



Bacteria Associated With a Commercial Mycorrhizal Inoculum: Community Composition and Multifunctional Activity as Assessed by Illumina Sequencing and Culture-Dependent Tools

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The implementation of sustainable agriculture encompasses practices enhancing the activity of beneficial soil microorganisms, able to modulate biogeochemical soil cycles and to affect soil fertility. Among them, arbuscular mycorrhizal fungi (AMF) establish symbioses with the roots of most food crops and play a key role in nutrient uptake and plant protection from biotic and abiotic stresses. Such beneficial services, encompassing improved crop performances, and soil resources availability, are the outcome of the synergistic action of AMF and the vast communities of mycorrhizospheric bacteria living strictly associated with their mycelium and spores, most of which showing plant growth promoting (PGP) activities, such as the ability to solubilize phosphate and produce siderophores and indole acetic acid (IAA). One of the strategies devised to exploit AMF benefits is represented by the inoculation of selected isolates, either as single species or in a mixture. Here, for the first time, the microbiota associated with a commercial AMF inoculum was identified and characterized, using a polyphasic approach, i.e., a combination of culture-dependent analyses and metagenomic sequencing. Overall, 276 bacterial genera were identified by Illumina high-throughput sequencing, belonging to 165 families, 107 orders, and 23 phyla, mostly represented by Proteobacteria and Bacteroidetes. The commercial inoculum harbored a rich culturable heterotrophic bacterial community, whose populations ranged from 2.5 to 6.1 × 10⁶ CFU/mL. The isolation of functional groups allowed the selection of 36 bacterial strains showing PGP activities. Among them, 14 strains showed strong IAA and/or siderophores production and were affiliated with Actinomycetales (*Microbacterium trichotecenolyticum*, *Streptomyces deccanensis/scabiei*), Bacillales (*Bacillus litoralis*, *Bacillus megaterium*), Enterobacteriales (*Enterobacter*), Rhizobiales (*Rhizobium radiobacter*). This work demonstrates for the first time that an AMF inoculum, obtained following industrial production processes, is home of a large and diverse

community of bacteria with important functional PGP traits, possibly acting in synergy with AMF and providing additional services and benefits. Such bacteria, available in pure culture, could be utilized, individually and/or in multispecies consortia with AMF, as biofertilizers and bioenhancers in sustainable agroecosystems, aimed at minimizing the use of chemical fertilizers and pesticides, promoting primary production, and maintaining soil health and fertility.

Keywords: arbuscular mycorrhizal symbionts, mycorrhizosphere, plant-growth promoting bacteria, siderophores production, indole acetic acid production, metagenomics

INTRODUCTION

Worldwide, a major shift is taking place in agriculture, in order to meet the growing global demand for a safe production of high-quality food, able to maintain or enhance environmental quality and to conserve natural resources for future generations. The implementation of sustainable agriculture encompasses practices enhancing the activity of soil biogeochemical cycles, at the basis of long-term soil productivity and health. The most important players of soil biological fertility are represented by beneficial soil microorganisms, able to modulate biochemical and physiological soil processes, and to affect its biological and nutritional characteristics (Barea et al., 2005). Among them, arbuscular mycorrhizal fungi (AMF, Glomeromycota) are recognized as ecologically and economically important elements of sustainable food production systems, given the key role played in plant nutrition and health, by reducing the input of chemical fertilizers and pesticides (Smith and Read, 2008).

AMF are obligate mutualistic biotrophs, establishing symbioses with the roots of most land plants, including the major food and feed crops, from cereals and legumes to fruits and vegetables, including also important industrial plants, such as sunflower, tobacco, cotton, and medicinal plants (Smith and Read, 2008). AMF symbionts facilitate plant nutrient uptake, mainly phosphorus (P), nitrogen (N), sulfur (S) potassium (K), calcium (Ca), copper (Cu), and zinc (Zn), by means of a large network of extraradical hyphae spreading from colonized roots to the surrounding soil and functioning as a supplementary absorbing system (Giovannetti et al., 2001; Avio et al., 2006). Moreover, they protect plants from biotic and abiotic stresses (Augé, 2001; Evelin et al., 2009; Sikes et al., 2009), provide essential ecosystem services (Gianinazzi et al., 2010), and affect the biosynthesis of beneficial plant secondary metabolites, contributing to the production of safe and high quality food (Sbrana et al., 2014; Avio et al., 2018). However, such beneficial services, encompassing improved crop performances and soil resources availability, are the outcome of the synergistic action of AMF and the vast communities of mycorrhizospheric bacteria living strictly associated with their mycelium and spores (Hildebrandt et al., 2006; Agnolucci et al., 2015). AMF-associated microbiota has been reported to promote mycorrhizal activity (Mayo et al., 1986; Xavier and Germida, 2003; Horii and Ishii, 2006; Giovannetti et al., 2010), to protect plants from soilborne pathogens (Citernes et al., 1996; Budi et al., 1999; Li et al., 2007; Bharadwaj et al., 2008a,b) and to provide nutrients

and growth factors (Barea et al., 2002; Xavier and Germida, 2003), thus being considered as plant growth promoting (PGP) bacteria (PGPB) (Philipot et al., 2013). Molecular investigations allowed the description of the complexity and diversity of bacterial communities associated to AMF spores belonging to different species and isolates, suggesting that their differential occurrence may affect the performance of the relevant taxa in terms of infectivity and efficiency, given their important functional roles as PGPB (Roesti et al., 2005; Long et al., 2008; Agnolucci et al., 2015). Other studies, aimed at isolating and functionally characterizing spore associated bacteria, reported the occurrence of bacteria showing antagonistic activity against plant pathogens (Budi et al., 1999; Bharadwaj et al., 2008a), phosphate-solubilizing and nitrogenase activity (Cruz et al., 2008; Cruz and Ishii, 2011), and indole acetic acid (IAA) production (Bharadwaj et al., 2008a). A recent work, using a culture-dependent approach, showed that bacterial strains isolated in pure culture from *Rhizophagus intraradices* spores were able to solubilize P from phytate and inorganic sources (69.7 and 49.2%, respectively), produce siderophores (65.6%), and IAA (42.6%) (Battini et al., 2016). The last two molecules are very important for plant growth and nutrition. Actually, IAA, a phytohormone of the auxin class, affects the morphology and physiology of roots, enhancing cell division and elongation, and the formation of lateral roots, thus improving water and nutrient uptake and playing a key role in the regulation of plant development (Khalid et al., 2004; Aloni et al., 2006; Duca et al., 2014). Siderophores are low molecular weight, high-affinity iron-chelating compounds able to bind soluble Fe₃, even at high pH when Fe solubility decreases (Mimmo et al., 2014), thus making it available to bacteria and plants (Colombo et al., 2014). Given the essential role played by iron in plant biochemical processes, such as photosynthesis and respiration (Kobayashi and Nishizawa, 2012), bacterial siderophores, facilitating plant Fe acquisition, represent important factors of plant growth and development (Crowley et al., 1988; Duijff et al., 1994a,b; Walter et al., 1994; Yehuda et al., 1996; Siebner-Freibach et al., 2003; Jin et al., 2006; Vansuyt et al., 2007; Robin et al., 2008). Moreover, siderophores have been reported to possess biocontrol activity against soilborne diseases, by means of iron competition (Thomashow et al., 1990; Glick, 1995; Whipps, 2001), inhibiting the development of deleterious plant pathogens (Davison, 1988; Arora et al., 2001).

Although the individual roles of AMF and their associated bacteria in optimizing plant performance are still to be

completely dissected, AMF are progressively more considered among the main factors of sustainable food (primary) production (Philippot et al., 2013; Roupheal et al., 2015). Two main strategies have been devised to exploit the benefits deriving from the mycorrhizal symbionts: the adoption of specific management practices and the use of AMF inoculation. The first one focuses on the improvement of the activity of native AMF, pursued by using crop rotation and mycotrophic cover crops, able to raise soil mycorrhizal potential and to shape native AMF communities (Kabir and Koide, 2002; Karasawa and Takebe, 2012; Lehman et al., 2012; Njeru et al., 2014, 2015; Turrini et al., 2016, 2017), and by reducing tillage intensity or chemical fertilizations, which affect AMF species composition, spore abundance and mycorrhizal colonization (Douds et al., 1995; Jansa et al., 2003; Oehl et al., 2004; Castillo et al., 2006; Brito et al., 2012; Avio et al., 2013). The second strategy focuses on the inoculation of selected AMF, either as single species or in a mixture, reported as efficient root colonizers and plant nutrition enhancers (Jeffries et al., 2003; Gianinazzi and Vosatka, 2004; Lekberg and Koide, 2005; Roupheal et al., 2015).

Many types of commercial AMF inoculum are available on the market, including sterile products obtained *in vitro* using genetically modified Ri T-DNA roots and the species *Rhizoglyphus irregularis* (synonym *Rhizophagus irregularis*, basionym *Glomus irregularis*). However, most of the commercial products are obtained from greenhouse multiplication on mycotrophic trap plants and represent a multipartite symbiosis, where a rich community of bacteria may thrive, associated with AMF propagules, and exert important functional activities, as PGPB. Here, for the first time, we explored the bacterial metagenome of a commercially available AMF inoculum by Illumina high-throughput sequencing, a method able to provide information about culturable and unculturable members of the inoculum microbiota. Moreover, we isolated and functionally selected culturable bacteria showing important PGP traits, as the ability to produce IAA and siderophores, to be utilized, individually and/or in multispecies consortia with AMF as beneficial biofertilizers/bioenhancers in sustainable agroecosystems.

MATERIALS AND METHODS

Biological Activity of the Commercial Inoculum

The commercial inoculum utilized consisted of the substrate where trap plants (*Allium ampeloprasum* var. *porrum* L.) were grown and of mycorrhizal root fragments, AMF spores, and extraradical mycelium of *Rhizoglyphus irregularis* BEG72 (synonym *Rhizophagus irregularis*, basionym *Glomus irregularis*). The substrate (vermiculite) and the seeds utilized for the inoculum production were sterilized prior to their utilization. The only microbial input in the commercial product arised from the AM fungus and its associated microbiota. The corresponding AMF inoculum is available on the market under the name "AEGIS" (Atens, Agrotecnologias Naturales S.L.). The percentage of mycorrhizal colonization of the roots contained within the inoculum was assessed on three 5 g samples by the gridline

intersect method, after clearing with 10% KOH and staining with 0.05% Trypan blue in lactic acid (Giovannetti and Mosse, 1980). The mycorrhizal potential of the commercial inoculum was assessed by using the Mycorrhizal Inoculum Potential (MIP) bioassay, as described in Njeru et al. (2014). Briefly, three replicate inoculum samples were sown with *Cichorium intybus* L. cv. Zuccherina di Trieste, put in sun-transparent bags and maintained in a growth chamber at 27°C and 16/8 h light/dark daily cycle until harvest. Roots were harvested 30 days after sowing, cleaned with tap water and cleared, stained, and examined for AMF colonization assessment, as described above.

Illumina MySeq Analysis of Bacteria Associated With the Inoculum

DNA Extraction

The composition of the bacterial community of three commercial inoculum samples was determined by Next-generation high-throughput DNA sequencing (NGS; Ansorge, 2009). Total community DNA was extracted from each sample using DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). In brief, 50 g of sample and 0.1 mL Tween 20 were suspended in saline phosphate buffer (100 mL) and homogenized in a paddle blender (BagMixer® 400, Interscience, Saint Nom, France) for three min at maximum speed. Substrate soil and root fragments were removed by low speed (1,000 g) centrifugation, then, for DNA extraction, cells were collected after centrifugation and lysed using the DNeasy PowerSoil reagents and Qiagen spin columns on a QIAcube automated station (Qiagen, Hilden, Germany).

Library Preparation

Three 16S rRNA gene amplicon libraries were prepared by PCR amplification of an approximate 630 bp region within the hypervariable (V3-V4) region of the 16S rRNA gene according to the Illumina 16S metagenomic sequencing library protocol. PCR amplification was performed with broad spectrum 16S rRNA primers (forward primer: 5'-TCGTCGGCAGCGTCA GATGTGTAT AAGAGACAGCCTACGGGNGGCWGCAG-3', reverse primer: 5'-GTCTCGTGGGCTCGGAGATGTGTA TAA GAGACAGGACTACHVGGGTAT CTAATCC-3') (Klindworth et al., 2013), using Kapa HiFi HotStart 2 × ReadyMix DNA polymerase (Kapa Biosystems Ltd., London, UK). Cycle conditions were: an initial step at 95°C for 3 min; 25 cycles of 95°C (30 s), 55°C (30 s), 72°C (30 s); a final extension of 5 min at 72°C. Libraries were purified using AMPure XP beads (LABPLAN; Naas, Ireland) according to the Illumina 16S metagenomic sequencing library protocol. Dual indices and Illumina sequencing adapters from the Illumina Nextera XT index kits v2 B and C (Illumina, San Diego, USA) were added to the target amplicons in a second Index PCR step using Kapa HotStart HiFi 2 × ReadyMix DNA polymerase (Kapa Biosystems Ltd.). Cycle conditions were: 95°C (3 min); 9 cycles of 95°C (30 s), 55°C (30 s), 72°C (30 s); a final extension of 5 min at 72°C. Libraries were again purified using AMPure XP beads (LABPLAN; Naas, Ireland) according to the Illumina 16S metagenomic sequencing library protocol. Libraries were quantified using a Qubit fluorometer (Life Technologies, Paisley, UK) and pooled in equal concentrations (4 nM) into a single pool,

according to their Qubit quantification measurement. The library pool was diluted and denatured according to the Illumina MiSeq library preparation guide. The amplicon library (8 pM) was spiked with 10% denatured and diluted PhiX Illumina control library (12.5 pM). The sequencing run was conducted on the Illumina MiSeq using the 600 cycle MiSeq reagent kit (version 3) with paired 300 bp reads.

Sequencing

Illumina sequencing was performed using MiSeq (Illumina, San Diego, CA). Paired-end sequencing used custom primers and a 600-cycle sequencing kit (V3), according to manufacturer instructions. Amplicon sequencing was carried out in the presence of 10% PhiX control (Illumina, San Diego, CA) to allow proper focusing and matrix calculations.

Bioinformatics

Raw data processing, run de-multiplexing and operational taxonomic unit (OTU) analysis were performed using the CLC Genomics Workbench (Version 11.0.1) with CLC Microbial Genomics Module (Version 3.5) (Qiagen Bioinformatics, Hilden, Germany). Such programme was used also for the estimation of alpha diversity (total richness in OTUs). Taxonomy attribution was performed against SILVA 16S v132 at the identity level of 97%.

Isolation and Characterization of Beneficial Bacteria Associated With the Inoculum

Bacterial Isolation

Three 40 g samples of the commercial inoculum were suspended in 360 mL of sterile physiological solution added with Tween 80 (0.36 μ L). The suspension was shaken for 30 min using a multi wrist shaker (Labline Instruments, Illinois, USA). Hundred microliter suspension for each sample were plated in triplicate onto Petri dishes containing different agar media. Culturable heterotrophic bacteria were isolated on Tryptic Soil Agar (TSA, 30 g L⁻¹ tryptic soy broth, 20 g L⁻¹ bacteriological agar, Oxoid, Milan, Italy), a medium which, given its non-selectivity, allows the recovery of a wide range of aerobic and facultative anaerobic gram-negative and gram-positive bacteria. In order to isolate specific functional bacterial groups, two additional selective media were used. The selective N-free Winogradsky medium (N-free W) (Tchan, 1984) was utilized for the isolation of putative nitrogen-fixing bacteria, able to grow on N-free medium. For the isolation of bacteria able to solubilize inorganic phosphate the National Botanical Research Institute's Phosphate growth (NBRIP) medium was used (Nautiyal, 1999). The three culture media were supplemented with 100 mg L⁻¹ of cycloheximide and 500 UI L⁻¹ of nystatin (Sigma-Aldrich, Milan, Italy) to inhibit possible fungal development. The number of colony forming units (CFU) was assessed after 2 and 7 days of incubation at 28°C for TSA and the other two media, respectively. Bacteria grown on N-free W and those showing halo zones formation on NBRIP, were selected and purified by streaking four times onto the same medium used for the isolation. In addition, bacteria grown in TSA medium were randomly selected on the basis of phenotypic colony characteristics, i.e., shape, size, edge

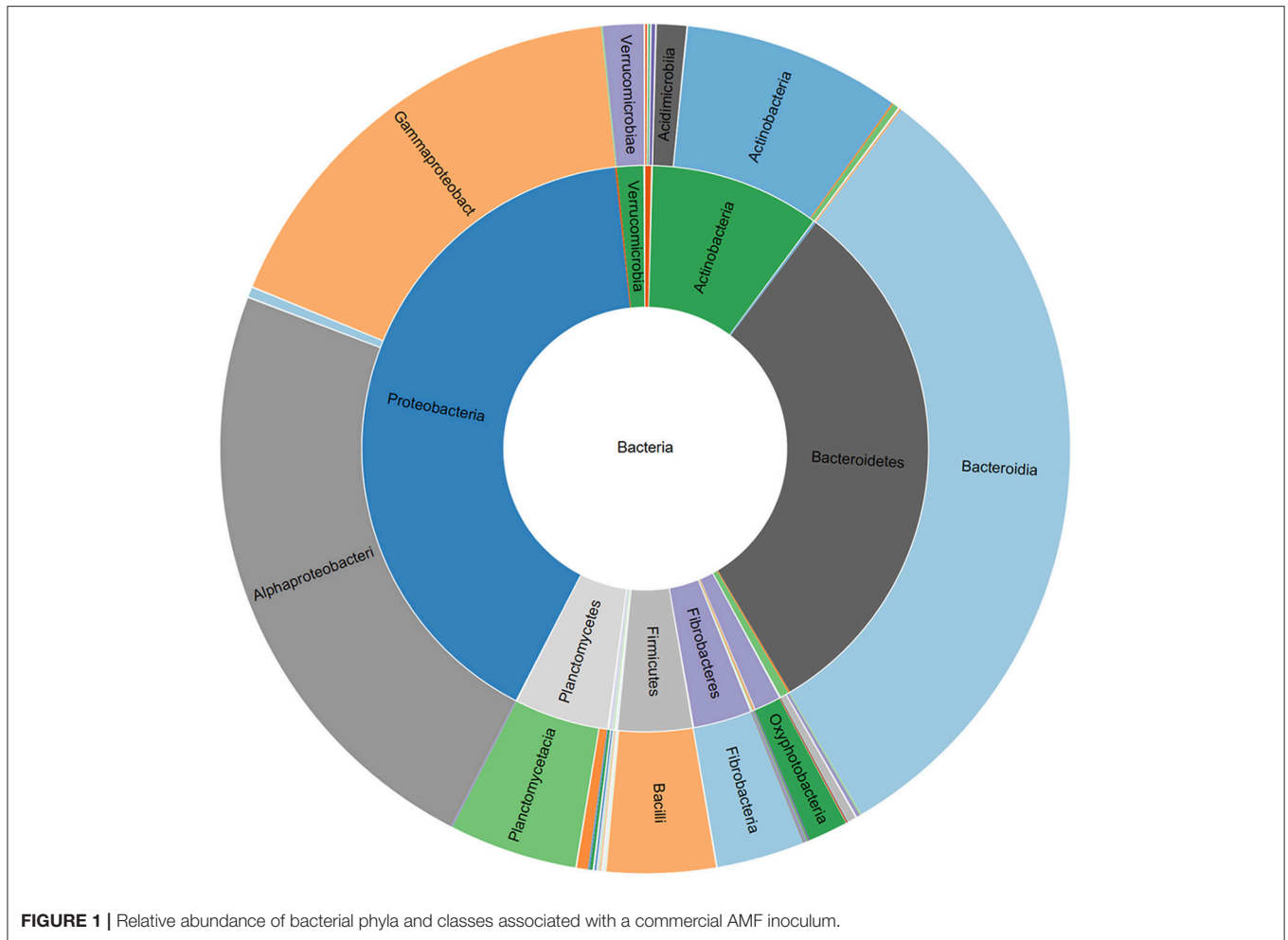
morphology, surface and pigment and inoculated onto N-free W and NBRIP media and then purified by streaking four times onto the same medium. The purified strains were maintained at -80°C in cryovials with 20% (v/v) of glycerol in the collection IMA (International Microbial Archives) of the Department of Agriculture, Food and Environment, University of Pisa.

Screening of the Selected Bacteria for PGP Traits

All the bacterial strains isolated and selected as described above, were screened *in vitro* for two functional traits linked to the promotion of plant growth and performance, i. e., the ability to produce IAA and siderophores. The production of IAA was assessed using Luria-Bertani Broth (LBB) (Bharadwaj et al., 2008a) and following the method described by Battini et al. (2016). Briefly, strains were inoculated in 4 mL of LBB amended with 1 mg mL⁻¹ of L-tryptophan (Sigma-Aldrich, Milan, Italy), incubated at 20°C until exponential growth phase was reached. They were centrifugated (7500 rpm for 10 min) and 1 mL of supernatant was transferred in a 24-well plate, mixed with 2 mL of Salkowski reagent (1.2% FeCl₃ in 37% sulfuric acid). The non-inoculated medium represented the negative control, and the medium amended with pure IAA the positive one. Development of red-purple color after 3 min incubation in the dark indicated positive strains for IAA production. Strains were classified using a rating scale as follows (Figure 1): -, no production (no color development); +/-, low production (pale pink); +, production (light purple); ++, moderate production (bright purple); + + +, high production (dark purple), considering color intensity of the positive controls, IAA (66 μ g/mL) representing the maximum value (10+) and IAA 1:2 the half (5+). The test was replicated three times. The ability to produce siderophores was investigated using the overlay Chrome Azurol S assay (CAS) described by Pérez-Miranda et al. (2007). CAS agar was prepared following the procedure described by Loudon et al. (2011). Siderophore-producing bacterial strains showed a change in color, from blue to yellow/orange, in the overlaid medium around the colonies. After 7 days the radius of the halo was measured (mm) from the colony edge to the edge of the colored halo. Strains were classified using a rating scale as follows: no production (halo = 0 mm), +/- = low production (halo < 2 mm), + = production (3 mm \leq halo \leq 8 mm), ++ = moderate production (9 mm < halo < 14 mm), + + + = high production (halo > 15 mm).

Molecular Identification of Cultured PGP Bacteria

The purified bacterial strains showing the best ability to produce IAA and siderophores 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" (Epicenter®), following the manufacturer's protocol. Bacterial 16S rRNA gene was amplified using the primers 27F (5'-GAGAGTTTGATC CTG GCT CAG-3') e 1495R (5'-CTA CGG CTA CCT TGT TAC GA-3') (Lane, 1991; Weisburg et al., 1991). The amplification reaction was carried out in a final volume of 25 μ L, containing:



μL of DyNAzyme buffer 10X (Finnzymes), $0.2\ \mu\text{M}$ of each primer, $0.2\ \text{mM}$ of each dNTPs (EuroClone), $0.625\ \text{U}$ of Taq DyNAzyme II DNA polymerase (Finnzymes) and $10\text{--}20\ \text{ng}$ of DNA. The samples were amplified using an iCycleriQ Multicolor Real-Time PCR Detection System (BIORAD), with the following PCR protocol: 95°C 2 min; 94°C 1 min and 20 s, 54°C 1 min, 72°C 1 min, and 30 s for 35 cycles; 72°C 5 min. PCR amplicons were analyzed by 1.5% agarose gel electrophoresis, stained with ethidium bromide, visualized and captured as TIFF format files by the UVITEC UV1-1D program for UVITEC Gel Documentation system Essential V6 (Cambridge, UK). The amplification products were purified by the Clean PCR CleanUp kit[®] (CABRU), quantified and 5' sequenced by Eurofins Genomics (Ebersberg, Germany), as reported in Palla et al. (2017). Sequences were analyzed using BLAST on the NCBI web (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences were aligned using MUSCLE, and phylogenetic trees were constructed using the Neighbor-Joining method based on Tamura 3-parameter method in MEGA10 (Kumar et al., 2018) software with 1,000 bootstrap replicates. The sequences were submitted to the European Nucleotide Archive under the accession numbers from LS999506 to LS999519.

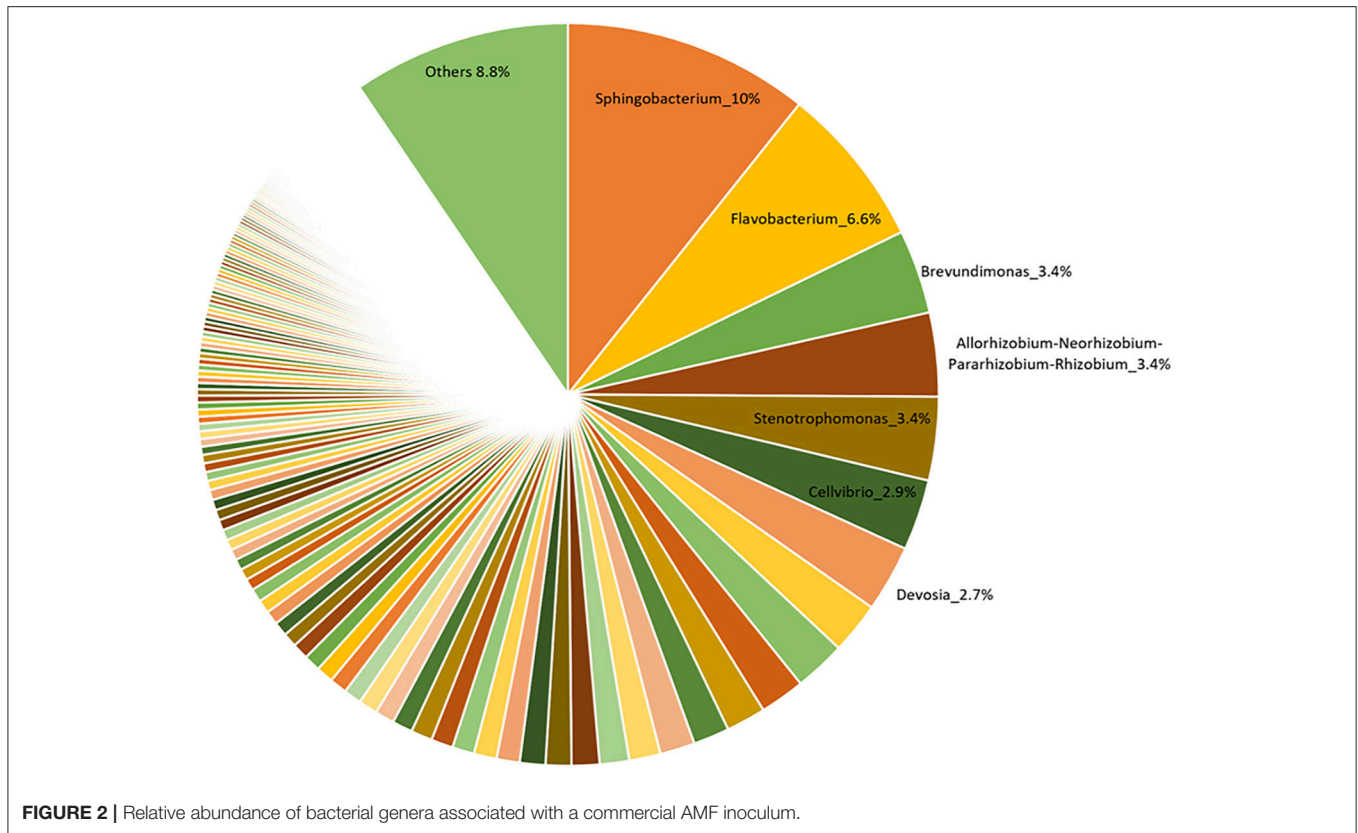
RESULTS

Biological Activity of the Inoculum

The percentage of colonized length of the root fragments contained within the inoculum was $77\pm 0.7\%$. The mycorrhizal potential of the inoculum ranged from 20 to 30%.

Illumina MySeq Analysis of Bacteria Associated With the Inoculum

The V3-V4 region of 16S rRNA gene was sequenced to analyze the composition of the bacterial microbiota associated with three different lots of AMF inoculum. NGS analysis allowed us to generate a number of reads per sample comprised between 3.1 and 3.9 million (**Supplementary Material 1**). Approximately 88% of raw reads per sample passed merging, trimming and chimera filtering steps and were analyzed for OTU search. The clustering produced a mean of reads in OTUs of $386,899 \pm 25,087$ with an average read length after trim of 232 bp. Alpha diversity (OTUs richness) value was 1485 ± 14 (**Supplementary Material 1**). In total, 23 phyla, 107 orders, 165 families, and 276 bacterial genera were identified in the samples. Nine phyla accounted for 95.8% of the sequence



reads across all samples with the majority being Proteobacteria (36.9%) and Bacteroidetes (29.3%; **Figure 1**). Other phyla that comprised $\geq 2.5\%$ of the bacterial communities were: Actinobacteria (8.4%), Planctomycetes (6.3%), Verrucomicrobia (3.7%), Firmicutes (3.3%), Patescibacteria (3.1%), Deinococcus-Thermus (2.6%), and Fibrobacteres (2.5%). The predominant orders were: Rhizobiales (23.6%), Caulobacterales (12.9%), Sphingomonadales (12.1%), and Cellvibrionales (9.0%) among Proteobacteria; Sphingobacteriales (45.1%) and Flavobacteriales (34.7%) among Bacteroidetes. A deeper phylogenetic classification of the reads revealed that the most represented genera were *Sphingobacterium* (10% of total bacteria), *Flavobacterium* (6.6%), *Brevundimonas* (3.4%), *Allorhizobium/Neorhizobium/Pararhizobium/Rhizobium* group (3.4%), *Stenotrophomonas* (3.4%), *Cellvibrio* (2.9%), and *Devosia* (2.7%) (**Figure 2**).

Isolation and Characterization of Beneficial Bacteria Associated With the Inoculum

Microbiological analyses allowed the determination of the bacterial cells associated with the inoculum. The CFU/ml number of heterotrophic bacteria ranged from 2.5 ± 0.2 to $6.1 \pm 1.4 \times 10^6$, while putative N-fixers and P-solubilizers ranged from $9.7 \pm 0.8 \times 10^5$ to $2.2 \pm 0.4 \times 10^6$ and from $9.2 \pm 0.3 \times 10^5$ to $2.4 \pm 0.9 \times 10^6$, respectively (**Table 1**). A total of 26 putative N-fixers and 9 P-solubilizers were obtained in pure culture. As an additional strain showed both characteristics, the total strains successively tested for their PGP traits were 36.

Among the 36 strains analyzed for IAA production, 6 showed the red/orange color similar to the positive controls. Such IAA producers were the isolates N-67 and N-92 within the putative N-fixers, and P-30, P-36, P-42, and P-57 within the P-solubilizers (**Supplementary Material 2**). The other isolates produced lower levels of IAA, as indicated by the golden yellow color of the substrate (**Supplementary Table S1**).

As to siderophores production, most strains showed the indicative clarification halo around the colonies, 5 of them producing a halo with a diameter higher than 5 mm, i.e. isolates N-21, N-75, N-78, and N-87 within the putative N-fixers and the isolate P-24 within P-solubilizers (**Supplementary Material 2**).

Three additional strains, P-23, N-P-27 and N-64, showed a moderate siderophore production, together with IAA production. The 14 bacterial isolates showing the best combination of PGP traits (production of IAA and siderophores) were 16S rDNA sequenced and affiliated to bacterial genera and species. Sequences were affiliated with Actinomycetales (*Microbacterium trichotecenolyticum*, *Streptomyces deccanensis/scabiei*), Bacillales (*Bacillus litoralis*, *Bacillus megaterium*), Enterobacteriales (*Enterobacter*), Rhizobiales (*Rhizobium radiobacter*, syn *Agrobacterium radiobacter/tumefaciens*) (**Table 2, Figure 3**).

DISCUSSION

In this work, for the first time, the microbiota associated with a commercial AMF inoculum was identified and characterized, using a polyphasic approach, i.e., a combination of traditional

TABLE 1 | Number of culturable bacteria isolated from three 40 g samples (A, B, C) of the AMF commercial inoculum (mean CFU/mL \pm SE) isolated from three different microbiological substrates.

Medium	A	B	C
TSA	6.1 \pm 1.4 $\times 10^6$	3.8 \pm 0.6 $\times 10^6$	2.5 \pm 0.2 $\times 10^6$
N-free W	2.2 \pm 0.1 $\times 10^6$	9.7 \pm 0.8 $\times 10^5$	2.2 \pm 0.4 $\times 10^6$
NBRIP	1.6 \pm 0.6 $\times 10^6$	2.4 \pm 0.9 $\times 10^6$	9.2 \pm 0.3 $\times 10^5$

Each value represents the mean of three replicates. TSA, Tryptic Soil Agar; N-free W, N-free Winogradsky medium; NBRIP, National Botanical Research Institute's Phosphate growth medium.

TABLE 2 | Phylogenetic identification of the 14 best performing plant growth promoting bacteria isolated from the mycorrhizal commercial inoculum (sequence accession numbers from LS999506 to LS999519).

Isolate	Identification	Identity (%)	Most closely related Genebank sequence
N-21	<i>Microbacterium trichotecenolyticum</i> DSM 8608	99	NR044937
P-23	<i>Bacillus megaterium</i> NBRC 15308	99	NR112636
P-24	<i>Bacillus megaterium</i> DSM 32	99	KJ476721
N-P-27	<i>Bacillus megaterium</i> XJGJ9	99	KR708952
P-30	<i>Enterobacter</i> sp. WP7	99	KU523560
P-36	<i>Enterobacter</i> sp. WP7	99	KU523560
P-42	<i>Enterobacter</i> sp. AJ2	99	KJ913658
P-57	<i>Streptomyces</i> sp. SCY301	99	GU045544
N-64	<i>Bacillus litoralis</i> KUDC 1714	99	KC414705
N-67	<i>Rhizobium radiobacter</i> (syn <i>Agrobacterium radiobacter/tumefaciens</i>) N70a	99	KM894180
N-75	<i>Microbacterium trichotecenolyticum</i> DSM 8608	99	NR044937
N-78	<i>Microbacterium trichotecenolyticum</i> DSM 8608	99	NR044937
N-87	<i>Microbacterium trichotecenolyticum</i> DSM 8608	99	KJ767329
N-92	<i>Enterobacter</i> sp. WP7	99	KU523560

Strains isolated from N-free Winogradsky and NBRIP, National Botanical Research Institute's Phosphate growth media are preceded by N and P, respectively.

microbiological culture-dependent analyses and metagenomic sequencing. A complex and highly diverse bacterial community was identified by Illumina high-throughput sequencing and several bacteria showing important PGP traits, as the ability to produce IAA and siderophores, were isolated and identified.

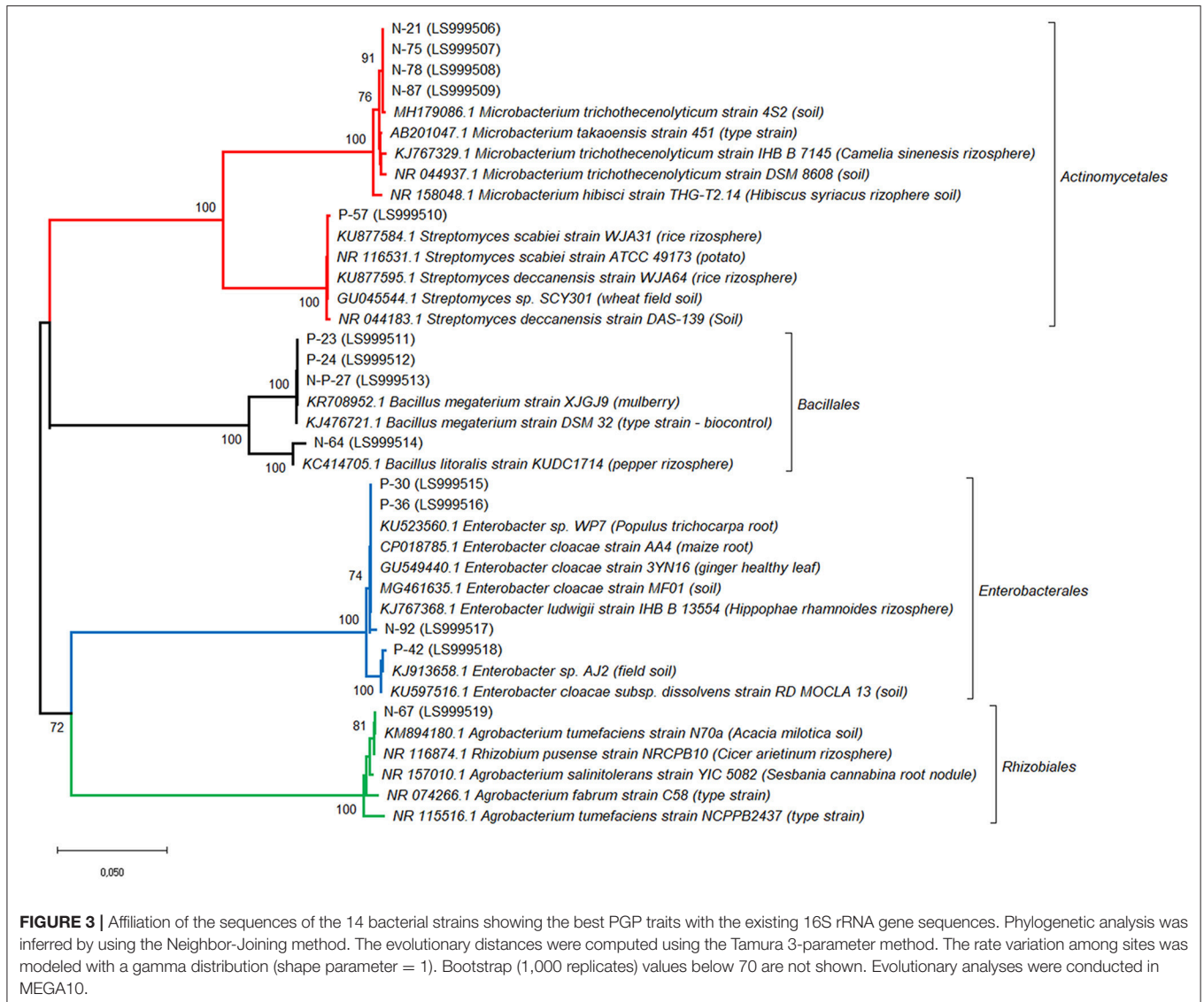
The assessment of mycorrhizal colonization of the roots contained in the inoculum and of the MIP was the necessary prerequisite for the feasibility of our study, given the recent data on the poor colonization of plant roots by a commercial AMF inoculum (Berruti et al., 2013). In our material, both roots contained in commercial inoculum and those of the plants used for the MIP bioassay were well colonized, showing that the commercial inoculum was highly infective and able to rapidly establish the mycorrhizal symbiosis.

The crude inoculum analyzed, consisting of the substrate where trap plants were grown (mycorrhizal root fragments, AMF

spores and mycelium) harbored a rich culturable heterotrophic bacterial community, whose populations ranged from 2.5 to 6.1 $\times 10^6$ CFU/mL. Such values are high, when considering the origin of the sampled material, which did not derive from living roots, but from a dry inoculum, and show that the rich bacterial community thriving in the particular ecological niche, rich in nutrients and exudates, represented by trap plants during AMF inoculum production, is able to maintain its vitality and activity through the different phases leading to the production of the commercial AMF inoculum, from plant harvest to substrate drying. Moreover, present data confirm previous molecular findings which detected large and complex bacterial communities associated with AMF spores (Roesti et al., 2005; Long et al., 2008; Agnolucci et al., 2015).

The culture-independent approach revealed the occurrence of 7 most represented bacterial genera known to include species isolated from a variety of environments that can be subjected to different environmental stresses. For example, bacteria belonging to *Sphingobacterium*, the most represented genus in the commercial inoculum, can survive at temperatures lower than 5°C (Shivaji et al., 1992) and higher than 65°C (Yoo et al., 2007), or can survive in soil contaminated with herbicides (Lü et al., 2006) or solvents (Mohammad et al., 2006). Some species of this genus have been reported to have PGP activities, such as inorganic phosphate solubilization, surfactant and IAA production (Marques et al., 2010; Ahmad et al., 2014; Ali et al., 2017), that can improve the efficacy of AMF inocula. Plant growth-promoting traits were also reported in bacteria belonging to other genera associated with the inoculum, including *Flavobacterium* (phosphate solubilization, production of phytohormones and antimicrobial compounds, Nishioka et al., 2016), *Brevundimonas* (production of IAA and ammonia, Kumar and Gera, 2014), *Stenotrophomonas* (production of antibiotics and plant growth regulators, Messiha et al., 2007) and *Devosia* (development of a nitrogen-fixing root-nodule symbiosis, Rivas et al., 2002). The potential contribution of these bacteria to the efficacy of AMF inocula is supported by recent findings reporting that inoculation with PGPB *Flavobacterium* and *Stenotrophomonas* can be effective in promoting plant growth under draft (Gontia-Mishra et al., 2016) or salinity stress (Singh and Jha, 2017). Interestingly, several sequences (2.9%) were assigned to *Cellvibrio*, a genus known for its cellulose and complex carbohydrate degradation potential, which was previously retrieved from AMF spores, where it was supposed to feed on components of the spore walls, thus facilitating AMF spore germination (Roesti et al., 2005). Many other genera were represented in the bacterial community associated with the commercial inoculum (**Supplementary Material 1**). Among them, several sequences occurring at low frequencies were ascribed to *Streptomyces* (0.22%), *Enterobacter* (0.24%), *Bacillus* (0.66%), *Microbacterium* (0.83%), genera to which our selected strains belonged.

Here, the inoculation and successive purification on selective media allowed the initial isolation of 36 bacterial strains, and their subsequent screening allowed the selection of the 14 best performing strains showing important PGP traits. Six and five strains were strong producers of IAA and siderophores,



respectively, while two of them (N-67 and N-92), displayed at high levels the two PGP traits. The occurrence of such bacterial functional groups in the commercial inoculum further supports our previous evidence that the beneficial microbiota associated with AMF maintains not only its vitality and activity, but also its functional properties during the different phases of the life cycle (Battini et al., 2016). The ability of such strains to produce IAA, a hormone enhancing cell division and boosting the development of plant root systems (Patten and Glick, 2002) and siderophores, able to facilitate plant acquisition of Fe, thus acting as potential biocontrol agents against soilborne plant pathogens (Glick, 1995; Arora et al., 2001; Whipps, 2001; Battini et al., 2016), confirms the need and utility of adopting culture-dependent methods in order to gain knowledge on functional traits of AMF-associated bacteria. The availability of such beneficial bacteria in pure culture allows their use in ecological studies aimed at investigating their mycorrhizospheric competence and role in plant growth promotion.

Fourteen bacterial strains showing the best combination of PGP traits were identified by 16S rDNA sequencing. Interestingly, 5 out of 14 strains (36%) belonged to Actinomycetales: among them, the *Microbacterium trichotecenolyticum* strains N-21, N-75, N-78, N-87, and the *Streptomyces* sp. strain P-57 were strong siderophores and IAA producers, respectively. Actinobacteria are ubiquitous in the soil and able to produce many biologically active secondary metabolites, including antibacterial, antifungal, antiparasitic, anticancer and immunosuppressant drugs (Wolf and Zähler, 1972; Weitnauer et al., 2001; Ritacco and Eveleigh, 2008; Qin et al., 2014) and/or to utilize a wide range of complex compounds (Vandera et al., 2015). They were previously reported to live in strict association with spores and hyphae of different AMF, including *F. coronatum*, *F. mosseae*, and *R. intraradices* (Walley and Germida, 1996; Andrade et al., 1997; Bharadwaj et al., 2008b; Agnolucci et al., 2015; Battini et al., 2016). Many Actinobacteria showed PGP traits, acting as antagonists against

plant pathogens, and mycorrhizal helper traits, enhancing mycorrhizal colonization and AMF functionality (Bharadwaj et al., 2008a; Hamdali et al., 2008; Giovannetti et al., 2010).

Members of the genus *Microbacterium* are ubiquitous in many environments and considered important players of biogeochemical cycles, due to their diazotrophic properties and endophytic behavior (Miliute et al., 2015). Consistent with our findings a *M. trichotecenolyticum* strain isolated from roots of wild *Dodonaea viscosa* L. was reported to possess multiple plant growth promoting activities, such as siderophore and IAA production (Afzal et al., 2017).

The genus *Streptomyces* is one of the main component of soil bacterial communities and is considered within the promising taxa to be investigated for PGP activity, given its ability to solubilize phosphates and produce growth regulators (Mohandas et al., 2013; Hamed and Mohammadipanah, 2015), two activities shown also by our strain P-57. Actually, two *Streptomyces* strains, W94 and W77, isolated from the spores of the AM fungus *R. irregularis* IMA6, significantly increased the uptake and translocation of ^{33}P in maize plants, and hyphal length specific ^{33}P uptake, respectively, compared with control plants (Battini et al., 2017). On the other hand, other IAA-producing bacteria isolated from AMF propagules were able to increase AMF development (Bidondo et al., 2011), in agreement with previous data reporting that *Streptomyces* spp. boosted AMF spore germination and hyphal growth (Mugnier and Mosse, 1987; Tylka et al., 1991; Carpenter-Boggs et al., 1995), thus showing mycorrhizal helper traits.

Four out of 14 strains (28%) were affiliated with Bacillales, and belonged to the species *Bacillus megaterium* and *Bacillus litoralis*. All of them produced siderophores, activity previously reported in other members of the order (Battini et al., 2016), known for their ability to control soilborne pathogens (Jeong et al., 2014) and to act as PGP and mycorrhizal helper bacteria, facilitating mycorrhizal establishment and improving plant growth (Budi et al., 2013; Pérez-Montañó et al., 2014; Zhao et al., 2014). The isolation of *Bacillus* species from our commercial inoculum represents a further confirmation of previous data obtained by culture-independent methods (Agnolucci et al., 2015).

One strain, *Rhizobium radiobacter* (syn. *Agrobacterium radiobacter/tumefaciens*) N-67, was affiliated to the Rhizobiales, an order thoroughly investigated for the ability of its members to fix nitrogen. This isolate was one of the two only strains able to produce both IAA and siderophores, confirming previous data on PGP ability of some rhizobia to boost plant nutritional status by producing phytohormones (Zahir et al., 2003; Chandra et al., 2007; Dodd et al., 2010). Its persistence in the AMF inoculum may be ascribed to the formation of biofilms containing exopolysaccharides which allow an efficient colonization of roots and mycorrhizal hyphae (Bianciotto et al., 1996; Toljander et al., 2006).

A very interesting finding is represented by the isolation of 4 strains, P-30, P-36, P-42, N-92, affiliated with Enterobacteriales (*Enterobacter cloacae/ludwigii*), which were strong producers of IAA, confirming previous data on the capacity of a strain of *E. cloacae* to produce as much IAA as a *Pseudomonas* strain (Imen et al., 2013). Recent works reported that a few

strains of the genus *Enterobacter*, isolated from legume plants, possessed multiple plant-growth promoting characteristics, such as phosphate solubilisation activity and IAA production, thus affecting plant growth and development (Ghosh et al., 2015; Khalifa et al., 2016). On the other hand, one of our isolates, N-92, produced also siderophores, activity already reported for members of the genus *Enterobacter* (Tian et al., 2009).

In conclusion, this work demonstrates for the first time that an AMF inoculum, produced following industrial production processes, is home of a large and diverse community of bacteria with important functional PGP properties, possibly acting in synergy with AMF and providing new services and benefits. The commercial AMF product could be enriched with the selected beneficial bacterial isolates utilized as an additional inoculum, further boosting plant growth, nutrition and health, in order to optimize plant performance in sustainable food production systems. Indeed, our findings imply a new perspective of AM symbiosis, that of a multipartite association - host plants, AMF and bacteria - where different microbial functional groups are active: for example, specific mycorrhizospheric bacteria, by solubilizing P and fixing N, may improve the availability of key mineral nutrients, then absorbed and translocated to the host plant by AMF extraradical hyphae, while other bacteria, by producing siderophores and IAA, may control plant pathogens and promote plant growth. Notwithstanding, so far only few works have been carried out either on the isolation and functional characterization of mycorrhizospheric microbiota, or on their occurrence and significance in AMF inocula. Yet, these studies are necessary and urgent, in the perspective of developing new strategies for sustainable intensification in agriculture, aimed at minimizing the use of chemical fertilizers and pesticides, promoting primary production and maintaining soil health and fertility. To this aim, the most diverse combinations of AMF and bacteria should be studied, in model experimental systems and in the field, to discover possible synergistic effects on different host plants, in order to select the best performing ones for their targeted use in sustainable food production systems in the years to come.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2018.01956/full#supplementary-material>

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