Multifunctionality and diversity of culturable bacterial communities strictly associated with spores of the plant beneficial symbiont *Rhizophagus intraradices*

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RUNNING HEAD: Multifunctional culturable bacteria from AMF spores

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ABSTRACT

Arbuscular Mycorrhizal Fungi (AMF) live in symbiosis with most crop plants and represent essential elements of soil fertility and plant nutrition and productivity, facilitating soil mineral nutrient uptake and protecting plants from biotic and abiotic stresses. These beneficial services may be mediated by the dense and active spore-associated bacterial communities, which sustain diverse functions, such as the promotion of mycorrhizal activity, biological control of soilborne diseases, nitrogen fixation, and the supply of nutrients and growth factors. In this work, we utilised culture-dependent methods to isolate and functionally characterize the microbiota strictly associated to *Rhizophagus intraradices* spores, and molecularly identified the strains with best potential plant growth promoting (PGP) activities by 16S rDNA sequence analysis. We isolated in pure culture 374 bacterial strains belonging to different functional groups - actinobacteria, spore-forming, chitinolytic and N_2-fixing bacteria - and screened 122 strains for their potential PGP activities. The

most common PGP trait was represented by P solubilization from phytate (69.7%), followed by siderophore production (65.6%), mineral P solubilization (49.2%) and IAA production (42.6%). About 76% of actinobacteria and 65% of chitinolytic bacteria displayed multiple PGP activities. Nineteen strains with best potential PGP activities, assigned to *Sinorhizobium meliloti*, *Streptomyces* spp., *Arthrobacter phenanthrenivorans*, *Nocardiodes albus*, *Bacillus* sp. *pumilus* group, *Fictibacillus barbaricus* and *Lysinibacillus fusiformis*, showed the ability to produce IAA and siderophores and to solubilize P from mineral phosphate and phytate, representing suitable candidates as biocontrol agents, biofertilisers and bioenhancers, in the perspective of targeted management of beneficial symbionts and their associated bacteria in sustainable food production systems.

Keywords: Plant growth promoting bacteria; AMF spores; culturable spore-associated bacteria; *Rhizophagus intraradices*

INTRODUCTION

The symbiosis between plants and Arbuscular Mycorrhizal (AM) Fungi (AMF, Glomeromycota) is the most widespread on Earth. AMF establish beneficial associations with the roots of the large majority of land plants, including the most important food crops, from cereals to legumes, vegetables and fruit trees (Smith and Read 2008), and contribute to key agroecosystem processes, such as nutrient uptake, soil aggregation and carbon sequestration (Gianinazzi et al. 2010). AM symbionts represent essential elements of soil fertility and plant nutrition and productivity, facilitating soil mineral nutrient uptake - mainly phosphorus (P), nitrogen (N), sulfur (S), potassium (K), calcium (Ca), copper (Cu) and zinc (Zn) - by means of an extensive extraradical network of fungal hyphae spreading from colonized roots into the soil (Giovannetti and Avio 2002). In addition, AMF protect plants from soilborne fungal pathogens and abiotic stresses, such as drought and salinity (Augé 2001; Evelin et al. 2009; Sikes et al. 2009) and affect the synthesis of beneficial

53 phytochemicals, contributing to the sustainable production of high-quality food (Giovannetti et al.
54 2012).
55 Such beneficial services may be affected by diverse factors, including agronomic practices (Njeru et
56 al. 2014) and further mediated by a third component of the symbiosis, the mycorrhizospheric
57 microbiota, represented by the dense and active bacterial communities living tightly associated with
58 AMF (Rambelli 1973). Mycorrhizospheric bacteria sustain diverse functions, driving plant growth
59 and health and nutrient acquisition, *i.e*. the promotion of mycorrhizal activity (Mayo et al. 1986;
60 Xavier and Germida 2003; Giovannetti et al. 2010; Hori and Ishii 2006), biological control of
61 soilborne diseases (Citernesi et al. 1996; Budi et al. 1999; Li et al. 2007; Bharadwaj et al. 2008a),
62 nitrogen fixation, and the supply of nutrients and growth factors (Barea et al. 2002; Xavier and
63 Germida 2003; Bharadwaj et al. 2008b).
64 A number of studies investigated spore-associated microbiota, as AMF spores have long been
65 known to harbor a wide diversity of bacterial species, living either intracellularly (Mosse 1970;
66 MacDonald and Chandler 1981; MacDonald et al. 1982; Bianciotto et al. 1996) or intimately
67 associated with the spore walls. Indeed, spore walls represent a privileged habitat where bacteria
68 can thrive on exudates and by hydrolyzing the relevant wall biopolymers, such as proteins and
69 chitin (Walley and Germida 1996; Filippi et al. 1998; Roesti et al. 2005) and survive before
70 colonizing the surface of germlings and extraradical hyphae (Lecomte et al. 2011). For this reason,
71 AMF spores are the preferential source of AMF-associated bacteria to be studied for their
72 prospective use as biocontrol agents, biofertilisers and bioenhancers, in order to develop strategies
73 able to minimize anthropogenic energy inputs and promote plant productivity and health, and soil
74 fertility. The isolation and functional characterization of Spore-Associated Bacteria (SAB) are of
75 key agronomical importance and represent a prerequisite for understanding entirely how the
76 complex network of microbial interactions in the mycorrhizosphere affects plant performance and
77 can be managed in sustainable plant production systems.

78 So far, a few works reported the isolation and characterization of SAB from a small number of
79 AMF. For example, ten bacterial species were recovered from *Glomus clarum* spores (Xavier and
80 Germida 2003), seven species from *Glomus irregulare* spores harvested from the field (Lecomte et
81 al. 2011), while a total of 36 species were obtained from *Glomus intraradices* and *Glomus mosseae*
82 spores extracted from the rhizosphere of *Festuca ovina* and *Leucanthemum vulgare* (Bharadwaj et
83 al. 2008a). Despite these studies, little information is currently available on the functional
84 significance of culturable bacterial communities associated with AMF spores, and on their possible
85 synergistic interactions. Some bacteria showed antagonistic activity against plant pathogens (Budi
86 et al. 1999; Bharadwaj et al. 2008b), phosphate-solubilizing and nitrogenase activity (Cruz et al.
87 2008; Cruz and Ishii 2011), and indole acetic acid production (Bharadwaj et al. 2008b), suggesting
88 their possible role as Plant Growth Promoting (PGP) bacteria.
89 Using a molecular approach, we recently reported the occurrence of specific and diverse microbial
90 communities tightly associated with spores of six different AMF isolates. In particular, sequencing
91 of DGGE bands lead to the identification of bacteria belonging to *Actinomycetales*, *Bacillales*,
92 *Rhizobiales*, *Pseudomonadales*, *Burkholderiales*, and *Mollicutes* related endobacteria (Mre).
93 Several strains belonged to species known to play important roles in promoting plant growth either
94 directly, by affecting nutrient availability - for example solubilizing phosphate and other nutrients
95 from insoluble sources, fixing nitrogen and producing phytohormones, mainly Indol Acetic Acid
96 (IAA) - or indirectly by protecting plants against pathogens through the production of siderophores,
97 antibiotics and extracellular hydrolytic enzymes.
98 As the different combinations of AMF and bacterial activities may result complementary and/or
99 synergistic and represent the basis of the differential symbiotic performance of AMF isolates, it is
100 crucial to gain knowledge on the functional significance of bacteria associated with AMF spores, in
101 order to exploit the potential of such multipartite association in sustainable food production
102 systems. In this work, we investigated the diversity and functional PGP potential of 122 culturable
103 bacterial strains strictly associated to spores of the AMF species *Rhizophagus intraradices*. To this

aim, we i) utilised culture-dependent methods to isolate the microbiota associated with *R. intraradices* spores, ii) functionally characterized bacterial strains in order to detect their beneficial role in plant growth and health, nitrogen fixation, P solubilization, siderophore and IAA production, iii) identified the strains with best potential PGP activities by 16S rDNA sequence analysis.

MATERIALS AND METHODS

Fungal material

The AM fungus used was *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & Schuessler isolate IMA6, obtained from pot-cultures maintained in the collection of Microbiology Labs (International Microbial Archives, IMA) of the Department of Agriculture, Food and Environment, University of Pisa, Italy. The isolate was maintained in sterilized calcinated clay (OILDRI, Chicago, IL, USA) and field soil (1:1, v/v) pot cultures, using *Trifolium alexandrinum* L. and *Medicago sativa* L. as host plants.

Spore collection and bacterial isolation

R. intraradices spores were extracted from three pot cultures using the wet-sieving and decanting technique, down to a mesh size of 50 μm (Gerdermann and Nicolson 1963). Spores retained on sieves were flushed into Petri dishes and manually collected with a capillary pipette under a dissecting microscope (Leica MS5, Milan, Italy). For each pot 200 intact and healthy spores were suspended in 1 mL sterile physiological solution (9 g L^{-1} NaCl) in a 1.5 mL Eppendorf tube and vigorously washed using a vortex mixer at 1500 rpm for 1 min. Spores were aseptically washed 15 times in succession, then crushed aseptically with a pestle and suspended in 3.5 mL of sterile physiological solution. Spores were not washed further, as previous experiments showed that 15 washings were effective in spore surface decontamination (Agnolucci et al. 2015).

An aliquot (1 mL) of spore suspension was heat-treated for spore forming bacteria isolation (80 °C for 10 min). 100 μL suspension for each sample were plated in triplicate onto different culture

130 media. Culturable heterotrophic and spore-forming bacteria were isolated on TSA (30 g L^{-1} tryptic
131 soy broth, 20 g L^{-1} bacteriological agar, Oxoid, Milan, Italy), a non-selective medium which allows
132 the recovery of a wide range of aerobic and facultative anaerobic gram-negative and gram-positive
133 bacteria. The media were supplemented with 100 mg L^{-1} of cyclohexymide and 500 UI L^{-1} of
134 nystatin (Sigma-Aldrich, Milan, Italy) to inhibit possible fungal development.
135 Selective media were used to isolate specific functional bacterial groups, *i.e.* actinobacteria,
136 chitinolytic and nitrogen-fixing bacteria. Actinobacteria were isolated using Waksman's agar
137 medium (10 g L^{-1} dextrose, 5 g L^{-1} sodium chloride, 5 g L^{-1} bacteriological peptone, 3 g L^{-1} lab-
138 lemco powder, 20 g L^{-1} bacteriological agar; Oxoid, Milan, Italy*)* supplemented with 5 mg L^{-1} of
139 polymyxin (Sigma-Aldrich, Milan, Italy) and with 100 mg L^{-1} of cyclohexymide and 500 UI L^{-1} of
140 nystatin (Sigma-Aldrich, Milan, Italy) to inhibit the growth of gram-negative bacteria and fungi,
141 respectively. Chitinolytic bacteria were isolated after plating samples on minimal medium
142 containing chitin as sole carbon source (Souza et al. 2009), while bacteria able to grow on N-free
143 medium were isolated from Winogradsky culture agar (Tchan 1984). Both media were
144 supplemented with 100 mg L^{-1} of cyclohexymide and 500 UI L^{-1} of nystatin (Sigma-Aldrich, Milan,
145 Italy) to inhibit fungal growth.
146 The number of Colony Forming Units (CFU) was assessed after 2 and 7 days of incubation at 28 °C
147 for TSA and the other media, respectively. From each isolation medium, representative bacterial
148 strains were randomly selected on the basis of phenotypic colony characteristics, *i.e.* shape, size,
149 edge morphology, surface and pigment, in order to include the most diverse strains, and purified by
150 streaking four times onto the same medium used for isolation. Each strain was named on the basis
151 of the acronym of the isolation medium, followed by a progressive number. Spore-forming bacteria
152 isolated on TSA were named as TSAT. Purified strains were maintained at –80 °C in cryovials with
153 20% (v/v) of glycerol.
154
155 *Screening for PGP traits*

156 *IAA production*

157 The production of IAA was investigated using Luria-Bertani Broth (LBB, 10 g L^{-1} bacto-tryptone, 5
158 g L^{-1} yeast extract, 5 g L^{-1} sodium chloride, pH 7.5, Oxoid, Milan, Italy) (Bharadwaj et al. 2008b).
159 The strains were inoculated in 15 mL tubes containing 4 mL of LBB amended with 1 mg mL^{-1} of L-
160 tryptophan (Sigma-Aldrich, Milan, Italy), incubated at 20 °C with continuous shaking (200 rpm)
161 until they reached exponential growth phase and then centrifugated at 7500 rpm for 10 min. After
162 centrifugation, 1 mL of supernantant was transfered in a 24-well plate, mixed with 2 mL of
163 Salkowski reagent (1.2% $FeCl_3$ in 37% sulphuric acid) and incubated in the dark for 30 min. The
164 non-inoculated medium was used as negative control, while the medium amended with pure IAA
165 was used as positive control. Development of red-purple color indicated positive strains for IAA
166 production. Strains were classified using a rating scale as follows: - = no production (no color
167 development), +/- = low production (pale pink), + = production (light purple), ++ = moderate
168 production (bright purple), +++ = high production (dark purple).
169
170 *Siderophore production*
171 Siderophore-producing strains were detected using the overlay Chrome Azurol S assay (CAS)
172 described by Pérez-Miranda et al. (2007). CAS agar was prepared following the procedure provided
173 by Louden et al. (2011) using 72.9 mg L^{-1} hexadecyltrimetyl ammonium bromide (HDTMA), 30.24
174 g L^{-1} piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), 1 mM $FeCl_3$ $6H_2O$ in 10 mM HCl 10 mL
175 and 0.9 g L^{-1} bacteriological agar. Four isolates per plate were inoculated on TSA and incubated 2-7
176 days at 28 °C. After incubation, 10 mL of CAS agar were spread as an overlay on the
177 microorganisms and incubated at room temperature. Siderophore-producing strains showed a
178 change in color, from-blue to yellow or from blue to orange, in the overlaid medium around the
179 colonies. After 7 days the radius of the halo was measured (mm) from the colony edge to the edge
180 of the colored halo. Strains were classified using a rating scale as follows: no production (halo = 0

mm), +/- = low production (halo < 2 mm), + = production (2 mm≤ halo ≤ 8 mm), ++ = moderate production (8 mm < halo < 14 mm), +++ = high production (halo > 15 mm).

P solubilisation from mineral phosphate and phytate

The ability of isolated bacteria to solubilize inorganic and organic phosphate was assayed on National Botanical Research Institute's Phosphate growth medium (NBRIP, 10 g L^{-1} D-glucose, 5 g L^{-1} $Ca_3(PO_4)_2$, 5 g L^{-1} $MgCl_2$ $6H_2O$, 0.25 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.2 g L^{-1} KCl, 0.1 g L^{-1} $(NH_4)_2$ SO_4, 15 g L^{-1} agar, pH 7) (Nautiyal et al. 1999), and Phytate Screening Medium (PSM, 10 g L^{-1} D-glucose, 4 g L^{-1} $C_6H_{18}P_6O_{24} \cdot 12Na \cdot xH_2O$, 2 g L^{-1} $CaCl_2$, 5 g L^{-1} NH_4NO_3, 0.5 g L^{-1} KCl, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.01 g L^{-1} $FeSO_4 \cdot 7H_2O$, 0.01 g L^{-1} $MnSO_4 \cdot H_2O$, 15 g L^{-1} agar, pH 7) respectively (Jorquera et al. 2008). In both assays, four strains were spot inoculated on each agar plate and incubated at 28 °C for 7 days. The formation of halo zones around bacterial colonies indicated phytate and phosphate solubilization capacity of the strains. After incubation, colony diameter and halo zones were recorded. The ability of bacteria to solubilize insoluble phosphate was evaluated as phosphate Solubilization Efficiency (SE), as described by Rokhbakhsh-Zamin et al. (2011): SE=[Halo zone (z)/Diameter of colonies (n)]×100. The Phosphate Solubilization Index (PSI) was also calculated according to the following formula: PSI = the ratio of the total diameter (colony + halo zone)/the colony diameter (Islam et al. 2007).

PCR amplification of NifH gene

Bacteria isolated from N-free medium were tested for the presence of nifH gene by PCR amplification. Genomic DNA was extracted from bacterial liquid cultures grown overnight at 28 °C using "MasterPure™ Yeast DNA Purification Kit" (Epicentre®) according to the manufacturer's protocols. The amplification of nifH gene 390 bp fragment was carried out using the specific primers 19F (5'-GCIWTYTAYGGIAARGGIGG-3') and 407R (5'-AAICCRCCRCAIACIACRTC-3') (Ueda et al. 1995). Amplification reaction was prepared in a final volume of 25 µl, with 10-20

ng of DNA, 1X Reaction buffer (Takara), 1.25 U of Takara ex Taq DNA polymerase, 0.2 mM of each dNTPs (Takara) and 0.5 µM of each primers (Primm). The reaction was carried out using an iCycler-iQ Multicolor Real-Time PCR Detection System (Biorad) with the following denaturation, amplification and extension procedure: 94 °C 1 min; 94 °C 30 sec, 56 °C 30 sec, 72 °C 30 sec for 35 cycles; 72 °C 5 min. The presence of amplicons was confirmed by electrophoresis in 1.5% (w/v) Agarose I (Euroclone®) in TBE 1 x buffer (Euroclone®) gels stained with ethidium bromide 0.5 µg mL^{-1}. All gels were visualized and captured as TIFF format files by the Liscap program for Image Master VDS system (Pharmacia Biotech).

Identification of bacterial strains with best potential PGP activities

Bacterial isolates showing multiple PGP activities were identified based on 16S rDNA sequencing. Genomic DNA was extracted from bacterial liquid cultures grown overnight at 28 °C using "MasterPure™ Yeast DNA Purification Kit" (Epicentre®) according to the manufacturer's protocols. The amplification of 16S rDNA was carried out using the primers 27f (5'-GAGAGTTTGACTCTGGCTCAG-3') and 1495r (5'-CTACGGCTACCTTGTTACGA-3') (Lane 1991; Weisburg et al. 1991). Amplification reaction was carried out in a final volume of 50 µl, using 10-20 ng of DNA, 1X Reaction buffer (EuroClone®), 2 mM $MgCl_2$ (EuroClone®), 1.25 U EuroTaq DNA polymerase (EuroClone®), 0.2 mM of each dNTPs (GeneAmp dNTP Mix, Applied Biosystem) and 0.2 µM of each primers (Primm). The reaction was carried out using an iCycler-iQ Multicolor Real-Time PCR Detection System (Biorad) with the following denaturation, amplification and extension procedure: 95 °C 2 min; 94 °C 1 min and 20 sec, 54 °C 1 min, 72 °C 1 min and 30 sec for 35 cycles; 72 °C 5 min. PCR amplicons were analysed by 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light as describe above. The amplification products were then purified with the EuroGold Cycle Pure Kit (EuroClone®) according to the manufacturer's protocol, quantified and 5' sequenced by BMR Genomics (Padova, Italy). Sequences were analyzed using BLAST on the NCBI web

(http://blast.ncbi.nlm.nih.gov/Blast.cgi). The related sequences were collected and aligned using MUSCLE (Edgar 2004a; 2004b), phylogenetic trees constructed using the Neighbor-Joining method and the evolutionary distances computed using the Kimura 2-parameter method (Kimura 1980) in Mega 6.0 software (http://www.megasoftware.net/) (Tamura et al. 2013) with 1,000 bootstraps replicates.

The sequences of 16S rRNA genes were submitted to the European Nucleotide Archive (ENA) under the accession numbers from LN871737 to LN871755.

RESULTS

Culturable bacteria strictly associated with R. intraradices spores

Heterotrophic bacteria strictly associated with *R. intraradices* spores ranged from 5.4±0.9 to 22.9 ±0.7 CFU spore^{-1}, spore forming culturable bacteria from 0.8±0.4 to 2.9±0.7 CFU spore^{-1}, actinobacteria from 3.7±0.4 to 23.0±1.5 CFU spore^{-1}, bacteria growing on N-free medium from 0.7±0.5 to 1.0±0.6 CFU spore^{-1}, and chitinolytic bacteria from 0.2±0.1 to 1.1±0.1 CFU spore^{-1} (Table 1). A total of 374 bacterial strains were isolated and purified. Among the isolated bacteria 50.5% was obtained from TSA, 36.1% from Waksman's agar, 7.2% from N-free Winogradsky medium and 6.2% developed on the selective chitin containing medium (Figure 1a).

Bacterial strains from TSA were morphologically examined and grouped into 16 morphotypes, according to their colony characteristics. Actinobacteria were categorized into 13 morphotypes, according to the color of the mature sporulated aerial mycelium and of the substrate mycelium (Fig. S1, supplementary electronic material). With the aim of detecting bacteria showing PGP traits, from the pool of the 374 isolated SAB a total of 122 strains were selected for further *in vitro* screening. Such strains included all the isolated chitinolytic and putative nitrogen fixers (23 and 27 strains, respectively), along with 38 heterotrophic bacteria and 34 actinobacteria, selected from the different morphotypes previously identified and chosen taking into account the number of isolates grouped in each morphotype.

Screening for PGP traits

The 122 selected bacterial strains were screened *in vitro* for the presence of PGP traits, *i.e*. the ability to produce IAA and siderophores, and to solubilize mineral phosphate and phytate. The most common PGP trait was represented by P solubilization from phytate (69.7%), followed by siderophore production (65.6%), phosphate solubilization (49.2%) and IAA production (42.6%). The different bacterial strains showed a large variability in their PGP abilities: for example, the halo zone produced by phytate solubilizing bacteria ranged from 0.1 to 1.80 cm, the SE of phosphate solubilizers from 10 to 150%, while for siderophore production the radius of color change ranged from about 0.2 cm to more than 1.5 cm (Tables 2-5).

The four functional bacterial groups (heterotrophs, actinobacteria, chitinolytic and putative N_2-fixing bacteria) showed different percentages of strains expressing single PGP activities. For example, the percentage of strains showing IAA production ranged from 70% of actinobacteria to 14% of putative N-fixers, while that of phosphate solubilizing bacteria (PSB) varied from less than 20% of heterotrophic isolates to 74% of actinobacteria and chitinolytic bacteria. Phytate solubilizing activity was detected in 50% of heterotrophic bacteria and in 100% of actinobacteria. Finally, siderophores were produced by 96% of bacteria growing on N-free medium, 69% of chitinolytic bacteria, and only 25% of the heterotrophic bacteria (Figure 1b). Interestingly, only 7 out of 38 heterotrophic strains showed high siderophore production activity *vs*. 19 out of 34 actinobacterial strains.

Interestingly, a large number of strains were able to express multiple PGP activities. Indeed, the percentage of strains displaying three to four PGP traits was about 76% in actinobacteria, 65% in chitinolytic bacteria, and 48% among bacteria growing on N-free medium (Figure 1c). Conversely, among heterotrophic bacteria no strain showed four PGP traits, and only 16% displayed three. Venn diagrams relevant to each functional group detected 17 actinobacterial strains (W1, W22,W39,W43N, W47, W54, W56, W58, W60B, W60R, W64, W68, W77, W90, W94, W115,

285 W132) and 8 chitinolytic strains (CH5, CH6G, CH10, CH11, CH14, CH15, CH17, CH20)
286 displaying the four PGP traits investigated. Among bacteria growing on N-free medium, only one
287 isolate (N24) showed the four activities, while 12 strains (N14, N18, N19, N20, N21, N22, N23,
288 N25, N26, N27, N28, N29) displayed three. Six heterotrophic bacteria (TSA3, TSA10, TSA26,
289 TSA41, TSA46, TSAT102B) showed three PGP activities (Figure 2).
290
291 *Amplification of NifH genes*
292 Bacterial strains isolated from N-free medium were further tested for the presence of nifH genes by
293 PCR amplification, using the specific primers 19F and 407R (Ueda et al. 1995). The expected 390
294 bp DNA fragment was obtained from 10 strains (N18, N19, N20, N21, N22, N23, N24, N25, N28,
295 and N29).
296
297 *16 S rDNA identification of selected PGP bacterial strains*
298 Representative strains from each functional group were selected on the basis of relevant PGP traits,
299 and identified by 16S rDNA sequencing. A total of 19 strains were sequenced and affiliated to
300 bacterial species using BLAST and phylogenetic trees analyses. Blast nucleotide searches of the
301 16S rDNA sequences showed at least 98% similarities to database entries (Table 6). Figure 3 shows
302 the related phylogenetic trees with the affiliation of sequences to bacterial species. Sequences were
303 affiliated with *Actinomycetales* (*Arthrobacter, Streptomyces* and *Nocardiodes*), *Bacillales* (*Bacillus,*
304 *Fictibacillus* and *Lysinibacillus*) and *Rhizobiales* (*Sinorhizobium*). Among the sequenced strains the
305 majority could be assigned to *Sinorhizobium meliloti* (47.4%), followed by *Streptomyces* spp.
306 (26.3%), while *Arthrobacter phenethrenivorans*, *Nocardiodes albus, Bacillus* sp. *pumilus* group.,
307 *Fictibacillus barbaricus* and *Lysinibacillus fusiformis* were represented by only one isolate each.
308
309 DISCUSSION

In this work we showed the multifunctional traits of culturable bacteria strictly associated with spores of the beneficial plant symbiont *R. intraradices* IMA6. We isolated 374 spore-associated bacterial strains belonging to different functional groups known to possess PGP activities - actinobacteria, spore-forming, chitinolytic and N_2-fixing bacteria. Strains with best potential PGP activities measured in pure culture, identified by 16S rDNA sequencing, showed the ability to produce IAA and siderophores and to solubilize P from mineral phosphate and phytate, representing suitable candidates as biofertilizers and bioenhancers.

Spore-associated culturable bacterial communities

Among the 374 bacterial strains strictly associated with *R. intraradices* spores isolated in this work, 189 were recovered from TSA medium, a general growth substrate for heterotrophic bacteria and 135 from Waksman's agar, a selective growth medium for actinobacteria. Quantitative analyses allowed us to confirm that heterotrophs and actinobacteria represented the majority of SAB, as bacterial counts reached a maximum of 23 and 22 CFU per spore, respectively. Such high numbers indicate that AMF spores represent a preferential ecological niche supporting bacterial metabolic activity as a result of their high levels of nutrients and exudates. Specific physiological groups of bacteria may be selectively favored, for their ability to hydrolyze spore wall biopolymers (Filippi et al. 1998; Roesti et al. 2005) or to form biofilms by producing exopolysaccharides, which allow them to adhere to spore walls (Toljander et al. 2006). Present results confirm our recent molecular findings on the occurrence of large bacterial communities intimately associated with spores of 6 different AMF isolates, including the isolate IMA6 of the species *R. intraradices* studied here (Agnolucci et al. 2015). Other authors have previously shown that the microbiota associated with AMF spores is a dynamic and complex community, whose structure has been molecularly dissected (Roesti et al. 2005; Long et al. 2008). However, isolation in pure culture of putatively beneficial microbiota represents an indispensable step in order to integrate knowledge of spore-associated bacterial communities obtained through molecular studies with data on their functional properties,

in the perspective of opening new avenues for targeted management of beneficial symbionts and their associated bacteria in sustainable food production systems.

Actinobacteria represented 36.1% of the total culturable bacteria recovered from *R. intraradices* spores, consistently with previous data on their widespread occurrence in the mycorrhizosphere and sporosphere (Ames et al. 1989; Filippi et al. 1998; Bharadwaj et al. 2008a). Recent findings, obtained using a culture-independent approach - PCR-DGGE analysis of the 16S rRNA gene - showed that DNA sequences affiliated with the order *Actinomycetales* were found in spore homogenates of six different AMF isolates originating from diverse geographical areas. Such AMF spores harbored different actinobacterial species, which were affiliated to the genera *Streptomyces*, *Arthrobacter*, *Amycolatopsis* and *Propionibacterium* (Agnolucci et al. 2015). Similar results were obtained by Long et al. (2008) who found *Streptomyces, Amycolatopsis*, and *Pseudonocardia* species associated with *G. margarita* spores. Their physiological characteristics, such as the ability to produce a vast array of enzymes that break down insoluble organic polymers, including chitin and chitosan, the major components of spore walls, may explain their intimate association with AMF spores. Indeed, 23 chitinolytic strains were isolated from *R. intraradices* spores, confirming previous findings on spore wall degrading activity of culturable bacteria embedded in spore walls of *Funneliformis mosseae*, where chitinolytic strains represented 72% of all the isolated microorganisms (Filippi et al. 1998). Other authors, using culture-independent methods, reported that most DNA sequences obtained from *Glomus geosporum* and *Glomus constrictum* spores were affiliated with bacterial strains able to hydrolyze biopolymers (Roesti et al. 2005). Such physiological traits may play important functional roles in the promotion of spore germination and germling growth, thus positively affecting AMF root colonisation and functioning (Mayo et al. 1986; Xavier and Germida 2003; Bharadwaj et al. 2008b; Giovannetti et al. 2010).

A very interesting result is represented by the isolation of 27 bacterial strains from N-free medium, 10 (37%) of which produced the expected nifH gene 390 bp amplicon, suggesting their possible role as biofertilizers by mediating the acquisition of nitrogen, a major plant nutrient. Such data confirm

362 our previous molecular findings, showing that nitrogen fixing bacteria, *Sinorhizobium meliloti*,
363 *Agrobacterium radiobacter*, *Rhizobium giardinii* and *Rhizobium rhizogenes*, were associated with
364 spores of different AMF isolates (Agnolucci et al. 2015). Bacteria belonging to *Rhizobiales* have
365 been rarely isolated from AMF spores (Bharadwaj et al. 2008a), though their co-inoculation with
366 AMF has been shown to positively affect nutrient uptake and photosynthetic rate in diverse plant
367 species and to promote mycorrhizal functioning, improving spore germination, mycelial growth and
368 mycorrhizal colonization (Gopal et al. 2012).
369
370 *Functional diversity of bacterial isolates*
371 Among the 374 bacterial strains isolated, 122 were selected for functional significance studies: 38
372 heterotrophs (including 15 spore forming bacteria), 34 actinobacteria, 23 chitinolytic and 27
373 putative N_2-fixing bacteria were further screened in pure culture for PGP traits, such as the ability to
374 produce IAA and siderophores, and to solubilize P from mineral phosphate and phytate.
375 We obtained a high percentage of IAA producing strains (42.6%), with 76% of positive isolates
376 among actinobacteria. As IAA produced by different bacterial strains is able to stimulate the
377 development of plant root systems (Glick et al. 1995; Patten et al. 2002), while displaying no
378 functions in bacterial cells, IAA-producing strains may play a beneficial role in the performance of
379 the complex plant-AMF interaction.
380 Siderophore-producing strains represented 65.6% of the total isolates, although their activity was
381 differentially expressed in the different bacterial groups. Such a trait may be functional to AMF
382 potential biocontrol activity against soilborne diseases, by means of bacterial siderophore-mediated
383 competition for iron (Thomashow et al. 1990; Glick 1995; Whipps 2001), thus indirectly
384 stimulating plant growth by inhibiting the development of deleterious plant pathogens (Davison
385 1988; Arora et al. 2001).
386 The ability to solubilize P was shown by 69.7% and 49.2% of SAB, when tested in the presence of
387 phytate and inorganic P, respectively. The majority of P-solubilizing strains were recovered from

388 Waksman's agar medium and minimal medium containing chitin as sole carbon source. Other
389 authors investigated the occurrence of P-solubilizing bacteria associated with *F. mosseae* spores in
390 the mycorrhizosphere of *Psidium guajava* L. and found that all the isolates showing high P-
391 solubilizing activity belonged to the genera *Streptomyces* and *Leifsonia* (Mohandas et al. 2013).
392 Since P is a key plant nutrient usually applied as fertilizer in the form of inorganic phosphates,
393 which are rapidly immobilized in the soil, thus becoming unavailable to plant roots, P-mobilizing
394 bacteria could act in synergy with AMF symbionts to enhance P availability to host plants
395 (Rodriguez and Fraga 1999). AMF are increasingly reported to play a fundamental role in P
396 biogeochemical cycle, improving P availability in the soil solution by means of the large
397 extraradical mycelial networks spreading from mycorrhizal roots into the soil environment and able
398 to absorb P far from the depletion zone surrounding the roots. As phosphate transporter genes are
399 differentially expressed on such hyphae, we could hypothesize that the higher P concentrations
400 found in AMF inoculated plants may be ascribed also to the activity of large communities of
401 mineral phosphate and phytate solubilizing bacteria (Harrison and van Buuren 1995; Fiorilli et al.
402 2013).
403 Finally, the highest number of high siderophore producing strains (halo zone> 15mm) (TSA20,
404 TSA44, TSA49, TSA108B and TSA120) and the two best phosphate solubilizing bacteria (TSA41
405 with SE =150 and TSA3 with SE =115.38), along with one out of the three best IAA producers
406 strains (TSA50, CH8, CH17) were found among heterotrophic bacteria (Tables 2 and 4).
407
408 *Identification of bacterial isolates*
409 Nineteen strains with best potential PGP activities were selected and identified by 16S rDNA
410 sequencing. Interestingly, 9 out of 19 strains (47,4%) were identified as *Sinorhizobium meliloti*:
411 three strains - TSA3, TSA26 and TSA41 - were able to produce IAA, six strains - TSA3, TSA26
412 and TSA41, N23, N28, N29 - were able to solubilize mineral and organic P *in vitro* and the three
413 chitinolytic strains - CH5, CH8, CH17 - showed siderophore and IAA production activity and

414 solubilized mineral and organic P *in vitro* (Tables 2, 4 and 5). The strict association of members of
415 the order *Rhizobiales* with AMF spores may be ascribed to their ability to form biofilms by
416 producing exopolysaccharides, allowing an efficient colonization of roots and mycorrhizal hyphae
417 (Bianciotto et al. 1996; Toljander et al. 2006). In addition to the beneficial effect in terms of
418 biological nitrogen fixation, rhizobia promote plant growth by producing phytohormones,
419 improving plant nutritional status and biocontrolling phytopathogens (Chandra et al. 2007). Here,
420 three strains of *S. meliloti* were isolated from a medium containing chitin as sole carbon source,
421 confirming previous data on the ability of several rhizobial strains isolated from root nodules to
422 produce chitinolytic enzymes (Sridevi et al. 2008). Such data highlight the multifunctionality of our
423 isolates, which could be further investigated in order to select the most efficient strains able to
424 degrade fungal cell walls, thus acting as biocontrol agents against fungal pathogens (Mazen et al.
425 2008). Indeed, two chitinase producing *Bradyrhizobium* strains were reported to inhibit mycelial
426 growth, sclerotia formation and germination of *Macrophomina phaseolina* (Tassi) Goid., a major
427 pathogen of more than 500 plant hosts (Dubey et al. 2012).
428 Five strains (26.3%) were assigned to *Streptomyces* (W43N, W64, W77, W94 and W115), a genus
429 representing a predominant component of the soil microbial population, capable of producing a vast
430 array of complex and biologically active secondary metabolites, including antibacterial, antifungal,
431 antiparasitic, anticancer and immunosuppressant drugs. These strains showed high growth
432 promoting potential exhibiting all the PGP traits tested. Among the species affiliated to our strains,
433 *Streptomyces phaeochromogenes* was reported to produce chloramphenicol, and one strain (LL-
434 P018) to produce phaeochromycins, a novel anti-inflammatory polyketides inhibitors of the
435 biosynthesis of a tumor necrosis factor (TNF-α) (van Pée and Lingens 1985; Ritacco and Eveleigh
436 2008), *Streptomyces collinus* strain Tü 365 to produce the antibiotic kirromycin (Wolf and Zähner
437 1972), *Streptomyces iakyrus* DSM 41873 actinomycin G (Qin et al. 2014) and *Streptomyces*
438 *viridochromogenes* Tü57 avilamycin A (Weitnauer et al. 2001). In addition, *Streptomyces* spp. are
439 considered promising taxa of PGP and mycorrhizal helper bacteria, due to their ability to solubilize

phosphates, produce chitinase and growth regulators (Mohandas et al. 2013; Hamedi and Mohammadipanah 2015) and stimulate AMF spore germination and hyphal growth (Mugnier and Mosse 1987; Tylka et al. 1991; Carpenter-Boggs et al. 1995). Interestingly, sequences affiliated to *Streptomyces flavogriseus* and *S. phaeochromogenes*, with a similarity of 99%, were recovered from *F. mosseae* and *R. intraradices* IMA6 , respectively (Agnolucci et al. 2015). The other two isolates affiliated with *Actinomycetales* were represented by *Arthrobacter phenanthrenivorans* (N17) and *Nocardiodes albus* (N13). Both isolates were able to grow on the N-free selective medium. For a long time it was believed that the ability to fix atmospheric nitrogen was limited to the actinomycetes *Frankia*, but lately, nitrogen fixation (nifH) genes were found in other non-*Frankia* actinomycetes including *Streptomyces*, *Arthrobacter* (Gtari et al. 2012; Sharon and Daniel 2013) and *Nocardiodes*.

Actinobacteria of the genus *Arthrobacter* are ubiquitous in all soil types and able to utilize a wide range of natural as well as xenobiotic compounds. In particular, *A. phenanthrenivorans* strain Sphe3 showed the ability to grow on phenanthrene as the sole carbon and energy source (Vandera et al. 2015). *Arthrobacter* species have been reported to be associated with AMF spores and hyphae of *F. mosseae* and *R. intraradices* (Bharadwaj et al. 2008a), although Andrade et al. (1997) found that they were most frequent in the hyphosphere, the zone of soil surrounding individual AMF hyphae (Artursson et al. 2006). It is interesting to note that *A. phenanthrenivorans* sequences were previously recovered from *F. coronatum*, *F. mosseae* and *R. intraradices*, including the isolate IMA6 analysed in the present study (Agnolucci et al. 2015). The strict association of *Arthrobacter* spp. with AMF spores may be functional to their role as PGP and mycorrhizal helper bacteria: indeed, some *Arthrobacter* strains have been reported to improve mycorrhizal colonization and root length, and to display antagonistic activity against plant pathogens (Bharadwaj et al. 2008b).

Three isolates were affiliated to *Bacillales*, namely to a species of *Bacillus pumilus* group (CH10), *Lysinibacillus fusiformis* (CH19) and *Fictibacillus barbaricus* (TSA50). *Bacillus* isolates closely related to *B. pumilus* are not easily distinguished from each other. Definitely, the *B. pumilus* group

466 contains 5 species, *B. pumilus*, *B. safensis*, *B. stratosphericus*, *B. altitudinis* and *B. aerophilus*,
467 which are nearly identical in 16S rRNA gene sequence, sharing similarity over 99.5% (Liu et al.
468 2013). *Lysinibacillus fusiformis* and *Fictibacillus barbaricus* originally assigned to the genus
469 *Bacillus* were reclassified respectively in 2007 (Trivedi et al. 2011) and in 2013 (Glaeser et al.
470 2013). *Bacillus* strains are among the major chitin decomposers, producing and secreting many
471 kinds of chitinase (Heravi et al. 2013). For example, *Bacillus pumilus* strain SG2 produces two
472 chitinases, namely ChiS and ChiL (Heravi et al. 2013), and *Lysinibacillus fusiformis* strain B-CM18
473 a purified chitinase of 20 Kd, possessing an *in vitro* strong antifungal activity (Singh et al. 2013).
474 Accordingly, our isolates assigned to *Bacillus pumilus* group and to *Lysinibacillus fusiformis* were
475 retrieved from the selective media for the isolation of chitinolytic bacteria. Interestingly, *Bacillus*
476 *pumilus* strains have a wide range of attributed applications, as PGP rhizobacteria and animal and
477 human probiotic (Branquinho et al. 2014). An interesting example of such application is represented
478 by the endophytic strain *Bacillus pumilus* INR7, which has been commercialized as a biological
479 control product, active by direct antagonism and induction of systemic resistance, against soilborne
480 pathogens as well as foliar pathogens (Jeong et al. 2014).
481 Interestingly, the isolation in pure culture of bacterial strains belonging to *Bacillales* support our
482 previous data on the occurrence of DNA sequences affiliated with *Paenibacillus castaneae* and
483 *Bacillus firmus* in spore homogenates of *F. mosseae* and *R. intraradices*, respectively (Agnolucci et
484 al. 2015). The role of members of *Bacillales* as PGP has long been known and their activity as
485 mycorrhizal helper bacteria, increasing mycorrhizal establishment and promoting plant growth, has
486 been recently reported (Budi et al. 2013; Zhao et al. 2014; Pérez-Montaño et al. 2014).
487 In conclusion, our work demonstrates that AMF spores are a privileged source of bacteria with
488 potential PGP activities, whose isolation and functional characterization represent a prerequisite for
489 their use as biocontrol agents, biofertilisers and bioenhancers. The emerging picture of
490 AMF/bacteria interactions suggests that different partners of tripartite associations - host plants,
491 AMF and bacteria - may act in synergy and provide new multifunctional benefits, improving plant

and fungal performances. SAB can be transferred from spores to soil-based hyphae, where they may enhance nutrient availability (phosphate solubilizing, nitrogen fixing and chitinolytic bacteria), control plant pathogens (siderophore producing bacteria) and promote plant growth (IAA producing bacteria).

Further investigations are in progress aimed at selecting the best performing AMF/bacteria combinations in the perspective of exploiting the potential of such multipartite association in a new food production system, aimed at maintaining and increasing soil biological fertility and protecting natural processes that are at the basis of energy flows and matter cycles in sustainable agroecosystems.

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720

721 **Table 1** Abundance of culturable bacteria isolated from three batches (A, B, C) of 200 spores of
722 *Rhizophagus intraradices* IMA6 on different media (mean CFU spore^{-1} ± SE). TSA, Tryptic soil
723 agar; TSAT, TSA plus thermic treatment; W, Waksman agar; N, nitrogen-free medium; CH, chitin
724 agar.
725

Media	A	B	C
TSA	22.9±0.7	7.9±1.6	5.4±0.9
TSAT	2.9±0.7	1.9±0.4	0.8±0.4
W	23.0±1.5	6.1±0.5	3.7±0.4
N	nd	1.0±0.6	0.7±0.5
CH	nd	0.2±0.1	1.1±0.1

726 nd = not determined.
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743 **Table 2** Plant growth promoting traits of heterotrophic bacteria isolated from spores of

744 *Rhizophagus intraradices* IMA6.

Isolate	IAA production	Siderophore production	Phosphate solubilization		Phytate solubilization
		Activity (after 7 days)	SE(%)	PSI	Halo zone (cm)
TSA2	+	-	-	-	-
TSA3	++	-	115.38	2.15	0.85
TSA10	-	+	35.71	1.36	0.85
TSA13	-	-	-	-	-
TSA20	-	+++	-	-	0.40
TSA25	-	-	-	-	0.20
TSA26	+	-	81.82	1.82	0.90
TSA32	-	-	-	-	-
TSA39B	-	-	-	-	-
TSA39G	-	++	-	-	0.40
TSA41	++	-	150	2.50	0.70
TSA43B	-	-	-	-	0.45
TSA44	-	+++	-	-	0.35
TSA46	-	+	11.76	1.12	0.25
TSA47	-	-	-	-	-
TSA49	-	+++	-	-	0.40
TSA50	+++	-	-	-	-
TSA58	-	+	-	-	-
TSA60	+	-	-	-	-
TSA108B	-	+++	-	-	0.65
TSA113	-	-	-	-	-
TSA136	-	-	-	-	-
TSA142	-	-	-	-	0.35
TSAT5G	-	-	-	-	-
TSAT7	-	-	-	-	-
TSAT11	-	-	-	-	0.15
TSAT14	-	+	-	-	-
TSAT28	-	-	-	-	-
TSAT38	+	-	-	-	-
TSAT50B	+	-	-	-	-
TSAT50T	+	-	-	-	-
TSAT51	-	-	-	-	0.25
TSAT60	-	-	-	-	-
TSAT92	+	-	-	-	0.30
TSAT101	+	-	-	-	0.20
TSAT102B	-	+++	16.67	1.17	0.35
TSAT113	-	-	-	-	-
TSAT115	+	-	-	-	-

745 IAA production: - = no production, +/- = low production, + = production, ++ = moderate production, +++ = high

746 production.

747 Siderophore production: - = no production (halo = 0 cm), +/- = low production (halo ≤ 0.2 cm), + = production (0.3

748 cm≤ halo ≤ 0.8 cm), ++ = moderate production (0.8 cm < halo < 1.4 cm), +++ = high production (halo > 1.5 cm).

749 Phosphate solubilization: SE = solubilization efficiency, PSI = phosphate solubilization index, - = absence of

750 solubilization.

751 Phytate solubilization: - = absence of solubilization.

752

753 **Table 3** Plant growth promoting traits of actinobacteria isolated from spores of *Rhizophagus*
754 *intraradices* IMA6.

Isolate	IAA production	Siderophore production	Phosphate solubilization		Phytate solubilization
		Activity (after 7 days)	SE(%)	PSI	Halo zone (cm)
W1	++	++	14.29	1.14	0.60
W2	-	+++	10.53	1.11	0.50
W19	-	++	11.11	1.11	0.20
W22	++	++	20	1.2	0.85
W27	++	++	-	-	1.10
W31	++	-	16.67	1.17	0.15
W39	++	++	28.57	1.29	0.75
W40	-	-	-	-	0.50
W41	-	++	-	-	0.05
W43N	++	++	63.64	1.64	0.80
W47	++	++	17.65	1.18	1.50
W49	++	+/-	-	-	0.45
W54	++	++	13.64	1.14	0.25
W56	++	++	18.18	1.18	0.25
W58	+	++	11.11	1.11	0.75
W60B	+	+++	11.11	1.11	0.85
W60R	+	+/-	57.14	1.57	0.35
W64	++	++	45.45	1.45	1.20
W65	-	-	23.53	1.24	0.95
W66	-	-	23.08	1.23	0.15
W68	++	++	13.33	1.13	0.70
W69	-	+	-	-	1.15
W71	-	++	25	1.25	0.90
W77	++	++	36.36	1.36	0.90
W85	-	-	-	-	1.80
W87	-	++	-	-	0.35
W90	+	++	20	1.2	0.80
W92	++	-	15.38	1.15	1.00
W94	++	+	54.55	1.55	1.15
W105	++	++	-	-	1.05
W115	++	++	38.46	1.38	0.50
W129	++	-	33.33	1.33	0.75
W132	++	+	35.71	1.36	0.80
W133	++	++	-	-	0.95

755 IAA production: - = no production, +/- = low production, + = production, ++ = moderate production, +++ = high
756 production.

757 Siderophore production: - = no production (halo = 0 cm), +/- = low production (halo ≤ 0.2 cm), + = production (0.3
758 cm≤ halo ≤ 0.8 cm), ++ = moderate production (0.8 cm < halo < 1.4 cm), +++ = high production (halo > 1.5 cm).
759 cm≤ halo ≤ 0.8 cm), ++ = moderate production (0.8 cm < halo < 1.4 cm), +++ = high production (halo > 1.5 cm).

760 Phosphate solubilization: SE = solubilization efficiency, PSI = phosphate solubilization index, - = absence of

761 solubilization.

762 Phytate solubilization: - = absence of solubilization.

763

764 **Table 4** Plant growth promoting traits of putative chitinolytic bacteria isolated from spores of
765 *Rhizophagus intraradices* IMA 6.

Isolate	IAA production	Siderophore production	Phosphate solubilization		Phytate solubilization
		Activity (after 7 days)	SE(%)	PSI	Halo zone (cm)
CH1	-	+	-	-	-
CH2	-	+	-	-	-
CH3	-	-	63.16	1.63	0.50
CH4	-	+	-	-	0.20
CH5	++	+	31.25	1.31	0.25
CH6B	-	+	30.77	1.31	0.30
CH6G	+	+	25	1.25	0.30
CH7	++	-	21.43	1.21	0.62
CH8	+++	+	-	-	0.15
CH9	-	-	15.38	1.15	0.15
CH10	+	+	69.23	1.69	0.25
CH11	+/-	+	63.64	1.64	0.35
CH12	-	+	36.36	1.36	0.25
CH13	-	+	46.15	1.46	0.20
CH14	+	+	50	1.5	0.35
CH15	+	+	44.44	1.44	0.30
CH16L	++	-	50	1.5	0.35
CH16B	-	-	20.83	1.21	-
CH17	+++	+	50	1.5	0.30
CH18	-	+	-	-	0.70
CH19	+	-	86.67	1.87	0.45
CH20	+	+	30	1.3	0.35
CH21	-	-	-	-	-

766 IAA production: - = no production, +/- = low production, + = production, ++ = moderate production, +++ = high
767 production.

768 Siderophore production: - = no production (halo = 0 cm), +/- = low production (halo ≤ 0.2 cm), + = production (0.3
769 cm≤ halo ≤ 0.8 cm), ++ = moderate production (0.8 cm < halo < 1.4 cm), +++ = high production (halo > 1.5 cm).

770 Phosphate solubilization: SE = solubilization efficiency, PSI = phosphate solubilization index, - = absence of
771 solubilization.

772 Phytate solubilization: - = absence of solubilization.

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778 **Table 5** Plant growth promoting traits of putative N_2-fixing bacteria isolated from spores of

779 *Rhizophagus intraradices* IMA6.

Isolate	IAA production	Siderophore production	Phosphate solubilization		Phytate solubilization
		Activity (after 7 days)	SE(%)	PSI	Halo zone (cm)
N1	-	+	-	-	-
N3	-	+	-	-	-
N4	-	+	-	-	-
N5	-	+	-	-	-
N6	-	+	-	-	-
N7	+/-	+	-	-	-
N8	-	+	-	-	-
N9	-	+	-	-	-
N10	-	+	-	-	-
N11	-	+	-	-	-
N12	-	+	10	1.1	-
N13	-	++	-	-	0.10
N14	+/-	+	-	-	0.10
N16	-	-	-	-	-
N17	-	++	-	-	-
N18	+	+	-	-	0.45
N19	-	+	41.67	1.42	0.50
N20	-	+	61.54	1.62	0.60
N21	-	+	81.25	1.81	0.55
N22	-	+	30.77	1.31	0.55
N23	-	+	71.43	1.71	0.65
N24	+/-	+	33.33	1.33	0.65
N25	-	+	58.33	1.58	0.70
N26	-	+	72.73	1.73	0.50
N27	-	+	64.29	1.64	0.60
N28	-	+	91.67	1.92	0.10
N29	-	+	84.62	1.85	0.60

780 IAA production: - = no production, +/- = low production, + = production, ++ = moderate production, +++ = high

781 production.

782 Siderophore production: - = no production (halo = 0 cm), +/- = low production (halo ≤ 0.2 cm), + = production (0.3

783 cm≤ halo ≤ 0.8 cm), ++ = moderate production (0.8 cm < halo < 1.4 cm), +++ = high production (halo > 1.5 cm).

784 Phosphate solubilization: SE = solubilization efficiency, PSI = phosphate solubilization index, - = absence of

785 solubilization.

786 Phytate solubilization: - = absence of solubilization.

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Table 6 Phylogenetic identification of the 19 best performing plant growth promoting bacterial strains.

Strain	Identification	Identity (%)	Most closely related GeneBank sequence
CH5	*Sinorhizobium meliloti*	100%	NR113670.1
CH8	*Sinorhizobium meliloti*	99%	NR113670.1
CH10	*Bacillus* sp. *pumilus* group	99%	KM087337.1
CH17	*Sinorhizobium meliloti*	99%	GU129568.1
CH19	*Lysinibacillus fusiformis*	99%	NR112569.1
N13	*Nocardioides albus*	99%	AF005004.1
N17	*Arthrobacter phenanthrenivorans*	100%	NR042469.2
N23	*Sinorhizobium meliloti*	100%	AB535689
N28	*Sinorhizobium meliloti*	99%	AB535689
N29	*Sinorhizobium meliloti*	99%	AB535689
TSA3	*Sinorhizobium meliloti*	99%	GU129568.1
TSA26	*Sinorhizobium meliloti*	99%	GU129568.1
TSA41	*Sinorhizobium meliloti*	99%	GU129568.1
TSA50	*Fictibacillus barbaricus*	99%	KJ831620.1
W43N	*Streptomyces* sp.	99%	NR041063.1
W64	*Streptomyces* sp.	98%	NR114792.1
W77	*Streptomyces* sp.	99%	NR112526.1
W94	*Streptomyces sp.*	99%	NR041231.1
W115	*Streptomyces* sp.	99%	JN969025.1

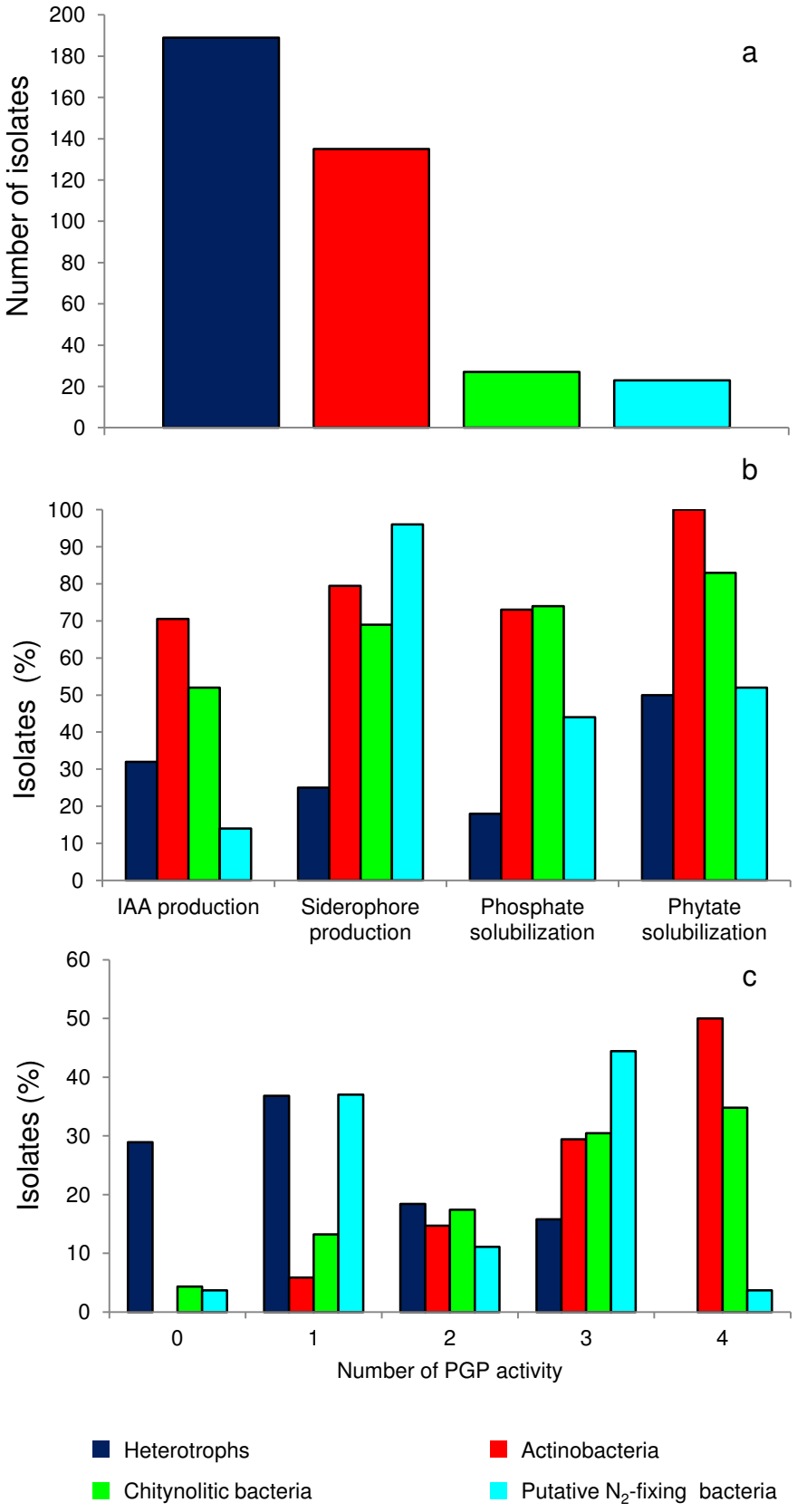
FIGURE LEGENDS

Fig. 1. (a) Number of bacterial strains isolated from spores of *Rhizophagus intraradices* IMA6 within each functional group (heterotrophs, actinobacteria, chitinolytic and putative N_2-fixing bacteria). (b) Percentage of selected isolates displaying plant growth promoting (PGP) traits within each functional group. (c) Percentage of selected isolates showing increasing numbers of PGP activities within each functional group.

Fig. 2. Venn diagram showing, within each functional group, the number of spore associated bacteria displaying plant growth promoting traits - IAA production, siderophore production, mineral phosphate solubilization and phytate solubilization.

Fig. 3. (a,b,c) Affiliation of the sequences of *Actinomycetales*, *Bacillales* and *Rhizobiales* isolated from spores of *Rhizophagus intraradices* IMA6 with the existing 16S rRNA gene sequences, using Neighbor-Joining trees. Bootstrap values below 70 are not shown. The sequences from the database are indicated by their accession numbers. The DNA sequences retrieved in this work are shown in boldface.

Figure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160
140
120
100
80
60
40
20
0
b
Isolates (%)
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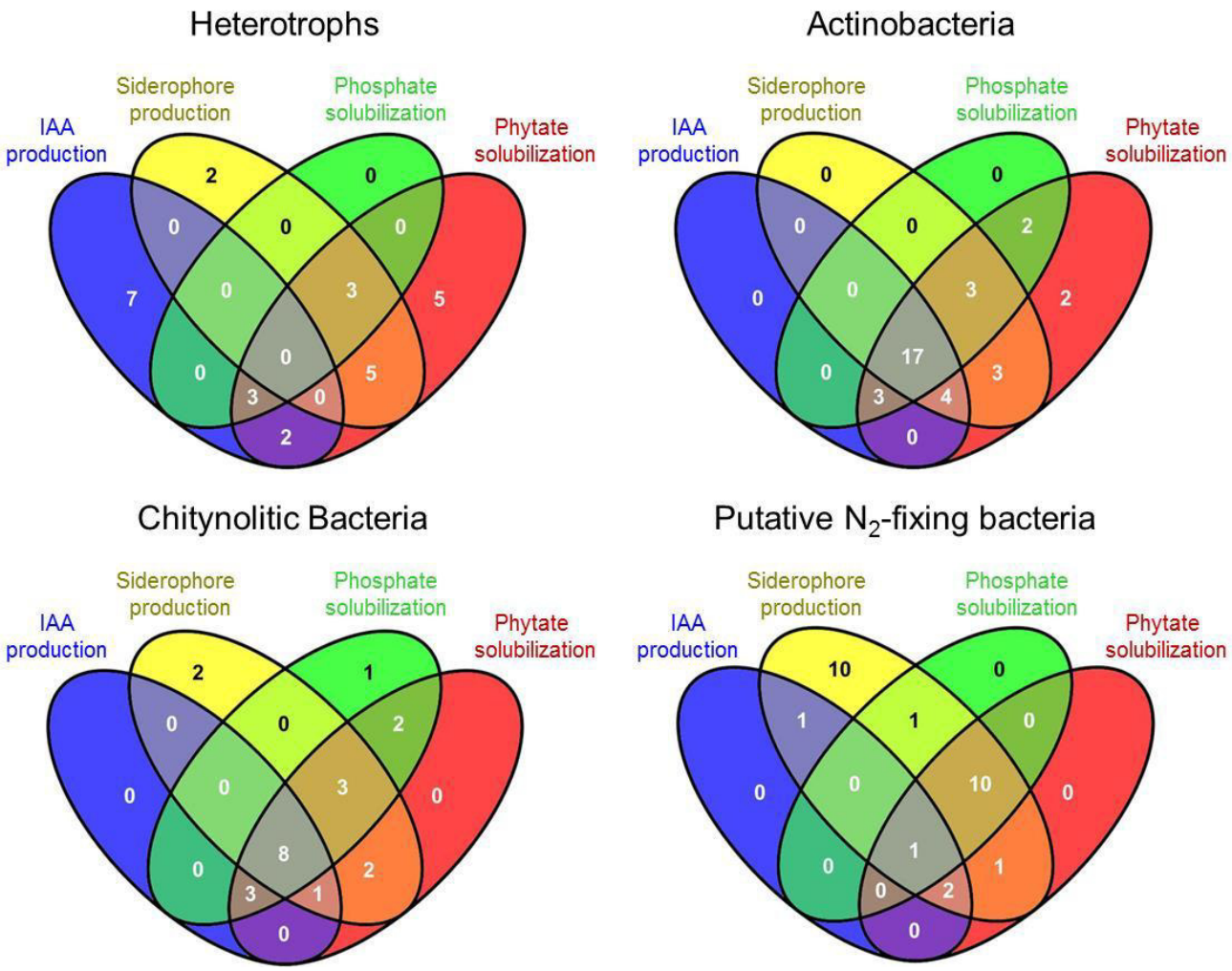
90
80
70
60
50
40
30
20
10
0
IAA production
Siderophore production
Phosphate solubilization
Phytate solubilization
c
Isolates (%)
60
50
40
30
20
10
0
0
1
2
3
4
Number of PGP activity
Heterotrophs
Actinobacteria
Chitynolitic bacteria
Putative N_2-fixing bacteria

Fig. 1


Heterotrophs
IAA production
Siderophore production
Phosphate solubilization
Phytate solubilization
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Actinobacteria
IAA production
Siderophore production
Phosphate solubilization
Phytate solubilization
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17
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Chitynolitic Bacteria
IAA production
Siderophore production
Phosphate solubilization
Phytate solubilization
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Putative N_2-fixing bacteria
IAA production
Siderophore production
Phosphate solubilization
Phytate solubilization
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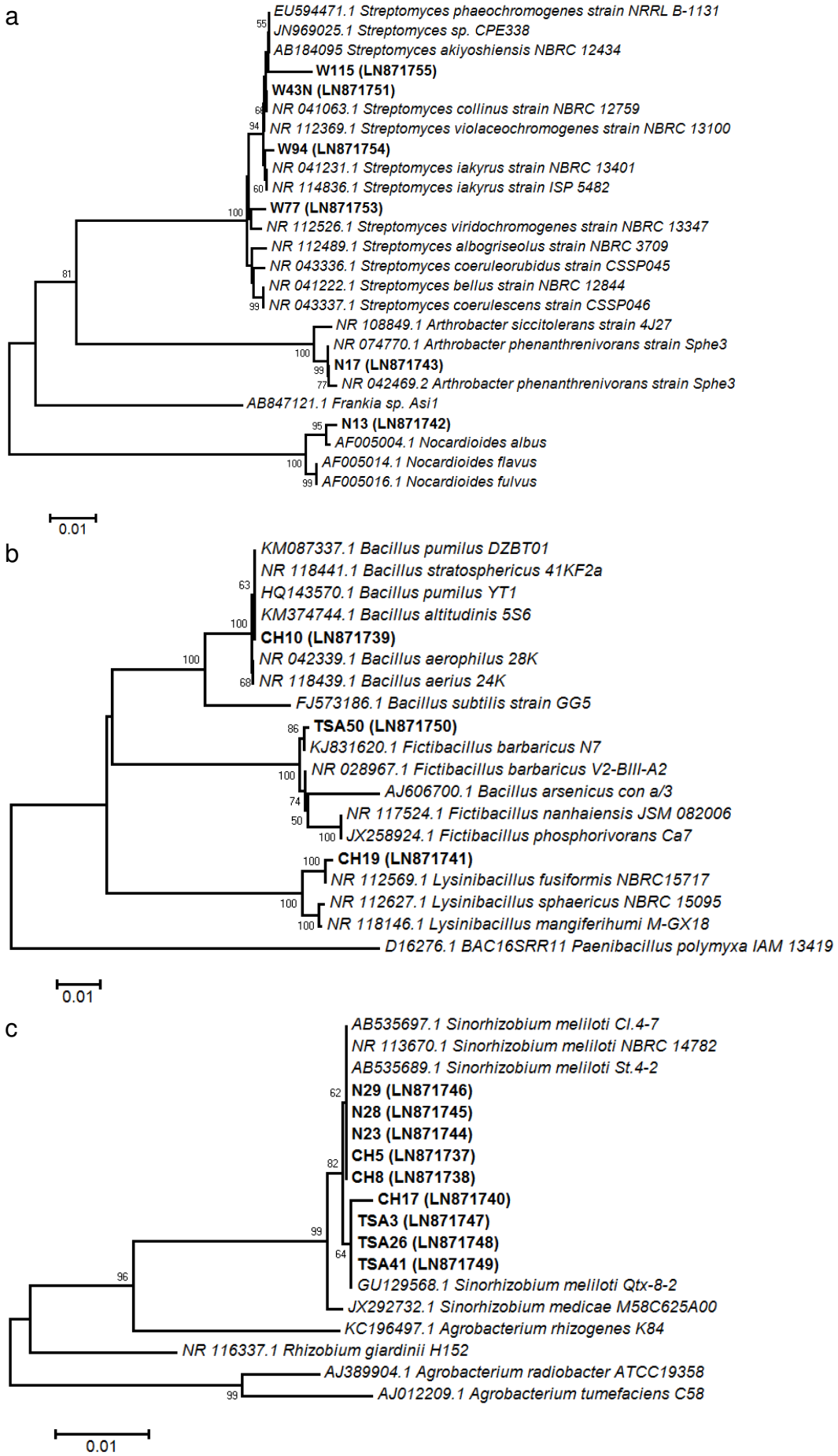
Fig. 2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AB184095 Streptomyces akiyoshiensis NBRC 12434
W115 (LN871755)
W43N (LN871751)
NR 041063.1 Streptomyces collinus strain NBRC 12759
NR 112369.1 Streptomyces violaceochromogenes strain NBRC 13100
W94 (LN871754)
NR 041231.1 Streptomyces iakyrus strain NBRC 13401
NR 114836.1 Streptomyces iakyrus strain ISP 5482
W77 (LN871753)
NR 112526.1 Streptomyces viridochromogenes strain NBRC 13347
NR 112489.1 Streptomyces albogriseolus strain NBRC 3709
NR 043336.1 Streptomyces coeruleorubidus strain CSSP045
NR 041222.1 Streptomyces bellus strain NBRC 12844
NR 043337.1 Streptomyces coerulescens strain CSSP046
NR 108849.1 Arthrobacter siccitolerans strain 4J27
NR 074770.1 Arthrobacter phenanthrenivorans strain Sphe3
N17 (LN871743)
NR 042469.2 Arthrobacter phenanthrenivorans strain Sphe3
AB847121.1 Frankia sp. Asi1
N13 (LN871742)
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AF005014.1 Nocardioides flavus
AF005016.1 Nocardioides fulvus
0.01
b
KM087337.1 Bacillus pumilus DZBT01
NR 118441.1 Bacillus stratosphericus 41KF2a
HQ143570.1 Bacillus pumilus YT1
KM374744.1 Bacillus altitudinis 5S6
CH10 (LN871739)
NR 042339.1 Bacillus aerophilus 28K
NR 118439.1 Bacillus aerius 24K
FJ573186.1 Bacillus subtilis strain GG5
TSA50 (LN871750)
KJ831620.1 Fictibacillus barbaricus N7
NR 028967.1 Fictibacillus barbaricus V2-BIII-A2
AJ606700.1 Bacillus arsenicus con a/3
NR 117524.1 Fictibacillus nanhaiensis JSM 082006
JX258924.1 Fictibacillus phosphorivorans Ca7
CH19 (LN871741)
NR 112569.1 Lysinibacillus fusiformis NBRC15717
NR 112627.1 Lysinibacillus sphaericus NBRC 15095
NR 118146.1 Lysinibacillus mangiferihumi M-GX18
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c
AB535697.1 Sinorhizobium meliloti CI.4-7
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N28 (LN871745)
N23 (LN871744)
CH5 (LN871737)
CH8 (LN871738)
CH17 (LN871740)
TSA3 (LN871747)
TSA26 (LN871748)
TSA41 (LN871749)
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KC196497.1 Agrobacterium rhizogenes K84
NR 116337.1 Rhizobium giardinii H152
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AJ012209.1 Agrobacterium tumefaciens C58
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Fig. 3