

Allelochemical Effects of Volatile Compounds and Organic Extracts from *Muscodor yucatanensis*, a Tropical Endophytic Fungus from *Bursera simaruba*

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Abstract *Muscodor yucatanensis*, an endophytic fungus, was isolated from the leaves of *Bursera simaruba* (Burseraceae) in a dry, semideciduous tropical forest in the Ecological Reserve El Eden, Quintana Roo, Mexico. We tested the mixture of volatile organic compounds (VOCs) produced by *M. yucatanensis* for allelochemical effects against other endophytic fungi, phytopathogenic fungi and fungoids, and plants. VOCs were lethal to *Guignardia mangifera*, *Colletotrichum* sp., *Phomopsis* sp., *Alternaria*

solani, *Rhizoctonia* sp., *Phytophthora capsici*, and *P. parasitica*, but had no effect on *Fusarium oxysporum*, *Xylaria* sp., the endophytic isolate 120, or *M. yucatanensis*. VOCs inhibited root elongation in amaranth, tomato, and barnyard grass, particularly those produced during the first 15 days of fungal growth. VOCs were identified by gas chromatography/mass spectrometry and included compounds not previously reported from other *Muscodor* species and the previously reported compounds octane, 2-methyl butyl acetate, 2-pentyl furan, caryophyllene, and aromadendrene. We also evaluated organic extracts from the culture medium and mycelium of *M. yucatanensis* on the same endophytes, phytopathogens, and plants. In general, extracts inhibited plants more than endophytic or phytopathogenic fungi. *G. mangifera* was the only organism that was significantly stimulated by both extracts regardless of concentration. Compounds in both organic extracts were identified by gas chromatography/mass spectrometry. We discuss the possible allelopathic role that metabolites of *M. yucatanensis* play in its ecological interactions with its host plant and other organisms.

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Introduction

Endophytic microorganisms live asymptotically and intercellularly within plant tissues (Saikkonen et al., 1998). Fungal endophytes have been found in every plant species examined and are largely unquantified components of fungal biodiversity. Endophytes are especially poorly understood in tropical

forest trees, where their abundance and diversity are greatest (Arnold et al., 2001).

The interaction between endophytes and their hosts varies from mutualistic to parasitic, depending on species, physiological state, chemistry of the host plant, environmental stress, and other abiotic conditions (Espinosa-García et al., 1993; Gilbert and Strong, 2007). These fungi may impact survival and fitness of plants in all terrestrial ecosystems, and may play a significant role in plant biogeography, evolution, and community structure (Rodríguez et al., 2009).

The secondary metabolites produced by endophytes or their infected hosts could be chemical defenses against herbivores, pathogens, or competitors (Schulz and Boyle, 2005; Strobel, 2006; Herre et al., 2007). *In vitro* competition experiments show that endophytic fungi inhibit the growth of other endophytic fungi (Espinosa-García et al., 1993) and phytopathogens (Yue et al., 2000; Arnold et al., 2003). Endophytes in the tissues of living plants are relatively unstudied as potential sources of novel natural products. These compounds offer an enormous potential for the discovery of new agrochemicals and drugs of natural origin (Tan and Zou, 2001; Schulz et al., 2002).

Muscodor has been isolated as an endophyte from tropical trees and vines in Central America, Australia, and Thailand. Four species of *Muscodor* are known: *M. albus* from small limbs of *Cinnamomum zeylanicum* in Honduras and *Myristica fragrans* in Thailand (Worapong et al., 2001; Sopalun et al., 2003); *M. roseus* from two monsoonal rainforest tree species, *Grevillea pteridifolia* and *Erythrophelum chlorostachys* in Northern Australia (Worapong et al., 2002); *M. vitigenus* from *Paullinia paullinioides*, a liana growing in the understory of the rainforests of the Peruvian Amazon (Daisy et al., 2002a); and *M. crispans* from *Ananas ananassoides*, a wild pineapple in the Bolivian Amazon Basin (Mitchell et al., 2008). Based on morphological and DNA sequence analyses, González et al. (2009) found that *M. yucatanensis* is a new species. Colonies of this fungus are whitish and produce a strong musty odor when grown on potato dextrose agar (PDA). They grow slowly and do not produce reproductive structures. *Muscodor* species produce a mixture of toxic volatile organic compounds (VOCs) that are lethal to a wide variety of plant and human pathogens. The mixture of VOCs consisted primarily of alcohols, acids, esters, ketones, and lipids, including 3-methyl butyl acetate (isoamyl acetate), which was the most active compound (Ezra et al., 2004a,b; Strobel, 2006).

This paper describes part of a long-term project on the chemical ecology of endophytic fungi and their possible role in plant defense mechanisms (González et al., 2007; Macías-Rubalcava et al., 2008). We investigated the allelochemical potential of the endophytic fungus *Muscodor yucatanensis* and cataloged the compounds it produces *in vitro*.

We performed bioassays to test the toxicity of VOCs and organic extracts of the mycelium and culture medium. We assessed the effects of the extracts on the growth of other endophytic fungi from El Eden plant species, on some important phytopathogens, and on dicotyledonous and monocotyledonous plants. We also evaluated the chemical composition of the VOCs mixture and of the organic extracts from mycelium and culture medium.

Methods and Materials

Fungal Material *M. yucatanensis* was isolated from surface sterilized segments of healthy leaves of *Bursera simaruba* (Burseraceae) ('chacah' or 'palo mulato') collected at the Ecological Reserve El Eden, Quintana Roo, Mexico in September 2004. A specimen of the plant was deposited in the University of California, Riverside Herbarium (G.P. Schultz & R. Palestina # 1092/UCR 110695). The fungus was cryogenically preserved in liquid nitrogen vapor 10% glycerol, and was deposited in the fungal collection of Herbario Nacional de México (MEXU), UNAM, accession number MEXU 25511.

Effects of VOCs Bioassays were performed using six endophytic fungi isolated from *B. simaruba* and other surrounding plants in the El Eden Ecological Reserve: *Colletotrichum* sp., *Phomopsis* sp., and *Guignardia mangifera* (isolated from the leaves of *Callicarpa acuminata*); the unknown isolate 120 and *M. yucatanensis* (isolated from *Bursera simaruba* leaves); and *Xylaria* sp. (isolated from *Pteridium aquilinum* leaves). As a complement to bioassays that used these endophytic fungi, we also tested VOCs on phytopathogenic microorganisms: fungi *Fusarium oxysporum*, *Alternaria solani*, and *Rhizoctonia* sp., and fungoids *Phytophthora parasitica* and *P. capsici*.

Bioassays were performed under sterile conditions using divided Petri dishes (9 cm) with two compartments containing PDA medium. The plastic walls dividing the plate prevented the diffusion of any soluble compounds produced by *M. yucatanensis* to the second compartment but did allow free exchange of VOCs. Due to the very slow growth of *M. yucatanensis* on PDA medium, an inoculum of it was placed in one of the compartments of the dish and grown at 25–27°C and a photoperiod of 12:12h fluorescent light (Octron 4100 K, Ecologic, 32 W) for 10 d before the bioassay. The inoculum from a test species was then placed in the other compartment of the dish, which was then wrapped with two layers of plastic wrap and incubated as above. Growth of the test organisms was assessed after different times of incubation, depending on individual growth rate. *Phytophthora capsici*, *P. parasitica*, *Colletotrichum* sp., *Phomopsis* sp., and *Rhizoctonia* sp. were examined after 3 d; *A. solani* and *F.*

oxysporum after 4 d, and *G. mangifera*, isolate 120, *Xylaria* sp. and *M. yucatanensis* after 10 d. The average of two perpendicular diameter measurements for each mycelium was recorded at the end of the bioassays and compared with the respective controls (Macías-Rubalcava et al., 2008). Experiments were performed following a complete randomized design with four replications per treatment.

Recovery of test fungi that were severely inhibited by *Muscodor* VOCs was evaluated by transferring an agar plug of the test microorganism to a new Petri dish with fresh PDA and incubating under the same conditions. Recovery was evaluated by measuring mycelial diameter as described above and comparing results with controls.

Phytotoxicity of the VOCs was tested on roots of two dicots, amaranth (*Amaranthus hypochondriacus*; Amaranthaceae) and tomato (*Lycopersicon esculentum* var. Pomodoro; Solanaceae), and one monocot, barnyard grass (*Echinochloa crus-galli*; Poaceae). We selected these test plants because we needed plants that have rapid, homogenous, high frequency germination, and because seeds of tropical plants including *B. simaruba* germinate poorly. Amaranth seeds were purchased at a local market at Tulyehualco, Mexico, D. F., tomato seeds in Semillas Berentsen, Celaya, Guanajuato, Mexico, and barnyard grass seeds were collected from plants growing in the greenhouse of Instituto de Ecología (UNAM).

The bioassays were performed with divided Petri dishes containing PDA and inoculated with *M. yucatanensis* in one compartment. The endophyte was grown at 28°C for different times (0, 3, 5, 10, 15, 20, 30, and 50 days) before introducing seeds into the other compartment that contained 1% water agar. At each time, 10 test seeds were sown and dishes were wrapped with two layers of plastic wrap and placed in a germination cabinet in the dark at 27°C. Root lengths of test plants were measured after 24 hr (amaranth), 48 hr (barnyard grass), or 72 hr (tomato) of exposure to *Muscodor* VOCs. Root lengths were compared to controls that did not have the *M. yucatanensis* inoculum. Experiments were performed following a complete randomized design with four replications per treatment.

We evaluated recovery of seeds and seedlings that were severely inhibited by *Muscodor* VOCs. Inhibited seeds and seedlings were removed from the divided Petri dishes and placed in new Petri dishes with fresh 1% water agar, and incubated as before. Root lengths were measured after 24 and 48 hr, and compared with their respective controls.

Organic Extracts of Culture Medium and Mycelium Fungi release some secondary metabolites into the environment while retaining others in the mycelium. *M. yucatanensis* was cultured in 18 Erlenmeyer flasks (1,000 ml), each containing 500 ml of potato dextrose broth (PDB). Each flask was inoculated with three agar plugs taken from a stock culture of *M. yucatanensis* on PDA, and was incubated with constant

agitation on an orbital shaker at 150 rpm for 6 wk at 25–27°C and a photoperiod of 12:12 hr (fluorescent light).

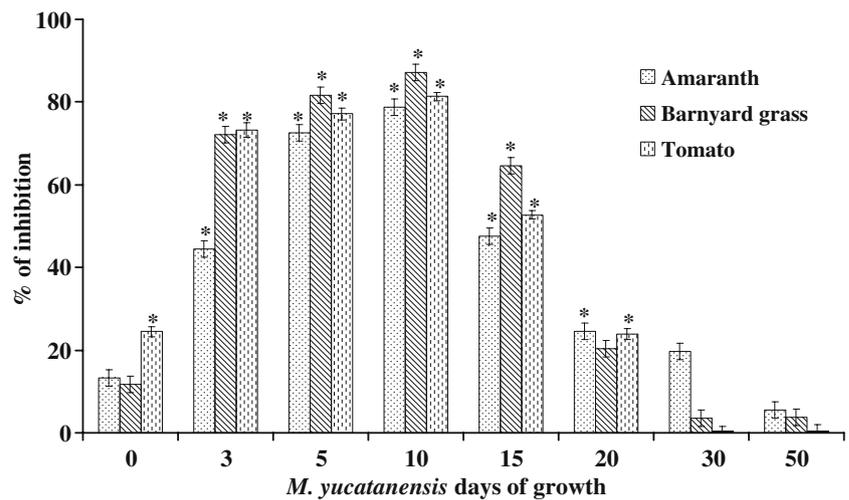
At the end of the fermentation process, culture medium in each flask was separated from the mycelium by filtration. Culture medium (9 L) was extracted $\times 3$ with 9 L of dichloromethane (CH_2Cl_2) and then $\times 3$ with 9 L of ethyl acetate (EtOAc). The combined organic phases were filtered over anhydrous Na_2SO_4 and concentrated *in vacuo* to give 306.9 mg of a yellowish resinous product. The mycelium was extracted with CH_2Cl_2 and EtOAc (3×2 L). The combined mycelium extracts were evaporated to yield 2.73 g of a yellowish resinous product.

Organic extracts of the culture medium and mycelium were tested with the same eleven test microorganisms used in the bioassays described above. Both extracts were evaluated at 125, 250, 500, and 1,000 $\mu\text{g/ml}$ in sterile PDA, added before the agar had solidified in 6-cm Petri dishes. The positive control was the commercial fungicide Prozycar[®] (carbendazim: methyl benzimidazol-2-yl-carbamate) added to agar at 200 $\mu\text{g/ml}$. This concentration of Prozycar completely inhibited test microorganisms in a preliminary bioassay. Unamended PDA was used as negative control. An inoculum (5 mm^2 agar plug) of each test microorganism was placed on the amended agar in a Petri dish and incubated in darkness at 27°C. The average of two perpendicular measurements of the mycelium was recorded after 3, 4, and 10 d of incubation depending on the growth rate of the test microorganism. The experiment used a complete randomized design with four replications per treatment.

A Petri dish bioassay was used to evaluate the effect of the organic extracts (culture medium and mycelium) on the root length of amaranth, tomato, and barnyard grass. Each extract was evaluated at 50, 125, 250, and 500 $\mu\text{g/ml}$ by adding the extract to 1% water agar before the agar had solidified. Unamended water agar was used as negative control, and agar with 1,000 $\mu\text{g/ml}$ of Rival[®] (Glyphosate: N-(phosphonomethyl) glycine) as positive control. This level of Glyphosate completely inhibited root growth in the test plants in a preliminary bioassay. Ten seeds of each test plant were sown directly on agar in 6 cm Petri dishes and incubated in the dark at 27°C. Root lengths were measured 24 hr after treatment for amaranth, 48 hr for barnyard grass, and 72 hr for tomato. The experiment used a complete randomized design with four replications per treatment.

Qualitative Analysis of VOCs *M. yucatanensis* mycelium was grown on PDA for 10 d in 15 ml SPME vials. A Solid Phase Micro Extraction (SPME) syringe was placed through a small hole drilled in the side of the vial through a Teflon septum and exposed to the vapor phase for 60 min. The SPME syringe (DVB–PDMS–Carboxen 50/30, Supelco Bormen, Belgium) was conditioned before use for 40 min at 250°C. After exposure to the VOCs, the syringe was inserted into the split (1:50 ratio) injection port of a gas

Fig. 1 Phytoinhibitory activity of VOCs produced by *Muscodor yucatanensis* after different periods of growth. The root lengths of amaranth, tomato, and barnyard grass seedlings were determined after 24 hr of exposure to VOCs (Amaranth): 48 hr of exposure (barnyard grass); or 72 hr of exposure (tomato). Vertical bars represent SD, $N=4$; $*P<0.05$



chromatograph (GC) (Agilent 6890N) interfaced to a LECO time of flight mass spectrometer (MS-TOF) operating at unit resolution with electron ionization (EI) operating at 200°C. Separation on a 10 m × 0.18 mm I.D. DB5 J&W capillary column with a film thickness of 0.18 micron used the following temperature program: 40°C for 0.5 min, followed by 280°C for 2.5 min at 70°C/min. The carrier gas was ultra high purity helium at 1 ml/min. A 3 min injection time was used to introduce the sample into the GC. The MS was scanned at a rate of 10 scans/s over a mass range of 30–450 Da. Data acquisition and data processing were performed with the LECO ChromaTOF software system. We established that the SPME syringe effectively trapped fungal volatiles in

preliminary tests. To evaluate background compounds, comparable analyses were conducted on vials containing only PDA, and the data were subtracted from the unknowns.

M. yucatanensis VOCs were identified by retention index (RI). Linear retention indices were calculated for all volatile constituents using a homologous series of *n*-alkanes with the parameters described above. Chemical identification was confirmed by comparison of mass spectra with the NIST database (National Institute of Standards and Technology).

Qualitative Analysis of the Organic Extracts Extracts were dissolved in CH_2Cl_2 , and one μl of each extract was directly injected into the gas chromatograph equipped and operated as

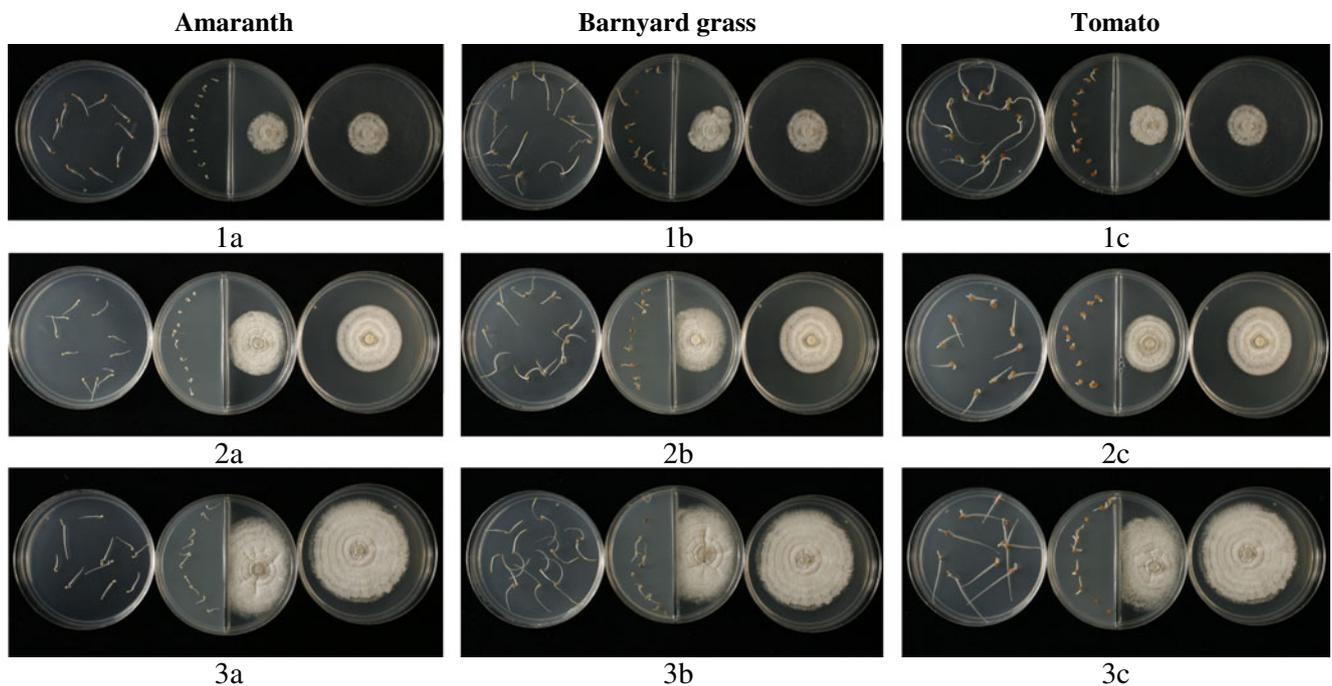


Fig. 2 Phytoinhibitory activity of VOCs from *Muscodor yucatanensis* grown for 5 (1), 10 (2), and 15 (3) days on the root length of amaranth (1a, 2a, and 3a), barnyard grass (1b, 2b, and 3b) and tomato (1c, 2c, and 3c)

Table 1 Percent recovery of the root length of amaranth, tomato and barnyard grass at 24 hr and 48 hr, after exposure to *Muscodor yucatanensis* VOCs

<i>Muscodor yucatanensis</i> days of growth	% of recovery ^a					
	Amaranth		Tomato		Barnyard grass	
	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
0	78.6	87.3	72.3	92.9	65.3	81.2
3	0	0	0	0	0	0
5	0	0	0	0	0	0
10	0	0	0	0	0	0
15	0	0	0	0	0	0
20	20.2	67.3	17.3	75.3	14.6	69.5
30	22.3	70.2	98.6	100	100	100
50	87.5	97.6	90.3	98.3	100	100

^a Percent of root length recovery compared with the respective control

described above, but with the column temperature programmed as follows: 40°C for 1 min, followed by 280°C for 5 min at 20°C/min. The injector was set at 250°C with a 1:50 split ratio and solvent delay of 100 sec. The MS was scanned at a rate of 10 spectra/s over a mass range of 33–600 Da. Background was established with an extract of PDA.

Components were identified by comparison of their RI, determined relative to the retention times (RT) of a series of n-alkanes with linear interpolation on the HP-5 MS capillary column. They were also confirmed by comparison of their mass spectra using the NIST database. The relative amounts of individual components of the organic extracts were expressed as percentages of the peak area relative to the total peak area.

Statistical Analysis Bioassay data were analyzed by ANOVA and Tukey's tests (Mead et al., 2002) using Statistica 6.0. IC₅₀ (50% inhibitory concentration) values for organic extracts were calculated by probit analysis (Zar, 2007) based on the average growth diameter of the mycelium and root length with SPSS 15.0 software. Data are represented as mean ± standard deviation (SD). A *P* value less than or equal to 0.05 was used to identify significant differences from controls.

Results

Effect of VOCs VOCs were lethal to the endophytes *Colletotrichum* sp., *Phomopsis* sp., and *Guignardia mangiferae*, and to the phytopathogens *Phytophthora capsici*, *P. parasitica*, *Rhizoctonia* sp., and *Alternaria solani*. *Fusarium oxysporum* remained alive and recovered 86 % of its growth after exposure to VOCs. The three endophytic fungi were not significantly inhibited by these VOCs: isolate 120 was inhibited only slightly (3.3 %), *M. yucatanensis* was not inhibited (0 %), and *Xylaria* was non-significantly stimulated (18 %).

The phytoinhibitory activity of VOCs increased proportionally from the first day of *Muscodor* growth, and was most

inhibitory after 10 d of endophyte growth (Fig. 1). However, phytotoxic activity decreased significantly from 15 to 50 d. Figure 2 is a qualitative example of phytotoxic activity after 5, 10, and 15 d of *Muscodor* growth. Roots of the most affected plants darkened before dying.

The recovery of germinating seeds after exposure to VOCs is shown in Table 1. VOCs produced by mycelium that was 3–15-d-old were very inhibitory to the three test plants. However, the roots of seedlings exposed to VOCs

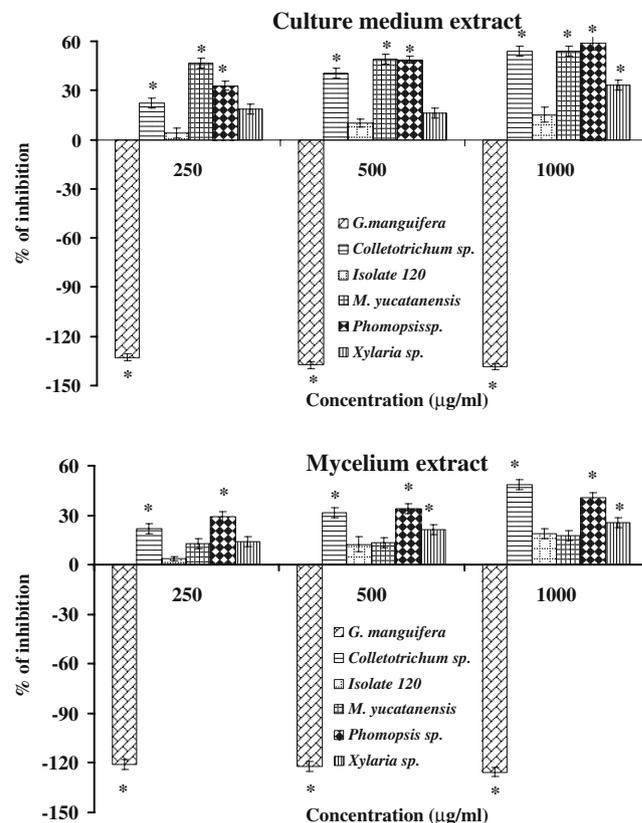


Fig. 3 Inhibitory activity of the culture medium and mycelium organic extracts from *Muscodor yucatanensis* against six endophytic fungi. Vertical bars represent SD, *N*=4; **P*<0.05

from 1–3-d-old mycelia or 20–50-d-old mycelia recovered 1 to 2 d after exposure.

Effects of Organic Extracts Figure 3 shows significant dose-dependent fungal growth inhibition caused by both extracts of *M. yucatanensis* on almost all test endophytic fungi. *Colletotrichum* sp. and *Phomopsis* sp. were significantly inhibited by both extracts at all concentrations. The culture medium extract from *M. yucatanensis* caused a significant reduction in its own growth at all concentrations. In contrast, the mycelium extract did not significantly inhibit the growth of *M. yucatanensis*. *Xylaria* sp. was inhibited only by the highest concentration of either extract. IC₅₀ values for the culture medium extract on the radial growth of *Colletotrichum* sp. was 794 µg/ml; for *M. yucatanensis*, 591 µg/ml; and for *Phomopsis* sp., 631 µg/ml. IC₅₀ for the mycelium extract for all endophytic fungi was >1,000 µg/ml.

G. mangifera was significantly stimulated by both extracts at all concentrations tested (Fig. 3). We estimated that the concentration required to stimulate a 50% increase in radial

growth (SC₅₀) for *G. mangifera* was 94 µg/ml for the culture medium extract and 104 µg/ml for the mycelium extract.

The effects of the culture medium and the mycelium extracts on the radial growth of phytopathogenic microorganisms are shown in Fig. 4. In general, extracts were inhibitory only at 500 and 1,000 µg/ml, and the IC₅₀ values for almost all phytopathogens were >1,000 µg/ml.

Both extracts inhibited root growth of the three test plants in a concentration-dependent manner (Fig. 5). The culture medium extract was more phytotoxic. The IC₅₀ values of the culture medium extract were 112 µg/ml for tomato, 132 µg/ml for amaranth, and 435 µg/ml for barnyard grass. The crude culture medium extract was more active than Glyphosate which had IC₅₀ values of 33 µg/ml for tomato, 234 µg/ml for amaranth, and 114 µg/ml for barnyard grass. All IC₅₀ values for mycelium extract were >500 µg/ml.

Chemical Composition The main volatile secondary metabolites produced by a 10-d-old *M. yucatanensis* culture comprised 38 compounds that were identified by GC/MS (Table 2). The culture medium extract contained 32 compounds, while the mycelium extract consisted of 23 compounds (Tables 3, 4). In general, the compounds present in

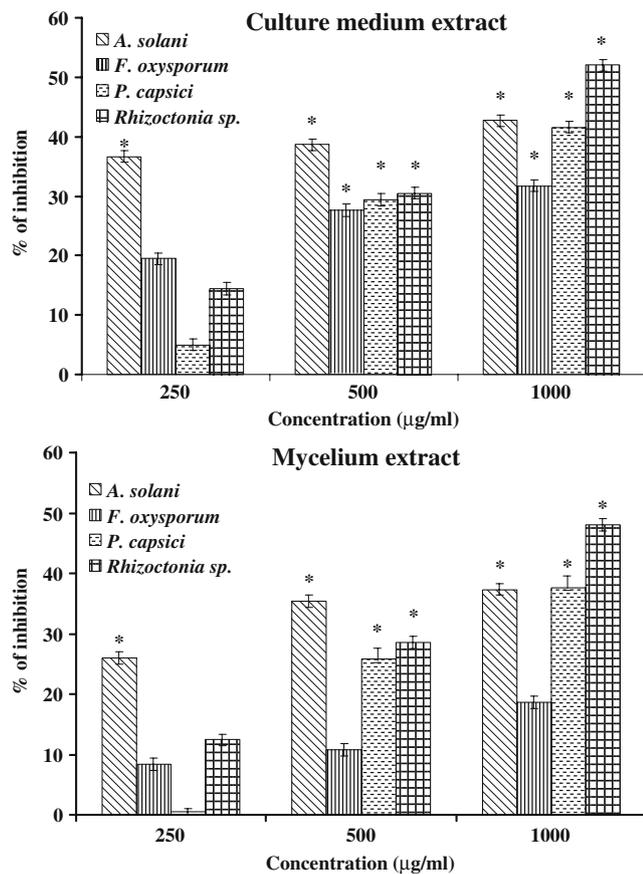


Fig. 4 Inhibitory activity of the culture medium and mycelium organic extracts from *Muscodor yucatanensis* against phytopathogenic microorganisms: *Alternaria solani*, *Fusarium oxysporum*, *Phytophthora capsici*, and *Rhizoctonia* sp. Vertical bars represent SD, N=4; *P<0.05

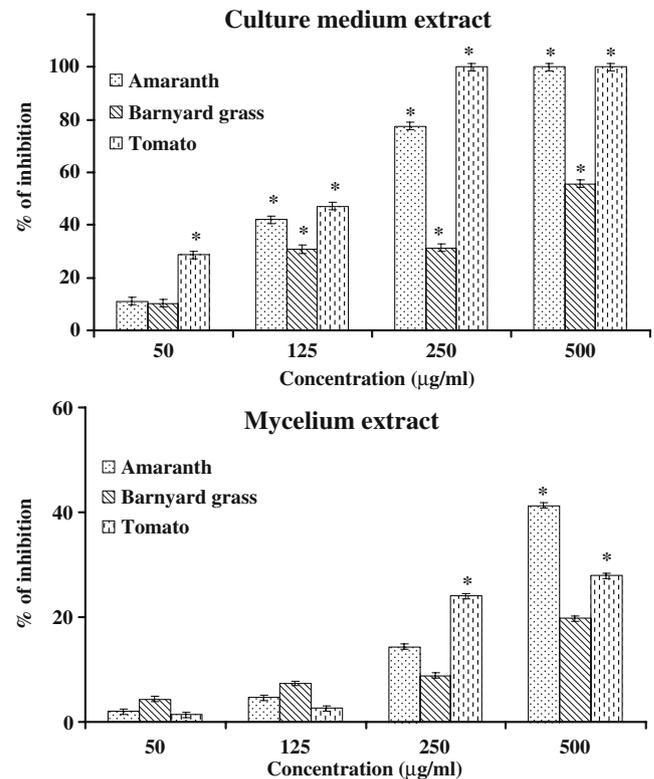


Fig. 5 Phytotoxic activity of the culture medium and mycelium organic extracts from endophytic fungus *Muscodor yucatanensis* on the root length of amaranth, tomato, and barnyard grass. Vertical bars represent SD, N=4; *P<0.05

Table 2 GC/MS analysis of the volatile compounds mixture (VOCs) produced by *Muscodor yucatanensis*

Compounds	Retention Index ^a	Total area (%)	MW	Molecular formula
2-methylbutan-1-ol	723.7	0.06	88	C ₅ H ₁₂ O
cyclohepta-1,3,5-triene	746.2	0.20	92	C ₇ H ₈
heptane	655.0	0.42	100	C ₇ H ₁₆
ethylbenzene	885.5	0.16	106	C ₈ H ₁₀
octane	794.9	0.16	114	C ₈ H ₁₈
propan-2-ylbenzene	988.3	0.04	120	C ₉ H ₁₂
4-ethylcyclohexan-1-one	1004.3	0.08	126	C ₈ H ₁₄ O
methyl 2,3-dimethylbutanoate	843.1	0.17	130	C ₇ H ₁₄ O ₂
2-methyl butyl acetate	874.9	0.49	130	C ₇ H ₁₄ O ₂
1-methyl-4-(1-methylethyl)-benzene	1020.1	0.43	134	C ₁₀ H ₁₄
1-methyl-3-(1-methylethyl)-benzene	1020.7	0.70	134	C ₁₀ H ₁₄
α-Phellandrene	1026.2	5.02	136	C ₁₀ H ₁₆
(1Z,5Z)-3,4-dimethylcycloocta-1,5-diene	1023.2	2.22	136	C ₁₀ H ₁₆
2-pentyl furan	985.2	1.04	138	C ₉ H ₁₄ O
(E)-7-methylundec-4-ene	793.8	1.00	168	C ₁₂ H ₂₄
3,4-dimethyldec-1-ene	1492.1	1.96	168	C ₁₂ H ₂₄
4,5-dimethyl-1,2,3,6,7,8,8a,8b-octahydrobiphenylene	1357.2	4.24	188	C ₁₄ H ₂₀
1-oxacyclotetradeca-4,11-diyne	1578.9	0.37	190	C ₁₃ H ₁₈ O
2-iodo-pentane	916.7	1.11	198	C ₅ H ₁₁ I
1-iodo-3-methyl-butane	919.2	0.07	198	C ₅ H ₁₁ I
4,5-dehydroisolongifolene	1500.6	0.71	202	C ₁₅ H ₂₂
2,5,9,9-tetramethyl-3,4,4a,5,8,9a-hexahydrobenzo[7]annulene	1398.8	5.32	204	C ₁₅ H ₂₄
aristolene	1323.3	3.04	204	C ₁₅ H ₂₄
caryophyllene	1376.8	15.84	204	C ₁₅ H ₂₄
elemene	1340.6	0.34	204	C ₁₅ H ₂₄
5,8a-dimethyl-3-prop-1-en-2-yl-2,3,4,4a,7,8-hexahydro-1H-naphthalene	1359.2	0.57	204	C ₁₅ H ₂₄
1R,4S,7S,11R-2,2,4,8-Tetramethyltricyclo[5.3.1.0(4,11)]undec-8-ene	1379.5	23.44	204	C ₁₅ H ₂₄
(Z)-4-(4,6,6-trimethyl-5-bicyclo[3.2.0]hept-3-enyl)but-3-en-2-ene	1400	2.29	204	C ₁₄ H ₂₀ O
(3Z,6E)-3,7,11-trimethyl-dodeca-1,3,6,10-tetraene	1422.9	0.20	204	C ₁₅ H ₂₄
(3Z)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec-3-ene	1805.6	0.96	204	C ₁₅ H ₂₄
2-methyl-2-(4-methyl-3-propan-2-ylpent-3-en-1-ynyl)cyclobutan-1-one	1321.8	2.94	204	C ₁₄ H ₂₀ O
aromadendrene	1375	12.19	204	C ₁₅ H ₂₄
cyclopentyl 4-ethylbenzoate	960.52	4.85	218	C ₁₄ H ₁₈ O ₂
5,6-dipropyldecane	1492.7	0.21	226	C ₁₆ H ₃₄
cyclohexyl 4-ethylbenzoate	954.9	1.00	232	C ₁₅ H ₂₀ O ₂
4-(butylsulfanylmethyl)-6-piperidin-1-yl-1,3,5-triazin-2-amine	971.29	0.05	281	C ₁₃ H ₂₃ N ₅ S
androstan-17-one, 3-ethyl-3-hydroxy-, (5a)-	1805	4.05	318	C ₂₁ H ₃₄ O ₂
pentacosanoic acid	1806	2.05	374	C ₂₅ H ₄₂ O ₂

Compounds found in the control PDA plate are not included in this table

^a Kovat's indices calculated from retention time data on an HP-5 MS capillary column

both extracts are alcohols, esters, and ketones of saturated and unsaturated compounds, as well as benzene derivatives.

Discussion

Muscodor yucatanensis, like other *Muscodor* species (Ezra et al., 2004a,b; Strobel, 2006), produces a mixture of volatile

compounds (VOCs) when cultured under *in vitro* conditions. These VOCs are selectively toxic to other endophytic and phytopathogenic fungi, and to plant roots. Strobel et al. (2001) found that VOCs of *M. albus* isolates had low inhibitory effects against fungi and bacteria. However, when these authors tested *M. albus* VOCs as a mixture they observed that they acted synergistically and killed a broad range of fungi and bacteria.

Table 3 GC/MS analysis of the compounds presents in the culture medium extract of *Muscodor yucatanensis*

Compounds	Retention Index ^a	Total area (%)	MW	Molecular formula
3-methyloxolan-2-one	950.7	0.03	100	C ₅ H ₈ O ₂
ethylbenzene	857.0	0.66	106	C ₈ H ₁₀
1,3-dimethylbenzene ^b	866.7	0.79	106	C ₈ H ₁₀
5-propan-2-ylidenecyclopenta-1,3-diene ^b	889.9	0.75	106	C ₈ H ₁₀
2-(hydroxymethyl)-2H-furan-5-one	1206.8	0.10	114	C ₅ H ₆ O ₃
ethyl butanoate ^b	796.7	2.65	116	C ₆ H ₁₂ O ₂
butyl acetate ^b	811.5	1.45	116	C ₆ H ₁₂ O ₂
5H-cyclopenta[b]pyridine	1302.3	1.63	117	C ₈ H ₇ N
2-phenylethanol ^b	1119.3	5.62	122	C ₈ H ₁₀ O
3,3-dimethyl-2-hexanone	1235.7	0.76	128	C ₈ H ₁₆ O
ethyl 2-methylbutanoate	848.7	1.58	130	C ₇ H ₁₄ O ₂
3-methylbutyl acetate	878.7	2.86	130	C ₇ H ₁₄ O ₂
4-hydroxy-4-methyloxan-2-one ^b	1313.0	2.96	130	C ₆ H ₁₀ O ₃
4-(2-hydroxyethyl)phenol ^b	1448.2	0.94	138	C ₈ H ₁₀ O ₂
nonanal	1106.6	0.35	142	C ₉ H ₁₈ O
2,2,6-trimethylcyclohexane-1,4-dione	1172.0	0.40	154	C ₉ H ₁₄ O ₂
1,7-dioxaspiro[5.5]undec-8-ene	1536.5	2.00	154	C ₉ H ₁₄ O ₂
undecane ^b	1099.9	1.01	156	C ₁₁ H ₂₄
8-hydroxy-3-methyl-3,4-dihydroisochromen-1-one	1542.6	5.60	178	C ₁₀ H ₁₀ O ₃
2-(3,3-dimethylbut-1-ynyl)-3-methoxy-1,1-dimethylcyclopropane	1454.9	1.31	180	C ₁₂ H ₂₀ O
3,8-dihydroxy-3-methyl-4H-isochromen-1-one	1963.3	2.71	194	C ₁₀ H ₁₀ O ₄
2,6-ditert-butyl-4-methylphenol ^b	1497.4	3.44	220	C ₁₅ H ₂₄ O
(E)-tetradec-9-enoic acid	2147.7	3.09	226	C ₁₄ H ₂₆ O ₂
8,14-cedrandiol	2105.5	10.49	238	C ₁₅ H ₂₆ O ₂
methyl octadecanoate	2141.3	31.00	298	C ₁₉ H ₃₈ O ₂
methyl (13E,16E)-octadeca-13,16-dienoate	2146.3	6.90	294	C ₁₉ H ₃₄ O ₂
hexadecanoic acid ^b	1982.9	0.40	256	C ₁₆ H ₃₂ O ₂
(9E,12E)-octadeca-9,12-dienal ^b	2141.3	1.21	264	C ₁₈ H ₃₂ O
tridecan-3-yl 2-methoxyacetate	2662.7	0.09	272	C ₁₆ H ₃₂ O ₃
octadecanoic acid ^a	2169.2	1.75	284	C ₁₈ H ₃₆ O ₂
tert-butyl hexadecanoate	2184.6	1.09	312	C ₂₀ H ₄₀ O ₂
(5-acetamido-3,4-diacetyloxy-6-carbamimidoylsulfanyloxan-2-yl)methyl acetate	2137.3	4.36	405	C ₁₅ H ₂₃ N ₃ O ₈ S

^a Kovat's indices calculated from retention time data on an HP-5 MS capillary column

^b Compounds presents in the culture medium and mycelium organic extract of *Muscodor yucatanensis*. Compounds found in the control PDB broth are not included in this table

M. yucatanensis VOCs emitted by 3–15-d-old fungus significantly inhibited germination and root elongation of the three test plants. Many damaged seeds and seedlings could recover from inhibition if they had been exposed to VOCs from fungi that had been cultured 1–3 d, or more than 15 d. The loss of toxicity in older cultures may be due to depletion of nutrients from the culture medium, which may result in a change in VOCs production. Ezra et al. (2004b) investigated the VOCs emission profile of *M. albus* from 14 to 19 d growth and found that concentration of volatiles increased continuously, reflecting the increased growth of the fungus. After day 19, *M. albus* VOCs production declined, probably because of the depletion of carbohydrates in PDA.

Our bioassays with VOCs showed that, in general, they were lethal to endophytic and phytopathogenic fungi. However, the VOCs were not inhibitory to *M. yucatanensis* or to the endophytes *Xylaria* sp. or isolate 120, or to the phytopathogen *F. oxysporum*. Only five compounds that have been reported previously in other *Muscodor* species were found in the *M. yucatanensis* VOCs: octane; 2-methyl butyl acetate; 2-pentyl furan; caryophyllene, and aromadendrene (Atmosukarto et al., 2005; Strobel, 2006; Strobel et al., 2007). We did not find naphthalene in the VOC mixture of *M. yucatanensis*, but we found some derivatives of naphthalene. These compounds were reported in *M. albus*, *M. roseus*, and *M. vitigenus* (Strobel et al., 2001; Daisy et al., 2002b; Ezra et

Table 4 GC/MS analysis of the compounds presents in the mycelium organic extract of *Muscodor yucatanensis*

Compounds	Retention Index ^a	Total area (%)	MW	Molecular formula
ethylbenzene	860.9	2.80	106	C ₈ H ₁₀
1,3-dimethylbenzene ^b	869.8	1.19	106	C ₈ H ₁₀
5-propan-2-ylidenecyclopenta-1,3-diene ^b	891.2	2.96	106	C ₈ H ₁₀
4-hydroxy-4-methylpentan-2-one	845.4	13.94	116	C ₆ H ₁₂ O ₂
ethyl butanoate ^b	804.7	7.98	116	C ₆ H ₁₂ O ₂
butyl acetate ^b	818.4	3.46	116	C ₆ H ₁₂ O ₂
2-phenylethylethanol ^b	1121.1	0.66	122	C ₈ H ₁₀ O
4-hydroxybenzaldehyde	1408.9	0.88	122	C ₇ H ₆ O ₂
4-hydroxy-4-methylxan-2-one ^b	1345.6	14.70	130	C ₆ H ₁₀ O ₃
4-(2-Hydroxyethyl)phenol ^b	1464.5	1.99	138	C ₈ H ₁₀ O ₂
undecane ^a	1100.6	7.10	156	C ₁₁ H ₂₄
methyl 1-methyl-2-oxocyclohex-3-ene-1-carboxylate	977.1	4.99	168	C ₉ H ₁₂ O ₃
butyl 2-phenylacetate	1280.7	1.36	192	C ₁₂ H ₁₆ O ₂
5,7,7-trichlorohept-6-en-2-one	1502.1	1.90	214	C ₇ H ₉ Cl ₃ O
2,6-ditert-butyl-4-methylphenol ^b	1500.5	10.04	220	C ₁₅ H ₂₄ O
3,5-di(phenyl)-1,2,4-trioxolane	963.0	4.10	228	C ₁₄ H ₁₂ O ₃
(Z)-hexadec-11-enoic acid	2155.8	3.13	254	C ₁₆ H ₃₀ O ₂
hexadecanoic acid ^b	1971.8	1.50	256	C ₁₆ H ₃₂ O ₂
(9E,12E)-octadeca-9,12-dienal ^b	2134.0	2.86	264	C ₁₈ H ₃₂ O
octadec-9-ynoic acid	2153.6	12.46	280	C ₁₈ H ₃₂ O ₂
octadecanoic acid ^b	2173.3	7.23	284	C ₁₈ H ₃₆ O ₂
pregnane-3,20-diol, diacetate, (3a,5a,20R)-	1659.7	2.13	404	C ₂₅ H ₄₀ O ₄
sitosterol	3162.8	1.51	414	C ₂₉ H ₅₀ O

Kovats' indices calculated from retention time data on an HP-5 MS capillary column

^a Compounds presents in the culture medium and mycelium organic extract of *Muscodor yucatanensis*

al., 2004a). The VOCs bioactivity suggested that *M. yucatanensis* plays an important mutualistic role by augmenting host defensive responses against pathogens and/or competitors and also provides defense against some of its own competitors.

The present study is the first to identify the chemical constituents of organic extracts from the *in vitro* culture medium and mycelium of a species of *Muscodor* and to report their *in vitro* bioactivity on the growth of other endophytic and phytopathogenic fungi and plants.

M. yucatanensis was not inhibited by the mycelial organic extract. However, its growth was inhibited 50% or more by the culture medium extract, apparently an autotoxic response.

The highly significant growth stimulation of *G. mangifera* by three concentrations of mycelial extract tested was unexpected. Rodrigues et al. (2004) estimated that *Guignardia* isolates were among the endophytes most frequently isolated from tropical woody plant species of Brazil. These authors thought that *Guignardia* species does not regularly co-evolve with hosts but instead 'jumps' to unrelated hosts. This species is polyphagous and occurs on many different and often unrelated plant species. We do not know if *Guignardia* grows more rapidly *in vivo* when it infects host plant tissues already

infected with *Muscodor*. Further studies that target this potential interaction could provide insight into fungal community dynamics and competitive and facilitative strategies.

The culture medium extract of *M. yucatanensis* was toxic to roots of test plants at higher concentrations than the mycelial extract. The culture medium had 23 compounds. Both extracts share 12 allelochemicals including benzene derivatives, phenolic compounds, cyclopentadienes, esters, lactones, alkanes, aldehydes, and carboxylic acids. It is possible that the most abundant compounds present in the culture medium extract are responsible for its strong allelochemical potential. This may provide a defense mechanism for *M. yucatanensis* against other endophyte competitors, as well as against some competitors and phytopathogens of *B. simaruba*. Given that both extracts of *M. yucatanensis* stimulate growth of *Guignardia mangifera*, there may be some metabolites that enhance symbiotic relationships with other endophytic fungi.

We have limited understanding of the mechanisms that regulate the integrated functions responsible for the success of endophyte-plant relationships. The ecological functions of the thousands of endophytic fungi associated with tropical plants are currently understudied. Dry tropical forests in Yucatan

Peninsula, and natural protected areas such as the Ecological Reserve of El Eden, Quintana Roo in Mexico, are sources of biological resources that consist of diverse macro and microorganisms. Further studies of endophytic relationships in plant communities will contribute to an increased knowledge of the biodiversity of microorganisms associated with plants, the allelochemicals they produce that gives them an aggregate value, and the tangled web of biotic and abiotic interactions they exhibit.

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