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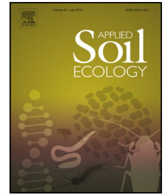

Short communication

Cultivable bacteria associated with infective propagules of arbuscular mycorrhizal fungi. Implications for mycorrhizal activity

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ABSTRACT

This study aimed to isolate and characterize bacteria associated with surface-sterilized germinated propagules of arbuscular mycorrhizal (AM) fungi. It also aimed to evaluate their activity as mycorrhization helper bacteria (MHB) on the AM fungus *Rhizophagus intraradices*, which is commonly used in the formulation of bioinoculants. Most isolated bacteria did not significantly affect the viability and subsequent growth of mycelia. *Azospirillum* sp., *Rhizobium etli, Bacillus megaterium*,*Bacillus* sp., and *Paenibacillus rhizosphaerae* significantly enhanced pre-symbiotic variables (the re-growth/germination and the mycelia formed from AM propagules). *P. rhizosphaerae*, *Azospirillum* sp., and *R. etli* also increased extraradical mycelial length, mycorrhization percentages and the number of newly formed spores. The isolated MHB were characterized based on their starch-degrading ability, indole acetic acid production, phosphate solubilization, and inhibition of phytopathogenic fungal growth. Results suggest that some of the MHB studied, in association with viable AM propagules, could be potentially used as complex microbial inoculants for plant growth promotion.

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1. Introduction

Most plant roots are colonized by arbuscular mycorrhizal (AM) fungi, obligate biotrophs that generally stimulate plant growth. After a symbiotic establishment with host roots, AM fungi produce a large number of infective propagules: spores, extraradical mycelium (ERM) and endophytic intraradical mycelium (IRM). These propagules are the source of inoculum for AM establishment in pure cultures, their viability is critical for successful cultivation. Fungal structures form the 'mycorrhizosphere', an additional habitat with characteristics that are different from those provided by roots. This habitat would selectively influence the presence of certain bacteria (Marschner and Timonen, 2005). The association of bacteria with AM propagules drastically influences the successful establishment of mycorrhization in both *ex vitro* and *in vitro* cultures.

Both culturable and non-culturable bacteria have been respectively isolated (Xavier and Germida, 2003) or detected by molecular methods (Roesti et al., 2005; Scheublin et al., 2010) in association with AM structures. The formation of bacterial biofilm-like structures on the surface of AM hyphae (Silvani et al., 2008; Lecomte et al., 2011) and the attachment of bacteria to AM spore walls (Cruz and Ishii, 2012) have been frequently observed. Mycorrhization 'helper' bacteria (MHB) include any strain capable of directly or indirectly promoting mycorrhizal symbiosis. MHB may increase propagule germination and hyphal growth, as well as stimulate spore production (Xavier and Germida, 2003). Many MHB are also plant growth-promoting rhizobacteria (PGPR) that enhance plant nutrient acquisition. Phosphate-solubilizing bacteria also show synergistic interactions with AM fungi (Fernández Bidondo et al., 2012). PGPR also produce auxins, which are able not only to stimulate differentiation and growth of plant tissues, but also to promote development of pre-symbiotic AM mycelium (Fernández Bidondo et al., 2011). Moreover, rhizospheric bacteria with antagonistic activity against fungal pathogens are able to promote AM formation (Budi et al., 1999).

The association of bacteria with AM fungi does not necessarily represent a benefit, as bacteria could take advantage of AM fungi in a trophic manner (Offre et al., 2006). Bacterial attachment to hyphae depends on the vitality of the fungal structures (Toljander

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et al., 2006). AM fungal inoculation stimulates chitinolytic activity in the rhizosphere (Abdel-Fattah and Mohamedin, 2000), and most bacteria attached to *Glomus* spp. spore walls are capable of degrading cellulose and chitin (Roesti et al., 2005). All these evidences indicate that AM fungal structures are a potential source of PGPR, but the compatibility among the isolated microbial strains should be tested, especially when complex biofertilizers (fungi and bacteria) are formulated, also including the host plant, to determine the effect of co-inoculations.

Sustainable agriculture maintains and enhances soil fertility and crop productivity through biological interactions and processes. Under sustainable crop management, AM fungi and plant growth-promoting MHB could be co-inoculated as biofertilizers, biocontrol agents and soil stabilizers. Since the development of an active rhizospheric community is essential for optimal plant productivity, knowledge of the interactions between functional microorganism groups is key to better understand plant-soil dynamics (Barea et al., 2005).

Although extensive studies have conducted on the benefits of the association between PGPR and AM fungi, very few studies have considered the diversity of PGPR naturally associated with the structures of these fungi. The impact of these bacteria on AM germination and infectivity dynamics, which are very significant events for AM *ex situ* germplasm conservation and for their effective application as biofertilizers, has also been scarcely discussed. Thus, the aims of this work were: i) to isolate and characterize rhizospheric and endophytic bacterial populations associated with infective AM hyphae and spores; ii) to analyze bacteria resistant to surface-sterilization processes, and their persistence upon the germination of fungal propagules (processes routinely involved in the isolation and conservation of AM fungal propagules); and iii) to assess their capacity as 'helper' bacteria in pre-symbiotic and symbiotic stages and their ability to promote plant growth, were also addressed to find possible candidates for complex biofertilizer formulations.

2. Materials and methods

2.1. AM fungi and bacterial strains

Bacteria were isolated from: (1) sporocarps of *Funneliformis mosseae* (strain G1), (2) spores of *Gigaspora margarita* (strain J5), and (3) IRM of *Rhizophagus/Glomus* spp. (BGIV collection of the School of Exact and Natural Sciences of the University of Buenos Aires (FCEyN, UBA)). One hundred sporocarps (G1) and one hundred spores (J5) were surface-decontaminated under axenic conditions (5%w/v Chloramine-T (Merck) solution for 30 min) and rinsed several times with sterile distilled water in line with Xavier and Germida (2003), sown on Petri plates with 0.35% w/v Gel-gro® (ICN Biochemicals, Aurora, OH, USA), and incubated for 10–15 days at 25 °C. Only the bacterial colonies growing in association with germinated spores/sporocarps after this time were transferred to tryptic soy agar (TSA) and re-streaked several times until pure cultures were obtained.

Bacteria associated with *Rhizophagus-Glomus* spp. strains were isolated from root segments (endophytic environment) from plants of several trap cultures (field soils) and processed as described by Silvani et al. (2008). Roots with visible intraradical AM fungal structures were selected under a stereomicroscope, surface-decontaminated under a laminar-flow bench with 3% v/v NaOCl for 3 min in Falcon® tubes, rinsed with sterile distilled water, cut into 3-mm pieces, and incubated on drops of 0.35% w/v Gel-gro® for 4 days at 25 °C. When re-growth of IRM from root fragments took place, bacteria associated with external hyphae were transferred to TSA medium as previously mentioned.

2.2. Characterization and identification of bacteria associated with AM fungi

Amylolytic, chitinolytic (Hankin and Anagnostakis, 1975), lipolytic (Sierra, 1957), proteolytic (Smibert and Krieg, 1994), cellulolytic, xylanolytic and pectolytic (Mikán Venegas and Castellanos Suárez, 2004) activities were detected using solid medium tests. A screening was performed to detect indole acetic acid (IAA) in the culture supernatants (Fuentes-Ramírez et al., 1993). The ability to solubilize inorganic phosphate was determined using NBRIP solid medium and sucrose or glucose as carbon source (Nautiyal, 1999) after 14 days of incubation. The *in vitro* antagonistic activity of isolated bacteria against *Macrophomina phaseolina* and *Fusarium solani* was screened. Diameters of fungal colonies in dual cultures were compared with those of the controls, and growth inhibition was calculated.

Bacteria were identified by 16S rDNA genes with the bacterial universal primers fD1 and rD1 (Weisburg et al., 1991). PCR-amplified 16S rDNA gene fragments (approximately 1.5 kb) were restricted with the endonuclease enzymes *Alu*I, *Hinf*I, *Dde*I, *Hha*I, *Hae*III, *Msp*I and *Rsa*I, and the lengths of the restriction fragments were determined by electrophoresis in 3% agarose gels. The restriction patterns (amplified rDNA restriction analysis: ARDRA) obtained from each isolate were compared. One strain of each ribotype was selected for bacterial identification. Sequencing was performed on an automated sequencer (ABI 3130xl Genetic Analyzer of Applied Biosystems) at the Sequencing and Genotyping Service of the FCEyN, UBA. Sequences were compiled using the BioEdit Sequence Alignment Editor 7.0 software, and compared to sequences from the GenBank database. Sequence similarity searches were performed using the BLAST server (http://www.ncbi.nlm.nih.gov).

2.3. Effect of bacteria on in vitro pre-symbiotic and symbiotic development

Viable and pure spores and mycelia of *Rhizophagus* intraradices strain GA5 (BGIV, http://www.bgiv.com.ar/strains/glomus-intraradices/ga5) were obtained from monoxenic cultures as described in Fernández Bidondo et al. (2011). Bacteria were grown in liquid BDN, centrifuged, and filter-sterilized (Millipore 0.2 μm pore size) to obtain diffusible substances (1). Pellets were re-suspended with 10 mM SO_4Mg to a final concentration of 10^9 cells ml^{-1} (2). Pre-symbiotic and symbiotic development of GA5 in response to 1 and 2 was evaluated.

2.3.1. Pre-symbiotic parameters

Groups of 30 spores or 10 colonized root fragments (1-cm long) previously removed from the GA5 monoxenic culture were transferred to Petri plates with 0.35% w/v Gel-Gro®. Each type of fungal inoculum was homogeneously mixed with 100 μl of (1) sterilized supernatants or (2) cell suspensions. Plates were incubated in the dark at 25 °C for ten days. All treatments were replicated in five Petri plates. The effect of bacterial strains on spore germination (%) and on re-growth of IRM from root fragments (%) was assessed under stereomicroscope (Nikon SMZ645). The ERM length (mm) was measured using the method proposed by Brundrett et al., 1994. Measurements were taken under a light binocular microscope (Nikon OPTIPHOT-T2) at 100x magnification.

2.3.2. Symbiotic parameters

A 1-cm^3 plug of a 3-month-old GA5 monoxenic culture, containing colonized roots (30% frequency and 50% intensity of colonization), approximately 250 spores and abundant ERM, was placed in proximity to fresh transformed carrot root explants in

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