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SHORT COMMUNICATION

Isolation and characterization of soil *Streptomyces* species as potential biological control agents against fungal plant pathogens

Zahaed Evangelista-Martínez

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Abstract The use of antagonist microorganisms against fungal plant pathogens is an attractive and ecologically alternative to the use of chemical pesticides. *Streptomyces* are beneficial soil bacteria and potential candidates for biocontrol agents. This study reports the isolation, characterization and antagonist activity of soil streptomycetes from the Los Petenes Biosphere Reserve, a Natural protected area in Campeche, Mexico. The results showed morphological, physiological and biochemical characterization of six actinomycetes and their inhibitory activity against *Curvularia* sp., *Aspergillus niger*, *Helminthosporium* sp. and *Fusarium* sp. One isolate, identified as *Streptomyces* sp. CACIS-1.16CA showed the potential to inhibit additional pathogens as *Alternaria* sp., *Phytophthora capsici*, *Colletotrichum* sp. and *Rhizoctonia* sp. with percentages ranging from 47 to 90 %. This study identified a streptomycete strain with a broad antagonist activity that could be used for biocontrol of plant pathogenic fungi.

Keywords Antagonist activity · Fungal plant pathogen · Biological control · *Streptomyces*

Introduction

Fungal plant diseases are a major concern to worldwide agricultural production. It has been estimated that total losses from plant diseases range from 25 % of the yield in western countries to almost 50 % in developing countries (Gohel et al. 2006). Control of fungal diseases is imperative to ensure a constant food supply to an ever-increasing world population (Oskay 2009). However, extensive use of chemical fungicides in agriculture can produce resistant fungal strains and the accumulation of compounds potentially hazardous to humans and the environment. Therefore, control of fungal plant diseases using naturally occurring non-pathogenic microorganisms represents a promising approach for the control of plant disease (Heydary and Pessarakli 2010) and an attractive alternative to chemical fungicides (Talubnak and Soytong 2010).

Of the microbial antagonists evaluated in the world, bacteria belonging to the *Streptomyces* genus are of particular interest (Hassan et al. 2011). Among the 10,000 antimicrobial compounds produced by microorganisms, over 50 % were isolated from actinomycetes (Anderson and Wellington 2001) and roughly 60 % of biologically active compounds developed for agricultural use originated from *Streptomyces* species (Ilic et al. 2007). Some members of the S*treptomyces* genus have been reported to significantly reduce the growth of fungal plant pathogens (Taechowisan et al. 2005; Errakhi et al. 2007; Maldonado et al. 2010). *S. aureofaciens* improved protection against post-harvest anthracnose in Mango caused by *Colletotrichum gloesporioides* (Haggag and Abdall 2011). Talc-based formulations of *S. griseus* were used to control *Fusarium* wilt in tomato (Anitha and Rabeeth 2009). *Streptomyces* sp. was used for biological control of silver scurf of potato tubers produced by *Helminthosporium solani* (Elson 1997). *S. rochei* in combination with *Trichoderma harzianum* was used to control root rot in pepper caused by *Phytophthora capsici* (Ezziyyani et al. 2007). Finally, *Streptomyces* strains were used to reduce the

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Z. Evangelista-Martínez (✉)
Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C. Unidad Sureste, Calle 30 No. 151 (7 y 7A). Col. García Ginerés, C.P. 97070 Mérida, Yucatán, México
e-mail: zevangelista@ciatej.mx

incidence of seed pathogen fungi *Aspergillus* spp. in maize (Bressan 2003).

Two of the most useful commercial products developed from *Streptomyces* strains are Actinovate® (Natural Industries, Inc) and Mycostop® (Verdara Oy). The first product is composed of spores of *S. lydicus* WYEC108 with activity against a wide range of foliar and root fungal diseases and the second one includes dried spores of *S. griseoviridis* strain K61 which control or suppress many root rot and wilt pathogenic fungi. These species were isolated from natural environmental soil in England (Crawford et al. 1993) and Finland (Lahdenperä 1987), respectively. Characterization of streptomycetes from different geographic regions is an important activity to identify new commercially valuable genetic and biochemical resources. Novel strains have been isolated from a variety of terrestrial habitats and ecological niches, including Indian natural protected areas (Thakur et al. 2007), Brazilian tropical regions (Gomes et al. 2000), desert ecosystems (Hozzein et al. 2011), plant roots and leaves (Cao et al. 2004), and wasp symbionts (Poulsen et al. 2011).

The Los Petenes Biosphere Reserve is a natural protected area with a low level of human activities located in southeastern Mexico. Los Petenes owes its name to this unique ecosystem, consisting of islands of forest vegetation in a matrix of wetlands, natural grasslands, mangrove or tropical forest associated with underwater springs from sinkholes or cenotes. The reserve covers an area of land and marine ecosystem which includes important wetlands designated under the Ramsar Wetlands Convention (http://www.ramsar.org). Due to the island distribution, the forest habitat presents a natural fragmentation (Mas et al. 2000). Nowadays, it is considered the most significant fragmented natural ecosystem in Mesoamerica with forest vegetation developed on soils of calcareous origin (León and Montiel 2008).

The present study seeks to isolate and characterize novel soil streptomycetes from Los Petenes Biosphere Reserve and screen for strains with antagonist activity. A selected isolate was identified by the 16S rDNA gene sequence analysis as a member of the *Streptomyces* genus. This isolate was further investigated for its potential to antagonize in vitro the growth of fungal pathogens such as, *A. niger*, *Fusarium* sp., *Curvularia* sp., *Helminthosporium* sp., *Alternaria* sp., *Rhizoctonia* sp., *Colletotrichum* sp., and *P. capsici*.

Materials and methods

Soil sampling

Six composite soil samples were taken from six locations positioned into a lineal transect of approximately 6.2 mi long at the Los Petenes Biosphere Reserve in Campeche State, Mexico. Each composite sample was prepared that consisted of six individual samples taken from a circular area 5 m in diameter. Approximately, fifty grams of individual samples were taken with an auger (up to 10 cm depth), after removing approximately 3 cm of the soil surface. The individual samples were mixed in sterilized plastic bag for 3 min. Composite soil samples were preserved at ambient temperature and processed within 36 h.

Reference fungal strains

The reference fungal strains *A. niger* NRRL-3 and *Fusarium* sp. (CDBB:1172) were obtained from National Culture Collection, CINVESTAV-IPN, México. *Curvularia* sp. and *Helminthosporium* sp. (isolated from leaves of *Chrysanthemum* sp.) were kindly provided by Dr. Jairo Cristobal Alejo from the Instituto Tecnológico de Conkal, Yucatán, México. *Alternaria* sp., *Rhizoctonia* sp., *Colletotrichum* sp. (all isolated from leaves of *Jatropha curcas*), and *Fusarium* sp. and *P. capsici* (isolated from root of infected plants of *Capsicum chinense*), were kindly provided by Dr. Alberto Uc Várguez from CIATEJ Unidad Sureste.

Culture media

International Streptomyces Project (ISP) agar media 2 and 3 (Shirling and Gottlieb 1966), Actinomycetes Isolation Agar medium (AIA, DIFCO), and Humic acid-vitamin agar medium (HV, Hayakawa and Nonomura 1987) were used for isolation of actinomycetes. All media were supplemented with 12.5 μg ml^{-1} of nalidixic acid and 21.5 μg ml^{-1} natamycin (Intra et al. 2011).

Isolation of actinomycetes

The composite soil samples were pre-treated at 70 °C for 1 h in order to select the population of sporulating actinomycetes in the sample (Takahashi and Omura 2003). Ten grams of pre-treated soil were suspended in 100 ml of sterile distilled water and mixtures were shaken vigorously for 1 h in an orbital shaker at 250 rpm and 29 °C. Samples were allowed to settle and serial dilutions up to 10^{-4} were prepared from the supernatants. An aliquot of 0.1 ml of each dilution was taken and spread evenly over the surface of medium in duplicate. The plates were incubated at 29 °C for 2–4 weeks and dilutions that gave 15–150 colonies were chosen for further isolation. Emerging colonies were selected based on streptomycete morphological aspect of the colony (Holt et al. 2000). Repeated streaking onto fresh ISP2 plates produced pure bacterial strains. Stored spore and mycelium suspensions were obtained as mentioned by Shirling and Gottlieb (1966). For long-term preservation,

Preservation Stocks (PS) of spore or mycelial suspensions was maintained at −20 °C in 20 % (v/v) glycerol.

Characterization of actinomycetes

Morphological and biochemical characterization of actinomycetes isolates was conducted according to Shirling and Gottlieb (1966), with slight modifications. For biochemical characterization, General Inoculums (GI) of spore or mycelium were prepared for each isolate from PS by transferring sufficient material to distilled water to make a suspension of 1.5×10^8 spores or cells ml^{-1}. Spore preparations were made by counting under a microscope with a Neubauer chamber. Mycelium inoculums were adjusted by turbidity to match to 0.5 McFarland scale. Two microliters of GI were used as inoculums. Carbon utilization was evaluated in ISP9 medium supplemented with different substrates in cell culture plates of 24 well. Melanine production and casein and starch degradation were also evaluated.

Enzymatic activity of preserved strains was determined by Petri plate-assay; lipase activity at 3.125 % v/v olive oil-rhodamine B plates (Kouker and Jaeger 1987), asparaginase activity at 1 % w/v L-asparagine-phenol red plates (Gulati et al. 1997) and protease activity at 1 % w/v of gelatine-amido black (Vermelho et al. 1996). All the assays were carried out by triplicate on base media ISP9 (Shirling and Gottlieb 1966).

Antagonist screening of isolates

Primary screening of antagonist activity was conducted by evaluation of each isolate against *Fusarium* sp. (CDBB:1172) and *C. albicans* on PDA agar plates according to the modified method of Crawford et al. (1993). The antagonist activity against *Fusarium* sp. was carried out by placing a block of 0.4 × 0.4 cm of agar covered with an actively growing fungal mycelium at the center of the plate and 2 microliters of GI deposited 1 cm away from the border of the Petri dish. Cultures were incubated at 29 °C for 7 days. Antagonist activity against *C. albicans* was evaluated by streaking a loopful of GI suspension along the center of the plate, and incubating the plates by 5 days at 29 °C. Then, a loopful of fresh *C. albicans* suspension equivalent to 0.5 McFarland scale (1.5×10^8 cells ml^{-1}) was streaked approximately 1 mm away from the actinomycetes growth. Petri dishes were incubated at 29 °C for additional 48 h. All experiments were done in triplicate. Antagonist activity was considered positive (+) if the halo of inhibition of the growth zone was at least of 3 mm.

In vitro antifungal activity

An antagonist plate-assay technique was developed to test the effects of isolates on the growth of the phytopathogenic fungi according to Bredholdt et al. (2007). Tests were conducted on Petri dishes of nutrient agar. An aliquot (2 µl) of GI suspension were inoculated 1.5 cm from the edge of the plate, and incubated at 29 °C for 0, 3 and 5 days. A 0.4 × 0.4 cm of agar block covered with actively growing fungal mycelium was transferred onto the center of each plate and incubation at 29 °C was continued for additional 7–10 days. Control cultures containing the fungus alone were used to compare the inhibition of fungal growth. All studies were conducted in triplicate. The percentage of inhibition (PI) was determined according to the formula: PI (%) = (FR − AR)/FR × 100, where FR represents the fungal growth radius (mm) of a control culture and AR the fungal growth radius distance (mm) in the direction of actinomycetes growth (Yuan and Crawford 1995).

Analysis of antagonist activity of selected *Streptomyces* sp. strain CACIS-1.16CA was conducted as outlined previously, by triplicate. As a control strain a non-antagonist *Streptomyces* sp. was chosen from the group of 151 of actinomycete isolated in the current study.

Molecular identification

The identity of the selected strain was determined based on partial length 16S rRNA gene sequence analysis. The genomic DNA used as template for PCR was prepared from 100 µl of PS using the Puregene Yeast/Bact Kit B (QIAGEN). The DNA purification protocol was modified at the step of cell lysis by the inclusion of acid-washed glass beads (0.5 % w/v) and vigorous vortexing. The integrity of chromosomal DNA was visualized by gel electrophoresis in 1 % (w/v) agarose. The complete 16S rRNA fragment was prepared by PCR amplification using Platinum Taq DNA polymerase (Invitrogen) and oligonucleotides fD1 (5′-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3′) (Weisburg et al. 1991). The PCR reaction was carried out in a total volume of 50 µl that contained 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 2 ng of chromosomal DNA, 0.4 µM each primer and 2 units of Taq DNA polymerase. PCR conditions consisted of an initial denaturation step at 94 °C for 5 min followed by 35 amplification cycles of 94 °C for 1 min, 55 °C for 45 s, and 72 °C for 1 min. The amplified fragment was purified using the kit PureLink PCR Purification Kit (Invitrogen) and was verified directly by nucleotide sequence determination of both strands. Sequencing was provided by LANGEBIO (National Laboratory of Genomics for Biodiversity, CINVESTAV-Irapuato, Mexico). Sequences were assembled and trimmed using CLC Main Workbench 5.6 (CLC Bio). A 641 bp fragment of sequence was analyzed for homology using the

BLASTN program and 16S rDNA gene sequences of type strains of various genera were retrieved from the nonredundant GeneBank database (Alschul et al. 1997; http://blast.ncbi.nlm.nih.gov/). These sequences, in conjunction with additional actinomycete strains, were used as reference in which unidentified and unpublished sequences were not included. Phylogenetic analysis was carried out at Phylogeny.fr web page: http://www.phylogeny.fr/version2_cgi/index.cgi. Selected sequences were aligned with MUSCLE (v3.7) configured for highest accuracy. After alignment, ambiguous regions (i.e. containing gaps and/or poorly aligned) were removed with Gblocks (v 0.91b). The phylogenetic tree was reconstructed using the neighbor joining method implemented in the BioNJ program. The K2P substitution model was selected for the analysis. The confidence of the grouping was verified by bootstrap analysis (1,000 replications). Graphical representation and editing of the phylogenetic tree were performed with TreeDyn (v 198.3). *Bacillus subtilis* subsp. *subtilis* was used as outgroup.

Partial sequence of 16S rDNA gene of *Streptomyces* sp. strain CACIS-1.16CA was deposited in GenBank database under accession number JQ400108.

Results

Isolation and selection of actinomycetes

Actinomycete like colonies was selectively isolated from culture plates (106 on ISP2, 13 from IPS3, 26 from AIA and 6 from HV). A total of one hundred-fifty-one strains were isolated and characterized based on morphological, cultural and physiological aspects, and the utilization of different carbon sources. All strains were evaluated for their ability to inhibit the growth of *Fusarium* sp. (CDBB:1172) and *C. albicans*; 18 (12 %) and 25 (17 %) isolates demonstrated antagonist activity against *Fusarium* sp. and *C. albicans,* respectively (data not shown). Six isolates were selected for further studies; five shown activity against both fungal strains and one (CACIS-1.5CA) only towards *Fusarium* sp.

Molecular identification

Analysis of the partial 16S rRNA gene sequence (641 bp) of CACIS-1.16CA exhibited a very high level of 16S rDNA sequence similarity (>98 %) with sequences related to different streptomycete species deposited in GenBank (NCBI). The above indicate the closest relation of CACIS-1.16CA to members of the genus *Streptomyces.* The phylogenetic tree based on the neighbor-joining method (Fig. 1) shown that strain CACIS-1.16CA conforms to branch with *S. albovinaceus* DSM 40136, *Streptomyces* sp. CNR918 PL04, *S. griseus* CB153, and other *Streptomyces* strains. The cluster of strains is represented by soil, marine, plant pathogens and others *Streptomyces* species.

Phenotypic characterization of actinomycetes isolates

The morphological characteristics of the six selected isolates were observed for 21 days. Initially, actinomycete colonies showed a smooth appearance that developed aerial mycelium that appeared floccose, granular or powdery (Shirling and Gottlieb 1966). Substrate mycelia were well developed, branched and mostly unfragmented. Colors on the reverse side of the culture growth ranged from beige to brown. Based on the color of the aerial mycelia, the strains were categorized in three color series: brown (CACIS-1.4CA), white (CACIS-1.16CA and CACIS-2.17CA) and red (CACIS-1.5CA, CACIS-2.19CA and CACIS-2.52CA) (Nonomura 1974). Diffusible pigments to the medium were only detected in CACIS-1.4CA and CACIS-1.16CA. Details of morphological, physiological, enzyme production and preliminary antagonist activity of selected strains are shown in Table 1.

Utilization of carbon sources by the strains highlights the pattern of development for substrate mycelium and sporulation process under different media conditions (Online Resource 1).

In vitro antagonist activity

Selected isolates were assayed against the plant pathogenic fungi *Curvularia* sp., *Helminthosporium* sp., *A. niger* and *Fusarium* sp. The isolates were inoculated and incubated for 3 and 5 days before the fungus inoculation; zero days means the simultaneous inoculation of actinomycetes and fungal strains. All isolates inhibited the growth of fungal mycelium to different degrees (Table 2; Online Resource 2). *Curvularia* sp. was inhibited at least by 40 % at zero days, except by strain CACIS-2.19CA. After 5 days, the inhibition of *Curvularia* growth was increased up to 70 % with isolates CACIS-1.4CA, CACIS-1.5CA and CACIS-1.16CA. The PI of *Helminthosporium* sp. mycelium growth was over 35 % (0 days) for isolates CACIS-1.5CA, CACIS-1.16CA and CACIS-2.52CA, but the PI values were increased between 62 to 71 % almost for all strains, except CACIS-2.19CA (5 days). *A. niger* was the most susceptible fungal strain to the antagonist activity of selected isolates; with exception of CACIS-2.19CA, the rest of isolates inhibit the fungal growth at least from 36 to 40 % for 0 days. It is clear that previous incubation of the isolates (3 and 5 days) increased the percentage of inhibition above 81 %. The pathogen with higher resistance to streptomycete antagonism was *Fusarium* sp., with a maximum

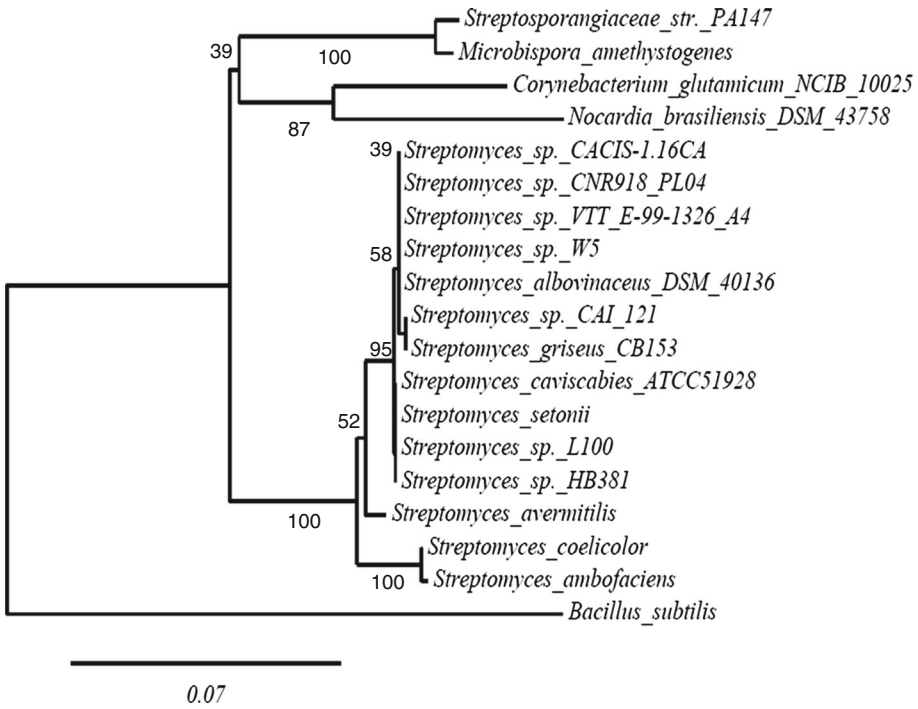

Fig. 1 Phylogenetic relationship based on the 16S rDNA gene between *Streptomyces* sp. CACIS-1.16CA with closely related members of the genus. Tree was constructed by the neighbor-joining method by using partial 16S rDNA sequence (641 bp) as mentioned in methods. The numbers at the nodes are percentages that indicate the levels of bootstrap support (n = 1,000 re-samplings). Except for the sequence determined in this study, all 16S rDNA sequences were retrieved from GenBank. The scale bar corresponds to 0.07 nucleotide substitution per site. The accession numbers for the sequences used are: *S. coelicolor* 14426 (EF371438.1), *S. avermitilis* (AB078897), *S. ambofaciens* (M27245.1), *Microbispora* amethystogenes (U48988), *Streptosporangium* str. PA147 (AF223347), *Nocardia brasiliensis* (AF430038), *Corynebacterium glutamicum* NCIB10025 (X84257), *Streptomyces* sp. VTT (E991326A4), *Streptomyces* sp. W5 (EU429478), *S. albovinaceus* DSM 40136 (GU383174), *S. griseus* CB153 (GU383191), *Streptomyces* sp. CAI 121 (JN400113.1), *S. caviscabies* ATCC 51928 (AF112160), *S. setonii* (D63872), *Streptomyces* sp. L100 (AM913952), *Streptomyces* sp. HB381 (GQ863932), *Streptomyces* sp. CNR918PLO4 (DQ448731). *Bacillus subtilis* subsp. *subtilis* was used as the outgroup (AB271744.1)

growth reduction of 62 % (CACIS-1.5CA for 5 days). Therefore, isolates CACIS-1.4CA and CACIS-1.16CA were the strains with a more constant inhibitory activity. However, isolate CACIS-1.16CA was chosen for further studies instead CACIS-1.4CA, considering its superior cultural characteristics, profuse spore production and abroad antagonistic activity over the fungal tested.

Antagonist activity of *Streptomyces* sp. CACIS-1.16CA was evaluated against *Fusarium* sp., *Alternaria* sp., *Curvularia* sp., *P. capsici*, *Colletotrichum* sp., *Rhizoctonia* sp. and *A. niger*. Broad antagonist activity was observed against all fungi tested as shown in Table 3. A PI greater than 70 % was observed against all pathogenic fungi after five days of streptomycete pre-inoculation; with the exception of *Alternaria* sp. The strain more susceptible to inhibition was *P. capsici*, with a PI ranging 61–90 % (0–5 days, respectively). Evidence of highest antagonism of CACIS-1.16CA against fungal pathogens was obtained by a simple serial of assays wherein the antagonist isolate was incubated in presence of fungal pathogen, together with a non-antagonist streptomycete strain, also isolated in this study. (Fig. 2; Online Resource 3). It is clear the broad antagonist activity of *Streptomyces* sp. strain CACIS-1.16CA.

Discussion

The searching of new natural products derived from microorganisms, especially from actinomycetes, has been focused on the isolation of species from unexplored niches to access to novel or endemic species. These natural environments are still either unexplored or under-explored status, and thus, can be considered as prolific resource areas for the isolation of diverse microorganisms. Many of them could be considered unique species or novel strains with potential to produce new metabolites and enzymes exhibiting different catalytic activities. In this sense, the actinomycetes isolated from Los Petenes are microorganisms localized at under-explored region, which are exposed to drastic environmental conditions, such as high salinity and alkalinity of soil, high temperatures, and to the process

Table 1 Characteristics of selected streptomycete isolates

	CACIS-1.4CA	CACIS-1.5CA	CACIS-1.16CA	CACIS-2.17CA	CACIS-2.19CA	CACIS-2.52CA
Test[a]						
Gram staining	+	+	+	+	+	+
Starch hydrolysis	−	−	+	+	+	−
Casein hydrolysis	−	−	+	+	+	+
Melanine production	−	−	−	−	−	−
Colony growth on ISP2[a]						
Colour of mycelium substrate (reverse)	Brown	Orange–red	Yellow pale	Beige	Orange–red	Orange–red
Colour of aerial mycelium	Brown	Orange–red	White	White	Orange–red	Orange–red
Colour of spore mass	Gray	White	Yellow	Grey	Red	Red
Diffusible pigments	Violet	None	Yellow light	None	None	None
Extracellular enzyme activity						
Lipase	−	−	−	−	−	−
Asparaginase	+	+	+	+	−	−
Gelatinase	+	−	+	+	+	+
Antifungal activity[b]						
Fusarium sp CDBB:1172	+	+	+	+	+	+
Candida albicans	+	−	+	+	+	+

[a] Assays were carried out as mentioned in Shirling and Gottlieb (1966)

[b] +, positive inhibition considers at least 3 mm of halo from the margin of actinomycetes colony to margin of fungal growth

Table 2 In vitro antagonist activity of selected *Streptomyces* strains against phytopathogenic fungi

Streptomyces isolate	*Curvularia* sp.			*Helminthosporium* sp.			*A. niger*			*Fusarium* sp. CDBB:1172		
	0 days[b]	3 days	5 days	0 days	3 days	5 days	0 days	3 days	5 days	0 days	3 days	5 days
Percentage of inhibition [PI (±SD)][a]												
CACIS-1.4CA	40 (2.4)	61 (1.4)	70 (1.4)	22 (6.6)	41 (2.3)	65 (1.7)	36 (2.9)	70 (3.6)	91 (3.6)	31 (1.0)	48 (1.8)	57 (0.7)
CACIS-1.5CA	44 (1.9)	64 (2.7)	73 (6.3)	37 (2.9)	53 (3.1)	62 (2.3)	40 (4.4)	59 (5.0)	86 (1.7)	42 (3.1)	53 (2.4)	62 (2.1)
CACIS-1.16CA	45 (3.3)	61 (2.3)	71 (1.4)	36 (4.9)	52 (2.6)	68 (4.0)	38 (2.1)	68 (3.5)	96 (0.7)	34 (1.2)	49 (1.6)	54 (3.9)
CACIS-2.17CA	43 (1.6)	44 (2.5)	55 (2.0)	27 (4.7)	39 (1.9)	71 (2.4)	38 (1.1)	63 (3.9)	96 (1.1)	28 (0.9)	37 (3.2)	43 (3.5)
CACIS-2.19CA	16 (6.7)	25 (6.4)	53 (2.7)	10 (2.5)	34 (7.7)	44 (3.7)	11 (3.0)	47 (6.7)	83 (1.4)	7 (2.7)	30 (2.0)	42 (2.1)
CACIS-2.52CA	48 (6.5)	63 (6.9)	63 (4.2)	35 (0.5)	51 (2.3)	69 (4.4)	41 (2.6)	54 (5.7)	81 (2.5)	38 (1.9)	45 (7.2)	55 (3.3)

[a] Control growth of plant pathogens on Petri-plate (radial rate). *Curvularia* sp. 34.6 ± 0.9 mm; *Helminsthosporium* sp. 42.36 ± 2.3 mm; *A. niger* NRRL-3 40.4 ± 2.2 mm; *Fusarium* sp. CDBB:1172 40.02 ± 0.7 mm

[b] Days to take place between actinomycete inoculation and fungal pathogens; at 0 days both microorganism were inoculated at the same time

of natural fragmentation of the site, which could increase the options to find different species with potential to be applicable to industrial methods.

In the present study, one hundred fifty-one actinomycetes were isolated from soil of the Los Petenes area, with a clear prevalence of streptomycetes. This prevalence is likely due to screening conditions, media culture and cultivation as suggested by Anupama et al. (2007), and also to the soil treatment conditions as mentioned in materials and methods. The isolates were initially characterized by the morphological, cultural and physiological aspects, and by their utilization of different carbon sources. Almost all isolated strains showed typical characteristics of streptomycetes (Shirling and Gottlieb 1966). Carbon utilization is critical for commercial utility to facilitate selection of the cheapest carbon sources for massive spore or mycelium production.

Six isolates were selected for more detailed study considering their ability to inhibit *Fusarium* sp. mycelium growth and *C. albicans* (five of them). All six strains

Table 3 Evaluation of antagonist activity of *Streptomyces* sp. CACIS-1.16CA against fungal plant pathogens

Fungal pathogen	Percentage of inhibition [PI (±SD)][a]		
	0 days[b]	3 days	5 days
Fusarium sp.	52.9 (1.8)	74.1 (4.2)	77.6 (1.0)
Alternaria sp.	47.6 (1.2)	60.6 (1.7)	61.4 (3.0)
Curvularia sp.	54.5 (3.6)	68.6 (3.6)	76.8 (1.7)
Phytophthora capsici	61.5 (0.4)	82.7 (1.3)	90.2 (1.6)
Colletotrichum sp.	59.1 (1.8)	69.8 (1.6)	80.8 (2.4)
Rhizoctonia sp.	55.2 (3.5)	70.5 (4.6)	76.4 (1.2)
Fusarium sp. CDBB:1172	54.8 (0.5)	64.8 (1.4)	71.0 (2.3)
Aspergillus niger NRRL-3	52.3 (0.3)	65.6 (5.1)	76.9 (1.7)

[a] Control growth of plant pathogens on Petri-plate (radial rate). *Fusarium* sp. 39.0 ± 0.6 mm; *Alternaria* sp. 40.3 ± 0.1 mm; *Curvularia* sp. 39.0 ± 0.6 mm; *P. capsici* 41.1 ± 0.8; *Colletotrichum* sp. 40 ± 0.9; *Rhizoctonia* sp. 39 ± 0.6; *Fusarium* sp. CDBB:1172, 40.0 ± 0.4 mm; *A. niger* NRRL-3, 40.4 mm ± 0.1 mm

[b] Days to take place between actinomycete inoculation and fungal pathogens; at 0 days both microorganism were inoculated at the same time

demonstrated the expression of extracellular protease and L-asparaginase activity in Petri-plate (Table 2). Enzymatic production is important for the biological activity of streptomycetes. In terrestrial habitats, streptomycetes are excellent saprophytes and prolific producers of extracellular enzymes required for the initial decomposition of organic soil material (Schrempf 2007). Asparaginases play an important role in N-mineralization (Frankenberger and Tabatabai 1991). Extracellular proteases are involved in the assimilation of extracellular proteinaceous nitrogen sources and are implicated in *Streptomyces* development (Chater et al. 2010).

Four of the six *Streptomyces* isolates, CACIS-1.4CA, CACIS-1.5CA, CACIS-1.16CA and CACIS-2.52CA, demonstrated high antagonist activity against *Curvularia* sp., *Helminthosporium* sp., *A. niger* and *Fusarium* sp. CDBB:1172. However, isolate CACIS-1.16CA inhibit in vitro additional fungal pathogens such as, *Colletotrichum* sp., *Alternaria* sp., *Rhizoctonia* sp., *P. capsici* and *Fusarium* sp. The above results strongly suggest that CACIS-1.16CA could be considered as a potential control agent to prevent and/or reduce plant diseases caused by fungal strains. Many researchers have studied the potential of several *Streptomyces* species to suppress or reduce plant pathogens such as *F. oxysporum,* which causes cucumber *Fusarium* wilt (Zhao et al. 2012), suppress *P. capsici* in red-peppers (Joo 2005), control of *R. solani* under field conditions by formulations based on two *Streptomyces* isolates, (Sadeghi et al. 2006), and antagonist activity against *C. gloesporioides* and *C, eragrostides*, both pathogens of Yam crops (Soares et al. 2006). There are reports related to the use of direct inoculation of *Streptomyces* spore solution in substrate inoculated with fungal

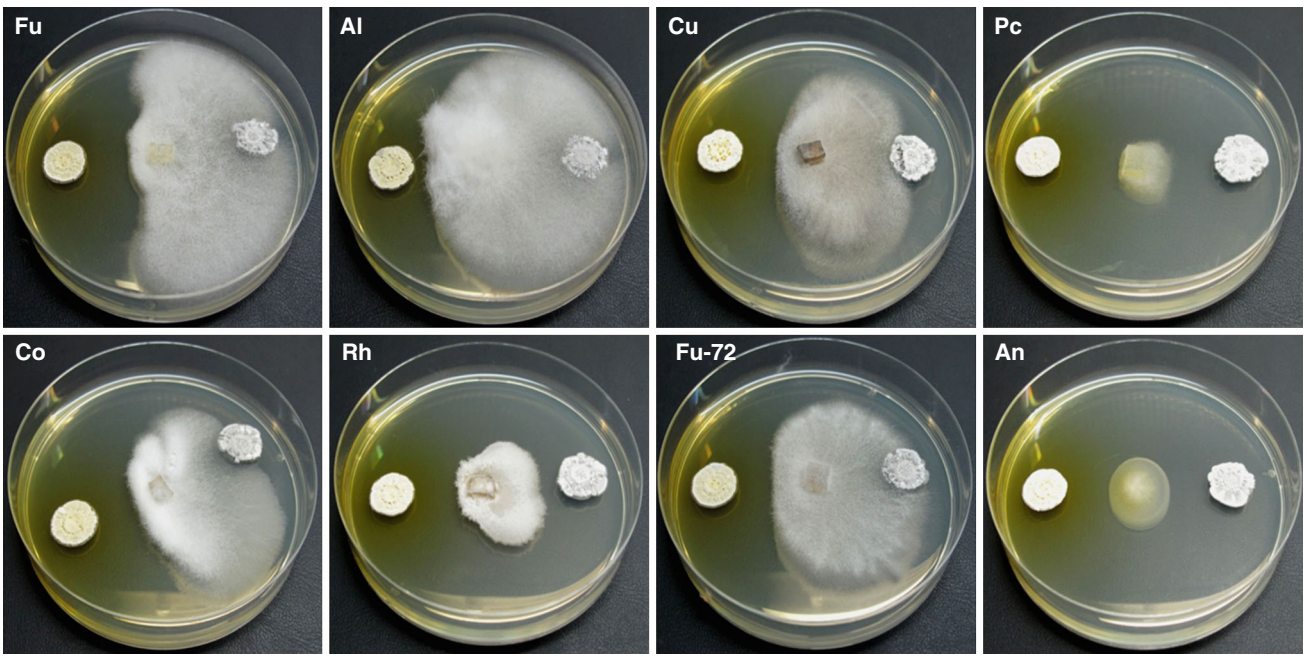

Fig. 2 In vitro antagonistic activity of *Streptomyces* sp. CACIS-1.16CA against diverse fungal plant pathogens. *Streptomyces* sp. CACIS-1.16CA (*left*), *Streptomyces* sp. (*right*) and fungal mycelium were inoculated at the same time (0 days of preincubation). Radial mycelia growth of fungi was determined after 7 days of incubation. Fungal pathogen from left to right: Fu, *Fusarium* sp.; Al, *Alternaria* sp.; Cu, *Curvularia* sp.; Pc, *Phytophthora capsici*; Co, *Colletotrichum* sp.; Rh, *Rhizoctonia* sp.; Fu72, *Fusarium* sp. CDBB:1172; An, *Aspergillus niger* NRRL-3

pathogen after planting seeds, which showed an enhanced resistance to *Phytophthora* root rots on alfalfa and soybean (Xiao et al. 2002). Alginate formulations of *Streptomyces* spore or biomass mycelium were effective for the suppression of *Rhizoctonia* damping-off in tomato (Sabaratnam and Traquair 2002). A formulation of *Streptomyces* spore or mycelium suspension mixed with chitosan solution, also produced a positive effect on growth promotion and enhanced disease resistance of seedlings of tomato to *Rhizoctonia* damping-off (Cao et al. 2004). Nowadays, a talc formulation of *Streptomyces* antagonist was used to coat seeds of pea, which resulted in a significant reduction of *Mycosphaerella* foot rot (Bencheikh and Setti 2010). Studies to develop a biological formulation with the *Streptomyces* sp. strain CACIS-116CA, and evaluate its utility under greenhouse and field conditions are in progress. In addition to the above, an evaluation and characterization of the active compound produced by strain CACIS-1.16CA are required.

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