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Evaluation of native bacteria and manganese phosphite for alternative control of charcoal root rot of soybean

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) are potential agents to control plant pathogens and their combined use with biopesticides such as phosphites may constitute a novel strategy to incorporate in disease management programs. In the present study, 11 bacterial isolates were selected on the basis of their antagonistic activity against *Macrophomina phaseolina* in dual-culture tests, and their plant growth promoting traits. Selected isolates were characterised on the basis of auxin and siderophore production, phosphate solubilisation and rep-PCR genomic fingerprinting. Two of these isolates, identified as *Pseudomonas fluorescens* 9 and *Bacillus subtilis* 54, were further evaluated for their inhibitory capacity against *M. phaseolina* using *in vitro* (on soybean seeds) and *in vivo* (greenhouse assay) tests. Both bacteria were applied individually as well as in combined treatment with manganese phosphite as seed treatments. Damage severity on soybean seeds was significantly reduced, compared with the untreated control, by both bacterial strains; however, the individual application of phosphite showed to be least effective in controlling *M. phaseolina*. Interestingly, the phosphite treatment improved its performance under greenhouse conditions compared to the results from the *in vitro* assays. In the greenhouse trials, the greatest reductions in disease severity were achieved when strain *P. fluorescens* 9 was applied singly or when strain *B. subtilis* 54 was combined with manganese phosphite, achieving 82% of control in both cases. This work is the first to report the control of *M. phaseolina* using combined treatment with PGPR and phosphite under greenhouse conditions.

1. Introduction

Soybean [*Glycine max* (L.) Merr.] is an economically very important crop in Argentina. However, the growing conditions under monoculture and no-tillage system have favoured the occurrence and the severity of a large number of diseases, some of which constitute serious constraints to production (Carmona et al. 2015).

Macrophomina phaseolina (Tassi) Goidanish root rot or charcoal root rot is the most common and widely spread root disease affecting soybean crop under conditions of high ambient temperatures and low soil moisture (Gupta et al. 2012; Kending et al. 2000). This disease can appear at any stage of plant growth affecting seeds, seedlings and adult plants. The aerial symptoms of charcoal rot in soybean generally appear after flowering (R1), specially between R5 and R8 growth stages (Almeida et al. 2003). The affected plants show leaf and stem blight and may prematurely die, with senesced leaves remaining attached to petioles (Mengistu et al. 2011). The brown discoloration of the pith in root and stems is well frequent in the diseased plants, with the presence of a lot of dark microesclerotia, specially on taproot and stems. Because of the wide host range of *M. phaseolina* and the long-term survival of its microsclerotia,

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the common management strategies, including seed treatment with fungicides, generally fail to provide adequate control of the disease (Hwang et al. 2009). It is therefore, necessary to search for novel antifungal agents that are cost effective, nontoxic and that eliminate or reduce the incidence of soil-borne diseases of soybean. Various studies have reported the capacity of diverse rhizospheric bacterial strains to inhibit or suppress fungal diseases, involving a wide range of biological control mechanisms, such as the competition for nutrients or niches on the root, production of inhibitory allelochemicals and induction of local or systemic resistance in host plants (Bhattacharyya and Jha 2012; Simonetti et al. 2012a). These biocontrol bacteria are more frequently isolated from disease suppressive soils where the expression of the disease is limited despite the presence of a virulent pathogen and a susceptible plant (Weller et al. 2002). On the other hand, plant defence activating compounds, known as chemical inducers, can provide other alternative disease management tool. Many of these compounds are frequently termed biopesticides and are environmentally friendly. Phosphites (Phi) are metal salts of phosphorous acid that are able to elicit systemic acquired resistance (SAR) in some plant species (Mersha et al. 2012; Percival et al. 2009) and can also exhibit direct toxicity against different pathogens, such as *Phytophthora* spp., *Streptomyces scabies*, *Rhizoctonia solani* and *Fusarium solani* (Dalio et al. 2014; Lobato et al. 2010). The application of Phi to plants protects them from infections, especially by oomycetes, and also against other plant pathogens, such as *Venturia inaequalis*, *V. pirina*, *F. solani* and *Erwinia carotovora* (Lobato et al. 2008; Percival et al. 2009). A novel alternative disease management approach is the combination of biocontrol agents with chemical inducers in order to achieve a better control of plant pathogens. The efficacy of combination treatments between antagonistic bacteria and chemical inducers has not been widely investigated. Yi et al. (2013) identified an additive effect on induced resistance, against bacterial spot in pepper, after a combination treatment composed of strain *Bacillus pumilus* INR7 with a chemical inducer benzothiadiazole (BTH) in the field. Myresiotis et al. (2012) found that the combination of different *Bacillus* strains with acibenzolar-S-methyl increased its suppression capacity against *Fusarium* crown and root rot on tomato plants.

Considering the above mentioned, the aims of the present work were (i) isolate native bacterial strains from soybean plants growing in disease-suppressive soils, (ii) determinate the presence of PGPR traits and *in vitro* effectiveness against *M. Phaseolina*, (iii) evaluate the efficacy of the candidate antagonists, when applied in individual or combined treatment with phosphite, in controlling charcoal root rot of soybean under greenhouse conditions.

2. Materials and methods

2.1. Bacterial isolation from soybean

Soil samples and healthy soybean plants were collected in the fields from different locations of Santa Fe Province, Argentina (Table 1). Soybean roots with firmly adhering soil were suspended in sterile 10 mM NaCl solution and the suspension was used for isolation of rhizospheric bacteria. The roots were surface sterilised with sodium hypochlorite (2.5%) for 2 min, cut into 2 cm long slices and macerated by three cycles of vortex (1 min each) in 20 ml sterile solution of Tween 80 (0.1%, v/v) plus glass beads. This suspension was used for isolation of endorhizospheric bacteria. The two suspensions obtained (from rhizospheric soil and endorhizosphere) were serially diluted, plated (100 μl) on nutrient agar (NA) and incubated for 48 h at 28 °C until observing colonies development. Distinct isolated colonies were picked up and streaked again on fresh King's B and NA media, and incubated similarly. This process was carried out thrice to get a pure single colony. Fluorescence of colonies was observed on King's B medium under UV exposure. *Bacillus*-like colonies were roughly identified on the basis of their morphology and Gram reaction. Bacteria were kept for long-term storage at −80 °C in nutrient broth (NB) with glycerol (15% v/v).

All isolated bacteria were tested for their inhibitory capacity against the fungal pathogen *M. phaseolina*. The fungal strain used in this work, was originally isolated from infected soybean plants showing root rot symptoms on potato dextrose agar medium (PDA, Merck) (Singleton et al. 1993). Each bacterial isolate was streaked as a thick band on the edge of a PDA plate and then a mycelial disc was placed onto the centre of the plate. The radial mycelial growth was registered after 3 days of incubation at room temperature in the dark. Due to the large number of bacteria assayed, the analysis was performed by grouping the isolates based on their origin (rhizospheric soil, endorhizosphere) and other characteristics like colony morphology, sporulation capacity, production of fluorescent pigment on King's B medium and Gram reaction. Isolates with antagonistic effect against *M. phaseolina* were selected for further experiments.

2.2. Determination of potential plant growth promoting traits of selected isolates

Auxin production was detected by the method described by Glickman and Dessaux (1995). Bacterial cultures were grown for 48 and 72 h on Luria-Bertani (LB) medium at 28 °C. Fully grown cultures were centrifuged at 10,000 g for 30 min and the supernatants were used for the colorimetric assay. Auxin produced by cultures was determined spectrophotometrically at 530 nm and the concentrations were estimated using indole-3-acetic acid (IAA) as standard.

The ability of bacterial isolates to solubilise insoluble mineral phosphate ($Ca_3(PO_4)_2$) was tested qualitatively by plate assay using Pikovskaya (PVK) and National Botanical Research Institute's phosphate (NBRIP) growth media (Nautiyal, 1999). Isolates were spot inoculated on the centre of PVK or NBRIP plates and incubated at 28 °C for 21 days. The plates were then examined for visual detection of a clear zone around the growing colonies.

Siderophore production was tested qualitatively through O-CAS assay method (Pérez-Miranda et al. 2007) in which chrome azurol sulphonate (CAS) medium was cast upon LB plates containing cultivated rhizobacteria. Development of yellow-orange halos around the colonies after 72 h of incubation was indicative of siderophore production. All these experiments were made at least three times with three replicates for each one.

2.3. Evaluation of strains for in vitro biological control

The isolates were tested for their ability to inhibit the growth of soil-borne fungal pathogen *M. phaseolina* using the *in vitro* dual-culture assay (Simonetti et al. 2012b). The fungus was maintained on PDA at 28 °C for a week. A 6 mm diameter mycelial plug was taken from the margin of a growing colony and placed centrally in a Petri dish containing PDA. Two drops (3 μl) of each bacterial culture were placed in a straight line 3 cm away from the centre of the plate and drops of sterile water served as control. After incubation for 3–4 days at 28 °C, mycelium growth inhibition percentage (I) was calculated as $I = [(C-T)/C] \times 100$, where C is mycelium diameter in control, and T mycelium diameter in bacteria-inoculated plates. All these experiments were made at least three times with three replicates for each one.

The antifungal activity of cell-free supernatants of the different antagonistic isolates was evaluated against the target fungus *M. phaseolina* using the *in vitro* test as previously described (Kumar