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gerardo.mata@inecol.edu.mx

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# Antagonistic properties of micromycetes isolated from sinkholes of the Yucatán Peninsula against fungal phytopathogens

Pablo Moreno-Pérez<sup>1</sup>, Marcela Gamboa-Angulo<sup>1</sup>, Gabriela Heredia<sup>2</sup>, Blondy Canto-Canché<sup>1</sup>  
Miguel Rosado-Vallado<sup>3</sup>, Irma L. Medina-Baizabal<sup>1</sup>, Raúl Tapia-Tussell<sup>1</sup>

<sup>1</sup>Unidad de Biotecnología, Centro de Investigación Científica de Yucatán, Mérida 97200, Yucatán, México.

<sup>2</sup>Red de Biodiversidad y Sistemática, Instituto de Ecología A.C., Km 2.5 antigua carretera a Coatepec No. 351, Xalapa 91070, Veracruz, México.

<sup>3</sup>Centro de Investigación Regional "Hidéyo Noguchi", Universidad Autónoma de Yucatán, México

## Propiedades antagonistas de micromicetos aislados de cenotes de la península de Yucatán contra hongos fitopatógenos

**Resumen.** En la búsqueda de alternativas naturales para el control de enfermedades fúngicas, los micromicetos con propiedades antagonicas son considerados una fuente valiosa para detectar modelos novedosos. En el presente estudio, un total de 41 micromicetos tropicales se aislaron a partir de restos vegetales sumergidos en cenotes de la Península de Yucatán. Todas las cepas se evaluaron en ensayos antagonistas contra cuatro hongos fitopatógenos (*Colletotrichum gloeosporioides*, *Corynespora cassiicola*, *Curvularia* sp. y *Fusarium* sp.). Los resultados de este ensayo detectaron que 17 aislamientos (41 %) provocaron  $\geq 50$  % de inhibición del crecimiento micelial (MGI  $\geq 50$  %) de al menos uno de los patógenos evaluados. La inhibición más alta fue ocasionada por las cepas *Hypocrea lixii* OSN - 37 (MGI = 61-77 %) y *Rhizoctonia solani* OSE- 73 (MGI = 55-64 %) contra todos los objetivos, mientras que *Pestalotiopsis mangiferae* OH - 02 (51 - 59%) causó la inhibición en tres de las cuatro cepas patógenas. Las cepas antagonistas con mejores resultados fueron cultivadas en arroz fermentado para obtener sus correspondientes extractos de acetato de etilo y metanol los cuales se evaluaron contra *C. gloeosporioides* en ensayo de microdilución. Los resultados indicaron que *H. lixii* OSN - 37 y *R. solani* OSE- 73 son productores de metabolitos antifúngicos como uno de sus modos de acción. En conclusión, tres cepas nativas promisorias como antagonistas de fitopatógenos se aislaron de los cenotes de la península de Yucatán, lo que representa una valiosa contribución para desarrollar alternativas eco-amigables en el control de enfermedades fúngicas que afectan los cultivos agrícolas en regiones tropicales.

**Palabras clave:** antagonista, *Hypocrea lixii*, micromicetos, *Pestalotiopsis mangiferae*, cenote, *Rhizoctonia solani*.

**Abstract.** In the search for natural alternatives to control fungal diseases, antagonistic fungi are valuable sources to find new models. In the present study, a total of 41 tropical micromycetes were isolated from plant debris submerged in sinkholes of the Yucatán Peninsula. All strains were tested in antagonist assays against four phytopathogenic fungi (*Colletotrichum gloeosporioides*, *Corynespora cassiicola*, *Curvularia* sp. and *Fusarium* sp.). Results of the antagonistic assays showed mycelial growth inhibition (MGI  $\geq 50$  %) by 17 isolates (41%) against at least one of the targets tested. The highest inhibition was exhibited by the *Hypocrea lixii* OSN-37 (MGI = 61 - 77%) and *Rhizoctonia solani* OSE-73 (MGI = 55 - 64%) strains against all targets while *Pestalotiopsis mangiferae* OH-02 (51 - 59%) caused inhibition on three of four pathogen strains. These three strains were cultured in fermented rice to obtain their ethyl acetate and methanol extracts which were tested against *C. gloeosporioides* using the microdilution assay. Results showed *H. lixii* OSN-37 and *R. solani* OSE-73 to be producers of antifungal metabolites as one of their modes of action. In conclusion, three promising antagonistic native strains were isolated from plant debris submerged in the Yucatan sinkholes, representing a valuable contribution to the development of ecofriendly alternatives to control fungal diseases in agriculture crops of the tropical regions.

**Keywords:** antagonist, *Hypocrea lixii*, micromycetes, *Pestalotiopsis mangiferae*, sinkhole, *Rhizoctonia solani*.

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Autor para correspondencia: Marcela Gamboa-Angulo  
mmarcela@cicy.mx

## Introduction

The control of pathogenic fungi in agricultural crops using synthetic agrochemicals has been well documented and proven to have adverse side effects on the environment (Alwathnani *et al.*, 2012). Therefore, there is an urgent need to find effective new strategies to replace them. One alternative is the use of antagonistic fungi that have demonstrated valuable abilities to inhibit the growth or development of fungal pathogen populations, with the additional advantage of reduced environmental and human health impacts (Whipps and Lumsden, 2001). For example, *Muscodor* sp. produces a mixture of volatile compounds with activity against bacteria, fungi, insects and nematodes (Strobel *et al.*, 2012), *Sphaerodes mycoparasitica* is an antifungal agent with wide spectra against members of the genera *Fusarium*, *Pythium*, *Rhizoctonia* and *Sclerotinia* (Vujanovic, 2012) and *Emericella rugulosa* is a biocontrol agent of *Fusarium* wilt of tomato (Sibounnavong *et al.*, 2012), among others.

In México, relatively few contributions dealing with the evaluation of antagonistic fungal native species for the control of phytopathogenic fungi have been published. Some examples are those of Quiroz-Sarmiento *et al.* (2008), Michel-Aceves *et al.* (2009), Galindo-Flores *et al.* (2005) and Cuervo-Parra *et al.* (2011).

In this context, our research group has been carrying out antimicrobial screening with extracts from tropical fungi of the southeast of México against very important phytopathogens belonging to the *Alternaria*, *Colletotrichum* and *Fusarium* genera, among others (Gamboa-Angulo *et al.*, 2012; Reyes-Estebanez *et al.*, 2011). These previous studies included isolation and identification of saprophytic fungal strains from sinkholes (cenotes in Spanish, from the Mayan word *dzonoʔ*), which were cultured in fermented rice, and organic extracts tested against a set of bacterial and fungal

phytopathogens. These investigations showed a broad antimicrobial spectrum in 53% of the saprophytic strains, and eight of which were also effective against fungal phytopathogens (Gamboa-Angulo *et al.*, 2012). The sinkholes are special freshwater ecosystems where the low availability of nitrogen and phosphorus sources induces strong competition for survival (Schmitter-Soto *et al.*, 2002). Around 2241 sinkholes have been reported in the state of Yucatán ([www.seduma.yucatan.gob.mx](http://www.seduma.yucatan.gob.mx)), most of them have not been investigated for their microbiota, and of course, in their antimicrobial abilities.

With these antecedents, the aim of the present work was to isolate native micromycetes from two different sinkholes of Yucatán to enrich our collection of fungal strains and to evaluate their antagonistic abilities against *Colletotrichum gloeosporioides*, *Corynespora cassiicola*, *Curvularia* sp. and *Fusarium* sp., four pathogenic fungi of agricultural importance.

## Materials and methods

### Isolation of saprophytic fungi

Two semi-closed cylindrical sinkholes named Oxola (N 20°40'41", W 89°14'30") and Kikal (in the Yalahau reserve, N 20°34'59", W 89°10'49") were selected. Both are located in the center of the state of Yucatán and are far from agricultural activities and human settlements. In order to detect a wider range of fungal species, besides collecting plant debris submerged in the study areas, litter traps (mesh bags of 20 x 30 cm, 1-mm mesh size) were installed with poles buried within the sinkholes. A set of litter traps was filled with non-sterile material gathered around the sinkholes, and a second one consisted in litter traps with plant debris sterilized and collected in the botanical gardens of the Scientific Research Center of Yucatán (CICY for its acronym in Spanish). In both sinkholes, after 7, 14 and 21 days the submerged bags were

collected. Samples were washed in laboratory with a slow water flow and then plant debris were gently dried with sterile paper towels and incubated ( $25 \pm 2^\circ\text{C}$ ) in damp chambers, made with glass Petri dishes with wet sterile filter paper. Every day the chambers were ventilated and monitored. With the aid of a stereo microscope structures of fungi were detected and transferred to Petri dishes containing PDA medium (Krug, 2006).

#### Identification of saprophytic fungi

Fungi were identified according to their morphological characteristics using taxonomic keys (Barnett and Hunter, 1998; Carmichael *et al.*, 1980). The fungal strains that showed antagonistic activity were submitted to molecular analysis. Genomic DNA (gDNA) was obtained according to Conde-Ferrández, (2008). The internal transcribed spacer regions, including the 5.8S rDNA, were amplified using universal primers ITS1 and ITS4 (White *et al.*, 1990). PCR products were analyzed by gel electrophoresis and the DNA bands carefully cut and purified with the Gene Clean II kit (Roche 1001-400). Samples were sequenced using 20 ng of PCR product and ITS1 primer. Sequences were obtained by MacroGen Inc, Korea. Alignment and edition were done with the BioEdit Program v 7.0.5 (Hall, 2005), and manually corrected. The sequences obtained were compared against those available in the GenBank database (GenBank, <http://www.ncbi.nlm.nih.gov>).

#### Target strains

*Colletotrichum gloeosporioides* Penz. & Sacc. (CICY-05) were isolated from *Carica papaya* L., *Corynespora cassiicola* Berk. & M. A. Curtis (ITC-03) from *Capsicum annum* L., *Curvularia* sp. (ITC-01) from *Zea mayz* L. and *Fusarium* sp. (CICY-04) from *Lycopersicon esculentum*. These phytopathogenic fungi were obtained from the CICY and Conkal Technological Institute (ITC) culture collection, and were identified by morphological characteristics using

taxonomic keys (Barnett and Hunter, 1998).

#### Antagonistic trial

Antagonistic activity against the phytopathogenic fungi was carried out by the *in vitro* dual culture assay (Asran-Amal *et al.*, 2010). All phytopathogenic and saprophytic fungal strains were maintained on potato dextrose agar (PDA), with natural light at  $25 \pm 2^\circ\text{C}$ . A mycelial disk of a seven day-old culture of the saprophytic strain (5 mm in diameter) was placed on one edge of the Petri dish (6 cm in diameter), while a mycelial disk of a seven day-old culture of the target (5 mm in diameter) was transferred to the opposite edge. For control, each target plant pathogen was confronted with a sterile disk of PDA (5 mm in diameter), and the mycelial growth was measured (negative control of inhibition); a PDA disk (5 mm in diameter) with antifungal Mirage<sup>®</sup> (Prochloraz 900  $\mu\text{g}/\text{mL}$ ) was also used as positive control of inhibition; experiments were performed in quadruplicate. All dishes were incubated at  $25 \pm 2^\circ\text{C}$  with continuous light, and growth was evaluated at the sixth day by measuring the radius of each mycelium, from the outer edge of the disk with the pathogen to the edge of the growth of the antagonist, using a Truper<sup>®</sup> millimeter gauge (CALDI-6MP model). Results were reported as percentage of mycelium growth inhibition (%MGI) of each pathogen, determined by Abbott's formula  $[(C - T/C) \times 100]$  (Abou *et al.*, 2002) where C is the hyphal extension of the target strain in single culture and T is the target fungus growth in dual culture.

#### Fungal extracts

Antagonistic strains *Hypocrea lixii* OSN-37, *Pestalotiopsis mangiferae* OH-02, and *Rhizoctonia solani* OSE-73 were inoculated into in fermented rice (160 g of rice was fermented with distilled water overnight and sterilized); these were then incubated at  $25 \pm 2^\circ\text{C}$ , using a light-dark photoperiod (12/12 h). After 40 days, cultures were frozen and lyophilized. They were then fragmented and subsequently extracted with ethyl acetate (three times, 50 mL, 24 h at  $25^\circ\text{C}$ , each) and methanol

(50 ml, 24 h at approximately 50°C). The solvents were eliminated by reduced pressure to obtain the corresponding fungal organic extracts (Soman *et al.*, 2001). These were dissolved in acetonitrile and defatted with hexane (three times, 2:1, 1:1, 1:1, v/v) obtaining the hexane and acetonitrile fractions and a precipitate. All organic samples were tested in broth microdilution bioassay.

### Broth microdilution assay on *Colletotrichum gloeosporioides*

The minimum inhibitory concentrations (MIC) of the extracts were determined by broth microdilution techniques according to the National Committee for Clinical Laboratory Standards (NCCLS, 2004) with slight modifications. Each organic extract, fraction, and precipitate was dissolved in dimethyl sulfoxide (DMSO) (40 µg/µL) and 10 µL were transferred to microwells containing medium Roswell Park Memorial Institute (RPMI-1640, 90 µL, www.sigmaaldrich.com). A conidial suspension of *C. gloeosporioides* (100 µL) was then added to each microwell to a final concentration of  $2.5 \times 10^4$  conidia/mL, 2000 µg/mL extracts and 5% DMSO. Hexane, acetonitrile fractions and precipitate of *P. mangiferae* OH-02, *T. lixii* OSE-37, and *R. solani* OSE-73, were tested at final concentrations of 2000, 1000 and 500 µg/mL, with 5, 2.5 and 1.25% of DMSO, respectively, while 25% DMSO was used as positive control, and negative controls were the spore suspension, RPMI-1640, 5% DMSO, and a blank of fermented rice. Tests were carried out in triplicate. The microdilution plates were incubated at  $25 \pm 2^\circ\text{C}$  in the dark for 96 hours, after which hyphal growth (HG) was, determined visually with a microscope (50×) following NCCLS standards (2004).

### Statistical analysis

Inhibition data were analyzed with *t*-Student statistical package SPSS.13.0, compared to negative control ( $P < 0.05$ ). Subsequently, a linear correlation analysis was performed

between percentage of mycelial growth of fungal saprophytic strains and fungal phytopathogen in dual culture (Figure 1).

## Results

### Isolation and identification of strains

Forty-one fungal isolates were obtained from submerged native plant debris and from the litter traps installed in the two sinkholes of Yucatán. Most of them were captured by litter traps containing *ex situ* sterilize plant debris (44%). Among these, 18 (44%) were identified as belonging to the genera *Bionectria*, *Clonostachys*, *Fomitopsis*, *Fusarium*, *Hypocrea*, *Monodictys*, *Penicillium*, *Pestalotiopsis*, *Pseudorobillarda*, *Rhizoctonia* and *Scopulariopsis*, and ten were identified at species level (Table 1). The remaining 23 strains (46%) were not taxonomically identified because they did not sporulate in the culture media used. On the other hand, since they were inactive in antagonistic assays, they were not analyzed by molecular techniques (Table 1).

### Antagonistic trial

Results of the antagonistic evaluation of fungal isolations against four fungal phytopathogens in dual culture assay showed statistical differences ( $P < 0.05$ ) after six days (Table 1). Seventeen of the 41 saprophytic strains were able to inhibit mycelial growth ( $\geq 50\%$ ) of at least one target tested, and six of them displayed good antagonistic properties (MGI values of  $> 60\%$ ). Half of these antagonistic fungi were obtained from the litter traps with *ex situ* sterilized material. The active fungi were *Fusarium solani* OE-80, *Hypocrea lixii* OSN-37 (teleomorph of *T. harzianum*), *P. mangiferae* OH-02, and *R. solani* OSE-73. The highest antagonist effect was produced by *H. lixii* OSN-37 (61 – 77%) and *R. solani* OSE-73 (55 – 64%), both were successful against all target phytopathogens (Table 1). Furthermore, *H. lixii* OSN-37 caused the highest inhibition of *C. gloeosporioides*, *Curvularia* sp. and

*Fusarium* sp. (MGI values of 67, 72 and 77%, respectively), and was notably active against *C. cassicola* (MGI values of 61%). Other attractive antagonist strain was *P. mangiferae* OH-02 that showed good inhibitory properties against *C. cassicola*, *Curvularia* sp. and *Fusarium* sp. (MGI values of 51–59%) (Table 1).

In this study, the most sensitive pathogen was *C. cassicola*, showing inhibition by 16 fungal saprophytes (MGI  $\geq$  50). On the other hand, *C. gloeosporioides* was the

most resistant target, being affected only by *H. lixii* OSN-37 and *R. solani* OSE-73 at 67 and 62 % (MGI  $\geq$  60%) respectively. *Curvularia* sp. was inhibited (MGI  $\geq$  60%) by *F. solani* OE-80, *H. lixii* OSN-37, *P. mangiferae* OH-02, *Pseudorobillarda sojae* OSN-36 and *R. solani* (Figure 1). Finally, *Fusarium* sp. was affected only by *H. lixii* OSN-37, *P. mangiferae* OH-02 and *R. solani* OSE-73 (MGI values of 55–77%).

Table 1. Antagonistic effect of saprophytic fungi isolated from sinkholes of Yucatán, México, against fungal phytopathogens

Strain	Site	Mycelial growth inhibition (%)			
		<i>C. cassicola</i>	<i>C. gloeosporioides</i>	<i>Curvularia</i> sp.	<i>Fusarium</i> sp.
<i>Bionectria ochroleuca</i> OE-81*	1	54 <sup>c</sup>	26 <sup>c</sup>	23 <sup>c</sup>	42 <sup>c</sup>
<i>Clonostachys</i> sp. OE-79	1	51 <sup>c</sup>	33 <sup>c</sup>	20 <sup>c</sup>	28 <sup>c</sup>
<i>Fomitopsis meliae</i> KSE-64*	2	37 <sup>c</sup>	5 <sup>c</sup>	48 <sup>c</sup>	23 <sup>c</sup>
<i>Fusarium solani</i> OSN-54*	1	55 <sup>c</sup>	33 <sup>c</sup>	26 <sup>c</sup>	45 <sup>c</sup>
<i>Fusarium solani</i> OE-80*	1	50 <sup>c</sup>	38 <sup>c</sup>	66 <sup>c</sup>	37 <sup>c</sup>
<i>Fusarium solani</i> KE-82*	2	61 <sup>c</sup>	48 <sup>c</sup>	44 <sup>c</sup>	47 <sup>c</sup>
<i>Fusarium</i> sp. OH-04	1	46 <sup>c</sup>	11 <sup>c</sup>	14 <sup>c</sup>	40 <sup>c</sup>
<i>Fusarium</i> sp. KS-15	2	38 <sup>c</sup>	31 <sup>c</sup>	12 <sup>b</sup>	29 <sup>c</sup>
<i>Fusarium</i> sp. OSN-32	1	53 <sup>c</sup>	48 <sup>c</sup>	43 <sup>c</sup>	40 <sup>c</sup>
<i>Fusarium</i> sp. KSN-65	2	52 <sup>c</sup>	25 <sup>c</sup>	30 <sup>c</sup>	37 <sup>c</sup>
<i>Hypocrea lixii</i> OSN-37*	1	61 <sup>c</sup>	67 <sup>c</sup>	72 <sup>c</sup>	77 <sup>c</sup>
<i>Monodictys</i> sp. OSE-75	1	32 <sup>c</sup>	3 <sup>c</sup>	-1 <sup>c</sup>	19 <sup>c</sup>
<i>Penicillium</i> sp. OSE-61	1	52 <sup>c</sup>	13 <sup>c</sup>	25 <sup>c</sup>	34 <sup>c</sup>
<i>Penicillium</i> sp. KE-83	2	23 <sup>c</sup>	-9 <sup>c</sup>	36 <sup>c</sup>	37 <sup>c</sup>
<i>Pestalotiopsis mangiferae</i> OH-02*	1	59 <sup>c</sup>	46 <sup>c</sup>	51 <sup>c</sup>	59 <sup>c</sup>
<i>Pseudorobillarda sojae</i> OSN-36*	1	33 <sup>c</sup>	1 <sup>c</sup>	60 <sup>c</sup>	29 <sup>c</sup>
<i>Rhizoctonia solani</i> OSE-73*	1	64 <sup>c</sup>	63 <sup>c</sup>	63 <sup>c</sup>	55 <sup>c</sup>
<i>Scopulariopsis</i> sp. KS-18	2	42 <sup>c</sup>	35 <sup>c</sup>	24 <sup>c</sup>	25 <sup>c</sup>
Unidentified OR-05	1	25 <sup>c</sup>	2 <sup>c</sup>	24 <sup>c</sup>	20 <sup>c</sup>
Unidentified OS-07	1	58 <sup>c</sup>	34 <sup>c</sup>	36 <sup>c</sup>	47 <sup>c</sup>
Unidentified KH-08	2	60 <sup>c</sup>	17 <sup>c</sup>	26 <sup>c</sup>	38 <sup>c</sup>
Unidentified KH-11	2	39 <sup>c</sup>	44 <sup>c</sup>	7 <sup>a</sup>	25 <sup>c</sup>
Unidentified KH-13	2	26 <sup>c</sup>	-2 <sup>c</sup>	26 <sup>c</sup>	23 <sup>c</sup>
Unidentified KS-17	2	21 <sup>c</sup>	8 <sup>c</sup>	40 <sup>c</sup>	30 <sup>c</sup>
Unidentified KS-19	2	38 <sup>c</sup>	24 <sup>c</sup>	25 <sup>c</sup>	38 <sup>c</sup>
Unidentified OH2-22	1	30 <sup>c</sup>	5 <sup>c</sup>	2	30 <sup>c</sup>



Continued Table 1

Unidentified OH2-23	1	30 <sup>c</sup>	9 <sup>c</sup>	16 <sup>c</sup>	31 <sup>c</sup>
Unidentified OH2-27	1	30 <sup>c</sup>	14 <sup>c</sup>	9 <sup>a</sup>	42 <sup>c</sup>
Unidentified OSN-35	1	44 <sup>c</sup>	39 <sup>c</sup>	-17 <sup>c</sup>	35 <sup>c</sup>
Unidentified OSN-38	1	39 <sup>c</sup>	0 <sup>c</sup>	23 <sup>c</sup>	25 <sup>c</sup>
Unidentified OSE-41	1	32 <sup>c</sup>	13 <sup>c</sup>	30 <sup>c</sup>	47 <sup>c</sup>
Unidentified OSE-43	1	46 <sup>c</sup>	25 <sup>c</sup>	10 <sup>a</sup>	29 <sup>c</sup>
Unidentified KSE-47	2	58 <sup>c</sup>	48 <sup>c</sup>	41 <sup>c</sup>	40 <sup>c</sup>
Unidentified OSE-52	1	36 <sup>c</sup>	23 <sup>c</sup>	43 <sup>c</sup>	34 <sup>c</sup>
Unidentified OSE-53*	1	52 <sup>c</sup>	39 <sup>c</sup>	35 <sup>c</sup>	49 <sup>c</sup>
Unidentified OSN-55	1	43 <sup>c</sup>	10 <sup>c</sup>	21 <sup>c</sup>	28 <sup>c</sup>
Unidentified KSE-59	2	51 <sup>c</sup>	16 <sup>c</sup>	27 <sup>c</sup>	29 <sup>c</sup>
Unidentified OSE-74	1	20 <sup>c</sup>	16 <sup>c</sup>	15 <sup>c</sup>	15 <sup>b</sup>
Unidentified OSE-76	1	32 <sup>c</sup>	8 <sup>c</sup>	45 <sup>c</sup>	35 <sup>c</sup>
Unidentified OSN-78	1	37 <sup>c</sup>	17 <sup>c</sup>	9 <sup>a</sup>	32 <sup>c</sup>
Unidentified KE-85	2	48 <sup>c</sup>	24 <sup>c</sup>	47 <sup>c</sup>	47 <sup>c</sup>
Positive Control		80 <sup>c</sup>	77 <sup>c</sup>	67 <sup>c</sup>	76 <sup>c</sup>
Negative control		0	0	0	0

Site 1) Oxola, 2) Kikal.

Negative control = Fungal phytopathogen disk vs sterile PDA disk

Positive control = Fungal phytopathogen disk vs Mirage ® plus disk (900 µl/ml).

Statistical analysis was performed by comparison of the negative control vs mycelial

growth (cm) of phytopathogen with saprophytic micromycete after six days in dual culture.

Different letters indicate statistical difference (*t*-Student) a)  $P < 0.05$  b)  $P < 0.01$  c)  $P < 0.001$

\* Molecular analysis was performed

### Antifungal assay of extracts on *Colletotrichum gloeosporioides*

The three most active antagonistic strains detected were *H. lixii* OSN-37, *P. mangiferae* OH-02, and *R. solani* OSN-73, all of them were cultured on fermented rice. Two extracts (ethyl acetate and methanol) and three fractions (Hexane, acetonitrile and precipitate) were obtained from each strain, and all were assessed against *C. gloeosporioides*.

Results showed that all samples of *H. lixii* OSN-37 were active, with the exception of the polar methanol extract and the acetonitrile fraction of *R. solani* OSE-73 (Table 2). The lowest MIC was displayed by the acetonitrile fraction of *H. lixii* OSN-37 with values of 1000 µg/mL.

### Discussion

The serial collections led to the isolation of 27 saprophytic fungal strains from Oxola (66%) and 14 (34%) from Kikal. Among these, strains belonging to the *Bionectria*, *Fomitopsis*, *Hypocrea*, *Monodictys*, *Pestalotiopsis*, *Pseudorobillarda* and *Scopulariopsis* genera corresponded to the first fungal contributions of Yucatán, México. The genus *Fusarium* was predominant in the sinkholes studied, with seven strains isolated, which is congruent with previous reports from other sinkholes (Gamboa-Angulo *et al.*, 2012).

All the strains were tested against four recognized fungal phytopathogens, the most active were *H. lixii* OSN-37, *P. mangiferae* OH-02, and *R. solani* OSE-73. *Hypocrea lixii* OSN-37 showed the most active and broadest antagonistic

effect against the targets tested. Although *H. lixii* is widely distributed in the world, (Druzhinina *et al.*, 2010), this is the first report from the state of Yucatán.

The antagonistic properties of *Trichoderma harzianum*, the asexual stage of *Hypocrea lixii*, have been extensively documented (Elgorban *et al.*, 2013; Asran-Amal *et al.*, 2010). At present several commercial formulations are available to control diverse fungal plant pathogens, such as Trianium® by Koppert Biological Systems (*Trichoderma harzianum* T22,), Trichodex® by AMC Chemical (*T. harzianum* T39) and Tusal® by NewBiotechnic (*T. harzianum* and *T. viride*) (Reino *et al.*, 2008; Xu, *et al.*, 2010), among others. On the other hand, it is widely known that *Trichoderma* possess several antagonistic mechanisms. In particular, it was observed that our native strain *H. lixii* OSN-37 displayed overgrowth on four phytopathogens tested herein, which suggests mycoparasitic action due to its rapid growth and abundance. This effect could be regulated by the production of enzymes such as chitinase,  $\beta$ -glucanase, cellulase and protease, and also by secondary metabolites (Harman, 2006; Reino *et al.*, 2008).

Therefore, the ability of *H. lixii* OSN-37 to produce antimicrobial metabolites was confirmed when this strain was cultured on fermented rice, and its ethyl acetate extract showed hyphal growth inhibition of *C. gloeosporioides*, as observed in dual culture (Table 2). This pathogen is one of the causal agents of anthracnose in various tropical crops in the world, such as *Annona muricata*, *C. papaya*, *Coffea arabica*, *Fragaria vesca*, *Lycopersicum esculentum*, *Mangifera*

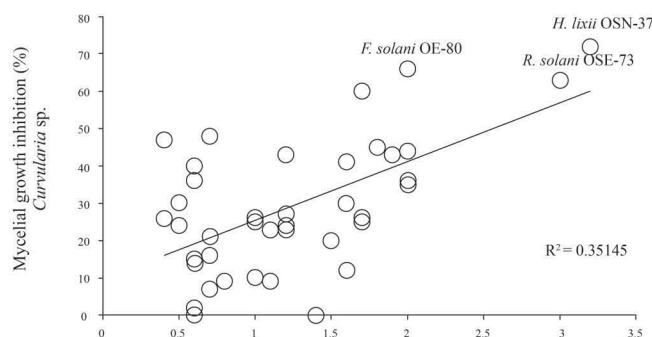


Figure 1. Correlation between mycelial growth of saprophytic strains and growth inhibition of the pathogen *Curvularia* sp. on the sixth day of cultivation.

*indica*, *Persea americana* and *Vitis vinifera* (Beltrán-Cifuentes and García-Jaramillo, 2006). This extract was later partitioned and all corresponding fractions displayed antifungal effect; the most effective was the acetonitrile fraction with MIC values of 1000  $\mu\text{g/mL}$  (Table 2). Thus, low and medium polarity compounds are implicated in the mechanism of action of *H. lixii* OSN-37. A great number of low molecular weight antibiotics with diverse structures have been isolated from several species of the *Trichoderma* genus (Reino *et al.*, 2008). Chemical purification and identification of these active metabolites, therefore, would be highly recommended.

Another antagonistic strain was *R. solani* OSE-73 which showed overgrowth on all target strains. This effect was similar to that displayed by *H. lixii* OSN-37, suggesting a mycoparasitism effect. *Rhizoctonia solani* is classified as a soil fungal pathogen, although it presents saprophytic and symbiotic associations (Bateman, 1970; Masuhara *et al.*,

Table 2. Minimal inhibitory concentration (MIC) of the fungal extracts and fractions of partition from ethyl acetate extracts against *Colletotrichum gloeosporioides* in broth microdilution assay

Strain	MIC ( $\mu\text{g/mL}$ )				
	EtOAc	Hexane	AcN	Precipitate	MeOH
<i>Hypocrea lixii</i> OSN-37	2000	2000	1000	2000	na
<i>Pestalotiopsis mangiferae</i> OH-02	na	na	na	na	na
<i>Rhizoctonia solani</i> OSE-73	na	na	2000	na	na

EtOAc= ethyl acetate. AcN= acetonitrile. MeOH = methanol. Na: no active with. MIC > 2000  $\mu\text{g/mL}$



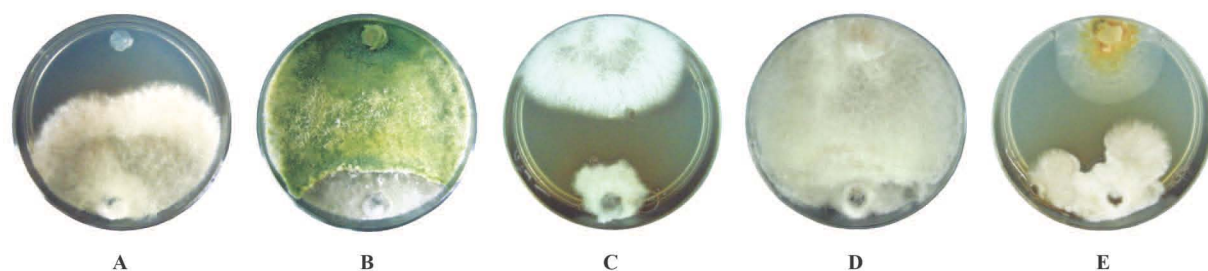


Figure 2. Growth inhibition of phytopathogenic *Curvularia* sp. confronted with: A. Negative control, B. *Fusarium solani* OE-80, C. *Pseudorobillarda sojae*. OSN-36, D. *Rhizoctonia solani* OSE-73, and E. *Hypocrea lixii* OSN-37.

1993). About organic extract and fractions of this fungus, only the acetonitrile fraction was able to inhibit the growth of *C. gloeosporioides*. This fact indicated the diffusion of medium polarity and low molecular weight compounds into the medium, as one of its strategies against fungal pathogens. In the literature, only one report was found dealing with metabolic fingerprinting studies of sclerotial extracts from *R. solani* (Aliferis and Jabaji, 2010).

In contrast, the *P. mangiferae* OH-02 isolate produced a notable inhibition zone without contact with the mycelium surrounding of *C. cassiicola*, *Curvularia* sp. and *Fusarium* sp. This would suggest an antibiosis phenomenon in which could be involved enzyme secretion, release of volatile compounds or diffusion of metabolites into the medium, inhibiting the development of other fungi. *Pestalotiopsis mangiferae* occur on multiple hosts (Yang *et al.*, 2012) and more recently, Subban *et al.* (2013) have reported a novel compound with antibacterial and antiyeast effect, named 4-(2,4,7-trioxa-bicyclo[4.1.0.]heptan-3-yl) phenol. As expected, the organic extract and fractions of *P. mangiferae* OH-02 showed no effect against *C. gloeosporioides*.

Finally, a correlation analysis was carried out between mycelial growth of saprophytic strains (cm) and the percentage of MGI obtained from each of the fungal phytopathogen studied in dual culture, on the sixth day of cultivation. With the exception of *H. lixii* OSN-37, results showed lower correlation in the interactions. This suggests

that the inhibitory effect is not due to direct contact among antagonists and phytopathogen mycelia, or competition for substrate or nutrients. In particular, this mechanism is presented by the *P. sojae* OSN-36 and *F. solani* OE-80 strains only against this pathogen (Figures 1 and 2).

In conclusion, *H. lixii* OSN-37, *P. mangiferae* OH-02 and *R. solani* OSE-73 are the most promising antagonistic saprophytic strains isolated in this work. These strains represent alternatives to control fungal diseases in tropical regions. Furthermore, *H. lixii* OSN-37 and *R. solani* OSE-73 produced metabolites effective against *C. gloeosporioides*. These saprophytic native fungal strains require further research to determine their active principles, mechanisms of action and to establish formulations that will allow the application of fungal extracts at greenhouse and field levels in the near future to protect crops in the tropical regions.

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