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Evaluation of actinomycete strains for key traits related with plant growth promotion and mycorrhiza helping activities

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ABSTRACT

Thirty actinomycete strains were isolated from the rhizosphere of field-grown plants (*Trifolium repens* L.) and identified by microscopic characteristics, biochemical tests and molecular techniques. The 16S rDNA sequence analysis showed that most of the isolates belong to the *Streptomyces* genus. These thirty isolates were tested for their capabilities of solubilizing/mineralizing sparingly phosphate sources, N_2-fixation and/or siderophore production, typical traits of the so-called Plant Growth Promoting Rhizobacteria (PGPR). Phosphate solubilizing ability was widely exhibited by the isolated. All of them produced acid phosphatase and thirteen of them alkaline phosphatase. Ten strains grew in N-free media. Almost all strains produced siderophores, however the production level was in general very low and only the strain *Thermobifida* MCR24 released considerable amounts of this metabolite. *Streptomyces* MCR9, *Thermobifida* MCR24 and *Streptomyces* MCR26 were selected to test their interactions with arbuscular mycorrhizal (AM) fungi because they produced the highest plant growth beneficial effects among ten isolates pre-selected as promissory PGPR. The inoculation of AM fungi improved early establishment of MCR9 in clover rhizosphere, and the late establishment of MCR24. The three actinomycete strains improved AM mycelial growth in absence of the test plant, and MCR9 and MCR26 also stimulate AM fungal spore germination. Inoculation of clover plants with either of the selected actinomycetes enhanced plant growth and N acquisition. Co-inoculation of actinomycetes and *Glomus mosseae* produced synergic benefits on plant growth and MCR9 and MCR24 also on P acquisition. The three selected actinomycetes improve AM formation by clover plants and *Glomus mosseae*.

1. Introduction

An increasing demand for low-input agriculture has resulted in a greater interest in soil microorganisms which are able to enhance plant nutrition and health, and to improve soil quality (Jeffries et al., 2003). Among the microbial groups, actinomycete bacteria and arbuscular mycorrhizal (AM) fungi are known to promote activities which can improve agricultural developments (Barea et al., 2005), thus these microorganisms appear as a research target with regard to sustainability purposes (Johansson et al., 2004).

Actinomycetes are one of the major components of the microbial populations present in soil. They belong to an extensive and diverse group of Gram-positive, aerobic, mycelial bacteria that play important ecological roles in soil nutrient cycling (Ames et al., 1984; Nonomura, 1989; Halder et al., 1991; Elliot and Lynch, 1995). In addition, these bacteria are known for their economic importance as producers of biologically active substances, such as antibiotics, vitamins and enzymes (de Boer et al., 2005). Actinomycetes are also an important source of diverse antimicrobial metabolites (Lazzarini et al., 2000; Basilio et al., 2003; Terkina et al., 2006). Historically the most commonly described actinomycete genera have been *Streptomyces* and *Micromonospora.* The genus *Streptomyces* is in fact known as one of the major sources of bioactive natural products (Bull et al., 1992; Basilio et al., 2003; Terkina et al., 2006). Particularly, it has been estimated that approximately two-thirds of natural antibiotics have been isolated from actinomycetes, and about 75% of them are produced by members of the genus *Streptomyces* (Newman et al., 2003; Jiménez-Esquilín and Roane, 2005). In the last decade research has focused on minor groups of actinomycetes, including species that are difficult to isolate and cultivate, and those that grow under extreme conditions, i.e. alkaline and acidic conditions (Lazzarini et al., 2000; Phoebe et al., 2001). However, most soil actinomycetes show their optimum growth in neutral and slightly alkaline conditions, thus their isolation procedures have been traditionally based on this neutrophilic character. Several selective isolation methods have been developed

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Table 1
Number of different actinomycete strains isolated from different location of Sabana de Bogota.

T (°C) of soil treated	Localization					
	Bogota *North* (CFU g^{-1})	Bogota *U. Nacional* (CFU g^{-1})	Zipaquirá (CFU g^{-1})	Guasca (CFU g^{-1})	Neusa (CFC g^{-1})	Villa de Leyva (CFU g^{-1})
45 °C for 2 h	0	2×10^7	0	2×10^7	1×10^7	1×10^7
50 °C for 10 min	2×10^7	5×10^7	4×10^7	2×10^7	6×10^7	2×10^7
60 °C for 30 min	0	1×10^7	0	0	1×10^7	0

Values are the means of values for 10 replicates samples from each one of ten plants from each place. $P = 0.05$.

(Goodfellow and OïDonnell, 1989; Edwards, 1993; Sabaou et al., 1998; Zakharova et al., 2003).

The AM fungi constitute another key group of soil-borne microorganisms known to play a critical role on agricultural sustainability after forming the AM symbiosis with roots of most plant species (Bagyaraj, 1984; Jeffries et al., 2003). These fungi influence, and are influenced by, the activities of other microorganisms in soil (Barea et al., 2005; Rillig et al., 2006; Lehr et al., 2008). Under natural conditions, mycorrhizal structures are surrounded by complex bacterial and fungal communities, which interact with the fungal–plant symbiosis at physical, metabolic and functional levels (Johansson et al., 2004; Finlay, 2008). Particularly, several studies have been conducted to identify bacterial isolates promoting the mycorrhizal symbiosis, the so-called "mycorrhizal helper bacteria" (Garbaye, 1994). Frey-Klett et al. (2007) reviewed the possible mechanisms underlying the mycorrhizal helper effect. The main mechanism involved is the direct effect of helper bacteria on the pre-symbiotic survival and growth of the mycorrhizal fungi in the soil.

As the AM symbiosis is a key component of agroecosystems it is important to increase our knowledge about ecological interactions with key groups of soil biota and AM fungi with a particular emphasis on research addressed toward managing these interactions (Barea and Jeffries, 1995; Elliot and Lynch, 1995). In this context, AM fungi have been used as rhizospheric biosensors of the impact from certain microbial inoculants such as the plant growth promoting rhizobacteria (PGPR) as reviewed by Barea et al. (2005).

Compatibility of actinomycetes inoculation with the formation and functioning of AM symbiosis has received some attention. For example, it has been found that certain *Streptomyces* strains which produce antimicrobial metabolites (El-Tarabily and Sivasithamparam, 2006) do not exhibit inhibitory effects on AM fungi, while other reports found inhibitory effects (Krishna et al., 1982; Ames, 1989). However, the information on actinomycetes, as PGPR, and their interactions with AM fungi is scarce.

The objective of the present study was to isolate and identify actinomycete strains with the aim of testing their abilities as plant growth promoting and/or mycorrhiza helper bacteria. Therefore, the isolated actinomycetes were screened for their ability to produce siderophore typical secondary metabolites involved in rhizosphere colonization competence, and tested for their phosphate solubilizing/mineralizing and/or N_2-fixing capabilities. Then the effects of selected actinomycete strains on AM formation and development, and their interactions with AM fungi on plant growth and nutrition, was also investigated. The Sabana de Bogotá, Colombia, and *Trifolium repens* L. were the target ecosystem and test plant species.

2. Materials and methods

2.1. Actinomycete isolation

Soil samples (pH 5.9, available P 1.13 ppm, total N 3.28 ppm, organic matter 17.26%) were collected from the rhizosphere of *T. repens* L. grown in the Sabana de Bogotá (North, U. Nacional, Ziquirá, Guasca, Neusa and Villa de Leyva), Colombia (lat 74°–73° and long 4°–5°) (Table 1). This clover species, a well-known model plant, with a ubiquitous distribution, had their roots mycorrhizal, as evidenced in the field-grown target plants by using the staining method of Phillips and Hayman (1970).

For actinomycete isolation, ten replicate samples from each one of ten plants from each one of the six target places (see Table 1) were taken. These samples were bulked and mixed, and then placed into plastic bags for the isolation and numeration of wild type actinomycete strains. Three batches of soil samples were prepared. These were heated at either 45 °C for 2 h, 50 °C for 10 min or 60 °C for 30 min, to promote growth on synthetic culture media of strains with complex growth cycles as it has been shown in the literature (Goodfellow, 1971). After the heat treatments, rhizospheric soil samples were suspended in sterile distilled water to prepare serial dilution (Nonomura and Ohara, 1969). Aliquots of each dilution (0.1 ml) were spread onto three different media, i.e. the Czapeck agar (g l^{-1}: K_2HPO_4 1, $NaNO_3$ 0.3, KCl 0.005, $MgSO_4 \cdot 7H_2O$ 0.005, $FeSO_4$ 0.0001, sucrose 30, agar 15), the casein starch agar (g l^{-1}: starch 10, casein 0.3, KNO_3 2, NaCl 2, K_2HPO_4 2, $MgSO_4 \cdot 7H_2O$ 0.05, $CaCO_3$ 0.02, $FeSO_4 \cdot 7H_2O$ 0.01 and agar 18) and the oat-meal agar (g l^{-1}:Oatmeal 60, agar 1.2), in Petri dishes. These media were supplemented with penicillin (25 mg ml^{-1}), nistatyn (0.1%) and cycloheximide (50 mg ml^{-1}) to inhibit growth of other bacteria and fungi, respectively. The plates were incubated at 22 °C for 15 days.

2.2. Morphological, physiological and chemotaxonomical identification

Morphology of different strain grown on tested media was analyzed for the mycelial organization and sporulation, under light microscopy. Cultural characteristics of actinomycetes were studied on various media as International *Streptomyces* Project (ISP) media (Sabaou et al., 1998), yeast extract-malt extract agar (ISP2), oat-meal agar (ISP3), inorganic salts-starch agar (ISP4) and oatmeal-yeast extract-glycerol (OYG) (Nonomura, 1989). Colors of aerial and substrate mycelia were determined with the ISCC-NBS centric color charts (National Bureau of Standards, 1964). Seventy-six tests were elaborated, including the utilization of 24 carbohydrate compound evaluated on BBL Crystal™ test (Becton, Dickinson and Company®) (Nonomura and Ohara, 1969), the degradation of organic compounds: adenine, guanine, xanthine, hypoxanthine, milk casein, tyrosine, testosterone (Goodfellow, 1971), in order to get physiological characteristics.

2.3. Molecular identification

Total genomic DNA from the bacteria was extracted using a modified protocol from J. Sanjuan (Com. Per). The actinomycete strains were grown during 4 days at 22 °C with agitation in 500 ml flasks containing 100 ml of ISP2 medium (Sabaou et al., 1998). The bacterial biomass was harvested by centrifugation (12,000 × *g*, 10 min) and cleaned three times with distilled water (50 ml). The mycelia were used for DNA extraction as follows: the sample was dispersed in 800 μl of the aqueous lyses solution (100 mmol l^{-1}

Tris–HCl, pH 7; 20 mmol l^{-1} EDTA; 250 mmol l^{-1} NaCl; 2%, w/v, SDS; 1 mg ml^{-1} lysozyme) and the suspension incubated at 37 °C for 60 min. Ten μl of a proteinase K solution (20 mg ml^{-1}) was added and the lyses solution was re-incubated at 65 °C for 30 min. The lysate was extracted with an equal volume of phenol and centrifuged (12,000 × *g*, 10 min). The aqueous layer was re-extracted with equal volume of phenol:chloroform. DNA was recovered from the aqueous phase by the addition of NaCl (150 mmol l^{-1} final concentration) and 2 volumes of cool 95% (v/v) ethanol. The precipitated DNA was cleaned with 50 μl of 70% (v/v) ethanol, centrifuged (12,000 × *g*, 10 min), resuspended in 50 μl of TE buffer (10 mmol l^{-1} Tris–HCl, pH 7.4; 1 mmol l^{-1} EDTA, pH 8), and stored at −20 °C.

For 16S RNA gene amplification and sequencing, the PCR technique was carried out in 50 μl of final volume containing 2 mM $MgCl_2$, 2U *Taq* polymerase (PROMEGA), 150 μM of each dNTP, 0.5 mM of each primer: Primer F1 (59-AGAGTTTGATCITGGCTCAG-39; I = inosine) and primer R5 (59-ACGGITACCTTGTTACGACTT-39), and 2 μl template DNA, using Cook & Meyers protocols (2003). These primers were used to amplify nearly full-length 16S rDNA sequences. The PCR program used was an initial denaturation (96 °C for 2 min), 30 cycles of denaturation (96 °C for 45 s), annealing (56 °C for 30 s) and extension (72 °C for 2 min), and a final extension (72 °C for 5 min). The PCR products were submitted to electrophoresis on 1% agarose gels, containing ethidium bromide (10 μg ml^{-1}), to ensure that a fragment of the correct size had been amplified. The 16S fragments were sequenced by Macrogen®. The sequences were grouped by the NCBI Taxonomy Browser (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtzx.cgi), and BLAST analysis was realized. The complete sequence of PCR fragments were compared in GenBank.

The restriction endonuclease digestion and analysis were realized with PCR amplified DNA. The *Sau*3AI digestion was purified using the QIAquick PCR Purification Kit (Qiagen). No pre-treatment of the DNA was required for the other restriction endonucleases (AsnI, KpnI, SphI, ScaI, PstI, HindIII, SnaBI, SalI, PvuII, AgeI, SstI). Restriction digestions were incubated at 37 °C for 3–4 h. Samples were electrophoresed on 1.5% agarose gels containing ethidium bromide (10 μg ml^{-1}). The restriction fragment patterns were manually compared with those from the in silica restriction endonuclease digestions and a series of dichotomous keys developed by Cook & Meyers (2003) only for actinomycetes were used.

2.4. Qualitatively phosphate solubilizing capacity

Actinomycete isolated were purified on oat-meal agar and then cultivated on minimal medium based on the Pikovskaya (PVK) medium described by Pikovskaya (1948). This medium contained (per liter): glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.2 g; $MgSO_4{\cdot}7H_2O$, 0.1 g; KCl, 0.2 g; yeast extract, 0.5 g; $MnSO_4{\cdot}H_2O$, 0.002 g; and $FeSO_4{\cdot}7H_2O$, 0.002 g, supplemented with 1.5% purified agar free from microbial inhibitors (Merck KGaA). The development of a clear zone around the colony on the culture plates was taken as an index of phosphate solubilization. After that the halo-forming bacteria were grown on SRSM-1 medium with the same composition of the PVK medium but supplemented with bromocresol purple as a pH indicator for determining the involvement of organic acid production in the tested phosphate solubilizing activity. The media pH was adjusted to 7.0 before autoclaving. Thirty-three strains per plate were stabbed in triplicate using sterile toothpicks. The phosphate solubilization halo and colony diameters were measured after 14 days of plate incubation at 22 °C. Halo size was calculated by subtracting colony diameter from the total diameter. The experiments were replicated three times and the results were average. The phosphate solubilizing capacity was determine positive as the halo diameter ≥5 mm.

2.5. Phosphate mineralization ability

This was estimated by measuring the production of either acid or alkaline phosphatases by all actinomycete isolates (Tabatabai and Bremner, 1969). The actinomycetes strains were grown in Oat Meal broth at 22 °C. The bacterial cultures were filtered through Whatman No. 42 filter paper and homogenized in a mortar at 4 °C using 0.02 M Tris buffer (1:1, w/v, pH 7.5). The macerate was centrifuged at 16,000 × *g* for 20 min and the supernatant was collected. For assaying alkaline phosphatase activity 0.1 ml of the culture supernatant were added to a reaction mixture consisting of 0.5 ml of a tris citrate buffer (pH 8.5/5.5 mM), 0.1 ml of $MgCl_2$ (0.1 M) and 1.0 ml of p-Nitrophenol phosphate (1 mg/ml) solution. The same procedure was followed for acid phosphatase activity but an acetate buffer (pH 4.5/0.1 M) was used instead of the tris citrate buffer. The test tubes were incubated at 22 °C for 30 min. After that, 5 ml of 0.5 M NaOH were added and the p-Nitrophenol released was measured by spectrophotometry at 405 nm. The measured values were converted to micromoles of p-Nitrophenol with reference to a standard curve. One enzyme unit was defined as the amount of enzyme that catalysed formation of 1 μmol of end product (p-Nitrophenol) in 1 min under experimental conditions (Tabatabai and Bremner, 1969).

2.6. Growth in N-free medium

The bacterial growth of the isolates actinomycetes in a nitrogen-free medium (NFB) was compared with that in a nitrogen-low medium (Ashby) and in nitrogen-standard media (ISP-2, ISP-3, Czapek agar). The composition of these media were as follows: (i) ISP medium 2 (yeast extract 0.4%, malt extract 1%, dextrose 0.4% and NH_4NO_3 0.01%); (ii) ISP medium 3 (oat-meal agar with NH_4NO_3 (0.01%)); (iii) Czapek agar medium with $(NH_4)_2SO_4$ (0.1%) (Atlas, 1993); (iv) Ashby (Manitol 0.2%, $CaCl_2$ 0.02%, KH_2PO_4 0.1%, $MgSO_4$ 0.02%, soil extract 10%, $FeSO_4$ 0.0005%, NaCl 0.02%); and (v) NFB (D-malic Acid 0.5%, KH_2PO_4 solution (10%) 5 ml l^{-1}, $MgSO_4$ solution (10%) 2 ml l^{-1}, NaCl solution (10%) 1 ml l^{-1}, $CaCl_2$ solution (10%) 2 ml l^{-1}, Bromothymol Blue solution (5%) in KON (2N) 2 ml l^{-1}, micronutrients solution 2 ml l^{-1} (per 200 ml: $Na_2MoO_4{\cdot}2H_2O$ 0.2 g, $MnSO_4$ 0.235 g, boric acid 0.280 g, $CuSO_4{\cdot}5H_2O$ 0.008 g, $ZnSO_4{\cdot}7H_2O$ 0.024 g), EDTA solution (1.64%) 4 ml l^{-1}, KOH 4.5 g). These media were supplemented with 1% purified agar free from microbial inhibitors (Merck KGaA) (without nitrogen source). Cultures were examined for growth characteristics in each medium after 2–3 weeks of incubation at 22 °C.

2.7. Siderophore assay

Isolated actinomycetes were inoculated on the medium of Simon and Tessman (1963), tryptone added, under severe iron limitation conditions. This medium contained (g l^{-1}): Sodium succinate 10, NaCl 5.8, KCl 3.7, $CaCl_2{\cdot}2H_2O$ 0.15, Tris–HCl 12.1, $MgCl_2{\cdot}7H_2O$ 0.1, NH_4Cl 1.1, $Na_2SO_4{\cdot}2H_2O$ 0.142, KH_2PO_4 0.272. The actinomycetes were grown in baffled shake flasks at 22 °C and 130 rpm, for 10 days. Cultures were sampled every day and samples centrifuged in a Sorvall centrifuge at 1600 × *g* and 5 °C for 15 min. One ml of supernatant was then carefully removed, and mixed with an equal volume of 5 mM ferric perchlorate in 0.14 M of perchloric acid. The reaction mixture was read at 480 nm of absorbance. The data were compared with a calibration curve using as control a strain of *Escherichia coli* (from Culture Collection of Dr. Mikan, Universidad Militar Nueva Granada, Bogotá-Colombia), reported as siderophore-producer strain. The siderophore concentration was measured from days 8 to 11 according with previous tests.

2.8. Effects of actinomycete inoculation on plant growth promotion

Ten actinomycete strains, namely, MCR3, MCR9, MCR10, MCR14, MCR16, MCR24, MCR25, MCR26, MCR27 and MCR32 were pre-selected to investigate their plant growth promotion effects. The reasons for selection, as explained in the Results Session, are the higher ability of these strains for phosphate solubilizing/mineralizing ability, to grow in N-free media and to produce siderophores. To investigate the plant promotion effects of these actinomycetes, a pot experiment was carried out. This involved clover plants (*T. repens* L.) that were grown in a sterilized soil-sand mixture (300-ml pot). The actinomycete strains were inoculated on the rootlets system of the clover plants at a concentration of 10^4 cells/pot. There were eleven treatments (ten actinomycete strains and one un-inoculated control) with five replications for each treatment. Surface-sterilized seeds of white clover were germinated on the sterilized sand-soil substrate and four seedlings were planted in each pot, watered as needed and grown for 30 days. Pots were placed in a growth chamber in a completely randomized design. After the growth period, the plants were harvested, dried at 60 °C for 1 day and weighed.

2.9. Interaction between actinomycetes and AM fungi

According to the results obtained in the pot experiment described under Section 2.8 (data not shown), three actinomycete strains, i.e. MCR9, MCR24 and MCR26, were selected for further interaction experiments involving AM fungi. The objectives of these interaction experiments were to ascertain the effects of the selected actinomycetes, in co-inoculation with AM fungi, on (i) AM fungal spore germination and mycelial growth both *in vitro* and in soil; (ii) plant growth, nutrient uptake and AM formation by clover plant; and (iii) changes in actinomycete populations in the rhizosphere of co-inoculated plants.

Experiments to test AM fungal spore germination and mycelial growth *in vitro* were based on the methodological approaches of Azcón-Aguilar et al. (1986). Spores of the AM fungi, *Glomus mosseae* were isolated from sporocarps obtained from rhizosphere samples of *T. repens* L. plants grown in pot cultures. The rhizosphere soil samples were kept in polyethylene bags at 4 °C, and the sporocarps were stored on damp filter paper at 4 °C. Resting spores freshly excised from the sporocarps were surface sterilized in a solution containing per liter chloramine T, 20 g; streptomycin, 200 mg; and Tween 80, 25 μl; for 20 min and then washed five times in sterile water (Mosse, 1962).

The three actinomycete strains, i.e. MCR9, MCR24 and MCR26 were grown at 22 °C for 5 days on Czapeck-Dox medium. Their cultures were centrifuged, and the cells washed three times in saline solution (0.1%) to prepare suspensions at a concentration of 10^6 cell ml^{-1}. Fifty μl portions were spread onto the agar surface in Petri dishes (diameter, 9 cm) containing water agar (0.8% Merck Agar) buffered with 10 mM MES [2-(*N*-morpholino) ethanesulfonic acid] (Calvet et al., 1992). The final pH after sterilization at 120 °C for 20 min was 7.0. Six surface-sterilized spores of *G. mosseae* were transferred individually to each actinomycete-inoculated Petri dish; these six spores were located at the vertices of an imaginary hexagon with sides approximately 3.5 cm long. Plates containing *G. mosseae* spores growing axenically were used as a control. The plates were sealed with Parafilm and incubated at 25 °C in the dark. Each treatment consisted of three replicate plates. Spore germination was checked thirty two days after inoculation. Hyphal growth from germinated resting spores was measured by a gridline intersect method (Azcón-Aguilar et al., 1986).

The effect of the test actinomycetes on AM fungal spore germination in soil was assessed following previous studies (Barea et al., 1998). Each experimental unit consisted of a slide frame that held two membrane filters (diameter, 45 mm; pore size, 0.45 μm). Twenty unsterilized *G. mosseae* spores were introduced between two membranes. Twenty-five grams of soil was placed in a 9-cm-diameter petri dish, and the sandwich units were then laid onto this soil layer. An agricultural soil collected from the Sabana de Bogotá (Colombia) was used. One milliliter of the corresponding actinomycete strain (MCR9, MCR24 and MCR26) suspension containing 10^7 cells ml^{-1} was applied to the membranes. Another 25 g of soil was added to cover the sandwich units. The soil was moistened to field capacity with distilled water, and the Petri dishes were sealed with Parafilm. The dishes (fifteen replicates for each treatment) were then incubated at 25 °C for 2 weeks. Upon removal from each dish sandwiches were opened, and germ tubes were stained with trypan blue (Phillips and Hayman, 1970). The percentage of germinated spores was calculated for each treatment. All experiments were conducted twice, and data for the level of germination for each treatment, including both *in vitro* and soil tests, was subjected to hypothesis test against control data. The *z* statistics tabulated at the 5% significance level. Mycelial growth data was processed by analysis of variance method and Duncan's multirange test. These experiments were repeated three times and the magnitudes of the responses were similar each time.

A pot experiment was carried out to investigate the effects of interaction of actinomycetes and AM fungi inoculation on plant growth and nutrition, AM colonization and dynamic population of actinomycetes in plant rhizosphere. The actinomycetes strains (10^4 cells/pot) and AM fungal (5 g/pot) treatment include: a control (sterile broth), MCR9, MCR24, MCR26, *G. mosseae*, MCR9 + *G. mosseae*, MCR24 + *G. mosseae*, and MCR26 + *G. mosseae*. There were eight treatments per experiment with five replications for each treatment. The soil-sand mix substrate was sterilized and all treatments received a sterilized *G. mosseae* inoculum. Surface-sterilized seed of white clover (*T. repens* L.) was germinated on the sterilized sand-soil substrate and four seedlings were planted in each pot, watered as needed and grown for 180 days. Pots were placed in a growth chamber in a completely randomized design.

After 180 d of growth, the plants were harvested. The plant material was washed free of soil, and, with exception of a 2 g root subsample, dried at 60 °C for 1 day. After drying and weighing, roots and leaves were grounded and analyzed for N and P content (Merzaeva & Shirokikh, 2006). At the end of the growing period, soil from the central portion of each pot was mixed thoroughly and sampled for study of AM hyphal density (Ames et al., 1984, 1987). Actinomycete populations were determined by dilution plating on oat-meal agar (Franco-Correa, 1999). The root subsamples were cleared and stained with trypan blue (Phillips and Hayman, 1970) and assessed for percentage of mycorrhizal root length (Giovannetti and Mosse, 1980). The data were subject to variance analysis. This experiment was repeated twice and the magnitudes of the responses were similar each time.

3. Results

3.1. Isolation and identification of actinomycetes

Thirty wild type actinomycete strains were isolated from the rhizosphere of *T. repens* L. grown in the Sabana de Bogotá, Colombia. The Oat-Meal agar medium added with penicillin, nistatyn and cycloheximide was selected for actinomycete isolation from rhizospheric soil samples. It was found that 50 °C for 10 min was the best heat regimen for recovering sporoformer actinomycetes after heat treatments of the rhizospheric soil (Table 1).

A number of actinomycete colonies with different morphological and cultural characteristics were picked from 1- to 4-week-old

Table 2
Identification of Actinomycetes. Molecular identification data (ARDRA, BLAST analysis) compared with morphological (microscopic characterization) and biochemical characterization.

Strain	Microscopic characterization	ARDRA	Biochemical characterization	BLAST analysis	Accession number
1	*Streptomyces*	*Streptomyces*	NI	–	AY917141.1
2	*Streptomyces*	*Saccharopo-lyspora*	NI	*Saccharopolyspora* (84%) *Streptomyces* (87%)	EU086556.1 DQ411818.1 EU080964.1
3	NI	*Microbispora*	*Streptomyces*	*Microbispora* (83%)	DQ530081.1
4	*Nocardia*	*Thermobifida*	NI	*Thermobifida* (83%)	AB006171
5	*Streptomyces*	*Streptomyces*	NI	–	EU080964.1 EF494217.1
6	*Streptomyces*	*Streptomyces*	NI	–	EU080964.1
7	*Nocardia*	*Thermonospo-ra*	NI	–	DQ343066.1
8	NI	*Thermobifida*	NI	–	DQ343066.1
9	*Streptomyces*	*Streptomyces*	*Streptomyces*	*Streptomyces* (98%)	EU080964.1 EF494217.1
10	*Streptomyces*	*Streptomyces*	*Nocardia*	*Streptomyces* (99%)	EU080964.1 EF494217.1
11	*Streptomyces*	*Streptomyces*	NI	*Streptomyces* (96%)	EU080964.1 EU273536.1
12	*Nocardia*	*Streptomyces*	NI	*Streptomyces* (96%)	EU080964.1 EU273536.1
13	NI	*Streptomyces*	NI	*Streptomyces* (99%)	EU080964.1 EF494217.1
14	*Streptomyces*	*Streptomyces*	*Streptomyces*	*Streptomyces* (99%)	EU080964.1
16	NI	*Streptomyces*	*Nocardia*	–	EU273536.1
17	*Streptomyces*	*Streptomyces*	NI	*Streptomyces* (97%)	EU080964.1
18	*Streptomyces*	*Streptomyces*	NI	*Streptomyces* (96%)	EU080964.1 EF494217.1
19	*Streptomyces*	*Streptomyces*	NI	*Streptomyces* (99%)	EU080964.1 EU273536.1
20	*Streptomyces*	*Streptomyces*	NI	*Streptomyces* (98%)	EU080964.1 EU273536.1
21	*Streptomyces*	*Streptomyces*	*Streptomyces*	*Streptomyces* (96%)	EU080964.1 EF494217.1
22	*Streptomyces*	*Thermobifida*	NI	–	DQ343066.1
23	*Streptomyces*	*Streptomyces*	NI		EU080964.1
24	*Nocardia*	*Thermobifida*	*Nocardia*	*Thermobifida* (84%)	AB006171
25	NI	*Saccharopo-lyspora*	NI	–	DQ343060.1
26	NI	*Streptomyces*	*Nocardia*	–	EU080964.1
27	NI	*Streptomyces*	*Pseudonocardia*	*Streptomyces* (96%)	EU080964.1
30	*Streptomyces*	*Streptomyces*	*Streptomyces*	–	EU080964.1
31	*Nocardia*	*Streptomyces*	NI	–	EU080964.1 EF494217.1
32	NI	*Kitasatospora*	*Nocardia*	*Nocardia* (85%) *Kitasatospora* (86%) *Streptomyces* (97%)	DQ343060.1 DQ343066.1 EF494217.1
CS	*Streptomyces*	*Streptomyces*	*Streptomyces*	*Streptomyces coelicolor* (99%)	EU080964.1 EU294136.1 EU257263.1

NI: Not identified. DR: Did not realize. CS: Control strain: from culture collection of Biomedical Laboratory of Universidad de Sao Paulo.

isolation plates and transferred to oat-meal agar medium for purification and morphology observation. Sixteen out of the thirty isolates were tentatively assigned to the genus *Streptomyces* according to their macro- and micromorphological and cultural characteristics. *Streptomyces* isolates had rich aerial mycelia and maturate to form short, long, spiral, or straight spore chain. Most of their colonies showed fuzzy surface because of sporulation. Some rare actinomycetes, such as *Nocardia* sp. with branched substrate mycelium, were tentatively identified. Molecular identification data compared with morphological and biochemical identification are recorded in Table 2.

The percentage (68%) of *Streptomyces* found in soil was very high compared with other genera of actinomycetes. The BLAST analysis was performed for all actinomycetes. However only with MCR2, MCR3, MCR4, MCR9, MCR10, MCR11, MCR12, MCR13, MCR14, MCR17, MCR18, MCR19, MCR20, MCR21, MCR24, MCR27, MCR32 and reference strains, an accession number was found which indicates a genus within the actinomycete group (Table 2). The other isolates can be non-identified taxa needing further studies for characterization. The results shown the confidence of sequencing analysis using the F1 and R5 primers correlating with those from the restriction endonuclease digestion technique used by Cook

Table 3
Qualitative phosphate solubilization in Pikovskaya and SRSM-1 media. Ability of actinomycete isolates to grow in culture media without nitrogen source. The isolates may be diazotrophs based on their ability to grow in N-free media.

Strain	Solubilization Pikovskaya (Halo ≥ 5 mm)	Acidification SRSM-1	Acidic phosphatase activity (enzyme unit: 15–25)	Alkaline phosphatase activity (enzyme unit: 10–15)	Growth on N free media	Siderophore production
MCR1	+	+	+	–	–	–
MCR 2	+	+	+	–	–	–
MCR 3	+	+	+	–	+	+
MCR 4	+	+	+	+	–	–
MCR 5	+	+	+	–	–	–
MCR 6	–	–	+	–	–	–
MCR 7	+	+	+	+	–	–
MCR 8	+	+	+	+	–	–
MCR 9	–	+	+	+	+	+
MCR10	+	+	+	–	+	+
MCR11	+	+	+	–	–	–
MCR12	+	+	+	+	–	–
MCR13	–	+	+	–	–	–
MCR14	–	+	+	–	+	+
MCR16	–	+	+	+	+	+
MCR17	+	+	+	+	–	–
MCR18	+	+	+	–	–	–
MCR19	–	+	+	+	–	–
MCR20	–	+	+	–	–	–
MCR21	–	+	+	–	–	+
MCR22	+	+	+	+	+	–
MCR23	+	+	+	+	–	–
MCR24	+	+	+	–	–	+
MCR25	+	+	+	+	+	–
MCR26	+	+	+	+	–	+
MCR27	–	+	+	–	+	+
MCR30	+	+	+	+	+	–
MCR31	+	+	+	–	+	–
MCR32	+	+	+	–	+	+
MCR33	+	+	+	–	+	–

and Meyers (2003). The amplification products were 1500–1540 bp fragments. The restriction endonuclease digestion and analysis results identified the strains MCR4, MCR8, MCR22 and MCR24 as *Thermobifida,* a genus established by Zhenshui et al. (1998) on the basis of phylogenetic and chemical taxonomic studies.

3.2. Qualitatively phosphate solubilizing ability

Qualitative estimation of solubilization by actinomycetes strains grown on Pikovskaya (PVK) (1948) and SRSM-1 media containing tri-calcium phosphate showed the development of a clear solubilization zone around the colony. Comparative studies on these media with the thirty bacteria indicated that twenty one of them exhibited phosphate solubilization ability (Table 3). However, in a plate assay with SRSM-1 medium, it was observed that ten strains produced acidification but these strains did not exhibit phosphate solubilization on PVK medium.

3.3. Phosphatase activity

The ability to produce acidic and alkaline phosphatase activity by the target actinomycetes is recorded in Table 3. All strains showed acidic phosphatase activity and thirteen of them alkaline phosphatase activity. The strains *Streptomyces* (MCR26), *Streptomyces* (MCR9) and *Thermobifida* (MCR24) exhibited the highest phosphatase activity.

3.4. Growth in N-free medium

Growth ability for the thirty actinomycetes strains on the N-free Ashby and NFB media are shown in Table 3. Ten strains were able to growth in these N-deficient media suggesting that they could be nitrogen fixing bacteria.

3.5. Siderophore production

With the strains that grew in nitrogen-free media were evaluated for the production of siderophores. Most strains produced siderophores except *Streptomyces cuspidosporus* (MCR30) as shown by the brown color due to the reaction of ferric cation chelation as a consequence of siderophore presence (Leong and Winkelmann, 1998). However, most strains produce very low amounts of this metabolite in comparison with the reference strain but MCR24 (*Thermobifida*) produce a highest amounts of siderophores (Table 3).

3.6. Interaction between actinomycetes and AM fungi

According to results summarized in Table 3, ten out the thirty isolated actinomycete strains (namely, MCR3, MCR9, MCR10, MCR14, MCR16, MCR24, MCR25, MCR26, MCR27 and MCR32), were pre-selected to investigate their plant growth promotion effects on clover plants. The corresponding pot experiment (results not shown) allowed us to select three out of these ten strains because they produced the higher plant growth promoting effects. Accordingly, three actinomycete strains, namely *Streptomyces* MCR9, *Thermobifida* MCR24 and *Streptomyces* MCR 26 were further selected and used to investigate some interactions with AM fungi (*G. mosseae*).

Interaction experiments not involving the presence of a host plant demonstrated that *Streptomyces* MCR9 and *Streptomyces* MCR26 strains stimulated germination and mycelial development from *G. mosseae* spores both *in vitro* and in soil while *Thermobifida* MCR24 inhibited spore germination but stimulated mycelial development, as summarized in Table 4. The results of other assays, which were designed to investigate any growth promotion effects using clover plants, can be summarized as follows. In general the inoculated actinomycetes, either alone or in co-inoculation with

Table 4
Effect of Actinomycete strains on *Glomus* spore germination *in vitro* and *in soil* and on mycelial growth in vitro.

Actinomycete strain	% Spore germination In vitro	In soil	Mycelial development (mm/germinated spore)
Control	71b	62b	13c
Streptomyces MCR9 sp.	81a	76a	48a
Streptomyces MCR26 sp.	85a	80a	32b
Thermobifida MCR24 sp.	25c	18c	28b

Values are the means for 18 replicates for spore germination *in vitro* and 300 replicates for spore germination in soil. Mean values in a column not followed by the same letter differ significantly at $P = 0.05$.

Table 5
Population dynamics of the selected actinomycetes inoculated either alone or in co-inoculation with *Glomus*. The population was measured each month during 6 months (3 replicates per treatment).

	Month	CFU/g rhizospheri c soil
MCR9	2	28 × 10E3 b
	4	35 × 10E3 b
	6	12 × 10E3 b
MCR9 + *Glomus*	2	73 × 10E3 b
	4	32 × 10E4 a
	6	95 × 10E3 b
MCR24	2	50 × 10E3 b
	4	15 × 10E4 a
	6	70 × 10E3 b
MCR24 + *Glomus*	2	75 × 10E3 b
	4	77 × 10E3 b
	6	17 × 10E4 a
MCR26	2	49 × 10E3 b
	4	17 × 10E4 a
	6	45 × 10E3 b
MCR26 + *Glomus*	2	15 × 10E3 b
	4	32 × 10E3 b
	6	51 × 10E3 b

For each experimental variable and sampling time mean values followed by the same letter are not significant different at $P = 0.05$.

G. mosseae, were established successfully in soil through time (Table 5). This experiment was repeated twice and the magnitudes of the responses were similar each time. Inoculation of AM fungi improved early establishment of *Streptomyces* MCR9 in clover rhizosphere, and the late establishment of *Thermobifida* MCR24. Inoculation with either actinomycetes or AM fungi increased plant biomass production. Co-inoculation of both types of microorganisms yielded significant plant growth (particularly shoot biomass at the end of the assay) effects over those of the individual inoculation with either organism alone. At the last harvest time, actinomycete inoculation increased the total mycorrhizal root length of *Glomus* inoculated plants (Table 6).

Inoculation with either actinomycetes or AM fungi, either alone or in combination, increased N acquisition by plants. Inoculation of MCR24 alone and *G. mosseae*, either alone or in combination with the tested actinomycetes MCR9 and MCR24, also benefited plant P acquisition with a significant synergic effect of the co-inoculation (Table 7).

4. Discussion

As in the case of other bacteria, the 16S rDNA sequencing approach is routinely used to identify actinomycete isolated after comparing with already described organisms in an rDNA sequence database (Edwards, 1993; El-Tarabily and Sivasithamparam, 2006). The F1 and R5 primers, used for amplification of 16S rDNA gene of the actinomycete isolates in here reported experiments, confirmed their efficiency to amplify all sequences of 16S rDNA gene of target bacteria (Cook and Meyers, 2003). Results here reported support the use of the Cook and Meyers methodology (2003) because a partial 16S rDNA sequence of a new actinomycete isolate can be obtained quickly and at low cost to give an unambiguous identification of the genus to which the isolate belongs.

Most of the actinomycete strains tested seems to have the ability of solubilizing sparingly available inorganic P sources or mineralizing some P from the organic P sources in soil. In fact, 70% of then solubilized inorganic P in the PVK medium and all of them acidified the SRSM-1. Among the several potential mechanisms for phosphate solubilization those involving the production of chelating compounds, like organic acids or by means of a modification of pH of the medium by the secretion of organic acids or protons are the more often described in the literature (Pikovskaya, 1948; Pareek and Gaur, 1973; Richardson et al., 2009). The secretion of acid phosphatase indicated that all the tested actinomycete strains would be able to mineralize organic P sources (Richardson et al., 2009). The potential for phosphate solubilization of the target actinomycetes supports future research for quantitative analysis (Pareek and Gaur, 1973; Halder et al., 1991).

The production of siderophores, a mechanism/strategy used by PGPR for rhizosphere colonization competence, has been shown to be, in some extent, related with nitrogen fixation. In fact, symbiotic nitrogen fixers such as *Bradyrhizobium japonicum*, *Rhizobium leguminosarum*, and *Sinorhizobium meliloti* were reported as producer of

Table 6
Effects of Actinomycete strains on mycorrhiza formation and biomass production by *Trifolium repens* L. plants growing during 6 month under greenhouse conditions (5 replicates per treatment).

	Shoot biomass production (mg[dry wt])* (month)			Root biomass production (mg[dry wt])* (month)			Mycorrhizal root length (%) (month)**		
	2	4	6	2	4	6	2	4	6
Control (none)	22d	45d	96d	5a	10c	22c	5c	8c	15c
Glomus sp.	98b	146b	185c	8a	24ab	36b	35b	46b	58b
Streptomyces MCR9	45c	90c	189c	7a	21b	46ab	3c	5c	10c
MCR9 + *Glomus* sp.	123a	257a	605a	7a	26ab	47ab	47a	68a	69a
Thermobifida MCR24	65c	132b	252b	6a	17b	35b	0c	7c	4c
MCR24 + *Glomus* sp.	135a	283a	584a	10a	27a	54a	37ab	52b	73a
Streptomyces MCR26	32c	85c	210c	8a	22b	41b	3c	5c	3c
MCR26 + *Glomus* sp.	106ab	238a	506a	12a	35a	68a	40a	74a	68a

For each experimental variable and sampling time mean values followed by the same letter are not significant different.
* $P < 0.05$ (Duncan test).
** $P < 0.001$ (Duncan test).

Table 7
N and P concentration (%) in plant (shoots and roots) of *T. repens* inoculated with actinomycete strains and/or *Glomus* sp. after 6 month of plant growth under greenhouse conditions (5 replicates per microbial treatment).

	Absolute control	*Glomus*	MCR9	MCR24	MCR26	*Glomus* + MCR9	*Glomus* + MCR24	*Glomus* + MCR26
N (ppm)	1.7b	2.8a	2.5a	2.7a	2.3a	2.6a	2.8a	2.5a
P (ppm)	0.19c	0.31b	0.18c	0.33b	0.15c	0.44a	0.48a	0.27b

For each nutrient mean values followed by the same letter are not significant different. $P < 0.001$ (Duncan test).

siderophores (Surange et al., 1997; Carson et al., 2000; El-Tarabily and Sivasithamparam, 2006). In the present study, 22 out of the 30 stains tested were either positive with regard to both siderophore production and ability to fix nitrogen, or negative in both activities.

There is also some evidence that diverse microbial communities may be selectively present in association with certain mycorrhizal mycelia (Read and Pérez-Moreno, 2003; Johansson et al., 2004; Finlay, 2008). Some of the actinomycetes strains tested improved AM mycelia development in soil (Table 4) and enhanced mycorrhizal formation by clover plants inoculated with the AM fungus *G. mosseae* (Table 6).

Numerous studies have shown that bacterial communities associated with fungal hyphae, fungal spores, or with the mycorrhizosphere of mycorrhizal plants display fungus-specific differential effects (Brule et al., 2001; Founoune et al., 2002; de Boer et al., 2005; Frey-Klett and Garbaye, 2005; Roesti et al., 2005). This suggests that rhizosphere bacteria are under selection to develop fungus-specific traits that confer a competitive advantage during colonization of fungal surfaces. Bacteria-induced alterations to fungal development differentiation have been described in previous studies (Barea et al., 2005). These effects include inhibition or promotion of germination, and alterations to foraging behavior, hyphal branching, growth, survival, reproduction, exudates composition and antibacterial metabolites production (de Boer et al., 2005).

Antagonistic interaction between an AM fungus and actinomycetes has been reported (Krishna et al., 1982). This was evident for *Thermobifida* MCR24 with regard to spore germination but not for AM mycelial development or for plant nutrition and growth promotion.

Activities that promote plant growth could be an indirect way to influence AM symbiosis development. Actinomycete isolates improved both shoots and roots biomass accumulation. These actinomycetes can be considered Mycorrhiza Helper Bacteria (MHB) because they may promote the mycorrhizal colonization rate at different stages of bacterium–fungus–plant interactions, including spore germination. This ability was shown by some MHB, as described by Tarkka and Frey-Klett (2008), and particularly by *Streptomyces* sp., (Meyer and Linderman, 1986; Mugnier and Mosse, 1987; Schrey et al., 2005).

5. Conclusion

The reported experiments show that the target actinomycete strains are able to improve plant growth and nutrition, and benefit root colonization by AM fungi. Co-inoculation with both types of microorganisms showed synergic effects at enhancing plant growth and nutrient acquisition. Here reported results support the use of actinomycetes as plant growth promoting and mycorrhiza helper bacteria.

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