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

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Total words: 6209. Introduction, 1087; Materials and methods, 2235; Results, 1051; Discussion, 1772; Acknowledgments, 64

Number of figures: 7

Supporting information: Tables, 3; Figures, 6

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

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

81 Several mechanisms have been proposed to explain cooperation (Nowak, 2006; West *et al*.,
82 2007). Kin selection is a widely accepted theory to explain cooperation formed in the same species
83 (Hamilton, 1963; West *et al*., 2002). For two unrelated species, reciprocity between two partners can
84 maintain their cooperation (Harcombe, 2010), and several lines of indirect evidence suggest that
85 reciprocity may maintain cooperation between AMF and associated soil microbes. AMF hyphae do
86 not benefit all microbes; indeed, they inhibit some (Nuccio *et al*., 2013; Bender *et al*., 2014). In
87 contrast, the stimulated microbes usually have potentially positive effects on AMF fitness (Scheublin

88 *et al*., 2010; Nuccio *et al*., 2013). These observations suggest that AMF may select microbes to
89 cooperate with. The excretion of metabolite products can provide a mechanism for the initiation of
90 reciprocation (Sachs *et al*., 2004). AMF produce extensive extraradical hyphae and transfer plant
91 derived C-rich compounds to the attached soil, providing them to microbes (Kaiser *et al*., 2015),
92 which usually face C scarcity (Blagodatskaya & Kuzyakov, 2013). However, AMF have no known
93 saprotrophic capability, which means that they cannot directly breakdown organic nutrients (Smith &
94 Read, 2008; Tisserant *et al*., 2013). In contrast, microbes are diverse in functions and play especially
95 important and varied roles within elemental (e.g., C, N, and P) biogeochemical cycles (Torsvik &
96 Øvreås, 2002; Nannipieri *et al*., 2003). Microbes can release various enzymes to decompose organic
97 matter, and in doing so can provide the AMF hyphae with inorganic nutrients (Hodge & Fitter, 2010;
98 Hodge, 2014; Zhang *et al*., 2014a). Therefore, microbes do not merely use AMF-released C but may
99 also pay back other benefits required by the fungi. Through cooperation, AMF and microbes can get
100 what they need from their partners and improve their own fitness.

101 Although P is the key nutrient that AMF acquire (Smith & Read, 2008), they lack the ability to
102 secrete phosphatases (Tisserant *et al*., 2013). Thus, AMF cannot utilize organic P directly, which
103 limits their contribution to plants P uptake, especially in forest soils with rich organic matters and
104 agricultural soils with large amounts of applied manure. However, more than 40% of culturable
105 bacteria are able to mineralize organic P (the so-called phosphate-solubilizing bacteria (PSB)) by
106 releasing numerous phosphatases into the surrounding soil (Jorquera *et al*., 2008). Although previous
107 studies have shown that AMF and PSB can interact to improve P acquisition for the AM host plant
108 (Toro *et al*., 1997; Kim *et al*., 1998), the mechanisms behind this nutritional benefit are unclear
109 (Artursson *et al*., 2006). In the present study we focus on the potential mechanisms behind the

110 synergy that exists between AMF and PSB by investigating the interactions of the two organisms
111 directly. PSB may rely on C released by AMF and in return provide hyphae with inorganic phosphate.
112 Here, we hypothesized that there was cooperation between AMF and PSB. More specifically we
113 conducted a series of experiments to address the following hypotheses:

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

120

121 **Materials and methods**

122 To test our hypotheses we performed four different experiments:

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

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

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

131 Experiment 4 (*In vitro* culture experiment): To determine if AMF hyphal exudates (collected

132 from Experiment 3) influenced growth of the PSB.

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

145 ## Microcosm experiment

146 The microcosm units each had two compartments, which were separated by a 30 μm mesh in the
147 middle. One compartment contained the plant, while the other was the hyphal compartment where
148 the AMF-PSB interaction could be investigated (see Fig. S3). Details of the experimental set-up are
149 given in the materials and methods section of the supporting information. The microcosm experiment
150 contained the following treatments: (1) two KH_2PO_4 levels, with 0 or 5 mg P kg^{-1} soil in the hyphal
151 soil, (2) with or without RIn in the plant compartment, and (3) with or without RA in the hyphal soil
152 which were applied in a factorial manner across the microcosms. Soil in the hyphal section also
153 contained 75 mg P kg^{-1} DW as Na-phytate (Sigma-Aldrich, St. Louis, MO, USA) because phytate P

154 is one of the main organic P forms in the soil (Turner *et al*., 2002). Each treatment had four replicates,
155 thus, there were 32 microcosms in total, which were arranged in a randomized block design in a
156 greenhouse. Plants in these microcosms were grown at China Agricultural University in Beijing from
157 12 May to 10 July 2011 at 24/30°C (night/day). The average photosynthetically active radiation at
158 plant level was 360 μmol m^{-2} s^{-1}. Soil gravimetrical moisture was kept at 18-20% (w/w, ~70% water
159 holding capacity) with deionized water by weighing the microcosm units every 2 days during the
160 experimental period.

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

168 Microbial biomass P (MBP) in the hyphal soil was extracted by the chloroform
169 fumigation-extraction method (Brookes *et al*., 1982), determined colorimetrically by a modified
170 ammonium molybdate-ascorbic acid method (Jackson, 1958) and calculated assuming a k_P value of
171 0.40 (Brookes *et al*., 1982). External mycorrhizal hyphae were extracted from two 5 g soil
172 sub-samples from the hyphal soil compartment using a modified membrane filter technique (Staddon
173 *et al*., 1999). Hyphal length was assessed using the gridline intercept method at × 200 magnification
174 and then converted to hyphal length density (m g^{-1} DW soil; Hodge, 2003).

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

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

182 Petri plate experiment 1

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

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

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

203 This Petri plate experiment examined three factors: (1) two RIr levels, with or without RIr in the
204 root compartment, (2) two RA levels, with or without RA in the hyphal compartment, and (3) two
205 harvest times, 2 weeks or 4 weeks after RA inoculation. Each treatment had four replicates, resulting
206 in a total of 32 plates. At harvest, the hyphae and medium in the hyphal compartment were separated.
207 The hyphae were put into a 2-ml tube using sterilized forceps and were immediately stored at -80°C
208 to determine the expression of the phosphate transport gene *GiPT*, according to the method by
209 Fiorilli *et al*. (2013). The bacterial attachments of RA to the hyphal surface were observed using an
210 Olympus BX51 fluorescence microscope (Olympus Optical, Tokyo, Japan). Some of the liquid
211 medium was used to immediately estimate bacterial numbers by determining the total number of
212 colony-forming units of RA (CFU per ml medium) in the medium using the plate count method of
213 Smit *et al*. (2001). The remainder of the medium was passed through a Acrodisc® Syringe Filter (0.2
214 μm Supor® Membrane, Pall Corporation, New York, USA) and stored at -20°C for acid and alkaline
215 phosphatase activity (μg *p*-nitrophenyl phosphate min^{-1} ml^{-1} medium) determination (as Neumann,
216 2006). Inorganic P concentration in the medium was measured with malachite green reagent (Irving
217 & McLaughlin, 1990).

218 Petri plate experiment 2

219 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,

242 USA) and stored at -20°C for subsequent analysis. The total C concentration of the medium was
243 determined by multi N/C® UV HS (Analytik Jena AG, Eisfeld, Germany), and the carboxylate
244 content was determined according to Shen *et al*. (2003). Sugar content was determined by ICS-3000
245 Ion Chromatography System (Dionex, California, USA). Total C of hyphal exudate in the medium
246 was calculated as following:

247 $$(C_{MR^+/RIr^+} \times V_{MR^+/RIr^+} - C_{MR^+/RIr^-} \times V_{MR^+/RIr^-}) / V_{MR^+/RIr^+}$$

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

249 $$(C_{MR^+/RIr^+} \times V_{MR^+/RIr^+} - C_{MR^+/RIr^-} \times V_{MR^+/RIr^-}) / m.$$

250 where:

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

255 *In vitro* culture experiment

256 RA was cultured in liquid LB medium for 12 h at 37°C at 180 rpm and then centrifuged at 8,000 rpm
257 for 6 min. The supernatant was discarded and the pellet was re-suspended using sterilized 0.85%
258 NaCl solution. After washing three times, the bacteria were diluted (OD_{600} = 0.1). In a 100-microwell
259 plate, 200 µl of 0.85% NaCl solution was added to the wells surrounding the plate without bacteria to
260 avoid potential border effects; 180 µl of 0.85% NaCl solution or medium collected from the Petri
261 plate experiment 2 (i.e., in the treatments of MR^-/RIr^-, MR^+/RIr^-, MR^+/RIr^+) in different KH_2PO_4
262 concentrations (i.e., 0 and 35 µM) was added to the other wells of the plate. Then, 20 µl of the
263 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

329 expression of *GiPT,* which was similar among the various treatments (Fig. 4b).

330 ## Petri plate experiment 2

331 Hyphal dry weight and total C in the hyphal exudate in the medium did not differ between the 0 and
332 35 μM KH_2PO_4 treatments. The dry weight of hyphae was 1.2-1.3 mg $plate^{-1}$ (Table S3), and the
333 concentration of total C of hyphal exudate in the medium was *c*. 4.0 mM (Fig. 5a). Thus, RIr
334 released approximately 30 mM C g^{-1} DW hyphae in 4 weeks (Fig. 5b). Sugars (galactose, glucose
335 and trehalose) were detected in the released exudate in both the KH_2PO_4 treatments tested (i.e., 0 and
336 35 μM KH_2PO_4). Two types of carboxylates were found in the treatment with zero KH_2PO_4
337 (aconitate and citrate), while three types of carboxylates were found in the treatment with 35 μM
338 KH_2PO_4 (aconitate, citrate and succinate).

339 ## *In vitro* culture experiment

340 In the *in vitro* bacterial incubation experiment, the medium collected from MR^-/RIr^- and MR^+/RIr^-
341 treatments from the Petri plate experiment 2 exhibited a consistent effect on bacterial growth at both
342 the tested KH_2PO_4 concentrations (i.e., 0 and 35 μM). The bacterial ODs in the 0.85% NaCl solution
343 (i.e., control) and the MR^-/RIr^- medium did not change over the time period 1 to 47 h, and, at each
344 time point, there was no difference in bacterial OD's between these two treatments (Fig. 6). In the
345 MR^+/RIr^- medium, the bacterial ODs increased from 5 to 13 h and then stabilized from 15 to 47 h. In
346 the MR^+/RIr^+ medium (which contained RIr hyphal exudate), the bacterial ODs increased from 1 to
347 47 h when the medium contained zero KH_2PO_4, while the ODs increased from 1 to 25 h and then did
348 not change from 27 to 47 h when the medium contained 35 μM KH_2PO_4. At each timepoint, the
349 bacterial OD in the MR^+/RIr^+ treatment was significantly larger than that in the other three

350 treatments (Fig. 6).

351

352 ## Discussion

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

364 Hyphal exudates are generally reported as mainly comprising sugars, carboxylates and amino
365 acids (Toljander *et al*., 2007; Bharadwaj *et al*., 2012). In this study, we found the sugars (galactose,
366 glucose and trehalose) and the carboxylates (aconitate, citrate and succinate) which were released by
367 the RIr hyphae, although succinate was only detected at the higher P level (Fig. 5). The occurrence of
368 trehalose is particularly striking given AMF-associated trehalose release has been implicated in
369 inducing shifts in the active bacterial population in the rhizosphere (Drigo *et al*., 2010). Furthermore,
370 previous Biolog analysis showed that RA could use these sugars and carboxylates as substrates
371 except aconitate (Chen, 2007). When the P level was altered RA cell counts were increased both

372 when it was inoculated near the hyphae and when it was incubated with collected hyphal exudates
373 (Fig. 4a; Fig. 6). Thus, our original hypothesis that the PSB could utilize compounds released from
374 the AMF hyphae was well supported (hypothesis 3). However, as we did not quantify the fungal
375 exudates released in this study, further research is required on how both quantitative and qualitative
376 differences in AMF exudates impact upon PSB growth.

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

392 PSB also benefitted the AMF by improving P availability, and these interactions had an indirect
393 benefit for the plant as shown by shoot P levels from the microcosm study. However, and counter to

394 our first hypothesis, the benefit to the plant of the AMF-PSB interaction only occurred when
395 additional P was also supplied (Fig. 1a). Toro *et al*. (1997) reported PSB aided AMF in acquiring P
396 from sources that were not otherwise accessible to the AMF. However, the main focus of that study
397 was on the resulting impact upon the plant; neither the impact on the AMF nor the potential
398 mechanisms behind the observed effect were evaluated. The results from our Petri plate experiments,
399 show inoculation of RA near the RIr hyphae increased both acid and alkaline phosphatase activity,
400 which hydrolyzed phytate-P in the medium to release inorganic P for AMF (Fig. S1, S5c, S5d).
401 Additionally, RIn hyphal growth was stimulated by the presence of RA (Fig. 3b). These results
402 indicated that PSB could benefit AMF by providing them with inorganic P. In the microcosm
403 experiment acid phosphatase activity in the RIn treatment was higher than the controls at both P
404 levels (Fig. S5a), and phytate-P levels lower than the controls (Fig. 1c). This result is rather odd
405 given AMF are thought to have no ability to secrete phosphatases (Smith & Read, 2008), a
406 suggestion supported by recent genomic sequencing data (Tisserant *et al*., 2013). Therefore, it may
407 have been due to air-borne microbial contamination of some units when in the glasshouse or possibly
408 as a result of microorganisms closely associated with the RIn AMF inoculum used in this
409 experimental phase being introduced into the units when the RIn inoculum was added. That this
410 result was an anomaly was supported by the finding that neither acid nor alkaline phosphatase
411 activity in the RIr treatments was higher than the controls in the Petri plate experiment conducted
412 under aseptic conditions (Fig. S5c, S5d). Utilization of phytate-P therefore depends on other soil
413 microbes (Zhang *et al*., 2014a). PSB, constituting up to 40% of all culturable bacteria (Jorquera *et al*.,
414 2008), can make up for this defect in AMF (see hypothesis 2). Similarly, soil saprobiotic microbes
415 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

438 the higher P level (i.e., 320 μM versus 32 μM). Following phosphate application to AMF hyphae the
439 genes responsible for phosphate, nitrogen and maintenance of cellular homeostasis were up-regulated
440 in the study by Kikuchi *et al*. (2014), although the levels of P application in their study were more
441 extreme (i.e., 1 mM KH_2PO_4 added to P-starved mycelia) compared to overall P levels in our study.

442 Collectively, our results demonstrate that beneficial interactions between an AMF and a PSB
443 occur, with each providing a key resource for the other (Fig. 7), but that the beneficial nature of the
444 interaction is altered by background P status. PSB are responsible for organic P hydrolysis by
445 releasing phosphatases (Fig. S5) while AMF can acquire the inorganic P subsequently released and
446 AMF hyphal growth was enhanced (Fig. 3). AMF release C compounds into the hydrosphere which
447 the PSB were demonstrated to utilize but the background P status modified the compounds released
448 (with succinate detected only at the higher background P level) and PSB growth was also altered (Fig.
449 6). However, before these interactions can be classified as cooperative behavior, key questions
450 remain to be addressed. First, there is the question regarding the mechanism underlying the release of
451 C compounds from the AMF hyphae: is this under AMF control or are the PSB simply benefitting
452 from C leakage? Secondly, we only used one AMF species and one PSB strain. Thus, there was no
453 opportunity for selection of 'best-partners' among different potential partners and so no
454 demonstration of reciprocal rewards. This reciprocal rewards mechanism has been proposed to
455 stabilize cooperation in the both the mycorrhizal and legume-rhizobia symbiosis (Kiers *et al*., 2003;
456 Hammer *et al*., 2011). In the one-to-one system (one fungus colonizes one plant root), the quantity of
457 C provided by the plant depends on the P contribution of its fungal partner, and *vice versa* (Hammer
458 *et al*., 2011). In the many-to-many system (many fungi colonize many plant roots), plants can detect,
459 discriminate, and reward the best fungal partners with more carbohydrates. In turn, their fungal

460 partners enforce cooperation by increasing nutrient transfer only to those roots providing more
461 carbohydrates (Kiers *et al*., 2011; Fellbaum *et al*., 2014). Unlike in the mycorrhizal symbiosis, where
462 both plants and fungi can select between multiple potential partners, in the hyphosphere, AMF may
463 obtain P from different PSB, but it is more likely AMF choice is more limited for the PSB due to
464 scale and non-filamentous growth issues thus each bacterium is likely dependent upon only a single
465 AMF hypha for its C support. Thus, this may be expected to make the PSB more open to cooperative
466 behavior, but in our study the PSB did not promote AMF hyphal growth at the lower P availability
467 (Fig. 3) suggesting a degree of control by the PSB also. The results from our study therefore suggest
468 the mechanisms behind resource exchange are complex but support ideas for further studies.
469

470 ## Acknowledgments

471 We thank associate professor Yanbin Guo from College of Resources and Environmental Sciences,
472 China Agricultural University for providing the bacterial strain *Rahnella aquatilis* HX2. This study
473 was supported by the National Natural Science Foundation of China (U1403285, 31372139), the
474 Specialized Research Fund for the Doctoral Program of Higher Education (20120008130001) and
475 the Innovative Group Grant of the National Science Foundation of China (31421092).
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676

677 ## Figure legends

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

683 **Fig. 2** Correlations between (a) acid phosphatase activity and phytate P ($y = –4.3x+10.4$; $R^2 = 0.699$,
684 $P = 0.010$), (b) phytate P and MBP ($y = –1.0x+7.0$; $R^2 = 0.576$, $P = 0.029$) and (c) phytate P and
685 shoot P content in the microcosm experiment. Open squares, treatments without KH_2PO_4 and RIn;
686 closed squares, treatments without KH_2PO_4 but RIn; open triangles, treatments with KH_2PO_4 but not
687 RIn; closed triangles, treatments with KH_2PO_4 and RIn. RIn, *Rhizophagus intraradices*.

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

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

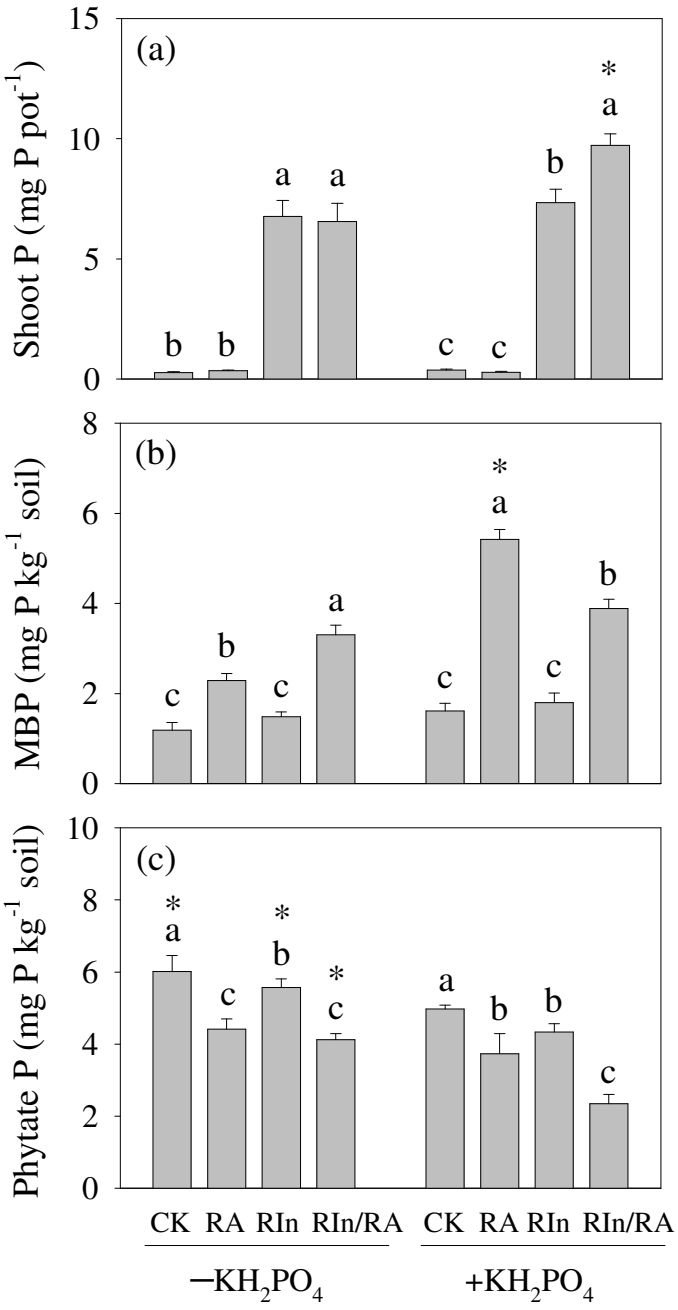
698 **Fig. 5** The concentration of (a) total carbon (C) of hyphal exudates in the medium and (b) C released

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.

711 Fig 1



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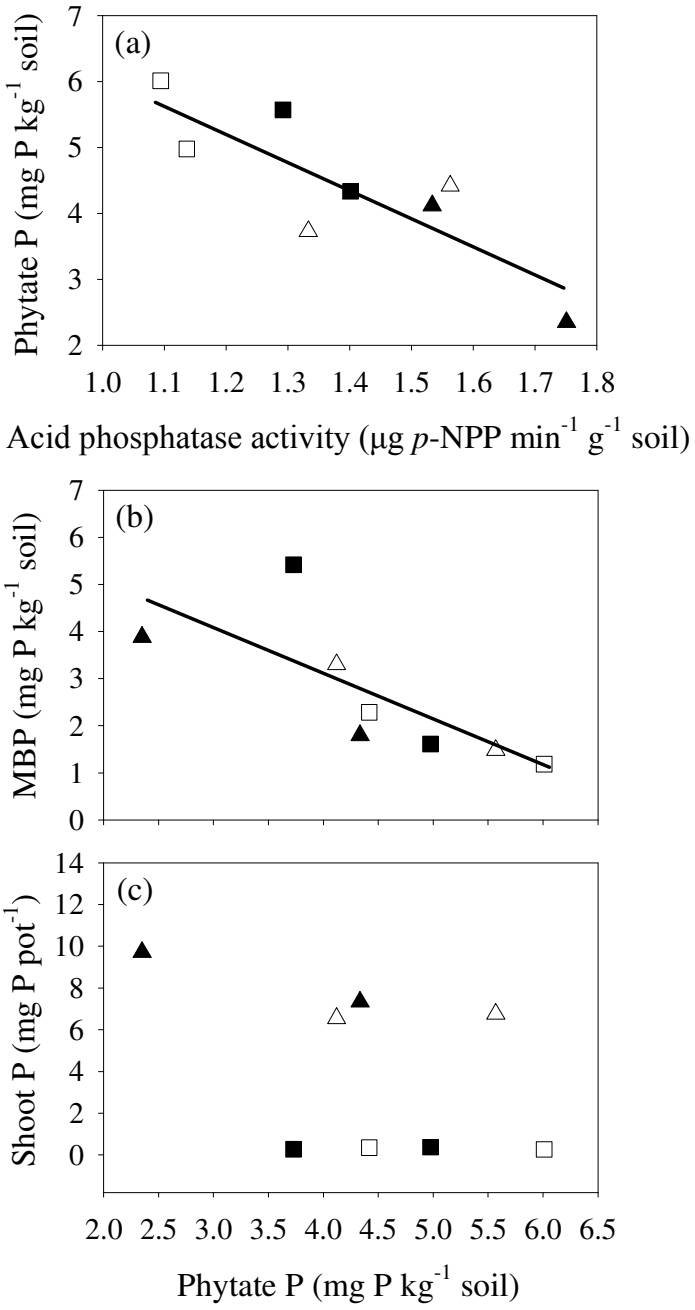
718 Fig 2



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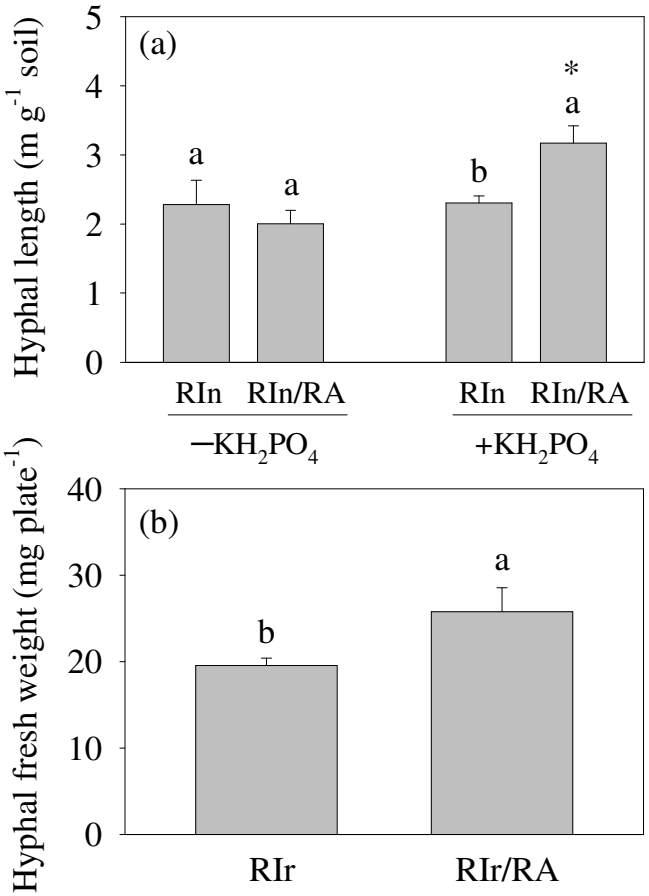
725 Fig 3



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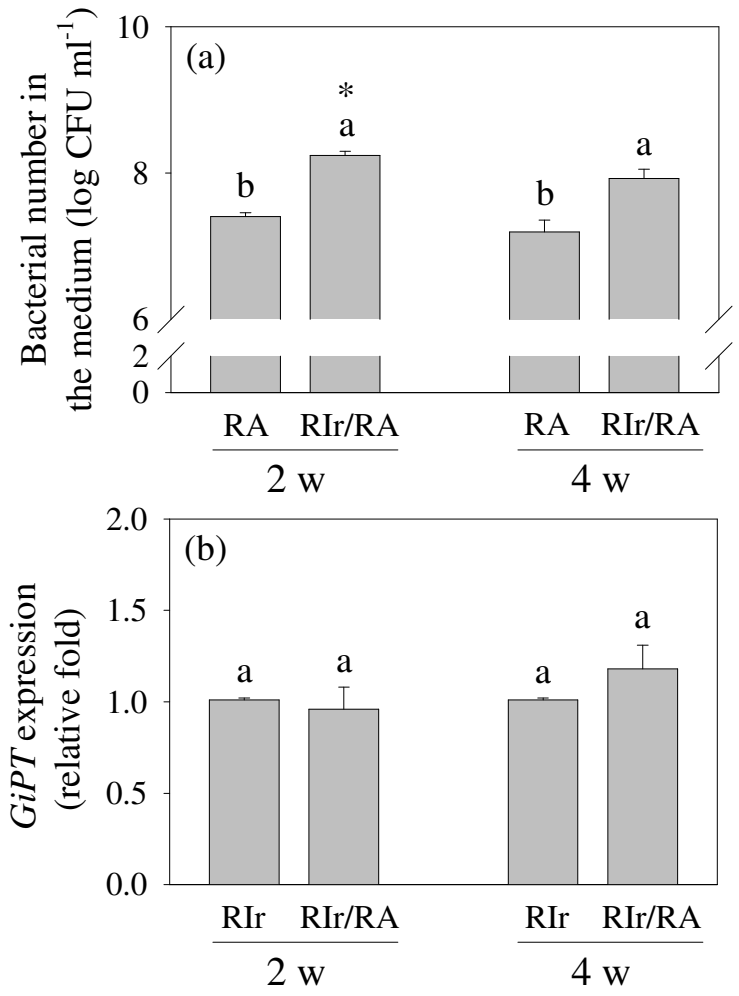

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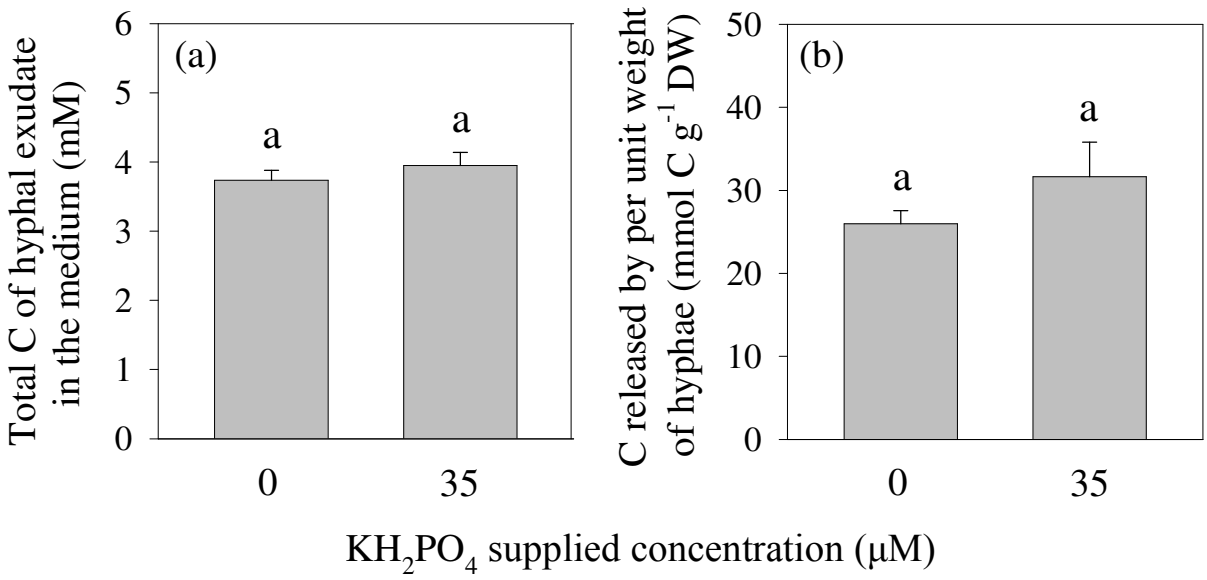

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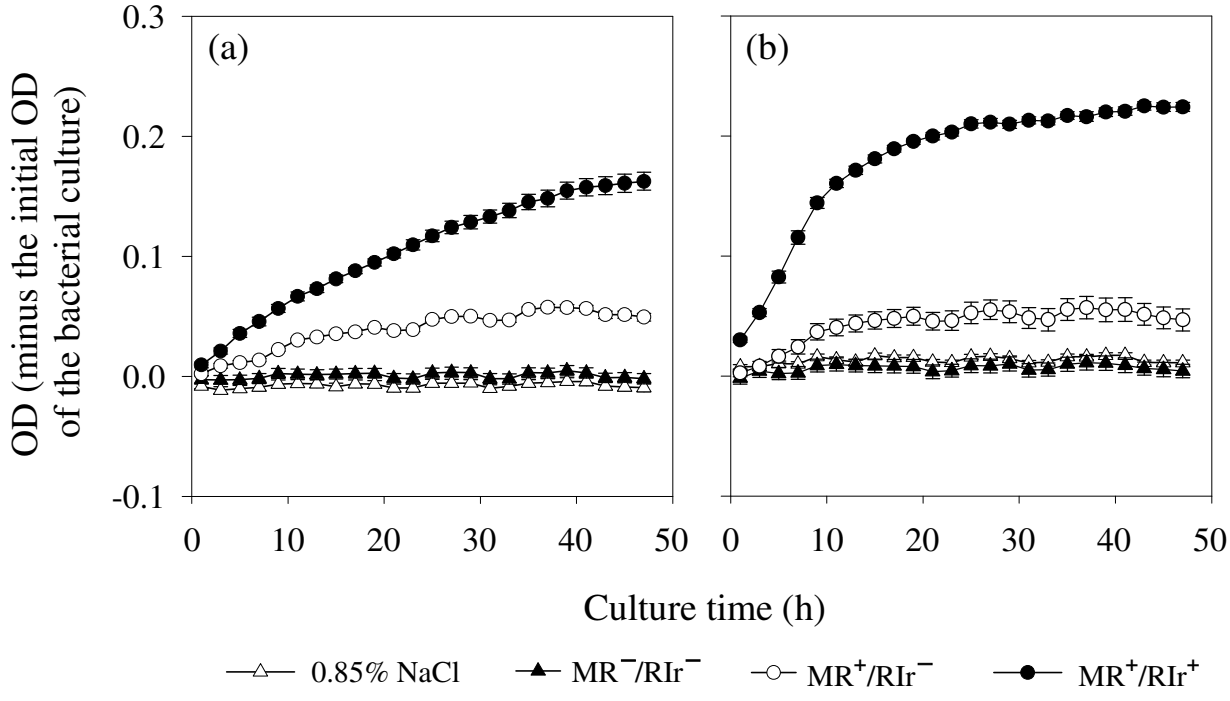

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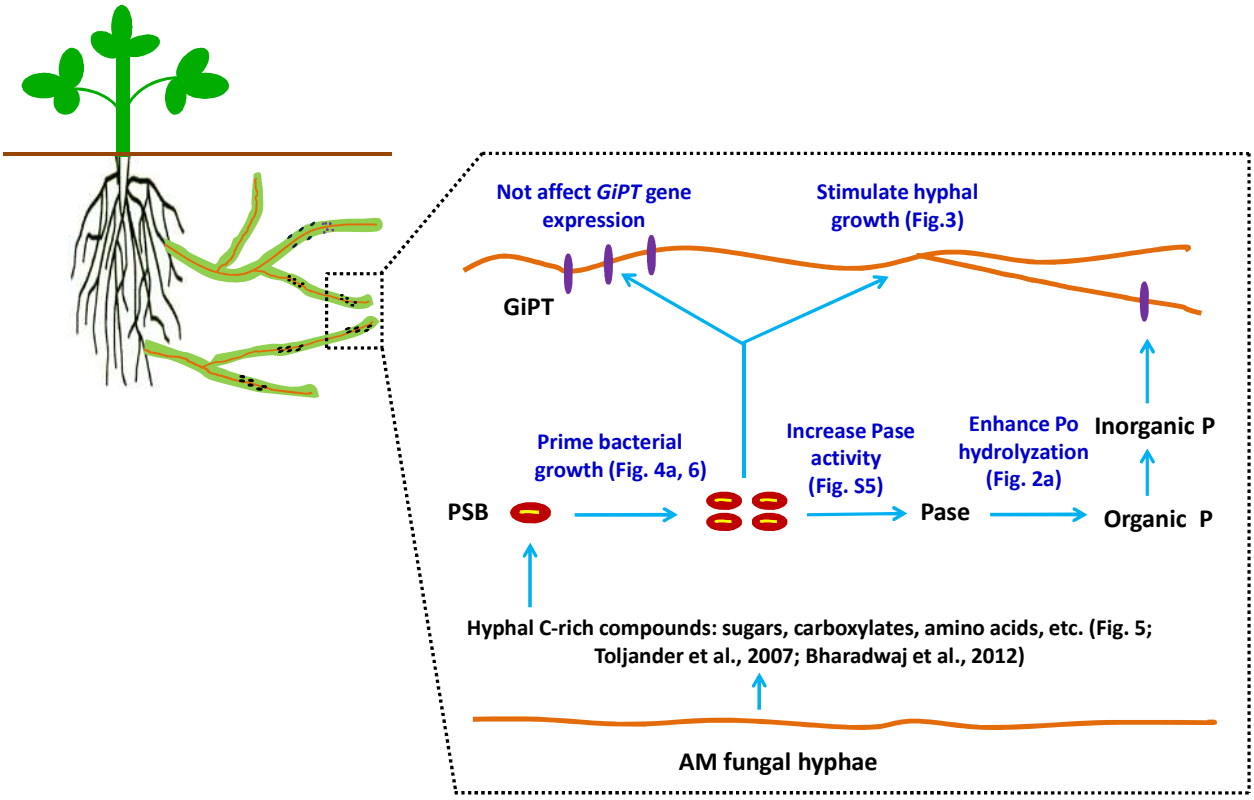

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Supporting information

Materials and Methods