

Short Communication

***In vitro* antagonistic potentials against fungal plant pathogens of soil actinomycetes species from the National Park “El Chico”, a protected forest in México**

ABSTRACT

Aims: Control of fungal plant diseases by using native microorganisms is a promising alternative to chemical fungicides. The present study aims to isolation and characterization of soil actinomycetes, and evaluation of their antagonistic activity.

Place and Duration of Study: National Park “El Chico”, Hidalgo State, and Laboratory of the Southeast Unit of CIATEJ, Yucatán, México, between June 2010 and May 2011.

Methodology: Actinomycetes species were isolated from six composite soil samples by microbiological standard procedures. All isolates were phenotypically characterized. Primary selection of antagonistic isolates was done by an *in vitro* screening of the effect of actinomycetes over the growth of *Fusarium* sp. and *Candida albicans*. Selected species were further evaluated against *Helminthosporium* sp., *Curvularia* sp., and *Aspergillus niger*. Antagonistic actinomycetes were identified by the analysis of the 16S rDNA gene sequence. An *in vitro* preliminary evaluation about the productions of diffusible inhibitory compounds by *Streptomyces* sp. CACIA-1.46HGO was carried out over *Phytophthora capsici*.

Results: 164 actinomycete strains were characterized by morphological and biochemical features. From them, six isolates inhibited the growth of *Fusarium* sp. and *C. albicans* by 5 to 10 mm between the border of the growth of the actinomycete colony and the border of growth of the fungal or yeast. Furthermore, the *in vitro* antagonism of the six actinomycetes over *Helminthosporium* sp., *Curvularia* sp., and *Aspergillus niger* showed a reduction of their growth from 50 to 83 %. The selected actinomycetes were identified by partial 16S rDNA gene sequence; four strains were enclosed into *Streptomyces* genus and one as *Microbacterium* sp. Results of inhibition of growth of *P. capsici* suggested that strain CACIA-1.46HGO produce diffusible compounds with an inhibitory effect over the fungal growth.

Conclusion: Actinomycetes isolates could be potentially used to control and prevent diverse fungal diseases affecting commercial plants. However, these new isolates needs be evaluated in fields.

Keywords: antagonism; actinomycetes; biocontrol; *Streptomyces*, fungal pathogen

1. INTRODUCTION

Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide [1]. For decades, control and manage of fungal

24 plant diseases has been dependent on the chemical synthetic fungicides. However, constant
25 use of fungicides may cause accumulation of toxic compounds potentially hazardous to
26 humans and environment.

27 Biological control of fungal plant diseases using native microorganisms are a promising
28 alternative to chemical fungicides [2]. The biocontrol agents are not limited to a specific
29 group, and given the microbial diversity of soil ecosystems it is probable that the full
30 spectrum of potentially effective strains has barely been explored [3]. Nowadays, a variety of
31 biopesticides based on bacteria and fungi are registered and available on the market and
32 have shown the potential to act as effective biocontrol agents of plant pathogens, by
33 suppressing its growth and promote overall plant vigor and yield [4].

34 The actinomycetes group enclosed species of gram positive bacteria broadly distributed in
35 both terrestrial and aquatic ecosystems, and well-known for the production of many
36 important bioactive metabolites [5]. The diversity and activity of terrestrial *Streptomyces*
37 species has been estimated on different ecosystems, such as beach and dune systems [6],
38 saline farmlands [7], and tropical soils [8].

39 Biological control products based on actinomycetes have been developed to suppress the
40 growth of pathogenic microorganisms in field. Different species has been evaluated *in vitro*
41 and *in vivo* against species of *Aspergillus*, *Fusarium*, *Trichoderma*, *Curvularia oryzae*,
42 *Pyricularia oryzae* and *Bipolaris oryzae* [9,10]. However, it is important search and identified
43 new isolates adaptable to different environment and soil conditions, considering the
44 molecular and physiological diversity of fungal pathogens and its host plants. Hence,
45 characterization and identification of potential biocontrol agents must be preceded to studies
46 about the mechanisms exerted to control the diseases. The present work aimed to isolation
47 and characterizations of soil streptomycetes and evaluates their antagonistic activity to some
48 fungal phytopathogens.

49 2. MATERIAL AND METHODS

50 51 2.1 Isolation of actinomycetes

52 Actinomycetes were isolated from six composite soil samples collected in six locations at "El
53 Chico", a National Park localized on Hidalgo State, Mexico. Each composite sample was
54 prepared from individual samples taken (with an auger and up to 10 cm depth), around an
55 area of 10 m in diameter. Samples were pretreated 1 hour at 70° C. 10 g of the soil sample
56 was deposited in 100 ml of sterile distilled water and serial dilutions up to 10⁻⁴ were
57 inoculated onto ISP2 and ISP3 agar media¹¹, supplemented with nalidixic acid (12.5 µg ml⁻¹)
58 and cyclohexamide (50 µg ml⁻¹). Incubation was carried out at 29°C by 2 to 4 weeks. Isolates
59 were stored as Preservation Stocks (PS) of spore or cells at -20°C in 20% (w/v) glycerol.
60 General Inoculums (GI) of spore or cells were prepared from PS to make a turbid
61 suspension of 0.5 McFarland standards (10⁸ CFU ml⁻¹).

62 63 2.2 Phenotypic characterization

64 Isolates were morphological and physiological characterized based on International
65 *Streptomyces* Project Methods [11]. Enzymatic activity of lipase [12], protease [13] and L-
66 asparaginase [14] were determined in Petri plate.

67 68 2.3 *In vitro* antagonistic activity

A dual culture and streaking assays on Petri plate were used to select antagonistic actinomycetes over *Fusarium* sp. (CDBB:1172) and *Candida albicans*. Antagonistic strains were detected as positive (+) if the zone of inhibition of growth between the borders of the actinomycete and fungi/yeast was greater than 5 mm. Furthermore, selected actinomycetes were tested by dual culture assays against *Aspergillus niger* NRRL-3, *Fusarium* sp. CDBB:1172, *Curvularia* sp. and *Helminthosporium* sp. For the above, 2 μ L of an actinomycete GI was pre-inoculated near the border of a PDA Petri plate at 0 and 5 days before fungal inoculation. Plates were incubated at 29°C. Afterward, an agar block of 0.6 X 0.6 cm with fungal mycelium was deposited at the center of the plate, and maintained at 29°C by additional 7 d. Zero days of incubation considers the inoculation at the same time of actinomycete and fungal pathogen. The percentage of inhibition (PI) was determined using the formula: $PI (\%) = [FR-AR]/[FR] \times 100$, where FR represents the fungal growth radius (mm) of a control culture and AR the distance (mm) of the fungal growth in the direction of actinomycetes [15]. All experiments were done by triplicate.

2.4 Production of diffusible substances by *Streptomyces* sp. CACIA-1.46HGO

Antifungal activity of diffusible substances produced by strain CACIA-1.46HGO was evaluated in Petri plate. An agar plug of 0.6 cm diameter was taken close to the border of a colony grown on ISP2 agar by 14 days, and deposited at the center of a PDA Petri plate together with two agar blocks (0.6 X 0.6 cm) with actively growing mycelium of *Phytophthora capsici*. Plates were incubated at 29° C for 7 days. As positive control, an agar plug of medium with the streptomycete mycelium growth was also inoculated. Assays were done by triplicate.

2.4 Molecular identification by analysis of the 16S rDNA sequence

Actinomycetes were identified by sequence analysis of 16S rRNA gene with oligonucleotides fD1 and rD1[16]. Partial sequences were analyzed for homology using the BLASTN program and the non-redundant GeneBank database (<http://blast.ncbi.nlm.nih.gov/>). Phylogenetic analysis was carried out at Phylogeny.fr web page: <http://www.phylogeny.fr/version2.cgi/index.cgi>. The partial 16rRNA gene sequences obtained in the present study were deposited in GenBank database under accession JQ400108, and JX534230 to JX534234.

3. RESULTS AND DISCUSSION

3.1 Phenotypic characterization

One hundred sixty-four putative actinomycetes were isolated with a predominance of spore producer isolates that could be likely due to isolation conditions [17]. All isolates were characterized by morphology, carbon source assimilation, physiology and their antagonism against *Fusarium* sp. and *Candida albicans* (data not shown). This last criterion was used to select six isolates for further antagonistic evaluation. Morphologically five isolates showed well-developed substrate mycelium, filamentous, branched and mostly unfragmented with a smooth appearance that develops to an aerial mycelium that either appeared floccose, granular or powdery. The sixth isolate (CACIA-1.29HGO) develops smooth, circular, convex, non-filamentous, transparent and yellow colonies with gram positive short-rod cells. Colors of colonies were also noted. Physiologically, some isolates degrade starch, casein and not at all produce melanin. The extracellular enzymatic activity of asparaginase and protease were also detected; lipase activity was only detected on strain CACIA-1.49HGO (Table 1). Enzymatic activity determination of isolates is a relevant characteristic of soil

microorganisms considering that in terrestrial habitats the actinomycetes are excellent saprophytes and prolific producers of extracellular enzymes for initial decomposition of organic material, N-mineralization and assimilation of extracellular nitrogen sources [18]. Phenotypic characterization of the six isolates was completed by data related to assimilation of carbon sources and its contribution to colonies growth, since highlights the pattern of development of mycelium and spore production (Supplementary Table S1).

Table 1. Characterization of soil actinomycetes isolates

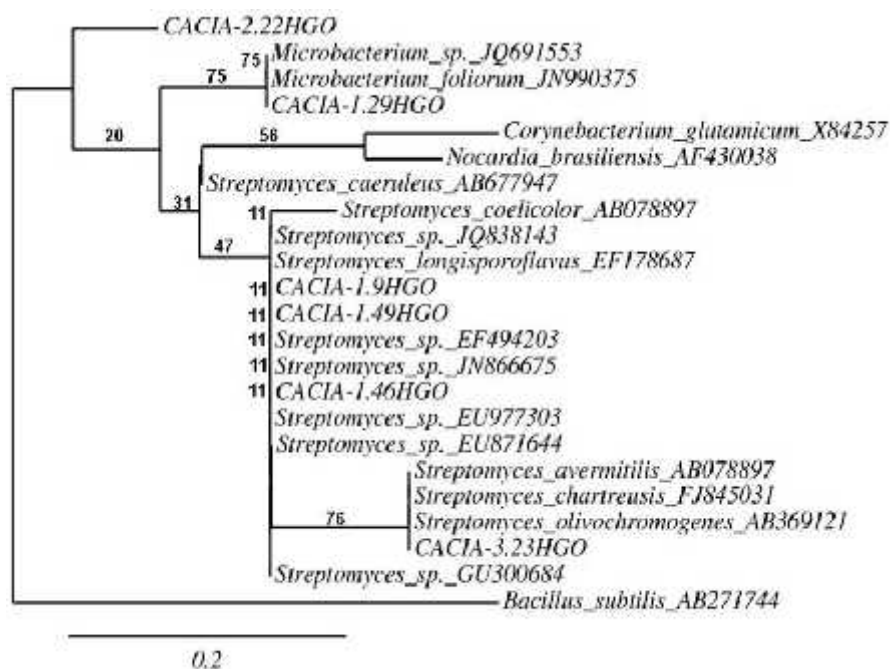
	CACIA-1.9HGO	CACIA-1.29HGO	CACIA-1.46HGO	CACIA-1.49HGO	CACIA-2.22HGO	CACIA-3.23HGO
Characteristics on ISP2						
SM ^a	White	Yellow	Brown	Beige	Brown	White
AM ^b	White	-	White	White	White	White
S ^c	Gray	-	Gray	Grey	Gray	Brown
Diffusible pigments	None	-	Yellow light	None	Violet	None
Growth	F ^d	S-R ^e	F	F	F	F
Test						
Gram	+	+	+	+	+	+
Starch	+	+	+	+	+	+
Casein	+	+	+	-	+	+
Melanin	-	-	-	-	-	-
Extracellular activity						
Lipase	-	-	-	+	-	-
Asparaginase	+	+	+	-	+	+
Gelatinase	+	-	+	+	+	+
Antifungal activity †						
<i>Fusarium</i> sp.	+	+	+	+	+	+
<i>C.albicans</i>	+	+	+	+	+	+

† Positive (+), considers at least 5 mm of inhibition of growth between the margin of margin of fungal growth to actinomycetes colony. ^aSM, color of substrate mycelium; ^bAM, color of aerial mycelium; ^cS, color of spores; ^dF, filamentous; ^eS-R, short-rod.

3.2 Phylogeny of native actinomycetes

Analysis of partial nucleotide sequence of 16S rRNA gene indicated that five isolates matched entries resembling *Streptomyces* and *Microbacterium* species (95 to 99% proximity). CACIA-2.22HGO showed a sequence divergent respect the actinomycete group, the closest match was with *Streptomyces* sp. G2_8 (84 %), which suggests that could be part of a novel actinomycetes group (Supplementary material Table S2). Therefore, the taxonomic position of the above strain remains uncertain. Phylogenetic tree of the isolates

133 further confirms the results (Fig. 1). Four isolates were sorted into *Streptomyces* cluster and
 134 CACIA-2.22HGO strains was clearly constrained in a separate branch. CACIA-1.29HGO is
 135 sorted into *Microbacterium* group, and its morphology fits to members of the genus [19].



136
 137 **Fig. 1. Phylogenetic tree of the 16S rRNA gene sequences from selected**
 138 **actinomycetes. Neighbour-joining analysis with bootstrap values from 1000**
 139 **replications. The scale bar corresponds to 0.2 nucleotide substitution per site**

140 3.3 In vitro evaluation of antagonistic activity

141 Evaluation of antagonist activity of isolates against *Curvularia* sp., *Fusarium* sp.,
 142 *Helminthosporium* sp. and *Aspergillus niger* was registered on Table 2. The isolates inhibited
 143 in different percentages the fungal growth, ranging from 20 to 82 percentages. The higher
 144 activity was detected with *Streptomyces* sp. strain CACIA-1.46HGO, which showed the
 145 highest PI values, principally the value registered at “zero days” of pre-inoculation (46 %),
 146 which could be considered an important strain attribute as potential biocontrol agent. In this
 147 sense, *Curvularia* sp., *Helminthosporium* sp. and *Fusarium* sp. were inhibited by 37 % and
 148 *A. niger* by 46 %. As expected, after five days of actinomycete pre-inoculation the inhibition
 149 reach to 70 % for *A. niger*, 82 % to *Curvularia* sp., 57 % to *Helminthosporium* sp., and 67 %
 150 to *Fusarium* sp. For *Microbacterium* strain, although new members are continuously
 151 determined from a wide range of environmental habitats [20], this is the first report related to
 152 its capacity to inhibit the growth of *Curvularia*, *Helminthosporim*, *Fusarium* and *Aspergillus*.

153 **Table 2. In vitro antagonist activity of actinomycetes**

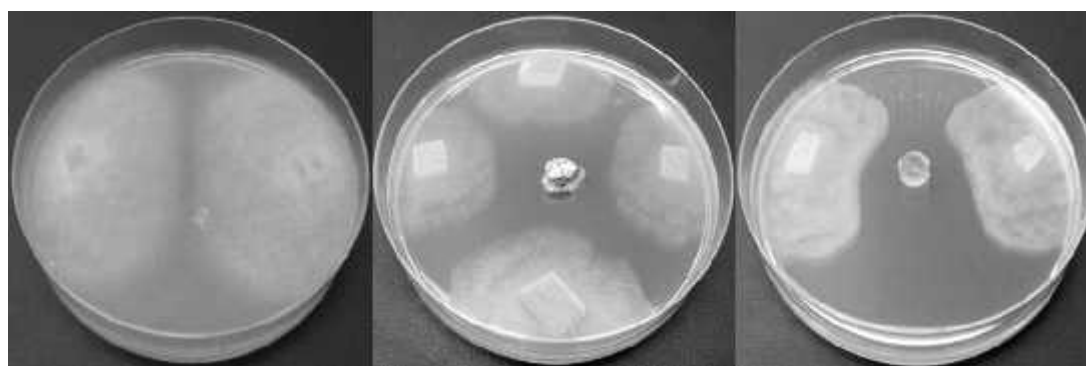
Isolate	Percentage of Inhibition (± SD)*			
	<i>Aspergillus niger</i>	<i>Curvularia</i> sp.	<i>Helminthosporium</i> sp.	<i>Fusarium</i> sp. CDBB:1172

	0**	5	0	5	0	5	0	5
1.9HGO	20 (± 5.4)	63 (± 4.1)	16 (± 6.9)	82 (± 5.3)	10 (± 5.7)	54 (± 1.1)	9 (± 2.5)	71 (± 3.6)
1.29HGO	33 (± 7.3)	72 (± 3.6)	28 (± 2.7)	83 (± 2.6)	21 (± 4.1)	54 (± 0.6)	6 (± 3.6)	68 (± 1.0)
1.46HGO	46 (± 2.5)	70 (± 3.1)	37 (± 7.7)	82 (± 7.2)	37 (± 1.7)	57 (± 4.5)	37 (± 6.4)	67 (± 1.6)
1.49HGO	21 (± 8.8)	60 (± 3.5)	7 (± 0.1)	72 (± 7.1)	15 (± 2.0)	62 (± 4.2)	10 (± 0.8)	71 (± 1.3)
2.22HGO	31 (± 3.0)	64 (± 2.4)	4 (± 2.4)	66 (± 7.7)	24 (± 4.7)	50 (± 2.9)	13 (± 3.8)	59 (± 1.1)
3.23HGO	32 (± 2.6)	59 (± 1.2)	35 (± 7.0)	64 (± 5.9)	25 (± 5.1)	48 (± 2.5)	13 (± 2.0)	60 (± 4.4)

*Control growth of plant pathogens on Petri-plate (radial rate) for *A. niger* NRRL-3, 39.8 ± 2.2 mm; *Curvularia* sp., 34.6 ± 0.9 mm; *Helminthosporium* sp., 42.36 ± 2.3 mm; *Fusarium* sp. CDBB:1172, 40.02 ± 0.7 mm. ** Days of actinomycetes pre-inoculation.

3.4 Preliminary evaluation of antifungal compounds

Considering that over 50 % of antimicrobial compounds produced by microorganisms have been isolated from actinomycetes, particularly from *Streptomyces* [21], it would be interesting evaluate if antagonistic activity of *Streptomyces* sp. CACIA-1.46HGO could be related to the production of substances diffusible to the agar medium. Fig. 2 showed the inhibition of growth of *Phytophthora capsici* by diffusible substances contained in an agar block of medium (right panel), with a PI of 15.9 % (SD ± 0.2). As a control, an agar block with streptomycete mycelium showed a PI of 13.4 % (center panel, SD ± 0.02). The above results suggest that strain CACIA-1.46HGO could be a potential candidate to be an agent to control fungal plant diseases.



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Fig. 2. Inhibitory activity of metabolites diffused into the agar media of growth from a fourteen days culture of *Streptomyces* sp CACIA-1.46HGO against *Phytophthora capsici*. Left, control Petri plate with *P. capsici*; center, inhibition of fungal growth by a disc of agar with streptomycete mycelium; right, inhibition of fungal growth by diffusible metabolites from a disc of agar media.

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4. CONCLUSION

The present works shows that soil actinomycetes from the National Park “El Chico” holds potential biocontrol agents to control fungal pathogens affecting commercial plants. These members of actinomycetes were isolated from an ecosystem characterized by the presence of trees vegetation (oyamel, cedar, oak, pine and tlascal) with epiphytes and ferns, which are mainly located in mountains, canyons and sites where conditions of humidity are favorable for decomposition of plant litter and residues to support a high microbial abundance at the topsoil’s surface. Under these environmental conditions new *Streptomyces* and *Microbacterium* species were isolated.

ACKNOWLEDGEMENTS

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Competing interests

“Author has declared that no competing interests exist”.

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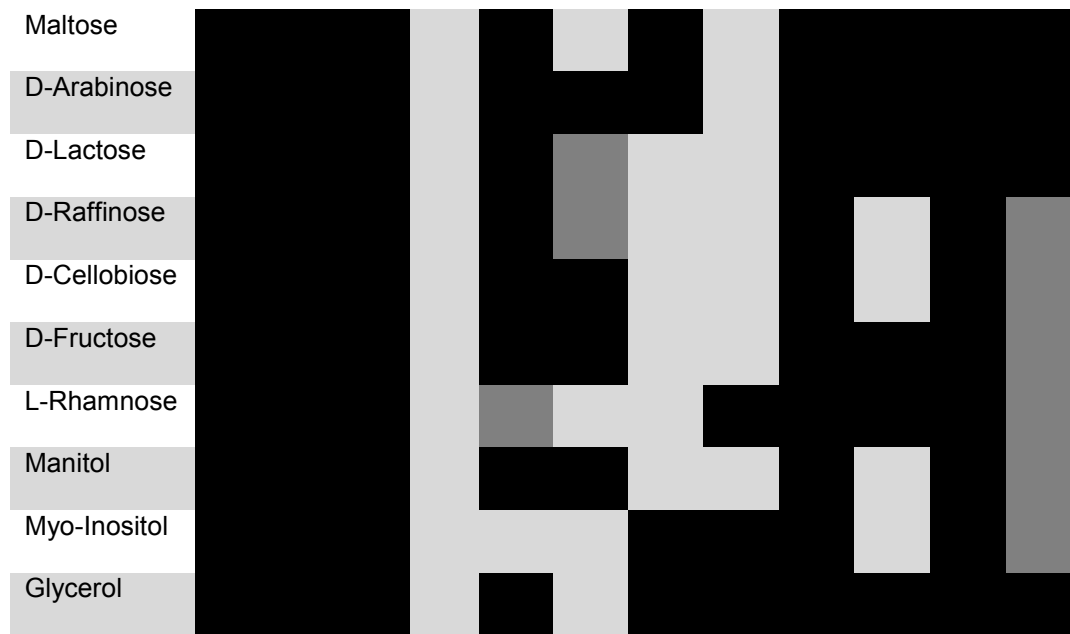
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Supplementary material Table S1

271 ***In vitro* antagonistic potentials against fungal**
272 **plant pathogens of soil actinomycetes species**

Carbon Source*	CACIA-1.9HGO		CACIA-1.29HGO		CACIA-1.46HGO		CACIA-1.49HGO		CACIA-2.22HGO		CACIA-3.23HGO	
	G †	S ‡	G	S	G	S	G	S	G	S	G	S
None												
D-Glucose												
Sucrose												
L-Xylose												
D-Xylose												



292 * ISP9 medium + 1% (w/v) of carbon source. † G, growth of substrate mycelium: ■, positive
293 utilization and vigorous develop of mycelium substrate; ■, positive utilization and little
294 develop of mycelium substrate; □, negative utilization and no develop of mycelium
295 substrate. ‡ S, Sporulation: ■, vigorous spore formation; ■, weak spore formation; □, no
296 spore formation.

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Table S2. Blast matches for the closest 16S rRNA gene sequence of the actinomycetes isolates

16S rDNA gene sequence match by BLASTN						
Isolates	Sequence ID	Organism	Sequence ID	Bits	(%)	References*
CACIA-1.9HGO	JX534230	<i>Streptomyces</i> sp. P911A	EU977303	605	95	Smith et al., 2008
CACIA-1.29HGO	JX534231	<i>Microbacterium</i> sp. FI1010	JQ691553	1478	99	Sanchez et al., 2012
CACIA-1.46HGO	JQ400108	<i>Streptomyces</i> sp. BA2	EF494203	1240	99	Yilmaz et al., 2008
CACIA-1.49HGO	JX534332	<i>Streptomyces longisporoflavus</i> NRRL_ISP-5165	EF178687	1186	97	Unpublished
CACIA-2.22HGO	JX534233	<i>Streptomyces</i> sp. G2_8	JN020512	329	84	Vetsigian et al., 2011
CACIA-3.23HGO	JX534234	<i>Streptomyces olivochromogenes</i> A018	AB369121	1137	99	Lee et al., 2008

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