Carbon and phosphorus exchange may enable cooperation between an arbuscular mycorrhizal fungus and a phosphate-solubilizing bacterium

Lin Zhang[1], Minggang Xu[2], Yu Liu[3], Fusuo Zhang[1], Angela Hodge[4*], Gu Feng[1*]

[1]College of Resources and Environmental Sciences; Research Center for Resources, the Environment and Food Safety, China Agricultural University, Beijing, China; [2]Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing, China; [3]College of Life Sciences, Zhejiang University, Hangzhou, China; [4]Department of Biology, University of York, Wentworth Way, York, YO10 5DD, UK

*Corresponding authors: Gu Feng and Angela Hodge

Tel: +86 10 62733885, Fax: +86 10 62731016 (Gu Feng); +44 (0) 1904 328562, Fax: +44 (0) 1904 328505 (Angela Hodge)

E-mail: fenggu@cau.edu.cn; angela.hodge@york.ac.uk

Addresses: College of Resources and Environmental Sciences, China Agricultural University, Beijing 100193, China (Gu Feng); Department of Biology, University of York, Wentworth Way, York, YO10 5DD, UK (Angela Hodge).

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Summary

- Arbuscular mycorrhizal fungi (AMF) transfer plant photosynthate underground which can stimulate soil microbial growth. In this study, we examined if there was a potential link between carbon (C) release from the AMF and phosphorus (P) availability via a phosphate-solubilizing bacterium (PSB).
- We investigated the outcome of the interaction between AMF and PSB by conducting a microcosm and two Petri plate experiments. An *in vitro* culture experiment was also conducted to determine the direct impact of AMF hyphal exudates upon growth of the PSB.
- AMF released substantial C to the environment, triggering PSB growth and activity. In return, PSB enhanced mineralization of organic P, increasing P availability for AMF. When soil available P was low, PSB competed with AMF for P, and its activity was not stimulated by the fungi. When additional P was added to increase soil available P, PSB enhanced AMF hyphal growth, and PSB activity was also stimulated by the fungi.
- Our results suggest that an AMF and a free-living PSB interacted to the benefit of each other by providing the C or P that the other microorganism required but these interactions depended upon background P availability.

Key words

Carbon-phosphorus exchange, cooperation, hyphal exudates, phosphatase activity, AMF-PSB interactions.

Introduction

Cooperation is a prevalent phenomenon in nature and occurs at a wide range of scales, from among genes in genomes to cooperation among nation states (Dorsky *et al*., 2003; Nowak, 2006). However, cooperative behavior is a difficult problem for biologists and ecologists to explain because from the point view of evolution, natural selection should favor selfish acts (West *et al*., 2007; Harcombe, 2010; Rainey & De Monte, 2014). Thus, much empirical and theoretical effort has been made to find a solution to this problem through the investigation of a wide range of organisms at both the same species and symbiosis scale (Keller & Chapuisat, 1999; Griffin *et al*., 2004; Douglas, 2008). It is perhaps surprising therefore, that the selection forces maintaining cooperation in the arbuscular mycorrhiza (AM) association, a 450-million-year-old symbiosis formed between AM fungi (AMF) and plant roots (Smith & Read, 2008), have only fairly recently been proposed (Bever *et al*., 2009; Kiers *et al*., 2011), although are not, as yet, fully resolved (see Walder *et al*., 2012; Walder *et al*., 2015).

AMF are obligate biotrophs that receive their C supply from their host plant; in return, the fungi compensate the plant through enhanced nutrient acquisition, particularly through the supply of poorly mobile phosphate ions (Smith & Read, 2008; Karasawa *et al*., 2012). Moreover, the AM symbiosis involves a complex series of interactions with multiple fungal strains and multiple hosts, and both plants and fungi can select the better partners that provide more resources (Bever *et al*., 2009; Kiers *et al*., 2011). These reciprocal rewards can stabilize cooperation by punishing selfish behaviors (Kiers *et al*., 2011; but see Walder *et al*., 2012).

AMF produce extensive extraradical hyphae in the soil, which are a habitat for other microbes (Gahan & Schmalenberger, 2015). Thus, cooperation may also exist between AMF and their associated microbes. Multiple lines of evidence suggest cooperation may occur. First, AMF hyphae are rapid conduits for recent plant photosynthates, which can attract microbes and stimulate their growth (Drigo *et al*., 2010; Kaiser *et al*., 2015). Second, microscopic and molecular analysis showing bacterial colonization on the surface of AMF hyphae and spores demonstrate that an intimate relationship between AMF and microbes exists (Toljander *et al*., 2006; Scheublin *et al*., 2010; Agnolucci *et al*., 2015). These bacteria can also influence AMF fitness (Frey-Klett *et al*., 2007) and ecological function (Hodge *et al*., 2001; Feng *et al*., 2003; Cheng *et al*., 2012; Zhang *et al*., 2014a). Consequently, microbes are recognized as a third part of the AM symbiosis, not just soil-borne 'free riders' (Jansa *et al*., 2013). Moreover, a plant-AMF-microbe model has been proposed to emphasize the coexistence and cooperation between AMF and microbes (Bonfante & Anca, 2009). However, a key question arises at this juncture: do AMF benefit by releasing C acquired from the plant to directly promote bacterial activity or is the C simply lost from the hyphae? In other words, is there cooperation, i.e., the investment of resources towards a common interest by the group members (Chase, 1980)? To our knowledge, no explanation for the AMF-microbe interaction from the aspect of cooperation has yet been offered.

Several mechanisms have been proposed to explain cooperation (Nowak, 2006; West *et al*., 2007). Kin selection is a widely accepted theory to explain cooperation formed in the same species (Hamilton, 1963; West *et al*., 2002). For two unrelated species, reciprocity between two partners can maintain their cooperation (Harcombe, 2010), and several lines of indirect evidence suggest that reciprocity may maintain cooperation between AMF and associated soil microbes. AMF hyphae do not benefit all microbes; indeed, they inhibit some (Nuccio *et al*., 2013; Bender *et al*., 2014). In contrast, the stimulated microbes usually have potentially positive effects on AMF fitness (Scheublin *et al.*, 2010; Nuccio *et al.*, 2013). These observations suggest that AMF may select microbes to cooperate with. The excretion of metabolite products can provide a mechanism for the initiation of reciprocation (Sachs *et al.*, 2004). AMF produce extensive extraradical hyphae and transfer plant derived C-rich compounds to the attached soil, providing them to microbes (Kaiser *et al.*, 2015), which usually face C scarcity (Blagodatskaya & Kuzyakov, 2013). However, AMF have no known saprotrophic capability, which means that they cannot directly breakdown organic nutrients (Smith & Read, 2008; Tisserant *et al.*, 2013). In contrast, microbes are diverse in functions and play especially important and varied roles within elemental (e.g., C, N, and P) biogeochemical cycles (Torsvik & Øvreås, 2002; Nannipieri *et al.*, 2003). Microbes can release various enzymes to decompose organic matter, and in doing so can provide the AMF hyphae with inorganic nutrients (Hodge & Fitter, 2010; Hodge, 2014; Zhang *et al.*, 2014a). Therefore, microbes do not merely use AMF-released C but may also pay back other benefits required by the fungi. Through cooperation, AMF and microbes can get what they need from their partners and improve their own fitness.

Although P is the key nutrient that AMF acquire (Smith & Read, 2008), they lack the ability to secrete phosphatases (Tisserant *et al.*, 2013). Thus, AMF cannot utilize organic P directly, which limits their contribution to plants P uptake, especially in forest soils with rich organic matters and agricultural soils with large amounts of applied manure. However, more than 40% of culturable bacteria are able to mineralize organic P (the so-called phosphate-solubilizing bacteria (PSB)) by releasing numerous phosphatases into the surrounding soil (Jorquera *et al.*, 2008). Although previous studies have shown that AMF and PSB can interact to improve P acquisition for the AM host plant (Toro *et al.*, 1997; Kim *et al.*, 1998), the mechanisms behind this nutritional benefit are unclear (Artursson *et al.*, 2006). In the present study we focus on the potential mechanisms behind the synergy that exists between AMF and PSB by investigating the interactions of the two organisms directly. PSB may rely on C released by AMF and in return provide hyphae with inorganic phosphate. Here, we hypothesized that there was cooperation between AMF and PSB. More specifically we conducted a series of experiments to address the following hypotheses:

1) That AMF would proliferate less hyphae and transfer less P when the PSB was absent, but the AMF reliance on the PSB would be less at higher background P levels.
2) That the PSB would increase P availability for the AMF particularly from organic P sources and this would increase phosphate transporter gene expression in the AMF hyphae.
3) The PSB would be able to utilize C compounds released from the AMF hyphae and that this would enhance PSB activity and function.

Materials and methods

To test our hypotheses we performed four different experiments:

Experiment 1 (Microcosm experiment): To determine how differing P levels influenced the AMF and/or PSB strain under realistic conditions, and the resulting impact in terms of P acquisition for the host plant.

Experiment 2 (Petri plate experiment 1): To quantify acid and alkaline phosphatase activities and bacterial number of PSB influenced by AMF under aseptic conditions. The expression of AMF hyphal phosphate transporter gene *GiPT* was also quantified.

Experiment 3 (Petri plate experiment 2): To enable collection of AMF hyphal exudates (under aseptic conditions) under two contrasting P levels.

Experiment 4 (*In vitro* culture experiment): To determine if AMF hyphal exudates (collected from Experiment 3) influenced growth of the PSB.

For the microcosm experiment, the host plant *Medicago sativa* cv. Aohan was selected because it has a relatively small biomass at the seedling stage and therefore allowed the effects of the AMF-bacterium interaction on the host plant to be readily observed. The AMF strain was *Rhizophagus intraradices* BEG 141 (RIn, formerly *Glomus intraradices*, kindly provided by Professor Vivienne Gianinazzi-Pearson, INRA, France). In the Petri plate experiments, *Daucus carota* roots transformed with T-DNA from a tumor-inducing plasmid were used as the host and the AMF strain was *Rhizophagus irregularis* DAOM 197198 (RIr), a widely studied strain which is often used as a 'model' AMF. The PSB strain used in all of the experiments was *Rahnella aquatilis* HX2 (RA), isolated from a vineyard soil in Beijing, China (Guo *et al*., 2012). In a preliminary experiment, this strain of RA was shown to be effective in mineralizing and utilizing phytin (calcium magnesium salt of phytic acid, a kind of phytate) as a P source (see Fig. S1) and was labeled with the plasmid pSMC21 containing a *gfp* gene. It can also colonize the hyphal surface of RIr (see Fig. S2).

Microcosm experiment

The microcosm units each had two compartments, which were separated by a 30 μm mesh in the middle. One compartment contained the plant, while the other was the hyphal compartment where the AMF-PSB interaction could be investigated (see Fig. S3). Details of the experimental set-up are given in the materials and methods section of the supporting information. The microcosm experiment contained the following treatments: (1) two KH_2PO_4 levels, with 0 or 5 mg P kg^{-1} soil in the hyphal soil, (2) with or without RIn in the plant compartment, and (3) with or without RA in the hyphal soil which were applied in a factorial manner across the microcosms. Soil in the hyphal section also contained 75 mg P kg^{-1} DW as Na-phytate (Sigma-Aldrich, St. Louis, MO, USA) because phytate P is one of the main organic P forms in the soil (Turner *et al*., 2002). Each treatment had four replicates, thus, there were 32 microcosms in total, which were arranged in a randomized block design in a greenhouse. Plants in these microcosms were grown at China Agricultural University in Beijing from 12 May to 10 July 2011 at 24/30°C (night/day). The average photosynthetically active radiation at plant level was 360 μmol m^{-2} s^{-1}. Soil gravimetrical moisture was kept at 18-20% (w/w, ~70% water holding capacity) with deionized water by weighing the microcosm units every 2 days during the experimental period.

At destructive harvest, soil samples were collected from the hyphal soil, and the top 2 cm of the soil from these samples were discarded to eliminate any possible surface effects. What remained of the sample was cut into small sections and then mixed in a blender to obtain a uniform matrix for subsequent analyses (see below). The plant material was separated into shoots and roots. The shoot material was oven-dried at 105°C for 30 min to arrest metabolic activity and then dried at 65°C for a further 2 days before being finally ground to a fine powder. Shoot P was determined following Thomas *et al*. (1967). The roots were washed with deionized water and then preserved at -20°C.

Microbial biomass P (MBP) in the hyphal soil was extracted by the chloroform fumigation-extraction method (Brookes *et al*., 1982), determined colorimetrically by a modified ammonium molybdate-ascorbic acid method (Jackson, 1958) and calculated assuming a k_P value of 0.40 (Brookes *et al*., 1982). External mycorrhizal hyphae were extracted from two 5 g soil sub-samples from the hyphal soil compartment using a modified membrane filter technique (Staddon *et al*., 1999). Hyphal length was assessed using the gridline intercept method at × 200 magnification and then converted to hyphal length density (m g^{-1} DW soil; Hodge, 2003).

Determination of acid and alkaline phosphatase activity (μg *p*-nitrophenyl phosphate min^{-1} g^{-1}

DW soil) in the hyphal soil was conducted according to Neumann (2006). The available phytate P for AMF and bacteria was measured by extracting the soil with 0.5 M $NaHCO_3$ solution (pH 8.5) for 30 min at a speed of 180 rpm at 25°C at a soil:solution ratio of 1:20. The phytate P in the $NaHCO_3$ solution was mineralized by commercially available phytase according to the technique of Hayes *et al*. (2000) to determine total P. Phytate P in the extract was calculated by subtracting inorganic P from total P.

Petri plate experiment 1

Two-compartment Petri plates (90 × 15 mm) with *D. carota* roots were used to study the AMF-PSB interaction under sterile conditions. In the first (root) compartment, 25 ml of solid M medium (modified from Bécard & Fortin, 1988 as Leigh *et al*., 2011) was added. In the other compartment, which only the AMF hyphae, and not roots, were permitted to grow into, 20 ml of solid M medium (but without sucrose and vitamin sources) was added. Non-mycorrhizal (only *D. carota* roots) or mycorrhiza-colonized (*D. carota* roots associated with *R. irregularis* DAOM 197198) roots were transferred to the root compartment and then cultured in an incubator at 27°C in the dark. Cultures were inspected on a regular basis, and the roots close to the plastic divider were removed before they were able to grow into the hyphal compartment.

Six weeks later, when the AMF hyphae had started to grow in the hyphal compartment, a block of phytagel (5 × 2 cm) was cut and removed from the hyphal compartment and replaced with 2 ml liquid M medium (without sucrose and vitamin sources). After another two weeks, when hyphae had visibly colonized the liquid compartment, the medium was removed and 2 ml of fresh liquid M medium (without sucrose and vitamin sources) was added. The liquid medium also contained 280 μM organic P in the form of Na-phytate for all treatments and 5×10^7 CFU ml^{-1} bacterial cells for the

+RA treatments. The RA inoculum was prepared as follows: bacteria were cultured in liquid LB medium with shaking at 180 rpm for 24 h at 37°C and then centrifuged at 8,000 rpm for 6 min. The supernatant was discarded, and the pellet was re-suspended and washed with sterilized 0.85% (w/v) NaCl solution three times. The supernatant was then adjusted to OD_{600}=1.0 with the sterilized 0.85% NaCl solution.

This Petri plate experiment examined three factors: (1) two RIr levels, with or without RIr in the root compartment, (2) two RA levels, with or without RA in the hyphal compartment, and (3) two harvest times, 2 weeks or 4 weeks after RA inoculation. Each treatment had four replicates, resulting in a total of 32 plates. At harvest, the hyphae and medium in the hyphal compartment were separated. The hyphae were put into a 2-ml tube using sterilized forceps and were immediately stored at -80°C to determine the expression of the phosphate transport gene *GiPT*, according to the method by Fiorilli *et al*. (2013). The bacterial attachments of RA to the hyphal surface were observed using an Olympus BX51 fluorescence microscope (Olympus Optical, Tokyo, Japan). Some of the liquid medium was used to immediately estimate bacterial numbers by determining the total number of colony-forming units of RA (CFU per ml medium) in the medium using the plate count method of Smit *et al*. (2001). The remainder of the medium was passed through a Acrodisc® Syringe Filter (0.2 µm Supor® Membrane, Pall Corporation, New York, USA) and stored at -20°C for acid and alkaline phosphatase activity (µg *p*-nitrophenyl phosphate min^{-1} ml^{-1} medium) determination (as Neumann, 2006). Inorganic P concentration in the medium was measured with malachite green reagent (Irving & McLaughlin, 1990).

Petri plate experiment 2

To facilitate the collection of hyphal exudates from the AMF, two-compartment Petri plates (90 × 15 mm), set up with separate root and hyphal compartments as before, were used. To the root compartment, 25 ml of solid M medium was added. To the hyphal compartment, 4 ml M medium with the carbon sources omitted (i.e., minus sucrose, EDTA and vitamin sources) was added to create a slope from the top of the plastic divider (Filion *et al*., 1999). Colonized (*D. carota* roots associated with *R. irregularis* DAOM 197198) roots were transferred to the root compartment (or not). In this experiment, three root and RIr culture treatments were examined, namely, (1) no roots or AMF hyphae in either compartment (MR^-/RIr^-); (2) mycorrhizal roots in the root compartment but no AMF hyphae from the hyphal compartment (MR^+/RIr^-), and (3) mycorrhizal colonized roots in the root compartment and AMF hyphae permitted into the hyphal compartment (MR^+/RIr^+). Mycorrhizal and non-mycorrhizal roots may release different volatile compounds from the root compartment (Schausberger *et al.*, 2012; Babikova *et al*., 2014); to avoid this potential confounding influence and ensure that the only substances detected at different levels in the hyphal compartment were those released from the AMF hyphae, we used a colonized mycorrhizal root in the MR^+/RIr^- treatment instead of a non-mycorrhizal root. The plates were cultured in an incubator at 27°C in the dark. Six weeks later, when the AMF hyphae began to cross the barrier and grow along the slope, 10 ml of liquid M medium (but without sucrose and vitamin sources) with 0 or 35 µM KH_2PO_4 was added to the hyphal compartment to permit the collection of hyphal exudates. There were a total of 6 treatments in this experiment, and each was replicated 5 times; thus, 30 plates were set up in total.

After 4 weeks, when most of the surface of the hyphal compartment was covered by actively growing AMF hyphae, the hyphae and medium were harvested. The hyphal material was dried and weighed using a Cubis® Ultramicro Balance (Sartorius, Goettingen, Germany). The medium was passed through Acrodisc® Syringe Filter (0.2 µm Supor® Membrane, Pall Corporation, New York, USA) and stored at -20°C for subsequent analysis. The total C concentration of the medium was determined by multi N/C® UV HS (Analytik Jena AG, Eisfeld, Germany), and the carboxylate content was determined according to Shen *et al*. (2003). Sugar content was determined by ICS-3000 Ion Chromatography System (Dionex, California, USA). Total C of hyphal exudate in the medium was calculated as following:

$$(C_{MR^{+}/RIr^{+}} \times V_{MR^{+}/RIr^{+}} - C_{MR^{+}/RIr^{-}} \times V_{MR^{+}/RIr^{-}}) / V_{MR^{+}/RIr^{+}}$$

and C released by per unit hyphal weight was calculated as:

$$(C_{MR^{+}/RIr^{+}} \times V_{MR^{+}/RIr^{+}} - C_{MR^{+}/RIr^{-}} \times V_{MR^{+}/RIr^{-}}) / m.$$

where:

$C_{MR^{+}/RIr^{+}}$ and $C_{MR^{+}/RIr^{-}}$ stand for the total C concentration of the medium; $V_{MR^{+}/RIr^{+}}$ and $V_{MR^{+}/RIr^{-}}$ represent the medium volume left in hyphal compartment in the MR^{+}/RIr^{+} and MR^{+}/RIr^{-} treatments; and m is the hyphal dry weight in the hyphal compartment in the MR^{+}/RIr^{+} treatment. For further details on these data, please see Table S3.

In vitro culture experiment

RA was cultured in liquid LB medium for 12 h at 37°C at 180 rpm and then centrifuged at 8,000 rpm for 6 min. The supernatant was discarded and the pellet was re-suspended using sterilized 0.85% NaCl solution. After washing three times, the bacteria were diluted ($OD_{600} = 0.1$). In a 100-microwell plate, 200 µl of 0.85% NaCl solution was added to the wells surrounding the plate without bacteria to avoid potential border effects; 180 µl of 0.85% NaCl solution or medium collected from the Petri plate experiment 2 (i.e., in the treatments of MR^{-}/RIr^{-}, MR^{+}/RIr^{-}, MR^{+}/RIr^{+}) in different KH_2PO_4 concentrations (i.e., 0 and 35 µM) was added to the other wells of the plate. Then, 20 µl of the prepared RA bacterial suspension was added to the wells and mixed uniformly with the medium (see

Fig. S4). Each treatment was replicated 8 times. Growth of the bacterial cultures at 37°C was monitored for 48 h by using a Bioscreen C MBR (Oy Growth Curves Ab Ltd, Helsinki, Finland). The OD_{600}s of the liquid cultures were determined every 2 h and reported as the mean of five different measurements.

Data analysis

A three-way analysis of variance was performed to compare the effects of KH_2PO_4, RIn, RA, and their interactions on shoot P content, MBP, phytate P and hyphal length in the microcosm experiment and the effects of harvest time, RIr, RA and their interactions on acid phosphatase activity, alkaline phosphatase activity, bacterial numbers and *GiPT* expression in the Petri plate experiment 1. All data were checked for normality using the Kolmogorov-Smirnov test, and Levene's test was used to test for the equality of variance. Prior to statistical analysis, bacterial numbers were log-transformed. Significant differences among the four treatments were evaluated by a Tukey's honest significant difference (HSD) test. When only two treatments were compared a *t*-test was performed. Differences referred to in the text were statistically significant at $P < 0.05$ unless otherwise stated. Statistical analyses were performed using SPSS v. 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Microcosm experiment

Shoot P content of *M. sativa* increased by 20-30 times due to the presence of the AMF RIn (RIn, $P < 0.001$; see Table S1); in contrast, the main effect of the bacterium RA on shoot P content was not significant (RA, $P = 0.066$; Fig. 1a). There was however, a significant three-way interaction among

RIn, RA and inorganic P level added (KH_2PO_4 × RIn × RA, $P = 0.021$) because the presence of both RIn and RA increased shoot P content only when 5 mg P kg^{-1} as KH_2PO_4 was also added (Fig. 1a). In the AMF hyphal soil, the presence of RA significantly increased MBP (RA, $P < 0.001$), whereas RIn had no effect (RIn, $P = 0.945$). Compared to the RA treatment alone, dual inoculation with both RIn and RA increased MBP when KH_2PO_4 was not added, but decreased MBP when KH_2PO_4 was added (KH_2PO_4 × RIn × RA, $P = 0.021$; Fig. 1b). Compared with the control, inoculation with RIn and RA, either singly or together, decreased soil phytate P significantly at both KH_2PO_4 levels (RA, $P < 0.001$; RIn, $P = 0.008$). Among the various treatments, phytate P was highest in the control and lowest in the dual RIn/RA inoculation treatment in both KH_2PO_4 levels. However, compared to the sole RA treatment, dual inoculation with RIn and RA decreased phytate P only when KH_2PO_4 was also added (Fig. 1c).

Soil phosphatase activities were increased by RA inoculation, and acid phosphatase activity was 2-3 times higher than that of alkaline phosphatase (Fig. S5a, S5b). Subsequent analyses showed that phytate P (i.e., that remaining from the original 75 mg P kg^{-1} soil Na-phytate after extraction by 0.5 M $NaHCO_3$) was significantly correlated with acid phosphatase activity ($R^2 = 0.699$ and $P = 0.01$, Fig. 2a), and MBP was significantly correlated with soil phytate P ($R^2 = 0.576$ and $P = 0.029$, Fig. 2b). However, there was no correlation between shoot P content and phytate P ($R^2 = 0.224$ and $P = 0.236$, Fig. 2c), indicating that plants could not acquire the mobilized phytate-P from hyphal compartment without AMF.

In the mycorrhizal treatments, roots of *M. sativa* were well colonized by RIn, and inoculation with RA did not affect this colonization (Table S2). In the −RIn treatment, some hyphae were observed that might have been dead fungal hyphae or non-mycorrhizal fungi, but their levels were low (0.10 ± 0.03 m g^{-1} soil). In the +RIn treatment, there was a significant interaction between KH_2PO_4 and RA (KH_2PO_4 × RA, $P = 0.032$) because AMF hyphal lengths were stimulated by the presence of RA when KH_2PO_4 was also added but not affected by RA when KH_2PO_4 was absent (Fig. 3a, Table S1). To confirm the effect of RA on hyphal growth of AMF, we conducted a Petri plate experiment (see the materials and methods section of the supporting information for details on experiment 3) that demonstrated how RA stimulated the growth of RIr under sterile conditions (hyphal fresh weight in the RIr-alone treatment was 20 mg $dish^{-1}$, but was 26 mg $dish^{-1}$ in the RIr/RA treatment; Fig. 3b).

Petri plate experiment 1

Under sterile conditions, while sole inoculation with RIr had no influence on either acid or alkaline phosphatase activity compared to the control, but inoculation with RA significantly increased activities of these enzymes (Fig. S5c, S5d). Acid phosphatase activity was much higher than alkaline phosphatase activity. Dual inoculation with RIr and RA increased acid and alkaline phosphatase activity compared to RA inoculation alone (RIr × RA, $P < 0.001$). Harvest timepoint also had a significant effect on acid ($P = 0.001$) and alkaline phosphatase activity ($P < 0.001$), with activities in the RA and RIr/RA treatments higher at 4 w than 2 w (Fig. S5c, S5d).

The growth of RA was significantly ($P < 0.001$) stimulated by the presence of RIr hyphae: bacterial counts in the RIr/RA treatment were *c.* 10^8 CFU ml^{-1} medium compared with *c.* 10^7 CFU ml^{-1} medium in the RA-only treatment at both 2 w and 4 w. In addition, bacterial counts were significantly ($P = 0.001$) higher at the 2 w harvest than at the 4 w harvest in both the RA and RIr/RA treatments (Fig. 4a). No bacteria were detected in the −RA treatments.

Neither harvest time ($P = 0.222$) nor RA presence ($P = 0.519$) had any influence on the relative expression of *GiPT,* which was similar among the various treatments (Fig. 4b).

Petri plate experiment 2

Hyphal dry weight and total C in the hyphal exudate in the medium did not differ between the 0 and 35 μM KH_2PO_4 treatments. The dry weight of hyphae was 1.2-1.3 mg plate^{-1} (Table S3), and the concentration of total C of hyphal exudate in the medium was *c*. 4.0 mM (Fig. 5a). Thus, RIr released approximately 30 mM C g^{-1} DW hyphae in 4 weeks (Fig. 5b). Sugars (galactose, glucose and trehalose) were detected in the released exudate in both the KH_2PO_4 treatments tested (i.e., 0 and 35 μM KH_2PO_4). Two types of carboxylates were found in the treatment with zero KH_2PO_4 (aconitate and citrate), while three types of carboxylates were found in the treatment with 35 μM KH_2PO_4 (aconitate, citrate and succinate).

In vitro culture experiment

In the *in vitro* bacterial incubation experiment, the medium collected from MR^{-}/RIr^{-} and MR^{+}/RIr^{-} treatments from the Petri plate experiment 2 exhibited a consistent effect on bacterial growth at both the tested KH_2PO_4 concentrations (i.e., 0 and 35 μM). The bacterial ODs in the 0.85% NaCl solution (i.e., control) and the MR^{-}/RIr^{-} medium did not change over the time period 1 to 47 h, and, at each time point, there was no difference in bacterial OD's between these two treatments (Fig. 6). In the MR^{+}/RIr^{-} medium, the bacterial ODs increased from 5 to 13 h and then stabilized from 15 to 47 h. In the MR^{+}/RIr^{+} medium (which contained RIr hyphal exudate), the bacterial ODs increased from 1 to 47 h when the medium contained zero KH_2PO_4, while the ODs increased from 1 to 25 h and then did not change from 27 to 47 h when the medium contained 35 μM KH_2PO_4. At each timepoint, the bacterial OD in the MR^{+}/RIr^{+} treatment was significantly larger than that in the other three treatments (Fig. 6).

Discussion

Similar to roots (Hodge & Millard, 1998; Hodge *et al*., 1998), AMF hyphae release C-rich compounds (Toljander *et al*., 2007; Bharadwaj *et al*., 2012) into the soil which can stimulate microbial growth and function (Filion *et al*., 1999; Leigh *et al*., 2011; Zhang *et al*., 2014a). Other studies have demonstrated AMF repress certain groups of bacteria and fungi in a microbial community (Filion *et al*., 1999; Nuccio *et al*., 2013; Bender *et al*., 2014) but enhance others (Nuccio *et al*., 2013; Bender *et al*., 2014). The exact mechanisms behind these interactions are unknown although several suggestions have been proposed including: niche competition for nutrients (Christensen & Jakobsen, 1993; Veresoglou *et al*., 2011), physical interactions including the ability to attach to AMF hyphae (Toljander *et al*., 2006; Scheublin *et al*., 2010) or manipulation of the community via direct or indirect influences of AMF hyphal exudation (Filion *et al*., 1999; Toljander *et al*., 2007). In this study we found evidence for the latter mechanism.

Hyphal exudates are generally reported as mainly comprising sugars, carboxylates and amino acids (Toljander *et al*., 2007; Bharadwaj *et al*., 2012). In this study, we found the sugars (galactose, glucose and trehalose) and the carboxylates (aconitate, citrate and succinate) which were released by the RIr hyphae, although succinate was only detected at the higher P level (Fig. 5). The occurrence of trehalose is particularly striking given AMF-associated trehalose release has been implicated in inducing shifts in the active bacterial population in the rhizosphere (Drigo *et al*., 2010). Furthermore, previous Biolog analysis showed that RA could use these sugars and carboxylates as substrates except aconitate (Chen, 2007). When the P level was altered RA cell counts were increased both when it was inoculated near the hyphae and when it was incubated with collected hyphal exudates (Fig. 4a; Fig. 6). Thus, our original hypothesis that the PSB could utilize compounds released from the AMF hyphae was well supported (hypothesis 3). However, as we did not quantify the fungal exudates released in this study, further research is required on how both quantitative and qualitative differences in AMF exudates impact upon PSB growth.

Although the term 'exudation' is frequently used to cover any compound released from roots or AMF hyphae, exudation is strictly the loss of water soluble compounds which leak from the roots (or hyphae) without the involvement of metabolic energy (Lynch & Whipps, 1990). Thus, it is not under plant or fungal control (unlike the release of secretions which is an active process dependent upon metabolic energy). Consequently, if these hyphal compounds are passively lost, although it represents a 'cost' to the fungus it does not support the 'reciprocity' theory, which is usually invoked to explain cooperation between different species (West *et al*., 2007; Harcombe, 2010). Moreover, other mechanisms normally associated with cooperative behaviour such as the imposing of sanctions on un-cooperative partners (Kiers *et al*., 2003; West *et al*., 2007; Kiers *et al*., 2011) could not be imposed. Intriguingly, recent evidence has suggested that AMF hyphal 'exudation' may not be a purely passive process, but instead a targeted response which occurs up-stream from the passive exudation processes of the root (Kaiser *et al*., 2015). Moreover, this C release via the AMF had implications for nutrient cycling dynamics in the rhizosphere of wheat plants (Kaiser *et al*., 2015). Future work is required to clarify the exact mechanisms that operate between AMF and PSB, but our results clearly suggest a key role for compounds released from the AMF hyphae.

PSB also benefitted the AMF by improving P availability, and these interactions had an indirect benefit for the plant as shown by shoot P levels from the microcosm study. However, and counter to our first hypothesis, the benefit to the plant of the AMF-PSB interaction only occurred when additional P was also supplied (Fig. 1a). Toro *et al*. (1997) reported PSB aided AMF in acquiring P from sources that were not otherwise accessible to the AMF. However, the main focus of that study was on the resulting impact upon the plant; neither the impact on the AMF nor the potential mechanisms behind the observed effect were evaluated. The results from our Petri plate experiments, show inoculation of RA near the RIr hyphae increased both acid and alkaline phosphatase activity, which hydrolyzed phytate-P in the medium to release inorganic P for AMF (Fig. S1, S5c, S5d). Additionally, RIn hyphal growth was stimulated by the presence of RA (Fig. 3b). These results indicated that PSB could benefit AMF by providing them with inorganic P. In the microcosm experiment acid phosphatase activity in the RIn treatment was higher than the controls at both P levels (Fig. S5a), and phytate-P levels lower than the controls (Fig. 1c). This result is rather odd given AMF are thought to have no ability to secrete phosphatases (Smith & Read, 2008), a suggestion supported by recent genomic sequencing data (Tisserant *et al*., 2013). Therefore, it may have been due to air-borne microbial contamination of some units when in the glasshouse or possibly as a result of microorganisms closely associated with the RIn AMF inoculum used in this experimental phase being introduced into the units when the RIn inoculum was added. That this result was an anomaly was supported by the finding that neither acid nor alkaline phosphatase activity in the RIr treatments was higher than the controls in the Petri plate experiment conducted under aseptic conditions (Fig. S5c, S5d). Utilization of phytate-P therefore depends on other soil microbes (Zhang *et al*., 2014a). PSB, constituting up to 40% of all culturable bacteria (Jorquera *et al*., 2008), can make up for this defect in AMF (see hypothesis 2). Similarly, soil saprobiotic microbes can improve available N for AMF by decomposing organic matter (Leigh *et al*., 2011; Herman *et al*., 2012; Nuccio *et al.*, 2013). Other microbes have additional mechanisms of increasing the fitness of AMF, e.g., by stimulating mycorrhizal colonization, as well as hyphal and spore production (Frey-Klett *et al.*, 2007).

Soil available P levels can determine the bacterial P contribution to plants by regulating the P mobilizing and immobilizing processes (Stevenson, 1986; Zhang *et al.*, 2014b). This principle formed part of our first hypothesis which we tested in the microcosm experiment by manipulating available P levels. Changes of hyphal length density or acid and alkaline phosphatase activity were used to measure the benefits that AMF and PSB gained from each other. In the P-limited soil without added KH_2PO_4, though soil phytate-P was mineralized, RA appeared to compete for the mobilized P with RIn (Fig. 1b) and hyphal length density was not increased (Fig. 3a), which was counter to our original first hypothesis. The acid and alkaline phosphatase activities were also not increased (Fig. S5a, S5b). In contrast, when 5 mg P kg^{-1} in the form of KH_2PO_4 was added, due to the lessened competition (Fig. 1b) and enhanced phytate-P mineralization (Fig. 1c), RIn hyphal length density was increased (Fig. 3a). As a result, RA acid and alkaline phosphatase activities also increased (Fig. S5a, S5b). Moreover, the hyphal exudate collected under 35 μM KH_2PO_4 promoted bacterial growth more effectively than under 0 μM KH_2PO_4 (Fig. 6). These results suggest that AMF could enhance the activity of PSB that successfully increased P availability and benefited fungal growth. However, *GiPT* expression was not up-regulated in our study due to the presence of the PSB despite the PSB impacting P availability (see hypothesis 2; Fig. S6). Using the same RIr isolate as the present study, Fiorilli *et al*. (2013) also found no difference in expression of *GintPT* (namely *GiPT*) due to external Pi levels in mycorrhizal roots of *Medicago.* When only cells containing arbuscules were examined by laser microdissection however *GintPT* expression was found to be down-regulated (by 2-fold) at the higher P level (i.e., 320 μM versus 32 μM). Following phosphate application to AMF hyphae the genes responsible for phosphate, nitrogen and maintenance of cellular homeostasis were up-regulated in the study by Kikuchi *et al*. (2014), although the levels of P application in their study were more extreme (i.e., 1 mM KH_2PO_4 added to P-starved mycelia) compared to overall P levels in our study.

Collectively, our results demonstrate that beneficial interactions between an AMF and a PSB occur, with each providing a key resource for the other (Fig. 7), but that the beneficial nature of the interaction is altered by background P status. PSB are responsible for organic P hydrolysis by releasing phosphatases (Fig. S5) while AMF can acquire the inorganic P subsequently released and AMF hyphal growth was enhanced (Fig. 3). AMF release C compounds into the hydrosphere which the PSB were demonstrated to utilize but the background P status modified the compounds released (with succinate detected only at the higher background P level) and PSB growth was also altered (Fig. 6). However, before these interactions can be classified as cooperative behavior, key questions remain to be addressed. First, there is the question regarding the mechanism underlying the release of C compounds from the AMF hyphae: is this under AMF control or are the PSB simply benefitting from C leakage? Secondly, we only used one AMF species and one PSB strain. Thus, there was no opportunity for selection of 'best-partners' among different potential partners and so no demonstration of reciprocal rewards. This reciprocal rewards mechanism has been proposed to stabilize cooperation in the both the mycorrhizal and legume-rhizobia symbiosis (Kiers *et al*., 2003; Hammer *et al*., 2011). In the one-to-one system (one fungus colonizes one plant root), the quantity of C provided by the plant depends on the P contribution of its fungal partner, and *vice versa* (Hammer *et al*., 2011). In the many-to-many system (many fungi colonize many plant roots), plants can detect, discriminate, and reward the best fungal partners with more carbohydrates. In turn, their fungal partners enforce cooperation by increasing nutrient transfer only to those roots providing more carbohydrates (Kiers *et al*., 2011; Fellbaum *et al*., 2014). Unlike in the mycorrhizal symbiosis, where both plants and fungi can select between multiple potential partners, in the hyphosphere, AMF may obtain P from different PSB, but it is more likely AMF choice is more limited for the PSB due to scale and non-filamentous growth issues thus each bacterium is likely dependent upon only a single AMF hypha for its C support. Thus, this may be expected to make the PSB more open to cooperative behavior, but in our study the PSB did not promote AMF hyphal growth at the lower P availability (Fig. 3) suggesting a degree of control by the PSB also. The results from our study therefore suggest the mechanisms behind resource exchange are complex but support ideas for further studies.

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Figure legends

Fig. 1 Variations in (a) shoot P content of 8-week-old *Medicago sativa* seedlings and (b) microbial biomass P (MBP) and (c) phytate P in soil inoculated with *Rhizophagus intraradices* (RIn) and/or *Rahnella aquatilis* (RA) in the microcosm experiment. Different letters indicate significant differences among inoculation treatments; asterisks indicate significant differences between the same inoculation treatment at the two different KH_2PO_4 levels (i.e., +/− KH_2PO_4).

Fig. 2 Correlations between (a) acid phosphatase activity and phytate P (y = –4.3x+10.4; R^2 = 0.699, P = 0.010), (b) phytate P and MBP (y = –1.0x+7.0; R^2 = 0.576, P = 0.029) and (c) phytate P and shoot P content in the microcosm experiment. Open squares, treatments without KH_2PO_4 and RIn; closed squares, treatments without KH_2PO_4 but RIn; open triangles, treatments with KH_2PO_4 but not RIn; closed triangles, treatments with KH_2PO_4 and RIn. RIn, *Rhizophagus intraradices*.

Fig. 3 Hyphal (a) length in the soil ± KH_2PO_4 in the microcosm experiment and (b) fresh weight in the medium in the Petri plate experiment 3 when the hyphal compartment ± inoculation with RA. Different letters indicate significant differences between inoculation treatments. The asterisk indicates significant differences between the same inoculation treatment + or − KH_2PO_4. RIn, *Rhizophagus intraradices*; RIr, *Rhizophagus irregularis*; RA, *Rahnella aquatilis*.

Fig. 4 Variations in (a) bacterial numbers in the medium and (b) *GiPT* expression of hyphae harvested at 2 w or 4 w following inoculation with RA in Petri plate experiment 1. Different letters indicate significant differences between inoculation treatments; asterisks indicate significant differences between the same inoculation treatment between 2 w and 4 w. RIr, *Rhizophagus irregularis*; RA, *Rahnella aquatilis*.

Fig. 5 The concentration of (a) total carbon (C) of hyphal exudates in the medium and (b) C released by per unit weight of hyphae with 0 or 35 μM KH_2PO_4 in Petri plate experiment 2.

Fig. 6 Effect of AMF hyphal exudates collected from the medium with (a) 0 or (b) 35 μM KH_2PO_4 in Petri plate experiment 2 on the bacterial growth of *Rahnella aquatilis* (RA). The data were calculated as the culture optical density (OD) from 1 to 47 hours minus the initial OD of the bacterial liquid cultures. Treatment codes are as follows: MR^{-}/RIr^{-}, no roots or AMF hyphae in either compartment; MR^{+}/RIr^{-}, mycorrhizal roots in the root compartment but AMF hyphae omitted from the hyphal compartment, MR^{+}/RIr^{+}, mycorrhiza-colonized roots in the root compartment and AMF hyphae permitted into the hyphal compartment.

Fig. 7 Schematic representation of the hyphosphere AMF-PSB interaction on organic P utilization for the host plant. PSB, phosphate-solubilizing bacteria; Pase, phosphatase.

Fig 1

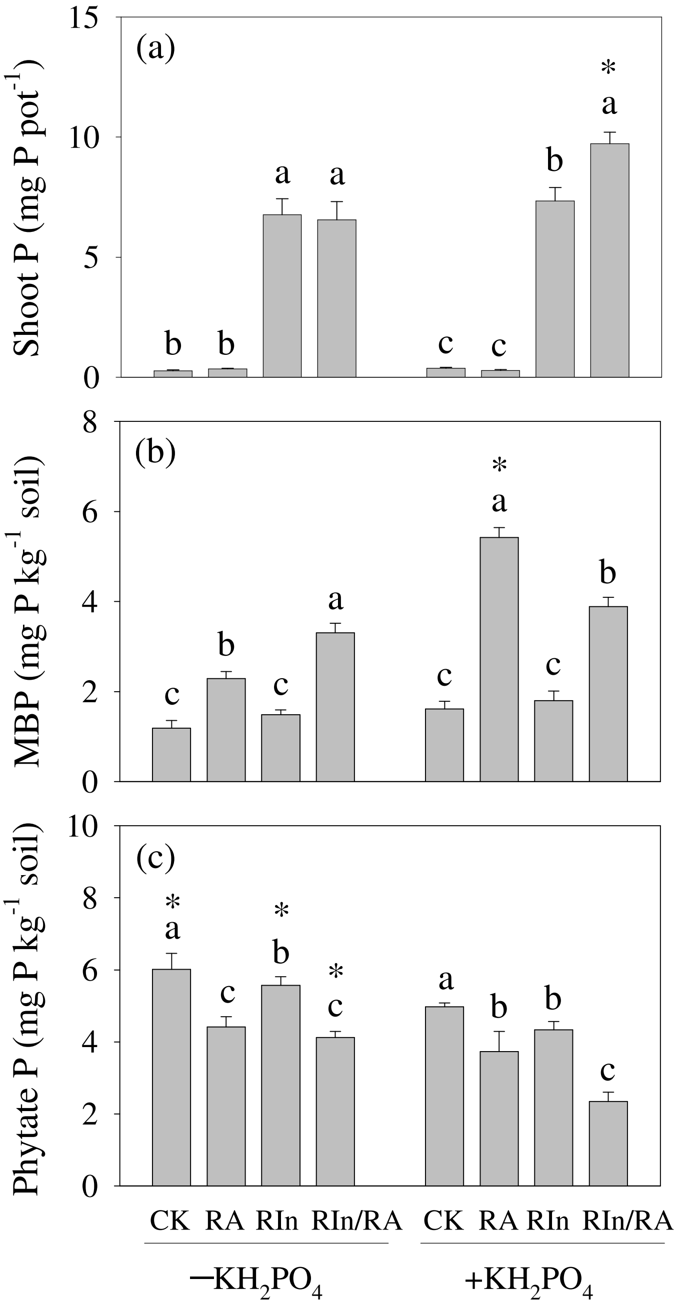

(a)
Shoot P (mg P pot^{-1})
(b)
MBP (mg P kg^{-1} soil)
(c)
Phytate P (mg P kg^{-1} soil)
CK RA RIn RIn/RA CK RA RIn RIn/RA
−KH_2PO_4
+KH_2PO_4

Fig 2

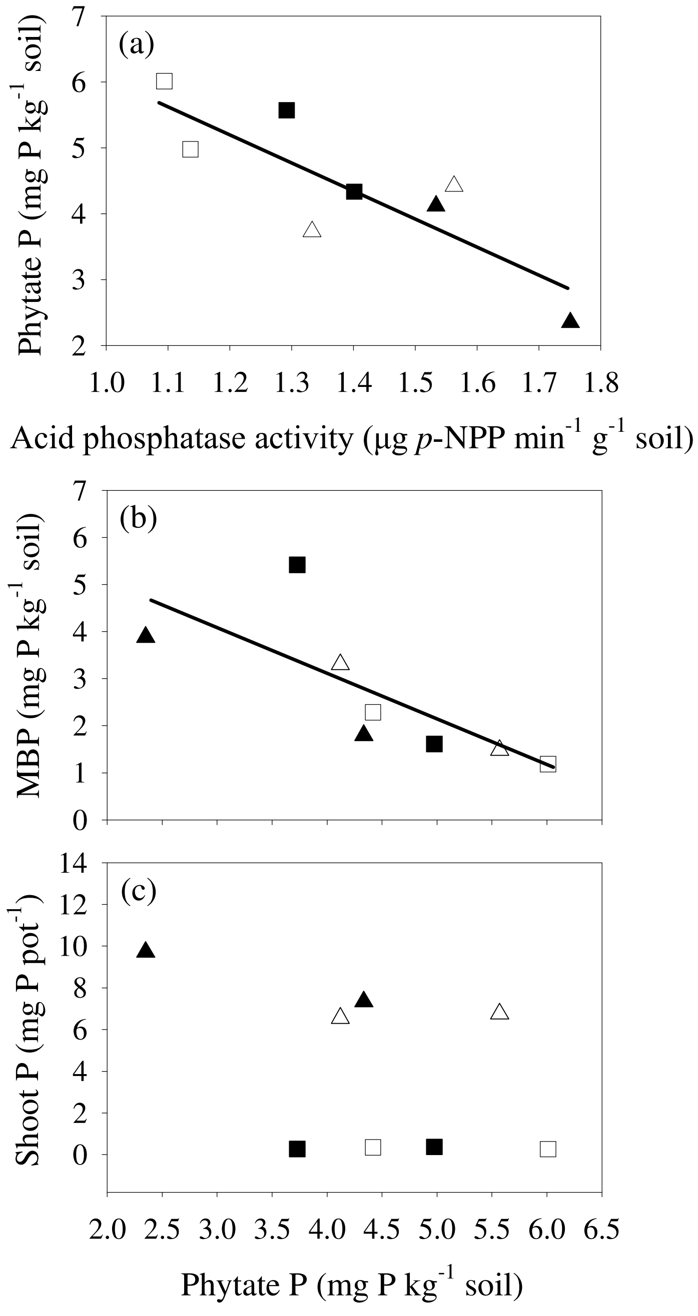
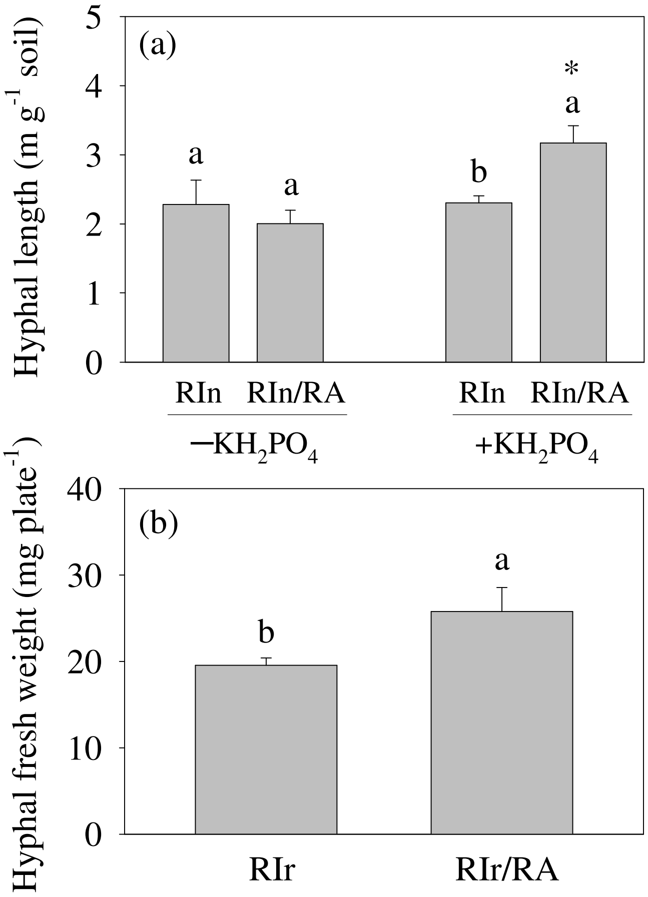

(a)
Phytate P (mg P kg-1 soil)
7
6
5
4
3
2
1.0 1.1 1.2 1.3 1.4 1.5 1.6 1.7 1.8
Acid phosphatase activity (µg p-NPP min-1 g-1 soil)
(b)
MBP (mg P kg-1 soil)
7
6
5
4
3
2
1
0
(c)
Shoot P (mg P pot-1)
14
12
10
8
6
4
2
0
2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5
Phytate P (mg P kg-1 soil)

Fig 3



(a)
Hyphal length (m g-1 soil)
0
1
2
3
4
5
a
a
b
*
a
RIn
RIn/RA
RIn
RIn/RA
−KH2PO4
+KH2PO4
(b)
Hyphal fresh weight (mg plate-1)
0
10
20
30
40
b
a
RIr
RIr/RA

Fig 4

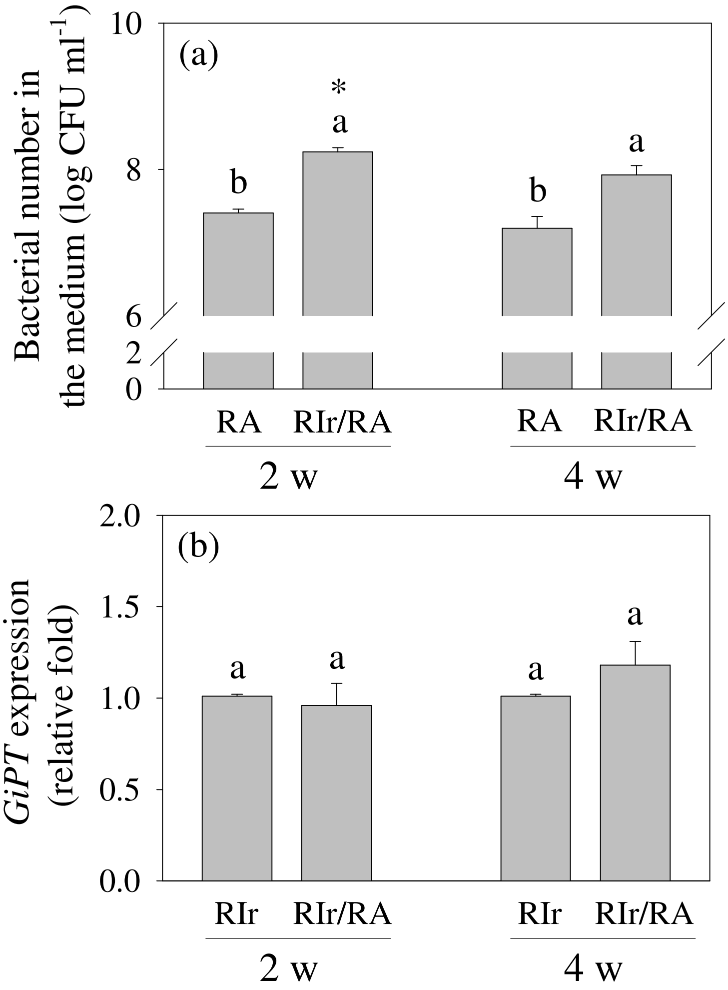

(a)
Bacterial number in the medium (log CFU ml-1)
10
8
6
2
0
*
a
b
b
a
RA
RIr/RA
RA
RIr/RA
2 w
4 w
(b)
GiPT expression (relative fold)
2.0
1.5
1.0
0.5
0.0
a
a
a
a
RIr
RIr/RA
RIr
RIr/RA
2 w
4 w

Fig 5

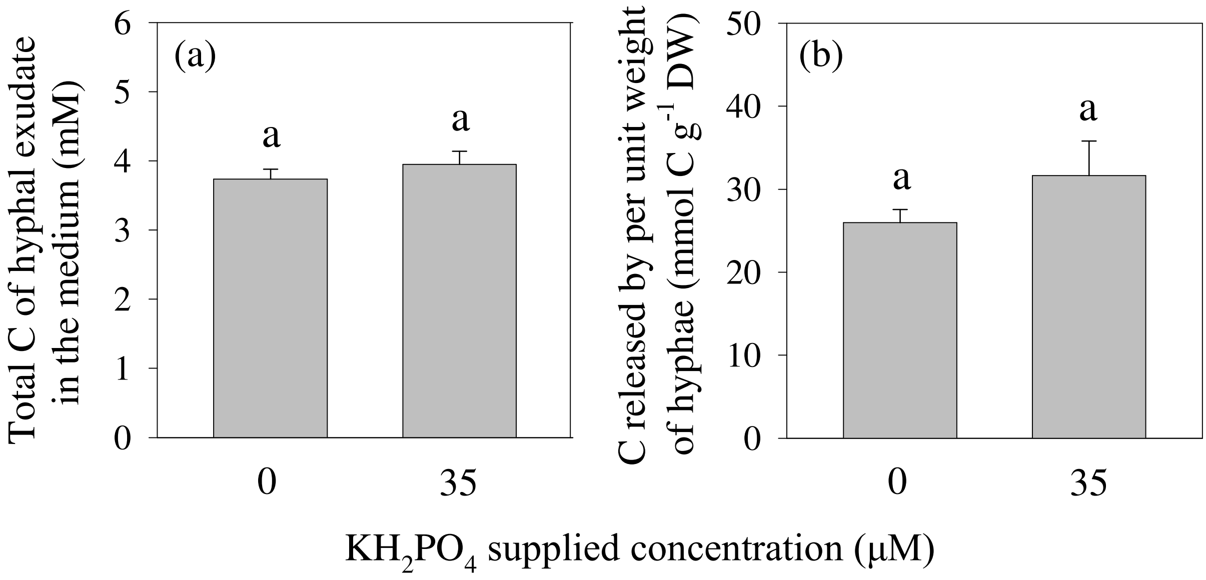

(a)
Total C of hyphal exudate in the medium (mM)
6
5
4
3
2
1
0
a
a
0
35
(b)
C released by per unit weight of hyphae (mmol C g-1 DW)
50
40
30
20
10
0
a
a
0
35
KH2PO4 supplied concentration (μM)

Fig 6

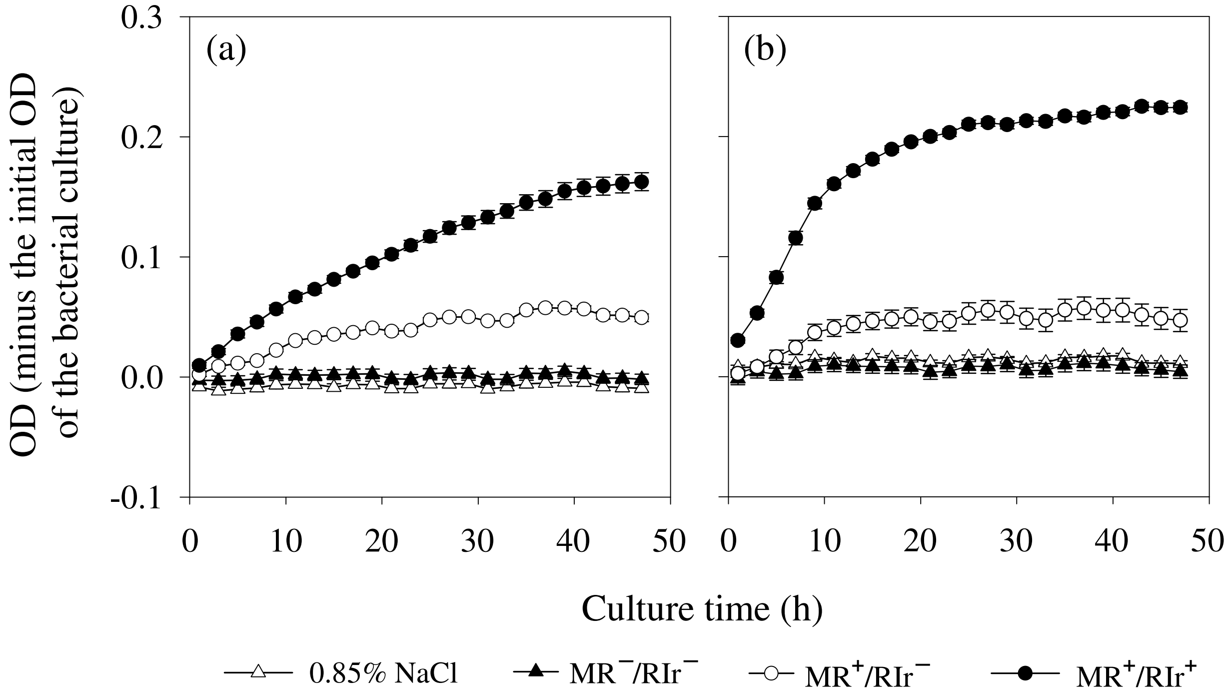
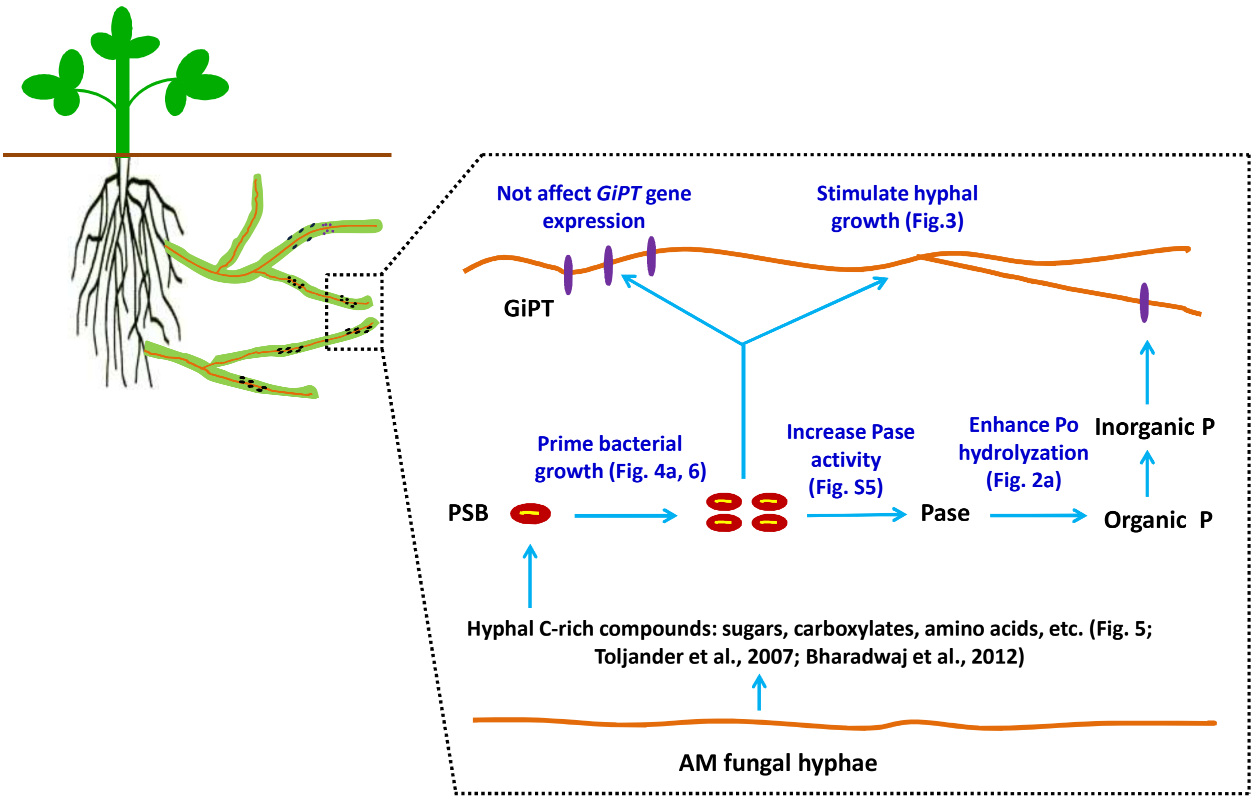

(a)
(b)
OD (minus the initial OD of the bacterial culture)
0.3
0.2
0.1
0.0
-0.1
0
10
20
30
40
50
Culture time (h)
0.85% NaCl
MR⁻/RIr⁻
MR⁺/RIr⁻
MR⁺/RIr⁺

Fig 7


Not affect GiPT gene expression
Stimulate hyphal growth (Fig.3)
GiPT
Prime bacterial growth (Fig. 4a, 6)
Increase Pase activity (Fig. S5)
Enhance Po hydrolyzation (Fig. 2a)
Inorganic P
PSB
Pase
Organic P
Hyphal C-rich compounds: sugars, carboxylates, amino acids, etc. (Fig. 5; Toljander et al., 2007; Bharadwaj et al., 2012)
AM fungal hyphae

Supporting information

Materials and Methods

Table S1 ANOVA output of the repeated-measures analysis

Table S2 Percentage (%) root length colonization (%RLC)

Table S3 Total carbon (C) concentration, volume of the liquid medium in the hyphal compartment

Fig. S1 A visual halo after 7 d growth in a 1.5% agar medium containing 2 g L^{-1} phytate-P and the inorganic P release over 72 incubation hours

Fig. S2 Fluorescent microscope observation pictures

Fig. S3 Schematic diagram of the experimental microcosm.

Fig. S4 Schematic representation of the 100-microwell plate *in vitro* incubation of *Rahnella aquatilis*

Fig. S5 Variations in acid phosphatase activity and alkaline phosphatase activity

Fig. S6 Variations in inorganic P concentration in the medium