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The interaction between *Rhizoglomus irregulare* and hyphae attached phosphate solubilizing bacteria increases plant biomass of *Solanum lycopersicum*

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ABSTRACT

The synergistic interaction between arbuscular mycorrhizal fungi (AMF) and phosphate solubilizing bacteria (PSB) can enhance growth and phosphorous uptake in plants. Since PSBs are well known hyphal colonizers we sought to understand this physical interaction and exploit it in order to design strategies for the application of a combined microbial inoculum. Phosphate-solubilizing bacteria strongly attached to the hyphae of *Rhizoglomus irregulare* were isolated using a two compartment system (root and hyphal compartments), which were separated by a nylon mesh through which AMF hyphae could pass but not plant roots. *Allium ampeloprasum* (Leek) was used as the host plant inoculated with *R. irregulare*. A total of 128 bacteria were isolated, of which 12 showed stable phosphate solubilizing activity. Finally, three bacteria belonging to the genus *Pseudomonas* showed the potential for inorganic and organic phosphate mobilization along with other plant growth promoting traits. These PSBs were further evaluated for their functional characteristics and their interaction with AMF. The impact of single or co-inoculations of the selected bacteria and AMF on *Solanum lycopersicum* was tested and we found that plants inoculated with the combination of fungus and bacteria had significantly higher plant biomass compared to single inoculations, indicating synergistic activities of the bacterial-fungal consortium.

1. Introduction

Phosphorous (P) is the second most limiting nutrient after nitrogen for plant growth (Wang et al., 2009). It is an important macronutrient required in many important metabolic processes such as photosynthesis, translocation of sugars, nucleic acid synthesis and energy generation (Shenoy and Kalagudi, 2005). Plants absorb P present in soils in the form of inorganic phosphorous (Pi). Most agricultural soils are deficient in soluble Pi since it readily binds to calcium (Ca), magnesium (Mg), aluminum (Al) or iron (Fe) ions, forming complexes that are not easily absorbed by plants (Hinsinger, 2001). Chemical fertilizers are commonly used to restore the adequate levels of Pi required by plants. However, the injudicious use of fertilizers over the last decade has led to negative impacts on the environment. Therefore, the need for sustainable agricultural practices has shifted the focus to exploring more environment friendly approaches to combat the problem of crop production in P-deprived soils. One of the alternatives to chemical fertilization is the use of biological agents, such as microorganisms (bacteria and fungi).

Arbuscular mycorrhizal fungi (AMF) are obligate biotrophs that improve the uptake of nutrients such as phosphorous and water in exchange for plant photosynthetic carbon (Smith and Read, 2009; Karasawa et al., 2012). The hyphae of AMF can extend beyond the zone from which plant roots can readily deplete P, thereby providing increased plant access to inorganic P (Smith and Smith, 2011). AMF extra-radical hyphae form an extensive network in the rhizosphere and not only act as hot spots for interactions with soil microbes, in particular mycorrhizospheric bacteria (Gahan and Schmalenberger, 2015), but can also influence the bacterial communities in the rhizosphere *via* exudation (Kaiser et al., 2015; Toljander et al., 2007, 2006; Wang et al., 2016). However, AMF can only take up soluble forms of P and transport it to plants, but the majority of P reserves in arable land is found in

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fixed insoluble forms. About 30–80% of P in agricultural land is present as organic P (Dalai, 1977) that cannot be accessed by plants. The most abundant form of organic P in the soil is phytate (Turner et al., 2002), which can be hydrolyzed by enzymes such as phytases. However, the transcriptome of the AMF *Glomus intraradices* (DAOM197198) indicates that this fungus may only have a low phytate-utilizing capacity since it lacks the phytase enzyme (Tisserant et al., 2013). Inorganically fixed forms of P cannot be solved in significant amounts by AMF (Joner and Johansson, 2000).

P turnover in the soil and its uptake by terrestrial plants is hugely influenced by phosphate solubilizing bacteria present in the rhizosphere. In a study by Jorquera and colleagues, phosphate solubilizing bacteria constituted > 40 % of cultivable bacteria in the rhizosphere (Jorquera et al., 2008), which were able to mobilize P from insoluble inorganic and organic P sources. These bacteria can increase soil available P by solubilizing inorganic phosphates through releasing enzymes such as phosphatases (Dobbelaere et al., 2003). Bacteria can also help to mineralize organic P reserves in the soil through the synthesis of enzymes such as phytases and phosphonoacetate hydrolases, which induce the hydrolysis of phosphoric esters (Rodríguez et al., 2006; Rodríguez and Fraga, 1999). Several studies have highlighted the close association of rhizospheric bacteria with AMF, where hyphae and spore surfaces were colonized by such bacteria (Scheublin et al., 2010; Toljander et al., 2006). In the rhizosphere, these two groups of microorganisms interact positively and it was recently shown that AMF hyphae promoted the growth of phytase producing bacteria by releasing carbon rich compounds, which in turn increased the soil P turnover and its acquisition by plants (Zhang et al., 2016, 2014). Bacteria associated with the spores of AMF were also shown to improve the P uptake of plants through promoting the development of fungal extra radical mycelium (Battini et al., 2017).

AMF can influence the bacterial communities *via* mycelial exudation (Toljander et al., 2007) and different bacteria show differential attachment to the fungal hyphae depending on the fungal species (Toljander et al., 2006), suggesting a regulated interaction between the organisms. It has also been shown that hyphospheric bacteria isolated from the AMF *R. irregulare* showed better P solubilization *in vitro* alone or in combination with the fungus (Taktek et al., 2015). Therefore, it is tempting to speculate that the combination of AMF and phosphate solubilizing bacteria (PSB) acts as a unit forming a channel between P reserves in the soil and their uptake by plants.

Previous studies have shown promising effects of dual inoculation with AMF and PSB on promoting plant growth and increasing mycorrhizal colonization rates (Singh and Kapoor, 1998). However, the bacteria were isolated from the rhizosphere and artificially combined with AMF, and were not a result of natural associations between AMF and PSB. Baiting for bacteria found naturally associated with AMF could lead to more stable synergism and have an advantage over the artificially combined microbes. We therefore hypothesize that bacteria isolated from AMF hyphae are highly efficient in solubilizing P from organic and inorganic sources and their interaction with AMF leads to improved P uptake and growth of plants.

In the present study we isolated phosphate solubilizing bacteria that are naturally associated and strongly attached to the hyphae of *Rhizoglomus irregulare*, characterized these bacteria, and studied their interaction with the fungus *in vitro*. According to our hypotheses, we particularly tested whether hyphae attached bacteria interact positively with AMF and whether co-inoculation of AMF and PSBs synergistically improves P uptake and increase biomass of the plants.

2. Materials and methods

2.1. Soil sampling

Phosphate solubilizing bacteria were isolated from soil samples collected from an agricultural field in Schnega (Lower Saxony, Germany) (52°54′18.0″N 10°50′01.8″E) at a depth of 20 − 30 cm. The soil samples were sieved through a mesh (500 µm) to remove any root fragments and stored at 4 °C for one week until the start of the experiment. The soil properties are listed in the supplementary data (Table S1).

2.2. Isolation of phosphate solubilizing bacteria attached to AMF- hyphae

Each 500 mL pot had two compartments (a root and hyphal compartment), which were separated by a 30 µm nylon mesh (Sefar GmbH, Edling, Germany) through which AMF hyphae could pass but not the plant roots (Fig. S1). Pots were filled with a mixture of sand and vermiculite (1:1 v/v), which was homogenously mixed with 350 mg L^{-1} of commercial inoculum containing *Rhizoglomus irregulare* strain QS69 (INOQ GmbH, Schnega, Germany). Each pot received 50 mL (10 % of the substrate) of the soil collected from Schnega (see above). The highly colonized plant (*Allium ampeloprasum*) was used as host. The plants were fertilized with Nutricote mini type (NPK 12 − 12-12) (Aglukon GmbH, Dusseldorf, Germany) solution at 0.8 g L^{-1} (mixed thoroughly in the substrate) and were watered with 150 mL water daily. The plants were grown in the greenhouse for 12 weeks under diurnal temperature of 22 °C during the day (14 h) and 18 °C during the night (10 h). At harvest (after 12 weeks), the hyphal compartments were collected and the fungal mycelium was harvested as follows: the dry hyphal soil (approximately 30 mL) was sieved through meshes sieves sized from 750 µm down to 50 µm. The sand particles were separated and the contents from the last sieve (50 µm) were mixed with 5 mL of sterile distilled water. The contents were inspected under a stereomicroscope for the presence of fungal hyphae, which were picked aseptically using sterilized forceps. The hyphae were washed twice in PBS buffer by centrifuging at 489 g, for 5 min to remove any loosely attached bacteria. Isolation of bacteria closely attached to hyphae was performed on National Botanical Research Institute's Phosphate (NBRIP) agar medium containing 5 g L^{-1} $MgCl_2.6H_2O$, 0.25 g L^{-1} $MgSO_4.H_2O$, 0.2 g L^{-1} KCl, 0.1 g L^{-1} $(NH_4)_2$ SO_4, 15 g L^{-1} Bacto Agar, 0.5 g L^{-1} yeast extract, 10 g L^{-1} glucose, 2.5 g L^{-1} tri calcium phosphate at pH 7 (Nautiyal, 1999). The plates were incubated at 28 °C for 2–3 days and colonies forming a clear halo, indicating phosphate solubilization were selected and sub cultured. Bacterial colonies showing stable colony morphology were recovered and stored in 50 % glycerol at − 80 °C.

2.3. Identification of bacteria

The bacteria showing the most pronounced phosphate solubilizing activity (indicated by the size of the solubilization halo) were selected for further analysis. After isolating the DNA, bacteria were identified *via* sequencing of the 16S rRNA gene. DNA was extracted from the bacterial colony using the DNeasy ultraclean microbial DNA isolation kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The universal bacterial primer pair 27f (5′ GAGAGTTTGATCCTGGCT CAG 3′) and 1492 r (5′ CTACGGCTACCTTGTTACGA 3′) (Lane, 1991) was used to amplify the gene coding for the 16S rRNA by a standard polymerase chain reaction (PCR). The PCR mix consisted of master mix reaction buffer (MyTaq™, Bioline, Luckenwalde, Germany), 0.25 mM of each primer and 0.2 U Taq polymerase (MyTaq™, Bioline, Luckenwalde, Germany). The following PCR conditions were used, 94 °C for 1 min, followed by 35 cycles of 95 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min followed by a final extension at 72 °C for 10 min. PCR products were examined by standard (1.5 % w/v) agarose electrophoresis, purified using QIAquick PCR Purification Kit (Qiagen) and sequenced *via* Sanger sequencing (Eurofins Genomics, Cologne, Germany). The BlastN program and gene bank database were used to compare the nucleotide sequences and the closest match on the basis of nucleotide identity was used to designate the taxonomic position of the isolated strains. Sequence data are available in NCBI database and were submitted to GenBank under the accession numbers MN944901– MN944912.

2.4. Characterization of the bacteria

2.4.1. Quantitative mineral phosphate solubilization assay

The selected PSBs were cultivated overnight in 10 % Tryptic Soy Broth (TSB; Sigma Aldrich, Munich, Germany) at 24 °C. To prepare a PSB inoculum, cells were collected and washed twice in sterile saline (NaCl, 0.1 %) by centrifugation for 5 min at 10,000 g and re-suspended in saline to a final concentration of 10^6 CFU mL^{-1}. Liquid NBRIP medium modified by the addition of tri calcium phosphate (5 g L^{-1}) received 1 mL of inoculum. Flasks containing 100 mL of inoculated media were incubated for 6 days on a rotary shaker (120 rpm, 24 °C). Uninoculated flask was used as a control. At different time intervals (0, 2, 4 and 6 days), 2 mL aliquots were aseptically sampled and used to estimate the pH and soluble phosphate using the method of Fiske and Subbarow (1925).

2.4.2. Phytate mineralization

The ability of the isolated bacteria to solubilize phytate was assayed using NBRIP medium containing 5 g L^{-1} $MgCl_2.6H_2O$, 0.25 g L^{-1} $MgSO_4.H_2O$, 0.2 g L^{-1} KCl, 0.1 g L^{-1} $(NH_4)_2$ SO_4, 15 g L^{-1} Bacto Agar, 0.5 g L^{-1} yeast extract, 10 g L^{-1} glucose, supplemented with 5 g L^{-1} of sodium phytate (Na-phytate, Sigma Aldrich) at pH 7 (Nautiyal, 1999). The bacterial strains were spot inoculated at the center of the Petri plate and incubated at 24 °C for 7 days. The formation of halo zones around the bacterial colonies indicated phytate solubilization capacity of the strains. The phytate solubilization efficiency of the isolates was calculated according to the following formula: SE = [Halo zone/diameter of colonies] × 100 (Rokhbakhsh-Zamin et al., 2011).

2.5. Motility

Swimming and swarming abilities of the isolated bacteria were evaluated on water agar plates containing 10 % TSB supplemented with 0.3 % or 0.5 % agar. In the first step all the PSBs were grown in 10 % TSB overnight at 24 °C under gentle shaking at 120 rpm. The bacterial cultures were washed twice in PBS solution by centrifugation at 1012 g for 10 min. The cell density was adjusted to 3×10^8 cells. Ten µl of each strain was spot inoculated onto the plates and incubated for 24 and 48 h at 24 °C before measuring the diameter of motility (in mm). The experiment was performed in triplicates.

2.6. Siderophore production assay

Siderophore production by the bacterial isolates was evaluated using the universal assay of Schwyn and Neilands using CAS blue Petri plates as described by Ji et al. (2014). Bacteria were grown overnight in LB media at 24 °C under gentle shaking at 120 rpm. The bacterial cells were washed twice in PBS and the OD was adjusted to 0.5 at 620 nm. Twenty µl of bacterial suspension was inoculated onto the center of the plate and incubated at 24 °C for 72 h. The change in color of CAS-blue to orange indicates the presence of a siderophore due to the chelation of bound iron.

2.7. Indole acetic acid production

Indole acetic acid (IAA) production was investigated using Luria Bertani broth (Sigma Aldrich). Fifty µl of overnight culture of the bacterial strains washed twice with 0.1 % NaCl were inoculated in 50 mL flasks containing 25 mL of LB supplemented with 1 mg mL^{-1} of L-tryptophan (Sigma Aldrich) and, incubated at 24 °C with continuous shaking (120 rpm) for 72 h. The bacterial cultures were centrifuged at 2024 g for 10 min. After centrifugation, 1 mL of supernatant was mixed with 4 mL of Salkowski reagent (1.2 % $FeCl_3$ in 37 % sulfuric acid) and incubated in the dark for 45 min. Absorbance of the resultant pink color was read at 535 nm in a spectrophotometer. The non-inoculated medium was used as a negative control, while the medium amended with pure IAA (Sigma Aldrich) was used as a positive control.

2.8. In vitro fungal hyphae colonization by the bacteria and its effect on fungal growth

Three PSBs (PSB1, PSB11 and PSB18) were tested for their effect on the AMF and their ability to colonize the AMF hyphae *in vitro*. For this purpose, two compartment Petri plates (90 × 15 mm; Greiner Bio-One GmbH, Baden-Württemberg, Germany) were used. Each treatment consisted of three replicates including the control without bacterial treatment, resulting in 12 root organ cultures (Fig. S2). Transformed carrot roots were used to propagate *R. irregulare in vitro* (St-Arnaud et al., 1996). The root compartment of the system contained M medium with the following composition: 80 mg L^{-1} KNO_3, 731 mg L^{-1} $MgSO_4.7H_2O$, 65 mg L^{-1} KCl, 4.8 mg L^{-1} KH_2PO_4, 288 mg L^{-1} Ca $(NO_3)_2.4$ H_2O, 8 mg L^{-1} NaFeEDTA, 0.75 mg L^{-1} KI, 6 mg L^{-1} $MnCl_2.4H_2O$, 2.65 mg L^{-1} $ZnSO_4.7H_2O$, 1.5 mg L^{-1} H_2BO_3, 0.13 mg L^{-1} $CuSO_4.5H_2O$, 0.0024 mg L^{-1} $Na_2MoO_4.2H_2O$, 3 mg L^{-1} glycine, 0.1 mg L^{-1} thiamine hydrochloride, 0.1 mg L^{-1} pyridoxine hydrochloride, 0.5 mg L^{-1} nicotinic acid, 50 mg L^{-1} myo-inositol, and 10 g L^{-1} sucrose. The distal compartment, where only AMF were allowed to grow, contained a medium with following composition: 80 mg L^{-1} KNO_3, 731 mg L^{-1} $MgSO_4.7H_2O$, 65 mg L^{-1} KCl, 4.8 mg L^{-1} KH_2PO_4, 288 mg L^{-1} Ca $(NO_3)_2.4$ H_2O, 8 mg L^{-1} NaFeEDTA, 0.75 mg L^{-1} KI, 6 mg L^{-1} $MnCl_2.4H_2O$, 2.65 mg L^{-1} $ZnSO_4.7H_2O$, 1.5 mg L^{-1} H_2BO_3, 0.13 mg L^{-1} $CuSO_4.5H_2O$, 0.0024 mg L^{-1} $Na_2MoO_4.2H_2O$. The pH of each compartment was adjusted to 5.5, and 0.4 % phytagel (Sigma Aldrich, Germany) was added as the solidifying agent before sterilization at 121 °C for 20 min. The medium was poured into two-compartment petri dishes in which the roots and AMF were allowed to grow for 90 days, after which the distal compartment was completely colonized by the AMF. To study the interaction between PSBs and AMF hyphae, four equidistant holes were made approximately 4 mm from the plastic barrier using a sterile pipette (Fig. S3) and each hole received 10 µL of PSB suspension and 10 µL of liquid M medium without sucrose or phosphorous. The control treatments received 10 µL of saline (NaCl, 0.1 %). The holes were left to be colonized by AMF, thereby allowing colonization of fungal hyphae by PSBs during the time of incubation. To prepare the bacterial suspension, the PSBs were grown overnight in 10 % TSB on a shaker at 1012 g, washed twice in saline (NaCl, 0.1 %), and the O.D of the culture solution was adjusted to 0.5 (10^8 cells per ml). The set up was incubated at 24 °C for 4 weeks. The plates were inspected weekly and the roots were trimmed so that they did not grow into the hyphal compartment. After 4 weeks, the length of extraradical hyphae, number of spores and colonization by the bacteria were measured (Ordoñez et al., 2016). Hyphal colonization by the bacteria was observed using a stereo microscope (Leica SZX 10, Wetzlar, Germany) at 10x magnification (Fig. 2) after 4 weeks of incubation with the fungus in the two-compartment petri dish.

2.9. Assessment of co-inoculation on plants

2.9.1. Biological materials and soil

Solanum lycopersicum (cultivar "Moneymaker") was used as host plant, and the AMF fungal strain *R. irregulare* plus each of the three bacterial strains PSB1, PSB11 and PSB18 were used for inoculation. A mixture of sand and vermiculite (2:1 v/v) was used as the substrate. The substrate was sterilized by autoclaving at 121 °C for 15 min twice before the start of experiment.

2.9.2. Experimental design

The experiment was designed to compare the effect between single and co-inoculations with PSB and AMF on *S. lycopersicum*. A total of eight treatments were tested. (1) No inoculation (control); (2) mono inoculation with *R. irregulare*; (3–5) mono inoculation with PSB1, PSB11or PSB18; (6–8) co-inoculation with *R. irregulare* and PSB1,

Table 1
Molecular identification of AM hyphae attached P solubilizing bacteria by 16S rRNA gene sequencing.

Isolate name	Accession number of 16S rRNA gene sequenced submitted to GenBank	Closest match based on the nucleotide identity	GenBank accession Number of closest match	% Gene identity
PSB1	MN944901	*Pseudomonas fluorescens F113*	NC_016830.1	99%
PSB2	MN944902	*Pseudomonas putida KT2440*	NC_002947.4	99%
PSB3	MN944903	*Pseudomonas fuscovaginae ICMP 5940*	NZ_BATG01000120.1	99%
PSB4	MN944904	*Pseudomonas putida KT2440*	NC_002947.4	99%
PSB8	MN944905	*Pseudomonas koreensis strain D26*	NZ_CP014947.1	99%
PSB10	MN944906	*Ochrobactrum anthropi ATCC* 49,188	NC_009668.1,	98%
PSB11	MN944907	*Pseudomonas koreensis strain D26*	NZ_CP014947.1	99%
PSB17	MN944908	*Ochrobactrum anthropi ATCC* 49,188	NC_009668.1	99%
PSB18	MN944909	*Pseudomonas fluorescens F113*	NC_016830.1	99%
PSB21	MN944910	*Ochrobactrum anthropi ATCC* 49,188	NC_009668.1	99%
PSB21b	MN944911	*Ochrobactrum anthropi ATCC* 49,188	NC_009668.1	99%
PSB23	MN944912	*Pseudomonas fluorescens F113*	NC_016830.1	99%

PSB11 or PSB18. Each treatment had six replicates, so a total of 48 microcosms were set up in a randomized block design. The plants were grown in a climate chamber from February 2 to March 30 at 20 °C/18 °C, 14 h/10 h (day/night) cycles. The soil moisture was kept at 18–20 % (w/w, approximately 70 % water holding capacity) with deionized water by weighing the pot every 2 days during the experiment.

2.9.3. Plant growth conditions

Two compartment microcosms were used to ensure a hyphal compartment and a root compartment, separated by a 30 µm nylon mesh (Sefar GmbH), which allowed the growth of fungal hyphae into the hyphal compartment but restricted the entry of plant roots. *S. lycopersicum* seeds were surface sterilized by soaking in 70 % ethanol for 1 min, followed by treatment with 2.5 % NaOCl for four min. The seeds were thoroughly washed with sterile water and germinated on moist filter paper for three days before planting. The PSBs were cultured overnight in LB medium at 24 °C. The bacteria were centrifuged at 1012 g for 10 min, washed twice in saline (NaCl, 0.1 %) and cell density was adjusted to 10^8 CFUs ml^{-1}. The seeds were immersed in this bacterial suspension for 30 min with frequent agitation. For the fungal treatments, the root compartment was inoculated with 120 mL of *R. irregulare* inoculum (INOQ GmbH). For the non-mycorrhizal treatment the inoculum was filtered with deionized water and autoclaved for 15 min at 121 °C. The filtrate and the sterilized inoculum were added to the pots in equal amounts (120 mL). The plants were fertilized with nutrient solution (De Kreij et al., 1997): 10.32 mM N; 0.07 mM Pi; 0.9 mM Po; 5.5 mM K; 1.2 mM Mg; 1.65 mM S; 2.75 mM Ca; 0.02 mM Fe; pH 6.2; EC 1.6 mS) containing 10 % of standard inorganic phosphate (Pi) in the form of KH_2PO_4 and 90 % organic P (Po) in the form of phytate (Na-phytate, Sigma Aldrich).

2.9.4. Harvest and sample analysis

At harvest, the plants were divided into stems, leaves and roots to make fresh weight measurements. The roots were carefully washed to remove adherent soil particles, blotted and fresh weights were determined. Samples were taken to determine the AMF colonization in all the treatments and roots were stored in 70 % ethanol. The percentage AMF colonization was measured using trypan blue staining according to Trouvelot et al. (1986). The dry weights were determined after drying the plant samples in an oven at 60 °C for 24 h. The dried stems and leaves were then ground into a fine powder to determine the phosphorous (P) content: 250 mg of the dry material was oxygenized for 20 min with 5 mL HNO_3 (65 %) and 3 mL H_2O_2 (30 %). After 1 h of microwave decomposition and filtration (MN 615, Macherey-Nagel, Munich, Germany) P concentrations in filtrates were analyzed using an EPOS analyzer (ascorbic-acid method, EPOS 5060 − 55, Eppendorf, Hamburg, Germany). The rest of the hyphal soil was oven dried at 50 °C for 24 h and two 10 g subsamples were taken to harvest extra radical hyphae and estimate hyphal length (Elmholt and Kjøller, 1987).

2.10. Data analysis

Statistical analysis was performed using STATISTICA version 12 software (StatSoft: 2013). The data was analyzed by one way analysis of variance (ANOVA), using treatments as factors, followed by a Tukey test with a cut off significance at $P \leq 0.05$.

3. Results

3.1. Isolation and identification of bacteria

A total of 128 bacteria were isolated from the hyphae of the AMF *Rhizoglomus irregulare*. All 128 isolates were screened for P solubilizing activity on modified NBRIP media supplemented with either phytate or tri-calcium phosphate as the sole P source. The isolates that produced 30 − 50 mm halo on plates were further tested for their P solubilizing efficiency in liquid culture. About 10 % of the strains showed P solubilizing activities on Petri dishes and in liquid cultures and were selected for further analysis (12 strains). The bacteria showed a rod shaped morphology and Gram negative staining. All the isolates produced round shaped raised colonies with a smooth surface. The selected bacteria were molecularly characterized using 16S rRNA partial gene sequencing (Table 1). The 12 selected bacteria, belonging to the phyla α- and γ-Proteobacteria, were identified as *Ochrobactrum anthropi* (33 %), *Pseudomonas fluorescens* (25 %), *Pseudomonas putida* (17 %), *Pseudomonas koreensis* (17 %) and *Pseudomonas fuscovaginae* (8%).

3.2. Mineral phosphate solubilization

All the 12 bacteria tested were able to solubilize phosphate in liquid media (Fig. 1a). The soluble phosphate concentration in the medium supplemented with tri calcium phosphate ranged between 38 (PSB10) and 59 (PSB11) mg phosphate L^{-1} 6 days after inoculation. The phosphate solubilization was associated with a significant decrease in the pH (Fig. 1b). The pH of the medium dropped within the range from 7–3.5 for the 12 bacteria tested. The non-inoculated control showed no phosphate solubilization or decrease in pH activity.

3.3. Phytate mineralization

All the 12 bacteria tested showed phytate solubilization (Fig. 1c). PSB1 (126 %), PSB3 (136 %), PSB11 (142 %), PSB21 (132 %) and PSB8 (115 %) were the most efficient solubilizers followed by PSB4 (98 %), PSB21b (96 %), PSB23 (88 %), PSB17 (75 %), PSB2 (71 %) and PSB18 (68 %). The phytate solubilizing efficiency varied from 62 % (PSB 10) to 142 % (PSB11) after 3 days of incubation.

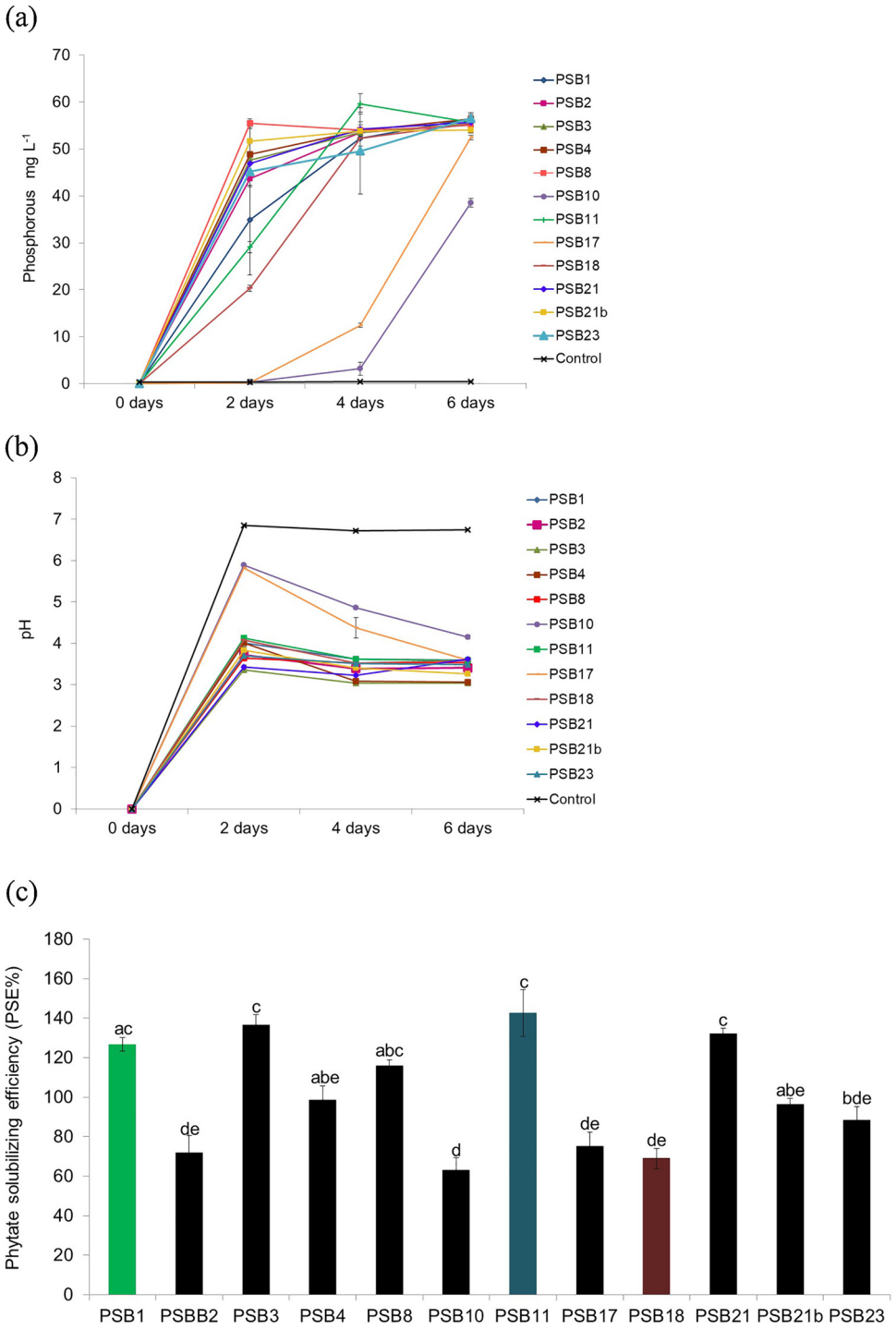

Fig. 1. (a) Phosphate solubilization activity of the PSBs in liquid culture when tri calcium phosphate was used as P source. (b) Variation in the pH of the supernatant of PSBs growing in NBRIP medium. (c) Phosphate solubilizing efficiency (%) of PSBs from phytate. The identities of PSBs are described in Table 2. Error bars indicate standard deviation of means of three replicates. Different letters above columns indicate significant differences between treatments according to one-way ANOVA followed by a Tukey test ($P < 0.05$).

3.4. Other characteristics of the PSBs

Besides phosphate solubilizing activities, a number of other characteristics were tested. All 12 PSB isolates showed different swimming and swarming patterns (Table 2). The swimming and swarming patterns were divided into slow swimmers which did not colonize the entire Petri dish in 48 h and fast swimmers which were able to colonize the entire Petri dish in 48 h or less. Interestingly 75 % of the isolates were slow swimmers whereas only 25 % comprised fast swimmers. All the 12 isolates were able to produce siderophores, as indicated by the formation of 8 − 14 mm orange/yellow circles around the colony margin. In the presence of tryptophan, all bacterial isolates produced indole acetic acid (IAA) *in vitro*. After 3 days of incubation, the IAA produced ranged between 1.49 mg L^{-1} (PSB3) and 24.16 mg L^{-1} (PSB 21b).

Table 2
Functional characterization of PSBs in terms of their swimming and swarming abilities, production of indole acetic acid (IAA) and siderophores *in vitro*. Different letters indicate significantly different values based on one-way ANOVA ($P < 0.05$) followed by a Tukey test. The error points represent standard deviations of the means of three replicates.

Isolate	Swimming (mm)	Swarming (mm)	Production of IAA and indole-related substances (μg/mL)	Siderophore production
PSB1	1.71 ± 0.05[a]	17.36 ± 0.19[a]	2.36 ± 0.04 k	+ +
PSB2	1.85 ± 0.05[a]	15 ± 0.05[a]	9.19 ± 0.01e	+ +
PSB3	1.96 ± 0.1[a]	14 ± 0.05[a]	1.49 ± 0.07 L	+ +
PSB4	1.81 ± 0.02[a]	14.16 ± 0.07[a]	6.30 ± 0.01 g	+ +
PSB8	1.75 ± 0.02[a]	13.83 ± 0.01[a]	3.37 ± 0.03 j	+ +
PSB10	4.13 ± 0.05[b]	54.16 ± 0.02[b]	17.78 ± 0.01 c	+ +
PSB11	1.88 ± 0.05[a]	14.5 ± 0.14[a]	8.08 ± 0.06 f	+ +
PSB17	7.96 ± 0.15[a]	19.66 ± 0.05[a]	17.97 ± 0.03 b	+ +
PSB18	8.4 ± 0.02[b]	59.83 ± 0.2[b]	3.38 ± 0.01 i	+ +
PSB21	2.68 ± 0.02[a]	18 ± 0.6[a]	17.23 ± 0.06 d	+ +
PSB21b	1.71 ± 0.5[a]	16.5 ± 0.05[a]	24.16 ± 0.01 a	+ +
PSB23	8.4 ± 0.05[b]	38 ± 0.1[b]	4.24 ± 0.03 h	+ +
One way ANOVA (P values)	–	–	< **0.001**	–
One way ANOVA (F values)	–	–	99,700	–

[a] Slow swimming bacteria.
[b] Fast swimming bacteria.

3.5. *In vitro* fungal hyphae colonization by the selected PSBs and its effect on fungal growth

Three bacteria, PSB1, PSB11 and PSB18 were selected based on their functional characteristics to further study their interaction with the AMF. The selected bacteria were able to grow in the distal compartment in the presence of AMF and were able to colonize the fungal hyphae forming a stable biofilm (Fig. 2). Growth of the AMF as measured by the length of extra radical hyphae remained unchanged when the fungus was grown alone (control) or with any of the three PSBs (Fig. S3a), but spore production was significantly increased in the case of PSB11, whereas no change was observed with PSB1 and PSB18 (Fig. S3b). Colonization of the AMF hyphae by the three bacteria did not differ significantly as determined by measuring the length of bacterial colonization of the fungal hyphae (Fig. S3c).

3.6. *Impact of co-inoculation on root colonization and plant biomass*

The roots of the plants inoculated with fungus were well colonized and the presence of all three bacteria improved AMF colonization (Fig. 3a). Compared to the AMF only treatment, the highest increase of 67 % was seen when the plants were inoculated with a combination of AMF + PSB1. However co-inoculation with AMF + PSB11 and AMF + PSB18 increased the fungal colonization by 65 %. The length of extraradical hyphae was measured in all the treatments. The presence of the bacteria significantly increased the hyphal length compared to mycorrhizal plants without bacterial inoculation by 60 % (PSB1), 48 % (PSB11) and 40 % (PSB 18) (Fig. 3b).

Plant shoot dry weight increased significantly when plants were co-inoculated with AMF and PSBs compared to single inoculations (Fig. 4a). Compared to the AMF only treatment the co-inoculations significantly increased the shoot dry weight by 8% (AMF + PSB1), 15 % (AMF + PSB11) and 1 % (AMF + PSB18). The improved plant growth was also supported by an increase in P uptake by the plants, but in contrast to growth, no significant difference was detected between

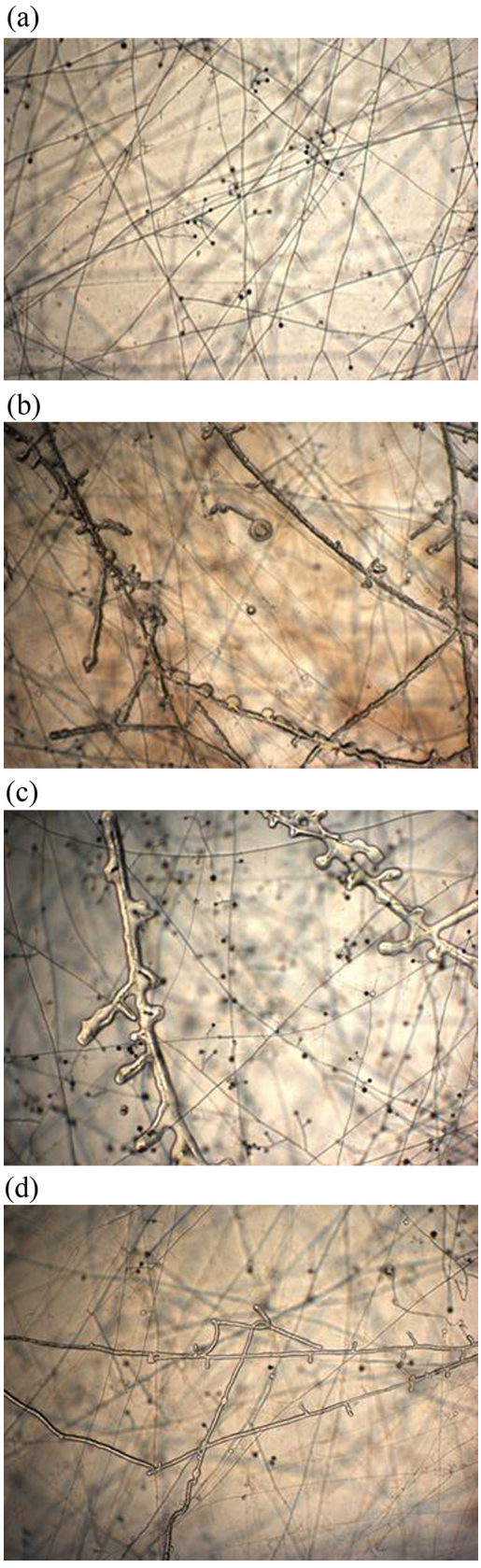

Fig. 2. Biofilm formation by 3 PSBs on the hyphae of the AM fungus *R. irregulare* in *in vitro* cultures. a) Hyphae of control treatment. b) AM hyphae colonized by PSB1. c) AM hyphae colonized by PSB11. d) AM hyphae colonized by PSB18.

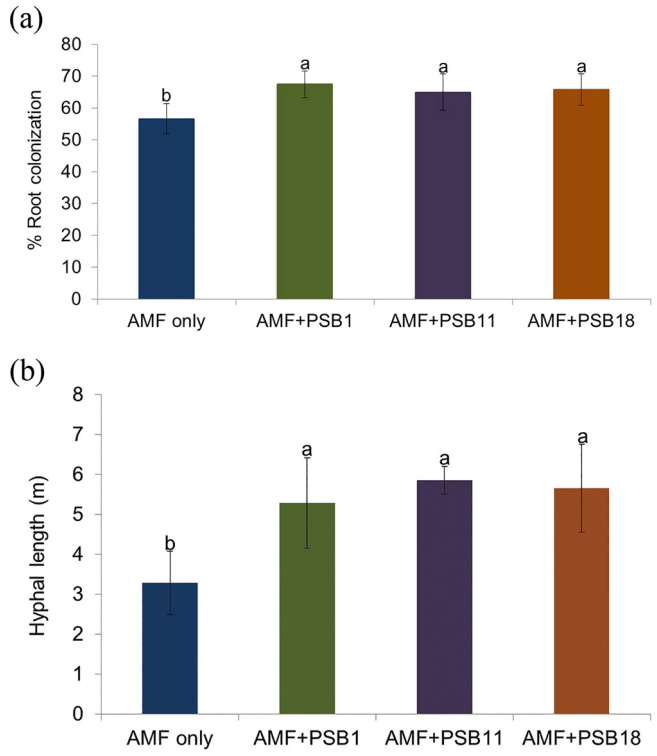

Fig. 3. (a) Mycorrhizal root colonization (%) in the tomato plants colonized by *R. irregulare* with or without PSB1, PSB11 and PSB18. (b) Length of the extra radical hyphae of the AM fungus when the plants were inoculated with fungus only or in combination with the bacteria. Different letters denote significant differences among treatments ($P < 0.005$) according to one-way ANOVA followed by a Tukey test. Error bars represent standard deviations of 3 replicates.

single and co-inoculations. Compared to the control, a significant increase was seen in the P uptake in the shoot under both single and co-inoculation of the microorganisms (Fig. 4b). The P uptake increased by 86 % (PSB1), 85 % (PSB11), and 63 % (AMF only), in case of single inoculations and by 73 % (AMF + PSB1) and 76 % (AMF + PSB11), in case of co-inoculations as compared to the control. There was also a significant increase in the number of flowers when the plants were co-inoculated with the bacterial-fungal consortium (Fig. S4). Compared to the AMF only treatment the co-inoculations significantly increased the number of flowers by 40 % (AMF + PSB1), 25 % (AMF + PSB11) and 30 % (AMF + PSB18).

3.7. Root growth and AMF abundance in roots and hyphal compartments

The root dry weight was significantly increased by co-inoculation (Fig. 4c). The dry weight of roots increased by 58 % (AMF + PSB1), 56 % (AMF + PSB11) and 53 % (AMF + PSB18) in the case of co-inoculation with the AM fungus and bacteria compared to AMF only treatments. The root systems were larger (Fig. S5), showed more lateral branching and root hair growth.

4. Discussion

The extraradical hyphae of AMF extend through the soil providing ample opportunity for interacting with phosphate solubilizing bacteria. The cooperation between AMF and PSB can be beneficial for both partners: AMF hyphae act as important conduits of mycelial exudates that are rich in carbon compounds, which can significantly influence the bacterial community composition (Kaiser et al., 2015; Toljander et al., 2006), while PSB can solubilize phosphorous from the sparingly soluble P reserves in the soil and provide it to the fungus (Zhang et al., 2014). It has long been speculated that these interactions can be beneficial for the plants (Calvo et al., 2014; Sharma et al., 2013). Therefore recently, the focus has shifted to understanding these beneficial interactions and exploiting them to their full potential. Synergistic interactions between AMF and plant growth-promoting rhizobacteria including phosphate solubilizers has been shown before, but our study makes an further step and lays the foundation for successful field application of fungus-bacterium consortia since this requires a stable, natural microbial association

To test the effects of AMF and PSB synergistic interactions it is important that the two groups of microorganisms interact positively with each other, or at least should not be antagonistic. In most previous studies AMF was combined with bacteria isolated from the rhizosphere. However, here we baited bacteria and isolated them when they were strictly attached to the hyphae of AMF.

We isolated hyphae attached P solubilizing bacteria by adapting techniques developed by Taktek (2015). We established a two compartment petri dish system that lead to easy harvesting of the fungal hyphae. The bacteria were isolated after careful washing, making sure that only the strongly attached bacteria were selected. This bacterial fungal interaction could be termed more “natural” as opposed to the conventional approach of “artificially” combining these two microorganisms. This “natural” approach selected the bacteria whose growth is supported by the fungus under normal environmental conditions. These “natural” bacteria-fungal consortia can have better stability and synergism as opposed to the artificial combinations of these microorganisms.

All our selected 12 PSBs that showed P solubilization activity were Gram negative, belonging to the phylum Proteobacteria as also observed by Taktek (2015). The bacteria isolated by Taktek belonged to *Burkholderia* genus. However, 65 % of the isolated bacteria in our study belonged to the genus *Pseudomonas* and were identified as *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas koreensis* and *Pseudomonas fuscovaginae*. The remaining 35 % of isolated bacteria belonged to the genus *Ochrobactrum* and were identified as *Ochrobactrum anthropi*. In previous studies it was also reported that *Pseudomonas* was one of the most predominant genera associated with AMF (Bonfante and Anca, 2009). Bacteria belonging to the genus *Pseudomonas* have been shown to be excellent P solubilizers that interact positively with AMF (Ordoñez et al., 2016; Zhang et al., 2014). Many studies have demonstrated that *P. fluorescens* and *P. putida* can act as a mycorrhizal helper bacteria and significantly improve the mycorrhizal colonization under different conditions (Meyer and Linderman, 1986; Gryndler and Vosátka, 1996; Ghorchiani and Etesami, 2018; Shinde et al., 2019). Recently *Pseudomonas koreensis* S2CB35 was isolated from the spore of Funneliformis mosseae (Selvakumar et al., 2017). Co-inoculation of P. koreensis with AMF belonging to *Gigasporaceae* family, significantly increased the mycorrhizal colonization and improved the growth of maize plants (Selvakumar et al., 2018).

The three PSBs chosen for closer inspection (PSB1, PSB11 and PSB 18) had no negative effect on the length of extra radical hyphae of AMF *R. irregulare* compared to the control treatment. These PSBs also did not affect the number of spores *in vitro* except PSB11, which significantly increased the spore numbers. The AMF chosen for this study was also colonized by a bacterial biofilm, which suggests that these interactions might be mutualistic. The growth of the bacteria along the fungal hyphae was not dependent on the bacterial strain since all three strains tested in this study showed similar fungal colonization behavior. The bacteria isolated in this study were able to colonize AMF hyphae *in vitro* and formed stable biofilms on the fungal hyphae which is in line with previous findings about the bacterial colonization of arbuscular mycorrhizal hyphae (Scheublin et al., 2010; Toljander et al., 2006). Our results concur with the previous finding that bacteria belonging to the Proteobacteria phylum show stable attachment to the fungal hyphae from which they were isolated (Frey-Klett et al., 2007).

Co-inoculation of *R. irregulare* and the three PSBs significantly

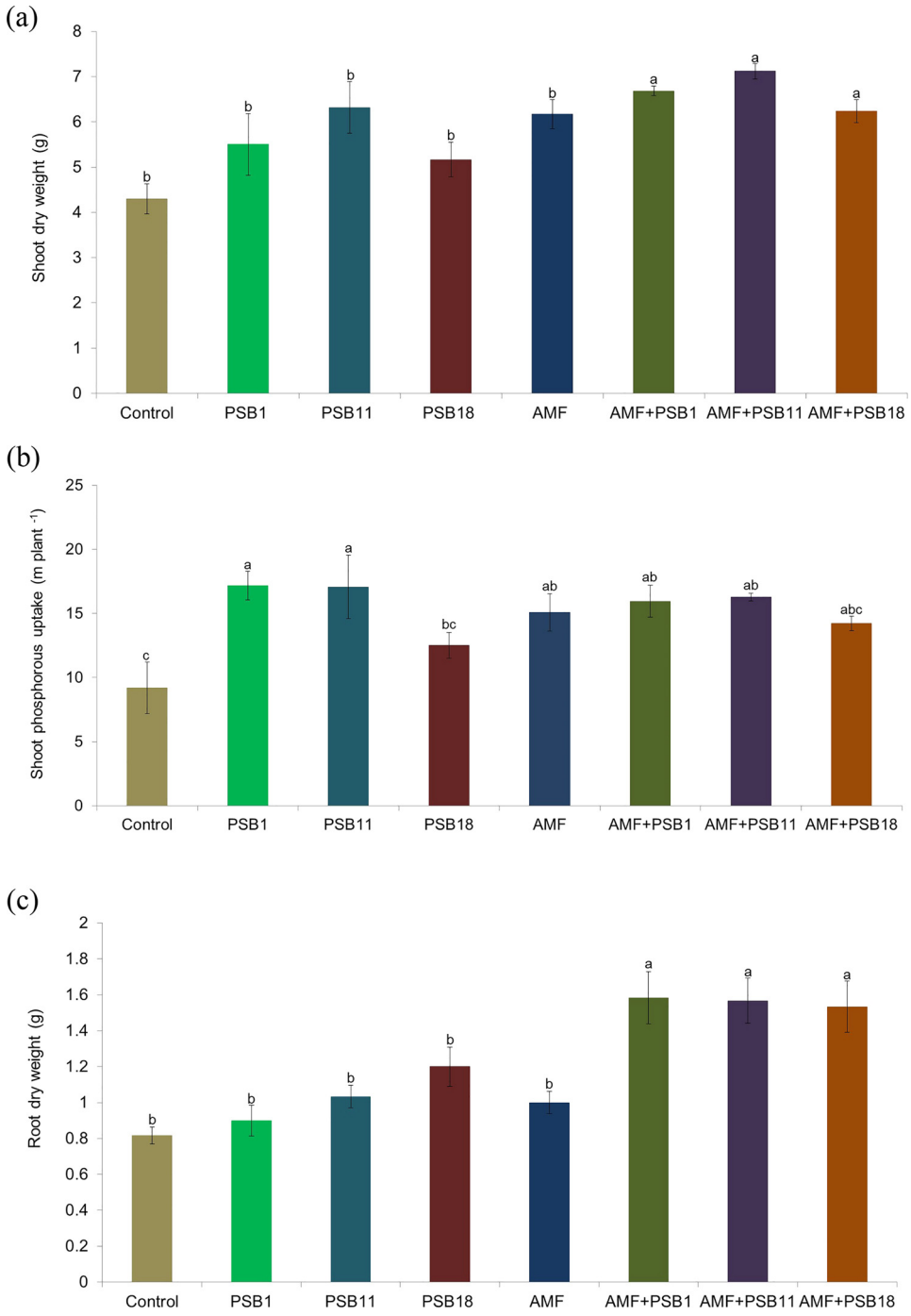

Fig. 4. Effect of co-inoculation on the (a) shoot dry weight, (b) shoot P uptake and (c) root dry weight. Different letters denote significant differences among treatments ($P < 0.005$) according to one way ANOVA followed by a Tukey test. Error bars represent standard deviation of 6 replicates.

improved the root and shoot dry weights of the tomato plants compared to the single inoculations or no inoculation control. The significant increase in the root biomass of the co-inoculated plants was particularly interesting since the dual inoculation increased the dry weight of roots by approximately 50–58 %. All three PSBs were able to produce phytohormone auxin such as indole-3-acetic acid (IAA) *in vitro*. IAA has been shown to enhance the production of root hairs and lateral roots, which are directly involved in nutrient uptake (Patten and Glick, 2002). Although certain microbial isolates can produce IAA independently of tryptophan, the majority of rhizobacteria require tryptophan, an amino acid found in root exudates, which functions as a precursor for IAA biosynthesis (Patten and Glick, 1996). Bacteria belonging to the genus *Pseudomonas* are efficient IAA producers (Ahemad and Khan, 2012; Dey et al., 2004; Tank and Saraf, 2009). The plant growth promoting effect of the tested bacteria PSB1, PSB11 and PSB18 in combination with AMF may be attributed to increased mineral uptake due to an increase in lateral roots and root hairs induced by the biosynthesis of auxin by these bacteria (Kapulnik et al., 1985).

All three PSBs increased the mycorrhizal colonization of *R. irregulare* in tomato roots, suggesting they behave as mycorrhiza helper bacteria (Singh and Kapoor, 1998; Toro et al., 1997). However, the improved mycorrhization in the co-inoculation treatments did not lead to a subsequent increase in P uptake by the plants compared to only AMF treatment. In fact plants inoculated with PSBs alone already showed a

significant increase in P uptake except with PSB18. Thus although P uptake was significantly increased compared to control plants when plants were co-inoculated with a AMF and PSB, it was not significantly higher than in the plants inoculated with PSB alone, indicating that AMF may compete with the plant for the available P. However the increase in the plant biomass under co-inoculation treatments could be attributed to other synergistic activities of the bacterial-fungal consortia. In order to exploit the full potential of the co-inoculations it is important to pay attention to the bacterial communities found attached with the fungal hyphae and characterize them on the basis of their functions. Different fungi may harbor different bacterial communities. These bacteria should be tested for their plant growth promoting traits such as nitrogen fixation, phosphate solubilization and IAA production.

In our experimental set up, although the microbial inoculations improved the plant phosphorous uptake, no synergistic effect of the bacterial-fungal consortium on P uptake by the plants was apparent, suggesting that the bacterial-fungal consortium improved the growth of the plants *via* other mechanisms. Bacterial-fungal biofilms can harbor different functional properties compared to their monoculture counterparts (Bandara et al., 2006; Seneviratne et al., 2009). In a study by Hettiarachchi et al. (2016), the authors concluded that bacterial-fungal biofilms were more effective in their biological performances such as IAA production, nitrogen fixation and phosphorous solubilization, than their monocultures. In another study, the nitrogenase activity of the bacterial-fungal biofilms was found to be significantly higher than that of their monocultures (Seneviratne et al., 2008; Seneviratne and Jayasinghearachchi, 2005). Under natural conditions, bacteria and fungi coexist in the rhizosphere and it is likely that such close associations between these two groups of microorganisms would be more common than the presence of their single counterparts. Therefore, when testing the effect of beneficial bacteria on plants, attention should be paid to their interaction with common soil fungi such as AMF. Since all three PSB's were able to produce IAA *in vitro*, it could be speculated that the root biomass was increased under co-inoculation because of increased IAA production in bacterial-fungal biofilms compared to the planktonic lifestyle. Further investigations are required to better understand the functional properties of AMF-PSB biofilms in order to fully elucidate the reasons for the increase in plant biomass due to the synergistic effect of the AMF-PSB inoculations observed in this study.

Collectively, our findings demonstrate that combining naturally co-occurring PSB and AMF can lead to increased plant growth. Following this approach, more compatible AMF-PSB consortia could be found that supports sustainable agricultural practices, more than single inoculations and co-inoculations or randomly selected plant growth-promoting microorganisms. In our study we used only one AMF species to bait the naturally associated phosphate solubilizing bacteria associated with the fungal hyphae. It is likely that different AMF species harbor different selections of bacteria depending on the mutual benefits provided by each partner. Further work is needed to screen for the bacterial communities found associated with different genera of AMF in order to understand whether a preference exists among the fungi for certain taxa of bacteria and *vice-versa*. This information could lead to the development of more stable, and AMF-specific AMF-PSB consortiums to be tested in the future. It is also important to understand the biological activities of such AMF-PSB biofilms, and more work should be done to elucidate these mechanisms. Such bacterial-fungal biofilms may have better chances of survival under field conditions, and could therefore improve the efficiency of microbial inoculants in the fields.

Author contribution statement

SS carried out the experiments and wrote the manuscript. SS, PF, MB, SR and SC designed the experiments. All the authors gave intellectual input and critically revised the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2020.126556.

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