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Trapping of phosphate solubilizing bacteria on hyphae of the arbuscular mycorrhizal fungus *Rhizophagus irregularis* DAOM 197198

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ABSTRACT

A simple method is described for trapping phosphate solubilizing bacteria (PSB) strongly attached to the hyphae of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus irregularis* (*Ri*). Bacteria were isolated from the hyphosphere of mycorrhizal leek plants growing on Turface previously inoculated with soil suspensions, obtained from the mycorrhizosphere of mycorrhizal plants growing in agricultural settings or maple forests in Quebec, Canada. Among the best PSB strongly attached to the hyphae of *Ri*, 26 isolates belonged to *Burkholderia* spp. and one was identified as *Rhizobium miluonense*. Four hyphobacteria exhibiting high potential of inorganic and organic P mobilization were further compared with four equivalent mycorrhizobacteria directly isolated from mycorrhizospheric soils sampled. In general, hyphobacteria were superior in mobilizing P from hydroxyapatite and from a low reactivity igneous phosphate rock from Quebec. Release of gluconic acid or the product of its oxidation 2-ketogluconic acid, are the main mechanisms involved in P solubilization. In a two compartments Petri plate system, *Ri* extraradical hyphal exudates, supported PSB growth and activity. In the absence of PSB *Ri* showed a negligible P solubilization activity. In the presence of PSB a substantial increase in P mobilization was observed, and the superiority of hyphobacterial activity was also observed under this system. Our results suggest that in developing a bioinoculant based on selected PSB, their interaction with AMF hyphae should not be overlooked.

1. Introduction

The dynamic processes that characterize relationships between plants and microbial communities are complex. Soil microorganisms have an important influence on soil fertility and plant growth (Andrade et al., 1997; Miransari, 2011).

Phosphorus (P) is a major essential macronutrient limiting crop yields and is required by plants in relatively large amounts. Most soils are frequently deficient in soluble orthophosphate (Pi), the P form directly available to plants (Richardson, 2001). Therefore, low Pi availability limits plant growth and agricultural productivity worldwide and to support and maintain crop production, P must be provided to plants as soluble chemical fertilizers. However, applied P is rapidly fixed in soil and it is estimated that only 10–20% of chemical P fertilizers are used by plants the year of application (Richardson, 2001). Phosphate rock (PR), the cheapest P-fertilizer, was recognized as a valuable alternative for sustainable agriculture (Vassilev et al., 2001; Reddy et al., 2002). Unfortunately, plants respond to fertilization with PR in an erratic way, and generally yields obtained with PR are lower than those produced with soluble phosphate fertilizers (Khasawneh and Doll, 1978).

In most natural terrestrial plant ecosystems, P is obtained from the soil with the help of symbiotic mycorrhizal fungi (Smith and Read, 1997). The arbuscular mycorrhizal fungi (AMF), forming the oldest and most widespread type of mycorrhizal association, are key components of the soil microbial community, influencing water and nutrients uptake (phosphorus, nitrogen and various micronutrients), and reducing the incidence of plant diseases to their

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host (St-Arnaud and Vujanovic, 2007; Bonfante and Anca, 2009). In addition, AMF can affect the diversity and structure of bacterial communities in the rhizosphere (Toljander et al., 2006). The soil zone influenced by both the mycorrhizal roots and the AMF hyphae extending into the soil from the colonized roots has been called the mycorrhizosphere (Linderman, 1988) and is formed of two principal components: the rhizosphere which is directly influenced by roots, and the hyphosphere, which refers only to the soil zone surrounding individual fungal hyphae (Andrade et al., 1997, 1998). The hyphae of AMF provide an increased area for interactions with other soil microorganisms, especially bacteria which may in turn synergistically interact with AMF and thereby promote plant growth (Johansson et al., 2004). AMF can only absorb phosphate ions from soil solutions but are unable to extract P by themselves from PR (Antunes et al., 2007). However, when associated with some bacteria (Villegas and Fortin, 2001) or fungi (Kucey, 1987), AMF can obtain P from PR and translocate it to their host plants.

Phosphate solubilizing bacteria (PSB) are considered to play an important role in P mobilization. They increase soil fertility through the solubilization of inorganic phosphates by releasing organic acids, and the mineralization of organic phosphates by producing phosphatases and phytases (Dobbelaere et al., 2003). PSB were already proposed as a viable solution to resolve the problem of precipitation of soluble superphosphate fertilizers (Omar, 1998; Narula et al., 2000; Whitelaw, 2000; Khan et al., 2007; Hameeda et al., 2008). Mycorrhizae can affect both the numbers and the composition of bacterial communities in the rhizosphere and the hyphosphere (Meyer and Linderman, 1986; Linderman, 1988; Paulitz and Linderman, 1991; Offre et al., 2007). In fact, qualitative and quantitative differences in bacterial communities between bulk soil and hyphosphere suggest preferential associations between some bacterial taxa and fungal hosts (Andrade et al., 1997, 1998). Interaction between AMF and PSB suggests mutual beneficial effects. The nutritional dynamics of bacteria and mycorrhizal fungi may be ecologically important. In fact, fungal exudates and other molecules could be used by bacteria as nutrients (Bonfante and Anca, 2009). On the other hand, hyphospheric bacteria are involved in the production of growth factors (affecting both plants and AMF), the reduction of stress and the inhibition of antagonists and competitors (Frey-Klett et al., 2007).

In order to establish efficient tandems between AMF and soil bacteria capable of extracting P from PR, we hypothesized that this beneficial interaction would be more effective with PSB strongly attached to hyphae, suggesting a close association with AMF hyphae, rather than PSB freely growing in the mycorrhizosphere.

The aim of this work is to isolate PSB closely attached to hyphae of the commercially used AMF *Rhizophagus irregularis* (*Ri*) DAOM 197198, hereafter called hyphobacteria. To reach this aim, a novel approach is described. We also present the first conclusive evidence that hyphospheric PSB display the ability to colonize AMF extraradical mycelium on a minimal growth medium and mobilize phosphates more efficiently than mycorrhizospheric bacteria, as a result of the PSB-*Ri* interaction.

2. Materials and methods

2.1. Soil sampling

In order to isolate PSB, thirteen soil samples were collected at a depth of 10–30 cm, from different sites near Quebec City, Canada and analyzed (Table S1). From each site, three subsamples were collected from adjacent plants and well mixed to form a composite sample. All tools used in soil sampling were surface disinfected using 70% ethanol and soils were placed in sterile Whirl-Pak® sample bags, transported on ice to the laboratory, stored at 4 °C and processed within a week. Each sample was homogenized in sterile saline (0.85% NaCl, w:v), serially diluted and used as described below.

2.2. Chemical composition and reactivity of Quebec phosphate rock

Igneous PR came from a phosphate rock open-pit mine located at Lac à Paul in the Saguenay–Lac-Saint-Jean region of Quebec, Canada and owned by Arianne Phosphate Inc., a Canadian mining exploration company. The chemical composition of the PR is presented in Table S2. The total P content of the PR used was 146 mg g^{-1} and its solubility was 3.4% in 2% formic acid and 5% in 2% citric acid, indicating a low reactivity.

2.3. Isolation of PSB strongly attached to hyphae or directly from the mycorrhizosphere

Leek (*Allium ampeloprasum* L., Norseco Inc.) seeds were planted in 200-cells seedling flats containing pasteurized and moistened Agro-mix substrate (Fafard, Saint-Bonaventure, Québec, Canada), homogeneously mixed with MYKE® PRO PS3, a commercial powdered inoculum containing spores of the AMF *R. irregularis* (*Ri*) DAOM 197198 (previously *Glomus irregulare*), to give a final concentration of 1200 spores L^{-1} of substrate. MYKE® PRO PS3 was kindly supplied by Premier Tech, Rivière-du-Loup, Québec, Canada, and was produced under aseptic conditions in a bioreactor. Plants were grown for 4 weeks in a greenhouse set to 14 h diurnal temperature of 22 °C and 18 °C at night. After 30 days, seedlings were transplanted into 15 cm diameter pots filled with Turface (Athletics™, Profile Products, Buffalo Grove, IL). This substrate, formed by calcined montmorillonite clay particles, allowed easily the separation and collection of AMF hyphae. Pots were placed in the greenhouse, watered daily and supplied twice a week for 15 d with a water soluble 20–2–20 (N–P–K) fertilizer diluted to a final concentration of 100 mg N L^{-1}, with 150 mg N L^{-1} for an additional 30 d, and finally with 200 mg N L^{-1} until the end of the experiment. After two-month, time required for the establishment of abundant extraradical AMF hyphae, each pot containing one mycorrhizal plant was inoculated with 10 mL of a diluted soil suspension in sterile saline, containing bacteria (approximately 10^6 CFU mL^{-1}) prepared from each of the 13 soil samples. Uninoculated control plants received 10 mL of sterile saline. The experiment was organized in a completely randomized block design with 14 treatments (13 soil samples and an uninoculated control) and 4 blocks. Additional set of pots were prepared as described above with or without inoculation with *Ri* DAOM 197198 and receiving diluted soil suspension or sterile saline. These pots were used to monitor leek plant colonization by *Ri* and the presence of PSB on hyphae. For the duration of the experiment no root colonization by AMF was observed in all treatments not inoculated with *Ri* DAOM 197198, and receiving soil suspension or saline. Plants were harvested two months after inoculation with the soil bacteria. Our observations indicated that this time was required for a good colonization of hyphae by PSB. At harvest, the percentage of root colonization by *Ri* ranged from 18 to 43%. From each pot, a 5 g subsample of Turface plus mycorrhizal roots was suspended in 50 mL of sterile dechlorinated tap water. Each suspension was vortexed three times for 5 min. Floating AMF hyphae were recovered by filtrating the supernatant using a synthetic nylon with a mesh size of 50 μm (Dynamic Aqua Supply Ltd, Surrey B.C., Canada) and then resuspended in 6 mL of sterile saline. The hyphae were washed three times with sterile water (2 min per wash) to remove loosely attached bacteria, and then ground using a Kontes Pellet Pestle (Fisher Scientific) to isolate the PSB strongly attached to AMF hyphae and endophytes. Ground hyphae were resuspended in 1 mL of sterile saline solution

and serially diluted. Isolation of PSB was performed on modified National Botanical Research Institute's Phosphate (NBRIP) agar medium containing 5 g $MgCl_2 \cdot 6H_2O$, 0.25 g $MgSO_4 \cdot H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, 15 g Bacto-Agar, 0.5 g yeast extract, 10 g glucose, 2.5 g hydroxyapatite and 50 μg mL^{-1} cycloheximide per liter at pH 7 (Nautiyal, 1999). NBRIP plates were incubated at 28 °C for 2–3 d, and colonies forming a clear solubilization halo were selected and streaked three consecutive times on the NBRIP medium to check for the stability of the dissolution phenotype. Colonies having a stable phenotype were recovered and stored in glycerol at −80 °C.

For comparison purpose, PSB were also isolated from the mycorrhizosphere soil of the plants initially sampled (Table S1). The serially diluted soil samples were plated on modified NBRIP medium and PSB were selected as described above.

2.4. Mineral phosphate solubilization assay

Selected PSB were cultivated overnight in 10% Difco Tryptic Soy Broth (TSB; BD, Franklin Lakes, N.J. USA) on a rotary shaker (200 rpm) at 28 °C. To prepare PSB inoculum, cells were collected and washed twice in sterile saline by centrifugation for 5 min at 10 000 *g* and resuspended in saline to a final concentration of 10^6 CFU mL^{-1}. Liquid NBRIP medium modified by the addition of glucose (10 g L^{-1}) and hydroxyapatite or Quebec PR (5 g L^{-1}) received 1 mL of inoculum. Flasks containing 100 mL of inoculated media were incubated for 7 d on a rotary shaker (200 rpm at 28 °C). At different time intervals (1, 3, 5 and 7 d), 2 mL aliquots were aseptically sampled and used for the estimation of the pH and the soluble phosphate by the method of Fiske and Subbarow (1925) adapted for microplates (Table S2). To appraise growth, bacterial cells were digested at 100 °C for 10 min in 1 M NaOH and protein content was determined with the Folin–phenol reagents (Lowry et al., 1951), using bovine serum albumin (Sigma–Aldrich) as a standard.

2.5. Identification of PSB

PSB exhibiting high phosphate dissolution activity in solid and liquid media were further investigated, but were first identified by amplifying and sequencing the 16S rDNA. The gene encoding the 16S rRNA was amplified by the polymerase chain reaction (PCR) using eubacterial universal primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) (Weisburg et al., 1991). The PCR mix consisted of deoxynucleotides at 200 mM each, 0.25 mM of each primer, 2.5 mM $MgCl_2$, 1 × PCR buffer and 0.2 U of Taq DNA polymerase (5 Prime GmbH). A suspension of cells in MilliQ water, coming from a fresh colony grown on 10% Tryptic Soy Agar (TSA; Difco), was used as target DNA. The following PCR conditions were used: 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 46 °C for 30 s and 72 °C for 2 min, and a final extension step at 72 °C for 10 min. The PCR products were purified and sequenced (CHUL Research Center, Québec City, Québec, Canada). The nucleotide sequences were compared using the BlastN program, and the closest match of known phylogenetic affiliation was used to assign the isolated strains to specific taxonomic groups.

2.6. Identification of organic acids

For the analysis of organic acids, bacterial cultures were filtered through 0.2 μm filter (Millipore®) and 10 μL of filtrates were injected in a Waters HPLC system (Pump Model 996). Organic acid separation was carried out on ICSep ICE-ION-300 column (7.8 × 300 mm) (Transgenomic) with 8.5 mM H_2SO_4 as mobile phase (0.4 mL min^{-1}). Organic acids were detected with a photodiode array detector (Waters model 600) at 210 nm and identified by comparison to standards purchased from Sigma–Aldrich (Oakville, Ontario, Canada).

2.7. Organic phosphate mineralization and phytase activity

A completely randomized block design with three replicates was used and each experiment was repeated three times.

To assess the phosphatase activity, PSB were grown to the stationary phase in 25 mL TSB broth at 28 °C on a rotary shaker (200 rpm). Cells from 2 mL aliquots culture were collected and washed in sterile saline by centrifugation (10,000 *g*, 10 min), and resuspended in 500 μL of Tris buffer (pH 7). Cells were sonicated eight times on ice for 5 s. Acid and alkaline phosphatase activities were determined in cell free extracts in 96 well microplates as described in the Sigma–Aldrich acid phosphatase assay kit (St. Louis, Mo; catalog # CS0740). For alkaline phosphatase, 0.05 M glycine buffer with 0.01% $MgCl_2.6H_2O$ and 5.5 mM of 4-nitrophenyl phosphate at pH 10.5 was used and one unit (U) of alkaline phosphatase will hydrolyze one μmol of 4-nitrophenyl phosphate per min at 37 °C and pH 10.5.

For phytase measurement, PSB were grown in modified NBRIP liquid medium supplemented with 5 g L^{-1} wheat bran as sole P source, for 5 d at 28 °C and 200 rpm. At the end of the culture, the crude supernatant (2 mL) obtained after centrifugation at 6 300 *g* for 15 min was used to determine the phytase activity (Farhat et al., 2008).

For all enzymes measurements, the specific activity (U mg^{-1} protein) was determined by measuring total soluble protein in the enzymatic extract (Lowry et al., 1951).

For each enzyme three different assays were performed, involving each three different measurements.

2.8. Interaction between PSB and AMF: phosphate solubilization and pH changes

Maintenance of the *Agrobacterium rhizogenes* transformed chicory (*Cichorium intybus*) roots cultured with *Ri* DAOM 197198 was performed in a minimal growth medium (Bécard and Fortin, 1988). Our experimental setup consisted of a two-compartment Petri plate system that allows the study of the interaction between *Ri* extraradical hyphae and PSB without interferences from the host roots (St-Arnaud et al., 1995). A proximal compartment contained a minimal medium containing the following (mg L^{-1}): 80 KNO_3, 731 $MgSO_4 \cdot 7H_2O$, 65 KCl, 4.8 KH_2PO_4, 288 $Ca(NO_3)_2 \cdot 4H_2O$, 8 NaFeEDTA, 0.75 KI, 6 $MnCl_2 \cdot 4H_2O$, 2.65 $ZnSO_4 \cdot 7H_2O$, 1.5 H_2BO_3, 0.13 $CuSO_4 \cdot 5H_2O$, 0.0024 $Na_2MoO_4 \cdot 2H_2O$, 3 glycine, 0.1 thiamine hydrochloride, 0.1 pyridoxine hydrochloride, 0.5 nicotinic acid, 50 myo-inositol, and 10,000 sucrose. A distal compartment contained the following (mg L^{-1}): 80 KNO_3, 731 $MgSO_4 \cdot 7H_2O$, 65 KCl, 4.8 KH_2PO_4, 288 $Ca(NO_3)_2 \cdot 4H_2O$, 8 NaFeEDTA, 0.75 KI, 6 $MnCl_2 \cdot 4H_2O$, 2.65 $ZnSO_4 \cdot 7H_2O$, 1.5 H_2BO_3, 0.13 $CuSO_4 \cdot 5H_2O$, 0.0024 $Na_2MoO_4 \cdot 2H_2O$ and 2500 of hydroxyapatite or Quebec PR. The pH of each compartment was adjusted to 5.5 and 0.4% of phytagel (Sigma–Aldrich) was added as the gelling agent before sterilization at 121 °C for 20 min. Mycorrhizal chicory roots were transferred in the proximal compartment and incubated for 3 weeks at 28 °C. Inocula of four selected PSB: *Rhizobium miluonense* (Rm3), *Burkholderia anthina* (Ba8), *Rahnella* sp. (Rs11) and *Burkholderia phenazinium* (Bph12) were prepared from an overnight shake culture in 10% TSB at 28 °C. Cultures were washed twice with sterile saline and resuspended to give a bacterial suspension of 10^8 CFU mL^{-1}. The distal compartment, where only the extraradical AMF mycelium was allowed to grow, received 10 μL of the bacterial suspension. An uninoculated treatment was run in parallel as control and

received 10 μL sterile saline. All treatments were replicated five times and the Petri dishes were incubated at 28 °C.

The amount of soluble P and pH changes in the distal Petri compartments was assessed as described by Villegas and Fortin (2002) with few modifications: six weeks after bacterial inoculation (9 weeks after the beginning of the experiment), to liquefy the gelled medium it was frozen at −20 °C for 24 h, thawed at room temperature and then centrifuged at 10,000 *g* for 30 min. Supernatant soluble P content and pH were measured as described above.

2.9. Statistical analysis

Data were tested for normality and variance homogeneity, and acid phosphatase, phytase and P-solubilization data were ln transformed to normalize skewed distributions. Analysis of variance was performed using the general linear model in SAS version 9.2 (SAS Institute). Means were compared using the Fisher's protected least significant difference (LSD) method at $P \leq 0.05$.

3. Results

3.1. Isolation and identification of bacteria

A total of 941 bacterial isolates were retained for their ability to produce 15–35 mm diameter P-solubilization clarification zones around colonies, on the modified NBRIP medium containing hydroxyapatite as a sole P source. There was 541 bacterial isolates obtained from hyphospheres and 400 from mycorrhizospheres (Table S3). Only 25% of PSB isolates showed a stable phosphate solubilization phenotype after 3 consecutive subcultures on solid modified NBRIP containing hydroxyapatite, while only 13% were able to solubilize hydroxyapatite in liquid modified NBRIP (Table S3). Based on their ability to mobilize more than 50 mg P L^{-1} from hydroxyapatite in liquid medium after 7 d of incubation, 51 PSB (Table S3), were selected and identified (Table 1).

The best eight PSB (four from the hyphosphere and four from the mycorrhizosphere) were identified as members of the phyla *Fermicutes*, *α*-, *β*- and *γ*-*Proteobacteria* (Tables 2 and S4). Hyphobacteria were identified as *R. miluonense* (Rm3), *B. anthina* (Ba8), and *Burkholderia* sp. (Bs9 and Bs13), while mycorrhizobacteria belonged to *Dyella japonica* (Dj1), *Bacillus pumilus* (Bp3), *Rahnella* sp. (Rs11) and *B. phenazinium* (Bph12).

3.2. Mineral phosphate solubilization ability of PSB and organic acids production

The soluble-P concentration in the medium containing hydroxyapatite ranged between 96 (Bp3) and 183 (Ba8) mg P L^{-1} after 7 d (Fig. 1A). In general after 7 d, hyphobacteria mobilized more P from hydroxyapatite than mycorrhizobacteria. In fact, 3 out of the 4 studied hyphobacteria exhibited a soluble P concentration in the medium higher than 177 mg P L^{-1} while with mycorrhizobacteria, the highest soluble P concentration was observed with *Rahnella* sp. Rs11 (Fig. 1A) with a value of 175 mg P L^{-1}. No soluble-P and no decrease in the pH were observed in the uninoculated controls.

The specific P-solubilization on NBRIP medium was determined by dividing the total P in the supernatant by the total protein concentration of the bacterial cells. Mycorrhizobacteria *Rahnella* sp. Rs11 showed the highest specific P-solubilization (1500 mg P mg^{-1} protein) after 3 d and hyphobacteria *B. anthina* Ba8 (1498 mg P mg^{-1} protein) after 5 d incubation. Specific P-solubilization activity decreased after 7 d indicating an important immobilization of soluble P by PSB (Fig. 1B). As observed with P-solubilization, hyphobacteria exhibited significantly higher specific P-solubilization than mycorrhizobacteria on NBRIP-hydroxyapatite medium (Fig. 1B).

Phosphate solubilization in liquid NBRIP-hydroxyapatite medium was accompanied by a significant drop in pH. In fact, after 1 d, the pH decreased significantly from 7 to 4.3 and 3.8 with mycorrhizobacteria Rs11 and Bph12, respectively (Fig. 1C). With the four tested hyphobacteria the pH dropped from 7 to 3.7.

The soluble-P concentration in the medium containing Quebec PR ranged between 3.5 and 19.6 mg P L^{-1} after 7 d (Fig. 2A). After 3 d, the mycorrhizobacteria *B. phenazinium* Bph12 mobilized the highest amount of P (22.4 mg P L^{-1}). Hyphobacteria Bs9 and Ba8 were the second best Quebec PR solubilizers (14.5 and 13.7 mg P L^{-1}, respectively) after 7 d of incubation, while mycorrhizobacteria *D. japonica* Dj1 showed the lowest soluble P in the supernatant (3.5 mg P L^{-1}). The maximum P-specific solubilization was recorded with mycorrhizobacteria Bph12 (195.1 mg P mg^{-1} protein) followed by hyphobacteria Ba8 (120.5 mg P mg^{-1} protein) after 3 d of incubation (Fig. 2B). Dj1 was the least efficient PSB with a maximum of 29.6 mg P mg^{-1} protein reached after 1 d of incubation (Fig. 2B). Quebec PR solubilization was also accompanied by a substantial decrease in culture media pH from 7 to 3 after 7 d of incubation (Fig. 2C).

Table 1
Origin of the 51 selected PSB trapped on AMF hyphae or isolated from the mycorrhizosphere soils used in this study.

Genera	Number of bacteria retained from		
	AMF hyphae (Hyphobacteria)		Mycorrhizosphere (Mycorrhizobacteria)
	Loosely attached	Strongly attached	
α-Proteobacteria			
Rhizobium	0	1	0
β-Proteobacteria			
Burkholderia	7	26	5
γ-Proteobacteria			
Rahnella	0	0	4
Pseudomonas	0	0	2
Ewingella	0	0	2
Dyella	0	0	1
Erwinia	0	0	1
Actinobacteria			
Leifsonia	1	0	0
Firmicutes			
Bacillus	0	0	1
Total	8	27	16

Table 2
Identification of the selected PSB isolates by sequencing of the 16S rDNA.

Isolate # (strain name)	Length of 16S rDNA gene sequenced	GenBank accession no.	Closest match	
			Strain	% Gene identity
PSB strongly attached to AMF hyphae (Hyphobacteria)				
3.1 (Rm3)	1293	KC241902	*Rhizobium miluonense* CC-B-L1	99
8.1 (Ba8)	1377	KC241903	*Burkholderia anthina* R7-112	100
9.1 (Bs9)	931	KC241904	*Burkholderia* sp.1 IT1	100
13.2 (Bs13)	1351	KC241905	*Burkholderia* sp.2 IT1	99
PSB from the mycorrhizosphere (Mycorrhizobacteria)				
1.39 (Dj1)	1366	KC241898	*Dyella japonica* NBRC 104185	99
3.10 (Bp3)	1066	KC241899	*Bacillus pumilus* R71	100
11.39 (Rs11)	985	KC241900	*Rahnella* sp.WMR15	99
12.21 (Bph12)	949	KC241901	*Burkholderia phenazinium* A 1	99

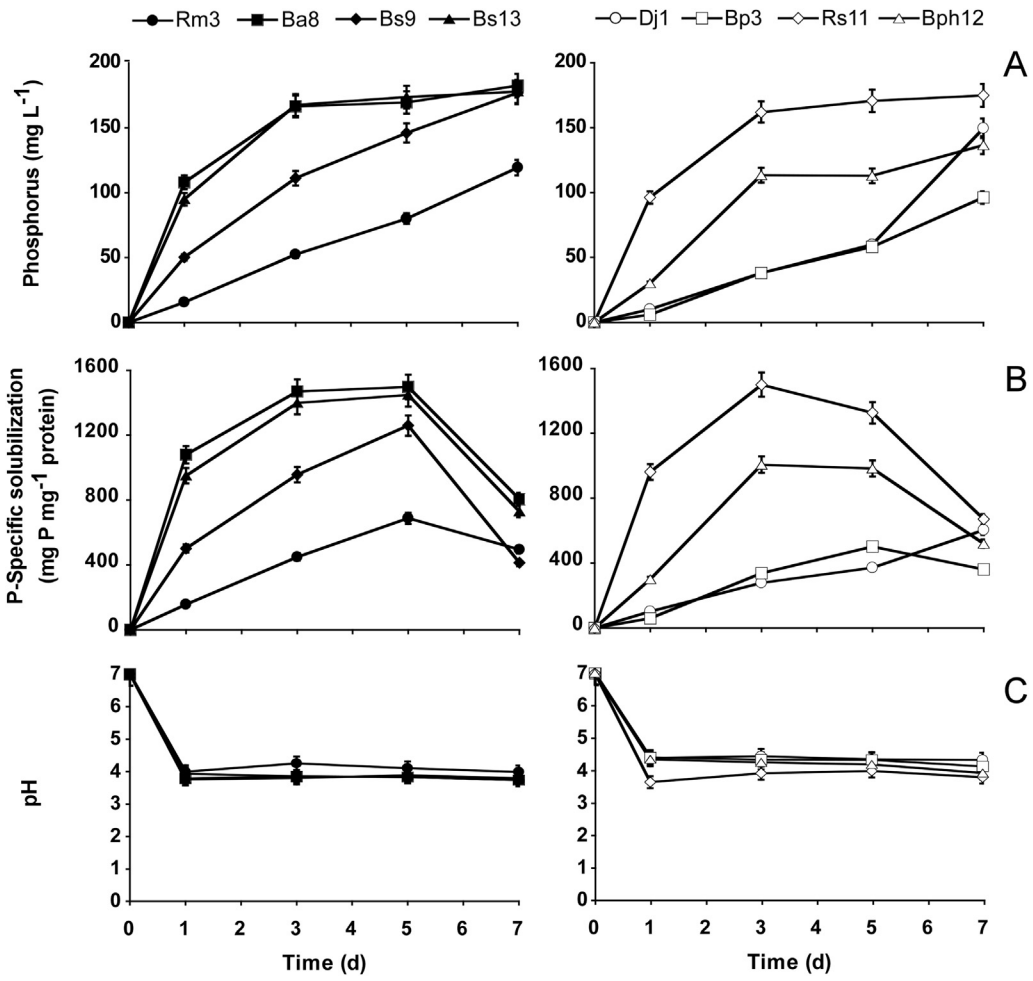

Fig. 1. Mobilization of P by PSB from hydroxyapatite (A) Soluble phosphate; (B) specific phosphate solubilization activity, $LSD_{0.05}$ values: d1 = 63.98, d3 = 177.78, d5 = 110.80, d7 = 55.28 mg P mg^{-1} protein; and (C) pH variation in the supernatant of PSB growing on NBRIP-hydroxyapatite. Hyphobacteria are shown on the left and mycorrhizobacteria on the right. PSB identity is described in Table 2. Error bars indicate standard deviation of means from three independent assays.

Three different organic acids, gluconic acid, 2-ketogluconic acid and pyruvic acid, were shown to be produced by the isolates (Table 3). After 7 d incubation the supernatant of *Burkholderia* sp. isolates (Bs9 and Bs13) grown on NBRIP-hydroxyapatite contained the highest organic acid concentrations (Table 3). *B. anthina* Ba8 and *Burkholderia* sp. Bs9 and Bs13 mostly produced 2-ketogluconic acid. *B. pumilus* Bp3 and *B. phenazinium* Bph12 produced only gluconic acid. *Rahnella* sp. Rs11 and *R. miluonense* Rm3 produced both gluconic acid and 2-ketogluconic acid. *D. japonica* produced only pyruvic acid.

On NBRIP-Quebec PR medium, all mycorrhizobacteria except *D. japonica* Dj1 produced only gluconic acid, although hyphobacteria produced mainly gluconic and 2-ketogluconic acids (Table 3). The highest production of gluconic acid was by *B. phenazinium* Bph12. *Burkholderia* sp. 1 Bs9 was the highest 2-ketogluconic acid producer.

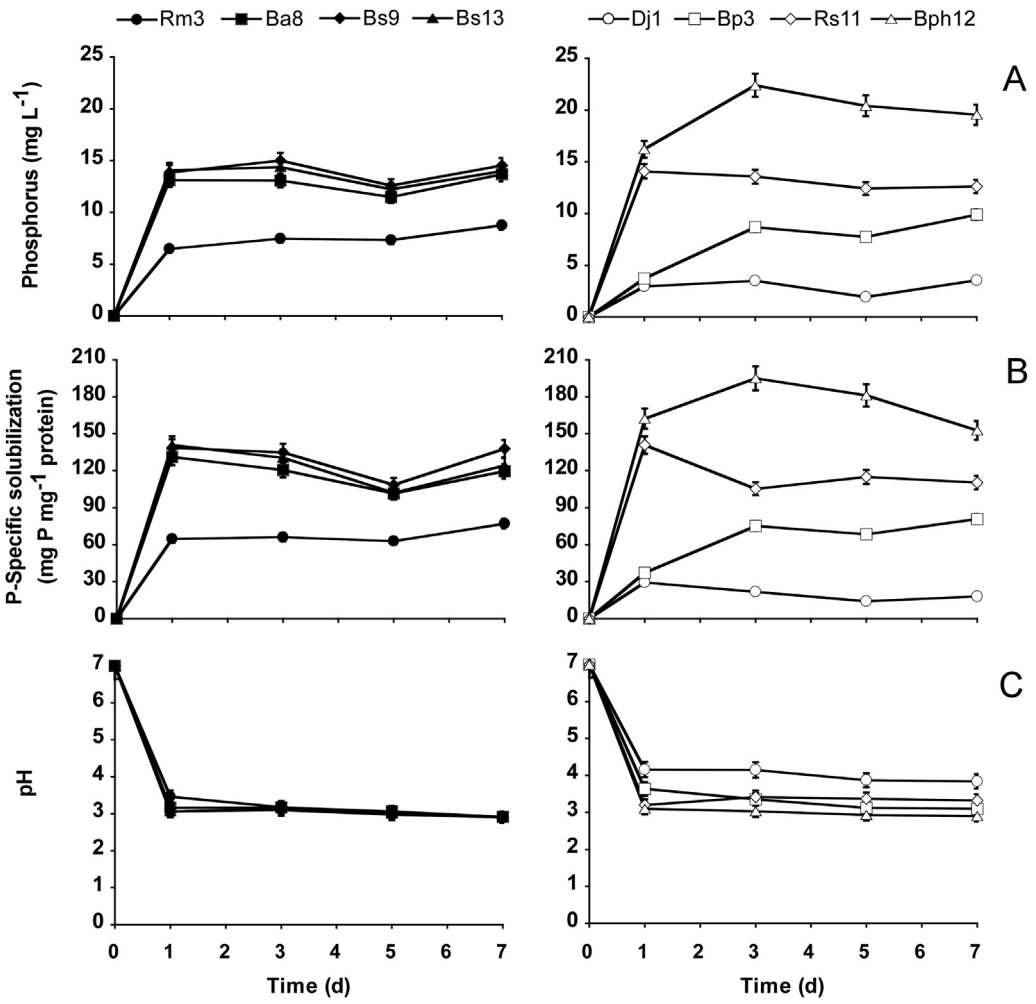

Fig. 2. Mobilization of P by PSB from Quebec phosphate rock (A) Soluble phosphate; (B) specific phosphate solubilization activity, $LSD_{0.05}$ values: d1 = 22.75, d3 = 23.61, d5 = 27.55, d7 = 26.59 mg P mg^{-1} protein; and (C) pH variation of PSB growing on NBRIP-Quebec PR. Hyphobacteria are shown on the left and mycorrhizobacteria on the right. PSB identity is described in Table 2. Error bars indicate standard deviation of means from three independent assays.

3.3. Organic phosphate mineralization

Important acid phosphatase activities were detected in all PSB tested except Bp3 (Table 4). Marked alkaline phosphatase activity was observed with PSB Rm3, Bs9 and Rs11 while Bp3 showed very low activity. Phytase activity was detected in all PSB selected and ranged from 28 to 86 mU mg^{-1} protein (Table 4).

3.4. PSB and AMF interaction

Two hyphobacteria (*R. miluonense* Rm3 and *B. anthina* Ba8) and two mycorrhizobacteria (*Rahnella* sp. Rs11 and *B. phenazinium* Bph12) were selected to study their interaction with AMF, based on their high P-solubilization and mineralization activities. The selected strains were unable to grow alone on the minimal growth

Table 3
Organic acid production after 7 days of incubation by selected PSB strongly attached to AMF hyphae (hyphobacteria) or isolated from the mycorrhizosphere (mycorrhizobacteria) growing on NBRIP medium containing hydroxyapatite or Quebec PR as sole P source.

PSB	Organic acids mg L^{-1}				
	Hydroxyapatite			Quebec PR	
	Gluconic acid	2-Ketogluconic acid	Pyruvic acid	Gluconic acid	2-Ketogluconic acid
Hyphobacteria					
Rm3	$1564^{b} \pm 324$	$898^{d} \pm 215$	3 ± 3	$294^{b} \pm 44$	$235^{b} \pm 14$
Ba8	–	$3643^{b} \pm 258$	$19^{cd} \pm 13$	$269^{b} \pm 97$	$226^{b} \pm 11$
Bs9	$152^{c} \pm 38$	$4345^{a} \pm 153$	$118^{b} \pm 7$	$305^{b} \pm 20$	$299^{a} \pm 19$
Bs13	–	$3855^{b} \pm 265$	$35^{c} \pm 22$	$201^{c} \pm 55$	$158^{c} \pm 0.4$
Mycorrhizobacteria					
Dj1	–	–	$455^{a} \pm 18$	–	–
Bp3	$1963^{b} \pm 232$	–	–	$351^{b} \pm 46$	–
Rs11	$2883^{a} \pm 366$	$2270^{c} \pm 160$	$17^{cd} \pm 8$	$204^{b} \pm 27$	–
Bph12	$3360^{a} \pm 223$	–	–	$1438^{a} \pm 55$	–

PSB identity can be found in Table 2. Values are means ± standard deviations of three independent assays. For each P source, in each column means followed by the same letter are not significantly different according to the Fisher's protected LSD test ($P \leq 0.05$). –: not detected.

Table 4
Activity of enzymes involved in organic phosphate mineralization.

PSB	Enzyme activity mU mg^{-1} protein		
	Acid phosphatase	Alkaline phosphatase	Phytase
Hyphobacteria			
Rm3	$439.6^{b} \pm 9.8$	$370.7^{b} \pm 8.9$	$205.5^{a} \pm 164.5$
Ba8	$360.2^{bc} \pm 9.8$	NA	$28.0^{c} \pm 13.5$
Bs9	$387.9^{bc} \pm 66.0$	$237.2^{c} \pm 45.3$	$32.1^{bc} \pm 7.1$
Bs13	$326.1^{bcd} \pm 177.9$	NA	$31.1^{bc} \pm 10.3$
Mycorrhizobacteria			
Dj1	$284.5^{cd} \pm 26.2$	NA	$85.9^{ab} \pm 43.1$
Bp3	NA	$5.3^{d} \pm 4.5$	$70.0^{abc} \pm 44.6$
Rs11	$752.8^{a} \pm 4.9$	$749.5^{a} \pm 3.6$	$54.4^{bc} \pm 45.3$
Bph12	$234.6^{d} \pm 75.0$	NA	$44.4^{bc} \pm 8.1$

PSB identity can be found in Table 2. Values are means ± standard deviations of three independent assays. In each column, means followed by the same letter are not significantly different according to the Fisher's protected LSD test ($P \leq 0.05$). NA: no activity detected. For phosphatases one unit (U) is the amount of enzyme catalyzing one μmol of 4-nitrophenyl phosphate per min at 37 °C. One unit (U) phytase releases 1 μmol of inorganic phosphate from phytic acid per minute at 55 °C.

medium of the distal compartment containing hydroxyapatite or Quebec PR without any carbon source or growth factors and thus no P-solubilization levels were detected. After 6 weeks of dual culture with *Ri* mycelium, the amount of solubilized-P was significantly ($P \leq 0.05$) increased compared with the AMF mycelium alone (Fig. 3A).

With Quebec PR or hydroxyapatite as sole P source, *B. anthina* Ba8 in association with *Ri* was the best P-solubilizer (1.84 and 3.51 mg P L^{-1}, respectively) followed by *R. miluonense* Rm3 (1.16 and 3.06 mg P L^{-1}, respectively). Lower P-solubilization levels were detected with *Rahnella* sp. Rs11 (0.88 and 2.72 mg P L^{-1}) and *B. phenazinium* Bph12 (0.77 and 2.75 mg P L^{-1}) in the presence of the AMF mycelium with Quebec PR and hydroxyapatite, respectively. *R. irregularis* (control) was barely able to solubilize phosphate. The pH of distal medium was initially adjusted to 5.5 and after 6 weeks of dual culture, *B. anthina* Ba8 produced the highest drop in pH (5.38 and 5.01 with Quebec PR and hydroxyapatite) (Fig. 3B). *Rahnella* sp. Rs11 caused little pH change (5.48 and 5.29 with Quebec PR and hydroxyapatite). The pH of the distal media containing only *Ri* hyphae did not change. In the uninoculated treatment no soluble-P and no decrease in the pH were observed.

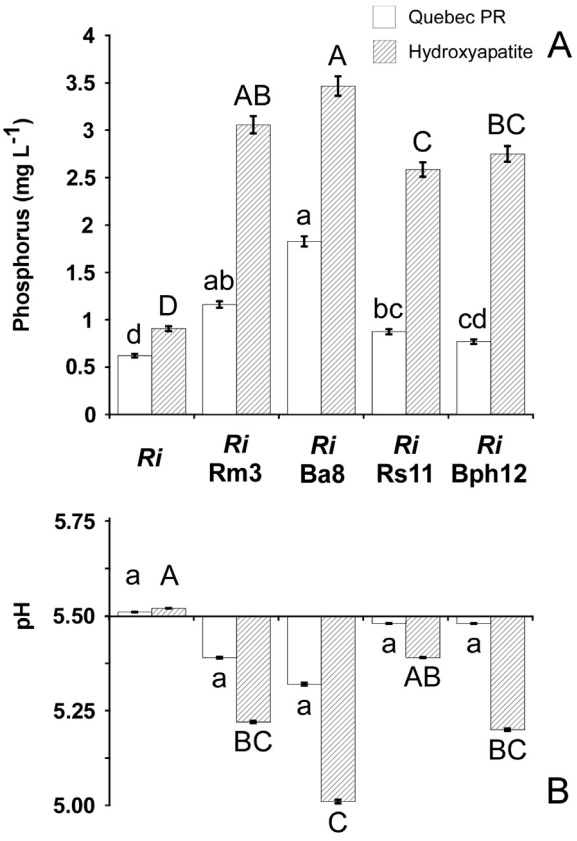

Fig. 3. Soluble phosphate and pH variation in the minimal medium extract obtained from the distal compartment containing hyphae of AMF *Rhizophagus irregularis* (*Ri*), *Ri* plus hyphobacteria *Rhizobium miluonense* (Rm3) or *Burkholderia anthina* (Ba8); *Ri* plus mycorrhizobacteria *Rahnella sp.* (Rs11) or *Burkholderia phenazinium* (Bph12) after 6 weeks of dual incubation. For each P-source means followed by the same letter (capital for hydroxyapatite or lowercase for Quebec PR) are not significantly different according to Fisher's protected LSD test ($P \leq 0.05$). Error bars are standard deviations of the means from three independent assays.

4. Discussion

Many genera of PSB showed a good potential to be used as biofertilizers for the improvement of plant P nutrition, because they mobilize P from sparingly inorganic and organic P sources in soil (Calvo et al., 2014). Several reports also highlighted the importance of beneficial interaction between PSB and AMF in P mobilization and improvement of uptake by plants (Sharma et al., 2013; Calvo et al., 2014). However, although AMF symbiosis with plants is ubiquitous, its interaction with PSB was overlooked during the development of biofertilizers (Sharma et al., 2013; Calvo et al., 2014), and this might explain in part the variability of the results obtained during inoculation trials (Sharma et al., 2013). In fact, it was clearly demonstrated that strains of bacteria exhibit different attachment patterns according to the AMF species involved (Toljander et al., 2006). Since AMF hyphae is an important interface for the signaling and exchange of metabolites with bacteria (Toljander et al., 2006), and as mycelial exudates significantly influence the composition of bacterial communities (Toljander et al., 2007), we hypothesized that hyphae competent PSB would be more effective to colonize the hyphosphere and would mobilize more P from PR than loosely attached PSB. Investigation of AMF hyphosphere *in vivo* requires the construction of elaborate compartmentalized plant growth systems (Mansfeld-Giese et al., 2002; Zhang et al., 2014). The use of T-DNA-transformed *Daucus carota* roots in two-compartment plates (St-Arnaud et al., 1995) proved to be a very useful *in vitro* tool to study bacterial attachments, however it has some limitations like the failure of production of enough fungal material in the liquid compartment as observed by Scheublin et al. (2010).

In this work, we developed a new simple method, inspired from the one described by Staddon et al. (2003), using mycorrhizal leek plants growing on Turface, which allowed the isolation of PSB from the hyphosphere. PSB strongly attached were obtained after sequential washing of *Ri* hyphae in saline to remove loosely attached bacteria. In this system, bacterial attachment to *Ri* hyphae is then a consequence of natural AMF-PSB interactions and mycorrhiza exudation patterns, without *a priori* assumptions on the specific species involved.

The present work was not planned to study all bacteria associated with *Ri* hyphae, but rather to select culturable bacteria closely attached to hyphae, having substantial ability to mobilize P from poorly soluble phosphates. Both Gram positive and negative bacteria include genera of PSB (Rodríguez and Fraga, 1999). We observed that Gram negative bacteria were the dominant PSB both on the hyphae of *Ri* and in the mycorrhizosphere. Except for one *R. miluonense* isolate, the 26 PSB strongly attached to *Ri* hyphae

belonged to the genus *Burkholderia*. *Pseudomonas*, *Burkholderia* and *Bacillus* have been reported as the most predominant species associated with AMF fungi (Bonfante and Anca, 2009), and *Burkholderia* species are frequently isolated as efficient PSB (Gupta et al., 2012; Calvo et al., 2014). However, since the last step of the method used to isolate strongly attached bacteria involved hyphal grinding, we cannot exclude the possibility that some of the isolates might be fungal endophytes. Thorough investigation revealed the presence of a nonculturable endophyte related to *Burkholderia* in spores and hyphae of *Glomus margarita* (Bonfante and Anca, 2009), however Cruz et al. (2008) isolated from surface sterile spores of *Gigaspora margarita* a culturable bacterial strain closely related to *Janthinobacterium lividum* (Burkholderiales) with P-solubilization trait. With the exception of a *Bacillus* isolate, the 15 mycorrhizobacteria mobilizing P retained from the 13 soils studied were Gram negative, and about one third belonged to *Burkholderia* (Table 1). This can explain in part the importance of *Burkholderia* strongly trapped on hyphae of *Ri*, as observed under our experimental conditions.

Organic P in soil represents 50% or more of the total soil P (Richardson, 2001). Therefore, in addition to their high inorganic P mobilization, four hyphobacteria and four mycorrhizobacteria were selected on the basis of their important phosphatase activity (Table S3). As expected, P was more readily mobilized from hydroxyapatite by PSB than from the Quebec PR, because of its very low reactivity (Table S2). Both hyphobacteria and mycorrhizobacteria solubilized hydroxyapatite and Quebec PR in a similar fashion, but hyphobacteria were superior solubilizers. The specific P-solubilization (mg P mobilized per mg protein) from hydroxyapatite decreased after 5 d incubation suggesting a decrease in solubilization activity or an important immobilization of P by bacteria, leaving less soluble P surplus in the supernatant (Fig. 1B). With Quebec PR, P specific solubilization reached rapidly a plateau indicating that the little P-solubilized is rapidly immobilized by the growing bacteria (Fig. 2B). Gluconic acid is the major organic acid involved in P-solubilization by bacteria (Rodríguez and Fraga, 1999), produced by the oxidation of glucose by glucose dehydrogenase. Gluconic acid can be further oxidized to 2-ketogluconic acid by gluconic acid dehydrogenase which can be further transformed to pyruvic acid (Buch et al., 2010). In this work, glucose was the carbon source used in NBRIP medium which explains the importance of gluconic and 2-ketogluconic acids detected in the supernatants of the PSB tested (Table 3). With the easily mobilized hydroxyapatite in addition to gluconic acid or 2-ketogluconic acid, all hyphobacteria produced pyruvic acid, probably reflecting a more rapid transformation of gluconic acid via the glucose catabolism phosphorylative pathway (Buch et al., 2010). Among mycorrhizobacteria, only Dj1 produced a significant amount of pyruvic acid in the presence of hydroxyapatite (Table 3), and the absence of gluconic or 2-ketogluconic acid suggests that pyruvic acid might be originating from the direct glucose oxidative pathway (Buch et al., 2010). Quebec PR was mobilized by PSB by producing gluconic or 2-ketogluconic, except for Dj1 for which no organic acids was detected (Table 3). In the NBRIP medium, ammonium sulfate was used as the sole nitrogen source, and the low amount of P mobilized from Quebec PR by Dj1 could be the results of the release of protons associated with ammonium assimilation, as previously observed with a *Penicillium rugulosum* mutant which do not produce organic acids (Reyes et al., 1999).

In addition to mineral P, soil contains a wide range of organic forms of P that may constitute 30–50% or more of the total P in most soils (Rodríguez and Fraga, 1999). Phytic acid is the most abundant form of organic P in soils, but it reacts with soil cations forming sparingly soluble phytates, not available for plants. Many soil microorganisms can produce phosphatases including phytases, able to hydrolyze organic P releasing orthophosphate the form of P absorbed by plants (Rodríguez and Fraga, 1999). All PSB tested except *B. pumilus* Bp3 produced acid phosphatase, however Bp3 produced phytase. In general, the high level of acid phosphatase observed (Table 4) suggests that the studied PSB selected for their high solubilization of inorganic phosphate, mainly by the production of organic acids, might also play a major role in organic phosphate mineralization in soils. Since organic acids improve significantly the bioavailability of P from phytate (Giles et al., 2014), more work is necessary to determine if the studied PSB can improve plant P uptake from phytates.

To study the interaction between PSB and *Ri* extraradical hyphae and their effect on Quebec PR solubilization, we used the two compartments plate system as previously described by Villegas and Fortin (Villegas and Fortin, 2001, 2002). However, in this work, no vitamins or carbon source were added in the distal compartment, and therefore all energy source required for bacterial growth and activity is totally derived from hyphal exudates. In fact we did not observe any bacterial growth or P-solubilization in the distal compartment medium, in the absence of *Ri* hyphae. This is in agreement with the results of Kaiser et al. (2015) showing that AMF extraradical hyphae is an active distributor of fresh plant carbon to soil microbes. We also used nitrate as the nitrogen source in the distal compartment to avoid solubilization by *Ri* hyphae resulting from medium acidification (Villegas and Fortin, 2001). After six weeks of incubation, *Ri* alone was a mediocre P-solubilizer, confirming previous observation made by Villegas and Fortin (2002). Hyphobacteria *B. anthina* Ba8 mobilized significantly more P from hydroxyapatite or Quebec PR (Fig. 3). The efficiency of this PSB strain might be explained by its ability to form important biofilm on *Ri* hyphae as indicated by our preliminary observations, and this close contact between the prokaryotic PSB and the eukaryotic AMF probably trigger interesting exchanges that merit further investigations.

This work illustrates the positive interaction taking place between PSB and AMF, in mobilizing P from sparingly soluble sources. This interaction has a great potential for use in the improvement of plant P nutrition. Our results also suggest that the hyphosphere might be a better source than the mycorrhizosphere for the isolation of efficient PSB. Because fungal species can influence bacterial attachment to AMF hyphae (Toljander et al., 2006), and our observations were made with the model isolate of AMF *Ri* DAOM 197198. Further work performed with other genera and species of AMF is necessary to properly appraise the potential of the selected PSB to be used in a commercial biofertilizer.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2015.07.016.

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