture (e.g. Matumoto-Pintro 1996 in Quiquampoix and Mousain 2005; Tibbett et al. 1998; Nygren and Rosling 2009). For example, the measurement of *p*NPPase activities in ten strains of Basidiomycetes (*Laccaria laccata*, *Suillus collinitus* (three strains), *Suillus granulatus*, *Suillus luteus*, *H. cylindrosporum* (two strains), *P. involutus* and *Rhizopogon rubescens*) after culture of the mycelia in a low-phosphate medium showed that only six of them had free extracellular *p*NPPase activity (one strain of *S. collinitus*, *S. granulatus*, *S. luteus*; both strains of *H. cylindrosporum* and *R. rubescens*). In addition, the two *H. cylindrosporum* strains presented exceptional high level of ACP activities they secreted into the external medium (Quiquampoix and Mousain 2005). The ability of *Hebeloma* species to increase the proportion of extracellular *p*NPPase compared to the surface-bound activity in the case of Pi depletion was also reported by Tibbett et al. (1998). The high ability to release acid phosphatases into the external medium was recently confirmed in another isolate of *H. cylindrosporum* by Louche et al. (2010). In this study, four fractions containing ACPase activity were separated from the culture medium, suggesting that this fungal species could be able to produce several isoforms of acid phosphatase. Each fraction was able to hydrolyse a range of phosphomonoesters, but the characteristics of the corresponding proteins remain to be determined. From a molecular point of view, a gene coding for an acid phosphatase (XP 001887867, <http://www.ncbi.nlm.nih.gov/>) is available from the genome of *L. bicolor* recently completely sequenced (Martin et al. 2008). The pre-

dicted protein is very close to the acid phosphatase AfPhoA (Q8X176) characterised in *Aspergillus fumigatus* (Bernard et al. 2002) as well as to two other acid phosphatases identified in the basidiomycete *Pholiota nameko* (PNAP1: BAD00139 and PNAP2: BAD00140; Fig. 2). Interestingly, the different polypeptides share three domains that are highly conserved among these acid phosphatases (Bernard et al. 2002; Fig. 2). However, no specific functions have been attributed yet to these conserved domains. Whether acid phosphatase fractions separated from the culture medium of *H. cylindrosporum* correspond to one gene homologous to PhoA or to four different genes remains to be determined.

Among the different forms of organic P extracted from soil, phytate, corresponding to the salt of *myo*-inositol hexakisphosphate (IP6), is often the dominant form of organic P (Turner et al. 2002). To be used as a P source, orthophosphate groups linked to the inositol ring with an ester bond must be released by the action of phosphatases named phytases. Saprotrophic fungi are able to release these enzymes that belong to the class of histidine acid phytase (HAPhy) that can be 6-phytase (EC 3.1.3.26) or 3-phytase (EC 3.1.3.8), depending on the position of the first phosphate group released by the enzyme (Mullaney and Ullah 2007). Alignment of available amino acid sequences of HAPhys show that the N-terminal motif RHGXRX and the C-terminal motif HD that compose the active site are the only ones conserved among the HAPhy identified in prokaryotes or eukaryotes (Mullaney and Ullah 2007).

These enzymes are acid phosphatases and have been shown to release up to five Pi and inositol monophosphate per inositol hexakisphosphate. Experimentally, phytase activity measurement is based on the release of Pi from phytate salt supplied in incubation medium because there is no artificial substrate as convenient as *p*NPP or MUP. However, the sensitivity of Pi release from phytate hydrolysis is higher when the green malachite method is used (Ohno and Zibilske 1991) as the minimal Pi concentration that could be detected with this method is around 2 µM. In ectomycorrhizal fungi, phytase activity has been measured in the ten ectomycorrhizal isolates cited above (Matumoto-Pintro 1996 in Quiquampoix and Mousain 2005). Only four isolates had free extracellular phytase activity (one strain of *S. collinitus*, *S. granulatus*, and both strains of *H. cylindrosporum*; Quiquampoix and Mousain 2005). However, the rate of Pi release was low compared to that measured for *para*-nitrophenol release, with values ranging from 1% to 8% in the four fractions separated from the culture medium of *H. cylindrosporum* (Louche et al. 2010).

Fig. 2 Comparison of the predicted amino acid sequences of four acid phosphatases identified in the ectomycorrhizal *L. bicolor*, *LbPhoA* (accession XP001887867); the ascomycete *A. fumigatus*, *AfPhoA* (accession Q8X176) and in the saprotroph basidiomycete *P. nameko*, *PNAP1* (accession BAD00139) and *PNAP2* (accession BAD00140). The sequences were retrieved from NCBI (<http://www.ncbi.nlm.nih.gov/>). Identical residues are indicated by *solid boxes*; similar residues are indicated by *grey boxes*. The *lines* indicate highly conserved motifs among the proteins

PNAP1	1	-----MIFSVVLFSSLVYSAQAIVPGVVFDRVVSIVLWE
LbPhoA	1	-----MHAKLVAFLLAGCIIINAFANSSPDSKHSKLSVVPCKAFDRFVTIWLWE
PNAP2	1	-----MLLSSSLICIGLLIGAESRLILPKPKPFKRFIVPGAVDFRFTIWLWE
AfPhoA	1	MKPSVATLLLATVSLVYAQIATAEKEPSLSAIESAAAASIQPYSPVSNVVEGVAFNRFQVWLWE
	*.....*
PNAP1	34	NTDFSKAAADPNFAALTKQGLQLTNTFFAVTHPSEPNIYVSVVGGHYFGMNDNLNINIPANI
LbPhoA	49	NTDFSSAMADPNLSALASQGITLNTYFALTHPSEPNIYVSVVGGHYFGMNDNLNINIPANI
PNAP2	47	NINIFASASADPNFAALTKQGITLNTYFAVTHPSEPNIYVSVVGGHYFGMNDNLNINIPANI
AfPhoA	61	NIDYEDAAADENMKWLASQGITLNTFYAVTHPSEPNIYCAAVGGITFGMNDNLNINIPANI
	*.....*.....*.....*.....*.....*.....*.....*
		1
PNAP1	94	SSVVDLLEDKGITWAEYQEDMPSTGFTGFSFS--KTSANDYVRKHNPLIIYDSVSTKPSR
LbPhoA	109	STIVDLLEDKGITWAEYEDMPSTGFTGLQFLNPRTGANDYVRKHNPLIIVYVSVGNPAR
PNAP2	107	SSVVDLLEKGIWGEYQEHMPTTGFQGFEMLN-PDGANDYVRKHNPLIIDSVANFSSR
AfPhoA	121	STVADLLDTRKNIWAGEYQEHLEYPGFQGFNYSNQETIYNDYVRKHNPLIYDSVSTKNSIR
	*.....*.....*.....*.....*.....*.....*.....*
		2
PNAP1	152	AANVKNFTLFCODLESNSLPQWMFITPNMTNDGHDNTNVAATSTWAINFLTPLLKKNPFNA
LbPhoA	169	SANIKNFTLFCODLAAGKLPQWIFITPNMTNDGHDNTNFAAKWVKGFLTPLLKKNPFNA
PNAP2	166	ANQVKNFTLFCODLADDNLPQWMFITPNMTNDGHDNTNLSFASIWAKDFLEPLLENPSFNA
AfPhoA	181	LRQIKNFTSFEEDDLANKKLPQWAFITPNMTNDGHDNTNIFGAKWERSNIAPLLENPSYFMN
	*.....*.....*.....*.....*.....*.....*.....*
		3
PNAP1	212	AKTLIVLTFDENGINSVANRDIIVLGSAPASLVGTODATVYNHYSEIATVEANWDLHT
LbPhoA	229	DRTLVLLTFDENETYGKQNRRAILLGSAIPSNLIGTDSNFYDHYSEISTIEANWGLHT
PNAP2	226	KKTLVLLTFDENGSTDPHNRVDSILLGTAVPRQLVGTODSTFYTHYSEIATVEANWGLHT
AfPhoA	241	-DTLIVLTFDEDDGTYSKSNKIFSVLLGGAIPDELKGTQDDTFYTHYSVIASVSNWGLPS
	*.....*.....*.....*.....*.....*.....*.....*
PNAP1	272	LGRYDVGANVYSFVAAKTGDIVDAPTNPILAKILLNLSYPGPFNSCAGCPPLPIPNTSLVV
LbPhoA	289	LGRYDVGANVFSFVAQRTHTDIRTLQNPPLSCQTFNLSYPGVFNASKTAPLPIPNTSLVV
PNAP2	286	LGRFDVGANVENFVAKHTHDVIRPAD---LEQSFLNLSYPGPFNSAPSCKFTVPTRLTV
AfPhoA	300	LGRWDCGANLEIVANKTYVYV---YVDVTNLRLENTYPGFMSAGEYSKYSVPWPNAIT
	*.....*.....*.....*.....*.....*.....*.....*
PNAP1	332	N----GRTVLPALASTWGSAAALQKCTTYTGSVNVPSNSNPPILPSSGC-----
LbPhoA	349	N----GRTVLPCTVNVWGSAAALQKCTTYACKLEIPASNPPVLPAGC-----
PNAP2	343	N----GRHVLPKIVETWGSLSHLQRCITLYTGALOPPSGLNPPITPQGC-----
AfPhoA	357	RGDCSACHGILDIVKETYANTEPTYNYSPPFPYDTASNYNTRVATKKNVTGTHRSSSSS
	*.....*.....*.....*.....*.....*.....*.....*
PNAP1		-----
LbPhoA		-----
PNAP2		-----
AfPhoA	417	SPSASSNAAVSAVAPAAGVSGLLLGLALNLL

When measured *in vitro*, an inverse relationship has generally been reported between Pi concentration in culture media and ACP, indicating that ACP is derepressed by Pi starvation (Tibbett 2002). Higher values of surface-bound ACP have also been measured in ectomycorrhizal roots (see, e.g. Ali et al. 2009; van Aarle and Plassard 2010) and hyphae (van Aarle and Plassard 2010) belonging to ectomycorrhizal plants grown in soil with a low level of available P, confirming the results obtained *in vitro*. In contrast, it seems that the presence of organic P did not activate the production of ACP in ECM fungi as no differences in enzyme production were found after growth on inorganic and organic P sources (Antibus et al. 1992; Nygren and Rosling 2009). The release of extracellular enzymes is costly and one can imagine that this strategy—synthesis

and release of ACP only in low P availability—will avoid a waste of energy for the fungus when Pi is available in the external solution. However, in addition to Pi availability, N fertilisation applied in the field appears also as an important factor to regulate ACP activity, as shown by a recent study made by Taniguchi et al. (2008). The authors used seven ECM fungi to inoculate *Pinus thunbergii* seedlings in control conditions: *C. geophilum*, *Rhizopogon* sp., *S. granulatus*, *Tomentella* sp. 1, *Tomentella* sp. 2, *Amanita* sp. and one unidentified ECM fungus T01. Once inoculated, the seedlings were cultivated in sterilised soil, whether or not supplemented with N supplied as ammonium nitrate. ACP was measured using *pNPP*, and the results showed that in non-fertilised conditions (control group), the activity was significantly higher in *Tomentella* sp. 1,

Tomentella sp. 2 or *Amanita* sp. than in *S. granulatus* or *Rhizopogon* sp., unidentified ECM fungus T01 and in the non-mycorrhizal root tips. In fertilised conditions (N group), activity of root tips was significantly higher in the seedlings inoculated with *Tomentella* sp. 1 or *Tomentella* sp. 2 than in the seedlings inoculated with *S. granulatus* or *Rhizopogon* sp. and in the non-inoculated seedlings. The application of N significantly increased the activity measured in NM and *S. granulatus* root tips. This increase of *p*-PNPase activity was attributed to the depletion of Pi due to better P uptake and accumulation in plant or fungal tissues following the enhanced N availability (Taniguchi et al. 2008).

Despite the fact that ECM fungi are able to release ACPase, especially when Pi availability is low, the efficiency of these enzymes to mobilise Pi from organic P in forest soils is still a matter of debate. As an example, studies using ectomycorrhizal roots and their external mycelium reported opposite conclusions regarding the use of inositol phosphate P by the mycorrhizal fungus (Antibus et al. 1997; Colpaert et al. 1997). These experiments were carried out in simplified conditions (perlite as growing medium and solution containing ^{32}P -labelled inositol phosphate, respectively) that overestimate the actual effect of ECM fungi as the availability of the substrate is not limiting. Indeed, it is important to keep in mind that the studies measuring P accumulation in mycorrhizal plants grown in substrate containing added soluble organic P compounds may overestimate the actual role of ECM fungi to use organic P in field conditions. This is particularly important in the case of inositol phosphate, a polyanion that can be easily combined with mineral surfaces (iron and aluminium oxides, calcium) and thus made unavailable to phytases (Giaveno et al. 2010). However, the ability to hydrolyse a large spectrum of phosphomonoesters, as shown by Louche et al. (2010), may be an advantage in soil conditions. The phosphatases could also be involved in recapturing of excreted plant or fungal compounds (Barrett-Lennard et al. 1993) or breaking down and recycling the phospholipids from old hyphae (Nygren and Rosling 2009), avoiding therefore the loss of P into the environment.

4 Diversity in P uptake

Considerable increased soil exploration by mycorrhizal fungi is thought to play a major role in overcoming the Pi depletion zone occurring around the roots. As an example, data obtained in young pot-grown *Pinus taeda* showed that the absorbing surface contributed by the hyphae of the ectomycorrhizal species *Pisolithus tinctorius* represented about 75% of the uptake potential absorbing area and over 99% of the absorbing length of the whole root system

(Rousseau et al. 1994). Inoculation of *P. taeda* plants with *C. geophilum* was by far less efficient to improve P nutrition of the host plant. This was attributed to a much lower hyphal growth of *C. geophilum* (2.80 mg^{-1} soil) compared with that measured in *P. tinctorius* (6.78 mg^{-1} soil), in combination with the absence of rhizomorphs formed by *C. geophilum* (Rousseau et al. 1994). These data illustrate the importance of hyphal growth and rhizomorph formation on P uptake and its net transfer to the ectomycorrhizal plant. However, it should be noticed that ectomycorrhizal species can display different patterns to explore the soil according to the classification proposed by Agerer (2001). Some examples of species belonging to different exploration types are given in Table 2 (Hobbie and Agerer 2010). In theory, species belonging to “long-exploration” type or to “medium-distance” should ensure a better soil exploration than the species of the “contact” type. However, determining the relationships between hyphal density, exploration type and the actual efficiency of ECM species to improve P uptake of trees in natural conditions is still a challenging issue.

From a functional point of view, studies carried out with intact plants grown in microcosms showed that extraradical hyphae and mycelial strands of *Suillus bovinus* interconnecting *Pinus contorta* and *Pinus sylvestris* plants were also able to take up ^{32}P -Pi and to translocate labelled P to the shoots (Finlay and Read 1986). This transport of P is unidirectional (from the fungal to the host cells) as no translocation of ^{32}P supplied to non-mycorrhizal roots was detected in *S. bovinus* mycelium (Finlay and Read 1986). External hyphae associated with young *P. sylvestris* grown in perlite strongly enhanced the Pi uptake capacity of the pine root system by decreasing the K_m and increasing V_{\max} of Pi uptake rates in ECM plants compared with non-mycorrhizal plants (Van Tichelen and Colpaert 2000). However, plants associated with *P. involutus* were more efficient to take up Pi than those associated with *S. bovinus* and *Thelephora terrestris* (Table 3), suggesting that the Pi transporters may exhibit different properties among the associated fungal species.

Regarding the identification of phosphate transporters in fungi, two types of high-affinity Pi transporters have been characterised, which are either Pi:H⁺ or Pi:Na⁺ transporters. In yeast, these two transporters are encoded by two genes, PHO84 (Bun-ya et al. 1991) and PHO89 (Martinez and Persson 1998), whose expression is activated when the cells meet a limitation in external Pi (Persson et al. 2003). The PHO84 transport system displayed K_m values for external Pi ranging from 1 to 15 μM , whereas PHO89 displayed a K_m for external Pi of 0.5 μM (Persson et al. 2003). In mycorrhizal fungi, the first data regarding the identification of phosphate transporters possibly involved in Pi uptake were obtained in the AM species *Glomus versiforme*

Table 2 Exploration type of different genera of ectomycorrhizal species

Exploration type	Genus
Long	<i>Boletus</i> , <i>Leccinum</i> , <i>Paxillus</i> , <i>Rhizopogon</i> , <i>Suillus</i> , <i>Tylopilus</i> , <i>Xerocomus</i>
Medium-mat	<i>Boletopsis</i> , <i>Gomphus</i> , <i>Hydnellum</i> , <i>Phellodon</i> , <i>Ramaria</i> , <i>Sarcodon</i>
Medium-fringe	<i>Cortinarius</i> , <i>Hebeloma</i> , <i>Hydnum</i> , <i>Lyophyllum</i> , <i>Tricholoma</i>
Medium-smooth	<i>Albatrellus</i> , <i>Amanita</i> , <i>Cantharellus</i> , <i>Craterellus</i> , <i>Gomphidius</i> , <i>Laccaria</i> , <i>Russula</i>
Short	<i>Inocybe</i> , <i>Rocites</i>
Contact	<i>Hygrophorus</i> , <i>Lactarius</i> , <i>Chroogomphus</i>

Exploration types were defined by Agerer (2001). Long is for long distance, medium is for medium distance, short is for short distance of emanating hyphae from ectomycorrhizal roots. Contact ectomycorrhizae have no emanating hyphae. Redrawn from Hobbie and Agerer (2010)

associated with *Medicago truncatula* (Harrison and van Buuren 1995). Pi uptake mediated by the transporter (named GvPT) in yeast is dependent on external pH, suggesting that it is operating via a proton-coupled symport and exhibited an apparent K_m value of 18 μM Pi (Harrison and van Buuren 1995). Furthermore, one partial cDNA (*GmosPT*) and one full-length cDNA (*GiPT*) putatively coding for Pi transporters have been identified in two AM species, *Glomus mosseae* and *Glomus intraradices*, respectively (Benedetto et al. 2005; Maldonado-Mendoza et al. 2001). Although the polypeptides encoded by *GmosPT* and *GiPT* were not functionally characterised yet, they all clustered with fungal Pi:H⁺ polypeptides (Tatry et al. 2009). All these transcripts have been predominantly detected in extraradical hyphae, with their expression level enhanced by low P availability, such as reported in *G. intraradices* (Maldonado-Mendoza et al. 2001; Olsson et al. 2006) and *G. mosseae* (Benedetto et al. 2005). Taken as a whole, these data suggest a role in Pi acquisition from the soil for all these Pi transporters despite the rather high value of K_m measured for Pi uptake mediated by GvPT expressed in yeast.

Regarding ECM fungi, data are available from three species: two Basidiomycetes, *L. bicolor* and *H. cylindrosporium*, and one Ascomycete, *Tuber melanosporum*. In *L. bicolor*, whose the genome has been completely sequenced, five genes possibly coding for Pi transporters belonging to the Major Facilitating Superfamily (MFS) of transporters have been identified and named *LbPht1;1* to *LbPht1;5*

(<http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html>). Two other genes, *HcPT1* and *HcPT2*, have been identified in *H. cylindrosporium* (Tatry et al. 2009). The peptide sequence of HcPT1 is very close to two *L. bicolor* predicted polypeptides (*LbPht1;4* and *LbPht1;5*) and HcPT2 is close to the three other ones (*LbPht1;1–3*). As in AM fungi, the seven polypeptides cluster fungal Pi:H⁺ transporters. However, the genome of *T. melanosporum* (<http://www.genoscope.cns.fr/externe/GenomeBrowser/Tuber/>) contains at least three genes coding for Pi transporters. Two of them encode putative Pi:H⁺ transporters belonging to the MFS, homologous to PHO84 (high affinity) and to PHO87 (low affinity) in yeast. The last gene should encode a protein belonging to another transporter family, the Inorganic Phosphate Transporter (PiT) family, very close to the high-affinity Pi:Na⁺ polypeptide NcPHO4 characterised in the mould Ascomycete *Neurospora crassa*.

The polypeptides encoded by the genes identified in *L. bicolor* or *T. melanosporum* have not been characterised yet. However, this was done for HcPT1 and HcPT2 (Tatry et al. 2009) which were shown able to mediate Pi:H⁺ symport with different affinities for Pi, the K_m values being 55 and 4 μM , respectively, for HcPT1 and HcPT2. The apparent K_m of HcPT2 was therefore comparable to that reported for PHO84 and lower than that of the gene GvPT (18 μM), which is the only value for a Pi transporter of AM fungi currently available for comparison (Harrison and van Buuren 1995). The value of K_m found for HcPT2 was in the same range as those found in intact plants of *P. sylvestris*

Table 3 Kinetic parameters of the high-affinity Pi uptake system in intact mycorrhizal and non-mycorrhizal *P. sylvestris* root systems

Plant age (weeks)	Inoculation treatment	Apparent K_m (μM Pi)	Apparent V_{max} ($\text{nmol g}^{-1} \text{s}^{-1}$)
7	<i>Paxillus involutus</i>	3.5 (2.4–4.5) ^a	0.57 (0.50–0.66)
	<i>Suillus bovinus</i>	7.5 (5.1–10.1)	0.49 (0.45–0.55)
	<i>Thelephora terrestris</i>	8.7 (6.5–10.1)	0.13 (0.09–0.17)
9	<i>Paxillus involutus</i>	5.9 (4.1–7.2)	0.62 (0.48–0.70)
	<i>Suillus bovinus</i>	10.2 (7.2–12.3)	0.52 (0.48–0.54)
	<i>Thelephora terrestris</i>	7.3 (4.6–9.9)	0.15 (0.10–0.19)
	None (non-mycorrhizal)	12.1 (7.8–16.4)	0.08 (0.07–0.10)

Adapted from van Tichelen and Colpaert (2000)

^aMeans and range of variation (between parentheses) are shown

(see Table 3), suggesting that this phosphate transporter could play a great role in Pi uptake into fungal cells. Expression levels of *HcPT1* and *HcPT2* were quantified as a function of external Pi availability in the hyphae grown in pure culture or associated with their host plant *P. pinaster*. Levels of *HcPT1* transcripts were always higher in fungal cells exposed to Pi starvation in solution or to low Pi availability in soil, suggesting that the regulation of this transport system is close to that of PHO84 in yeast. In contrast, transcript levels of *HcPT2* were less dependent on Pi availability in fungal cells grown in vitro and were upregulated in ectomycorrhizal roots grown in soil with high P availability (Tatry et al. 2009). Taken as a whole, these results indicate that *H. cylindrosporum* might use *HcPT1* to mediate Pi uptake when soil P availability is low and *HcPT2* when soil P availability is high. It is therefore intriguing to find such rather high apparent affinity value measured for *HcPT1* expressed in yeast and its functioning when Pi availability is low. As proposed by Tatry et al. (2009), this could be due to heterologous expression that may modify the actual rates of Pi uptake and particularly the apparent K_m value. Indeed, one would expect a low value of apparent K_m of Pi uptake in ectomycorrhizal fungi (see Table 3), close to that found in *HcPT2*. It should be noticed that the heterologous expression of *HcPT1* required starving the yeasts for Pi (Tatry et al. 2009), a feature that was not observed for *HcPT2*. These data suggest that *HcPT1* expression may have been significantly modified by the yeast machinery, contrary to *HcPT2*, leading to the overestimated apparent K_m value. In this context, it will be very interesting to carry out the functional characterization of Pi transporters identified in other ectomycorrhizal fungi, particularly those close to *HcPT1*. These data will be helpful to get a better quantification of the actual capacities of Pi transport by these proteins. Also, the functional characterization of the polypeptides encoded by the genes discovered in *T. melanosporum* genome will confirm whether this fungus is able to take up Pi in alkaline conditions, where protons are not available.

5 Concluding remarks

From this review, it is clear that mycorrhizal fungi exhibit a large diversity in their ability to mobilise mineral or organic P from the soil, as well as in their efficiency to explore the soil and to take up Pi from the soil solution. It is clear also that most of the data have been obtained by carrying studies on a small number of fungal species. The challenge will be now to find tools to extend our studies in situ to increase our comprehension of the functioning of forest ecosystems. Regarding insoluble mineral P mobilisation, it seems that the release of LMWOAs such as oxalate is of great

importance to enhance P availability. However, we need to know better what the diversity is and how this production of oxalate is regulated by environmental factors such as nitrogen source. There is no molecular tool available yet, but measurement of oxalate production capacities by ectomycorrhizal tips (Rineau et al. 2008) in various environmental conditions, together with the determination of available sources of N (that could be identified by incubating field soil samples in laboratory conditions), should help us improve our knowledge of this regulation in the field. However, despite the importance of the accompanying cation on the actual efficiency of LMWOA release, determining whether oxalate—or other LMWOAs—are released with or without protons in situ remains a challenging issue.

Regarding the mobilisation of organic P, the extensive use of microplate assay (Pritsch et al. 2004; Courty et al. 2005; Pritsch and Garbaye 2011, this issue) for measuring acid phosphatase activities on individual root tips, together with the molecular identification of the fungal species forming the ectomycorrhizal tip, should be a valuable tool to extend our knowledge about the factors that regulate the abundance of phosphatases released into the soil. Alternatively, molecular tools could be designed to study the diversity of these enzymes in the field. Finally, regarding soil exploration and Pi uptake efficiency, the diversity of Pi transporters among ectomycorrhizal species is still a challenging question in the field. It should be useful to combine studies dealing with exploration types and variability of Pi transporters that could be assessed using molecular tools.

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