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Growth benefits provided by different arbuscular mycorrhizal fungi to *Plantago lanceolata* depend on the form of available phosphorus

Roel Pel, Simon Dupin, Henk Schat, Jacintha Ellers, E. Toby Kiers, Nico M. van Straalen*

Institute of Ecological Science, Vrije Universiteit Amsterdam, De Boelelaan 1085, 1081HV, Amsterdam, Netherlands

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ABSTRACT

Strains of arbuscular mycorrhizal fungi (AMF) differ markedly in the growth benefits they provide to plants. We investigated whether these differences depend on the chemical form of inorganic phosphorus. The closely related AMF *Glomus custos* and *Rhizophagus irregularis* were compared using *Plantago lanceolata* as the host plant, grown in quartz sand with either soluble orthophosphate or sparingly soluble hydroxyapatite as a sole source of phosphorus. In a growth experiment with AMF-inoculated plants in a climate chamber, sampling at 3-wk intervals enabled a detailed time-resolved analysis of shoot and root phosphorus concentrations and growth performance of *P. lanceolata*. The ability of AMF to enhance plant growth and deliver phosphate depended strongly on the identity of the available phosphorus source. In orthophosphate-amended substrate only modest differences in plant growth performance (dry matter accumulation and allocation, phosphorus acquisition) were observed between the two AMFs, despite evident AMF root colonization as shown by strain-specific mtLSU qPCR analysis. The treatment with hydroxyapatite however, created stringent growth-limiting conditions and significantly increased the growth benefit provided by *R. irregularis* over *G. custos* and the non-mycorrhizal treatment. Plants with *R. irregularis* could acquire much more phosphorus from apatite compared to *G. custos*. There were also differences in shoot-to-root dry matter allocation and plant tissue phosphorus concentrations between the *R. irregularis* and *G. custos* treatments. Our observations suggest that in experiments on the symbiosis between plants and mycorrhizal fungi more attention should be paid to the chemical form of phosphorus in soil.

1. Introduction

Arbuscular mycorrhizal fungi (AMF) are important root symbionts in the majority of terrestrial plants because of their ability to enhance nutrient uptake [1]. It is generally accepted that both mycorrhizal and non-mycorrhizal plants rely on orthophosphate in the soil pore water that is exchanged with phosphorus complexes in the soil [2,3]. Due to the very low mobility of phosphate (P) in solution, replacement does not usually keep pace with local uptake, leading to so-called depletion zones in the rhizosphere [4]. The effectiveness of AMF to increase phosphate acquisition of their hosts can largely be attributed to the ability of their hyphae to grow beyond these depletion zones with greater ease than plant roots, thus scavenging larger volumes of soil for P [5].

It is well established that AMF strains show a large functional diversity [6–9], with some fungi appearing to transfer less P to the host than others. Indeed, AMF have been shown to differ in the efficiency with which they explore soil, and this has been related to differences in morphology of the extraradical mycelium (ERM), the density and extension of the ERM network in the soil, the efficacy of the mycorrhizal pathway in the uptake of P (density and K_m value of P_i-transporters) and the transfer of P to the host root [7,10–12]. Apart from the differences in soil exploration efficiency, other factors, biotic as well as abiotic, may determine the benefits for the plant. For example, the carbon to phosphate exchange ratio between the host plant and its fungal partner is important in explaining the variation in host carbon investments for specific plant-fungus combinations [8,13–15]. Likewise, the interaction between light and soil nutrients can affect preferential bidirectional allocation patterns of C and P [16,17]. As a result, reported effects of AMF on plant growth fall within a broad range of outcomes with fungal symbiont qualifications varying from 'high quality' to 'antagonistic' [18].

However, little is known about how mycorrhizal 'quality' is determined by the ability of the fungus to access and utilize different forms of P present in soils. Along with organic P pools [19,20], sparingly soluble (crystalline) P minerals such as apatites ($Ca_{10}X(PO_4)_6$, where $X = F^-$, Cl^-, OH^- or CO_3^{2-}), may contribute significantly to the total phosphorus content of a soil, up to 75–90%, especially in the

* Corresponding author.
E-mail address: n.m.van.straalen@vu.nl (N.M. van Straalen).

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more calcareous soil types [21–24]. These Ca-phosphate species are often denoted by the term rock phosphates if they originate from a marine diagenetic mineral deposit [4,22]. A number of plant-mycorrhiza studies have demonstrated that AMF can successfully mobilize the phosphorus in apatites or at least, can significantly contribute to the solubilization of these P minerals, *e.g.*, *Glomus manihotis* [25]; *Glomus fasciculatus* and *Glomus tenuis* [26], *Glomus clarum* [27] and *Glomus margarita* [28]. In root organ cultures it was shown that the extraradical mycelium (ERM) of *Rhizophagus irregularis* (formely known as *Glomus intraradices*) was able to release orthophosphate from hydroxyapatite, and to a lesser extent, also from a low-reactive rock phosphate mineral [29]. So while P acquisition is clearly important for mycorrhizal species, there have been no quantitative comparisons of how the ability to mobilize phosphate from apatite affects the quality of AMF to their hosts.

Our aim was therefore to establish if the ability to release P from a resilient apatite matrix differed between the two closely related fungal species *Glomus custos* and *Rhizophagus irregularis*. We were interested in a comparison between these species because in previous research we found that *G. custos* was a poorer quality symbiont compared to *R. irregularis* (~24% reduction in shoot dry weight and shoot P content of *Plantago lanceolata* [30]. This lower quality has been confirmed with respect to vegetative growth and P status of a diversity of hosts, including *Allium porrum* and *Medicago truncatula* [13], and *Glycine max* [31]. However, these studies used readily available phosphate species such as orthophosphate as a P source. In this study we wanted to investigate whether the differential beneficial properties of AMF were also evident when using a more natural, recalcitrant P source, such as apatite. Since the reactivity of phosphate in apatites largely depends on the (variable) level of residual carbonate in the crystal lattice [32,33] we used in this study a well-defined model apatite: a synthetically produced crystalline/crystallite hydroxyapatite mineral ($Ca_{10}(PO_4)_6(OH)_2$ rather than rock phosphate [34,35].

An important aspect of characterising the functional diversity in fungal species is the time of harvest in relation to the growth cycle of the plant. In most studies of arbuscular mycorrhizal systems, conclusions are based upon a single harvest because of experimental constraints. This means that critical changes in the life-cyle of the plant may go unnoticed [36]. In this study we monitored the P acquisition ability of the two AMF species in a time course experiment by harvesting *P. lanceolata* at 3-week intervals over a period of approximately 4 months. We hypothesized that the quality differences between *G. custos* and *R. irregularis* noticed in previous studies can be attributed to – at least partly – the differential capabilities of these strains to solubilize the more recalcitrant inorganic P species present in (calcareous) natural soils. In addition to total P acquisition by *P. lanceolata*, other parameters reflecting the impact of the AMF on the host plant were also monitored, *i.e.*, dry matter yields with the shoot-to-root mass allocation ratio, and the tissue P concentrations in shoots and roots. Quantitative PCR (qPCR) using strain-specific mtLSU DNA markers was applied to assess and compare the relative abundances of *R. irregularis* and *G. custos* biomass in the roots within and between the two time course experiments.

2. Materials and methods

2.1. General experimental setup

Two experiments were conducted, one using orthophosphate and the other using apatite as sources of phosphorus. Each involved three different AMF inoculation treatments and 120 pots, of which 7 (1st experiment) or 8 (2nd experiment) replicates per treatment were sacrificed every 3 weeks over a period of 15 weeks. Because the available climate chamber facility did not provide sufficient bench space to accommodate them simultaneously the experiments were conducted consecutivily. This was considered a minor constraint since growth conditions were stringently controlled and closely reproduced with respect to temperature, air humidity, mineral nutrients supplement regime and seed germination (see 2.3).

2.2. Growth substrate and nutrient additions

Pure high-grade quartz sand, autoclaved at 120 °C, ($SiO_2 \geq 99.7\%$, P-free, S60 size fraction 150–300 µm) from Sigrano BV Maastricht, Netherlands, was applied as the sole growth substrate. No constituents other than quartz sand were used in order to maintain defined growth nutrient conditions with respect to the available P source. Also, the substrate was not microbially inoculated with a suspension of native soil (the so-called 'microbial wash'), because such extracts may contain colloidal or fine-sized Ca-phosphate conglomerates [37,38].

Plants were grown in 0.88 l plastic pots (11 cm diameter) with 750 g substrate, which was maintained at a 15% (w/w) water content by weighing. In the orthophosphate treatment, a ½-P strength pH 6.0 Hoagland nutrient solution [39] was added once every two weeks at a rate of 4 ml/kg wet quartz sand, starting at t = 0 (wk0) right after the transplanting of seedlings. The nutrient solutions were injected below the quartz sand surface (over a depth of approximately 1–6 cm) at four equidistant positions around the root (~2 cm away from the pot wall) to achieve an even distribution of the nutrients over the pot volume. This resulted in a cumulative amount of 24 µmol P per pot over the course of the experiment. This rate of mineral nutrient supplementation was similar to that in previous mycorrhiza studies [30].

In the hydroxyapatite experiment, fine-powdered $Ca_{10}(PO_4)_6(OH)_2$ (purity ≥ 99.9%; Acron Organics, Belgium) was thoroughly mixed with quartz sand (1 g per kg sand, equivalent to ~3.8 mmol P per pot) prior to moistening and autoclaving. The level of hydroxyapatite was chosen based on the concentration range of low soluble P minerals encountered in calcareous dune soils of the so-called Renodunaal coastal district in the Netherlands [40], and reported apatite contents measured in other (non-cultivated) lime-rich soils [23,24]. Pots in the hydroxyapatite treatment received mineral nutrients at the same rate as in the orthophosphate treatment, but with all phosphate omitted from the Hoagland nutrient solution. In these modified nutrient solutions, $NH_4H_2PO_4$ was substituted by an equimolar amount of NH_4NO_3.

The starting pH of the substrates was in the range of 7.8–7.9, irrespective of amendment with soluble orthophosphate or the solid hydroxyapatite. Bulk substrate pH values were measured at each timepoint before harvesting shoot and root of the pots.

2.3. Plant culturing and mycorrhizas

Plantago lanceolata L. (Ribwort Plantain) seeds were obtained from a large *c.* 5-year old stock collection, originally collected from open field (low fertile) grown plants (Cruydthoek, Assen, the Netherlands), and thereafter permanently stored in the dark at 4–5 °C. Seeds were sterilized using diluted bleach (2.5% NaOCl) for 10 min, washed with sterilized water and germinated on quartz sand (17% w/w water content) under transparent plastic foil at 20 °C in the climate chamber also used for the P addition treatments (see below). Two weeks after sowing, one randomly selected seedling was planted in the middle of each pot. Roots of the seedlings were inoculated with liquid spore material procured from *in vitro* root organ culture by Mycovitro S.L. Biotechnología Ecológica (Granada, Spain). The spore suspension also contained some hyphae and *Daucus carota* root parts. Inoculation treatments were as follows: (1) *Rhizophagus irregularis*, strain 09 [41], previously *Glomus intraradices*, (2) *Glomus custos*, strain 010 [42], and (3) a heat-sterilized (120 °C) 50:50 mixture of *R. irregularis* and *G. custos* as the non-mycorrhizal (control) treatment. In all cases, a total of approximately 1000 spores were applied.

Pots were randomly positioned on the bench and reshuffled two times a week upon watering. Five destructive samplings, at 3 week intervals, were conducted with the first harvest 3 weeks after planting.