

RESEARCH ARTICLE

Hyphosphere interactions between *Rhizophagus irregularis* and *Rahnella aquatilis* promote carbon–phosphorus exchange at the peri-arbuscular space in *Medicago truncatula*

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Funding information

Beijing Natural Science Foundation, Grant/Award Number: 6212020; National Natural Science Foundation of China, Grant/Award Numbers: 42277112, 32011530457; Program of Advanced Discipline Construction in Beijing

Abstract

Arbuscular mycorrhizal (AM) fungi form a continuum between roots and soil. One end of this continuum is comprised of the highly intimate plant–fungus interface with intracellular organelles for nutrient exchange, while on the other end the fungus interacts with bacteria to compensate for the AM fungus' inability to take up organic nutrients from soil. How both interfaces communicate in this highly complex tripartite mutualism is widely unknown. Here, the effects of phosphate-solubilizing bacteria (PSB) *Rahnella aquatilis* dwelling at the surface of the extraradical hyphae of *Rhizophagus irregularis* was analysed based on the expression of genes involved in C–P exchange at the peri-arbuscular space (PAS) in *Medicago truncatula*. The interaction between AM fungus and PSB resulted in an increase in uptake and transport of Pi along the extraradical hyphae and its transfer from AM fungus to plant. In return, this was remunerated by a transfer of C from plant to AM fungus, improving the C–P exchange at the PAS. These results demonstrated that a microorganism (i.e., a PSB) developing at the hyphosphere interface can affect the C–P exchange at the PAS between plant and AM fungus, suggesting a fine-tuned communication operated between three organisms via two distantly connected interfaces.

INTRODUCTION

Microorganisms establish complex interactions with other organisms and their environment (Zengler & Zaramela, 2018) to evolve in nature (diCenzo et al., 2020). Many of these interactions, in particular cooperation, have been addressed with theoretical tools and concepts (Nowak, 2006), lacking experimental verification. One of the most emblematic examples of inter-kingdom cooperation is the symbiosis established between arbuscular mycorrhizal (AM) fungi and 72% of terrestrial plants (Brundrett & Tedersoo, 2018), characterized mainly by an exchange of carbon (C) and minerals (especially phosphorus—P) between both partners (Kiers et al., 2011; Smith & Read, 2008). In an

attempt to explain the continued cooperation between AM fungi and plants, the market economy theory has been applied (Kiers et al., 2011). In this theory, cooperation is maintained by reciprocal trading of C–P (Bever et al., 2009; Kiers et al., 2011, 2016). However, in their response to the work of Kiers et al. (2016), van der Heijden and Walder (2016) pointed out that, to be a universal model of resources exchange, the specific traits of mycorrhizal symbiosis (e.g. plant sink strength, resources exchange surpluses and environmental conditions) must be taken into consideration, as this would help explain the exchange of resources between the two symbionts. Both plants and AM fungi can detect, discriminate, and reward the best partners with more C or P, respectively (Fellbaum et al., 2014; Kiers

et al., 2011). This is influenced by species and genotypes of plants (Engelmoer & Kiers, 2015; Lekberg et al., 2010) and AM fungi (Ji & Bever, 2016; Kiers et al., 2011).

Recent studies have uncovered the molecular processes involved in C-P exchange between the plant and AM fungus. The peri-arbuscular space (PAS), a compartment connecting the arbuscules to the root cells, was determined as the key interface for C-P exchange (Doidy et al., 2012; Hijikata et al., 2010; Parniske, 2008). In this process, fatty acids synthesized by the host plant are transferred to the AM fungus as the major C and energy source to sustain root colonization, hyphae development and spore production (Jiang et al., 2017; Luginbuehl et al., 2017). In the plastids of arbuscule-containing cells, acetyl-coenzyme A first synthesizes long-chain fatty acids (palmitoyl-acyl carrier protein [ACP]) via fatty acid synthase (KASI, and KASIII, Keymer et al., 2017). The palmitoyl-ACP is then converted into free palmitic acid by the palmitoyl-ACP thioesterase FatM, which in turn produces 2-monoacylglycerol with the aid of the glycerol 3-phosphate acyltransferase RAM2 (Bravo et al., 2017; Jiang et al., 2017). Subsequently, the 2-monoacylglycerol is transferred to the AM fungus through the peri-arbuscular membrane (PAM)-localized ATP-binding cassette transporter STR and STR2 (Jiang et al., 2017). The transcription factor WRI5a and its two paralogues WRI5b and WRI5c control fatty acid synthesis and transfer as well as PAM formation during AM symbiosis by regulating the expression of several key genes (e.g. FatM, STR, MtPT4; Jiang et al., 2018). At the same time, as compensation, the AM fungus supplies P to the plant. Phosphate (Pi) transporter (i.e. MtPT4) is localized in the PAM and mediate Pi uptake from the PAS in *Medicago truncatula* (Breuillin-Sessoms et al., 2015; Harrison, 2002). A SPX domain-containing Pi transporter, RiPT7, located on membranes of the fungus at the symbiotic interface, is responsible for arbuscular development and symbiotic Pi delivery during AM symbiosis (Xie et al., 2022). The Pi from the AM fungus is exported to the PAS through the RiPT7 and further into plant cells through the MtPT4. The plasma membrane H⁺-ATPase MthA1 energizes the nutrient uptake, specifically Pi, by creating a proton gradient across the PAM in *M. truncatula* during AM symbiosis (Wang et al., 2014).

The C-P exchange between the plant and AM fungus is highly dependent on biotic and abiotic factors. For the host plant nutrients demand, the availability of soil nutrients and environmental factors (e.g. light intensity) can change the overall trading strategy (Bücking & Shachar-Hill, 2005; Konvalinková et al., 2015, 2017; van't Padje et al., 2021). For example, short periods of shade influence the Pi transfer from the AM fungus to plant, with less Pi taken up by the plant via the mycorrhizal pathway (Konvalinková et al., 2015). If soil Pi

levels are high, the plant inhibits the formation of arbuscules in roots, minimizing the amount of C allocated to the AM fungus (Konvalinková et al., 2017). Moreover, when the AM fungus is unable to supply Pi, the plant is able to reduce the AM fungal colonization to weaken trade (van't Padje et al., 2020). In a similar manner, the alterations of environmental conditions impacting the development of the AM fungal extraradical hyphae as well as species composition within the mycorrhizal network and nutrient availability in the hyphosphere can also result in shifts in nutrient trading strategies (Argüello et al., 2016; van't Padje et al., 2020; Wang et al., 2022; Whiteside et al., 2019). When externally available nutrient resources (i.e. mineral P) suddenly rise, the AM fungus is able to store the surplus Pi absorbed by extraradical hyphae until the plant's demand for Pi increases, rather than immediately trading it.

Interestingly, the bipartite cooperation between plant and AM fungus can extend to a third partner—bacteria—developing in close vicinity of the hyphae (Zhang et al., 2016, 2018). Indeed, various bacteria have been found to colonize the surface of AM fungal hyphae to access hyphal exudates (Emmett et al., 2021; Scheublin et al., 2010). Over the last decade, substantial progress has been made in comprehending these interactions in the hyphosphere (Duan et al., 2022; Jiang et al., 2021; Zhang et al., 2014, 2018), the most prominent one being between the AM fungus *Rhizophagus irregularis* and the phosphate-solubilizing bacterium (PSB) *Rahnella aquatilis*. The hyphal exudates of the AM fungus were found to represent both a source of C for the bacterium as well as signalling and effector molecules stimulating bacterial growth and activity (Zeng et al., 2018; Zhang et al., 2016, 2018). More specifically, it was shown that the AM fungus stimulated the capacity of the PSB to secrete phosphatases, increasing the mineralization of organic P into Pi accessible for the AM fungus (Zhang et al., 2014, 2016). In another study, it was reported that the extraradical hyphae of *R. irregularis* can act as a 'superhighway' for bacteria to migrate to organic P patches, therefore changing the distribution of bacteria and enhancing utilization of organic P in the soil (Jiang et al., 2021). Finally, multitrophic interactions were observed in the hyphosphere (e.g. the interactions between soil bacteria and protists), improving the utilization of organic nutrients by AM fungi (Rozmoš et al., 2022).

Taken together, the plant-AM fungus-bacterium interaction thus represents a continuum with two distantly located interfaces: the PAS connecting plant cells to fungal cells (i.e. the arbuscules) in the roots and the hyphosphere, connecting extraradical hyphae of AM fungi with the microbial community dwelling in the soil. These two interfaces are connected by the extraradical hyphae of the AM fungus. However, how the

hyphosphere interaction influences the C–P exchange at the PAS level is still unknown. To shed light on these processes, we constructed an experimental system associating *Medicago truncatula* with the two microorganisms above to address the following questions: (1) Does the interaction between AM fungi and PSB at the hyphosphere interface improve Pi absorption and Poly-P synthesis in the extraradical hyphae of AM fungus? (2) does the increased Pi uptake and transport by hyphae to the plant cell promote the C–P exchange between plants and AM fungi at the PAS interface? and (3) does the interaction between the two interfaces enhance plant growth and Pi uptake efficiency? To address these questions, the expression of genes involved in (i) Pi uptake and transport of the extraradical and intraradical hyphae of *R. irregularis* (*PHO84p*, *VTC4*, *PHO91*, and *RiPT7*), (ii) lipid biosynthesis (*FatM* and *RAM2*) and regulation (*WRI5a*, *WRI5b*, *WRI5c* and *RAM1*) of *M. truncatula* and (iii) C–P exchange between *M. truncatula* and *R. irregularis* at the PAS interface (*MiPT4*, *MiHA1*, *STR*), was measured. The functions and details of these genes can be found in Table S1.

EXPERIMENTAL PROCEDURES

Experimental materials

For our experiments, we used the model plant *Medicago truncatula* L. cv. Paraggio. The seeds were surface-sterilized with 10% (v/v) H_2O_2 for 10 min and 70% (v/v) ethanol for 3 min and then rinsed eight times with sterile deionized water. The seeds were subsequently placed in the dark on Petri plates containing water agar (1%) for vernalization (72 h at 4°C) and germination (12 h at 27°C).

The AM fungus used was *Rhizophagus irregularis* (Błaszk., Wubet, Renker and Buscot) C. Walker and A. Schüßler as ‘irregulare’ MUCL 43194, isolated from *Fraxinus americana* L. in Pont Rouge, Québec, Canada (Chabot et al., 1992). The fungus was grown in bi-compartmented Petri plates on Ri T-DNA transformed roots of carrot (*Daucus carota* L.).

The bacterium used was *Rahnella aquatilis* HX2, isolated from a vineyard soil in Beijing, China (Guo et al., 2012). This strain was shown to mineralize organic P and solubilize mineral P (Chen et al., 2007; Guo et al., 2009). It was reported to colonize the extraradical hyphae surface of AM fungi (Zhang et al., 2016) and to efficiently mineralize and use phytate (Zhang et al., 2016, 2018).

An inceptisol (according to the USDA classification system) was collected from Changping (116°23 E, 40°22 N), Beijing, China. The soil was alkaline (pH_{water} 7.68; water/soil = 5/1), containing 21.53 g organic matter kg⁻¹, 1.0 g total N kg⁻¹, 4.18 mg Olsen-P kg⁻¹ (extracted by 0.5 M NaHCO₃), and 214.2 mg

CH₃COONH₄ exchangeable K kg⁻¹. The soil was sieved (2 mm) and air dried until use.

Three-compartment microcosm pots were constructed, consisting of a central root and hyphae compartment (RHC), separated from an external hyphae compartment (HC) by a buffer compartment (BC) (Figure 1). The pots had a diameter of 13 cm (top) and 9 cm (bottom) and a height of 10 cm. The RHC was delimited by a PVC tube of 10 cm in height and 5 cm in diameter. This PVC tube was placed in a second PVC tube, slightly larger (7.5 cm diameter), forming the BC separating the RHC from the HC. The sides of the two tubes were covered with a nylon mesh of 30 µm pore size (Anping Wire Mesh Industrial Ltd, Hebei, China) preventing the outgrowth of roots from the RHC to the BC and HC but allowing the spreading of hyphae in both compartments. The BC was intended to prevent the translocation of NSP and SOP (see below), added into the HC, from HC to RHC. The substrate in the RHC was a mixture of inceptisol with river sand, thoroughly rinsed with deionized water, in the ratio of 4:1 (w:w). As substrate in the BC, we used pure inceptisol. The substrate in the HC was a mixture of inceptisol with glass beads (diam. 1 mm; ratio 1:1, w:w). The inceptisol was passed through a 30 µm sieve

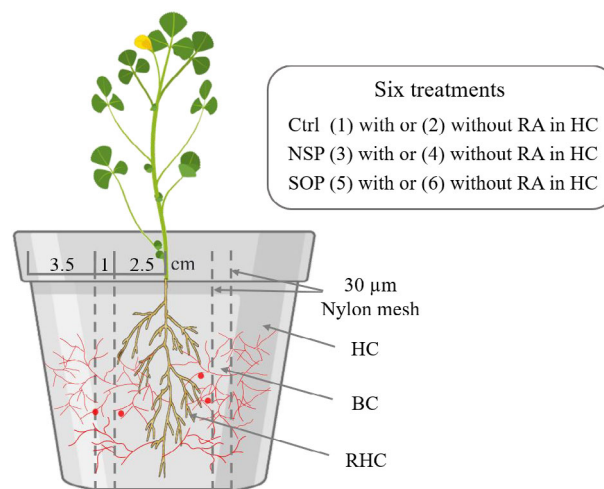


FIGURE 1 Schematic diagram of the three-compartment experimental set-up. The inner compartment contains the roots and fungal hyphae (RHC), the middle compartment is a buffer compartment (BC) and the outer compartment is the hyphal compartment (HC) only containing the extraradical hyphae. The BC, with a width of 1 cm, was set between the two compartments to prevent the movement of non-soluble P or soluble organic P from HC to RHC. A nylon mesh of 30 µm separating the RHC from the BC and the BC from the HC was used to avoid roots to extend from RHC to HC, while allowing the hyphae to extend from RHC to HC. The extraradical hyphae of *R. irregularis* were inoculated with or without the bacterium *R. aquatilis* in the HC. Each treatment consisted of three replicates with three *M. truncatula* plants per RHC. Red lines, extraradical hyphae of *R. irregularis*; red dots, spores of *R. irregularis*. Ctrl, control; NSP, non-soluble P; SOP, soluble organic P; RA, *R. aquatilis*

by washing with deionized water. The glass beads were first washed with 1% HCl, followed by deionized water until the pH of washed water became neutral (6.8–7.2). Inceptisol and glass beads were then dried in an oven at 60°C for 2 days and 90°C for 1 day. The substrates in the RHC, BC and HC were sterilized by γ -ray (25 kGy, 60 Co γ -ray, Beijing Atomic Hi-Tech Jin-hui Radiation Technology Application Co. Ltd., Fangshan, Beijing, China). The following nutrients were added per kg of substrate in RHC and HC (no additional nutrients were added to the glass beads of substrate in the HC and substrate in the BC): 200 mg N (NH_4SO_4), 200 mg K (K_2SO_4), 50 mg Mg ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), 5 mg Zn ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), 5 mg Mn ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), and 5 mg Cu ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$). In addition, 15 mg P kg^{-1} (KH_2PO_4), to facilitate normal growth of *M. truncatula*, and 1% nitrification inhibitor (3,4-dimethylpyrazole phosphate, Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) were added to the RHC. A total of 250, 200 and 650 g of substrate was added into the RHC, BC and HC, respectively.

Experimental design

Two hundred gram of substrate was added into the RHC and topped with 5 ml suspension containing approximately 1000 spores, extracted from a culture Petri plate by using the citrate buffer to dissolve the gel, which contained carrot root segments colonized by *R. irregularis* (Doner & Bécard, 1991). Then, 50 g of substrate was added on top of the layer of spores. The substrate was wet up to 70% of field capacity by adding deionized water. Four germinating seeds were placed on the top of the RHC and after 1 week thinned to three seedlings showing homogenous growth. During the growth of *M. truncatula*, soil moisture was kept at 18%–20% (w/w, c. 70% of field moisture capacity) as determined gravimetrically by weighting the pots every day and adding water when necessary. The experiment was conducted at China Agricultural University (Beijing) in the greenhouse 28/22°C (day/night) for a total of 11 weeks.

At Week 8, a pre-harvest was conducted in two pots (not included in the experimental design) to confirm the presence of hyphae in the HC following the method of Zhou et al. (2020). Numerous hyphae (approximately 60 mg, FW) were detected and therefore at Week 10, 25 ml of liquid suspension containing NSP (200 mg kg^{-1} , Ca_3PO_4 , Sigma-Aldrich, Saint Louis, USA) or SOP (200 mg kg^{-1} , Na-phytate, Sigma-Aldrich, Saint Louis, USA) and *R. aquatilis* was added to the HC. Ca_3PO_4 and Na-phytate are the most common sources of NSP and SOP in the soil, respectively, which cannot directly be utilized by AM fungi. Part of the Pi available to the AM fungi is released from the organic acids and phosphatases secreted by PSB. The

bacterial supernatant (5 ml) contained an approximate of 10^8 CFUs ml^{-1} mixed uniformly with 20 ml liquid suspension containing NSP or SOP. The NSP was injected into the HC through a 50 ml syringe and the treatments without P or bacteria supernatant were replaced with an equal volume of sterile deionized water. The *R. aquatilis* inoculum was prepared as follows: bacteria were cultured in liquid Luria–Bertani medium with shaking at 180 rpm at 28°C until OD_{600} reached 0.4–0.6 (logarithmic phase) and then centrifuged at 5878g for 6 min. The supernatant was discarded, and the pellet was re-suspended and washed three times with sterilized 0.85% (w/v) NaCl solution. The supernatant was then adjusted to $\text{OD}_{600} = 0.8$ with the autoclaved 0.85% NaCl solution and stored for 4 h before use. The experiment consisted of three replicates: NSP with or without *R. aquatilis* in the HC ($\text{NSP}^{+\text{RA}}$ and $\text{NSP}^{-\text{RA}}$, respectively), SOP with or without *R. aquatilis* in the HC ($\text{SOP}^{+\text{RA}}$ and $\text{SOP}^{-\text{RA}}$, respectively) and the control in the absence of NSP and SOP with or without *R. aquatilis* in the HC ($\text{Ctrl}^{+\text{RA}}$ and $\text{Ctrl}^{-\text{RA}}$, respectively) with four harvesting times for a total of 72 pots.

Hyphae sampling in the HC and evaluation of shoot biomass, P concentration, P content, root mycorrhizal colonization and arbuscular abundance of *R. irregularis* in the RHC

The experiment was conducted for one additional week after bacteria inoculation in the HC. The substrate in the HC was harvested at 0, 1, 3 and 7 day post inoculation (dpi) with the bacterium. At harvest, the top 2 cm of soil in the HC was discarded to eliminate any surface effects and the remaining substrate was harvested and immediately frozen with liquid nitrogen to stop metabolic activity of the extraradical hyphae. The substrate was then thawed and placed in a 30 μm stainless steel sieve, rinsed continuously with sterilized distilled water and filtered off with water, leaving glass beads and hyphae. The hyphae were carefully separated from the glass beads with tweezers, poured in a 2 ml sterile centrifuge tube and stored at -80°C for the expression analysis of genes involved in uptake and transfer of inorganic Pi.

Plants in the RHC were separated into shoots and roots. The shoots were oven-dried at 105°C for 30 min to stop metabolic activity and then at 65°C for 2 days to determine shoot dry weight. The shoots were finally ground to a fine powder. Determination of shoot P content and concentration was conducted as detailed in the study by Thomas et al. (1967). Roots were washed with sterile PBS buffer (NaH_2PO_4 – Na_2HPO_4 , pH = 7). One fifth of the roots was frozen with liquid nitrogen to stop metabolic activity and then preserved at -80°C for

evaluation of the relative abundance of AM fungus and gene expression analysis of P transport in intraradical hyphae as well as fatty acid biosynthesis, transport and regulation in roots. The remaining roots were used for evaluation of total mycorrhizal colonization and arbuscular abundance using the method of Trouvelot et al. (1986). Substrates in both the BC and RHC were discarded.

Gene expression analysis and relative contribution of PSB to C–P exchange

Total RNA was extracted from the frozen roots (from the RHC) or hyphae (from the HC) using the RNeasy Plant Mini Kit (Catalogue number: 74904, QIAGEN GmbH, Hilden, Germany). For single-strand cDNA synthesis, 120 ng of total RNA in roots or 50 ng of total RNA in hyphae was reverse-transcribed at 42°C for 2 min, 37°C for 15 min and 85°C for 5 s in a final volume of 20 µl using PrimeScript™ RT Reagent Kit with gDNA Eraser (Catalogue number: RR047A; TaKaRa Bio Inc, Japan). The products were then diluted to 200 µl.

Quantitative RT-PCR (qRT-PCR) was performed using a CFX96™ Real-Time System (BIO-RAD, America). Each PCR reaction was conducted in a total volume of 25 µl containing 2 µl cDNA, 13 µl TB Green® Premix Ex Taq™ II (Catalogue number: RR820A, TaKaRa Bio Inc, Japan), 1 µl of each primer (10 µM) and 9 µl ddH₂O. The following PCR program was run: 95°C for 300 s, 40 cycles of 95°C for 15 s, 52°C for 15 s and 72°C for 45 s with roots and 95°C for 300 s, 40 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 45 s with hyphae. A melting curve was recorded at the end of each run to rule out non-specific PCR products (Ririe et al., 1997). All reactions were performed on three technical replicates and their mean value used. Baseline range and threshold cycle (Ct) values were automatically calculated using LightCycler® 96 software. The Δ Ct was calculated by subtracting the Ct value of a reference gene from the Ct value of each target gene. Relative fold-change of each target gene was normalized by the $2^{-\Delta\Delta C_t}$ method, with reference to the Δ Ct value in Ctrl^{−RA} treatment harvested at Day 0 of sampling time. The optimal reference genes *MtEF-1* in *M. truncatula* (Jiang et al., 2018) and *RDN5.8s* in *R. irregularis* (Li et al., 2012) were used as the reference genes for expression analysis. Primer sequences for real-time PCR are listed in Table S1.

The relative contribution of *R. aquatilis* to C–P exchange is defined as the average values of genes expression directly involved in lipid (*STR*) or Pi (*MtPT4*) transport at 1, 3 and 7 dpi in the presence of the bacteria versus the average values of genes expression directly involved in lipid (*STR*) or Pi (*MtPT4*) transport at 1, 3 and 7 dpi in the absence of the bacteria.

DNA isolation and real-time q-PCR analysis of AM fungal relative abundance

DNA was isolated from the frozen roots (from the RHC) using the DNAsecure Plant Kit (Catalogue number: DP305, Tiangen Biotech Co. Ltd., Beijing, China). As described in the manufacturer instructions, DNA was eluted with 50 µl elution buffer and quality detected with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, Wilmington, DE, USA).

DNA isolated from the roots of each treatment was quantified in triplicate by real-time q-PCR using a CFX96™ Real-Time System (BIO-RAD, America) with *R. irregularis* specific primer (forward primer 5'-GGTGCGATTCTGTGGAGTGTGAGG-3' and reverse primer 5'-CAAGCTTTCGGCACCAGAGCAACG-3'; Jansa et al., 2003). Each q-PCR reaction was conducted in a total volume of 25 µl containing 2 µl DNA, 13 µl TB Green® Premix Ex Taq™ II (Catalogue number: RR820A, TaKaRa Bio Inc, Japan), 0.5 µl of each primer (10 µM) and 9.5 µl ddH₂O. The following reaction conditions were used: 95°C for 300 s, 40 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 45 s. Melting curve, baseline range and Ct values were recorded and calculated as described above.

From the Ct values, we calculated the copy numbers, which can be directly related to AM fungal biomass present in the sample using standard curves generated from samples with a known quantity of target DNA (Engelmoer et al., 2014). The standard curve was determined in triplicate using five serial 10-fold dilutions, and copy numbers calculated by determining the starting copy number by considering the concentration of the plasmid and number of base pairs (vector plus primer). Subsequently, we calculated DNA isolation efficiency referring to the Ct values of standard curves and normalized the relative abundance of *R. irregularis* for the DNA isolation efficiency (Engelmoer et al., 2014; Kiers et al., 2011).

Statistical analysis

Statistical analyses were performed using SPSS v. 16.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was conducted to compare the effect of different treatments. Mycorrhizal colonization, arbuscular abundance, *R. irregularis* relative abundance, shoot biomass, P concentration, P content, and the relative contribution of PSB to C–P exchange among the three treatments (Ctrl, NSP and SOP) at a specific time were evaluated by a Tukey's honest significant difference (HSD) test at $p \leq 0.05$. Significant differences among the two treatments inoculated with or without *R. aquatilis* (i.e. Ctrl^{+RA} and Ctrl^{−RA}, NSP^{+RA} and

NSP^{-RA} or SOP^{+RA} and SOP^{-RA}) were assessed at different time points by *t*-test at $p \leq 0.05$. Two-way ANOVA was conducted to compare the effects of harvest time (Time) and Ctrl^{-RA}, NSP^{-RA}, SOP^{-RA}, Ctrl^{+RA}, NSP^{+RA} and SOP^{+RA} treatment (Treatment) on the expression of the 13 genes. Significant differences in expression of a specific gene among the six treatments (Ctrl^{-RA}, NSP^{-RA}, SOP^{-RA}, Ctrl^{+RA}, NSP^{+RA} and SOP^{+RA}) at a specific time and among different harvest times in a specific treatment (Ctrl^{-RA}, NSP^{-RA}, SOP^{-RA}, Ctrl^{+RA}, NSP^{+RA} or SOP^{+RA}) were evaluated using a Tukey's honest significant difference (HSD) test at $p \leq 0.05$.

RESULTS

Expression of genes involved in C-P exchange between plant and AM fungus

The expression of three genes (*MtPT4*, *MtHA1*, *STR*) involved in C-P exchange between the plant and AM fungus at the PAS was measured. Independent of the harvesting time (0, 1, 3 and 7 dpi with the bacterium), the expression of the three genes was not significantly different between the Ctrl^{-RA}, NSP^{-RA}, SOP^{-RA} and

Ctrl^{+RA} treatments (Figure 2A–C). Conversely, in the NSP^{+RA} and SOP^{+RA} treatments, the expression of *MtPT4*, *MtHA1* and *STR* was significantly increased at 1, 3 and 7 dpi with the bacterium compared to the other four treatments (Figure 2A–C). Furthermore, the relative contribution of *R. aquatilis* to C-P transport at the PAS was significantly increased in the NSP and SOP treatments compared to the Ctrl treatment. (Figure 2D).

Expression of genes involved in Pi uptake and transport by the extraradical and intraradical hyphae of *R. irregularis*

The expression of four genes (*PHO84p*, *VTC4*, *PHO91*, and *RiPT7*) involved in Pi uptake and transport by the extraradical and intraradical hyphae of *R. irregularis* was measured. Independent of the harvesting time (0, 1, 3 and 7 dpi with the bacterium), the expression of the four genes was not significantly different between the Ctrl^{-RA}, NSP^{-RA}, SOP^{-RA} and Ctrl^{+RA} treatments (Figure 3A–D). Conversely, in the NSP^{+RA} and SOP^{+RA} treatments, the expression of *PHO84p*, *VTC4* and *PHO91* at 1, 3 and 7 dpi with the bacterium and *RiPT7* at 3 and 7 dpi with the bacterium was significantly higher compared to Day 0. No other differences were noticed between harvest

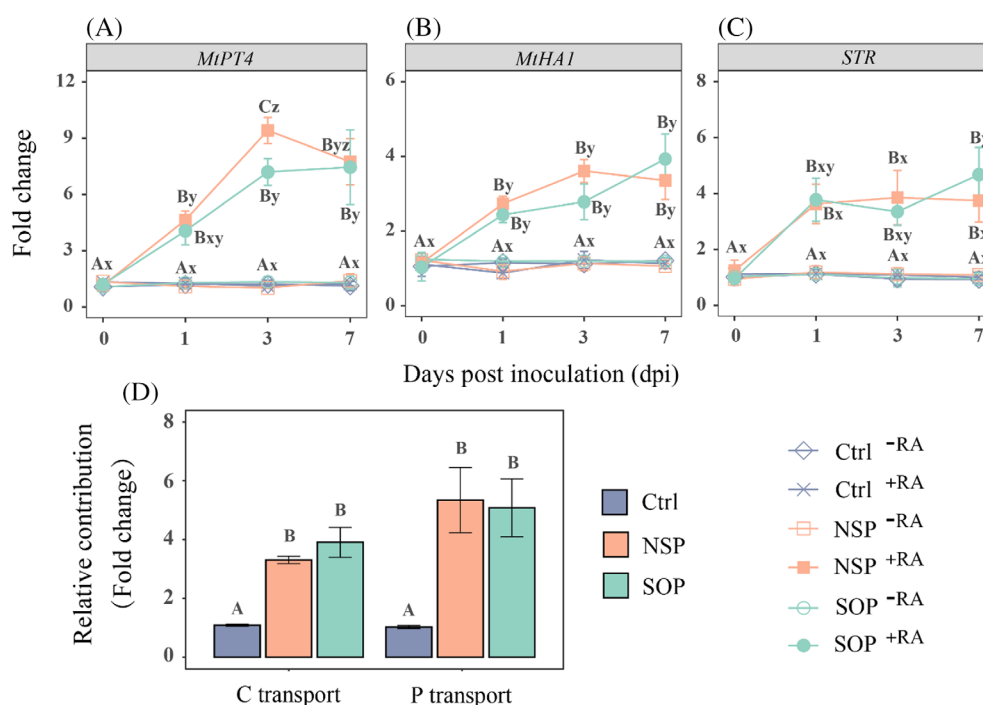


FIGURE 2 The relative expression of (A) Pi transporter gene *MtPT4*, (B) plasma membrane H⁺-ATPases gene *MtHA1* and (C) lipid transporter gene *STR* in *M. truncatula* colonized by the AM fungus *R. irregularis* at 0, 1, 3 and 7 days post inoculation (dpi) with *R. aquatilis*. (D) The relative contribution of *R. aquatilis* to C-P exchange at the arbuscular interface. The relative contribution of *R. aquatilis* to C-P exchange is defined as the average values of genes expression directly involved in lipid (*STR*) or Pi (*MtPT4*) transport at 1, 3 and 7 dpi in the presence of the bacteria versus the average values of genes expression directly involved in lipid (*STR*) or Pi (*MtPT4*) transport at 1, 3 and 7 dpi in the absence of the bacteria. The error bar represents the standard errors. For the same harvesting time point (i.e., 0, 1, 3 and 7 dpi with the bacterium), different capital letters were used to indicate a significant difference among the treatments. Within the same treatment, different lowercase letters represent a significant difference among the harvesting time points (i.e., 0, 1, 3 and 7 dpi with the bacterium). ($p \leq 0.05$ by Tukey's HSD test). Ctrl, control; NSP, non-soluble P; SOP, soluble organic P; RA, *R. aquatilis*

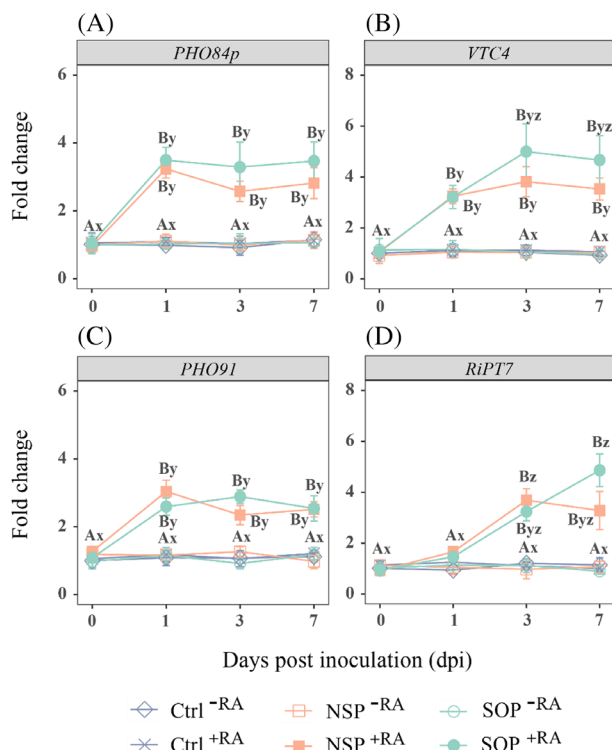


FIGURE 3 The relative expression of (A) the high-affinity Pi transporter gene *PHO84p* and (B, C) the vacuolar Pi transporter genes *VTC4* and *PHO91* in extraradical hyphae of *R. irregularis*, and (D) the Pi transporter gene *RiPT7* in intraradical hyphae *R. irregularis* during mycorrhizal symbiosis at 0, 1, 3 and 7 days post inoculation (dpi) with *R. aquatilis*. The error bar represents the standard errors. For the same harvesting time point (i.e., 0, 1, 3 and 7 dpi with the bacterium), different capital letters were used to indicate a significant difference among the treatments. Within the same treatment, different lowercase letters represent a significant difference among the harvesting time points (i.e., 0, 1, 3 and 7 dpi with the bacterium). ($p \leq 0.05$ by Tukey's HSD test). Ctrl, control; NSP, non-soluble P; SOP, soluble organic P; RA, *R. aquatilis*.

times. In addition, the expression of *PHO84p*, *VTC4* and *PHO91* at 1, 3 and 7 dpi with the bacterium and *RiPT7* at 3 and 7 dpi with the bacterium were significantly higher compared to the other four treatments (Figure 3A–D).

Expression of genes involved in lipid biosynthesis and regulation of *M. truncatula*

The expression of six genes involved in lipid biosynthesis (*FatM* and *RAM2*) and regulation (*WRI5a*, *WRI5b*, *WRI5c* and *RAM1*) of *M. truncatula* was determined. Independent of the harvesting time (0, 1, 3 and 7 dpi with the bacterium), the expression of the six genes was not significantly different between the Ctrl^{−RA}, NSP^{−RA}, SOP^{−RA} and Ctrl^{+RA} treatments (Figure 4A–E). In contrast, in the NSP^{+RA} and SOP^{+RA} treatments, gene expression varied with harvesting time. For *FatM*, *RAM2* and *WRI5a*, the expression increased steadily from Day 0 to 7, and the highest

expression was noticed at Day 7 (Figure 4A–C). For *WRI5b* and *RAM1*, the expression first increased between Day 0 and 1, decreased from Day 1 to 3, and then continued to increase until Day 7 (Figure 4D,F). For *WRI5c*, it was only significantly higher at Day 7 (Figure 4E). The expression of *FatM*, *RAM2*, *WRI5a*, *WRI5b* and *RAM1* at 1, 3 and 7 dpi with the bacterium and *WRI5c* at 7 dpi with the bacterium were significantly greater in the NSP^{+RA} and SOP^{+RA} treatments compared to the other four treatments (Figure 4).

Mycorrhizal colonization, arbuscular abundance, *R. irregularis* relative abundance in roots, shoot biomass, P concentration and P content of *M. truncatula*

Independent of the harvesting time (0, 1, 3 and 7 dpi with the bacterium), the root mycorrhizal colonization and arbuscular abundance did not differ significantly between the Ctrl^{−RA}, NSP^{−RA}, SOP^{−RA} and Ctrl^{+RA} treatments. Similarly, no significant differences were noticed at 0, 1 and 3 dpi with the bacterium in the NSP^{+RA} and SOP^{+RA} treatments, while at 7 dpi, the root mycorrhizal colonization and arbuscular abundance were significantly increased in both treatments as compared to the Ctrl^{+RA} treatment (Figure 5A,B; Figure S1). In addition, the relative abundance of *R. irregularis* in roots was significantly increased at 7 dpi in the same two treatments (Figure 5C).

Independent of the harvesting time (0, 1, 3 and 7 dpi with the bacterium), the shoot biomass, P concentration and P content of *M. truncatula* did not differ significantly between the Ctrl^{−RA}, NSP^{−RA}, SOP^{−RA} and Ctrl^{+RA} treatments. In the NSP^{+RA} and SOP^{+RA} treatments, the shoot biomass, shoot P concentration and P content were significantly increased at 3 and 7 dpi with the bacterium. Moreover, the shoot biomass, shoot P concentration and P content were significantly higher at 3 and 7 dpi with the bacterium in the SOP^{+RA} treatment compared to the NSP^{+RA} treatment (Figure 5D–F).

DISCUSSION

The plant–AM fungus association functions like a market economy in which C–P are exchanged, with a preferential allocation to the partner offering the best exchange rate. This trade has commonly been measured by stable isotope labelling (Bever et al., 2009; Fellbaum et al., 2014; Kiers et al., 2011), which can quantify the amount of C–P exchanged in a given time and thus calculate the trade price under different conditions. Using this method, Kiers et al. (2011) demonstrated that plants can detect, discriminate and reward the best AM fungal partners with more carbohydrates and reversely, that fungal partners can allocate more Pi

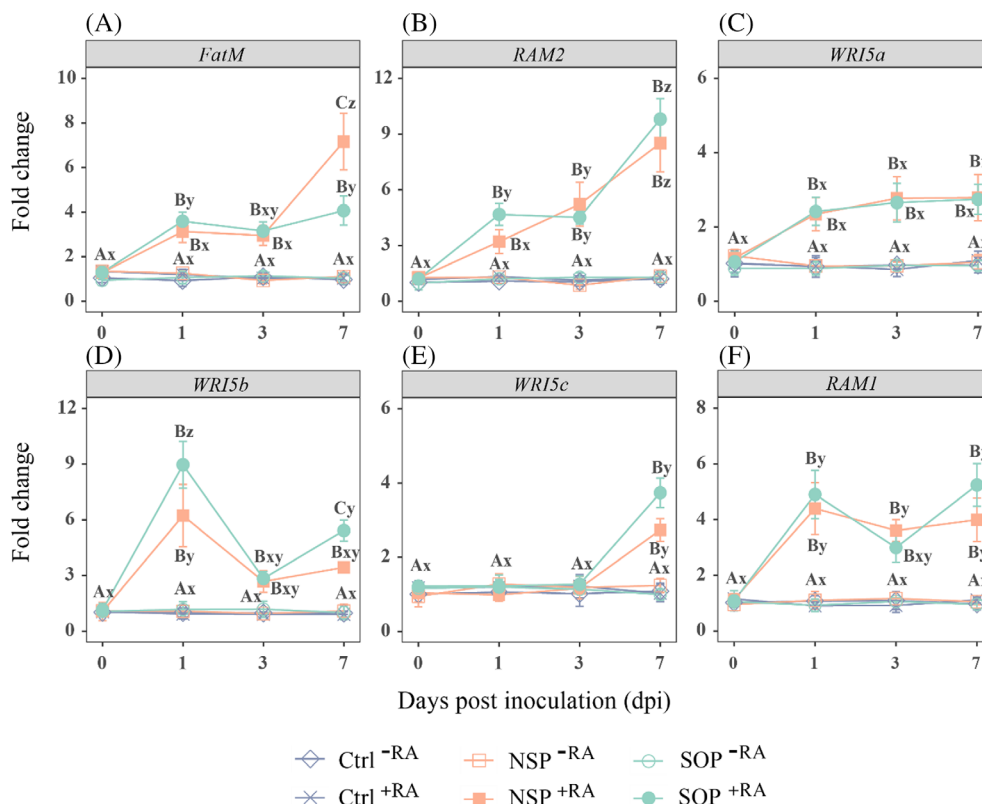


FIGURE 4 The relative expression of (A, B) the fatty acid biosynthesis genes *FatM* and *RAM2*, (C–E) the genes involved in regulating lipid biosynthesis, lipid and Pi transport *WRI5a*, *WRI5b* and *WRI5c*, and (F) the transcription factor *RAM1* in *M. truncatula* colonized by the AM fungus *R. irregularis* at 0, 1, 3 and 7 days post inoculation (dpi) with *R. aquatilis*. The error bar represents the standard errors. For the same harvesting time point (i.e., 0, 1, 3 and 7 dpi with the bacterium), different capital letters were used to indicate a significant difference among the treatments. Within the same treatment, different lowercase letters represent a significant difference among the harvesting time points (i.e., 0, 1, 3 and 7 dpi with the bacterium). ($p \leq 0.05$ by Tukey's HSD test). Ctrl, control; NSP, non-soluble P; SOP, soluble organic P; RA, *R. aquatilis*

to those host plants providing more carbohydrates. However, stable isotopes cannot reveal the physiological mechanisms and gene networks involved in regulating C-P exchange between both partners of the symbiosis, hence molecular biological approaches are required.

In the recent decade, several genes encoding C-P transporter proteins have been found at the PAS (Breuillin-Sessoms et al., 2015; Gutjahr et al., 2012; Jiang et al., 2017; Wang et al., 2017). Here, the expression of six genes involved in Pi uptake and transport in *R. irregularis* and *M. truncatula* as well as seven genes involved in lipid synthesis, transfer and regulation in *M. truncatula* were analysed. The interaction between *R. irregularis* and *R. aquatilis* in the hyphosphere interface resulted in the solubilization/mineralization of non-soluble P/organic P in the growth medium, increasing the uptake and transport of Pi along the extraradical hyphae and its transfer from the AM fungus to the plant. This was supported by a significant increase in the expression of Pi transporter genes. The transfer of P from the AM fungus to the plant resulted concomitantly in the transfer of C from the plant to the AM fungus, and improved the C-P trade at the PAS. This was evidenced by the significant increase in the expression of

genes involved in lipid biosynthesis, transport and regulation in *M. truncatula*. However, gene expression and product activity are not always in direct correspondence due to the occurrence of post-transcriptional and post-translational events. Therefore, it is crucial to combine stable isotope labelling and even develop new approaches to further increase our understanding of the physiological mechanism and whole spectrum of C-P exchange between plants and AM fungi. Importantly, our results suggested that the C-P exchange between the plant and AM fungus is enhanced by the PSB developing in the hyphosphere. The PSB improved the flux of P from the AM fungus to the plant and, in turn enhanced the flux of C from the plant to the AM fungus, providing new insights on the molecular mechanism behind the hyphosphere interaction to enhance plant P nutrition.

***Rhizobium aquatilis* in the hyphosphere stimulate C-P tradeoff between plant and AM fungus**

Although AM fungi are dependent on the host plant for their C supply, the amount of Pi that AM fungi allocate

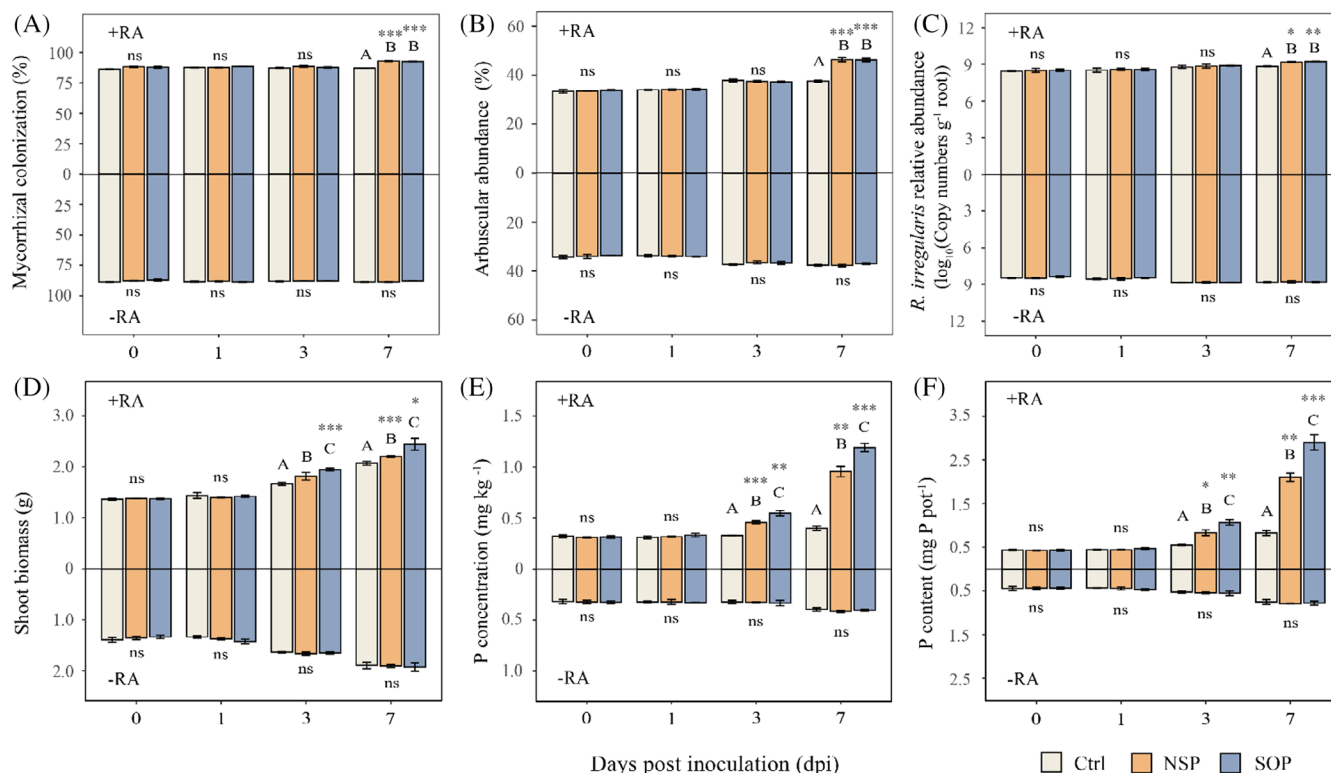


FIGURE 5 The (A) root mycorrhizal colonization, (B) arbuscular abundance, (C) *R. irregularis* relative abundance, (D) shoot biomass, (E) P concentration and (F) P content of *M. truncatula* are shown. The error bar represents the standard errors. For the same harvesting time point (i.e., 0, 1, 3 and 7 days post inoculation with *R. aquatilis*), different capital letters were used to indicate a significant difference among the treatments. ($p \leq 0.05$ by Tukey's HSD test; ns, $p > 0.05$). The asterisks represents a significant difference with or without *R. aquatilis* in a specific treatment (Ctrl, NSP or SOP) at a specific time point (t-test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Ctrl, control; NSP, non-soluble P; SOP, soluble organic P; RA, *R. aquatilis*

to the plant varies with the environmental conditions (Merrild et al., 2013; Roger et al., 2013; van't Padje et al., 2020; Whiteside et al., 2019). For instance, it has recently been shown that extraradical hyphae, when exposed to high inequalities of inorganic P, stimulate trade with increasing P distribution to the host roots (Whiteside et al., 2019). Zhang et al. (2018) further demonstrated that the presence of hyphosphere PSB promoted the Pi uptake by the AM fungus from P patches, further affecting C-P trade between plant and AM fungus. When the plant's demand for Pi increases, the AM fungus transfers a higher amount of Pi to the host plant, in exchange for more C (van't Padje et al., 2020). In the present study, we found that the expression of genes (*MtPT4*, *MtHA1* and *STR*) involved in C-P exchange between *R. irregularis* and *M. truncatula* was significantly increased at 1, 3 and 7 dpi by the presence of PSB in both NSP and SOP treatments (Figure 2A–C). Moreover, an expression analysis of genes directly involved in C (*STR*) and P (*MtPT4*) exchange revealed a substantially higher relative contribution of PSB to C-P exchange in both treatments (Figure 2D). These results indicate that the ability of PSB to mineralize SOP and solubilize NSP in the hyphosphere was stimulated in presence of NSP or SOP, both sources of P which cannot be directly

absorbed and utilized by AM fungi (Tisserant et al., 2013), thus enhancing the C-P exchange between plants and AM fungi. Interestingly, some genera of bacteria (e.g. *Streptomyces*) dwelling in the hyphosphere have been shown to promote the hyphal growth of AM fungi (Garbaye, 1994). This corroborates our results, which show that root colonization and growth of *R. irregularis* were facilitated by *R. aquatilis*, likely caused by the promotion of C-P exchange at the PAS (Figure 5A–C). Therefore, these findings demonstrate that a microorganism (i.e. a PSB) developing at the hyphosphere interface can affect the C-P exchange at the PAS.

***Rahnella aquatilis* in the hyphosphere improve P supply from AM fungus to plant**

In the last decade, several studies have shown that the PSB *R. aquatilis* developing in the hyphosphere can assist AM fungi in the uptake of Pi from soil via the secretion of phosphatases (Zhang et al., 2014), subsequently increasing the amount of P transferred from fungus to plant. Interestingly, the presence of this bacterium induced the expression of genes involved in Pi uptake by the AM fungus *R. irregularis* (Zhang

et al., 2016, 2018). However, these studies were mainly focused on the molecular mechanisms at the hyphosphere level, but less on the uptake and transport of Pi within the AM fungus or on the gene expressions at the PAS level.

The uptake and transport of Pi within the AM fungus is regulated by a number of genes in the following cascade: Pi is first taken up through the high-affinity Pi transporter PHO84p driven by H⁺-ATPases under low Pi conditions in extraradical hyphae (Kikuchi et al., 2014). It is then polymerized into Poly-P through the vacuolar transporter chaperon (VTC) complex and translocated towards the host in vacuoles (Ezawa & Saito, 2018; Kikuchi et al., 2016). In the highly branched arbuscular structures, Poly-P is further hydrolyzed to Pi and exported to the cytosol through the vacuolar Pi exporter PHO91 (Kikuchi et al., 2014). Finally, Pi enters the PAS through the Pi transporter RiPT7 on the membranes of the AM fungus and is taken up by the plant (Xie et al., 2022). The promotion of C-P trade at PAS may enable AM fungi to provide more P to plants. Hence, we analysed the expression of genes involved in Pi uptake, Poly-P synthesis and transport within the extraradical and intraradical hyphae. Our results showed that the expressions of *PHO84p*, *VTC4*, and *PHO91* were significantly increased at 1, 3 and 7 dpi and *RiPT7* was significantly increased at 3 and 7 dpi by the presence of PSB in both NSP and SOP treatments (Figure 3). It suggests that the presence of *R. aquatilis* in the hyphosphere enhance the ability of the AM fungus to obtain Pi from P patches and supply P to the plant. *M. truncatula* rapidly increased their shoot biomass in a short time (from 1 to 7 dpi) when they received a large amount of Pi from the AM fungus.

AM fungal P supply drive the C distribution from plant to the AM fungus

During the C-P trade at the PAS, the main form of C supplied from plants to AM fungi is fatty acids (Jiang et al., 2017; Luginbuehl et al., 2017). Plants may deliver more lipids to AM fungi to maintain trade stability in case of an increase of P supply from AM fungi to plants. We used the expression of genes involving in lipid synthesis (*FatM* and *RAM2*) and regulation (*WRI5a*, *WRI5b*, *WRI5c* and *RAM1*) as read-out to characterize the ability of *M. truncatula* to supply C to the AM fungus when a higher amount of Pi was acquired. The results show that, except for *WRI5c*, the expression of all genes involved in fatty acid biosynthesis and regulation were significantly increased in both NSP and SOP treatments at 1, 3 and 7 dpi with the bacterium in the hyphosphere (Figure 4). Jiang et al. (2018) demonstrated that overexpression of *WRI5a* in *M. truncatula* hairy roots resulted in a significant increase in the expression of the genes *FatM*, *RAM1*, *STR* and *MtPT4*, with more palmitic acid production during AM

symbiosis. In addition, the 2-monoacylglycerol content in colonized roots of *M. truncatula* was significantly decreased when *FatM* or *RAM2* was knocked out (Bravo et al., 2017). Our results indicate that the P supply from the AM fungus to *M. truncatula* facilitates the C exchange process. The 2-monoacylglycerol can be converted into triacylglycerol in intraradical hyphae and further transported through the hyphal network (Wang et al., 2017), facilitating the growth and development of the AM fungus. In the present study, the root mycorrhizal colonization, arbuscular abundance and *R. irregularis* relative abundance were significantly increased at 7 dpi with the bacterium in both NSP and SOP treatments (Figure 5A–C; Figure S1), confirming that *R. irregularis* received more C from the host plant. The C flow from host plant to AM fungus and P from AM fungus to host plant happened simultaneously and the hyphosphere bacteria influenced this process.

Rahnella aquatilis enhances C-P exchange at the PAS to promote plant growth

Cooperation between species belonging to the same phylum or to different kingdoms (e.g. plants vs. fungi) or even to different domains (eukaryotes vs. prokaryotes) is beginning to be gradually understood (Venice et al., 2020). An emblematic example of such cooperation is the symbiosis established between AM fungi and more than two thirds of plant species on one side and the beneficial bacterial (e.g. PSBs) dwelling at the surface of the extraradical hyphae on the other side. Two interfaces involved in nutrient exchange, the PAS and hyphosphere, thus coexist at reasonable distance, in different environments (the root cortex and the soil), connected via intraradical and extraradical hyphae (Keymer & Gutjahr, 2018; Zhang et al., 2022). However, the interaction between the two distant interfaces and their effects on nutrient efficiency of the system are still poorly understood. At the hyphosphere interface, the PSB secrete phosphatases for the mineralization of organic P and gluconic acid for the solubilization of non-soluble P (Zhang et al., 2016, 2018), increasing Pi uptake and transport by the AM fungus, further enhancing C-P exchange at the arbuscular interface between plant and AM fungus (Figure 2). This process ultimately facilitates plant Pi uptake efficiency, further promoting plant growth in P deficient soils. Specifically, the PSB developing at the hyphosphere interface can affect the C-P exchange at the PAS between plant and AM fungus and further promote plant growth. Our results also showed that the PSB made Pi available to the AM fungus which otherwise would not be available. More importantly, a fine-tuned communication at two distant interfaces occurred via the hyphae of the AM fungus, and root-inaccessible Pi could never have been utilized by plants in the absence of the AM fungal hyphae. Recently, a study showed that *Candidatus*

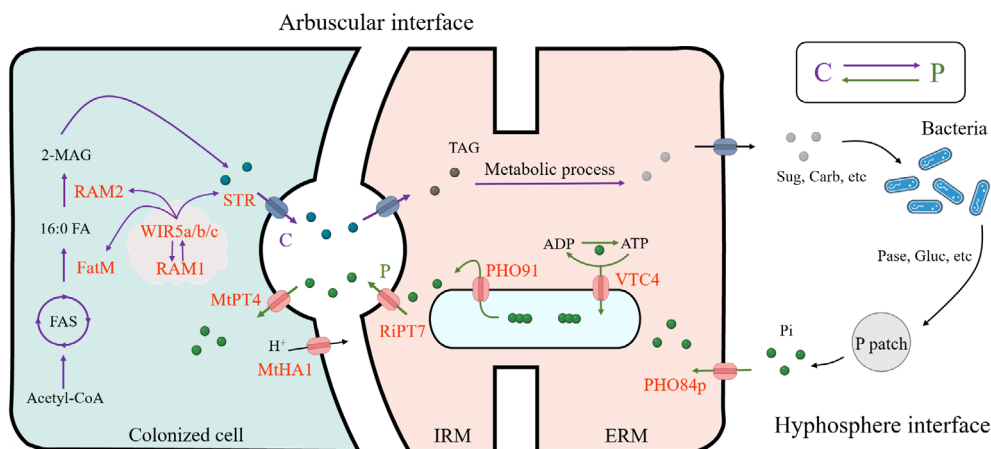


FIGURE 6 Diagrammatic representation of C-P exchange between the plant (*M. truncatula*) and the AM fungus (*R. irregularis*). The C-P exchange is facilitated by the presence of the PSB *R. aquatilis* in the hyphosphere of the AM fungus. The PSB interacting at the hypha surface increase the secretion of phosphatases for the mineralization of organic P, and gluconic acid for the solubilization of non-soluble P, increasing Pi uptake and transport in the AM fungus in exchange for carbohydrates exuded by the hyphae. This further enhanced the C-P exchange at the arbuscular interface between plant and AM fungus. Genes with up-regulated expression are highlighted in red. Carb, carboxylate; ERM, extraradical mycelium; FAS, fatty acid synthase; FA, fatty acids; Gluc, gluconate; IRM, intraradical mycelium; 2-MAG, 2-monoacylglycerol; Pase, phosphatase; Sug, sugar; TAG, triacylglycerol

Glomeribacter gigasporarum, growing as an endosymbiont of *Gigaspora margarita*, can also affect plant lipid metabolism and enhance C flux from plant to AM fungus (Venice et al., 2021). This suggest that regardless of their location (i.e. in the fungal cells as endobacterium or on the surface of hyphae as PSB), bacteria can impact plant responses to AM fungi. The plant responds to changes in fungal Pi transfer by modifying the amount of C allocated to the AM fungus, which can be predicted by reciprocal rewards between plants and AM fungi (Kiers et al., 2011). The acquisition of Pi may enable plants to provide more C to the AM fungus. One part of this C is used for fungal growth and development, increasing the relative abundance of AM fungus in root (Figure 5A–C), while the other part is released into the hyphosphere in the form of exudates used by the bacteria, stimulating their growth and activity, further enhancing the solubilization/mineralization of non-soluble P/organic P in soil (Figure 6).

AUTHOR CONTRIBUTIONS

Shilong Duan: Data curation (equal); writing – original draft (equal); writing – review and editing (equal). **Stéphane Declerck:** Data curation (equal); writing – original draft (equal); writing – review and editing (equal). **Gu Feng:** Writing – original draft (equal); writing – review and editing (equal). **Lin Zhang:** Data curation (equal); writing – original draft (equal); writing – review and editing (equal).

ACKNOWLEDGEMENTS

The authors thank associate Professor Yanbin Guo from College of Resources and Environmental Sciences, China Agricultural University, for providing the bacterial strain *R. aquatilis* HX2. The authors thank

Felix Richter from the Department of Bioengineering, Imperial College London, London, United Kingdom for revising the manuscript. This study was financially supported by the National Natural Science Foundation of China (42277112, 32011530457), Beijing Natural Science Foundation (6212020) and the Program of Advanced Discipline Construction in Beijing (Agriculture Green Development).

CONFLICT OF INTEREST

All authors declare that they have no competing financial interests.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the online publication file (See Data Tables S1–S5).

ETHICS STATEMENT

This work does not involve any ethical issues.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Duan, S., Declerck, S., Feng, G. & Zhang, L. (2023) Hyphosphere interactions between *Rhizophagus irregularis* and *Rahnella aquatilis* promote carbon–phosphorus exchange at the peri-arbuscular space in *Medicago truncatula*. *Environmental Microbiology*, 1–13. Available from: <https://doi.org/10.1111/1462-2920.16333>