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Running title: P efflux and polyP dynamics of *H. cylindrosporum*

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Abstract

Ectomycorrhizal (ECM) association can improve plant phosphorus (P) nutrition. Polyphosphates (polyP), synthesized in distant fungal cells after P uptake may contribute to P supply from the fungus to the host plant if they are hydrolyzed to phosphate in ECM roots then transferred to the host plant when required. In this study, we addressed this hypothesis for the ECM fungus *Hebeloma cylindrosporum* grown *in vitro* and incubated without plant or with host (*Pinus pinaster*) and non-host (*Zea mays*) plants, using an experimental system simulating the symbiotic interface. We used ^{32}P labelling to quantify P accumulation and P efflux and *in vivo* and *in vitro* NMR spectroscopy and cytological staining to follow the fate of fungal polyP. Phosphate supply triggered a massive P accumulation as newly synthesized long-chain polyP in *H. cylindrosporum* if previously grown under P-deficient conditions. P efflux from *H. cylindrosporum* towards the roots was stimulated by both host and non-host plants. However, the host plant enhanced ^{32}P release compared to the non-host plant and specifically increased the proportion of short-chain polyP in the interacting mycelia. These results support the existence of specific host-plant effects on fungal P metabolism able to provide P in the apoplast of ectomycorrhizal roots.

Summary

In this work we examined whether a host or a non-host ectomycorrhizal (ECM) plant can enhance P efflux and affect polyphosphate (polyP) metabolism of an ECM fungus. In a system simulating the symbiotic interface *in vitro*, we used ^{32}P to quantify P fluxes and ^{31}P NMR to follow the fate of fungal polyP. For the first time, this paper shows that an ECM host plant has a direct effect on polyP hydrolysis to promote fungal Pi release.

Keywords: *Pinus pinaster*, *Zea mays*, *Hebeloma cylindrosporum*, fungal P efflux, polyphosphates, ^{32}P labelling, *in vivo* ^{31}P NMR spectroscopy.

Introduction

Ectomycorrhizal (ECM) symbiosis is a mutual association between fungal symbionts and lateral roots of trees. This symbiosis is the most widespread association in temperate and boreal biomes where it is formed by 95 % of forest trees (Smith & Read 2008). ECM fungi are known to stimulate host plant growth, mainly by enhancing soil nutrient uptake. The positive effect of ECM symbiosis on plant phosphorus (P) nutrition has been demonstrated (Smith & Read 2008; Plassard & Dell 2010; Cairney 2011; Smith *et al.* 2015) although more knowledge is available regarding the effect of ECM fungi on plant nitrogen (N) nutrition (Smith *et al.* 2015). The positive effect on phosphate (Pi) uptake has been attributed primarily to the external mycelium of the fungus that functions as an extension of the root system allowing the exploration of a larger volume of soil beyond the depletion zone formed around actively absorbing roots (Smith & Read 2008; Cairney 2011; Smith *et al.* 2015). Pi is absorbed from the soil solution by Pi transporters in the extraradical hyphae (Tatry *et al.* 2009). Once absorbed, Pi may be incorporated in the active P fungal pool, including phosphorylated primary metabolites, structural molecules and nucleic acids necessary for fungal growth and metabolism or transferred to the host-plant, as demonstrated by pioneer labelling experiments (Melin & Nilsson 1950). Pi can also be transferred to the vacuoles and condensed into polyphosphates (polyP) to constitute a storage pool (Ashford *et al.* 1994, 1999). Cairney (2011) proposed a model of ECM functioning, and suggested that these polyP are probably translocated to the host *via* a motile tubular vacuolar system (Ashford & Allaway 2002; Darrah *et al.* 2006; Bücking & Heyser 1999) before being hydrolyzed within the Hartig net to provide Pi efflux from the fungal cells to the interfacial apoplast. Pi could then be recovered by some plant P transporters whose expression is enhanced in ECM roots (Loth-Pereda *et al.* 2011; Kariman *et al.* 2014) or in host cells containing arbuscules (Harrison *et al.* 2002; Loth-Pereda *et al.* 2011; Rausch *et al.* 2001; Volpe *et al.* 2016) in arbuscular mycorrhizal (AM) plants.

The synthesis of polyP by the ECM fungi appears therefore to be a key element of mycorrhizal functioning. In living systems, linear inorganic polyP may contain up to 1000 Pi units (Wood & Clark 1988). Chen (1999) proposed the classification of polyP as short-chain (chain length < 20 units) or long-chain (chain length > 50 units). To date, several methods have been used to reveal and to analyze polyP in cells. For example *in vivo* NMR spectroscopy of ³¹P can give information on the forms and locations of P metabolites by detecting freely mobile inorganic phosphorus in the living cells such as orthophosphate and

polyphosphate, and small organic phosphate metabolites (esters of phosphate or phosphonate). PolyP occurrence in ECM fungi has been intensively studied (reviewed by Pfeffer *et al.* 2001) and, when grown *in vitro*, the chain length of NMR-visible polyP ranged from 11 to 15 P residues (Martin *et al.* 1983, 1985; Ashford *et al.* 1994), suggesting that the polyPs visible by *in vivo* ^{31}P NMR are mainly short-chain polyP. In addition to ^{31}P NMR, polyPs can be observed as metachromatic granules following the application of cationic dyes such as toluidine blue O to chemically fixed and dehydrated samples (Chilvers *et al.* 1985; Ashford *et al.* 1986, 1999). Energy dispersive X-ray microanalysis applied to cryofixed material showed spherical electron-opaque deposits containing high amounts of P associated with potassium (K) (Bücking & Heyser 2000). Both structures are localized in the vacuoles of ECM fungi and have been defined as polyP granules (Chilvers *et al.* 1985; Ashford *et al.* 1986; Bücking & Heyser 1999). In contrast to ^{31}P NMR, the latter methods can reveal either mobile or immobile polyP consisting of short- and long-chain polyP.

Within the Hartig net of ECM roots, where the exchanges between the fungal and the host cells take place, there is no direct symplastic continuity between both partners. Therefore, Pi must pass through the interfacial apoplast before it can be absorbed (Peterson & Bonfante 1994). P transfer from fungal hyphae must be sufficient to meet host plant demand (Smith & Smith 1990). Such conditions would therefore be a prerequisite for effective symbiotic association. P efflux rates have been measured from fungal hyphae grown *in vitro* but their values were much lower than the flows of P across the apoplasmic space and the estimated P influx rates into plant cells (Smith *et al.* 1994). It has been suggested that specific conditions at the mycorrhizal interface enhance P efflux (Smith *et al.* 1994). Among them, specific signalling by the host cells in the Hartig net could enhance the P flow from the fungal cells. Interestingly, direct contact between the fungus and the plant cells is not necessary for the induction of changes in fungal partner behavior (Horan & Chilvers 1990; Kim *et al.* 1999; Martin *et al.* 2001; Plett *et al.* 2011) suggesting that signalling occurred between the ectomycorrhizal fungus and its host plant before the formation of ectomycorrhizal roots. Such interactions could also impact fungal P metabolism and lead to P efflux from the fungus toward the external medium.

In this work, we hypothesize that the host plant is able to exert specific effects on P efflux from the fungal cells through the hydrolysis of fungal polyP. We addressed this hypothesis using an *in vitro* system enabling the plant and the ECM fungus to interact in the same bathing solution taken as an experimental model of the symbiotic interface. We used the ECM fungus *Hebeloma cylindrosporum* grown *in vitro* to obtain mycelia enriched in long-chain polyP. The mycelia were then incubated without plant or with host (*Pinus pinaster*) or non-host (*Zea mays*) plant species. Maize was considered to be representative of AM-forming plants, so this experimental design should help to assess the specificity of the effect of plants forming ECM or AM symbiosis.

The first step of our study was to determine whether two P conditions, without P or with non-limiting P supply, could affect the rate of P accumulation and polyP formation in the hyphae of the ECM species, using ^{32}P labeling, *in vitro* and *in vivo* ^{31}P NMR and cytological staining. Our findings demonstrated that P application triggered a massive accumulation of P as newly synthesized long-chain polyP in *H. cylindrosporum* if previously grown under P-deficient conditions. We used therefore these polyP-enriched mycelia to carry out the rest of our study.

The second step was to investigate the influence of a host plant (*P. pinaster*) or a non-host plant (*Z. mays*) on the fungal P efflux and the fate of fungal polyP during an *in vitro* time-course experiment. ^{32}P labelled mycelia were used to quantify fungal P fluxes towards the plant compartments and the interaction medium without added P or carbohydrates, which was taken as a proxy for the interfacial apoplast. Also the fate of fungal P compounds, mostly polyP, was followed by *in vivo* ^{31}P NMR in unlabelled mycelia. The findings allowed a better understanding of the role of the host plant in the regulation of the fungal P metabolism at the root-fungus interface.

Material and Methods

Solutions

Three solutions were used in this study, namely (1) a complete nutrient solution to grow the fungus, (2) a mineral solution to grow the plants and (3) an interaction medium (IM) to incubate the plants and the fungus together. The complete fungal nutrient solution was based on N6 medium described by Louche *et al.* (2010) and contained 6 mM KNO₃, 4 mM KCl, 1 mM CaCl₂, 1 mM MgSO₄, 50 µg l⁻¹ thiamine hydrochloride, 0.5 ml l⁻¹ 1% Fe citrate solution, 0.2 ml l⁻¹ micronutrients of Morizet & Mingeau (1976) solution and 55 mM glucose, pH 5.5. This solution was used either without any added P (-P solution) or with 1 mM NaH₂PO₄ (instead of 3 mM in Louche *et al.* 2010) (+P solution). Where indicated +P solution was labelled with ³²P orthophosphate (KH₂³²PO₄ in water from Amersham, France, pH 4.5). The radiolabelled source was diluted to obtain an initial activity of 3.3 10⁴ Bq per µmol Pi in the solution. The plant mineral solution had the same pH and concentrations of thiamine hydrochloride, Fe citrate, micronutrients and MgSO₄ as the fungal nutrient solution. It did not contain any glucose, and other salts were 0.2 mM Ca(NO₃)₂, 0.6 mM KNO₃, 0.2 mM KCl and 0.2 mM KH₂PO₄ (Irshad *et al.* 2011). The IM was made without any P or carbon source. It contained only 0.2 mM MgSO₄ and 0.5 mM CaCl₂ and was buffered at pH 5.5 with 5 mM MES (2-N-morpholino-ethanesulfonic acid, 4-morpholineethanesulfonic acid). All these solutions were used after sterilization (20 min, 121°C). Where stated radioactive ³²P was added to the fungal complete nutrient solution under sterile conditions.

Biological material

Ectomycorrhizal fungal material

A dikaryotic strain (D2) of the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum* Romagnesi resulting from the plasmogamy of two compatible homokaryotic strains h1 and h7 (Debaud & Gay 1987) was used in this study. The fungus used in all experiments originated from 3-week-old stock cultures grown in the dark at 25 °C on agar nutrient medium (N6 medium) as described by Louche *et al.* (2010). The mycelia were always grown in glass flasks containing 40 ml of complete nutrient solution, without shaking (Scheromm *et al.* 1990) (Supporting Information Fig. S1). The fungal plug (8 mm diameter) was maintained at the surface of the medium with a nichrome wire. After 7 days in the complete nutrient solution containing 1 mM Pi the mycelia were transferred into new flasks containing the

complete nutrient solution without added Pi (-P) or with 1 mM Pi (+P). Before being transferred in the -P solution, mycelia were rinsed several times in the nutrient solution without P to eliminate the carryover solution from the hyphae, as described in Fig. S1. The fungus was grown for 5 additional days to obtain P-starved or P-sufficient mycelia, respectively (Supporting Information Fig. S2). Unless stated mycelia were then transferred for 16 h into the complete nutrient solution containing 1 mM Pi, whether labelled or not with ^{32}P (P-resupply or ^{32}P -resupply period). Afterward, mycelia were suspended, using the nichrome wire, inside 60 ml syringes equipped with a tap at their bottom and filled with 15 ml of IM. Each fungus was rinsed with successive withdrawn and addition of 15 ml of IM. For all experiments except one (experiment 1, ^{32}P rinsing experiment, see below), IM was renewed 5 times at 1, 15, 30, 45 and 90 min. This procedure allowed the Pi solution adhering to the fungal material to be discarded.

Plant material

The host plant was maritime pine (*Pinus pinaster* Soland in Ait. from the Médoc, Landes-Sore-VG source, France). The non-mycorrhizal seedlings were obtained from seeds surface-sterilized with 30 % (w/w) H_2O_2 solution for 40 min and soaked in sterile distilled water for 48 h at 4 °C. Germination was performed on agar-solidified media (10 g l⁻¹) containing 2 g l⁻¹ glucose to reveal any contamination. Plants were grown in test tubes (Plassard *et al.* 1994) and watered every week with 20 ml of mineral solution described above. After two months the mineral solution was removed and replaced by 0.2 mM CaSO_4 sterile solution for 1 week. Finally the CaSO_4 solution was removed and substituted by the IM for 24 h for root acclimatization to the medium. Maize (*Zea mays* L. var. Volga) seedlings were obtained from seeds surface-sterilized with 6 % H_2O_2 solution for 30 min and rinsed with sterile distilled water. Germination was carried out at 30 °C between two sterile sheets of filter paper previously wetted with deionized sterile water. After 6 days of germination plants were transferred to test tubes with 0.2 mM CaSO_4 solution for 3 days before undergoing an acclimatization period of 24 h in IM. All test tubes were placed in a growth chamber under a 16 h/8 h light/dark cycle at 25 °C/20 °C, 80 % /100 % RH, CO_2 concentration of about 350 $10^{-6} \text{ dm}^3 \text{ dm}^{-3}$ and a PAR (Photosynthetically Active Radiation) of approximately 400 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ (400–700 nm). At the beginning of the experiment pine and maize plants had similar total biomass.

Experimental approaches

Fate of P in H. cylindrosporum mycelia in pure culture

Four independent experiments were carried out to follow the fate of P taken up by *H. cylindrosporum* mycelia during a P-resupply period according to their previous P status (Supporting Information Fig. S2, Table S1)

Experiment 1. P-starved and P-sufficient ^{32}P labeled mycelia (6 replicates for each condition) were transferred from the radioactive solution (^{32}P -resupply period) to 60 ml-syringes filled with 15 ml of IM. Mycelia were rinsed about 18 times with 15 ml of IM during 360 min. After each rinsing period, IM was collected and substituted by fresh IM solution before being assayed for ^{32}P radioactivity. The ^{32}P remaining in the mycelia at the end of the experiment was also measured and the amount of Pi taken up by each mycelium calculated using the specific activity of phosphate in the nutrient solution.

Experiments 2 and 3. *In vivo* and *in vitro* ^{31}P NMR measurements were used to examine P-starved (n=6) and P-sufficient (n=6) non-labelled mycelia sampled without or after a P-resupply period (24 mycelia in total).

For *in vivo* ^{31}P measurements (experiment 2), the six mycelia were introduced into the 10-mm NMR tube as described by Roby *et al.* (1987). Mycelia were continuously perfused with IM and aerated with a stream of pure oxygen (Lee & Ratcliffe 1983). Acquisition conditions were: 90° radio frequency pulses angle, 20-s inter-scan delay giving a fully relaxed spectrum (Quiquampoix *et al.* 1993), 180 scans giving a total accumulation time of 1 h. The probe temperature was 25°C . Before Fourier transformation, *in vivo* spectra were apodized using a 10 Hz exponential function and zero-filled to 16 K points, then phased and baseline corrected. An aqueous solution of methylene diphosphonate (MDP) contained in a 1-mm capillary was centered in the inlet tube, as described by Roby *et al.* (1987). Peak assignments were carried out according to chemical shifts referenced to MDP at 16.38 ppm and from spectra of perchloric extracts that contained soluble, low-molecular weight constituents (see below). Vacuolar and cytoplasmic Pi were distinguished because of the pH dependent chemical shift. For quantification the peaks of both intracellular Pi and polyP were integrated and the sum fixed to 100 to give relative values. We also integrated the peak area of polyP and Pi which were then normalized relative to that of MDP.

For *in vitro* ^{31}P measurements (experiment 3), mycelia were fixed with liquid nitrogen and stored at $-80\text{ }^{\circ}\text{C}$ before extraction. For each treatment six frozen mycelia were crushed together with 1 ml of 70 % (v/v) perchloric acid. The frozen powder was then treated as described by Roby *et al.* (1987) to obtain a supernatant without HClO_4 that was freeze-dried and stored at $-80\text{ }^{\circ}\text{C}$ until required for NMR measurements. Perchloric acid extracts were obtained as follows: the freeze-dried material was dissolved in 1 ml of 40 mM MOPS (3-N-morpholino-propansulfonic acid) buffer, pH 7.8, containing 60 mM CDTA (1,2-diaminocyclohexanetetraacetic acid monohydrate) and 10 % D_2O (Roby *et al.* 1987). ^{31}P NMR spectra were recorded on a Varian UNITY INOVA 500 MHz spectrometer operating at 202.4 MHz for ^{31}P , equipped with a 10-mm broad band probe. The deuterium resonance of $^2\text{H}_2\text{O}$ added into the extract was used as a lock signal. Spectra were acquired at $25\text{ }^{\circ}\text{C}$ using a 90° pulse angle, a 4-s inter-scan delay and a ^1H decoupling. *In vitro* spectra were apodized using a 2 Hz exponential line broadening, zero-filled to 16 K points then phased and baseline-corrected. A sufficient number of accumulations were used to give an acceptable signal to noise ratio. Peak assignments were established from chemical shifts of standard solutions containing known phosphorylated molecules and confirmed by direct addition of commercially available compounds.

Both experiments were repeated twice independently (Table S1). For each experiment the six replicates were pooled together to increase the signal to noise ratio and to decrease the time required to get NMR spectra of good quality after 1 h. ^{31}P spectra were processed and analyzed using both VnmrJ and ACDlabs softwares.

Experiment 4. PolyP granules were examined in P-starved ($n=3$) and P-sufficient ($n=3$) mycelia, without or after the 16 h P-resupply period, for a total of 12 mycelia (Supporting Information Fig. S2). An independent experiment (denoted exp 4*) was carried out with P-starved mycelia to assess the dependence of polyP granule formation on P_i concentration and incubation time. Those mycelia were incubated in complete nutrient solution supplied with 0.01 or 0.1 mM NaH_2PO_4 for 1 and 16 h (3 replicates per condition, giving 12 mycelia in total). For the observation of polyP, mycelia were placed in a solution of 95 % ethanol to precipitate polyP before staining. The fungal material was stored in ethanol at $4\text{ }^{\circ}\text{C}$ until analysis (Ashford *et al.* 1999).

Plant interaction experiment

Experiments 5 and 6. The 60 ml syringe, closed at the bottom by a valve and equipped at the top with a teflon tube for the supply of sterile air, was used as an *in vitro* experimental device to study the interaction between the fungus and the plant (Supporting information Fig. S3). Independent plant interaction experiments were carried out with P-starved mycelia using either ^{32}P labelled (experiment 5) or unlabelled P (experiment 6) (Fig. 1). After the 16 h P-resupply period, mycelia were transferred from the glass flasks to the syringes filled with 15 ml of IM. Mycelia were rinsed 5 times for a total of 90 min as described above. The syringes were filled with 60 ml of a new IM (t0) and the fungi were incubated alone (no plant) or with roots from three maize plants (with *Z. mays*) or three pines (with *P. pinaster*). This incubation was carried out under continuous light for up to 48 h and six syringes per treatment (no plant, with *Z. mays* and with *P. pinaster*) were destructively sampled after 6, 24, 48 h recorded t6, t24 and t48, respectively (Fig. 1). The total number of syringes was 54 (corresponding to 3 conditions x 3 sampling times x 6 replicated syringes). At each sampling time fresh weights of mycelia and plants and volume of IM were recorded. For the radiolabelled experiment (experiment 5), amounts of radioactivity in mycelia, plants and IM were assayed as described below. For the non-radiolabelled experiment (experiment 6), mycelia were pooled and introduced in the 10-mm NMR tube, and *in vivo* ^{31}P NMR measurements were carried out using the same conditions as described above. Both experiments were repeated twice, giving 4 plant-interaction experiments in total (Table S1).

Chemicals and chemical assays

Unless stated, all chemical compounds were of analytical grade and were purchased from Sigma. Radioactivity was measured in a scintillation counter (Beckman LS5801). ^{32}P released from the fungal material was measured using the Cerenkov effect by mixing 1 ml of IM with 4 ml of demineralised water in a scintillation vial. For solid samples, 5 ml of scintillation liquid were added to the vial to increase the radioactivity counting efficiency. The ratio of counting efficiencies in water and scintillation liquid was measured by comparing counts for the replicate samples in either water or scintillation liquid. Data were corrected for decay and the efficiency ratio obtained was then used in calculations to normalize all radioactivity measurements.

For observation of polyP granules, mycelia were rinsed with distilled water before staining with Toluidine Blue O (Ref T3260, from Sigma) as described by Ashford *et al.* (1999). Three fungal pieces per mycelium were mounted with water and observed under an optical microscope (Olympus BX 61). Images were taken with a Color View II® camera using Analysis® software (Soft Imaging System, Munster, Germany). Images were analysed using ImageJ (<http://imagej.nih.gov/ij/>) software to determine the number of metachromatic granules in individual hyphae (n=12 per treatment).

Statistical treatment

Excepted for ^{31}P NMR measurements, all other results are given as means calculated from 6 (^{32}P labelling experiments) to 12 (image analysis for polyP granules) measurements and SEM. The differences between means were analysed by one-way ANOVA followed by Tukey's HSD post-hoc test using Statistica 7.1 (StatSoft Inc., Tulsa, OK, USA). Normality was tested using the Kolmogorov Smirnov test and, where necessary, the data was either square root or log10 transformed prior to analysis to meet the assumptions of ANOVA. For ^{31}P NMR measurements, data in percentage were transformed ($\arcsin \sqrt{x}$) as described in Legendre & Legendre (1998) before ANOVA.

Results

Effect of P-resupply on P accumulation in *H. cylindrosporum* hyphae using ^{32}P and ^{31}P NMR

Figure 2a shows the amounts of ^{32}P measured in the IM of P-starved and P-sufficient *H. cylindrosporum* mycelia after P-resupply period with the ^{32}P tracer (experiment 1, Fig. S2). The amounts of radioactivity were measured over 6-h rinsing experiments. Whatever the P status of the mycelia, they released huge amounts of radioactivity during the first 40 minutes of rinsing, suggesting important nonspecific binding of the tracer at the surface of the fungal cells. Thereafter, only small increases in the amount of ^{32}P were measured in the IM either in P-starved or P-sufficient mycelia. These findings indicate that about 40 minutes were required for the complete removal of unbound ^{32}P from free space and cell walls. At the end of the rinsing period, the total amounts of ^{32}P released in IM by P-starved and P-sufficient mycelia were not significantly different (Fig. 2a). In contrast, ^{32}P values remaining in P-starved and P-sufficient mycelia at the end of the rinsing period were markedly different (Fig. 2b). The corresponding values of P accumulation calculated using ^{32}P phosphate specific

activity in the culture medium were also different with 3.6 ± 0.7 and $0.8 \pm 0.2 \mu\text{mol g}^{-1}$ fungal FW in P-starved and P-sufficient mycelia, respectively. These data indicated that P-starved mycelia accumulated four times more P than P-sufficient mycelia during their exposure to the P-resupply period.

Figure 3 shows *in vivo* ^{31}P NMR spectra from P-starved and P-sufficient mycelia without P-resupply (experiment 2, Fig. S2). Signal identification was carried out according to chemical shifts reported in other ectomycorrhizal fungi (Gerlitz & Werk 1994; Martins *et al.* 1999). We also used the spectra of *H. cylindrosporum* perchloric extracts obtained from P-starved and P-sufficient mycelia without and after P-resupply (experiment 3, Supporting Information Fig. S4). The *in vivo* NMR spectra of P-starved and P-sufficient mycelia exhibited same ^{31}P resonance signals. The first three signals visible in all spectra were assigned to cytoplasmic Pi (peak b, 1.6 ppm), vacuolar Pi (peak c, 0.4 ppm) and to glycerophosphocholine (peak d, 0.05 ppm). Peaks e (-11 ppm) and f (-13 ppm) were attributed to P groups of UDP-N-acetylglucosamine. The last peak (g) at -22 ppm corresponded to the central Pi residues of NMR-visible polyP chain (Martin *et al.* 1983). Vacuolar Pi represented the major part of NMR-visible P. As shown in Table 1, the sum of cytoplasmic and vacuolar Pi represented always a great proportion of intracellular P with a value of about 87 %. In contrast, the proportion of NMR-detectable polyP was very low, whatever the P pre-treatment applied to the mycelia. The polyP fraction reached a maximum of 14 % of internal Pi in P-starved mycelia. When the external Pi was increased, the polyP content slightly decreased to 10 and 7 % of the total internal Pi in P-starved and P-sufficient mycelia, respectively. The 6-h rinsing period did not change the proportion of NMR-visible polyP in P-starved and P-sufficient mycelia (data not shown).

Effect of P-resupply on the P pool in *H. cylindrosporum* hyphae observed by cytological staining

Figure 4 shows the accumulation of metachromatic granules in P-starved and P-sufficient *H. cylindrosporum* hyphae, without and after a P-resupply period of 16 h (experiment 4, Fig. S2). Without P-resupply it was not possible to detect any metachromatic granules in P-starved mycelia whereas P-sufficient mycelia contained numerous granules in their hyphae. Exposure to P-resupply led to the formation of polyP granules in previously P-starved mycelia to reach the number measured in P-sufficient mycelia. In contrast, the density of polyP granules in P-sufficient mycelia was not significantly modified after P-resupply (Fig.

4). In a parallel experiment (experiment 4*, Fig. S2), we studied time and P concentration dependence of polyP granules formation during P-resupply by incubating P-starved mycelia for 1 and 16 h in a nutrient solution containing concentrations of NaH₂PO₄ of 0.01 or 0.1 mM instead of 1 mM. Table 2 shows that *H. cylindrosporium* hyphae began to accumulate *de novo* synthesized metachromatic after only one hour of P-resupply. Thereafter, granule densities were not significantly different between P concentrations. Increasing incubation time up to 16 h lead to significantly higher densities of granules measured in hyphae, whatever the Pi concentration supplied to the nutrient solution. Interestingly, the densities of polyP granules observed at low Pi concentrations (such as 0.01 mM) were very close to those observed when Pi is supplied at 1 mM (compare Fig. 4 and Table 2). Therefore, P-starved mycelia were able to synthesize *de novo* a large amount of total polyP during P-resupply, even at very low Pi concentrations.

The impact of *P. pinaster* and *Z. mays* on ³²P release from *H. cylindrosporium* mycelia

The effect of a host or a non-host plant on P fluxes from *H. cylindrosporium* mycelia was investigated using the ability of P-starved mycelia to accumulate a massive amount of P as polyP after the resupply of P. Figure 5 shows the amounts of ³²P released by *H. cylindrosporium* incubated alone (no plant) or with *Z. mays* or *P. pinaster* (experiment 5, Fig. 1) and measured in plants or in the IM. For both plant species accumulated ³²P increased continuously with incubation time (Fig. 5, upper part). Measurements of ³²P in the IM showed that the fungus incubated alone (no plant) released low amounts of ³²P that did not increase continuously over time (Fig. 5, lower part). In contrast to ³²P accumulation in plant tissues, the amounts and the patterns of ³²P release in IM when plants are interacting with the fungus were markedly different between the two plant species. With the non-host plant (*Z. mays*), the amounts of ³²P were first smaller (6 h), not different (24 h) and higher (48 h) than those measured with no plant. Considering the whole duration of incubation, the ³²P release by *H. cylindrosporium* mycelia interacting with *Z. mays* increased slightly with time but remained low after 48 h (91 Bq g⁻¹ fungal FW) (Fig. 5, lower part). Thus, most of fungal ³²P released (about 75 %) was accumulated in maize plants. By comparison, the amounts of fungal ³²P released into the IM resulting from the co-incubation with the host plant (*P. pinaster*) were much greater than the values measured with no plant after 24 and 48 h of interaction. In *P. pinaster* treatments, fungal ³²P in IM increased regularly with interaction time to reach about 348 Bq g⁻¹ fungal FW at 48 h, representing approximately half of total ³²P released by the fungus incubated with *P. pinaster* (Fig. 5). Finally, as shown in

supplementary Table S2, the total amount of ^{32}P released by *H. cylindrosporum* increased with *Z. mays* and *P. pinaster*, indicating that P efflux was stimulated by the presence of plants. More remarkably, when the fungus was incubated with its host plant *P. pinaster* fungal release of ^{32}P was enhanced two-fold compared to *Z. mays* treatments (Table S2). The total amount of ^{32}P released by the fungus corresponded to 0.35 %, 5.3 % and 9.6 % of initial radioactivity for no plant, with *Z. mays* and with *P. pinaster* after 48 h, respectively.

The impact of *P. pinaster* and *Z. mays* roots *in vitro* on ^{31}P NMR-visible polyP in *H. cylindrosporum* mycelia

Figure 6 shows *in vivo* ^{31}P NMR spectra of *H. cylindrosporum* P-starved mycelia re-supplied with ^{31}P and then incubated over 48 h without seedlings (no plant) or with *Z. mays* or *P. pinaster* (experiment 6, Fig. 1). Peak identification was carried out according to chemical shifts and from spectra of perchloric extracts (Supporting Information Fig. S4). The four expected signals for P metabolites that are cytosolic and vacuolar Pi, UDP-N-acetylglucosamine and central polyP (Fig. 3), were present in the spectra of *H. cylindrosporum* mycelia either incubated alone (no plant) or with plants (*Z. mays* and *P. pinaster*) (Fig. 6). However, the peak attributed to glycerophosphocholine (0.05 ppm) in P-starved and P-sufficient mycelia (Fig. 3) was observed in no plant-spectra but disappeared from *Z. mays* and *P. pinaster*-spectra (Fig. 6). Considering the whole duration of experiment, the most obvious changes in ^{31}P NMR spectra involved mainly central polyP and intracellular (cytoplasmic and vacuolar) Pi pools. The changes in relative central polyP and Pi contents, estimated as the intensity ratio of these signals, were first integrated in spectra from each condition and incubation period and are given in Table 3a.

In agreement with our previous results (see Table 1), the proportion of NMR-visible central polyP chains in no plant-mycelia was low, ranging from 7 to 9 %. When incubated with maize seedlings, *Z. mays*-mycelia contained also a small proportion of polyP, ranging from 6 to 10 %. By contrast, in *P. pinaster*-mycelia NMR-detectable polyP proportion was increased up to between 24 and 26 %.. This increase was constant over the 48 h-period (Table 3a). When the values were averaged over the whole incubation period, the proportion of NMR-detectable polyP in *P. pinaster*-mycelia was significantly increased compared to no plant- and *Z. mays*-mycelia. This increase corresponded to a decrease in the proportions of Pi (Table 3a). As the same reference (MDP) was used in all the NMR experiments, we also integrated the peak area of polyP and Pi relative to that of MDP arbitrarily fixed to 1 (Table 3b). When

averaged over the whole incubation period, only the polyP fraction in *P. pinaster*-mycelia was significantly higher than the means calculated in no plant- and *Z. mays*-mycelia whereas the Pi fractions were not significantly different among the three treatments (Table 3b).

Discussion

P starvation and increased fungal accumulation of P: what is the role of polyP?

The labelling experiment carried out with *H. cylindrosporum* mycelia grown in pure culture showed clearly that P starvation had a major impact on the subsequent accumulation of P by the fungal cells. P-starved mycelia accumulated about 4 times more P than P-sufficient mycelia when measured after the rinsing period. The over-accumulation of P by P-starved microbial cells has previously been reported and attributed to the capacity for synthesizing molecules of polyP through the “polyP overplus” (see Harold 1966). As “polyP overplus” has been demonstrated in yeast (Harold 1966; Trilisenko *et al.* 2002) and in AM fungi (Ezawa *et al.* 2004; Hijikata *et al.* 2010; Kikuchi *et al.* 2014), one should expect its occurrence in ECM fungi. Surprisingly, our results showed that the relative contribution of *in vivo* ^{31}P NMR-visible polyP over total inorganic P did not vary with P-status of mycelia without and after P-resupply. In contrast, dye application revealed the presence of metachromatic granules synthesized by P-starved fungal cells during the P-resupply period and this *de novo* synthesis of polyP could explain the difference of ^{32}P accumulation observed in P-starved and P-sufficient mycelia. Our results are in agreement with those obtained by Lapeyrie *et al.* (1984) who showed that the ECM fungus *P. involutus* did not contain any metachromatic granules after P-starvation and abundant populations of granules after the transfer of these P-starved mycelia in P containing solution. The discrepancy between the results obtained with *in vivo* ^{31}P NMR and dye application may be explained if the mechanism of polyP synthesis in ECM fungi is similar to that described in yeast (Langen *et al.* 1962, cited in Harold 1966). Using differential extraction, four distinct polyP fractions were identified in yeast, whose average length were 260 (fraction 4), 55 (fraction 3), 20 (fraction 2) and 4 (fraction 1) Pi residues. Using radiolabelled P supply, it was shown that ^{32}P was most rapidly incorporated into the high molecular weight fraction 4, whereas incorporation into other fractions was slower (Langen *et al.* 1962, cited in Harold 1966). In addition, the same authors demonstrated that P starvation increased the rate of polyP synthesis. Therefore, our results could be explained by a preferential incorporation of the newly absorbed Pi in starved cells in long-chain polyP of high molecular weight that are not visible using *in vivo* ^{31}P NMR because they are not freely

mobile. The chain length of these polyPs could be greater than 200 Pi residues as measured in yeast (Vagabov *et al.* 2008) or AM fungi (Ezawa *et al.* 2004). The size of ^{31}P NMR visible polyP can be calculated if the peak corresponding to the resonance of the end or the penultimate phosphates is higher than the signal-to-noise ratio. In *H. cylindrosporum*, such calculation can be made only using *in vitro* spectra, giving a chain length around 10 Pi residues. In contrast, *in vivo* spectra displayed only internal P of polyP, suggesting that they are longer than 10 Pi residues. The same situation was observed in spectra from *C. graniforme* by Martin *et al.* (1985). These authors estimated the minimal chain length of polyP *in vivo* to be not less than 30 P residues. Our results suggest that *H. cylindrosporum* may contain at least two pools of polyP, the first comprised of rather long-chain polyP not visible by *in vivo* ^{31}P NMR that is specifically increased by P-resupply in P-starved fungal cells and the second comprised of polyP of short-chain that could be around 30 Pi residues long in living mycelia that is relatively stable and did not seem to depend on P status of fungal cells.

External Pi concentration can also affect the rate of polyP accumulation. In our study we supplied 1 mM Pi during the P-resupply, an extremely high concentration when compared with Pi concentration in soil solution, which is usually considered to be in the range 1-10 μM (reviewed by Hinsinger 2001). However our results showed that the synthesis of new polyP revealed by cytochemistry was not restricted to this high P supply (1 mM) since metachromatic granules were detected after exposure to Pi concentrations as low as 0.01 mM. Our results are comparable to those reported in *P. involutus* P-starved mycelia which also started to accumulate metachromatic granules in solutions as dilute as 0.005 mM Pi (Lapeyrie *et al.* 1984). However, these authors reported that at least 4 hours were required to observe granules in *P. involutus* hyphae whereas one hour of incubation was sufficient for *H. cylindrosporum*. These differences could be explained by the use of older biological material and by the radial cutting of the mycelia leading to cellular damage in the case of experiments done with *P. involutus*. Taken together, these results strongly suggest that polyP synthesis operates in P-starved ECM species such as *H. cylindrosporum* and *P. involutus* at external P concentrations similar to those that can be found in the soil solution that are in the micromolar range. Such ability to synthesize new polyP by P-starved fungal cells would increase the capacity of the fungus to exploit patches of P rich soil that can be found in natural conditions.

The host plant *P. pinaster* enhanced ^{32}P efflux from *H. cylindrosporium* mycelia

Calculated over the whole period of incubation (48 h), *H. cylindrosporium* hyphae released a very low proportion (on average 0.35 %) of the ^{32}P accumulated during the P-resupply period when incubated alone in the IM. This value is much lower than those measured in three ECM species (*P. tinctorius*, *P. involutus* and *H. crustuliniforme*) by Cairney & Smith (1993a). These authors found a release of ^{32}P ranging from 7 to 11 % of absorbed ^{32}P when rinsed mycelia were transferred for 8 h in distilled water. Depending on the fungal species, these values were multiplied by 2 or 3 by the presence of external KCl supplied at 20 mM compared to KCl 0.35 mM (Cairney & Smith 1993b). These authors found also that the addition of divalent cations such as Ca^{2+} (CaCl_2 1 mM) decreased five times this KCl-enhanced ^{32}P efflux from the mycelia of *P. tinctorius* whereas Mg^{2+} (MgCl_2 1 mM) had no effect (Cairney & Smith 1993b). The IM used in the present study contained no KCl and 0.5 mM CaCl_2 and these ionic conditions may therefore explain the low rate of ^{32}P efflux from the mycelium incubated alone. The low proportion of ^{32}P absorbed by the mycelia and lost by the fungal cells incubated without plants also indicates that our experimental system was not stressful for the fungal cells, making it suitable to study the effect of the plant on fungal ^{32}P loss and P metabolism.

In contrast to the results obtained with the fungus incubated alone, the presence of plants dramatically increased total ^{32}P efflux from the fungus by a factor 15 and 25 for maize and pine plants, respectively, after 48 h of incubation. At a first glance, the P efflux appeared not to be ECM host plant specific, because roots of the non-host plant *Z. mays* could also induce efflux. Remarkably, the IM bathing maize roots contained much less ^{32}P than pine roots, suggesting that maize roots were able to take up ^{32}P as soon as it is released by the fungus. Such an immediate absorption by maize roots would maintain a concentration gradient between intracellular P concentration in fungal cell and the external medium. This gradient would maximize P efflux and permit a net movement of P to the plant (Cairney & Smith 1993a). In contrast, the ^{32}P efflux caused by the host plant *P. pinaster* was two times greater than that measured for *Z. mays*. Therefore, at least a part of the P efflux from *H. cylindrosporium* mycelia was closely related to the presence of host plant roots. More significantly, pine roots appeared to increase strongly the ^{32}P release into the IM, while the accumulation of ^{32}P by *Z. mays* and *P. pinaster* was similar suggesting that the P efflux exceeded even the instant requirements of pine. Such an accumulation of ^{32}P in the IM is less in favor of a passive release of P from the mycelium. These results suggest a host plant

specific efflux of ^{32}P independently or in addition of a gradient between the fungus and the external medium containing the plant.

PolyP metabolism of *H. cylindrosporum* mycelia was specifically modulated by *P. pinaster* to mobilise the P pool for enhanced P efflux

In our experiment, it was not possible to calculate the absolute size of polyP and Pi pools in the mycelia. However, the variation of peak area normalized against the reference signal (MDP) in the spectra reflects the changes in the size of polyP and Pi pools. The values showed that the signals of polyP in *P. pinaster*-mycelia were on average 3.6 times higher than those found in no plant- or *Z. mays*-mycelia (Table 3b). This supports the hypothesis that the relative changes of polyP and Pi percentages (Table 3a) are due to an increase of short-chain polyP and not to a decrease of the vacuolar Pi pool in *P. pinaster*-mycelia. In addition, the proportions of polyP and Pi (in %) in each plant treatment were constant from 6 to 48 h, indicating that a controlled polyP/Pi homeostasis occurs in the vacuole. Therefore, the maintenance of this homeostasis in the vacuole of mycelia incubated with *P. pinaster*, together with a continuous Pi efflux, could be mainly explained by the enhanced hydrolysis of long-chain polyP into NMR visible short-chain polyP.

Therefore, only the host plant *P. pinaster* was able to specifically enhance polyP hydrolysis in *H. cylindrosporum* mycelia. Grellier *et al.* (1989) also found that the amounts of polyP in the fungal cells from ectomycorrhizal roots of *P. involutus* associated with *Betula pendula* were higher than those detected in pure cultures of this fungus. More significantly, the proportions of NMR-detectable polyP calculated in our experiments for *P. pinaster*-mycelia are comparable with the values reported in excised ectomycorrhizas of this association, which ranged from 10 to 40 % (Grellier *et al.* 1989). The appearance of NMR-visible polyP, assumed to be short-chain polyP, in *P. pinaster*-mycelia could be explained by the following sequence of events. The first step would be the release by pine roots of a signal able to elicit a specific response targeting the fungal polyP metabolism, although the nature of this signal has not yet been elucidated (Garcia *et al.* 2015). The signal appears to be specific of ECM plants as we did not observe the same response with maize plants. Then the second step would be to increase the activity of endopolyphosphatases acting on long-chain polyP previously synthesized during the P-resupply period and resulting in the appearance of short-chain polyP. This hypothesis is supported by the observations reported in AM plants showing that AM roots of *Gigaspora margarita* contained a greater amount of short-chain polyP than

did the extraradical hyphae (Solaiman *et al.* 1999). These authors proposed that long-chain polyP might be partly hydrolyzed into shorter chains by endopolyphosphatase and further hydrolyzed with exopolyphosphatase to release Pi. The third step would be to increase the activity of exopolyphosphatases, as proposed by Solaiman *et al.* (1999), acting on short-chain polyP resulting in the production of free Pi, which can then be released in the IM representing the apoplast of ECM. Such enzyme activities involved in polyP hydrolysis have been measured in arbuscular mycorrhizal symbiosis (Ezawa *et al.* 2002) but have not yet been determined in ECM fungi.

In conclusion, the combination of all the results from the different approaches used in this work lead us to propose a schematic representation regarding the fate of Pi taken up by the fungal cells and its subsequent transfer to a non-host or host-plant (Fig. 7). During P-resupply, P-starved mycelia accumulated preferentially newly absorbed Pi in long-chain polyP. When the mycelia are incubated alone or with non-host plant, this pool of long-chain polyP was only marginally hydrolyzed, suggesting that the pool of free Pi was the main source of Pi released into the IM. In contrast, when the mycelia are incubated with host-plant, the pool of long-chain polyP is hydrolyzed to increase the pool of short-chain polyP, which is then hydrolyzed to supply the free Pi pool and the efflux of Pi in the IM. The activation of the polyP hydrolysis to sustain Pi efflux from the fungal cells is probably under the dependence of a specific signal released by the roots of the host-plant. This signal appears to be strictly specific of an ECM-forming plant as an AM-forming plant was not able to activate the polyP hydrolysis of *H. cylindrosporum*. Taken as a whole, our results suggest that the synthesis and transport of long-chain polyP and their subsequent hydrolysis in ECM might sustain fungal Pi release into the apoplast of Hartig net. They demonstrate also the usefulness of our simple experimental device that could be used in further studies aiming at elucidating the molecular mechanisms involved in P transfer from the fungus to the host during the ECM association.

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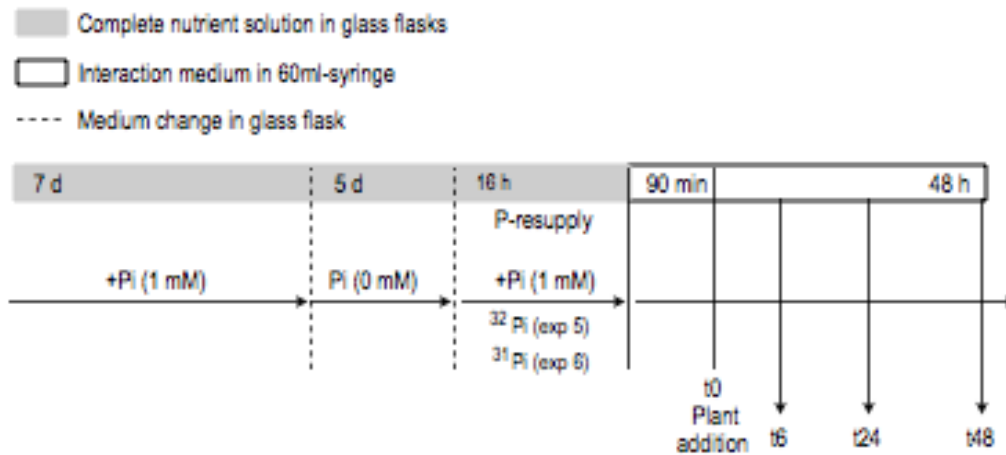


Figure 1. Experimental diagram of the plant interaction experiments.

Experiment 5 and 6 were run with ^{32}P labelled or ^{31}P non-labelled *H. cylindrosporum* mycelia, respectively. For both experiments, only P-starved mycelia were used. Mycelia were rinsed with IM for 90 min before addition of fresh IM (60 ml) and plants into the syringes (t0). For each experiment, syringes (18 per period) were collected after 6 (t6), 24 (t24) and 48 (t48) h of incubation. For one experiment, 54 syringes were set up to receive 54 mycelia (one per syringe), 54 maize plants (3 seedlings per syringe x 6 replicates x 3 periods) and 54 pine plants (3 seedlings per syringe x 6 replicates x 3 periods).

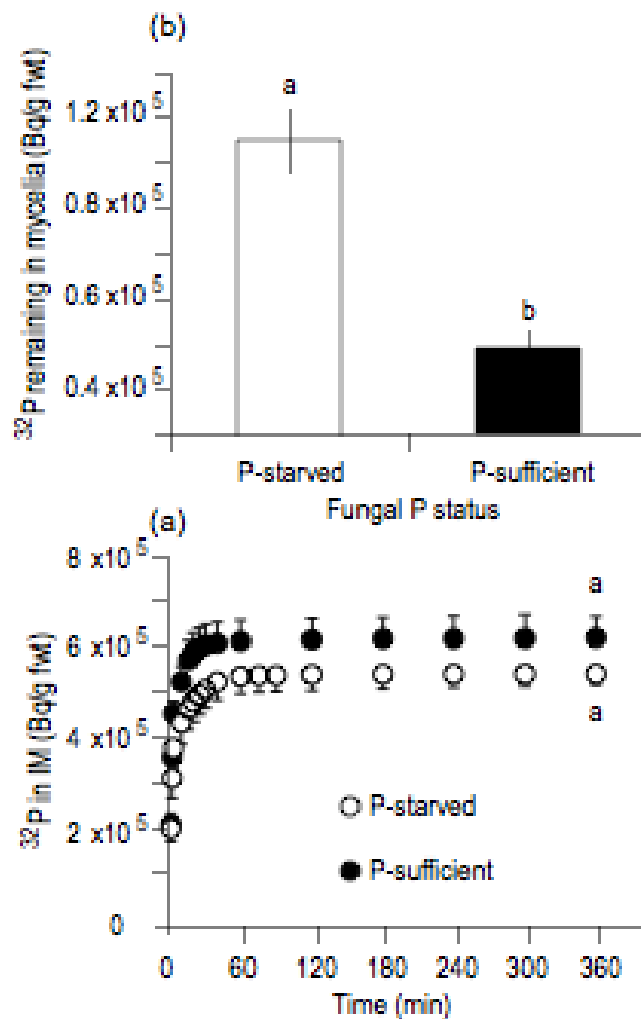


Figure 2. Fate of ^{32}P radioactivity (Bq g^{-1} fungal FW) in P-starved or P-sufficient *H. cylindrosporium* mycelia after ^{32}P -resupply.

(a) Cumulative amounts of radioactivity measured in the interaction medium (IM). (b) Amounts of radioactivity remaining in the mycelia at the end of the 360 min-rinsing period. The values are means with SEM ($n=6$). Different letters denote significant differences between means at $p<0.05$ (one-way ANOVA, Tukey's post-hoc test).

Mycelia were grown in glass flasks (see Fig. S1) with nutrient solutions and conditions described for experiment 1 (see Fig. S2).

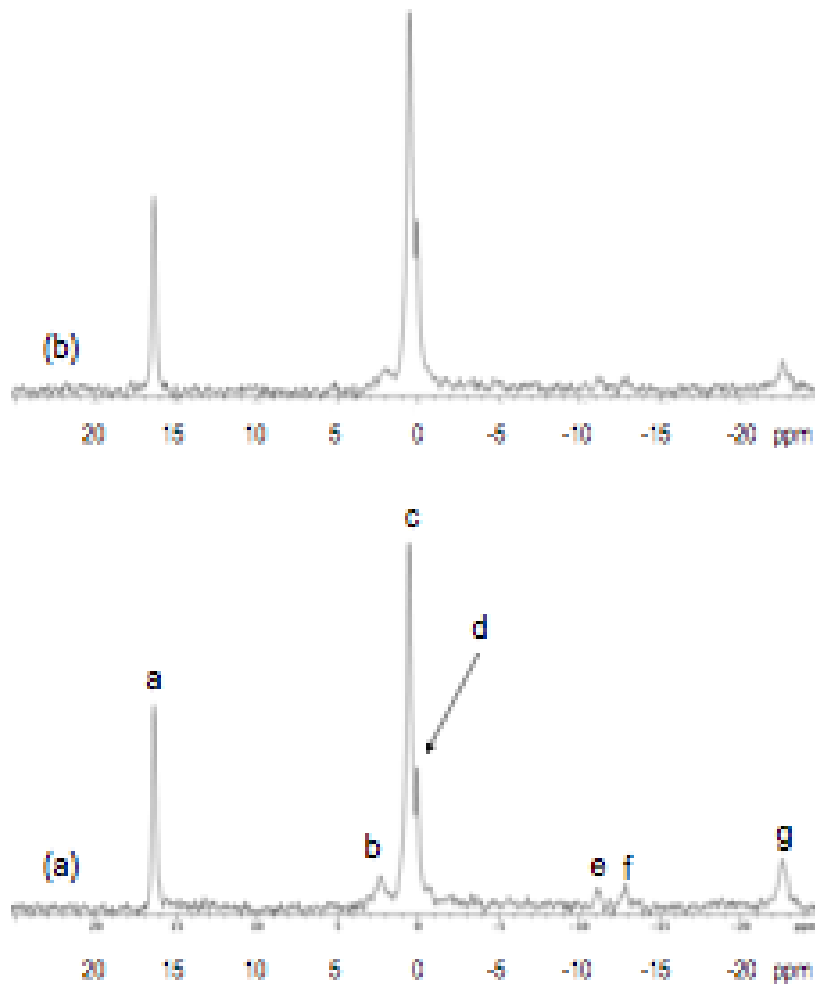


Figure 3. *In vivo* ^{31}P NMR spectra of *H. cylindrosporium* mycelia showing resonances for Pi and mobile polyP in P-starved (a) and P-sufficient (b) mycelia without P-resupply.

Peak assignments were as follows: a, external standard (MDP); b, cytoplasmic orthophosphate (Pi); c, vacuolar Pi; d, glycerophosphocholine; e, f, UDP-N-acetylglucosamine; g, soluble polyphosphate.

Mycelia were grown in glass flasks (see Fig. S1) with nutrient solutions and conditions described for experiment 2 (see Fig. S2). Spectra were recorded from six mycelia pooled together in the 10 mm-NMR tube.

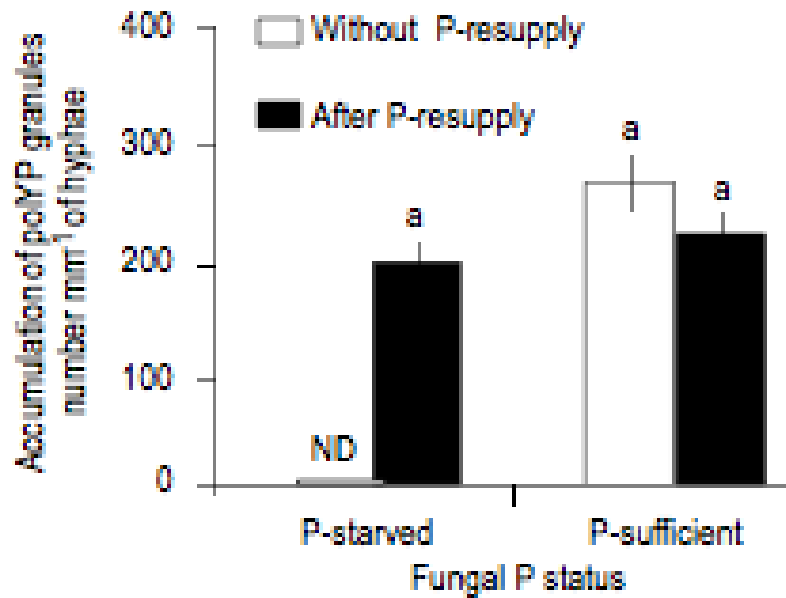


Figure 4. Number of polyP metachromatic granules in P-starved and P-sufficient *H. cylindrosporum* mycelia without P-resupply (white bars) and after P-resupply (black bars). Bars correspond to mean values with SE (n=12). ND means not detected. Different letters denote significant differences at $P < 0.05$

Mycelia were grown in glass flasks (see Fig. S1) with nutrient solutions and conditions described for experiment 4 (see Fig. S2).

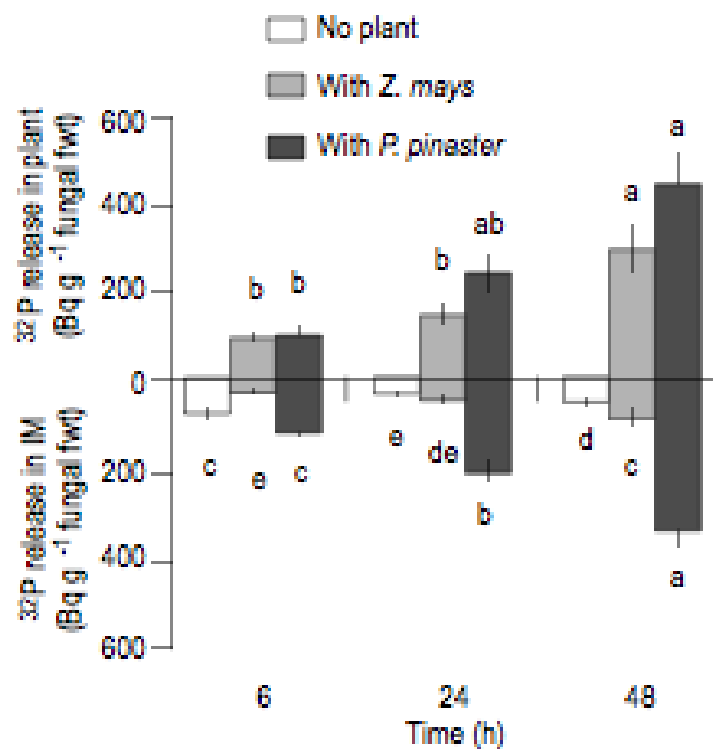


Figure 5. ³²P release by the ectomycorrhizal fungus *H. cylindrosporum* incubated with no plant or with *Z. mays* or *P. pinaster* and accumulated in plants (upper part) or in the IM (lower part). The amount of ³²P is expressed per gram of fungal FW. Error bars represent SE (n = 6). Letters indicate significant differences ($P < 0.05$) between treatments (no plant, with *Z. mays* and with *P. pinaster*) and incubation time (6 h, 24 h and 48 h).

Mycelia were prepared with nutrient solutions and conditions described in Fig. 1 (experiment 5).

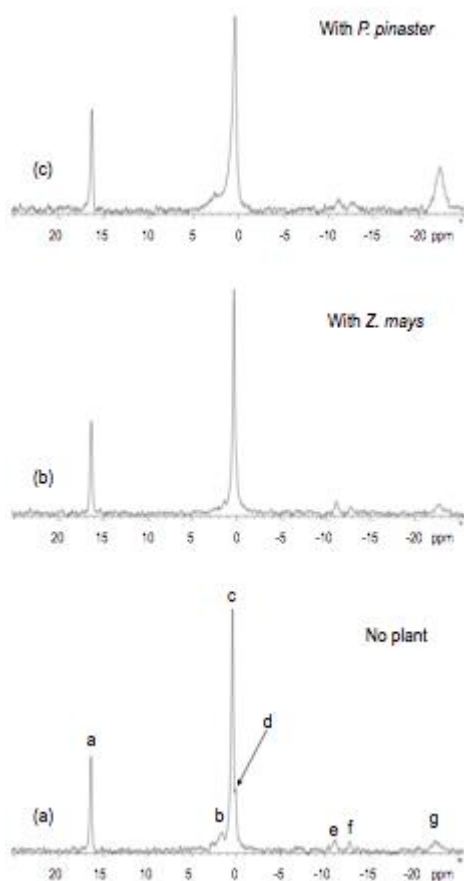


Figure 6. ^{31}P NMR spectra of *H. cylindrosporium* mycelium showing resonances from Pi and mobile polyP incubated with no plant (a) or with *Z. mays* (b) or *P. pinaster* (c).

Peak assignments were as follows: a, external standard (MDP); b, cytoplasmic orthophosphate (Pi); c, vacuolar Pi; d, glycerophosphocholine; e, f, UDP-N-acetylglucosamine; g, soluble polyphosphate. No glycerophosphocholine peak was observed in mycelia interacting with *P. pinaster* or *Z. mays*.

Mycelia were prepared with nutrient solutions and conditions described in Fig. 1 (experiment 6).

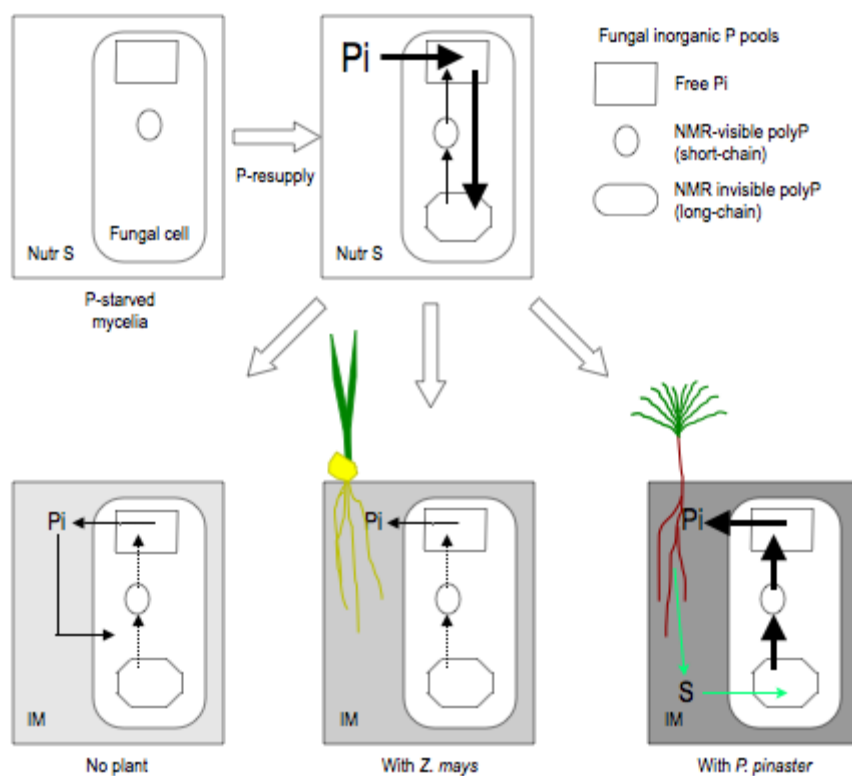


Figure 7. Schematic representation of the fate of Pi absorbed by P-starved mycelia during P-resupply and subsequent incubation of mycelia in IM either alone (no plant), or with *Z. mays* or with *P. pinaster* roots.

The intensity of grey in IM denotes the amount of fungal ^{32}P measured after 48 h of incubation. The weight and width of each black arrow indicates the magnitude of fluxes between P pools. Nutr S: nutrient solution, IM: interaction medium, S: unknown soluble plant signal.

Table 1. Proportion of NMR-visible polyphosphate (polyP) and cytoplasmic and vacuolar Pi (Pi) in P-starved and P-sufficient *H. cylindrosporium* mycelia determined from *in vivo* NMR ^{31}P spectra.

Mycelia were grown in glass flasks (see Fig. S1) with nutrient solutions and conditions described for experiment 2 (see Fig. S2).

Time	Signal	Proportion (%) in:	
		P-starved mycelia	P-sufficient mycelia
Without P-resupply	polyP	14	11
	Pi	86	89
After P-resupply	polyP	10	7
	Pi	90	93

Table 2. Density of metachromatic granules (expressed in number of granules per mm of hyphae) in P-starved *H. cylindrosporum* mycelia transferred in a complete nutrient solution containing different Pi concentrations during 1 h or 16 h (P-resupply period). Values are means \pm SEM (n=12). Effect of Pi concentration and time were analyzed with one-way ANOVA.

Mycelia were grown in glass flasks (see Fig. S1) with nutrient solutions and conditions described for experiment 4* (see Fig. S2).

Time (h)	P concentration (mM)		<i>P</i>
	0.01	0.1	
1	150 \pm 4	151 \pm 3	> 0.05
16	206 \pm 13	229 \pm 10	> 0.05
<i>P</i>	0.03	0.01	

Table 3. Variation of Pi accumulation in visible signals of ^{31}P NMR spectra in *H. cylindrosporum* P-starved mycelia incubated with no plant or with *Z. mays* or *P. pinaster*. Peak areas are expressed as (a) the percentage of total area of NMR visible P or (b) relative to the reference peak of methyl diphosphonate (MDP). Means correspond to the average of values recorded after 6, 24 or 48 h and different letters denote significant differences between means (one-way ANOVA, Tukey's post-hoc test).

Mycelia were prepared with nutrient solutions and conditions described in Fig. 1 (experiment 6).

Peak area	Signal	Plant treatment	Incubation period (h)			Mean
			6	24	48	
(a) Fraction (%) of total fungal P	PolyP	No plant	9	8	7	8 ^b
		With <i>Z. mays</i>	6	10	8	8 ^b
		With <i>P. pinaster</i>	26	25	24	25 ^a
		<i>P</i>				<0.001
(b) Fraction relative to MDP area	Pi	No plant	91	92	93	92 ^a
		With <i>Z. mays</i>	94	90	92	92 ^a
		With <i>P. pinaster</i>	74	75	76	75 ^b
		<i>P</i>				<0.001
(b) Fraction relative to MDP area	PolyP	No plant	0.45	0.39	0.41	0.42 ^b
		With <i>Z. mays</i>	0.29	0.48	0.37	0.38 ^b
		With <i>P. pinaster</i>	1.60	1.32	1.46	1.46 ^a
		<i>P</i>				<0.01
(b) Fraction relative to MDP area	Pi	No plant	4.32	4.76	5.39	4.82
		With <i>Z. mays</i>	4.58	4.18	4.56	4.44
		With <i>P. pinaster</i>	4.61	3.88	4.64	4.37
		<i>P</i>				>0.05