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Research Paper

Potential biocontrol actinobacteria: Rhizospheric isolates from the Argentine Pampas lowlands legumes

Mariana Solans[1], Jose Martin Scervino[1], María Inés Messuti[1], Gernot Vobis[1] and Luis Gabriel Wall[2]

[1] INIBIOMA, UNComahue, CONICET. CRUB, Quintral 1250, S. C. Bariloche 8400, Argentina
[2] Departamento de Ciencia y Tecnología, Programa de Interacciones Biológicas, UNQuilmes, CONICET, R. Saénz Peña 352, B1876BXD Bernal, Argentina

Control of fungal plant diseases by using naturally occurring non-pathogenic microorganisms represents a promising approach to biocontrol agents. This study reports the isolation, characterization, and fungal antagonistic activity of actinobacteria from forage soils in the Flooding Pampa, Argentina. A total of 32 saprophytic strains of actinobacteria were obtained by different isolation methods from rhizospheric soil of *Lotus tenuis* growing in the Salado River Basin. Based on physiological traits, eight isolates were selected for their biocontrol-related activities such as production of lytic extracellular enzymes, siderophores, hydrogen cyanide (HCN), and antagonistic activity against *Cercospora sojina*, *Macrophomia phaseolina*, *Phomopsis* sp., *Fusarium oxysporum*, and *Fusarium verticilloides.* These actinobacteria strains were characterized morphologically, physiologically, and identified by using molecular techniques. The characterization of biocontrol-related activities *in vitro* showed positive results for exoprotease, phospholipase, fungal growth inhibition, and siderophore production. However, none of the strains was positive for the production of hydrogen cyanide (HCN). *Streptomyces* sp. MM140 presented the highest index for biocontrol, and appear to be promising pathogenic fungi biocontrol agents. These results show the potential capacity of actinobacteria isolated from forage soils in the Argentine Pampas lowlands as promising biocontrol agents, and their future agronomic applications.

Keywords: Actinobacteria / Antagonistic activity / Biocontrol / Potential agronomic applications

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Introduction

The most important region for livestock and agriculture in Argentina are the Pampas lowlands, a vast plain that extends to the northeast and southwest Tandilia hills in the province of Buenos Aires, and includes the areas known as the Salado depression in northeast and the Southwestern Region of Laprida. These natural and cultivated grasslands are the main forage resource devoted to beef and dairy production [1], and are the so-called Salado River Basin in (the province of) Buenos Aires.

Plant pathogens are responsible for many crop plant diseases resulting in economic losses. In many cases, pathogenic fungi are responsible for anthracnose (caused by *Colletotrichum* spp.), frogeye leaf spot (caused by *Cercospora sojina*), charcoal rot (caused by *Macrophomina phaseolina*), pod and stem blight (caused by *Phomopsis* spp.), and root, stem, and/or spike rot (caused by *Fusarium* spp.). These economically important crop diseases are known in the Flooding Pampa, and are difficult to control, even with chemical substances [2].

Recently, biological control by using microorganisms to suppress plant disease has offered a powerful alternative to the use of synthetic chemicals in eco-friendly and sustainable agricultural practices [3, 4]. Therefore, there is greater interest in soil microorganisms that reliably promote plant health and nutrition,

Correspondence: Dr. Mariana Solans, INIBIOMA, UNComahue, CONICET. CRUB, Quintral 1250, S. C. Bariloche 8400, Argentina
E-mail: marianasolans2005@hotmail.com

and improve soil quality. Increasing the abundance of strains in the vicinity of a plant – preferably in the rhizosphere – can suppress disease without producing lasting effects on the rest of the community in the ecosystem.

Antagonistic mechanisms can comprise diverse effects such as competition for iron by means of the production of siderophores, antibiotics, and secretion of lytic enzymes [3, 4]. Soil microorganisms produce a variety of degrading enzymes such as chitinases, β-1,3-glucanases, lipases, cellulases, and exoproteases, which allow them to attack and parasitize fungi. In addition, the release of non-specific volatile inhibitors, such as HCN, may hamper the activity of pathogenic microorganisms but it can also restrict plant growth [4].

In the rhizosphere, diverse microbial groups such as plant growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal (AM), cyanobacteria, and actinobacteria produce bioactive substances to benefit growth and/or protect plants against pathogens [4, 5]. Among these microorganisms, the actinobacteria [6] are one of the major components of the microbial populations present in soil. Particularly, *Streptomyces* species are well-known saprophytic bacteria quantitatively and qualitatively significant in the rhizosphere where they may influence plant growth and protect plant roots against invasion by root pathogenic fungi [7–9], and 80% of biologically active compounds are produced by this genus [6].

Historically, actinobacteria have been the origin of the largest number of new antibiotic drug candidates and molecules such as antibiotics, enzymes, enzyme inhibitors, and pharmacologically active agents with applications in many other therapeutic areas [6, 8–12]. Besides the great metabolic versatility, they may represent an under-explored reservoir of novel species of potential interest in the discovery of new compounds useful for agricultural technology and pharmaceuticals industry [11, 13].

There have been previous studies on the antifungal effects of three rhizosferic actinobacteria strains from the actinorhizal N-fixing plant *Ochetophila trinervis* in Patagonia, Argentina, represented by *Streptomyces*, *Actinoplanes*, and *Micromonospora* genera. [14]. These strains showed antifungal activity by fungal growth inhibition causing a halo in the contact area of the fungal culture. Strain *Streptomyces* sp. MM40 presented the most antagonistic effect against the tested fungal strains: *Alternaria* sp. VP60, *Cladosporium* sp. VP61, and *Pestalotiopsis* sp. VP59 [14]. Moreover, these same strains showed a positive effects on actinorhizal (*O. trinervis*) and legumes (*Medicago sativa*, *Glycine max*, and *Lotus tenuis*) symbioses [15–17]. *L. tenuis* is a model forage legume of the Flooding Pampa. Actinobacteria have potential agronomic applications that should be studied in depth.

Based on these findings, and keeping in mind the importance of actinobacteria as significant secondary metabolite producers, promoters of N-fixing symbioses, and their role as biocontrol agents, the present study was focused on the isolation, characterization of actinobacteria from forage soil, and the screening *in vitro* assays for antagonistic activities of actinobacteria from forage soil against pathogenic fungi with agricultural value.

Materials and methods

Soil sampling

Soil samples were collected in the Pampas lowlands, in the Salado River Basin in the province of Buenos Aires, 140 km south of Buenos Aires city (Chascomús, Argentina) at the Experimental Station "Manantiales" (INTA-Ministry of Agricultural Affairs of the Province of Buenos Aires), S 35° 44′ 45.54″–O 58° 03′ 02.76″ in autumn 2011. Three different sites were selected in this area, with three subsamples per site. The rhizospheric soil samples from *L. tenuis* (Waldst. and Kit., syn. *L. glaber*) plants were taken from the upper 15 cm and stored at 4 °C for 7 days until isolation assays were performed.

Isolation of actinobacteria

Three isolation methods were applied: (i) soil-dilution-plate, using 1 g of rhizospheric soil; (ii) stamping technique with 1 g of small pieces of roots and nodules, making imprints on agar; and (iii) chemotactic method, with 1 g of soil. Emersońs yeast extract-starch agar (YpSs) and artificial soil agar (KEHE) were routinely used for culture media at 28 °C for 7 days, and characterization of the strains [18]. The isolates obtained are listed in Table 1. All strains were sub-cultured in YpSs slants, incubated at room temperature for 7–10 days to achieve good sporulation, and stored at the culture collection of the Herbarium BCRU, Department of Botany, Centro Regional Universitario Bariloche, UNComahue, 8400 Bariloche, Argentina. (Website: http://www.sciweb.nybg.org/science2/IndexHerbariorum.asp). Also, all strains were stored in 20% glycerol stocks at −80 °C.

Identification of actinobacteria

Morphological, physiological, and molecular techniques were used to identify the isolates. Colonial development, pigmentation and consistence, presence or absence of aerial mycelium, and spore formation types of each strain (Table 1) were studied [19–21] by using an Olympus SZH stereomicroscope and an Olympus BX50 optic microscope with Lumenera Infinity camera equipment.

Table 1. Morphological characterization of actinobacteria isolated from *Lotus tenuis* rhizosphere.

Strain number	Genus	Spore formation	Colour of aerial mycelium	Pigment production
ME28	Acp	sporangium	light orange*	–
ME29	Acp	sporangium	pale orange*	–
MM123	**Stm**	**rectiflexibiles**	**white and beige**	+
MM124	Stm	rectiflexibiles	white	+
MM125	Stm	rectiflexibiles	white–gray	–
MM126	**Stm**	**rectiflexibiles**	white and beige	+
MM127	Stm	rectiflexibiles	yellowish white	+
MM128	Stm	retinaculiaperti	white and gray	+
MM129	Stm	spira	gray, beige and white	–
MM130	Stm	rectiflexibiles	grayish white and green	+
MM131	Stm	spira	pinkish gray	+
MM132	Stm	rectiflexibiles	grayish white and green	+
MM133	Stm	rectiflexibiles	white and gray	+
MM134	Stm	rectiflexibiles	grayish light green	+
MM135	Stm	spira	pinkish gray	+
MM136	Stm	rectiflexibiles	olive and white	+
MM137	Stm	spira	pinkish white	+
MM138	Stm	rectiflexibiles	gray and white	+
MM139	Stm	spira	gray–blue and white	+
MM140	Stm	spira	gray–blue	+
MM141	Stm	retinaculiaperti	pinkish white	+
MM142	Stm	spira	gray–blue and white	+
MM143	Stm	rectiflexibiles	gray–white	+
MM144	Stm	rectiflexibiles	yellowish gray	+
MM145	Stm	spira	gray	–
MM146	Stm	spira	light blue–gray and white	+
MM147	Stm	spira	white	–
MM148	Stm	spira	light violet and white	+
MM149	Adm	short, irregular	white	–
MM150	Stm	rectiflexibiles	light green and white	+
MM151	Stm	rectiflexibiles	greenish gray–white	+
MM152	Stm	spira	white and gray	+

Genus: Acp, *Actinoplanes*; Adm, *Actinomadura*; Stm, *Streptomyces*.
Production of melanin pigment: +, positive; –, negative.
*Substrate mycelium.

Regarding the physiological characterization, the following tests were performed (Table 2): (i) degradation of cellulose and hemicellulose by using cellulose crystalline and carboxymethyl cellulose (CMC) as sources of insoluble and soluble cellulose respectively, and XED medium for the detection of hemicellulose degradation [22]; (ii) production of auxin by the colorimetric method with Salkowski reagent (ferric chloride in perchloric acid) following a similar protocol to that described by Sarwar and Kremer [23]; and (iii) biological N fixation by using solid/semisolid/liquid N-free NFb media [24] generating a defined growth ring at a certain depth of the semisolid medium. Based on these characteristics, the eight most active strains (ME28, MM136, MM140, MM141, MM142, MM145, MM146, MM147) were selected for testing additional biocontrol-related activities.

Identification of selected strains

To confirm the systematic affiliation of these strains, they were identified by 16S rRNA gene sequencing. DNA of each strain was isolated by using Ultraclean Microbial DNA isolation kit (Mo Bio Laboratories, Carlsbad, USA) following the manufacturer's instructions. For these isolates, the region from positions 27 to 1492 of the 16S rRNA gene was amplified with 27f and 1518r (5′-TACGGYTACCTTGTTACGACTT) primers. The PCR reactions were performed in a 25 μl PCR mixture containing 5 × GoGreen Buffer (5 μl), 10 mM of each PCR nucleotide mix (1.6 μl), 10 μM of upstream (1 μl), and 10 μM downstream primer (1 μl), 5u μl^{-1} GoTaq DNA polymerase (Promega, USA) (0.125 μl), 2 ng of template DNA and nuclease-free water to final volume (25 μl). PCR amplification was carried out in a Veriti 96-well thermal cycler (Applied Biosystems Inc., USA) using the following profile: one cycle of 2 min at 94 °C; 35 cycles of 1 min at 94 °C, 55 s at 53 °C, 3 min at 72 °C; one cycle of a final extension for 10 min at 72 °C. DNA fragments were detected by staining with SYBR® Safe DNA gel stain (Invitrogen, Life Technologies, USA). PCR-generated amplicons (1,466 bp product) were sequenced by the INTA Castelar (Argentina) sequencing

Table 2. Physiological characteristics of actinobacteria isolated from the Flooding Pampa.

Strain number	Cellulose crystalline	Cellulose CMC	Hemicellulose (xylan)	IAA production ($\pm\mu g\,ml^{-1}$)	Biological N fixation in NFb
ME28	+	−	−	7.0	++
ME29	−	+	+	≤2.5	−
MM123	−	−	−	≤2.5	−
MM124	+	+	−	2.5	−
MM125	−	−	−	3.0	−
MM126	+	+	−	≤2.5	+
MM127	+	+	+	≤2.5	+
MM128	+	−	−	≤2.5	+
MM129	+	−	−	≤2.5	+
MM130	−	+	+	2.5	−
MM131	−	−	+	≤2.5	−
MM132	−	+	+	≤2.5	−
MM133	−	−	−	≤2.5	−
MM134	+	+	+	≤2.5	+
MM135	−	−	+	≤2.5	+
MM136	−	−	+	≤2.5	++
MM137	−	−	-	≤2.5	−
MM138	+	−	+	≤2.5	+
MM139	−	−	−	≤2.5	+
MM140	−	−	−	2.8	++
MM141	−	−	−	≤2.5	++
MM142	+	−	−	≤2.5	++
MM143	+	+	−	≤2.5	+
MM144	−	−	+	≤2.5	+
MM145	−	−	+	≤2.5	++
MM146	+	−	−	≤2.5	++
MM147	−	+	+	≤2.5	++
MM148	−	+	+	≤2.5	−
MM149	−	−	−	≤2.5	−
MM150	+	−	−	≤2.5	−
MM151	−	+	+	≤2.5	−
MM152	−	+	−	≤2.5	−

+, poor activity; ++, moderate activity; −, no activity; ≤, less than 2.5 $\mu g\,ml^{-1}$.
CMC, carboxymethyl cellulose.

service. The resultant 16S rRNA gene sequences were compared to the GenBank databases by using BLASTN, and then deposited into GenBank under the following accession numbers: KX499441 for *Actinoplanes* sp.ME28; KX530965–KX530970 for *Streptomyces* spp. MM136, MM140, MM141, MM142, MM146, and MM147, respectively.

Fungal strains

The antagonistic potential of actinobacteria isolates was evaluated against five plant pathogenic fungi: *C. sojina* MOR70 (code CEREMIC 172 2009); *Macrophomia phaseolina* 131 (code CEREMIC CCC131-2010); *Phomopsis* sp. 134 (code CEREMIC CCC134-2010); *Fusarium oxysporum* 126 (code CEREMIC CCC126-2010); *Fusarium verticilloides* 128 (code CEREMIC CCC128-2010). The cultures were maintained on potato dextrose agar (PDA) slants. CCC code given at Culture Collection of CEREMIC (Reference Center Mycology), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario.

In vitro characterization of biocontrol-related activities

Qualitative plate assays were used for detection of lytic extracellular enzymes. Exoprotease and phospholipase activities were analyzed in skimmed milk agar and in egg yolk agar, respectively. Actinobacteria strains were streaked on 5% w/v skim milk agar and 15% v/v emulsion egg in TSA ($4\,g \times l^{-1}$), yeast extract ($0.5\,g \times l^{-1}$), and agar ($12\,g \times l^{-1}$), and incubated at 28 °C for 7 days. Subsequently, the plates were observed. Phospholipase activities were detected as either opaque (phospholipase C) or clear (phospholipase A) zones surrounding the colonies. Proteolytic activities were detected as a clear zone around the colonies [25]. The relative extracellular protease and phospholipase

activities were calculated as (halo diameter/colony diameter) ×100 according to Fernández et al. [26].

Siderophore production was estimated on cromo azurol S (CAS) agar plates [27]. The development of yellow–orange halo around the growth was considered as positive for siderophore production. The relative production was expressed as described above.

Cyanhidric acid (HCN) production was detected qualitatively by the picrate-filter paper method according to Egan et al. [28]. The change in color of the filter paper from yellow to orange was recorded at 5 days as an indication of cyanogenesis. Reactions from inoculated plates were visually compared with those from non-inoculated control plates and *Pseudomonas protegens* CHAO was used as positive control [25, 26]. All assays were carried out by triplicate.

Based on the results of the average biocontrol-related activities, the biocontrol traits index (BTI) was calculated according to Agaras et al. [29] as follows: $([a_i/a_{\max\ i}])/n$. In this formula, a_i is the average activity of the biocontrol trait i for a given actinobacteria isolate, $a_{\max\ i}$ is the maximum average activity detected among all tested isolates, and n is the total number of activities evaluated.

In order to estimate the global ability of isolates to act as biocontrol agents, we calculated the biocontrol potential index (BPI) as (API + BTI)/2 [29].

Primary screening: Antagonistic activities in dual culture assay

In this assay, two agar blocks (6 × 6 mm) with actively growing mycelium of 7-day-old actinobacteria grown on yeast extract and starch were placed on opposite sides of 9 cm PDA plates, 2 cm away from the edge. After 5 days, a fungal mycelia disc (6 mm diameter) from growing margin of fungal cultures was placed in the center of each plate. Control cultures containing fungi alone were used to compare fungal growth inhibition. All plates were incubated at 28 °C for 14 days, and colony growth inhibition (%) was calculated by using the formula PI (%) = [(C-T)/C] × 100, where C is the colony growth of pathogen in control, and T is the colony growth of pathogen in dual culture [12]. Assays were done by triplicate. Based on the results of the growth inhibition generated by a single isolate against all tested fungi, we calculated the antagonism potential index (API) according to Agaras et al. [29] with modifications as follows: $([hi/h^{max}\ i])/n) \times$ (number of inhibited fungi/n). In this formula, hi is the % inhibition of actinobacteria isolates over each of the tested fungi ($n = 5$, i.e., the total number of fungi tested; $i = 1$–5), and $h^{\max}\ i$ is the % inhibition of actinobacteria isolates with the highest antagonistic activity in the collection against each of the tested fungi. The isolates, which inhibited fungal growth, were selected for further screening in fermentation broth.

Secondary screening: Diffusible substances produced by actinobacteria

The effect of diffusible substances produced by actinobacteria against fungal growth was studied in the following assays: well diffusion and agar blocks.

The active isolates selected from primary screening were further subjected to secondary screening in the fermentation broth for well diffusion assay. The isolates were cultured for 7 days in liquid YpSs medium at 28 °C. Three discs (6 mm diameter) of actinobacteria cultures grown on agar were transferred to 250 ml Erlenmeyer flasks containing 50 ml of YpSs, and incubated for 7 days at 28 °C in a rotary shaker at 125 rpm. Mycelium was removed by centrifuging at 10,000×*g* for 20 min and supernatant was used for bioassay. The PDA plates inoculated with fungal mycelia discs placed in the center of the Petri dish were punctured with a sterile cork borer to make wells (9 mm diameter), and 0.2 ml of actinobacteria culture (supernatant filtered with free-cells or non-filtered supernatant) was transferred to each well under aseptic conditions and incubated at 28 °C for 7 days. The antifungal activity of the isolates was detected as clear zones of inhibition around wells.

In the other trial, antifungal activity of diffusible substances produced by selected isolates were evaluated by agar blocks, placing in the center of a PDA Petri dish an agar block (6 mm diameter) with actively growing mycelium of fungal strains, and two agar blocks (6 mm diameter) of a 7 old days colony of actinobacteria grown on starch agar medium, taken close to the border. The dishes were maintained for 7 days at 28 °C. As positive control, an agar plug of starch agar medium without the actinobacteria growth was used. Assays were done by triplicate.

Statistical analysis

Statistical analysis was done by using statistical STATISTICA 7 program. After normality assumptions were tested, differences identified by one-way ANOVA were compared with Fisher's LSD test.

Results

Morphological and physiological characterization of actinobacteria strains

The isolated actinobacteria were identified to genus level based on morphological characteristics (Table 1). Also, some physiological traits were studied (Table 2). Based on both kinds of characterizations we have organized the

isolates in a collection covering the whole diversity isolated. For instance, two isolates that appear similar in Table 1 (i.e., MM123 and MM126) are different in Table 2.

The colonies showed a coloration that varied from gray to brown, green to blue, violet to pink, orange, and white to cream-colored. During this study, 25 strains presented melanin pigmentation in YpSs medium.

A total of 32 different actinobacteria isolates were obtained from a wild forage legume of the Flooding Pampa, *L. tenuis* (syn. *L. glaber*), and the majority belonging to the genus *Streptomyces* (91%), followed by *Actinoplanes* (6%) and *Actinomadura* (3%).

Table 1 also shows that, from 32 actinomycetes isolated, the sporulating aerial hyphae of *Streptomyces* can be differentiated into the following main types: 46.8% showed the spore chain morphology as *rectiflexibiles*, 37.5% as *spira* type, and 6.2% as *retinaculiaperti* type. The rest of the isolates showed sporangia and irregular short chains from *Actinoplanes* and *Actinomadura* genus, respectively (Table 1).

Physiological characterization showed that some of our isolates exhibited a positive activity for cellulose (15 strains) and hemicellulose degradation (12 strains). All strains produced indole acetic acid, and *Actinoplanes sp.* ME28 presented the highest value (7 $\mu g\,ml^{-1}$) (Table 2). Based on the qualitative assay to determine the ability to fix atmospheric nitrogen in free-living conditions, and consequently, grow in an N-free medium (NFb), we detected 18 isolates capable of growing in NFb, eight of which were selected for growth assays at greater depth (Table 2). Based on this feature and their IAA producing trait, eight strains were chosen for further physiological tests related to biocontrol activities.

Characterization of biocontrol-related activities

The following eight actinobacteria strains were identified by sequencing the 16S rRNA gene, and selected for *in vitro* assays of activities related to biocontrol of fungal pathogens and antifungal activity: *Actinoplanes* sp. ME28, and *Streptomyces* spp. MM136, MM140, MM141, MM142, MM145, MM146, and MM147 (Table 3).

All selected strains were positive for phospholipase A, showing clear halos of activity. Strains MM136, MM140, MM141, MM146, and MM147 exhibited clear halos corresponding to the presence of phospholipase A, and opaque halos corresponding to phospholipase C. These strains were positive for both phospholipases. *Streptomyces* sp. MM140 showed the higher activity for phospholipase A and high activity for phospholipase C. However, the highest phospholipase C activity was observed in the positive control, *P. protegens* CHAO (Table 3, $p \leq 0.05$).

For exoprotease activity, five actinobacteria strains produced clear halos around the colonies on skim milk agar plates, which is indicative of extracellular protease activity and the highest activities were observed in CHAO, *Actinoplanes* sp. ME28 and *Streptomyces* sp. MM147 ($p \leq 0.05$).

All strains were positive for the production of siderophores, with the exception of MM141. *Streptomyces* sp. MM142 showed the highest activity (Table 3). None of the selected isolates produced sufficient HCN to be detected by the picrate impregnated filter paper. However, a positive reaction was observed in the control CHAO strain.

Antifungal activities

In the first screening, all selected isolates were active against at least one of the five pathogenic fungi, and *Streptomyces* strains MM140 and MM146 presented antagonistic effect (23–78% inhibition) for all fungi studied (Table 4). In general, the highest antifungal activity was observed in strain MM140. For *C. sojina*, all isolates showed activity with varying degree of inhibition ($p \leq 0.05$), whereas for the rest of the pathogenic

Table 3. Biocontrol-related activities of selected actinobacteria.

Strains	Phospholipase A	Phospholipase C	Exoprotease	Siderophores
ME28	164.4 (2.2)^{b}	0.0^{e}	225 (14.4)^{a}	161.7 (0.31)^{a}
MM136	196.6 (10.7)^{bc}	149 (9.6)^{cd}	170 (17.3)^{b}	134.8 (0.02)^{a}
MM140	381.3 (29.7)^{a}	325.9 (29.6)^{b}	0.0^{c}	160 (0.28)^{a}
MM141	183.3 (2.4)^{b}	142.8 (0.0)^{b}	165 (8.6)^{b}	0.0 (0)^{b}
MM142	121.9 (12.6)^{b}	0.0^{d}	0.0^{c}	191.7 (0.58)^{c}
MM145	159.9 (20.3)^{b}	0.0^{d}	106.9 (0.1)^{b}	140.4 (0.13)^{a}
MM146	232.1 (17.4)^{cd}	206.5 (17.8)^{c}	0.0^{c}	149.2 (0.15)^{a}
MM147	288.1 (39.6)^{d}	199.5 (36.2)^{cd}	204.1 (2.4)^{a}	137.5 (0.17)^{a}
Control	0.0^{e}	438.8 (30.9)^{a}	245.3 (24.9)^{a}	ND

Positive control: *P. protegens* strain CHA0 [25].
ND, not determined.
Means (±SE) with different letters in the same column differ significantly at $p \leq 0.05$ according to the Fisher LSD.

Table 4. Percentage inhibition of fungal growth by actinobacteria after 9 days post-inoculation in dual assay.

Strains	*C. sojina*	*M. phaseolina*	*Phomopsis* sp.	*F. oxysporum*	*F. verticilloides*
ME28	25.2 (3.4)^{ac}	0.0^{b}	0.0^{d}	0.0^{b}	0.0^{b}
MM136	21.2 (9.2)^{ac}	0.0^{b}	0.0^{d}	0.0^{b}	0.0^{b}
MM140	36.5 (3.3)^{ac}	72.2 (0.0)^{a}	78.8 (1.1)^{b}	75.8 (4.1)^{a}	72.7 (1.6)^{a}
MM141	32.5 (7.5)^{ac}	71.6 (2.8)^{a}	93.3 (1.1)^{a}	0.0^{b}	30.5 (30.5)^{bc}
MM142	14 (7.8)^{b}	0.0^{b}	0.0^{d}	0.0^{b}	63.8 (2.7)^{ac}
MM145	38.8 (5.5)^{a}	0.0^{b}	0.0^{d}	0.0^{b}	0.0^{b}
MM146	37.5 (0)^{ac}	71.3 (4.7)^{a}	64.5 (2.0)^{c}	23.6 (23.6)^{b}	57.7 (2.2)^{ac}
MM147	39.8 (13.3)^{a}	0.0^{b}	0.0^{d}	0.0^{b}	0.0^{b}

Data represents mean (±Standard Error). Means with different letters in the same column differ significantly at $p \leq 0.05$ according to the Fisher LSD.

fungi, only some actinobacteria strains exhibited inhibition. *Streptomyces* MM140 showed the highest percentage inhibition for *Fusarium* species (*F. oxysporum* and *F. verticilloides*), and for *M. phaseolina* (Fig. 1), together strains MM145 and MM147. Also, strain *Streptomyces* MM141 presented the highest activity against *Phomopsis* sp. and *Streptomyces* MM147 exhibited the highest inhibition against *C. sojina* (Table 4).

The antagonistic potential index (API) is shown in the Table 5 together with biocontrol traits index (BTI) and biocontrol potential index (BPI). The highest index (API, BTI, and BPI) values were exhibited by strains MM140 and MM146. Table 5 shows the results in descending order of BPI.

In general, *Streptomyces* sp. MM140 presented the highest halos of inhibition and index (Tables 4, 5 and Fig. 1). Figure 1 shows the antifungal activity of this strain against fungi in different assays.

The effect observed in agar blocks assays shows that this antagonism is due to the production of extracellular metabolites present in the culture agar (Fig. 1d, i, and n). Perhaps, the effect was less than that observed in well-diffusion assays (Fig. 1c, h, and m). Strain *Streptomyces* sp. MM140 was also able to inhibit the growth of *Fusarium* spp. (Fig. 1k–n).

Discussion

Regarding the isolation of actinobacteria strains from *L. tenuis* rhizosphere, our results agree with the concept that genus *Streptomyces* is the predominant actinobacteria in soil – present in all soil types and environments. This ubiquity relates to the fact that *Streptomyces* have a fast growth rate [6].

Streptomyces are known to produce lytic enzymes such as amylases, cellulases, chitinases, glucanases, hemicellulases, and ligninases [14, 30]. Some of our isolates showed positive activity for amylase, cellulose, and hemicellulose degradation, presenting halo around the

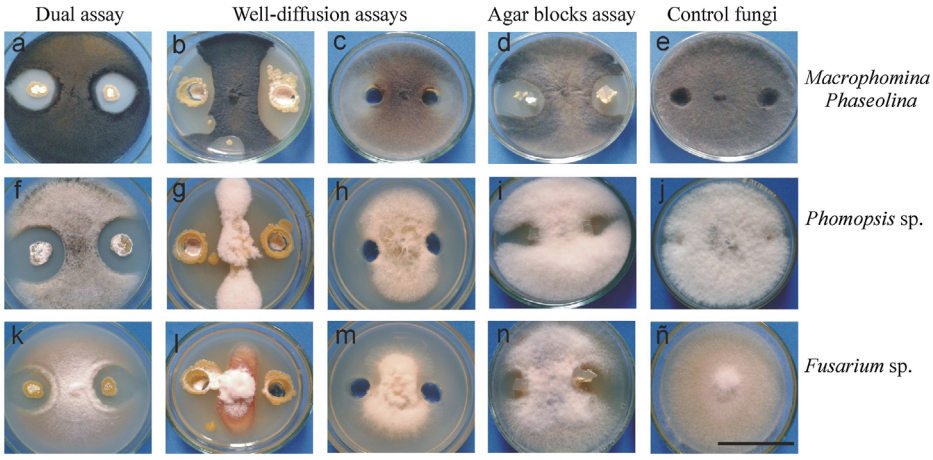

Figure 1. *In vitro* antifungal activity of *Streptomyces* sp. MM140, grown on potato dextrose agar in different tests: dual assay (1st column), well-diffusion assay with micelium + supernatant (2nd column), well-diffusion assay with cell-free supernatant (3rd column), agar blocks assay (4th column), and control fungi (5th column). (a–e) Against *M. phaseolina*; (f–j) *Phomopsis* sp.; (k–n) *Fusarium* sp. Scale: 25 mm.

Table 5. Ranking of *in vitro* antagonism potential against fungal pathogens by actinobacteria.

Actinobacteria isolates	Number of fungi inhibited[a]	API[b]	BTI[c]	BPI[d]
MM140	5	0.952	0.708	0.83
MM146	5	0.745	0.504	0.625
MM141	4	0.516	0.413	0.465
MM147	1	0.04	0.748	0.394
MM136	1	0.021	0.618	0.319
ME28	1	0.025	0.568	0.297
MM145	1	0.039	0.406	0.222
MM142	2	0.098	0.329	0.213

[a]Number of the fungi inhibited *in vitro* from the total of five fungi tested.
[b]Antagonism potential index, calculated with the formula explained in Materials and Methods section [29].
[c]Biocontrol traits index, calculated with the formula explained in Materials and Methods section [29].
[d]Biocontrol potential index, calculated with the formula explained in Materials and Methods section [29].

colonies. These enzymes help in preventing the crops from plant pathogens and deleterious microbes by degrading their cell walls. In this study, 14 isolates of 32, showed activity for the hemicellulose (xylan), and only three of eight selected strains were positive (MM136, MM145, and MM147) for this substrate.

Ramesh et al. [31] showed that the marine *Streptomyces fungicidicus* MML1614 can be exploited as a hyper producer of thermostable alkaline protease as it produces higher protease than the reference protease producing strain, *Bacillus subtilis* MTCC1789. In our study, *Actinoplanes* sp. ME28 produced similar protease as the positive control, *P. protegens* CHAO ($p \geq 0.05$).

The production of high levels of extracellular lytic enzymes such as chitinases and glucanases by some actinobacteria prevents the growth of plant pathogenic organisms [32, 33], and it is known that chitinases are present in a wide range of organisms such as *Streptomyces* spp. [30]. However, no halo of activity was observed in mineral media containing chitin from *Suillus luteus* fungi powder or shrimp shell crushed, as sole carbon source. Nevertheless, good growth was observed in these media.

In relation to other enzymatic activities related to biocontrol, Sacherer et al. [25] demonstrated that strain *P. fluorescens* CHA0 (currently named as *P. protegens*) produces extracellular protease, phospholipase C, and lipase, and protects several plants from root diseases caused by soil borne fungi. In our study, this strain was used as positive control for enzymatic activities and for HCN production. Although in this research the HCN production by actinobacteria was negative, the production of volatile substances by many bacteria including actinomycetes have been reported, and some of these substances have been implicated as a biocontrol mechanism [34, 35].

In this study, all of the isolates but strain MM141 grew and produced siderophores. A color change was observed in the medium, exclusively surrounding producer microorganisms, from blue to orange (as reported for microorganisms that produce hydroxamates). Several reports in pathogen suppression, indicate the production of diverse types of siderophores by actinobacteria [36–38]. Khamna et al. [12] showed that three actinobacteria produced both types of siderophores, hydroxamate, and catechols in *Streptomyces* spp. and *Actinomadura* sp. Laboratory assays, such as the production of chitinases and the determination of siderophores-types, are required to complement these preliminary results.

It is known that differences in the quantitative and qualitative compositions of root excretions provide different impacts on the rhizosphere microbiota and attract more or less bacterial antagonists responsible for natural soil suppression. Plant root exudates stimulate growth of rhizosphere actinobacteria that are strongly antagonistic to fungal pathogens, while utilize root exudates for growth and synthesis of antimicrobial substances. Evangelista-Martínez [39] showed that four of the six *Streptomyces* isolates demonstrated high antagonistic activity against different fungi, and these results strongly suggest that *Streptomyces* CACIA-1.46HGO could be considered as potential control agents to prevent and/or reduce plant diseases caused by fungal strains.

Strains *Streptomyces* MM140 and MM146 showed the highest API and BPI values, and MM147 together with MM140 presented the highest BTI index. Thus, these strains offer great potential for suppressing fungal pathogens and appear as promising biocontrol agents.

From the present study, it could be demonstrated that rhizosphere soil from *L. tenuis* provided a rich source of actinobacteria, and had the ability to produce antifungal compounds, siderophore and hydrolytic enzymes, which are all activities that could contribute to potential

biocontrol. The next step will be to perform *in-plant* experiments to demonstrate the potential of these organisms as biocontrol agents of pathogenic fungi. In the immediate future, these *in vitro* assays will be needed to prove or justify the antagonistic effect of actinobacteria, and its potential use in the field as biocontrol, and consequently as PGP actinobacteria.

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Conflict of interest

The authors have not conflict of interest.

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