Methods in Rhizosphere Biology Research

1. Title

Molecular and functional characterization of beneficial bacteria associated with AMF spores

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1.1. Abstract

In the years to come, a major challenge for agriculture will be the implementation of sustainable intensification of agricultural pratice, to ensure sufficient food production for the growing global population and to reduce chemical and energy inputs. This aim may be pursued by promoting the efficient use of beneficial soil microorganisms, that play fundamental roles in plant growth and health. Among them, arbuscular mycorrhizal fungi (AMF), and their associated microbiota, can be considered biofertilizers, bioenhancers and biocontrol agents, showing diverse plant growth promoting (PGP) properties. Here we focus on approaches for the study of the identity and function of bacteria associated with AMF spores, referred to as spore-associated bacteria (SAB). Culture-independent methods are essential for the identification of their diversity, however, only culture-dependent approaches allow the determination of SAB functional roles, and the selection of the best performing strains, to be tested in laboratory experiments, as well as in the field. The discovery of SAB functional activities, e.g. phosphate solubilization and nitrogen fixation, as well as production of phytohormones, siderophores and antibiotics, is opening new avenues for their targeted management in agriculture. In this chapter the approaches, techniques and results relevant to culture-independent and culture-dependent studies on beneficial SAB will be reviewed. Significant case studies dealing with SAB utilization as inoculants in experimental trials will be discussed, with the aim of prospecting their utilization, individually or in specially designed multifunctional consortia, in sustainable and innovative food production systems.

1.2. Introduction

In the years to come one of the major problems to tackle will be represented by food production for a growing global population, while minimizing chemical inputs to the soil and adverse environmental impacts. This objective can be pursued by promoting sustainable methods for intensified agriculture, founded on the efficient use of natural soil resources, such as beneficial microorganisms, that are the fundamental components of soil nutrient flows, playing key roles in the completion of biogeochemical cycles. Among beneficial soil microorganisms, arbuscular mycorrhizal (AM) fungi (AMF) represent a key functional group, facilitating the uptake and transfer of mineral nutrients, such as phosphorus (P), nitrogen (N), sulfur (S), potassium (K), calcium (Ca), copper (Cu) and zinc (Zn), from the soil to the host plants, in exchange for plant carbon, on which they depend as chemoheterotrophic organisms (Smith and Read, 2008). AMF are important in agroecosystem processes, as they enhance carbon sequestration and soil aggregation, and plant tolerance to biotic and abiotic stresses (Gianinazzi et al., 2010). Moreover, AMF can also increase the content of healthy secondary metabolites, an essential property for the production of sustainable high-quality foods (Sbrana et al., 2014).

Recent studies reported that the services provided by AMF are often facilitated by the abundant and various microbiota living in association with spores, sporocarps and extraradical mycelium. Such beneficial microbiota play many plant growth promoting (PGP) roles, including nitrogen fixation, P solubilization and mineralization, the production of indole acetic acid (IAA), siderophores and antibiotics (Barea et al., 2002; Rouphael et al., 2015). AMF spores have been identified as a rich source of bacteria (spore associated bacteria, SAB) to be investigated for their potential PGP activities, with the aim of selecting the best performing strains to be used as biofertilisers and bioenhancers in innovative and sustainable food production systems. The aim of this chapter is to review the developments which contributed to disclose the previously underestimated networks of functional interactions occurring in and around AMF spores. This review will focus on the approaches, techniques and results that allowed the isolation and selection of SAB strains with specific functional traits.

1.2.1. Arbuscular mycorrhizal fungi

AM fungal symbionts belong to the subphylum Glomeromycotina (Spatafora et al., 2016) and show a very low host specificity, establishing mutualistic symbioses (mycorrhiza) with the roots of more than 80% of the species within all major land plant taxa, including the most important food crops, such as cereals, pulses, potatoes, fruit trees, vegetables and officinal herbs (Smith and Read, 2008). AMF are obligate biotrophs, as their life cycle cannot be completed in the absence of host plants. When their spores germinate, AMF produce hyphae able to recognize host roots and to differentiate specialized structures on the root surface, the appressoria, which give rise to hyphae growing intercellularly within the root cortex, eventually forming intracellular structures similar to haustoria, the arbuscules. Arbuscules are formed by successive dichotomous hyphal branching and are the key structures of the symbiosis, which are required for nutrient exchange between the two partners: AMF obtain carbon from the host plant and release mineral nutrients absorbed and translocated by the large mycelial network spreading from colonized roots into the surrounding soil (Smith and Read, 2008).

After reaching their sources of energy and carbon in the host cells, AMF can complete their life cycle producing new spores (Giovannetti, 2000) and intraradical vesicles, spore-like storage structures containing lipids. Some AMF species produce spores in the roots, which, in the juvenile stage are very difficult to distinguish from vesicles.

Two types of AM colonization are known: *Arum*-type and *Paris*-type (Gallaud, 1905, quoted in Smith and Read, 2008). The *Arum*-type is characterized by the spread of fungal symbiont between cortical root cells. Vesicles, when present, are intercellular or intracellular and arbuscules are terminal on intracellular hyphal branches (Smith and Smith, 1997). In the *Paris*-type intercellular hyphae are not produced, as the fungus spreads directly from cell to cell within the cortex and forms intracellular hyphal coils and intercalary arbuscules along the coils. Most of the experimental works have been carried out on the *Arum*-type mycorrhizas, which are widely distributed in natural and agricultural ecosystems.

The extraradical mycelium (ERM), consisting of a large network of hyphae extending from colonized roots into the soil, represents the key element of the symbiosis, as its structure, extent and interconnectedness are of fundamental importance for the flow of mineral nutrients absorbed from the soil and translocated to the root cells of host plants. The efficient functioning of such auxiliary absorbing system is determined by the high surface-to-volume ratio of the hyphae, by hyphal P absorption beyond the P

depletion zone around roots and by the occurrence and differential expression of nutrient transporter genes on ERM hyphae (Karandashov and Bucher, 2005; Casieri et al., 2013; Pepe et al., 2017).

AMF produce asexual, multinucleate spores, whose phenotypic characteristics, such as shape, color, size, spore walls, subtending hyphae, sporocarp occurrence, and mode of spore germination, are utilized for their morphological identification. AMF spores, whose diameters range from about 50 to 600 μm, develop from extraradical hyphae, either single or aggregated to form more complex structures, the sporocarps, and live tightly associated with highly diversified microbiota. Some unculturable endobacteria were detected inside the spore cells either by ultrastructural studies (Mosse, 1970; MacDonald and Chandler, 1981; MacDonald et al., 1982; Bianciotto et al., 1996), or by molecular methods (Naumann et al., 2010; Desirò et al., 2014). Besides these unculturable intracellular organisms, a highly diverse microbial community lives on the spore surface, sometimes sandwiched between the outer and inner spore walls or in the microniches formed by the peridial mycelium surrounding spores and sporocarps (Ames et al., 1989; Walley and Germida, 1996; Filippi et al., 1998; Maia and Kimbrough, 1998; Artursson and Jansson, 2003).

1.2.2. Bacteria associated with AMF spores and their functional roles

SAB were studied by culture-dependent and independent approaches. Molecular studies, such as PCR denaturing gradient gel electrophoresis (PCR-DGGE) method, allowed the detection of bacteria associated with *Funneliformis geosporus*, *Septoglomus constrictum* and *Gigaspora margarita* spores (Roesti et al., 2005; Long et al., 2008). We recently identified, by PCR-DGGE and band sequencing, many different bacterial taxa living in close association with the spores of six AMF isolates, belonging to Actinomycetales, Bacillales, Burkholderiales, Pseudomonadales Rhizobiales, and Mollicute-related endobacteria (Mre). Interestingly, most of them fall in clades containing PGP bacteria, as capable of increasing nutrient availability, by P solubilization, nitrogen fixation and phytohormones production, and protecting plants against fungal pathogens by the production of antibiotics, siderophores and hydrolytic enzymes (Agnolucci et al., 2015).

With the aim of exploiting PGP bacteria, culture-dependent investigations were carried out, utilizing AMF spores as a source of culturable bacteria, isolated from the spores and spore walls of *Glomus versiforme*, *Rhizophagus clarum* NT4, *G.*

margarita, *Rhizophagus irregularis* (formerly *Rhizophagus intraradices* and *Glomus irregulare*) and *Funneliformis mosseae* (Mayo et al., 1986; Xavier and Germida, 2003; Cruz et al., 2008; Bharadwaj et al., 2008b; Lecomte et al., 2011). In a recent study 374 bacterial strains were isolated in pure culture from *R. intraradices* spores, with numbers ranged from 5 to 23 CFU per spore (Battini et al., 2016b).

Isolated mycorrhizospheric bacteria were characterized for their functional properties, in order to understand how their interaction, either as individual strains or as a consortium, with AMF could affect plant performance. The first functional trait to be assessed was the ability to improve spore germination and boost mycorrhizal activity (Mayo et al., 1986; Xavier and Germida, 2003; Giovannetti et al., 2010), which lead to the description of such bacteria as "mycorrhiza helpers" (Frey-Klett et al., 2007). Several studies had previously reported that diverse soil microorganisms affected spore germination and hyphal extension (Mosse, 1959; Azcòn, 1987; 1989). For example, several *Streptomyces* species*, Pseudomonas* sp. and *Corynebacterium* sp. increased the germination of *F. mosseae*, *Glomus versiforme* and *G. margarita* spores (Mayo et al., 1986; Mugnier and Mosse, 1987, Tylka et al., 1991; Carpenter-Boggs et al., 1995). *Klebsiella pneumoniae* and *Trichoderma* sp. enhanced hyphal extension of *Glomus deserticola* and *F. mosseae* germlings (Will and Sylvia, 1990; Calvet et al., 1992), while one bacterium of the Oxalobacteriaceae was able to enhance spore germination, germling growth and root colonization (Pivato et al., 2009). Recent work confirmed that bacterial taxa belonging to Oxalobacteriaceae (Burkholderiales) lived tightly associated with hyphae and spores of diverse AMF species and genera (Scheublin et al., 2010; Agnolucci et al., 2015). The mechanism underlying the important functional role of spore germination enhancement was ascribed to the capacity of some of the bacterial taxa to degrade chitin, the main component of AMF spore walls, thus facilitating spore germination (Roesti et al., 2005). Indeed, chitinolytic bacteria were isolated from washed, healthy spores of *Glomus macrocarpum* and *F. mosseae* (Ames et al., 1989; Filippi et al., 1998), and from the inner layers of *R. intraradices* spore walls (Battini et al., 2016b).

Besides facilitation of spore germination, the microbiota of the sporosphere may play the role of "mycorrhiza helper" by improving the growth of AMF extraradical mycelium (ERM). For example, *Paenibacillus rhizosphaerae*, *Azospirillum* sp., *Rhizobium etli* and several *Pseudomonas* strains significantly improved ERM growth in *R. irregularis in vitro* (Bidondo et al., 2011; Ordoñez et al.,

2016), while the strains DF57 of *Pseudomonas fluorescens* and Bc2 of *Burkolderia cepacia* enhanced mycelial development of *Glomus caledonium* and *G. intraradices in vivo*, respectively (Ravnskov and Jakobsen, 1999; Ravnskov et al., 2002). Recently, by quantifying the length of AMF hyphae in the soil, *Sinorhizobium meliloti* TSA41 and *Streptomyces* sp. W43N were reported to increase hyphal growth by 24%, compared with hyphal lengths assessed in AMF plants without bacterial inoculation (Battini et al., 2017). The mechanisms underlying this growth promotion could be related to the production of IAA and indole butyric acid (IBA), as the exogenous application of these phytohormones was reported to promote hyphal growth of *Diversispora versiformis* (Liu et al., 2016).

Another fundamental feature of mycorrhizospheric bacteria investigated by many authors was their biocontrol activity against phytopathogens, putatively attributed to their capacity to produce antibiotics (Citernesi et al., 1996; Budi et al., 1999; Li et al., 2007; Bharadwaj et al., 2008a). Actually five *Streptomyces* isolates, obtained from *R. intraradices* spores, were molecularly affiliated to strains able to produce the antibiotics chloramphenicol, kirromycin, actinomycin G and avilamycin A (Battini et al., 2016b). However, also siderophore-producing strains, which in the quoted work represented 66% of all isolates, could play a role in the biocontrol of fungal diseases, due to their ability to inhibit pathogens growth by means of siderophore-mediated competition for iron (Davison, 1988; Thomashow et al., 1990; Glick, 1995; Arora et al., 2001; Whipps, 2001).

SAB display other multifunctional PGP activities: they can mediate the uptake of major plant nutrients, such as P and N (Barea et al., 2002). Recent studies reported that highly active P-solubilizing bacteria associated with *F. mosseae* and *R. intraradices* spores belong to *Streptomyces* and *Leifsonia* (Mohandas et al., 2013) and to *S. meliloti* (Battini et al., 2016b), respectively. Such bacteria could represent a very important factor in plant nutrition, acting synergistically with AMF to increase P availability, as P is rapidly immobilized and in many soils is unavailable to plant roots. Other studies, utilizing both culture-independent and culture-dependent methods, revealed that diverse bacterial species known as N-fixers lived tightly associated with AMF spores and that many strains belonging to Rhizobiales could be isolated, some of which possessing the *nifH* gene amplicon, confirming the key multifunctional roles played by SAB in mediating the acquisition of major plant nutrients (Bharadwaj et al., 2008b; Agnolucci et al., 2015; Battini et al., 2016b).

In the years to come, further research should thoroughly dissect the complex networks of interactions occurring among AMF, associated bacteria, and host plants, in order to reveal the new properties emerging from their possible synergies. To this aim, the data on the diversity and composition of AMF-associated bacterial communities obtained by molecular studies should be integrated with those on their functional roles, in the perspective of utilizing the best-performing consortia of AMF symbionts and their associated bacteria in innovative food production systems.

1.3. Approaches, techniques, and results

1.3.1. Fungal material and spore collection

Whatever the approach to the study of SAB, the first and indispensable step is represented by spore rinsing, as many and different taxa of generalist bacterial contaminants occur on the surface of spores, either collected from the field or pot-cultures. Spores extracted from soil (Gerdermann and Nicolson, 1963) were selected under a dissecting microscope, suspended in 1 mL of physiological solution (PS) (9 g L^{-1} NaCl), rinsed using a vortex mixer at 1500 rpm for 1 min, then aseptically successively rinsed 15 times in PS. Spores were not rinsed further, as 15 washings were effective for spore surface decontamination.

1.3.2. Culture-independent approaches for the detection of bacteria tightly associated with AMF spores

1.3.2.1. Techniques

Culture-independent approaches are particularly useful when studying SAB, as they are able to overcome the problem of underestimation due to the limitations of cultivation substrates and conditions, and of the occurrence of bacteria in viable but non-culturable state. One of the most utilized method is PCR- DGGE analysis of the 16S ribosomal RNA (rRNA) gene, able to obtain the complete fingerprinting of SAB microbiota (Fig. 1).

Fig 1. Simplified schematic representation of the detection of the different bacterial species living tightly associated with AMF spores, carried out by using the culture-independent method PCR-DGGE.

The washed spores were homogenized in sterile water, the homogenate centrifuged at 11,700 g for 20 min, and the supernatant molecularly analyzed. DNA was extracted using a kit, such as MasterPureTM Yeast DNA Purification kit. Bacterial populations were analyzed by amplification of the V3-V5 of 16S rDNA, utilizing the primers 341 F (CCTACGGGAGGCAGCAG) and 907R (CCGTCAATTCCTTTRAGTTT) (Yu and Morrison, 2004). An additional 40-nucleotide GC-rich tail was added at the primer 341 F 5′ end. Amplification was performed in 50 µL, with 10–20 ng of DNA, 5 µL of 10× Gold Buffer ($MgCl_2$-free), 2 mM of $MgCl_2$, 1.25 U of AmpliTaq Gold (Applied Biosystem), 0.2 mM of each dNTP and 0.5 µM of each primer. The reactions were performed with a thermocycler with the following cycle parameters: 95 °C for 10min; 94°C for 30s, 55°C for 30s, 72°C for 60s (for 35 cycles); 72 °C for 10 min. Amplicons of 560 bp were detected by electrophoresis in 1.5 % (w/v) agarose gel.

For DGGE and fingerprinting analysis, 20 µL of amplicons, supplemented with 20 µL of buffer 2× made with 70 % glycerol, 0.05 % xylene cyanol and 0.05 % bromophenol blue were loaded on a 8 % polyacrylamide-bisacrilamide (37.5:1) gel with an urea-formamide denaturing gradient ranging from 30 to 65 %. A combination of 16S rDNA from several bacterial species was added in the middle and at both ends of each gel as DGGE markers. Gels were run at 80 V and 60 °C for 16 h and stained for 30 min in 500 mL of TAE 1× buffer containing 50 µL of SYBR Gold Nucleic Acid Gel Stain. DGGE profiles may be digitally processed and analyzed with BioNumerics software, as reported in Agnolucci et al. (2015), in order to obtain data on the diversity of SAB populations, obtained through clustering and multivariate analyses, determination of richness, dominance and evenness diversity indices. In addition, the identification of the individual bacterial species was carried out by sequencing the DNA of DGGE bands excised from the gels, using the same primers described above, devoid of the GC-rich tail. Amplicons were purified, quantified and 5' sequenced. Sequence similarities were determined using the Basic Local Alignment Search Tool (BLASTn). Sequences were aligned with those corresponding to the closest matches from GenBank using MUSCLE as implemented in MEGA software (Edgar, 2004a, b), and phylogenetic trees were inferred using the maximum likelihood method based on the Kimura 2-parameter model (Kimura, 1980) in

MEGA. The confidence of branching was assessed using 1000 bootstrap replicates. The DGGE band sequences were submitted to an official nucleotide archive, such as EMBL or NCBI.

1.3.2.2. Results

After PCR-DGGE the profiles obtained from spore homogenates were analyzed. In the case that spores from different AMF species or isolates were investigated, it was possible to compare the banding patterns, analyze them by unweighted pair group method using arithmetic average (UPGMA) and obtain a dendrogram showing the relationships among the different samples, based on similarity and evaluated by the Dice coefficient (Fig. 1).

If the bands are excised from the DGGE gel and sequenced, it is possible to identify the bacterial species and estimate their relative abundance in the different samples. Fig. 2 shows the results obtained in a work investigating the microbiota associated with the spores of six different AMF: each isolate was characterized by a diverse bacterial community composition. Species of the genus *Arthrobacter* and *Streptomyces* (Actinomycetales) were retrieved, together with members of the orders Burkholderiales, Rhizobiales, Bacillales and Pseudomonadales and with two different endobacteria related to Mollicutes and Burkholderiaceae (Agnolucci et al., 2015).

Fig. 2. Relative abundance (%) of the microbiota associated with six geographically different AMF isolates belonging to one isolate of *F. coronatus*, two isolates of *R. intraradices* and three isolates of *F. mosseae*.

The high diversity and richness of the bacteria tightly associated with AMF spores have been ascribed to the abundance of nutrients occurring in the sporosphere, a privileged niche where bacteria are able not only to establish and thrive, but also to multiply and play multiple key roles, as biofertilizers, (phosphate solubilizing, nitrogen fixing and chitinolytic bacteria), biocontrol agents (siderophore and antibiotic producing bacteria) and as bioenhancers (PGPB).

1.3.3. Culture-dependent approaches for the quantification of bacteria associated with AMF spores

1.3.3.1. Techniques

The washed spores (see 1.3.1) were homogenized and suspended in sterile physiological solution. 100 µL suspension were inoculated onto different microbiological substrates. Spore-forming bacteria were isolated from 1 mL of heat-treated (80°C for 10 min) spore suspension. The medium Tryptic Soy Agar (TSA), supplemented with 500 UI L^{-1} of nystatin and 100 mg L^{-1} of cyclohexymide, was utilized to isolate heterotrophic and spore-forming bacteria.

1.3.3.2. Results

SAB abundance was assessed by counting the number of colonies developed after 2 days at 28°C. Then, the selection of bacterial isolates was performed based on phenotypic colony characteristics, i.e., shape, size, edge morphology, surface and pigment. The isolates should be purified by streaking several times onto the same media utilized for isolation. The pure culture strains can be maintained at –80° C. It is important to mention that from a single spore it is possible to retrieve 5-23 CFUs (on TSA medium) (Bharadwaj et al., 2008b; Battini et al., 2016b).

1.3.4. Culture-dependent approaches for the detection of SAB showing specific functional traits

1.3.4.1. Techniques

Specific bacterial groups or SAB with particular functional properties were isolated using selective media. For example, Actinobacteria are isolated from Waksman's agar medium supplemented with 5 mg L^{-1} of polymyxin and with 100 mg L^{-1} of cyclo-hexymide and 500 UI L^{-1} of nystatin to inhibit the growth of gram-negative bacteria and fungi. Chitinolytic bacteria are isolated from minimal medium containing chitin as the only source of carbon (Souza et al., 2009), and putative nitrogen-fixers are isolated from Winogradsky agar (Tchan, 1984). 100 mg L^{-1} of cyclohexymide and 500 UI L^{-1} of nystatin were added to inhibit the growth of moulds.

The bacterial isolates may be further characterized by assessing their PGP activities, such as IAA and siderophore production, P solubilisation and nitrogen fixation ability, and then identified by the sequencing of 16S rDNA (Fig. 3).

Fig. 3. Simplified scheme for isolating and selecting PGP bacterial strains living tightly associated with AMF spores.

IAA production by SAB isolates was assessed by inoculating the bacteria in 4 mL of Luria–Bertani Broth (LBB), supplemented with 1 mg mL^{-1} of l-tryptophan, incubated at 20°C in aerobiosis and centrifuged at 7500 rpm for 10 min. Then, 1 mL of supernatant was added to 2 mL of Salkowski reagent (1.2% $FeCl_3$ in 37% sulfuric acid) and placed in the dark for 30 min. The non-inoculated medium and the medium supplemented with pure IAA represent the negative and positive controls, respectively. Samples developing a red/purple color indicate IAA production.

The production of siderophores can be assessed by the over-lay Chrome Azurol S assay (CAS) (Pérez-Miranda et al., 2007). CAS agar is prepared following Louden et al. (2011) using 30.24 g L^{-1} piperazine-1,4-bis(2- ethanesulfonic acid) (PIPES), 72.9 mg L^{-1} hexadecyltrimetyl ammonium bromide (HDTMA), 1 mM $FeCl_3$ $6H_2O$ in 10 mM HCl 10 mL and 0.9 g L^{-1} bacteriological agar. The bacterial strains, inoculated on TSA, were incubated at 28°C for 2–7 days. Then, 10 mL of CAS agar were overlaid on the bacterial colonies and incubated at 25°C. The strains producing siderophores showed a yellow/orange halo around the colonies, which was measured after 7 days.

The capacity of solubilizing organic and inorganic phosphate by SAB is assessed using the National Botanical Research Institute's Phosphate growth medium (NBRIP) (Nautiyal, 1999), and Phytate Screening Medium (PSM) (Jorquera et al., 2008). In the two tests, the bacterial isolates were spotted onto agar plates and grown at 28°C for 7 days. Phytate and phosphate solubilization ability of the relevant bacteria were indicated by halo zones around bacterial colonies, that are recorded, as well as colony diameter. Bacterial P solubilization capacity is evaluated as phosphate Solubilization Efficiency (SE), as described by Rokhbakhsh-Zamin et al. (2011). The Phosphate Solubilization Index (PSI) was calculated according to Islam et al. (2007).

Putative N-fixers can be screened by PCR amplification of *nifH* genes. DNA was extracted from microbial cultures grown overnight at 28°C using a kit, such as MasterPureTM Yeast DNA Purification kit. The degenerate primers 19F (5'-GCIWTYTAYGGIAARGGIGG-3') and 407R (5'-AAICCRCCRCAIACIACRTC-3') were used to amplify a 390bp fragment of *nifH* gene (Ueda et al., 1995). Amplification was carried out in 25 µl, with 10–20 ng of DNA, 1× Reaction buffer, 0.2 mM of each dNTPs, 0.5 µM of each primers and 1.25 U of Takara ex Taq DNA polymerase. The reaction was carried out in a thermocycler with the following cycles: 94°C 1 min; 94°C 30 s, 56°C 30 s, 72°C 30 s for 35 cycles; 72°C 5 min. Amplicons were revealed by electrophoresis in 1.5% (w/v) agarose in TBE 1× buffer gels stained with ethidium bromide 0.5 µg mL^{-1}. The gels were captured as TIFF format files.

The selected PGP bacteria were identified by 16S rDNA sequencing. DNA was extracted from liquid cultures grown overnight at 28°C using the MasterPureTM Yeast DNA Purification kit. 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). PCR was performed in 50 µL, with 10‑20 ng of DNA, 1× Reaction buffer, 2 mM MgCl2, 1.25 U EuroTaq DNA polymerase, 0.2 mM of each dNTPs and 0.2 µM of each primers, using a thermocycler with the following cycles: 95◦C 2min; 94◦C 1 min and 20s, 54°C 1 min, 72°C 1 min and 30s for 35 cycles; 72°C 5 min. PCR amplicons were analyzed, then purified and sequenced as described above.

1.3.4.2. Results

The number of SAB isolated per spore on TSA medium ranged from 5 to 23 CFUs, comprising on average 1-3 CFUs of spore-forming bacteria, 4-23 CFUs of actinobaceria, 1 CFU of putative N-fixers and 0.2-1 CFU of chitinolytic bacteria (Bharadwaj et al., 2008b; Battini et al., 2016b). The results obtained from the *in vitro* screening for PGP traits of strains isolated from TSA and all the other specific media may be expressed: a) as the number or the percentage of bacterial isolates displaying specific PGP traits, b) as the percentage of bacterial isolates expressing multiple PGP properties.

406 The bacterial isolates producing IAA were discriminated on the basis of the
407 developed levels of intensity in the red/purple color. Accordingly, the radius of the
408 halo of color change allowed the differentiation variable levels of siderophores of
409 SAB producing. For phytate and phosphate-solubilizing bacteria the diameter of the
410 halo zone formed around the colonies differentiated the activity of SAB from low to
411 high (Battini et al., 2016b).
412 Further analyses were carried out on the data obtained, such as the
413 construction of Venn diagrams to visualize all possible intersections among the
414 relevant functional traits. The sequenced bacterial strains were assigned to species
415 using BLASTn and phylogenetic analyses. Results from BLASTn searches with the
416 16S rDNA sequences were considered as a match when they showed at least 98%
417 similarity to the query. Affiliation of the sequences with the database 16S rRNA gene
418 sequences may be carried out using Neighbor-Joining phylogenetic analysis in order
419 to build the relevant phylogenetic trees. Table 1 shows the data obtained by the
420 quoted study, with the affiliation of the different SAB strains to the relevant species.
421
422 Table 1. Plant growth promoting traits of bacteria isolated from spores of *R.*
423 *intraradices* IMA6
424

Isolate	IAA	Siderophore Activity	P solubilization SE (%)	Phytate solubilization Halo zone (cm)
Sinorhizobium meliloti TSA3	++	-	115,38	0.85
S. meliloti TSA26	+	-	81.82	0.90
S. meloloti TSA41	++	-	150	0.70
S. meliloti CH5	++	+	31.25	0.25
S. meliloti CH8	+++	+	-	0.15
S. meliloti CH17	+++	+	50	0.30
S. meliloti N23	-	+	71.43	0.65
S. meliloti N28	-	+	91.67	0.10
S. meliloti N29	-	+	84.62	0.60
Streptomyces W43N	++	++	63.64	0.80
Streptomyces sp. W77	++	++	36.36	0.90
Streptomyces sp. W94	++	+	54.55	1.15
Sreptomyces sp. W115	++	++	38.46	0.50
Arthrobacter phenanthre-	-	++	-	-

nivorans N17				
Bacillus pumilus CH10	+	+	69.23	0.25
Fictibacillus barbaricus TSA50	+++	-	-	-
Lysinobacillus fusiformis CH19	+	-	86.67	0.45
Nocardioides albus N13	-	++	-	0.10

425
426
427 **1.4. Discussion**

428 The utilization of culture-independent approaches allowed the detection and
429 identification of specific SAB and the characterization of their diversity, as affected
430 by AMF identity, plant genotype and environmental conditions. Moreover, SAB
431 molecular identification at the genus/species level represented the first and essential
432 step for proposing their relative contribution to sporosphere communities, and their
433 putative roles in this peculiar ecological niche. However, only culture-dependent
434 approaches allow scientists to investigate SAB functional roles, to study their
435 physiological interactions and to select the best performing strains, among hundreds
436 of isolates, to be further evaluated as biofertilizers and bioenhancers.

437 The regular detection of many *Actinobacteria* (genera *Amycolatopsis*,
438 *Arthrobacter*, *Propionibacterium*, *Streptomyces*) by both methods (culture-
439 independent and -dependent), confirmed their predominance in the mycorrhizosphere
440 (Ames et al., 1989; Filippi et al., 1998) and was correlated with the ability to degrade
441 chitin, a main component of the AMF spore wall, and to hydrolyze biopolymers
442 (Roesti et al., 2005). In particular, species of *Arthrobacter* and *Streptomyces* were
443 often retrieved, able to produce a number of enzymes and to biodegrade complex
444 polymers, including chitin and chitosan (Mongodin et al., 2006; Seipke et al., 2012).
445 Such physiological traits were considered essential to AMF beneficial activity, as
446 different authors hypothesized that the partial digestion of AMF outer walls might
447 increase spore germination and germling growth, thus promoting AMF root
448 colonization and symbiosis functioning (Mayo et al., 1986; Carpenter-Boggs et al.,
449 1995; Xavier and Germida, 2003; Roestli et al., 2005; Bharadwaj et al., 2008a;
450 Hamdali et al., 2008; Giovannetti et al., 2010). Accordingly, also the presence of SAB
451 taxa affiliated to the *Bacillales* may represent an important functional trait, as some

strains are strong chitin decomposers, producing many kinds of chitinases (Heravi et al., 2014), and may promote mycelial development (Hildebrandt et al., 2006).

The isolation and molecular detection of rhizobia from AMF spores, such as *Rhizobium* and *Sinorhizobium*, suggest their possible beneficial role as biofertilizers, as they, by nitrogen fixation, in legume plants, can mediate plant acquisition of nitrogen, a major plant nutrient (Bharadwaj et al., 2008b; Agnolucci et al., 2015; Battini et al., 2016b). Accordingly, when spore associated rhizobial strains were used as inocula, together with AMF, they promoted mycorrhizal functioning by enhancing spore germination, mycelial growth and mycorrhizal colonization (Gopal et al., 2012). Likewise, *S. meliloti* increased the growth of AMF extraradical mycelium by 19-25% over the levels measured in mycorrhizal plants without bacterial inoculation, they and improved plant mineral nutrition (Battini et al., 2017).

An important PGP trait of SAB is the ability to solubilize P from mineral phosphate and phytate, as P is a major plant nutrient, occurring at high concentrations in agricultural soils, but being poorly available for plants due to immobilization and precipitation reactions with soil minerals. Moreover, current agriculture is dependent on chemical fertilizers, in particular on phosphate rock P, which is a non-renewable, finite resource, whose reserves may be depleted in ca.100 years (Cordell et al., 2009). The few works on the occurrence of SAB with P solubilizing activity reported that strains showing this ability, isolated from *F. mosseae* spores, belonged to the genera *Streptomyces* and *Leifsonia* (Mohandas et al., 2013), while strains isolated from *R. intraradices* spores belonged to *Streptomyces* spp., *Bacillus pumilus*, *Lisinobacillus fusiformis* and *S. meliloti* (Battini et al., 2016b). Such P-mobilizing bacteria, when inoculated together with AMF, could show synergistic activity and enhance P availability to the host plants. Indeed, a recent study reported that some *Streptomyces* strains facilitated P uptake in maize plants and enhanced the growth of extraradical hyphae, which represent the fungal key structure spreading from mycorrhizal roots, absorbing and translocating P from the surrounding soil to plant roots (Battini et al., 2017).

A direct role in the promotion of plant growth may be played by bacteria producing phytohormones, mainly IAA, which positively affect many functional activities, such as cell division, elongation, root initiation and the development of plant root systems (Patten and Glick, 2002; Duca et al., 2014). IAA-producing strains were isolated from *R. intraradices* and *F. mosseae* spores: most of them were

represented by actinobacteria species, followed by *S. meliloti*, *Fictibacillus barbaricus* and *Paenibacillus favisporus* (Bidondo et al., 2011; Battini et al., 2017). As two of such strains, belonging to the species *S. meliloti* and *P. favisporus*, were reported to promote the elongation of AMF extraradical hyphae, the mechanisms underlying such outcome could be ascribed to the alteration of root architecture induced by IAA.

The production of siderophores by SAB has been assessed only recently, on *R. intraradices* spores (Battini et al., 2016b). Such a trait may play an indirect role in the promotion of plant growth, by protecting plants against soil-borne pathogens, as a result of bacterial siderophore-mediated competition for iron (Glick, 1995; Whipps, 2001).

It is important to note that a number of SAB possess multifunctional traits: for example 17 actinobacterial and 8 chitinolytic strains were able to produce IAA and siderophores and to solubilize P from inorganic and organic forms (Battini et al., 2016b), thus representing good candidates for further tests aimed at evaluating their performance as biocontrol agents, bio-fertilisers and bio-enhancers. Moreover, recent findings highlighted the ability of some SAB to enhance plant food quality by producing health-promoting phytochemicals (Battini et al., 2016c) and affecting gene expression of key enzymes involved in their biosynthetic pathway (Battini et al., 2016a), in accordance with previous works carried out using PGP rhizobacteria (Copetta et al., 2011; Lingua et al., 2013; Berta et al., 2014; Bona et al., 2015).

1.5. Conclusions & Outlook

In the years to come, a major challenge for agriculture will be the development and implementation of management practices for sustainable intensification of primary production, in order to guarantee enough food crops for the growing global population. Sustainable intensification of agriculture should aim at improving biological soil fertility, which underwent a drastic decline due to the continuous applications of chemical fertilizers and pesticides (Gruhn et al., 2000; FAO, 2011). This aim may be pursued by promoting the efficient use of beneficial soil microorganisms, that play fundamental roles in biogeochemical cycles and plant nutrition. Among them, the most important group is represented by AMF and their associated bacteria, whose activities enhance the functioning of mycorrhizal symbioses.

Culture-independent methods for the study of bacterial communities associated with AMF spores improved our knowledge of their diversity and will contribute to a better understanding of their roles in this peculiar ecological niche. However, only culture-dependent methods allowed to study the functional roles of SAB, aimed at identifying the most efficient strains, to be further selected as the best performing not only in laboratory experiments, but also in the field.

The detection of their functional activities, e.g. phosphate-solubilization, nitrogen fixation, and production of phytohormones, siderophores and antibiotics, is opening new avenues for their targeted management in sustainable food production systems. To this aim, the possible synergistic interactions among SAB and among diverse AMF and their SAB, should be deeply investigated, in order to understand the functioning of the complex network of microbial interactions and how they affect plant performance. The identification and selection of the most active bacterial strains, inoculated individually or in specially designed multifunctional consortia, will lead to the development of microbial inocula to be used as biofertilizers, bioenhancers and biocontrol agents in sustainable and innovative food production systems.

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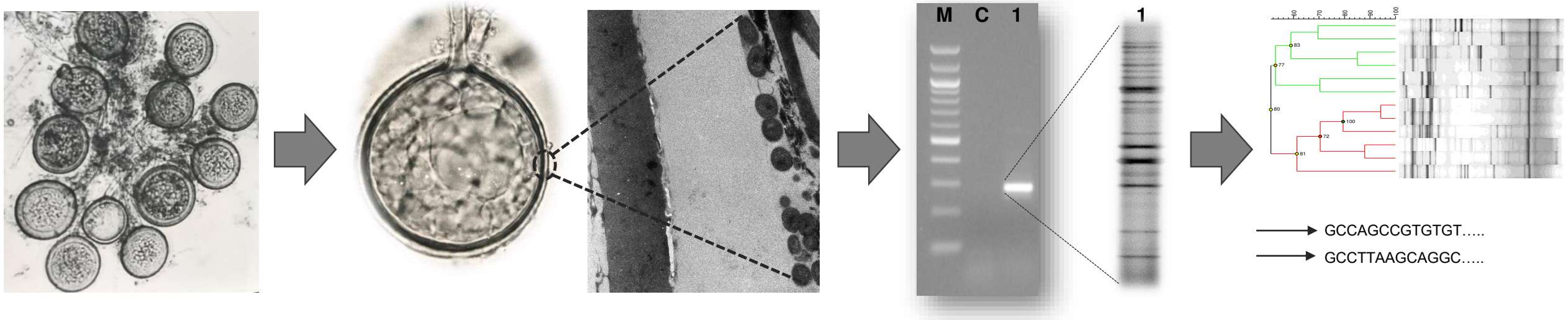
Keywords: Beneficial bacteria; plant growth promoting bacteria, PGPB; spore-associated bacteria, SAB; phosphate-solubilising bacteria; biofertilizers; biostimulants; bioenhancers; arbuscular mycorrhizal fungi; mycorrhizal symbiosis; mycorrhizosphere; sporosphere; siderophore production.



Spore collection and washing

DNA extraction and 16S rDNA PCR amplification

DGGE Analysis

Cluster analysis and species identification by sequencing

Fig. 1

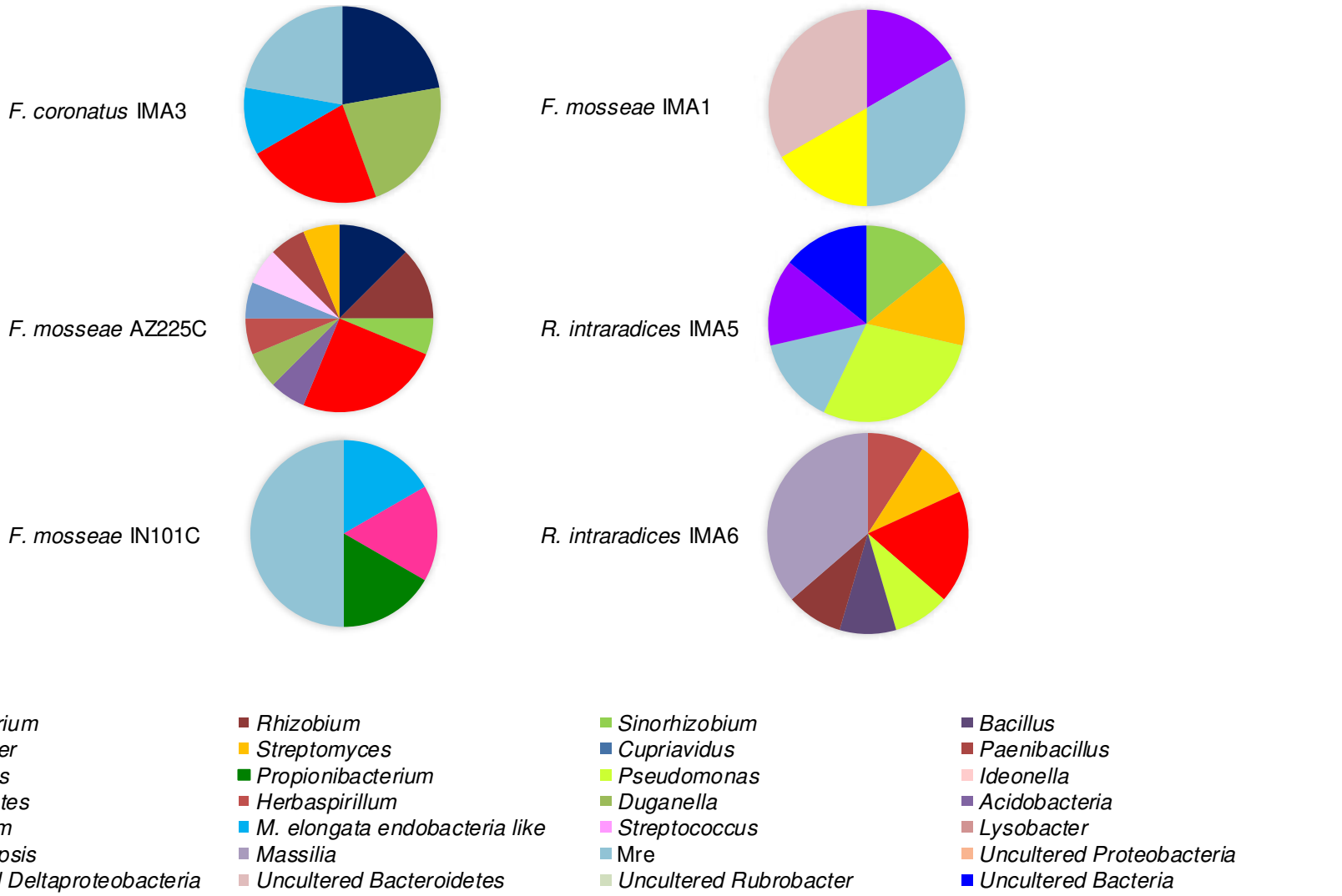
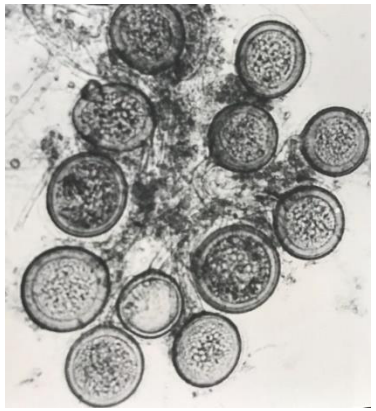
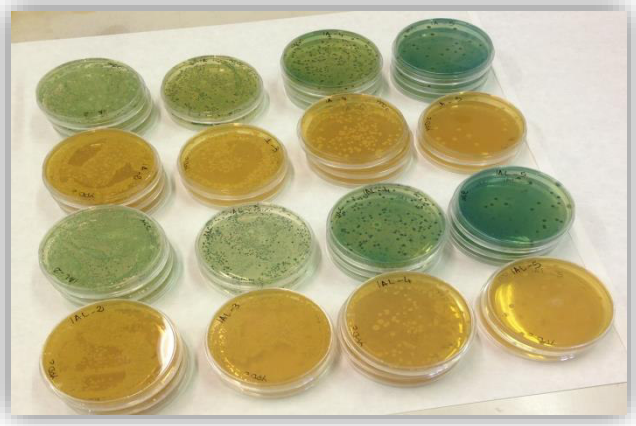

F. coronatus IMA3
F. mosseae IMA1
F. mosseae AZ225C
R. intraradices IMA5
F. mosseae IN101C
R. intraradices IMA6
Agrobacterium
Rhizobium
Sinorhizobium
Bacillus
Arthrobacter
Streptomyces
Cupriavidus
Paenibacillus
Geobacillus
Propionibacterium
Pseudomonas
Ideonella
Bacteroidetes
Herbaspirillum
Duganella
Acidobacteria
Methylibium
M. elongata endobacteria like
Streptococcus
Lysobacter
Amycolatopsis
Massilia
Mre
Uncultered Proteobacteria
Uncultered Deltaproteobacteria
Uncultered Bacteroidetes
Uncultered Rubrobacter
Uncultered Bacteria

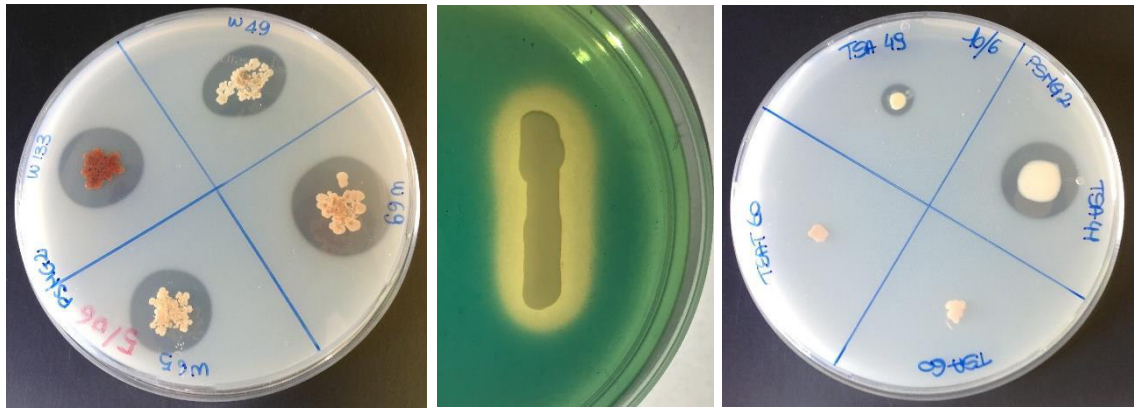
Fig. 2



Spore collection, washing and crushing



Inoculation on selective media and isolation in pure culture



Screening for PGP traits, such as phosphatase and phytase activities and production of siderophores and IAA

Fig. 3