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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

**Table 1**
Summary of identification results and plant growth promoting characteristics of selected bacterial isolates.

| Strain ID | Coordinates[a] | Source of isolation | 16S rRNA gene accession number | Species assignment | Auxin production[b] | Phosphate solubilisation[c] | Siderophore production[b] | Fungal inhibition[d] |
|---|---|---|---|---|---|---|---|---|
| **7** | 32°40'S, 61°31′W | Rhizosphere | KM887990 | *Bacillus cereus* | + | – | + | ++ |
| **9** | 32°40'S, 61°31′W | Rhizosphere | KM887989 | *Pseudomonas fluorescens* | + | +++ | + | +++ |
| **13** | 32°48'S, 61°23′W | Endorhizosphere | KM887991 | *Bacillus cereus* | + | – | + | ++ |
| **48** | 32°25'S, 61°53′W | Endorhizosphere | KM887995 | *Stenotrophomonas maltophilia* | + | – | + | + |
| **54** | 32°25'S, 61°53′W | Endorhizosphere | KM887996 | *Bacillus subtilis* | + | ++ | + | +++ |
| **110** | 32°48'S, 61°23′W | Rhizosphere | KM887997 | *Chryseobacterium vietnamense* | + | + | + | + |
| **115** | 32°40'S, 61°31′W | Endorhizosphere | KM887988 | *Pseudomonas fluorescens* | + | +++ | + | +++ |
| **116** | 32°40'S, 61°31′W | Endorhizosphere | KM887992 | *Bacillus cereus* | + | – | + | ++ |
| **117** | 32°40'S, 61°31′W | Rhizosphere | KM887998 | *Chryseobacterium indologenes* | + | + | + | + |
| **123** | 32°48'S, 61°23′W | Endorhizosphere | KM887993 | *Bacillus cereus* | + | – | + | ++ |
| **125** | 32°40'S, 61°31′W | Endorhizosphere | KM887994 | *Bacillus cereus* | + | – | + | ++ |

[a] Geographical origin of bacterial isolates
[b] +: positive result, –: negative result
[c] +++: solubilisation halo ≥ 10 mm; ++: 5mm < halo < 10 mm; +: halo < 5 mm; –: no solubilisation halo
[d] *Macrophomina phaseolina* growth inhibition in dual plate assays + : low (<20%); ++: moderate (~40%); +++: strong (>55%).

et al. 2012). Bacteria were grown on NB at 28 ± 2 °C for 48 h, cells were removed by centrifugation and supernatants were filtrated through 0.22 μm MILLEX®GP filter units (Millipore, Carrigtwohill, Co. Cork, Ireland). Then, 100 μl aliquots of sterilised supernatant samples were dispensed in four wells (performed with a sterile cork borer, 6 mm diameter) equidistant from the centre of a PDA plate inoculated previously with an actively growing mycelia disc (6 mm diameter) of *M. phaseolina*. These plates were performed in triplicate and incubated at 28 ± 2 °C for 15 days and examined for zones of inhibition of colony growth.

### 2.4. Genotypic characterisation and identification of selected isolates

Genomic DNA was extracted from pure cultures of each isolate using the DNeasy Tissue kit (Qiagen) according to manufacturer's instructions. In order to determine whether or not isolates were members of a clone, the selected isolates were characterised by repetitive sequence-based genomic fingerprinting (rep-PCR) using (GTG)5 primers (Versalovic et al. 1994) as previously described (Montecchia et al. 2002).

Bacterial identification was performed by 16S rRNA gene sequence analysis. The 16S rRNA gene was amplified by PCR using the eubacterial universal primers 27F and 1492R (Lane, 1991). Each 50 μl PCR reaction mixture contained 5 ng of template DNA, 1 × Taq reaction buffer with a final magnesium concentration of 2 mM (Fermentas), 5% DMSO, 200 μM dNTPs, 0.5 μM of each primer, and 1 U of Dream Taq DNA Polymerase (Fermentas). The thermocycling conditions consisted of an initial denaturation at 95 °C for 5 min, 30 amplification cycles of denaturation at 94 °C, 1 min; annealing at 65 °C, 40 s; extension at 72 °C, 2 min. Cycling was completed with a final elongation step of 72 °C for 5 min. Then, amplicons (~1500 bp) were purified using QIAquick PCR purification kit (Qiagen) and sequenced by Unidad de Genómica (Instituto de Biotecnología, INTA, Buenos Aires, Argentina) in both directions with the same universal 16S rRNA primers. The obtained 16S rDNA sequences were compared with the sequences in the GenBank nucleotide database using BLAST (blastn) program and phylogenetic analysis of sequences were conducted in MEGA version 6 (Tamura et al. 2013).

The 16S rDNA sequences obtained in this study were submitted to the GenBank/EMBL/DDBJ database under the accession numbers KM887988 to KM887998.

### 2.5. Effect of bacterial inoculation and Phi treatment against M. phaseolina on soybean seeds

The two strains employed in these assays (strains 9 and 54) were selected on the basis of *in vitro* antibiosis dual plate assays against the test pathogen (*M. phaseolina*) together with potential plant-growth promoting properties (Table 1). Biocontrol tests were conducted with the cultivar NIDERA A 4990RG susceptible to *M. phaseolina*.

To determinate the effects of seed inoculation with the bacteria, surface-sterilised seeds were immersed in either sterile water (negative control) or in a standardised suspension of strains 9 or 54 ($10^7$ CFU $ml^{-1}$ in sterile water) for 15 min at room temperature. Some of these seeds were subsequently treated with manganese phosphite (MnPhi, ULTRA PLUS Mn, Spraytec), at a dosage of 200 mL/100 kg of seeds. Untreated and pretreated seeds were air dried and placed on PDA plates completely covered by mycelia and microsclerotia of *M. phaseolina*. These plates were incubated at 28 °C for 4–5 days, in a completely randomised design, including the following treatments: (1) untreated seeds (2) seeds with MnPhi (3) seeds inoculated with strain 9 (4) seeds inoculated with strain 9 and treated with MnPhi (5) seeds inoculated with strain 54 (6) seeds inoculated with strain 54 and treated with MnPhi. These treatments were carried out in duplicate with PDA plates without the fungus, maintained as uninoculated controls. The disease was assessed only in the plates previously incubated with the fungus. Damage severity on seeds was estimated according to the following visual scale 0: healthy seed; 1: seed teguments invaded by mycelium; 2: seed teguments and radicle invaded by mycelium; 3: seed teguments, radicle and interior of the seed invaded by mycelium; 4: seed teguments invaded by mycelium and sclerotia, without internal mycelium; 5: seed teguments invaded by mycelium and sclerotia with the presence of internal mycelium; 6: seed teguments, radicle and/or interior invaded by mycelium and sclerotia and 7: seed not germinated and completely colonised by the fungus. These experiments were repeated three times with six biological replicates for each treatment. In turn, each biological replicate consisted of a PDA plate with six seeds.

### 2.6. Colonisation assay

In order to verify if the colonisation of soybean roots by strains 9 and 54 was affected by the MnPhi treatment, the colonisation

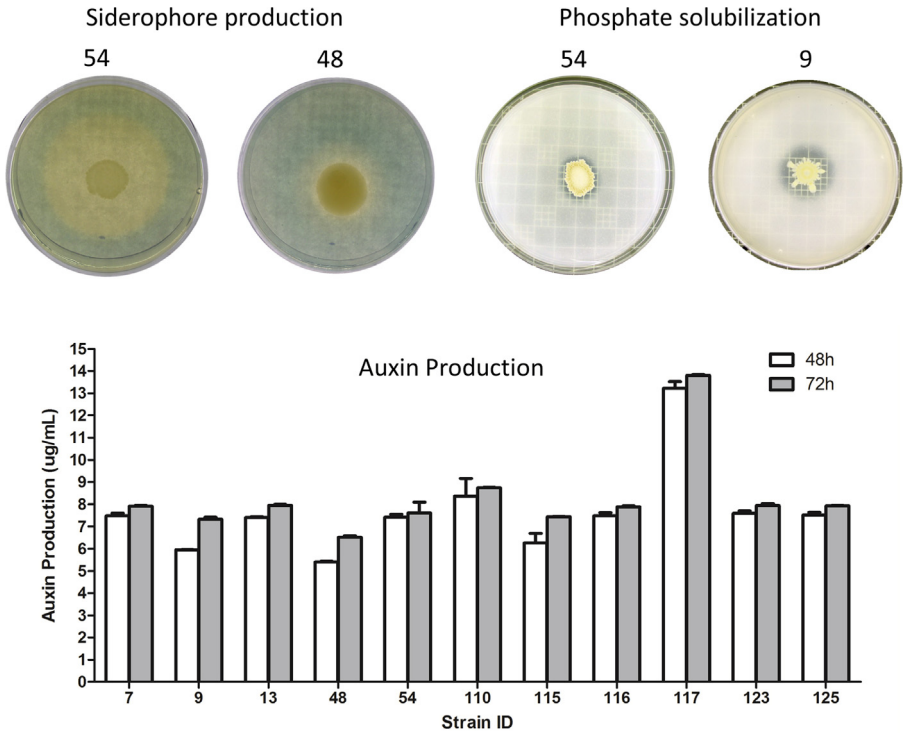


**Fig. 1.** Potential plant growth promoting traits of selected bacterial isolates. Siderophore production was assessed through O-CAS assay method. Phosphate solubilisation activity was tested qualitatively by plate assay using Pikovskaya agar medium. Auxin production was determined by a colorimetric assay using the Salkowski reagent. All experiments were performed in triplicate with three independent trials.

capacity of both bacterial isolates was evaluated on treated (as previously described, Section 2.5) and untreated seedlings after 3 days of germination onto wetted-filter paper in a Petri dish. The roots from three seedlings per treatment were submerged in 3 ml sterile water and macerated with mortar. Each bacterial suspension was serially diluted and plated on NA and CFU per gram of root was quantified. The colonisation assay was repeated three times in a completely randomised design with three biological replicates for each treatment.

### 2.7. Effect of bacterial inoculation and Phi treatment against charcoal root rot under greenhouse conditions

Inoculum of *M. phaseolina* was prepared in laboratory by multiplying the pathogen on rice (*Oryza sativa*). The rice grains (250 g) were soaked in distilled water overnight in autoclavable bags. Then they were autoclaved twice (121 °C for 30 min) and inoculated with five agar plugs (6 mm diameter) cut from the margin of an actively growing pathogen colony. The bags were incubated at 28 °C in the dark for 2–3 weeks and shaken daily. Rice seeds containing mycelial fragments plus sclerotia served as inoculum. A mix (3:1 v/v) of tyndallised nursery substrate (Grow Mix, Multipro, Terrafertil S.A., Buenos Aires, Argentina) and sand was placed in 8.5 × 7 cm plastic pots. A layer of infested rice seeds (12 g per pot) was distributed in half of the experimental pots and covered with 2 cm of the same substrate. Soybean seeds inoculated with bacterial strain 9 or 54 and subsequently treated with MnPhi, as previously described, were planted and maintained in the greenhouse (25 ± 5 °C) for 45 days. Each treatment was run with six replications in a completely randomised design and the experiment was repeated two times. An experimental unit was one soybean plant per pot. Thus, the disease severity was evaluated in the plants from seeds subjected to the same six treatments as previously described in Section 2.5. The correlation between disease severity and CFU was confirmed in previous studies (Mengistu et al. 2007), so CFU in soybean root was used as a measure of disease severity. At the plant growth stage of R1, plants were carefully uprooted to determine CFU of the pathogen. The roots were dried at 40 °C and ground with a sample mill. For each sample, 5 mg of ground tissue was placed in a centrifuge tube with 1 ml of sodium hypochlorite (2%) for 3 min, subsequently washed with sterile distilled water and then added to 20 ml of autoclaved PDA amended with rifampicin (100 mg $L^{-1}$) and metalaxyl (224 mg $L^{-1}$). After 3 days of incubation at 28 °C, *M. phaseolina* CFUs were counted and converted to CFU per gram of root. Severity Control was estimated as [(Mean of CFU/g root in untreated control)-(Mean of CFU/g root in treated plants)]/(Mean of CFU/g root in untreated control) × 100

### 2.8. Data analysis

The data on the effects of the bacterial isolates on the growth of the *M. phaseolina* and disease severity (from tests on soybean seeds and greenhouse trials) were subjected to one-way analysis of variance (ANOVA). Data on fungal colony diameters from dual culture tests were $\log_{10}$-transformed before conducting ANOVA. Statistical analysis was performed using Infostat software (Balzarini et al. 2008). Mean separations were performed by Duncan's multiple range test. Differences at $P < 0.05$ were considered significant.

## 3. Results

### 3.1. Screening of selected isolates for their potential plant growth promoting traits

A total of 120 bacteria, isolated from rhizospheric soil and endorhizosphere, were assayed for mycelial inhibition of *M. phaseolina* on PDA. In the preliminary screening, 11 representative isolates that exhibited the highest *M. phaseolina* inhibition in radial mycelial growth, were selected for further investigation.

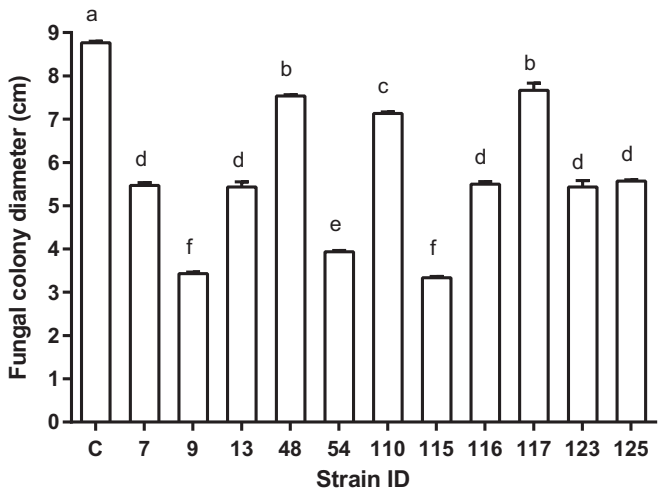


**Fig. 2.** Effects of PGPR isolates against *Macrophomina phaseolina* tested by following the dual plate assay method. Colony diameter was measured after incubation for 3–4 days at 28 °C. **C**: Control without bacteria. Data represent the average of three independent experiments with standard error bars. Different letters on the columns indicate significant differences among treatments according to Duncan's multiple range test ($P < 0.05$).

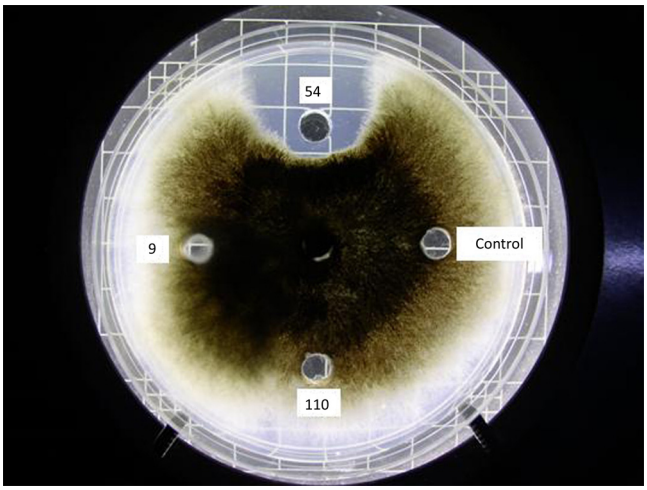


**Fig. 3.** Effects caused by the cell-free supernatants from different PGPR isolates on the mycelial growth of *Macrophomina phaseolina*. A clear zone of inhibition was observed for strain 54. **C**: Control without bacteria.

Table 1 summarises the results of the determined plant growth promoting traits of the 11 strains. All of them were able to produce siderophores, which was confirmed by appearance of yellow-orange halos around their colonies in the O-CAS assay (Table 1 and Fig. 1). Strains 54 and 117 appeared to produce the largest halos, while strain 48 exhibited the smallest one (Fig. 1). The isolates 9, 115, 117, 110 and 54 were able to solubilise mineral phosphate on PVK agar medium. Strains 9 and 115 appeared to produce the largest solubilisation halos (translucent areas around the colonies), followed by isolate 54 (Table 1 and Fig. 1). The quantity of auxins produced by these strains ranged from $13.8 \pm .04$ to $6.52 \pm 0.06$ μg $ml^{-1}$ after 72 h of growth in LB medium. The highest production was observed for strain 117, while the lowest value was detected for strain 48 (Fig. 1). The bacteria with the highest *M. phaseolina* inhibition percentage (*I*) of about 55, 62 and 61% were strains 54, 9 and 115, respectively. Some isolates showed weaker inhibition activity, which was still significant compared to the negative control (Fig. 2, $P < 0.05$). Strains 7, 13, 116, 123 and 125 exhibited intermediate and similar mycelium growth inhibition percentage ($I \sim 39\%$). The isolates 117, 48 and 110 showed the lowest inhibition percentage ($I \sim 19$, 15 and 13%, respectively).

The antifungal activity of bacterial extracellular metabolites was studied by *in vitro* test using the cell-free culture supernatants. As shown in Fig. 3, only the cell-free supernatant of strain 54 significantly inhibited the mycelial growth of *M. phaseolina*.

### 3.2. Genotyping of selected bacterial isolates by rep-PCR and 16S rRNA gene sequencing

In order to achieve differentiation of isolates at strain level, rep-PCR genomic fingerprinting was performed using the (GTG)5 primer, since this method was proven to be a reliable tool for typing a wide range of Gram-negative and several Gram-positive bacteria at intraspecific level (Versalovic et al. 2013). As shown in Fig. 4, all isolates showed distinctive banding profiles and were considered distinct strains.

The identification of bacterial strains-based on 16S rRNA gene sequence similarities is presented in Table 1. Strains 9 and 115 belonged to the genus *Pseudomonas* and were closely related to *P. fluorescens* Pf0-1 (99% identity). Strain 54 belonged to the genus *Bacillus* and was most closely related to *B. subtilis* JCM 1465$^T$ (99% identity). Similarly, the sequences of strains 7, 13, 125, 123 and 116 were identical among them and had the highest similarities with *Bacillus* genus, but in this case with *B. cereus* ATCC 14579$^T$. The closest relatives of strains 110 and 117 were the type strains of *Chryseobacterium vietnamense* GIMN1.005$^T$ (99% identity) and *Chryseobacterium indologenes* NBRC 14944$^T$ (99% identity), respectively, therefore, they were assigned to those species. Finally, the strain 48 exhibited the highest 16S rRNA gene sequence similarity (99% identity) with *Stenotrophomonas maltophilia* LMG 958$^T$.

### 3.3. Effect of bacterial inoculation and MnPhi treatment against M. phaseolina on soybean seeds

Only the strains *P. fluorescens* 9 and *B. subtilis* 54 were selected to carry out soybean inoculation assays. Both isolates demonstrated the highest antifungal activity against the test pathogen *M. phaseolina in vitro*, together with the presence of desirable plant growth promoting properties. Despite their promising characteristics, the strains identified as *S. malthophilia*, *C. indologenes* and the isolates closely related to *B. cereus*, were not included in any further experiments, because of their genetic relationship with potentially hazardous bacteria (Bottone 2010; Brooke 2012; Lin et al. 2010).

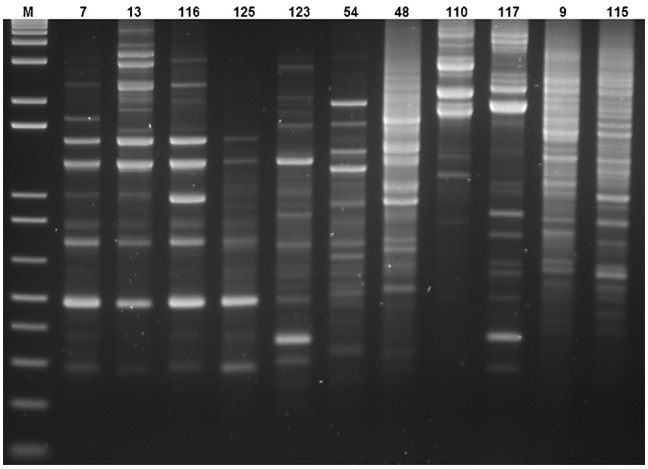


**Fig. 4.** Rep-PCR genomic fingerprints of selected isolates obtained with (GTG)5 primers. M: 1Kb Plus DNA Ladder (Invitrogen).

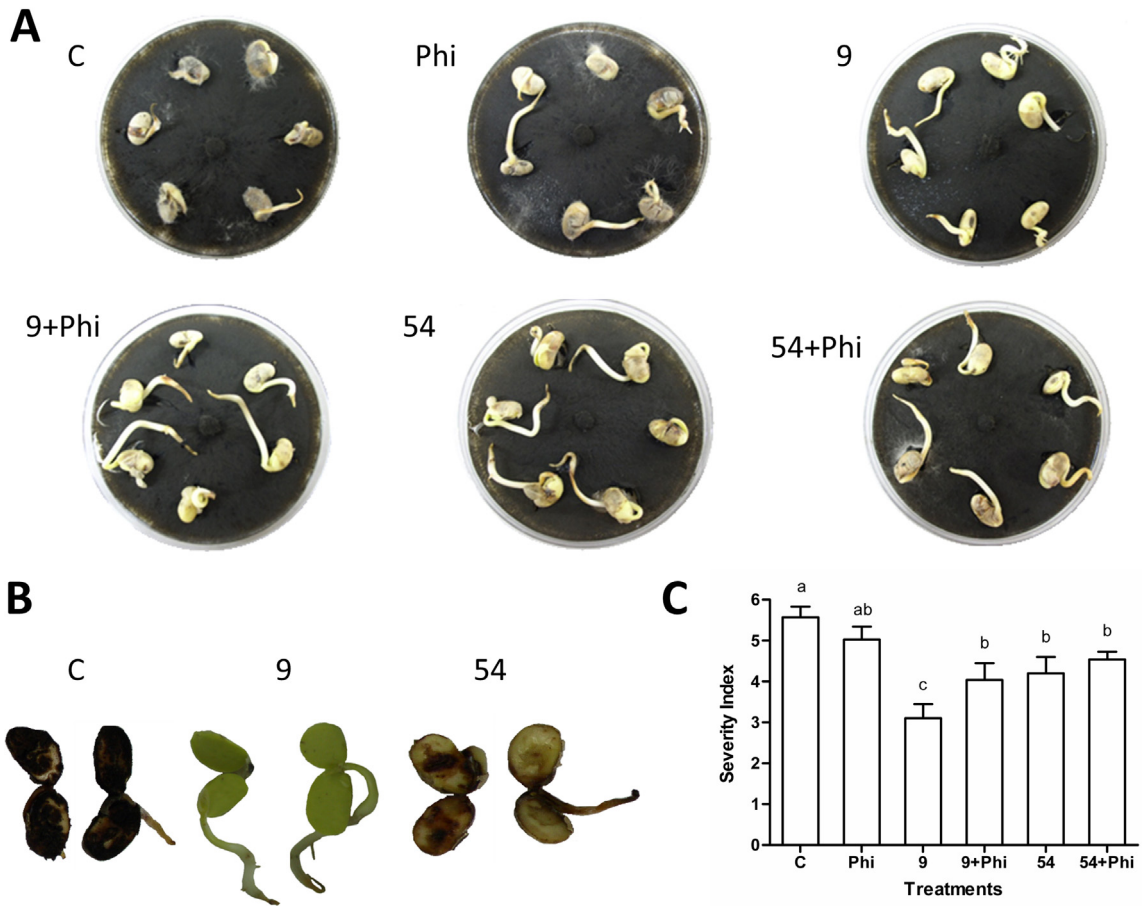


**Fig. 5.** *In vitro* biological control of *Macrophomina phaseolina* in soybean seeds treated with PGPR strains, individually or in combination with manganese phosphite. (A) Examples of soybean seeds with 5 days of germination in plates in the presence of a 7-day-old colony of *M. phaseolina* on PDA medium **C**: untreated; **Phi**: treated with manganese phosphite; **9**: inoculated with *Pseudomonas fluorescens* 9; **9** + **Phi**: treated with *P. fluorescens* 9 and manganese phosphite; **54**: inoculated with *Bacillus subtilis* 54; **54** + **Phi**: treated with *B. subtilis* 54 and manganese phosphite. (B) Examples of soybean seeds exhibiting different damage severity levels. **C**: untreated seeds with interior completely colonised by mycelium and microsclerotia; **9**: *P. fluorescens* 9 inoculated seeds with minimal fungal internal colonisation; **54**: *B. subtilis* 54 inoculated seeds with internal mycelium. (C) Severity index from three independent experiments with standard error bars. Different letters on the columns indicate significant differences among treatments according to Duncan's multiple range test ($P < 0.05$).

Damage severity on soybean seeds was significantly reduced, compared with the non-treated control, by all treatments, with the exception of seeds only treated with MnPhi. The reduction in disease severity was highest when the bacterial strain *P. fluorescens* 9 was applied individually (Fig. 5). In contrast, the combined application of this bacterium with Phi, led to a severity index significantly higher than that of treatment with strain 9 separately ($P < 0.05$). Furthermore, the reduction in disease severity by strain *B. subtilis* 54, was not affected when the MnPhi was applied in combination (Fig. 5).

### 3.4. Colonisation assay

The two evaluated bacterial strains, *P. fluorescens* 9 and *B. subtilis* 54, were able to effectively colonise the soybean seedling roots after 3 days of germination (levels of $1.8 \times 10^9$ and $7.7 \times 10^7$ CFU/g root, respectively). In addition, the levels of colonisation by both bacteria, were not affected by subsequent treatment with MnPhi (levels of $3.5 \times 10^9$ and $7.5 \times 10^7$ CFU/g root, respectively, $P < 0.05$).

### 3.5. Effect of bacterial inoculation and MnPhi treatment against charcoal root rot under greenhouse conditions

The CFU density of the pathogen *M. phaseolina* on soybean plants from seeds pretreated with MnPhi, in individual application or in combination with PGPR isolates, was significantly reduced, compared with the untreated control ($P < 0.05$, Fig. 6). The separately application of MnPhi reduced the disease severity caused by *M. phaseolina* by 37%, while the inoculation with the strains *P. fluorescens* 9 and *B. subtilis* 54, showed 82 and 64% of severity control, respectively. Notably, the reduction of CFU density was highest when strain 54 was combined with MnPhi ($P < 0.05$, Fig. 6), leading to 82% of control. By contrast, combined treatment of strain *P. fluorescens* 9 with MnPhi led to a significantly higher CFU per gram of root than that of individual treatment with strain 9 ($P < 0.05$, Fig. 6), obtaining only a 41% of the control.

## 4. Discussion

In the present study, 120 bacterial isolates were obtained from healthy soybean plants sampled from crop fields in the Province of Santa Fe, Argentina. The primary screening resulted in a group of 11 bacteria able to efficiently inhibit the mycelial growth of *M. phaseolina in vitro*. We focused on bacterial genera that are often found in large populations in disease suppressive soils (Mercado-Blanco et al. 2004), such as spore-forming Gram-positive species belonging to the *Bacillus* genus and Gram-negative ones from the *Pseudomonas* genus. In this context, the potential PGPR isolates belonged to *P. fluorescens*, *B. cereus*, *B. subtilis*, *Chryseobacterium vietnamense*, *Chryseobacterium indologenes* and

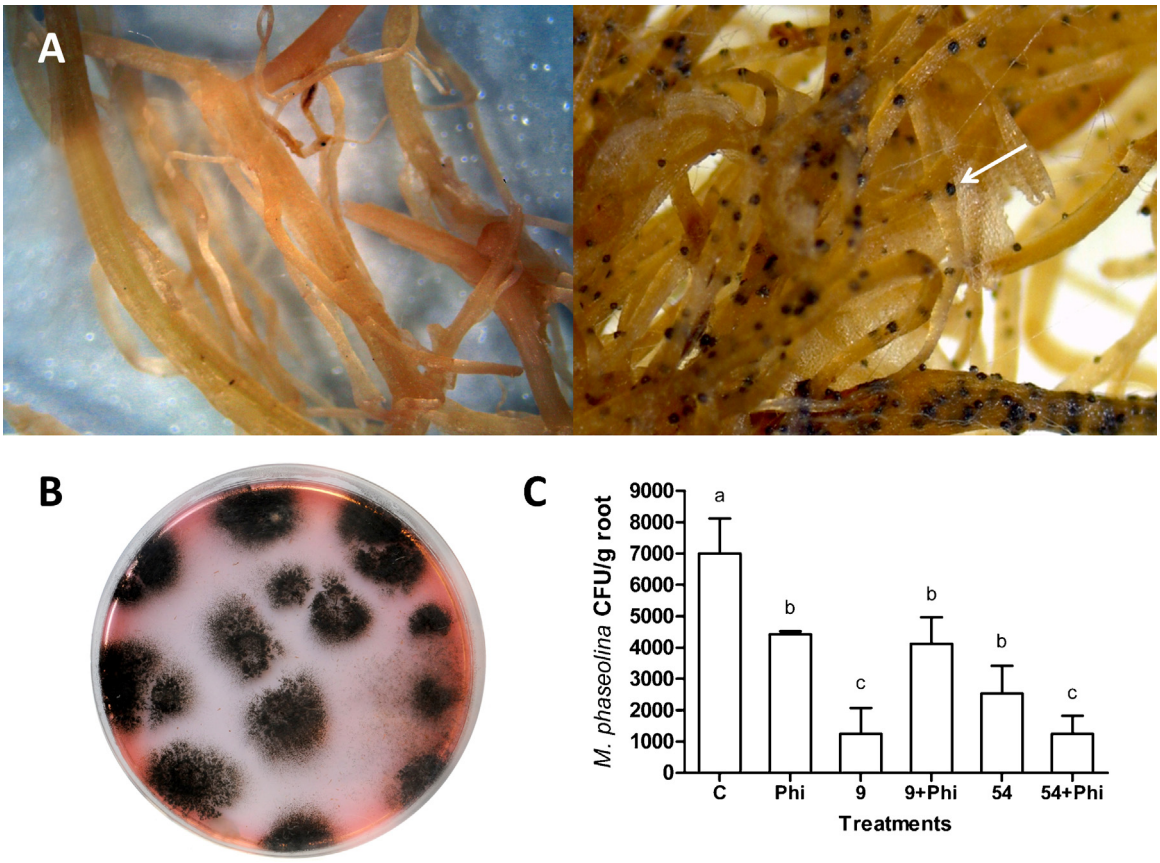


**Fig. 6.** Biological control of *Macrophomina phaseolina* in soybean plants treated with PGPR strains, individually or in combination with manganese phosphite, under greenhouse conditions. (A) Examples of soybean roots from infected and uninfected plants observed under stereoscopic microscope. White arrow indicates presence of microsclerotia. (B) CFU of *M. phaseolina* from root tissues of infected plants on semi-selective medium. This counting was used as a measure of disease severity. (C) Estimation of disease severity from two independent experiments with standard error bars. **C**: untreated; **Phi**: treated with manganese phosphite; **9**: inoculated with *Pseudomonas fluorescens* 9; **9 + Phi**: treated with *P. fluorescens* 9 and manganese phosphite; **54**: inoculated with *Bacillus subtilis* 54; **54 + Phi**: treated with *B. subtilis* 54 and manganese phosphite. Different letters on the columns indicate significant differences among treatments according to Duncan's multiple range test ($P < 0.05$).

*Stenotrophomonas maltophilia*. Our results are consistent with the reported by Kuklinsky-Sobral et al. (2005), which have focused on the endorhizospheric bacteria from soybean in the South American region. Although the bacterial isolates belonging to the *B. cereus*, *Chryseobacterium* spp. and *S. maltophilia* displayed interesting phenotypic characteristics as potential PGPR, due to the reasons previously outlined, only the *P. fluorescens* 9 and *B. subtilis* 54 strains were selected to carry out the inoculation assays on soybean seeds. Both isolates demonstrated the highest antifungal activity against the test pathogen *M. phaseolina in vitro*, together with other important plant growth promoting characteristics, such as mineral phosphate solubilisation, siderophore and auxin production (Table 1). Furthermore, the *in vitro* test carried out with the cell-free culture supernatant of strain *B. subtilis* 54, confirmed that the extracellular metabolites secreted by this isolate could inhibit the growth of the fungus. This finding is consistent with the well-established ability of *B. subtilis* to secrete a number of antibiotics, microbial lipopeptides and hydrolytic enzymes such as chitinases and proteases that can be harnessed for the control of phytopathogens (Cazorla et al. 2007; Romero et al. 2007).

Various studies have demonstrated that Phi can act as SAR inducers, mediating resistance to pathogens in a wide range of cultivated species (Mersha et al. 2012; Percival et al., 2009). In addition they can also exhibit direct toxicity against certain pathogens (Lobato et al. 2010; Lobato et al. 2011). Although we previously demonstrated the inhibitory effect of manganese phosphite on the fungal mycelial growth of *M. phaseolina in vitro* (Ravotti 2012), in the present study the MnPhi has shown to be least effective in controlling *M. phaseolina* when applied individually on soybean seeds. Its performance was significantly improved, compared with the untreated seeds, by its combined application with the strains *P. fluorescens* 9 and *B. subtilis* 54. The low efficacy provided by single MnPhi applications could be explained by different factors: (1) the high inoculum pressure prevailing under the *in vitro* conditions. In this way, the better performance of Phi when combined with the antagonistic bacteria may be explained by a reduction in the pathogen inoculum by the PGPR strains; (2) the expression of the induced resistance probably requires of the absorption of the Phi by the radicle into the soil and more time of incubation before the direct exposure to the pathogen.

The colonisation capacity of both bacterial strains was not affected by the subsequent treatment with Phi. Furthermore, the soybean plants from seeds treated with Phi, grown under greenhouse conditions, did not show phytotoxic effects. The separately application of Phi showed to be more effective in controlling *M. phaseolina* under greenhouse conditions than when applied on soybean seeds *in vitro*. This result could be due to the absorption of the Phi by roots, followed by a fast translocation in the xylem and phloem and probably by the induction of defense responses in the Phi-treated plants. These plants, only treated with Phi, exhibited a 37% of control. A similar result was obtained in a trial against

*Fusarium tucumaniae*, the primary causal agent of sudden death syndrome of soybean, where the Phi treatment reduced the soybean root rot severity evaluated at the end of the trial (unpublished results).

In the greenhouse trials, the two evaluated bacterial strains exhibited different behaviour when applied in combination with MnPhi. The greatest severity control was achieved when *P. fluorescens* 9 was applied singly or when *B. subtilis* 54 was combined with Phi. In a previous study, other *Pseudomonas* strain (GRP3) was characterised for its plant growth promotion activities against the charcoal rot disease (Choudhary 2011). In soybean seeds, treated with the strain GRP3 together with a spore suspension of *M. phaseolina*, a decrease in disease intensity was observed, accompanied by an enhanced level in the amount of jasmonate and ethylene dependent enzymes. Although the MnPhi did not affect the colonisation capacity of the strain *P. fluorescens* 9 it could probably affect any of the mechanisms involved in *M. phaseolina* biocontrol leading to only a 41% of severity control in the combined treatment.

By contrast, the application of strain *B. subtilis* 54 in combined treatment with Phi showed an additive effect, significantly reducing CFU density of pathogen on soybean plants compared to the treatment with strain *B. subtilis* 54 or with Phi separately (Fig. 6). To investigate whether the additive effect on plant protection by the combination of strain *Bacillus* 54 and MnPhi is related to the two widely described induced resistance pathways (SAR triggered by Phi and induced systemic resistance elicited by PGPR) the biochemical changes used as indicators of both pathways should be studied and quantified in future researches.

In conclusion, the results of the present study suggest that the combination of native PGPR strains with chemical inducers, such as Phi, would contribute to the development of novel strategies for integrated control of charcoal root rot of soybean. However, further research is required to investigate possible interactions with environment and another crop management practices such as soybean genotype and application of chemical fungicides. These interactions should be investigated under both laboratory and field conditions in order to provide an efficient alternative approach to control *M. phaseolina* root rot.

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