Title: Tit for Tat? A Mycorrhizal Fungus Accumulates Phosphorus Under Low Plant Carbon Availability

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Abstract

The exchange of carbohydrates and mineral nutrients in the arbuscular mycorrhizal (AM) symbiosis must be controlled by both partners in order to sustain an evolutionarily stable mutualism. Plants downregulate their carbon (C) flow to the fungus when nutrient levels are sufficient, while the mechanism controlling fungal nutrient transfer is unknown. Here, we show that the fungus accumulates nutrients when connected to a host that is of less benefit to the fungus, indicating a potential of the fungus to control the transfer of nutrients. We used a monoxenic in vitro model of root organ cultures associated with Glomus intraradices, in which we manipulated the C availability to the associated roots. We found that G. intraradices accumulated up to seven times more nutrients in its spores, and up to nine times more in its hyphae, when the C pool available to the associated roots was halved. The strongest effect was found for phosphorus (P), considered to be the most important nutrient in the AM symbiosis. Other elements such as potassium and chlorine were also accumulated, but to a lesser extent, while no accumulation of iron or manganese was found. Our results suggest a functional linkage between C and P exchange.

Introduction

Earth’s primary production depends to a great extent on the mycorrhizal symbiosis, as mycorrhizal fungi supply the plants with essential mineral nutrients, in exchange for photosynthetically fixed carbohydrates (Smith & Read, 2008). Arbuscular mycorrhizal fungi (AMF) are believed to have coevolved with the first plants colonizing land (Remy et al., 1994; Redecker et al., 2000), enabling the early plants with their simple roots to cope with the poor nutrient conditions in soil, in particular with low phosphorus (P) availability. In today’s agriculture, P supply is often ensured by the excessive application of mineral fertilizers. However, Earth’s P resources are limited because P is a nonrenewable resource, and P fertilizers are being lost continuously by leaching into the sea. In the future, the use of biotechnological applications such as AMF inoculation for more efficient P uptake may therefore become more important in agriculture and horticulture, and the mode of its transfer needs to be understood.

Up to 100% of the P in plants may be derived from the mycorrhizal pathway (Pearson & Jakobsen, 1993; Ravnskov & Jakobsen, 1995; Smith et al., 2003). Plants seem to rely on fungal P supply when colonized, as AM formation can lead to the downregulation of plant P transporters (Maldonado-Mendoza et al., 2001; Smith et al., 2003). In return, around 20% of host photosynthates can be allocated to the fungus (Jakobsen & Rosendahl, 1990; Douds et al., 2000; Bago et al., 2003). AMF are believed to be completely dependent on their host plant for their carbon (C) supply, because they are unable to complete their life cycle without the host plant (Smith & Read, 2008) and cannot take up sugars through their extraradical mycelium (Nakano et al., 1999; Pfeffer et al., 1999; Bago et al., 2000).

The exact regulation of the nutrient exchange still remains obscure. A model of the interface between the arbuscule and the root cell of the host plant is illustrated in Fig. 1. The root cortical cells release sucrose into the interfacial apoplast (Smith et al., 1994; Bücking & Schachar-Hill, 2005), which is hydrolysed by an acid invertase into hexoses (Schaarschmidt et al., 2006) that can be taken up by the fungus (Schüßler et al., 2006; Fig. 1, [1]). Once taken up by the fungus, the C is incorporated into lipids and transported throughout the fungal mycelium (Pfeffer et al., 1999; Bago et al., 2002). The
Lipids are metabolized through the glyoxylate cycle (Bago et al., 2000; Lammers et al., 2001) and represent the major C source for supporting the extension of the extraradical mycelium in the soil (Bago et al., 2000; Douds et al., 2000). The hyphal tips take up P via high-affinity inorganic P (Pi) transporters, which is transported within the hyphae in the form of polyphosphate. The polyphosphate is depolymerized by alkaline phosphatases, and Pi is released into the interfacing apoplast in an as yet unknown manner. Plants take up the Pi by mycorrhiza-specific Pi transporters in the peri-arbuscular membrane. C release to the fungus declines under high plant P levels, and plant peri-arbuscular Pi transporters are downregulated. Premature arbuscule senescence is induced under low P delivery. Fungal nutrients can stimulate plant C release. Hyphal P uptake is P induced, as the P transporter gene is regulated by the environmental P concentration, but C flow to the fungus does not influence P uptake from the medium. We hypothesized that the fungus can control the P transfer to plants that provide low C benefit.

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Fig. 1. A conceptual model of the nutrient exchange between plants and AMF. The fluxes are marked by numbers and (possible) regulation mechanisms as letters in square brackets: [1] Cortical cells release sucrose into the interfacing apoplast, which is hydrolysed by an acid invertase into hexoses that can be taken up by the fungus. [2] The extraradical hyphal tips take up P via high-affinity inorganic P (Pi) transporters, which is transported within the hyphae in the form of polyphosphate. [3] The polyphosphate is depolymerized by alkaline phosphatases, and P, is released into the interfacing apoplast in an as yet unknown manner. [4] Plants take up the P, by mycorrhiza-specific P, transporters in the peri-arbuscular membrane. [a] C release to the fungus declines under high plant P levels, and plant peri-arbuscular P transporters are downregulated. Premature arbuscule senescence is induced under low P delivery. [b] Fungal nutrients can stimulate plant C release. [c] Hyphal P uptake is P induced, as the P transporter gene is regulated by the environmental P concentration, but C flow to the fungus does not influence P uptake from the medium. [d] We hypothesized that the fungus can control the P transfer to plants that provide low C benefit.

For each partner in symbiosis, it is most beneficial to achieve the highest possible gain per unit investment. Hence, both parties need to have control mechanisms that prevent the other from developing parasitic behaviour (Johnson et al., 1997) that would lead to the collapse of the mutualistic association in an evolutionary perspective. There are examples of achlorophyllous plants cheating on their mycorrhizal fungi (Bidartondo, 2005), and also mycorrhizal fungi can cause growth reductions of the host plant compared with nonmycorrhizal individuals (Klironomos, 2003). However, plants growing in soils with high nutrient levels usually show reduced mycorrhizal colonization (Mosse, 1973; Olsson et al., 1997), indicating that they control the C transfer to the fungus, or that they control attraction of AMF via control of root signals (Akiyama et al., 2005), and only trade when they are in need of nutrients.

Under conditions of high plant P levels, the C release to the fungus declines (Olsson et al., 2002; Fig. 1, [a]), and plant peri-arbuscular P transporters are downregulated, reducing the P uptake via the fungal pathway (Nagy et al., 2009). Premature arbuscule senescence is induced under low P delivery (Javot et al., 2007). Plants must therefore be able to control either their C release through the membrane, their invertase activity or the reuptake of C from the apoplast. Fungal nutrients can stimulate plant C release (Harrison, 1996; Fig. 1, [b]), and C supply increases the cytoplasmic P levels within the IRM of the fungus (Bücking & Shachar-Hill, 2005). At the fungus–soil interface, hyphal P uptake is P induced, as the expression of the P transporter gene is regulated by the P concentration in the environment.
of the external hyphae (Fig. 1, [c]) (Maldonado-Mendoza et al., 2001), but C flow to the fungus does not influence P uptake from the medium (Olsson et al., 2006).

We hypothesized that the C–P exchange is functionally linked at the plant–fungus interface (Fig. 1, [d] in addition to [b]), and that the fungus can control the P transfer to plants that provide low C benefit. This would result in an increase of P within the fungal tissue under conditions of low plant C unless the fungal P uptake does not decrease (Olsson et al., 2006). We used a root organ culture model system, in which we could precisely regulate the amount of C available to the plant. We examined the changes in the elemental composition of the fungus resulting from reduced C availability to the host plant by using proton-induced X-ray emission (PIXE) analysis, which enabled us to quantify elements in spores and hyphae and locate them at a resolution of about 5 \( \mu \)m (Pallon et al., 2007).

Materials and methods

Experimental design

The experiment was performed in a model system consisting of root organ cultures of *Daucus carota* L. inoculated with *Glomus intraradices* Schenck & Smith (DAOM 197198; Biosystematics Research Center, Ottawa, Canada) (Bécard & Fortin, 1988; StArnaud et al., 1996), which was recently recommended to be renamed to *Glomus irregulare* (Stockinger et al., 2009). Two-compartment split-plates were used. One side, denoted the ‘root compartment’, was filled with minimal medium (Bécard & Fortin, 1988) containing 0.3% Phytagel\textsuperscript{TM} for stabilization (Sigma Chemical Co., St. Louis, MO). Half of the plates were provided with the full sucrose addition of 10 g L\(^{-1}\) of the external hyphae (Fig. 1, [c]) (Maldonado-Mendoza et al., 2001), but C flow to the fungus does not influence P uptake from the medium (Olsson et al., 2006).

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Main experiment

Sixty-five days after the addition of the liquid medium to the fungal compartment, plates subjected to the full-C and the half-C treatment were selected based on vigorous growth and the mycelium was harvested (\( n = 5 \)). The liquid medium in the fungal compartments was removed, the intact mycelium was washed six times thoroughly with Millipore water, then cut off with a scalpel, and mounted over a slit in a tape attached to a mounting ring, as described by Olsson et al. (2008). In this way, the fungal material could be analysed without background material. The rings were freeze-dried and stored at -20 °C until analysis. The dry weight (d.wt.) of the roots from the root compartment and of the external mycelium (ERM) from the fungal compartment was recorded. Root length colonization was determined on trypan blue-stained roots (Brundrett et al., 1996) using the on-axis(off-axis) geometry simultaneously with the PIXE analysis.

And 80 days, and from half-C plates 65 and 80 days after the addition of the liquid medium. Mycelium was prepared for PIXE analysis as described above.

**PIXE analysis**

Particle-induced X-ray emission is a multi-elemental analytical technique based on the detection of characteristic X-rays produced by MeV ions (protons or heavier ions). PIXE is analogous to energy-dispersive X-ray analysis, but its sensitivity is much higher, allowing elements to be detected at the p.p.m. level. The analyses were performed at the Lund Nuclear Microprobe Laboratory, where a focused, micro-metre-sized beam was scanned across the sample to collect spatially resolved data for elemental analysis. Scanning transmission ion microscopy (STIM) measurements (Lefevre et al., 1987; Overley et al., 1988) were performed in on-axis/off-axis geometry simultaneously with the PIXE analysis (Pallon et al., 1987, 2004; Ryan & Jamieson, 1993).
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STIM is based on the detection of energy loss when protons pass through the sample. A 2.5 MeV proton beam with a current of 300 pA was focused to about 5 μm. Each sample was typically scanned accumulatively over an area of 256 × 256 μm for 25–35 min, or until the data collected yielded sufficiently detailed elemental maps of at least P, S, K, Ca and Fe. Several spores and hyphae from a particular sample were analysed after analysis, quantitative images were created using the CSIRO Dynamic Analysis method (http://www.nmp.csiro.au/dynamic.html), which enables true elemental images to be derived from the generally complex PIXE energy spectrum (Ryan & Jamieson, 1993). The program is based on the software package IDL 6.2 (Research Systems, 2002: http://www.ResearchSystems.com).

The elemental images were examined to identify regions for a more detailed quantitative evaluation. Detailed PIXE spectra were constructed by re-sorting the data, and the spectra were fitted to provide quantitative information. The local mass per unit area of the sample was determined from the corresponding STIM spectra, and the elemental content per unit dry weight (freeze-dried) was calculated.

Statistical analysis

The basis of each replicate is a growth system, the plate, n = 5 in the main experiment. Data on hyphae and spores from the same plate were pooled, typically three spores and five hyphae. ANOVA was performed using the statistical software JMP 7.1 (SAS, Cary, NC). Because of differences in the variance between treatments, statistical analysis was performed on log-transformed data. SE of ratios and multiplications of replicated data were obtained accounting for error propagation of independent samples.

Results

The proportion of root length colonized by G. intraradices was not influenced by the C availability (Table 1), but roots with low C availability had lower arbuscule and vesicle density. The availability of C to the carrot roots strongly influenced the elemental composition of the associated fungus G. intraradices (see Supporting Information, Table S1; Fig. 2). The concentration of several elements increased in response to reduced C, the most pronounced effect being seen for P. In spores, the P concentration increased by the factor 7.4 from 530 to 3900 μg g⁻¹ (P = 0.03), which corresponds to 0.4% of the spore’s dry weight. Other elements that increased significantly in spores were chlorine (Cl), potassium (K) and calcium (Ca) (P = 0.01, 0.04 and 0.04, respectively), while no significant accumulation was found for sulphur (S), manganese (Mn) or iron (Fe) in spores. The P accumulation was even more pronounced in hyphae, with 9.7 times higher concentration in the half-C treatment than in the full-C treatment (Fig. 2). Cl, K, Ca and silicon (Si) were also found at significantly higher amounts in hyphae in the half-C treatment. The spore-to-hyphae ratios of elemental concentrations were not influenced by a reduction in C.

Halving the medium C content resulted in a lower root biomass: 52 ± 2 mg d.wt. per plate in the full-C treatment compared to 28 ± 1 mg d.wt. per plate in the half-C treatment (P < 0.001), and a lower ERM biomass, 1.1 ± 0.2 mg in full-C plates and 0.6 ± 0.1 mg in half-C plates (P < 0.05). In total, 28.7 μg P (53% of the total amount in the plate) was recovered from root and mycelium biomass in full-C plates, and 23.9 μg (44%) in half-C plates. The main fraction of this P was contained in the biomass of the root compartment including roots, IRM and the remains of ERM (Fig. 3a). In full-C plates, only 2% of the P taken up was contained in the ERM in the fungal compartment, while as much as 10% were remaining in the fungal compartment’s ERM in half-C plates. The concentration of P in the roots (including IRM and ERM remains) differed by a factor of 1.4 from 540 ± 27 μg g⁻¹ in the full-C

<table>
<thead>
<tr>
<th>AM (%)</th>
<th>Vesicles (%)</th>
<th>Arbuscles (%)</th>
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<tbody>
<tr>
<td>Full-C 32.2 (2.1)</td>
<td>2.5 (0.35) b</td>
<td>17.8 (1.4) a</td>
</tr>
<tr>
<td>Half-C 32.7 (2.1)</td>
<td>0.66 (0.43) b</td>
<td>8.3 (1.3) b</td>
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Fig. 2. The ratios of elemental concentrations in the half-C treatment compared with the full-C treatment, in plant roots (filled bars), mycorrhizal spores (hatched bars) and hyphae (open bars). The horizontal line indicates a ratio of 1, i.e. the same concentrations following both kinds of treatment. Means and SE, n = 5.
treatment compared with 770 ± 63 μg g⁻¹ in the half-C treatment (P < 0.05). The ratio of P concentration in the roots to P concentration in the fungus was 2.5 in full-C plates, but < 0.4 in the half-C treatment (Fig. 3b).

The concentration of other elements in the roots was also influenced by C availability, and significant changes were found for aluminium (Al), Ca, Mn and S (Table S1). The differences were, however, much smaller than in the fungal mycelium, being between 1.7 and 2.4 times higher in half-C plates compared with full-C plates (Fig. 2).

The elemental maps obtained with PIXE revealed that P accumulated in certain areas within the spores, together with K (Fig. 4). In the half-C treatment, P accumulation appeared spot-wise in vacuole-like structures. Measurements on a cross-section of two individual spores from mycelium of an 80-day-old plate revealed that the concentrations of K and Ca were the highest near the spore wall in the full-C treatment (Fig. 5a). In the cross-section of a spore from an 80-day-old plate of the half-C treatment, the concentration of Ca was still the highest near the spore wall, while P, even more so than in the main experiment, accumulated in the centre of the spore, together with K (Fig. 5b). A 25-fold higher accumulation of P, leading to concentrations of about 4% dry weight, was found in the mycelium of 80-day-old plates in the half-C treatments compared with the full-C.

In the supplementary experiment, we examined the effect of age on the elemental composition of the fungus. Only Ca, Cl and iron (Fe) concentrations in spores were influenced by mycelium age, the amounts increasing with increasing age, but the amounts of P or K were unaffected in the full-C plates. In the half-C plates, P increased with spore age, indicating that the measured P accumulation in the main experiment was underestimated.

**Discussion**

The AMF *G. intraradices* accumulated P and other nutrients in spores and hyphae when plant C availability was low. The seven- to 25-fold increase in tissue P concentrations resulted in a P share of up to 4% of the fungal dry weight biomass when the amount of C available to the plant was halved. In contrast, a 100-fold increase in the availability of P to the ERM of the fungus has been found to have a much less pronounced effect on the P concentration, where a sixfold increase was found and the highest P levels were 1% of the spore dry weight (Olsson et al., 2008). High accumulation of P has been found for example in the yeast *Saccharomyces cerevisiae*, where > 10% of the cells’ dry weight can be P, mainly as vacuolar polyP (Kornberg et al., 1999). Our hypothesis, that low plant C status will lead to a nutrient accumulation in its fungal symbiont, was thus confirmed. The observation of this strong effect strengthens the hypothesis that there is a link between C and P transfer – as incorporated into a number of models (e.g. Fitter, 2006) – and that the fungus can control the transfer of P (Fig. 1, [b]): a host that cannot provide C to the fungus may not gain significant amounts of P from the symbiosis.

Decreased AMF colonization has been found in shaded plants (Gehring, 2004; Heinemeyer et al., 2004). In this study, the hyphal length colonization rates (which might be controlled by and limited in a C-saturated plant) were not reduced under low-C availability to the plant, but the number of arbuscules was lower, indicating that P transfer was reduced. Similar results for root colonization were found in a recent study by Lekberg et al. (2010), which demonstrated by isotope probing that mycorrhizal 33P transfer was strongly reduced to root organs that were C-starved (in a manner similar to this experiment) in a common mycorrhizal network.

Possible mechanisms for the observed P accumulation could be: (1) reduced active release through putative P transporters in the IRM, or reduced P leakage, (2) reuptake of P from the apoplast or (3) controlled release of P from the polyP chains within the arbuscules (Fig. 1, [d]). Benedetto et al. (2005) found that P transporters of *Glomus mosseae* were also expressed in IRM, which might enable the controlled release of P to the plant. Increased carbohydrate availability triggers P efflux from the IRM (Solaiman & Saito, 2001) and translocation from storage to transport molecules, i.e. from polyphosphates to P₀, within the extraradical mycelium (Bücking & Shachar-Hill, 2005). Funamoto et al. (2007) found enhanced activity of alkaline phosphatases in mature arbuscules. A possible mechanism for C–P exchange could therefore also be a C-triggered activation of the release of P₀ from the storage polyP via alkaline phosphatases. This implies, as also found previously (Olsson et al., 2006), that reduced C availability does not lead to the downregulation of the high-affinity P transporter in the extraradical mycelium of the same fungus.
Other elements such as K also accumulated under conditions of low C availability to the plant. We have observed previously that increasing the P availability to the fungus leads to an increase in other elements (Olsson et al., 2008). Therefore, the increased concentrations of elements such as K in the fungus may be an indirect effect of increased P accumulation, as K was suggested to be important for the charge balance of polyP (Ashford et al., 1994). The fungal uptake of many other elements, such as Zn, Cu, Fe or Mn, is important in the mycorrhizal symbiosis (Marschner, 1995). No accumulation of micronutrients such as Fe or Mn was found in the fungus, but instead in the roots, where the concentrations of both elements were 1.7 times higher in the half-C roots.

An alternative explanation to the increased fungal P accumulation at low C availability could be a reduced plant uptake from the fungus or reduced sink strength in the roots. Half-C roots did have higher P concentrations, but both the half-C and the full-C treatments had a low P status, lower than usually found in low P plants (Marschner, 1995; Pessarakli, 1999). Plants usually continue to accumulate P under high P availability (e.g. Sharma & Sahi, 2005), and in a study by Sharda & Koide (2010), D. carota accumulated five times more P as an effect of high P availability in the soil. The strong concentration gradient in P falling from the fungal tissue towards the roots suggests a tight fungal control of P release into the apoplast, because accumulation of P in the apoplast would enable plant uptake and reduce P concentration differences between the tissues of the two partners.

Another possible explanation for the higher P levels in the more slowly growing low-C mycelium might be that younger hyphae could contain higher element concentrations than older hyphae. It has been found earlier that in Gigaspora margarita, the P concentrations were 2.5 times higher in younger hyphae, although not in spores (Solaiman & Saito, 2001). The spore content results of our study cannot be explained by a decrease in the P content with age because the P content was equal or higher in older spores in our experiment.

Extrapolation of our results to intact plants must be carried out with caution because the root organ cultures are missing the nutrient sink of the shoots as well as shoot-
borne phytohormones. We, however, believe that these systems can provide insights into the fundamental mechanisms of fungal physiology, and root organ cultures have previously been an important tool for physiological studies of AMF (as reviewed by Fortin et al., 2002) including the symbiotic C exchange in root organ cultures (e.g. Bago et al., 2000, 2003).

Spores are the storage organs of AMF and are mainly considered to be a C reservoir for the growth of the germ tube when establishing a new colonization. We found previously (Olsson et al., 2008) that spores also accumulate considerable amounts of mineral nutrients. These nutrients could be important in supporting the growth of a new mycelium from a detached spore before finding a new plant host (and maybe even act as an 'entrance fee' for a new colonization). The present results show that the AMF mycelium could function as a storage unit for plant nutrients in the soil. During periods of low C availability, P may be accumulated in spores and hyphae, for later redistribution in the common mycorrhizal network. Although the mass of the mycorrhizal mycelium may be small compared with that of the roots, by the strong concentration of available P within the fungus, the fungal storage of P may be significant from the perspective of the plant.

Our results have implications for the understanding of how AM plants interact in nature. The C status of a plant depends on its rate of photosynthesis in connection with its capability to use the light, water and nutrients in its environment. Hence, different plants can have different qualities as hosts for mycorrhizal fungi. The same fungal symbiont may form an extensive mycorrhizal network in plant communities, connecting individual plants of different species (Whitfield, 2007). In order to optimize its growth, the fungus should colonize the plants that supply the highest amount of C, while ensuring an optimal C supply during the entire year. If AMF are able to reduce the delivery of P to plants with a limited supply of C, they possess an important tool to actively increase their fitness by choosing the best plant partner. This, meanwhile, could increase selection pressure on the plant individuals within a community.

We suggest a fungal control mechanism of the P transfer to the plant, and that C and P exchange between the symbionts is closely linked. This is a mycorrhizal view of symbiosis, but we believe it is necessary in order to explain the evolutionarily stable mutualistic relationship that exists between these symbionts.

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References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Element concentrations in spores and hyphae of *Glomus intraradices*, and in roots of *Daucus carota*, following the half-C treatment and the full-C treatment.

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