

# Bioprospecting of fungi with antiproliferative activity from the mangrove sediment of the Tampamachoco coastal lagoon, Veracruz, Mexico

## Bioprospección de hongos con actividad antiproliferativa provenientes del sedimento del manglar de la Laguna de Tampamachoco, Veracruz, México

Homero Lumbreras-Martínez<sup>1</sup>, César Espinoza<sup>2</sup>, José J. Fernández<sup>3</sup>, Manuel Norte<sup>3</sup>, Irene Lagunes<sup>2,3</sup>, José M. Padrón<sup>3</sup>, Jorge López-Portillo<sup>4</sup>, Ángel Trigos<sup>2</sup>

<sup>1</sup> Doctorado en Ciencias en Ecología y Biotecnología, Instituto de Biotecnología y Ecología Aplicada (INBIOTECA), Universidad Veracruzana. Xalapa, Veracruz, México.

<sup>2</sup> Laboratorio de Alta Tecnología de Xalapa, Universidad Veracruzana. Calle Médicos 5, Col. Unidad del Bosque, 91010, Xalapa, Veracruz, México.

<sup>3</sup> Instituto Universitario de Bio-Orgánica "Antonio González", Universidad de La Laguna. Avenida Astrofísico Francisco Sánchez 2, 38206, Tenerife, Spain.

<sup>4</sup> Red de Ecología Funcional, Instituto de Ecología A. C. Apartado Postal 63, 91070, Xalapa, Veracruz, México.

### RESUMEN

**Antecedentes:** Los hongos de los bosques de manglar representan un gran potencial para la bioprospección de nuevos metabolitos bioactivos. La rizósfera de varias regiones del mundo ha sido ampliamente estudiada, no obstante, no existen estudios sobre la biodiversidad de los manglares mexicanos.

**Objetivos:** Evaluar la actividad anti-proliferativa de extractos de hongos aislados de sedimentos acumulados en la rizósfera de tres especies de mangles distribuidos en tres zonas con diferentes grados de salinidad en México.

**Métodos:** Los hongos aislados se identificaron a nivel de género, mediante claves taxonómicas. Se determinó la actividad anti-proliferativa mediante el ensayo SRB y se identificaron molecularmente las cepas con valores de  $GI_{50} < 100 \mu\text{g.mL}^{-1}$ .

**Resultados y conclusiones:** Seis extractos de cuatro cepas presentaron valores de  $GI_{50} < 100 \mu\text{g.mL}^{-1}$  en al menos una línea celular cancerosa. Una cepa de *Aspergillus pulverulentus* aislada de una zona perturbada de *Avicennia germinans* mostró la mayor actividad anti-proliferativa con seis líneas celulares de cáncer diferentes. Los valores de  $GI_{50}$  variaron de 40 a 83  $\mu\text{g.mL}^{-1}$  en extractos de biomasa y de 6 a 28  $\mu\text{g.mL}^{-1}$  en extractos de caldo de cultivo. Este trabajo es el primer estudio de bioprospección de hongos que muestran actividad anti-proliferativa en sedimentos de manglar en México.

**Palabras clave:** bioprospección, hongos microscópicos, suelo de manglar, niveles de salinidad, efecto anti-proliferativo.

### ABSTRACT

**Background:** Fungi from mangrove forests represent a great potential for the bioprospecting of new bioactive metabolites. The rhizosphere of several regions of the world has been widely studied, however, there are no studies on the biodiversity of Mexican mangroves.

**Objectives:** To evaluate the anti-proliferative activity of fungal extracts isolated from accumulated sediments in the rhizosphere of three mangrove species distributed in three zones with different degrees of salinity in Mexico.

**Methods:** The isolated fungi were identified at genus level by taxonomic keys. The anti-proliferative activity was determined by the SRB assay and the strains with values of  $GI_{50} < 100 \mu\text{g.mL}^{-1}$  were identified molecularly.

**Results and conclusions:** Six extracts from four strains exhibited  $GI_{50}$  values  $< 100 \mu\text{g.mL}^{-1}$  in at least one cancer cell line. A strain of *Aspergillus pulverulentus* isolated from a disturbed area of *Avicennia germinans* showed the highest anti-proliferative activity against six different cancer cell lines. The  $GI_{50}$  values ranged from 40 to 83  $\mu\text{g.mL}^{-1}$  in biomass extracts and 6 to 28  $\mu\text{g.mL}^{-1}$  in culture broth extracts. This paper is the first study of bioprospecting fungi showing anti-proliferative activity in mangrove sediments in Mexico.

**Keywords:** bioprospecting, microscopic fungi, mangrove soils, salinity levels, anti-proliferative effect.

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### CORRESPONDING AUTHOR

✉ Ángel Trigos, atrigos@uv.mx

ORCID: 0000-001-6112-2288

## INTRODUCTION

Among marine environments, mangroves ecosystems are a habitat of a great diversity of microorganisms (Kathiresan & Bingham, 2001; Kathiresan *et al.*, 2013), and bioactive metabolites from mangrove plants (Bandaranayake, 2002) and microscopic fungi (Couttolenc *et al.*, 2016; Thatoi *et al.*, 2013) have been reported, representing a great potential for the development of future drugs. In this respect, pharmacological interest from fungal sources started when siccayne was isolated from the marine basidiomycete *Halocyphina villosa* (Kupka *et al.*, 1981). Since then, the number of new reported bioactive compounds of mangrove-related fungi has increased steadily (Jones, 2011; Debbab *et al.*, 2013). However, most of these bioactive fungal compounds come primarily from Asia (Huang *et al.*, 2008; Debbab *et al.*, 2012; Wang *et al.*, 2013). Many of these substances have been obtained mainly from endophytic fungi found on roots, leaves and branches, and from soil fungi (Sosa-Rodríguez *et al.*, 2009).

The most sought after metabolites are those with potential anti-proliferative activity, since cancer has become a major health problem because of its high mortality rate (Rizo-Ríos *et al.*, 2007). Mexico has a great untapped potential in this field with approximately 890,000 ha of mangrove forest (CONABIO, 2009). In this work, we report the first bioprospecting study of fungi from soil of the rhizosphere in three species of mangrove from Tampamachoco Lagoon Tuxpan-Veracruz, Mexico. As a model for biological activity we selected a panel of representative human solid tumor cell lines.

## MATERIALS AND METHODS

### Collection area and biological material

Fungal strains were isolated from rhizosphere sediment collected from the mangrove forest at the Tampamachoco lagoon, Tuxpan, Veracruz, Mexico (21° 0.9' N, 97° 47' W). This mangrove forest has been affected in some areas by high salinity concentration due to the construction of levees that blocked of fresh water flow from the Tuxpan River, resulting in areas ranging from 35 ‰ (preserved area), to 65 ‰ (semi-preserved, chronically affected zone) and up 140 ‰ (massive mangrove death; Vovides *et al.*, 2011). Therefore, five replicate samples were collected from the rhizosphere with a hand auger at 10-15 cm depth and at 30 m intervals among samples. We

collected a complete set (15 samples per species) from *Rhizophora mangle* L. (red mangrove), *Avicennia germinans* L. (black mangrove) and *Laguncularia racemosa* (L.) CF Gaertn (white mangrove). Each undrained sample was kept in sterile polyethylene bags and sealing them for transport inside a cooler. Once in the laboratory, the bags were stored at 4 °C for no more than five days.

### Isolation of fungal strains

Ten grams from each sample were diluted in a sterile solution of 0.1 % peptone and 0.4 % NaCl. From this initial stock, three successive dilutions were made to reach a concentration of 10<sup>-3</sup>. Afterwards, one mL of each dilution was seeded in duplicate in Petri dishes containing marine agar (BD, Difco™ Marine Agar 2216) and malt extract (BD, Difco™ Malt Extract Broth) prepared according to the manufacturer's instructions with 0.2 g.L<sup>-1</sup> of chloramphenicol and incubated at 25 ± 2 °C until the development of fungal colonies was observed. The fungal colonies were then individually purified by transferring mycelium to new Petri dishes with the same medium, until pure cultures were obtained.

### Fermentation and preparation of extracts

Fermentation of 500 mL from each of the pure cultures was performed using Wickerham 's medium (Kjer *et al.*, 2010) and then transferred to five 100 mL Erlenmeyer flasks. This medium was incubated in a dark room at 25 ± 2 °C, first at 150 rpm for 7 days and later for 7 days in a static mode. After the incubation stage, the biomass and culture broths were separated by vacuum filtration. Both fractions were lyophilized and the generated residues were extracted with a Chloroform-Methanol (1:1) mix. Subsequently, the extracts were concentrated to dryness using vacuum evaporation. The extracts from each fungal strain thus obtained were then tested for their anti-proliferative activity.

### Cell lines and culture

The human solid tumor cell lines A-549, HBL-100, HeLa, SW1573, T-47D and WiDr, donated by Prof. G. J. Peters (VU Medical Center, Amsterdam, The Netherlands), were used in this study. The cells were maintained in 25 cm<sup>2</sup> culture flasks in RPMI 1640 supplemented with 5 % heat-inactivated fetal calf serum and 2 mM L-glutamine in an incubator at 37 °C, 5 % CO<sub>2</sub> and 95 % air humidity. Exponentially growing cells were trypsinized and re-suspended in an antibiotic-containing medium

(100 units penicillin G and 0.1 mg of streptomycin per mL). Single cell suspensions were counted using an Orflow's MoxiZ automated cell counter (Ketchum, ID) and dilutions were made to give the appropriate cell densities for the inoculation onto 96-well microtiter plates. Based on their doubling times, cells were inoculated in 100  $\mu$ L per well at 10 000 (A-549, HBL-100, HeLa and SW1573), 15 000 (T-47D), and 20 000 (WiDr) cells per well.

#### Anti-proliferative activity

Dry extracts were initially dissolved in DMSO at 400 times the desired final maximum test concentration, i.e. 10 mg.mL<sup>-1</sup> and diluted in the culture media until they reached an assay concentration of 250  $\mu$ g.mL<sup>-1</sup>. Control cells were exposed to an equivalent concentration of DMSO but with no extract (0.25% v/v, negative control). The extract (i.e. drug) treatment began on the first day after plating. The extracts were incubated for 48 h and after that cells were precipitated with 25  $\mu$ L ice-cold TCA (50 % w/v) and fixed for 60 min at 4 °C. Then the SRB assay was performed (Skehan *et al.*, 1990) sensitive, and inexpensive method for measuring the cellular protein content of adherent and suspension cultures in 96-well microtiter plates. The method is suitable for ordinary laboratory purposes and for very large-scale applications, such as the National Cancer Institute's disease-oriented in vitro anticancer-drug discovery screen, which requires the use of several million culture wells per year. Cultures fixed with trichloroacetic acid were stained for 30 minutes with 0.4% (wt/vol.) The optical density (OD) of each well was measured at 492 nm using BioTek's PowerWave XS Absorbance Microplate Reader (Winooski, VT). The percentage growth was calculated as the OD difference between the start and end of each treatment level corrected for background OD of the control wells and compared with untreated control cells. The results were expressed as the concentration of extract causing 50 % reduction in the proliferation of cancer cells (GI<sub>50</sub>). We selected the most bioactive extracts, with GI<sub>50</sub> values less than 100  $\mu$ g.mL<sup>-1</sup>.

#### Strain identification

The identification of the strains was carried out by the observation of reproductive fungi structures under the microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and employing taxonomic keys (Barnett and Hunter, 1998; Watanabe, 2002). The mycelial cultures

were observed after seven days of incubation at 25  $\pm$  2 °C employing lacto-phenol as a staining medium.

#### Molecular identification

The identity of the fungal strains that showed anti-proliferative activity was confirmed by obtaining ITS-rDNA nuclear sequence data. Genomic DNA was isolated following the protocol proposed by Yu *et al.* (2011), and the nuclear rDNA-ITS regions were amplified using ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3', TM = 55° C) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3', TM = 53° C) primers by direct PCR technique. The PCR products were purified with a Wizard kit (Pro-Mega®, Madison, WI, USA) following the manufacturer's instructions, and were then sequenced at the Institute of Biotechnology, National Autonomous University of Mexico (UNAM) using an Applied Biosystems (model 391) DNA sequencer.

The identity of the species was confirmed using the search tool Blast in the NCBI GenBank. For phylogenetic analysis, the sequences were aligned using the ClustalW algorithm of MegAlign from MEGA6 software (Tamura *et al.*, 2013) which currently contains facilities for building sequence alignments, inferring phylogenetic histories, and conducting molecular evolutionary analysis. In version 6.0, MEGA now enables the inference of timetrees, as it implements the RelTime method for estimating divergence times for all branching points in a phylogeny. A new Timetree Wizard in MEGA6 facilitates this timetree inference by providing a graphical user interface (GUI). The phylogenetic tree was constructed using the Maximum Likelihood algorithm (K2+G) of the MEGA6 software. The bootstrap analysis was performed with 1000 replications.

## RESULTS

#### Sampling, isolation and identification of fungi

From soil samples collected from mangrove at the Tampamachoco lagoon, 32 fungal strains were isolated, of which 15 were isolated from *Rhizophora mangle*, 10 strains were obtained from *Avicennia germinans*, and 7 strains from *Laguncularia racemosa*. Regarding the degree of salinity of the mangroves, 12 strains were obtained from the area with 35 %, 15 from the area with 65 %, and 5 from the area with 140 %.

The microscopic preparations of reproductive structures were compared with those reported by Barnett

and Hunter (1998) and Watanabe (2002). We identified 16 possible genera according to their macroscopic and microscopic characteristics: *Fusarium*, *Alternaria*, *Phytophthora*, *Trichoderma*, *Penicillium*, *Aspergillus*, *Sepedonium*, *Mucor*, *Acremonium*, *Nectria*, *Monacrosporium*, *Geotrichum*, *Phomopsis*, *Blastomyces*, *Humicola* and *Talaromyces* (Table 1).

Four extracts with anti-proliferative activity were assigned to the genus *Aspergillus* and two to the genus *Fusarium*. The morphological characteristics observed for the genus *Aspergillus* were fast growing cottony white mycelium, with production of black spores from the center to the edges, and microscopically we identified the characteristic aspergilar head composed of a conidiophore with termination in a vesicle covered with phialides, in which the conidia are formed. *Fusarium* genus was characterized by its purple mycelium, short phialides, conidia added in false heads and abundant microconidia.

Of the isolated strains, 22 % were from the genus *Aspergillus*, 22 % were from the genus *Fusarium*, 13 % from genus *Penicillium*, 6 % from the genus *Acremonium*, and the remaining genera accounted for 37 %.

### Anti-proliferative activity

Of the 32 fungal isolates we obtained from the rhizosphere, 64 extracts (broth and biomass per strain) were prepared and assayed against human cancer cell lines. The chemosensitivity assay indicated that six extracts showed 50% growth inhibition ( $GI_{50}$ ) to one or more cancer cell lines at concentrations  $<100 \mu\text{g}\cdot\text{mL}^{-1}$  (Table 2). The bioactive fungi were identified as *Aspergillus niger* (RmS2-2a), *A. tubingensis* (AgC1-2), *A. pulverulentus* (AgS4), and *Fusarium oxysporum* (RmS4-3), which were isolated from the rhizosphere of *Avicennia germinans* and *Rhizophora mangle*. The active extracts consisted of three biomass extracts and three culture broths. In broth extracts,  $GI_{50}$  values were  $<50 \mu\text{g}\cdot\text{mL}^{-1}$  in all tested cell lines. In contrast, biomass extracts displayed activity below this threshold only in HeLa cells.  $GI_{50}$  values obtained ranged from 40 to  $180 \mu\text{g}\cdot\text{mL}^{-1}$  in the biomass extracts and from 6 to  $41 \mu\text{g}\cdot\text{mL}^{-1}$  in the culture broth (Table 2).

### Molecular identification

The isolates RmS4-3, AgS4, AgC1-2 and RmS2-2a were identified morphologically and as belonging to

TABLE 1. Fungal strains isolated from the rhizosphere sediment from three mangrove species in three site types: 35, 65 and 140% of salinity, at the Tampamachoco lagoon, Veracruz, Mexico

GENUS	NUMBER OF ISOLATED STRAINS								
	<i>Rhizophora mangle</i>			<i>Avicennia germinans</i>			<i>Laguncularia racemosa</i>		
	35	65	140	35	65	140	35	65	
<i>Fusarium</i>	2	3			1				1
<i>Acremonium</i>						1	1		
<i>Trichoderma</i>	1								
<i>Sepedonium</i>		1							
<i>Nectria</i>				1					
<i>Phomopsis</i>			1						
<i>Humicola</i>					1				
<i>Aspergillus</i>		1		1	1		2		2
<i>Penicillium</i>		2			1	1			
<i>Talaromyces</i>							1		
<i>Geotrichum</i>			1						
<i>Blastomyces</i>					1				
<i>Alternaria</i>	1								
<i>Monacrosporium</i>				1					
<i>Phytophthora</i>	1								
<i>Mucor</i>			1						

TABLE 2. Antiproliferative activity of extracts of fungal strains obtained from the rhizosphere of mangrove species at the Tampamachoco lagoon, Veracruz, Mexico.

FUNGAL ISOLATED (STRAIN NUMBER)	RHIZOSPHERE SOURCE	EXTRACT	CANCER CELL LINES					
			A-549 (LUNG)	HBL-100 (BREAST)	SW1573 (LUNG)	HELA (CERVIX)	T-47D (BREAST)	WiDR (COLON)
<i>Aspergillus niger</i> (RmS2-2a)	<i>Rhizophora mangle</i>	Biomass	66	42	58	41	80	75
<i>Aspergillus pulverulentus</i> (AgS4)	<i>Avicennia germinans</i>	Biomass	83	52	64	42	69	79
		Broth	25	6	14	12	28	28
<i>Aspergillus tubingensis</i> (AgC1-2)	<i>Avicennia germinans</i>	Broth	41	34	30	37	41	33
<i>Fusarium oxysporum</i> (RmS4-3)	<i>Rhizophora mangle</i>	Biomass	74	54	73	40	152	180
		Broth	40	29	34	37	40	24

Values are expressed as  $GI_{50}$  ( $\mu\text{g mL}^{-1}$ )

*Fusarium* and *Aspergillus* in the case of the last three strains accordingly. A phylogenetic analysis of the four isolates is shown in the phylogenetic tree (Figure 1). Isolate RmS4-3 had a 99 % similarity with the *Fusarium oxysporum* Schltldl (Nectriaceae) ITS sequences deposited in the GeneBank and was located in the *F. oxysporum* clade (Figure 1), clearly separated from a *Gibberella intermediate* and *G. moniliformis*, which is a synonymy of *Fusarium verticillioides* (Sacc.) Nirenberg. Isolate RmS2-2a shared a sequence similarity of 99 % with *Aspergillus niger* Tiegh (Aspergillaceae), corresponded to the *A. niger* clade (Figure 1). Isolate AgS4 presented a 99 % similarity with *Aspergillus pulverulentus* (McAlpine) Wehmer, Bot (Aspergillaceae), corresponded to the *A. pulverulentus* clade (Figure 1). This species is considered synonymous with *A. niger* var. *pulverulentus* (McAlpine) Kozak. Isolate AgC1-2 shared a 100 % similarity with *A. tubingensis* Mosseray (Aspergillaceae), corresponded to the *A. tubingensis* clade (Figure 1). This last species is considered synonymous with *A. niger* var. *tubingensis* (Mosseray) Kozak. Both isolates were obtained from the rhizosphere soil supporting *A. germinans* in the semi-preserved and preserved zones respectively. Therefore, we conclude that AgS4 and AgC1-2 belong to complex strains of the *A. niger* species.

## DISCUSSION

There are few studies on fungi bioactivity, thus it is difficult to compare our results concerning the level of disturbance in mangroves, therefore it is believed we have set a precedent in this regard.

Several reports in the literature indicate that genera such as *Fusarium*, *Aspergillus*, *Alternaria*, *Penicillium*, *Talaromyces* and *Phomopsis* isolated from mangrove produce bioactive metabolites that inhibit the growth of different cancer cell lines (Debbab et al., 2013; Gao et al., 2013; Wang et al., 2013; Nicoletti et al., 2018). We managed to isolate and identify these genera, although in the anti-proliferative tests only *Aspergillus* and *Fusarium* showed values similar or better than those reported in the literature, for example, at extracts level, *Aspergillus*, has shown  $GI_{50}$  values above  $150 \mu\text{g.mL}^{-1}$  (Abreu et al., 2015), which differs with the values shown by our extracts, which were below  $50 \mu\text{g.mL}^{-1}$  in at least one cancer cell line. In addition and in agreement with Monks et al. (1991), who suggest a cut-off value of  $50 \mu\text{g.mL}^{-1}$  to consider an extract as suitable for the search of the metabolites responsible for its bioactivity, the six extracts reported here comply with this parameter.

On the other hand, extracts from *Fusarium oxysporum* strains isolated from marine environments, have been shown to possess strong anti-proliferative activity against various cancer cell lines, with  $GI_{50}$  values  $<50 \mu\text{g.mL}^{-1}$  (Couttolenc et al., 2016). The bioactivity of *F. oxysporum* was again confirmed in our study, since a strain of this species was among the most bioactive, against the same cell lines used by Couttolenc et al. (2016); additionally, a strain of *F. oxysporum* was isolated from *Rhizophora annamalayana*, from which Taxol was obtained (Elavarasi et al., 2012), which is used as a treatment for several types of cancer, this compound could be responsible for the anti-proliferative activity of our strain.

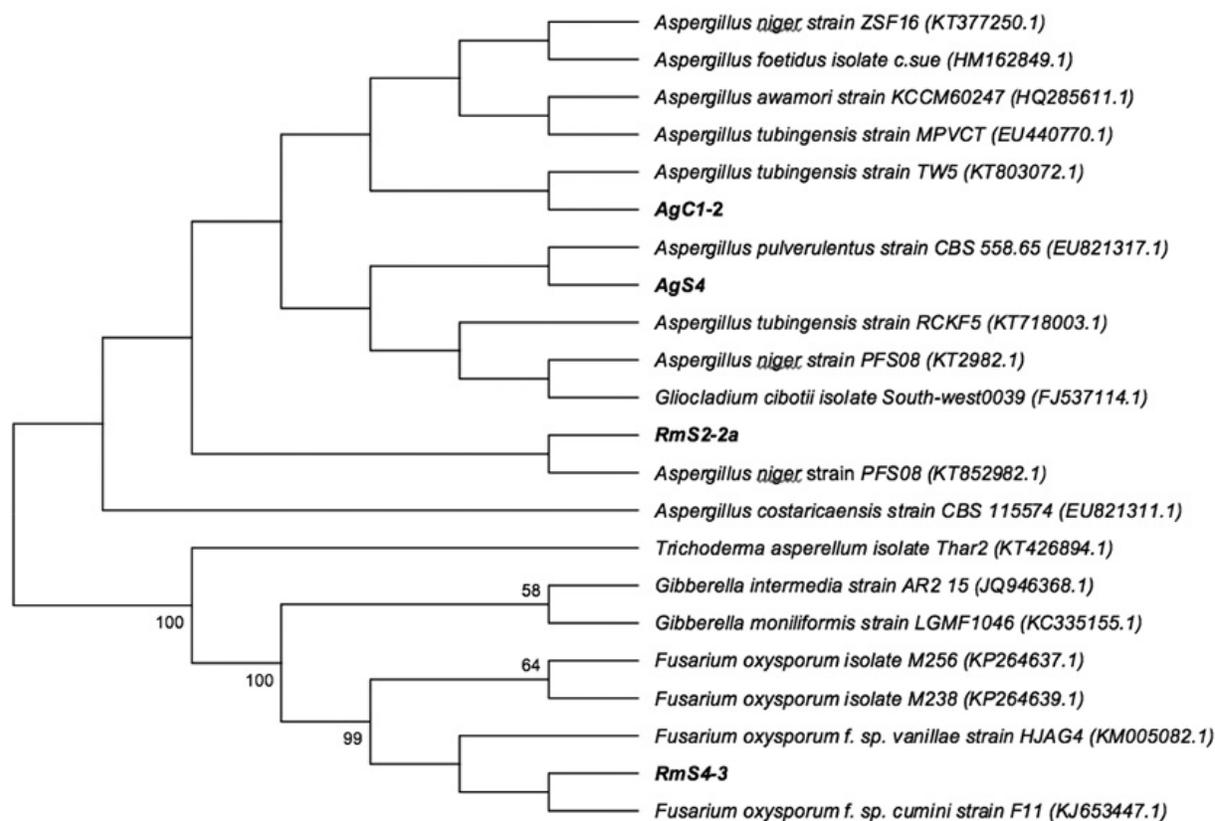


FIGURE 1. Maximum-likelihood phylogenetic tree based on ITS sequences of the fungi extracted from the rhizosphere sediment and reference strains. Numbers at the nodes are bootstrap support values for 1000 replicates.

The genus *Aspergillus* is characterized by producing several bioactive metabolites, which are generated when there is nutrient deficiency in the medium, when the growth of the fungus ends or when the conditions of the environment are not favorable (Diaz, 1996), these conditions are present in the semipreserved site of our study area, from which three strains with strong anti-proliferative activity were isolated. Thus, the anti-proliferative activity of various *Aspergillus* strains isolated from mangroves has been reported in several studies (Cai et al., 2011).

Among the bioactive metabolites of endophytic *Aspergillus* strains of mangrove species, we can mention the compounds called rubasperone D, TMC 256 A1, rubrofusarin B and flavasperone isolated from *A. tubingensis* (GX1-5E) endophytic of *Pongamia pinnata*, and which showed activity antitumor against several types of cell lines (Huang et al., 2011). On the other hand, from the strain of *A. niger* MA-132 endophytic of *Avicennia*

*marina*, the compounds called Nigerasterols A and B were obtained, which showed a potent activity against the tumor cell lines HL60 and A549 (Liu et al., 2013). As in *F. oxysporum*, these compounds could be responsible for the anti-proliferative activity of our strains.

The presence of strains with higher bioactivity in the area with 65 % of the salinity (semi-preserved area) may be a result of the fact that this area is in the middle part of the salinity concentration scales and suggests a great capacity of the species to respond to the changing conditions, producing and accumulating different metabolites that may be associated with the regulation of internal osmotic pressure (Ramírez et al., 2006).

In summary, a series of fungal isolates from the rhizosphere of *Rhizophora mangle* and *Avicennia germinans* present at the mangrove forest of the Tampamachoco lagoon were studied for their anti-proliferative activity against human solid tumor cell lines. The results sug-

gest a higher metabolic activity of fungal strains isolated from semipreserved (65 %) and preserved (35 %) areas of said mangrove. To the best of our knowledge, this work represents the first bioprospecting study of mangrove fungi in Mexico. The results justify the search for bioactive fungal strains in other mangrove sites as well as to support the conservation of these ecosystems. Based on the anti-proliferative effects, the reported *Aspergillus* and *Fusarium* strains are suggested as good candidates for further analysis of its metabolites, without leaving aside the rest of the strains isolated for other possible bioassays.

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