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Full Length Research Paper

Antimicrobial and nematicidal screening of anamorphic fungi isolated from plant debris of tropical areas in Mexico

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Forty seven fungal strains were isolated from plant debris in the tropical regions of Mexico, where fifteen of them were identified to species and twenty two to genus level. All isolates were grown in fermented rice and their EtOAc extracts screened against ten targets, four bacteria (*Bacillus subtilis*, *Erwinia carotovora, Staphylococcus aureus* and *Xanthomonas campestris*), the yeast *Candida albicans*, three phytopathogenic fungi (*Alternaria tagetica*, *Colletotrichum gloeosporioides* and *Fusarium oxysporum*), the Oomycete *Pythium aphanidermatum* and the nematode *Meloidogyne incognita*. Antimicrobial activity was detected in 18 isolates against at least one of the target strains tested. Seven of these isolates with broad spectrum activity, which were defatted and their minimum inhibitory concentrations (MICs) were determined by microdilution assay. The greatest antagonistic action was produced by *Cylindrium elongatum* with broad spectrum activity while *Corynespora cassiicola* and *Memnoniella* sp. MR33 showed moderate antimicrobial properties. On other hand, *in vitro* nematotoxic activity was clearly detected only in *Selenosporella* sp. GH26 with 91 (LD_{50}) and 147 µg/ml (LD_{90}). This is the first report on the isolation and biological evaluation of anamorphic fungi from some Mexican tropical regions, demonstrating their potential as a source of biologically active natural metabolites for use in future applications in agriculture or pharmacy.

Key words: Anamorphic fungi, antimicrobial, *Corynespora, Cylindrium,* fungal extracts, nematicide, *Selenosporella*, Yucatan peninsula.

INTRODUCTION

Eight decades after the discovery of penicillin, the intense efforts to find new and more effective antimicrobial agents continue. One of the main causes is attributed to the misuse of antibiotics which has led to the development of resistance in pathogens (Demain and Sanchez, 2009). This situation is similar with pest such as insects, protozoa, and nematodes both in agriculture and medicine (Daam and Van den Brink, 2010; Dayan et al., 2009). Faced with this urgent need, different strategies are being used where microbial products continue to be a major source of new natural models to study. In particular, fungi have been recognized as a very important resource of secondary metabolites with a tremendous structural diversity and useful activities for agricultural and pharmaceutical purposes (Misiek and Hoffmeister, 2007; Tulp and Bohlin, 2004). However, most of the fungal metabolites have been obtained

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mainly from macro and micro-fungi isolated in temperate regions (Bills et al., 2002). Although tropical ecosystems are recognized for their high biodiversity, there are relatively few explorations dealing with their chemical characterization and as a consequence, their ability to produce important novel compounds remains unknown (Bills et al., 2002; Gloer, 2007). During the last 10 year several papers were published about active metabolites produced by fungi from tropical countries as Costa Rica, Venezuela, Canary Islands (Spain), China and so on (Castillo et al., 2000; Badhury et al., 2006; Dong, et al., 2009; Ayers et al., 2010). The biotechnological potential of tropical fungi is evident with some remarkable examples such as apicidine, an antiprotozoary metabolite isolated from a strain of *Fusarium pallidoroseum* collected in Costa Rica (Park et al., 1999); and flutimide from *Delitschia confertospora*, isolated from dung in Namibia, which is able to inhibit influenza A virus transcription (Tomassini et al., 1996).

With approximately 2, 600 genera and 15, 000 species, anamorphic fungi represent a rich biota resource to be explored (Cannon and Sutton, 2004). In Mexico, there are very few reports regarding the biological profile of the native tropical anamorphic fungi (Reyes-Estebanez et al., 2008; Torres-Barragán et al., 2004; Trigos et al., 2005). With these antecedents, the purpose of our work was to isolate anamorphic fungi from unexplored tropical regions in the south-east of Mexico in order to create a fungal collection and determine their antimicrobial and nematicidal activities. A set of ten pathogenic microorganisms considered recurrent diseases of medical or agricultural importance were selected for this study. These include polyphagous nematode *Meloidogyne incognita*; the species ranked as the most pathogenic and evolved species of the genera, which affects a large number of agricultural crops (Cid del Prado-Vera et al., 2001). This study represents a contribution to the search for natural alternatives to control pathogens in agriculture and pharmacy.

MATERIALS AND METHODS

Fungal strains

Isolation and identification

Fungi were isolated from plant debris collected in the following locations in the Mexican south-east; (a) La Venta National Park, Villahermosa, Tabasco; (b) Rancho Guadalupe, Xalapa, Veracruz; (c) Dzibilchaltun Eco-Archeological Park, Merida, Yucatan; and (d) Xiitbal Neek Botanical Garden of the Centro de Investigación Científica de Yucatán, A.C. (CICY), Mérida, Yucatán. Plant debris was incubated in damp chambers at room temperature to induce sporulation (Krug, 2004). After five days, fungal structures were isolated and cultured in potato dextrose agar (PDA) plates. All strains were incubated at 25°C, with light-dark photoperiod (12/12 h) until they covered the plate surface. Fungi were identified according to their morphological characteristics using taxonomic keys (Barnett and Hunter, 1972; Carmichael et al., 1980), and/or by molecular taxonomy.

Molecular identification

Genomic DNA (gDNA) was obtained according to Johanson and Jeger (1993). Universal ITS1 (on 18S rDNA) and ITS4 (on 28S rDNA) primers (White et al., 1990) were used to amplify from ITS1 to ITS2. PCR products were analyzed by gel electrophoresis and the DNA bands were carefully cut with a razor blade and purified with the High Pure PCR Product Purification Kit (Roche Applied Science). Samples were sequenced in a Davis sequencing facility (Davis, CA, http://www.davissequencing.com) using 100 ng of PCR product and ITS1 primer.

Culture conditions and fungal extraction

Rice (20 g) was fermented with distilled water overnight (30 ml), sterilized at 121°C for 30 min and inoculated with hyphal fragments-spore suspension (2 ml) of each strain. Two flasks per strain were inoculated and maintained at 25°C, using a light-dark photoperiod (12/12 h). After 40 days, cultures were fragmented and subsequently extracted three times using ethyl acetate (50 ml each time). The solvent was evaporated under vacuum obtaining the fungal crude extract (Soman et al., 2001). The active fungal extracts were dissolved in acetonitrile and defatted with hexane (3 x 2:1, 1:1, 1:1, v/v) resulting in two fractions (acetonitrile and hexanic) which were then evaluated in microdilution assays and gas chromatography-mass spectroscopy (GC-MS) analyses.

Chemical profile

GC-MS analyses were performed on an Agilent Technologies 6890N chromatograph [0.4 µl of sample at 2%, HP DB-5MS column [(5% phenyl)-methyl polisiloxane, 30 m long, 0.32 mm i.d., 0.5 µm film thickness], helium at flow rate = 1.2 ml/min, T_1 = 140°C, T_2 = 300°C, gradient = 8°C/min] coupled to an Agilent Technologies 5975B mass selective detector. The majority of components remained unidentified due to the lack of library spectra for the corresponding compounds.

Bioassays

Target strains: All fungal species were tested against ten target strains which included four bacteria: *Bacillus subtillis* (ATCC 6633), *Erwinia carotovora* subp*. carotovorum* (ATCC 138), *Staphylococcus aureus* (ATCC 6536), and *Xanthomonas campestris* pathovar. *carotae* (ATCC 10547); a yeast *Candida albicans* (ATCC 10231); three fungal phytopathogens: *Alternaria tagetica* (ATCC 58771), *Colletotrichum gloeosporioides* (CICY002), *Fusarium oxysporum* (CICY003), the Oomycete *Pythium aphanidermatum* (CICY006), and the nematode *Meloidogyne incognita* (ITCN001).

Antimicrobial activity assay: Antimicrobial activity was determined by the disk diffusion assay (Bauer et al., 1966). Tripticasein soy agar (TSA) plates were inoculated with bacteria and yeast target strains (final concentration 1.5 x 10^8 cfu/ml) and 700 µl of pathogenic fungi spore suspension (8 x 10^5 spores/ml) were applied to PDA plates (Petrikkou et al., 2001). Subsequently, each fungal extract (500 µg) was dissolved with acetone (1%), impregnated onto a paper filter disk (6 mm in diameter) and placed on the inoculated agar surfaces. Amykacin (5 µg) was used as positive control for bacteria, Neomicol (1 µg) for *C. albicans*, Alliette (3 µg) for *P. aphanidermatum* and Ricoil (25 µg) for the other phytopathogens. The plates were incubated for 24 h at 25 and 37°C for phytopathogenic and zoopathogenic bacteria, respectively; 48 h at 37°C for yeast; and 72 h at 25°C, for fungal phytopathogens (12/12 h light-dark). All tests were performed in triplicate and the antagonism was expressed by an inhibition zone around the disk.

Broth microdilution assay: The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the active defatted fractions were determined as previously described (Andrews, 2001). Activated bacteria were inoculated on Müeller-Hinton broth (MHB) and incubated for 24 h at 37°C (*B. subtilis* and *S. aureus*), at 25°C (*E. carotovora* and *X. campestris*) and at 37°C *C. albicans*. The cultures were adjusted to 0.5 McFarland standards to approximately 1 x 10^8 cfu/ml. Dilutions of the fractions were dissolved in 5% dimethyl sulfoxide (DMSO) to 400, 200, 100 and 50 µg/ml and added to MHB. Tests were carried out in duplicate, using indicated positive and negative controls. After 24 h, 50 µl of 1% triphenyl tetrazolium chloride (TTC) were added to each well, positive test was detected as colorless (dead bacteria) and negative as red color (bacterial growth). The MIC was the lowest concentration preventing visible growth. The MBC was considered to be the complete absence of bacterial growth. To confirm the results of MBC, 1 µl was sub-cultured on Müeller-Hinton agar (MHA) and incubated for 24 h at the temperature previously indicated (Taylor et al., 1983). Each test was performed in triplicate

Nematotoxic assay: The nematode inoculum was prepared as previously described (Cristobal-Alejo et al., 2006). All crude extracts (300 µg/ml) were dissolved in tween 20 (0.5%,). Twenty freshly hatched J_2 were placed in suspension, and incubated at room temperature in special dishes for J_2 mortality studies. Vydate (Oxamyl) was used as positive control and negative control included water (100%). Each extract was replicated four times Dose-inhibitory response curves using a dilution series (100, 200, 300, 400 and 500 µg/ml) were prepared for *Selenosporella* sp. extract using approximately one hundred J_2 larvae. Each extract was replicated four times. Dead larvae of *M. incognita* were counted (Cristobal-Alejo et al., 2006) and the percentage of J_2 mortality was obtained by variance analyses, previous transformation with arcosin [y = arcosin (sqrt y/100)] followed by multiple medias comparisons (Tukey p = 0.05) (Steel and Torrie, 1988). Effective concentrations (LD_{50} and LD_{95}) were obtained by transforming to "Probit" and ten-base logarithms the calculated percent mortality of second nematicidal assay (Thorne et al., 1995).

RESULTS

Isolation and identification of fungal strains

Fungal strains were selectively isolated from plant debris collected in the Mexico States of Tabasco (14), Veracruz (6), and Yucatan (27). Among all isolates, 15 were identified at species and 22 at genus level, the other 10 strains were sterile mycelia (Table 1).

Antimicrobial evaluation of the fungal extracts

A total of 47 fungal extracts were tested in the antimicrobial assay, 18 of which (38%) were detected with inhibitory effect against at least one target strain (Table 2). A broad activity spectrum was detected for seven isolates (*Beltraniella japonica, Brunfelsia portoricensis, Beltraniopsis* sp. GH19*, Corynespora cassiicola, Cylindrium elongatum, Memnoniella* sp. MR33 and *Phaeobotrys* sp. MR14) being able to inhibit Gram-positive and Gram-negative bacteria and/or fungal phytopathogens. The active defatted extracts were then tested by broth microdilution assay against *B. subitilis*, *C. albicans, E. carotovora, S. aureus* and *X. campestris* (Table 3). The results of the MICs showed that the defatted extracts of *C. cassiicola* and *C. elongatum* were more active against *S. aureus* (100 µg/ml) and *B. subtilis* (400 and 200 µg/ml, respectively). Both exhibited significant inhibitory effect with MBC at 200 µg/ml, where *C. cassiicola* produced bactericidal and *C. elongatum* bacteriostatic effects. The remainder fractions presented activity at concentrations greater than 100 µg/ml.

Inhibitory action against *C. albicans* was observed in *C. elongatum*, *Memnoniella* sp. and *Phaeobotrys* sp. crude extracts, the lowest MIC (100 µg/ml) corresponded to the second extract. Further, the extracts of *B. portoricensis* and *C. elongatum* were able to affect the mycelial growth of *A. tagetica* and *C. gloeosporioides.* The mycelium growth of phytopathogen *P. aphanidermatum* was limited only by the extract of *C. elongatum* with a good inhibition halo (> 20 mm). In the present contribution, the most resistant pathogen was *F. oxysporum* with none of the extracts being able to inhibit it (Table 2).

Nematotoxic test

Results of the nematotoxic assay (Table 2) of the fungal extracts showed statistical differences (P = 0.01). Only the extract of *Selenosporella* sp.GH26 was able to induce 82% of mortality in *M. incognita* with 300 µg/ml, after 48 h. When this fungal extract was tested at different concentrations (100, 200, 300, 400 and 500 µg/ml) against *M. incognita,* it was possible to determine that the LD_{50} and LD_{90} were 91 and 147 µg/ml, respectively.

DISCUSSION

Our results revealed that 38% of the fungal extracts have the ability to produce inhibitory substances against one or more targets tested. Most of the antimicrobial effect was observed against bacteria with 92% of the fungal extracts, whereas 22% were active against *C. albicans*, 4% against phytopathogenic fungi and only 2% against nematode (Table 2).

The strongest and broadest antimicrobial activity was displayed by the *C. elongatum* strain, against six target evaluated. The *Cylindrium* genus has 34 species documented, but no chemical or biological studies have been reported from them, so far. Furthermore, *B. japonica*, *Beltraniopsis* sp. GH19*, Memnoniella* sp. MR33, and *Phaeobotrys* sp. MR14 showed moderate activity against bacteria and/or fungi. It is interesting to note that the *Memnoniella* sp. MR33 extract displayed high specificity and good MIC (100 µg/ml) against the yeast *C. albicans*, a widespread human opportunist pathogen, with very few safe drugs available to control it (Natarajan et al., 2007). From the 10 species included in

Table 1. Anamorphic fungi isolated from tropical areas in the South-east of Mexico.

Anamorphic Fungi	Substrate	Localities
Beltraniella japonica Matsush., GH18	L	1
Beltraniella portoricensis (F. Stevens) Piroz. and S.D. Patil, MR42	L	2
Beltraniopsis sp. GH19	L	1
Chaetopsina sp. GH20	L	1
Corynespora cassiicola (Berk. and M.A. Curtis) C.T. Wei, MR01	L	3a
Curvicladiella sp. GH29	L	1
Cylindrium elongatum MR45	B	2
Cylindrosympodium sp. MR38	L	3b
Dactylaria sp. MR03	G	3a
Dactylaria sp. MR05	B	3a
Dactylaria sp. MR07	B	3a
Dactylaria sp. GH17	L	1
Geniculisporium sp. MR32	L	3a
Gliocladium sp. MR41	L	2
Gliomastix sp. MR37	L	3b
Helicosporium indicum P.Rag. Rao and D. Rao, MR49	L – B	3a
Helicosporium talbotii Goos, MR48	L – B	3a
Hemibeltrania malaysiana Matsush., GH24	L	1
Kutilakesa sp. MR46	B	2
Memnoniella sp. MR33	B	3a
Perelegamyces parviechinulatus W.B. Kendr. and R.F. Castañeda, GH25	L	1
Periconia sp. MR10	L	3a
Phaeobotrys sp. MR14	L	1
Phaeoisariopsis bataticola (Cif. and Bruner) M.B. Ellis, MR50	B	3b
Phaeoisaria clematidis (Fuckel) S. Hughes, MR53	T	3a
Phialophora verrucosa Medlar, MR54	L	3a
Ramichloridium apiculatum (J.H. Mill., Giddens and A.A. Foster) de Hoog, MR39	L	3b
Redbia sp. MR34	L	3b
Selenodriella fertilis (Piroz. and Hodges) R.F. Castañeda and W.B. Kendr., GH23	L	1
Selenosporella sp. GH26	L	1
Selenosporella sp. MR31	L	3a
Spadicoides sp. MR52	T	3b
Sporidesmium sp. MR40	L – B	3a
Thozetella havanensis R.F. Castañeda, MR43	L	2
Vermiculariopsiella sp. MR06	L	3a
Veronaea coprophila (Subram. and Lodha) M.B. Ellis, MR02	L	3a
Zygosporium sp. GH12	L	1
Unidentified MR09	L	3a
Unidentified GH11	L	1
Unidentified GH15	L	1
Unidentified GH16	L	1
Unidentified MR30	L	3b
Unidentified MR35	L	3b
Unidentified MR44	L – B	2
Unidentified MR47	T	3a
Unidentified MR51	L	3a
Unidentified MR55	L	3a

L: Leaves, G: Grass, B: Branch, T: Trunk, 1. Villahermosa, Tabasco; 2. Xalapa, Veracruz; 3a. CICY, Merida, Yucatan; 3b. Dzibilchaltun, Merida, Yucatan.

Table 2. Anamorphic species with antimicrobial (500 µg/disk assay) and nematotoxic (300 µg/ml) activity against *Meloidogne incognita* J_2 larvae (percentage of mortality after 48 h).

Fungi	*B. s*	*E. c*	*S. a*	*X. c*	*C. a*	*A. t*	*C. g*	*F. o*	*P. a*	*M. i* (%) 48 h*
Beltraniella japonica GH18	7	10	8	16	—	—	—	—	—	16 c
Beltraniella portoricensis MR42	13	—	7	—	—	11	18	—	—	Nt
Beltraniopsis sp. GH19	11	11	11	22	—	—	—	—	—	10 c
Corynespora cassiicola MR01	9	8	11	8	—	—	—	—	—	13 c
Curvicladiella sp. GH29	—	—	8	—	—	—	—	—	—	9 c
Cylindrium elongatum MR45	11	13	12	15	12	18	22	—	22	Nt
Helicosporium talbotii MR48	—	—	9	—	—	—	—	—	—	Nt
Hemibeltrania malaysiana GH24	—	—	7	—	—	—	—	—	—	20 c
Memnoniella sp. MR33	—	—	—	—	17	—	—	—	—	18 c
Perelegamyces parviechinulatus GH25	—	—	7	—	—	—	—	—	—	12 c
Phaeobotrys sp. MR14	7	13	8	7	9	—	—	—	—	21 c
Phialophora verrucosa MR54	8	—	—	—	—	—	—	—	—	15 c
Ramichloridium apiculatum MR39	—	—	8	—	—	—	—	—	—	Nt
Selenosporella sp. GH26	—	—	—	—	—	—	—	—	—	82 b
Thozetella havanensis MR43	—	—	8	—	—	—	—	—	—	8.5 c
Vermicularopsiella sp. MR06	7	—	—	—	—	—	—	—	—	14 c
Unidentified GH15	—	—	—	11	—	—	—	—	—	22 c
Unidentified MR44	—	—	14	8	—	—	—	—	—	9 c
Positive control	25	25	25	25	25	24	24	24	24	100 a
Blank (extract of fermented rice)	—	—	—	—	—	—	—	—	—	0 d
Negative control	—	—	—	—	—	—	—	—	—	0 d

Bs = Bacillus subtillis Sa = Staphylococcus aureus Xc = Xanthomonas campestris Ca = Candida albicans Ec = Erwinia carotovora, *At = Alternaria tagetica Cg = Colletotrichum gloeosporioides Pa = Pythium aphanidermatum Mi = Meloidogyne incognita*, Positive control: amykacin (5 µg) for bacteria; Neomicol (1 µg) for yeast; Ricoil (25 µg) for fungi, Alliete (25 µg) for *P. aphanidermatum*; Vydate (oxamyl 10 %) for *Meloidogyne incognita*, Negative control: acetone (12 µl) for bacteria and fungi; water for nematode, and viability indicator

Nt = No tested, *SD^2 (300 µg) = 0.1076.

Table 3. MIC's of the deffated fractions obtained from the active antimicrobial extracts.

Species	Minimum inhibitory concentration (MIC) µg/ml				
	B. s.	*E. c*	*S. a*	*X. c*	*C. a*
Beltraniella japonica	>400	400	200	>400	nt
Beltraniella portoricensis	>400	nt	400	nt	nt
Beltraniopsis sp.	>400	>400	400	>400	nt
Corynespora cassiicola	400	>400	100	>400	nt
Cylindrium elongatum	200	400	100	400	400
Memnoniella sp.	>400	nt	nt	nt	100
Phaeobotrys sp.	400	>400	>400	>400	400
Amykacin	<100	<100	<100	<100	<100
Blank (Fermented rice)	nt	nt	nt	nt	nt

Bs = Bacillus subtillis Ec = Erwinia carotovora Sa = Staphylococcus aureus Xa = Xanthomonas campestris Ca = Candida albicans nt = no tested.

the *Memnoniella* genus, antibacterial and cytotoxic metabolites have only been isolated in *M. echinata* (Hinkley et al., 1999; Jarvis et al., 1996; Vertesy et al., 2001).

Antifungal activity against the Oomycete and filamentous phytopathogens was exhibited only for *B. portoricensis* and *C. elongatum*. This last being the most promising strain of this screening, with a broad spectrum activity and the highest inhibition zones against *C. gloeosporioides* and *P. aphanidermatum* (22 mm). Although fungi with rhombic or rostrate conidia such as *Beltraniella* and *Beltraniopsis* genera are commonly found on tropical leaf litter (Heredia et al., 2002) they had not yet been screened.

Undoubtedly, of special importance was the discovery that *Selenosporella* sp. GH26 extract able to cause a nematotoxic effect against *M. incognita* (J_2), with a promissory LD_{50} of 91 µg/ml. In general, from anamorphic fungi we found that very few nematicidal pure compounds have been obtained. Among them are phomalactone (Khambay et al., 2000), 3-hydroxypropionic acid (Schwarz et al., 2004), and 4-(4´-carboxy-2´-ethyl-hydroxypentyl)-5,6-dihydro-6-methyl-cyclobutan[b]pyridine-3,6-dicarboxylic acid (Liu et al., 2009). These compounds showed LD_{50} values between 471 to 12 µg/ml. Then, the active principles produced by *Selenosporella* sp. GH26 could be stronger to control *M. incognita*. Twelve species are reported belonging to *Selenosporella* (Castañeda et al., 2009), where the strain isolated in this study is the first tested in any bioassay.

As expected, the chemical profile (GC-MS) of the most active fungal extracts revealed a complex mixture of compounds. This data suggests that some of the isolates obtained might be able to produce metabolites that have remained unknown until now. Finally, the taxonomic identification of the active fungi was supported with molecular analyses; *C. elongatum* and *C. cassiicola* were identified by BLAST analysis in databases at NCBI (http://blast.ncbi.nlm.nih.gov), CBS Fungal Biodiversity Centre (http://www.cbs.knaw.nl) and NITE Biological Resource Center (http://www.nbrc.nite.go.jp). Other active fungal strains were confirmed at genus, family or order level because no sequences are available in the ITS-rDNA database for these particular species. Analyses of ITS and 28S for leaf litter-related fungi are beginning to be conducted and many descriptions found in databases use the terms “saprophyte fungi”, “leaf litter fungi” or simply “fungal sp” (Smith et al., 2008; U'ren et al., 2009). Although, molecular techniques are strong tools in modern biotechnology, classical taxonomy is also required to support identifications, especially when databases are insufficient to identify the species or genus of families under study.

In conclusion, *C. cassiicola, C. elongatum* and *Selenosporella* sp. GH26 are the most promising strains in this study to continue searching friendly antimicrobials and nematicidal to the environment and humans. Toxicity studies, isolation and/or structural identification of the active principles from the most active strains are now in progress. Hence, this is an important contribution to the knowledge and exploration of the biotechnological potential of the anamorphic fungi from tropical areas in Mexico.

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