Agrociencia

BIOACTIVITY OF *Pseudomarasmius nidus-avis* AND OTHER WILD FUNGI FROM MESOPHYTIC MOUNTAIN FOREST IN MEXICO IN CONTROL OF PHYTOPATHOGENS

J. Francisco **Castillo-Esparza**[1], Leticia **Montoya**[1*], Damaris **Desgarennes**[1], Gloria **Carrión**[1], Antero **Ramos**[1], Enrique **César**[2], Randy **Ortiz-Castro**[3], Victor M. **Bandala**[1]

[1] Instituto de Ecología A. C. Red de Biodiversidad y Sistemática. Carretera Antigua a Coatepec No. 351, El Haya, Xalapa, Veracruz, Mexico. C. P. 91073.
[2] Instituto de Ecología A. C. Herbario XAL. Carretera Antigua a Coatepec No. 351, El Haya, Xalapa, Veracruz, Mexico. C. P. 91073.
[3] Instituto de Ecología A. C. Red de Estudios Moleculares Avanzados. Carretera Antigua a Coatepec No. 351, El Haya, Xalapa, Veracruz, Mexico. C. P. 91073.
* Author for correspondence: leticia.montoya@inecol.m

ABSTRACT

The mesophytic mountain forest is one of the most biodiverse ecosystems in Mexico, in which there is a diverse mycobiota of which many aspects of its biology and properties are unknown. *Pseudomarasmius nidus-avis* is a recently discovered and described fungus that forms rhizomorphs used in the nesting habits of birds of the family Tyrannidae (*Mionectes oleagineus* and *Tolmomyias sulfurescens*). In this work, the bioactive properties of *P. nidus-avis*, seven basidiomycetes and two wild ascomycetes from the mesophytic mountain forest of central Veracruz state, Mexico, were evaluated in terms of their effect against phytopathogenic fungal (PF) species such as *Fusarium oxysporum* (ATCC-417 and CBF-185), *Sclerotium cepivorum* (CBF-414) and *Leptosphaerulina australis* (CBF-433), which generate important economic losses in several crops. *In vitro* confrontation tests of mycelium isolated from the macrofungi against the PFs were developed, where 50 % of the wild fungi, including *P. nidus-avis*, showed effect on the phytopathogens. Furthermore, the crude extract obtained from the mycelium and supernatant of *P. nidus-avis* was evaluated against PF, showing an inhibition of 16.08 % against *F. oxysporum* (ATCC-417). These results show that *P. nidus-avis* has bioactive properties with potential for use in the control of some PF diseases of agricultural and forestry importance.

Keywords: Antagonistic fungi, biological activity, Basidiomycota, Ascomycota.

INTRODUCTION

The mesophytic mountain forest (MMF) is considered one of the most biodiverse terrestrial ecosystems in Mexico, but it is also one of the most threatened in the country. The central region of Veracruz, in eastern Mexico, presents an archipelago of MMF remnants, which persist under strong pressure from changes in land use. However, despite their current state, these forest fragments still harbor a rich biota, which represent important carbon resource pools (Liu *et al*., 2018) and include a rich

Citation: Castillo-Esparza JF, Montoya L, Desgarennes D, Carrión G, Ramos A, César E, Ortiz-Castro R, Bandala VM. 2023. Bioactivity of *Pseudomarasmius nidus-avis* and other wild fungi from the mesophytic mountain forest of Mexico in the control of phytopathogens. **Agrociencia** https://doi.org/ 10.47163/agrociencia.v57i3.2864

Editor in Chief:
Dr. Fernando C. Gómez Merino

Received: July 21, 2022.
Approved: January 30, 2023.
Published in Agrociencia:
May 04, 2023.

diversity of fungal metabolites with unknown and scarcely investigated properties in Mexico (Espinosa-García *et al*., 2021).

The Bosque de Niebla Sanctuary (BNS), a peri-urban natural protected area under the care of the Instituto de Ecología A.C., corresponds to one of these MMF patches in central Veracruz. It presents a rich community of macrofungi, among which new species of different taxonomic groups have been discovered (Bandala *et al*., 2016; César *et al*., 2018; Montoya *et al*., 2019), most of them endemic and not yet studied in terms of their bioactivity. Such is the case of *Pseudomarasmius nidus-avis*, a recently discovered endemic species of this site, of special interest due to its ecological function (César *et al*. 2018).

This species like other gymnopiodes fungi, forms rhizomorphs, which are structures built by aggregates of linear hyphae with an apical growing point and a melanized cortex (Yafetto, 2018). These structures, resembling long, black horsehair, are important decomposers that bind litter particles and move nutrients in forest soils (Peay *et al*., 2016). Furthermore, several bird species actively collect rhizomorphs to build their nests (Aubrecht *et al.*, 2013), the additional value of this behavior has been explained in terms of a putative antibacterial and antifungal activity of rhizomorphs, which increases the chances of survival of the bird's offspring (Aubrecht *et al*., 2013). However, the potential bioactivity of *P. nidus-avis* has not yet been tested.

There are reports of wild fungi (WF) species with different types of bioactivity, such as antibacterial, antifungal, antiviral, anti-allergenic, anti-inflammatory, antiatherogenic, immunomodulatory, hallucinogenic, and plant growth promoter (Suay *et al*., 2000; Finimundy *et al*., 2014; Sandargo et al., 2019; Castillo-Esparza *et al*., 2021). On the other hand, there are reports of macrofungal species with potential activity against economically important phytopathogenic fungi (PF) (Anusiya *et al*., 2021). Some important PFs in Mexico are *Fusarium oxysporum*, *Sclerotium cepivorum* and *Leptosphaerulina australis*, fungi of common occurrence in agriculturally important crops. *Fusarium* species are soil-borne plant pathogenic fungi that cause vascular wilt and root diseases in a wide range of economically important crops, such as tomato (Gamboa-Becerra *et al*., 2021). *Sclerotium cepivorum* is an ascomycete that can cause white rot, which is one of the most destructive diseases, affecting both onion and garlic bulbs in most parts of the world (Amin *et al*., 2014). On the other hand, *Leptosphaerulina australis* causes serious diseases in economically important crops such as maize, rice, banana, wheat and sugarcane (Phookamsak *et al*., 2013).

This work explores the potential of some species of wild fungi present in the MMF of Veracruz, Mexico, as a control of phytopathogenic fungi that cause damage and economic losses in crops of global importance. In addition, it was considered important to evaluate the possible bioactive properties of *P. nidus-avis* based on César *et al*. (2018). Two types of experiments were conducted: 1) essays using dual culture technique with mycelium, and 2) response of phytopathogenic strains against methanolic extract obtained from the fruiting body, mycelium, and supernatant of the wild fungi culture.

MATERIALS AND METHODS

Sampling and taxonomic identification of wild fungi

Fruiting bodies of 10 wild fungi species were collected during a period between March and June 2019 at BNS, a MMF protected area located at an altitude of 1343 m in Xalapa, central region of the state of Veracruz, on the east coast of Mexico (19° 30′ 46.38″ N, 96° 56′ 32.64″ W). Samples were recorded, labeled and herborized for further study following the recommendations of Lodge *et al.* (2004). Taxonomic identification of the collected specimens was based on macro- and microscopic morphological characteristics according to Moser (1983) and Pegler (1983), among other specialized literature. Microscopic observations were made on dry material mounted with 3% KOH, 1% Congo red aqueous solution and Melzer's solution (Largent *et al*., 1977).

Mycelium isolation

For mycelial isolation, an internal fragment of both the petiole and stipe of the collected fruiting bodies was placed on potato dextrose agar (PDA) medium under axenic conditions (Suay *et al*., 2000). After transferring the fungal tissue to Petri dishes, the cultures were incubated at 20-25 °C in dark conditions for 7-30 days until complete colonization.

Molecular identification

DNA extraction from mycelium isolated from fruiting bodies was carried out according to César *et al.* (2018). Molecular identification was performed by amplification of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA using primers ITS1F, ITS5/ITS4 (Gardes and Bruns, 1993). PCR conditions and amplified products were sequenced according to César *et al.* (2018). The sequences obtained were analyzed using the Blast program and deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank).

Dual tests against phytopathogenic fungi

Tests against PF with WF isolates were performed using a dual culture technique (Kumar and Kaushik, 2013) on PDA, as this medium favors PF growth. Four PF strains were used, provided by the Laboratory of the Pilot Plant for the Development of Biological Control Agents, Instituto de Ecología A.C., which come from three crops of agricultural importance in the region. Two strains of *Fusarium oxysporum* (CBF-185 and ATCC-417) were isolated from tomato (*Solanum lycopersicum* L.). *Stromatinia cepivora* (*Sclerotium cepivorum*) (CBF-414) was isolated from garlic (*Allium sativum* L.) and *Leptosphaerulina australis* (CBF-433) from a local variety of semi-wild bell pepper (*Capsicum annuum* L.) grown in coffee plantations under the shade of MMF tree species. The fungi were cultured and confronted in Petri dishes. Fragments of mycelium of 5 mm in diameter from each fungus, previously growing for seven days, were placed in Petri dishes, keeping 4 cm between the two fungi. Each confrontation was carried out in triplicate. The boxes were incubated at 25 °C in dark conditions for seven days. Subsequently, the plates were observed, and the antagonistic effect was recorded as an inhibition zone was present at the point of interaction of the two strains (Kumar and Kaushik, 2013). Based on Luo *et al.* (2015), six scales were established for the width of the growth inhibition zone (T): T= negative (-) when the PFs exceed the WF, T= positive (+) when there is a barrier between the two fungi, T= (++) if the separation is ≤ 2 mm, T= (+++) when ≤ 5 mm and > 2 mm, T= (++++) when > 5 mm, and ND= not determined.

Selection of culture medium for mycelium growth

Due to the slow growth of some of the WF on PDA, seven different liquid culture media were tested. The *Lentinus crinitus* strain (Cesar 16) was used to select the appropriate medium and obtain higher biomass in less time (Table 1). All culture media were adjusted to a pH of 5.8 before sterilization (121 °C for 20 min). Flasks containing 100 mL of culture medium were inoculated with 15-day-old mycelium culture fragments of 5 mm in diameter. Cultures were incubated at 25 °C for 30 days under dark conditions, with three replicates per treatment. Subsequently, the contents of the flasks were filtered and centrifuged at 4500 rpm (Hermle Z 326 K, Germany) and the biomass weight was recorded.

Extraction of active compounds from fruiting bodies, mycelium and fungal supernatant

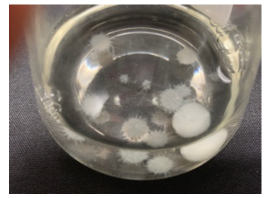
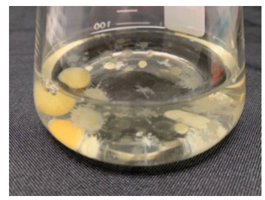
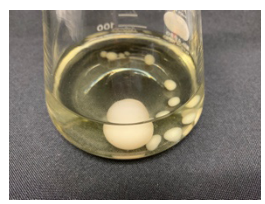
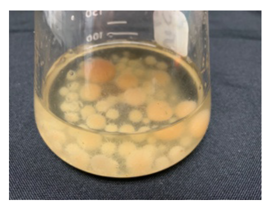
Fruiting bodies were air-dried at 45 °C, ground to a fine powder using the Tissue Lyser II (Qiagen, USA) at 30 Hz s^{-1}, and stored until use. Alternatively, flasks were inoculated with 250 mL of V8 liquid culture medium [30 % V8 juice (Campbell's®) 4 g $CaCo_3$], with 5 mm diameter mycelial fragments from 15 days of previous growth. Cultures were incubated at 25 °C for 30 days under dark conditions and were subsequently filtered and centrifuged at 4500 rpm (Hermle Z 326 K, Germany) to separate the broth from the mycelium (Aqueveque *et al.*, 2016).

The mycelium of the isolated strains was freeze-dried and ground, the supernatant of each culture was frozen and freeze-dried. The crude extract of the samples (fruiting bodies, mycelium and supernatant) was extracted by shaking with methanol (1 mL per 50 mg of sample) at room temperature overnight at 100 rpm, then filtered through Whatman paper (#1). The filtered extracts were concentrated in a Centrivap (Labconco, USA) under vacuum at 30 °C to remove methanol and then lyophilized and weighed. The freeze-dried powders were stored until use (Castillo-Esparza *et al.*, 2021).

Antifungal activity of the methanolic extract of wild fungi

The crude extract of fruiting bodies, mycelium and supernatant of each WF species sample was evaluated against phytopathogenic fungal strains using the agar well diffusion method (Samuel *et al*., 2011). A 5 mm diameter hole was made in Petri dishes with 25 ml of PDA and 100 μL of the crude extract was poured into the hole. Likewise, 100 μL of methanol and dimethyl sulfoxide (DMSO) was used as a negative control, and 100 μL of ketokonazole (16 μg mL^{-1}) was used as a positive control. The extract

Table 1. Mycelium production of Lentinus crinitus in different culture media.

Culture medium	Medium composition (g/L)	Yield in lyophilized weight (mg)a	Figure
Modified Melin-Norkrans medium (Version 1)	$CaCl_2$ 0.05, $MgSO_4.7H_2O$ 0.0358, KH_2PO_4 0.50, NaCl 0.025, malt extract 3.0, sucrose 10.0 and glucose 4.0	19.4 ± 0.77	
Modified Melin-Norkrans medium (Version 2)	Glucose 14.0, malt extract 3.0, $CaCl_2$ 0.05, NaCl 0.025, KH_2PO_4 0.5, $MgSO_4.7H_2O$ 0.15, $FeCl_3$ 1.2	18.6 ± 0.97	
Modified Pridham-Gottlieb medium.	Glucose 10.0, casein peptone 3.33, yeast extract 0.67, KH_2PO_4 0.264, K_2HP0_4 0.628, $MgSO_4.7H_2O$ 0.33, $CuSO_4.5H_2O$ 0.002, $MnCl_2.4H_2O$ 0.0006; $ZnSO_4.7H_2O$ 0.0005 y $FeSO_4.7H_2O$ 0.0004	444.4 ± 8.82	
Medium V8	30 % V8 juice (Campbell's®), $CaCo_3$ 4.0	991 ± 21.08	
Potato and dextrose broth medium	Dextrose 20 and potato infusion 6.5	134.8 ± 7.21	
Modified Melin-Norkrans medium (Version 2) with 10 % V8 juice (Campbell's®)	Melin-Norkrans (Version 2) and 10 % of V8 juice	211.9 ± 25.43	
Pridham-Gottlieb modified with 10 % of V8 juice (Campbell's®)	Pridham-Gottlieb and 10 % of V8 juice	529.5 ± 55.51	

and the controls were diffused in the culture medium overnight at 4 °C. After the diffusion period, an agar disk with 7-day-old mycelia of the phytopathogenic strains was placed on the plates. All tests were conducted in triplicate.

Statistical data analysis

For all experiments, data analysis was performed in SPSS 10 program (SPSS, Chicago, MI, USA). A one-way analysis of variance (ANOVA) with a Tukey's post hoc test was used to test for differences in radial growth responses of PFs.

RESULTS AND DISCUSSION

Wild fungi studied

A total of 30 WF specimens were collected from which 10 pure strains were obtained for confrontation tests against PF (Table 2). It is worth noting that one of the isolates, according to the molecular results, corresponds to the filamentous fungus *Clonostachys* sp., an ascomycete mycoparasite isolated from *Gymnopus iocephalus* that generates interest in the biological control of pests.

Table 2. Wild fungi studied and their antifungal activity against phytopathogenic fungi using the dual culture technique.

Strain	Accession	Taxon	Potential antifungal activity			
			F. oxysporum CBF-185	*F. oxysporum* ATCC-417	*S. cepivorum*	*L. australis*
César 44	MT232391	*Marasmiellus diaphanus* César, Bandala & Montoya	+++	ND	-	ND
César 45	MW173021	*Pseudomarasmius nidus-avis* (E. César, Bandala & Montoya) R.H. Petersen	++++	++++	-	++++
César 47	MW173022	*Marasmius* sp.	-	ND	-	ND
César 49	MT232389	*Gymnopus brunneiniger* César, Bandala & Montoya	++	ND	-	ND
César 16	MW165498	*Lentinus crinitus* (L.) Fr	+++	+++	+	+
Ramos 822	MW173023	*Artomyces pyxidatus* (Pers.) Jülich	-	-	-	-
Ramos 823	MW173024	*Auricularia nigricans* (Sw.) Birkebak, Looney & Sánchez-García	-	-	-	-
Dorantes 06	MW173025	*Daldinia* sp.	-	-	-	-
Montoya 5471b	MW173026	*Clonostachys* sp.	+	+	+	+
Montoya 5472	MW173027	*Panus conchatus* (Bull.) Fr.	-	-	-	-

Width of the growth inhibition zone (T): T= negative (-) when PFs exceed WF; T= (+) when there is a barrier between both fungi; T= (++) when ≤ 2 mm; T= (+++) when ≤ 5 mm and >2 mm; T= (++++) when > 5 mm; ND= not determined.

Effect of wild fungi against phytopathogens

Antifungal activity was present in 5 of the 10 WF evaluated (50 %) by the dual culture test (Table 2), which affected the growth of the phytopathogenic strains. One of the fungi that showed effect is *Clonostachys* sp. (Montoya 5471b), which is characterized by being endophytic and saprotrophic in soil. It can produce volatile organic compounds that are toxic to some fungi, bacteria and even insects (Flores *et al*., 2015). In dual confrontations, *Clonostachys* sp. exhibited a weak inhibitory effect against *Fusarium oxysporum* (CBF-185), *F. oxysporum* ATCC-417, *Leptosphaerulina australis* and *Sclerotium cepivorum* (Table 2). In a previous dual antagonism study (Flores *et al*., 2015), *Clonostachys* sp. showed some antagonism effect against *F. oxysporum*. Their colonization was similar in our study, filling 75 % of the Petri dishes, but our results were recorded considering the width of the inhibition barrier. However, considering the width of the inhibition barrier, it presented a much lower inhibitory effect against *Fusarium* than other species of macrofungi studied, such as *Marasmiellus diaphanus* (César 44) and *Gymnopus brunneiniger* (César 49), although they only inhibited the development of *F. oxysporum* (CBF-185) (Figure 1).

The strains of *Pseudomarasmius nidus-avis* (César 45) and *Lentinus crinitus* (César 16) showed the highest range of inhibition against the phytopathogenic fungal strains used in the present study, compared to the rest of the isolates obtained. These two strains (*P. nidus-avis* and *L. crinitus*) showed that there was a difference in the behavior and growth rate of the phytopathogens: *Fusarium oxysporum* CBF-185, *F. oxysporum* ATCC-417, *Leptosphaerulina australis* and *Sclerotium cepivorum*, in the presence or absence of mycelium of *P. nidus-avis* and *L. crinitus* for nine days (Figures 1 and 2).

In the dual essay, *Lentinus crinitus* (César 16) showed a weak inhibition effect against *Sclerotium cepivorum* and *Leptosphaerulina australis*. This result was superior in the dual confrontation against ATCC-417 and CBF-185 strains of *Fusarium oxysporum* (Table 2). Considering the intrinsic growth rate of each PF, a stronger inhibitory effect was observed against *S. cepivorum* followed by *F. oxysporum* ATCC-417, *F. oxysporum* CBF-185 and *L. australis* (Figures 1 and 2). After nine days of incubation, the phytopathogens were unable to overcome the mycelium of *L. crinitus.*

In the case of *Pseudomarasmius nidus-avis*, a significant effect on the growth rate of three phytopathogens was recorded. The greatest effect was observed against *Fusarium oxysporum* CBF-185, followed by *F. oxysporum* ATCC-417 and *Leptosphaerulina australis*. Confrontations were measured for nine days but were observed for more than 15 days and the phytopathogens did not cross the mycelial barrier of *P. nidus-avis.* The results obtained with the *P. nidus-avis* strain are relevant for the antifungal effect against *Fusarium oxysporum* and *Leptosphaerulina australis*, supporting the observation of César *et al*. (2018) on the probable antagonistic behavior of fungal rhizomorphs of this species on nests of birds belonging to the family Tyrannidae.

Lentinus crinitus and *Pseudomarasmius nidus-avis* showed an effect against three and two PF species, respectively. In the case of *L. crinitus*, despite having a broad spectrum of activity with the three species of PF (Table 2), it is clearly shown that in both strains

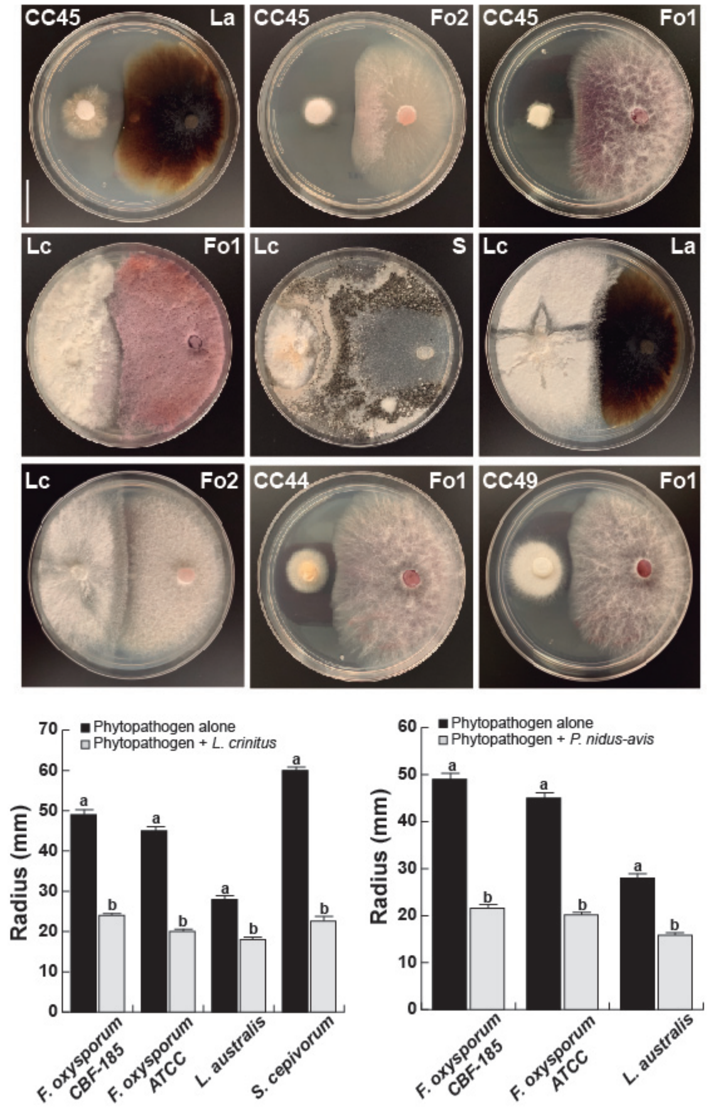


Figure 1. Dual *in vitro* assays between wild fungi isolated from mesophytic forest against phytopathogenic fungi of agricultural interest. CC45: *Pseudomarasmius nidus-avis*; La: *Leptosphaerulina australis*; Fo2: *Fusarium oxysporum* ATCC-417; Fo1: *F. oxysporum* CBF-185; Lc: *Lentinus crinitus*; S: *Sclerotium cepivorum*; CC44: *Marasmiellus diaphanus*; CC49: *Gymnopus brunneiniger*. Different letters indicate significant differences ($p \leq 0.05$).

of *F. oxysporum* (CBF-185 and ATCC-417) there is a greater zone of inhibition with respect to the other two species, so it could be suggested that *L. crinitus* is a candidate for the control of *F. oxysporum*, with the possibility of acting against other non-specific PFs. Therefore, a thorough investigation of the metabolites of this species is necessary to determine which ones are involved in PF inhibition and their possible mechanism of action. On the other hand, *Pseudomarasmius nidus-avis* had the same effect in the zone of inhibition for both PF species (*F. oxysporum* and *Leptosphaerulina australis*).

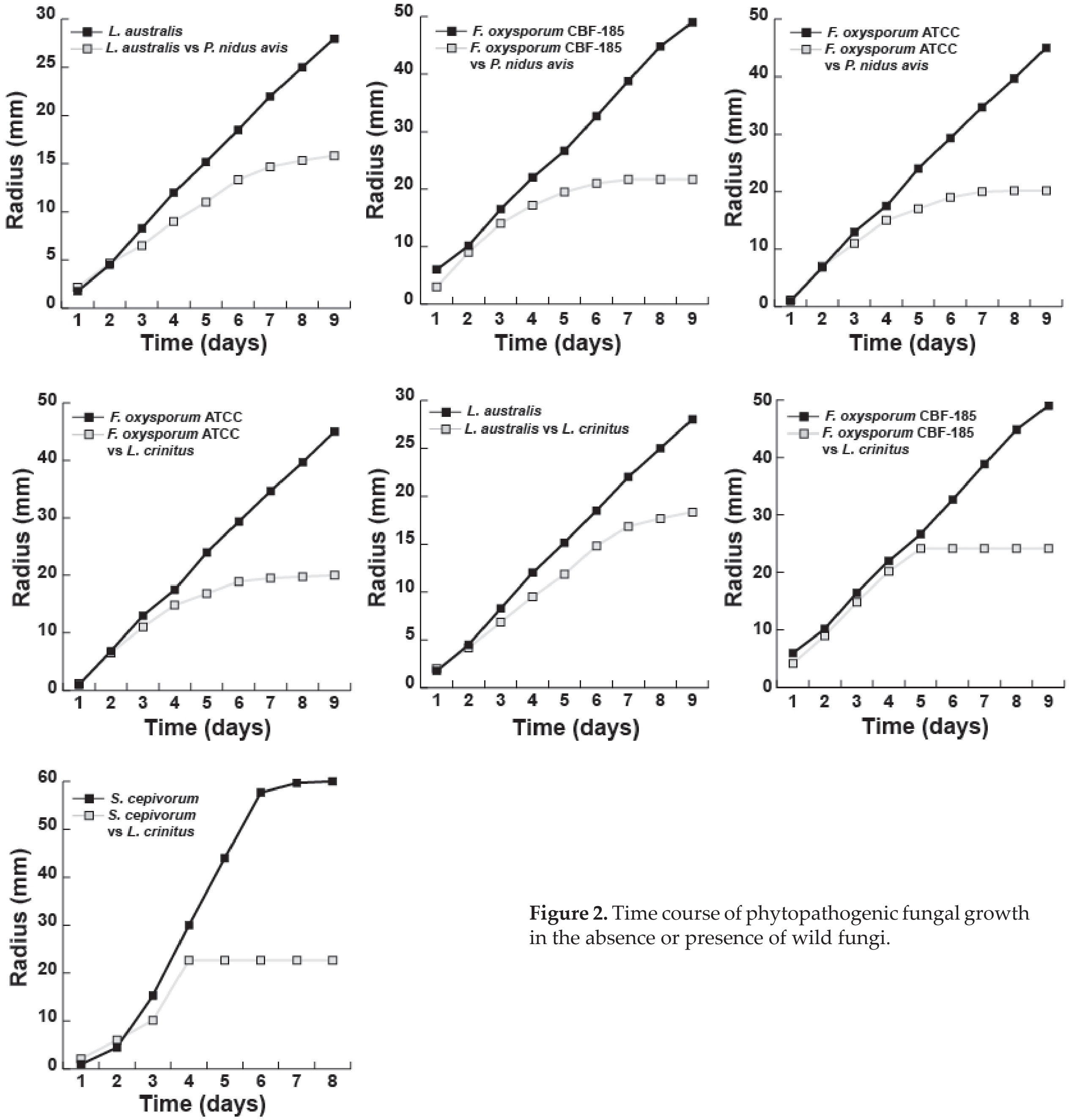


Figure 2. Time course of phytopathogenic fungal growth in the absence or presence of wild fungi.

However, it had no effect on *Sclerotium cepivorum*, suggesting some specificity and efficacy against PF species that may share some characteristics. Therefore, further investigation of the bioactive properties of *P. nidus-avis* and a detailed analysis of the metabolic profile for its use in the control of PF are required.

Mycelium production in liquid culture

The biomass obtained from César strain 16 (*Lentinus crinitus*) was analyzed in seven different liquid culture media, with the V8 medium at 30 % being the one with the highest mycelial yield recorded. Furthermore, the addition of a percentage of V8 to the other media contributed to increased biomass (Table 1). This was clearly observed in the mycelium of César strain 16, with a >10-fold increase in biomass on modified Melin-Norkrans medium (Version 2) with 10 % V8 juice (Campbells), relative to Melin-Norkrans medium (Version 2). Similarly, there was a slight increase in biomass of mycelium grown on modified Pridham-Gottlieb medium with 10 % V8 juice compared to Pridham-Gottlieb medium without V8 juice. With respect to Melin-Norkrans medium (Version 1 and 2) and potato dextrose broth, there was no mycelium production (biomass) comparable to the 30% V8 juice culture medium (Table 1).

Effect of the crude extract against phytopathogenic fungi.

To test the effect of metabolites present in crude extracts against PFs, higher amounts of biomass were produced by probing in different liquid culture media (Table 1). Of the 10 WF, the strains *L. crinitus* (César 16) and *P. nidus-avis* (César 45) were selected for presenting the highest values in the dual clashes against PFs. Due to the scarcity of biomass of the minute fruiting bodies of *P. nidus-avis*, only the isolated mycelium and culture supernatant were analyzed. Methanolic extracts of the fruiting body, supernatant and mycelium of *Lentinus crinitus*, as well as mycelium and supernatant of *Pseudomarasmius nidus-avis*, were tested against four PF strains.

There was no effect of extracts obtained from the fruiting body, mycelium, and supernatant of *L. crinitus* on PF. This could be due to the fact that some secondary metabolites produced by *L. crinitus* are only expressed in the presence of competition for the culture medium, as there are reports of some *Lentinus* species with inhibitory effect against phytopathogens (Rojas-Ramírez, 2013; Vásquez *et al.*, 2018). On the other hand, it should be noted that there is a possibility that the lack of effect of *L. crinitus* extract on PFs is also due to the type of solvent used, since the solvent determines the type of metabolites extracted. Dual confrontation assays revealed that *L. crinitus* has activity against various PFs, which makes it a candidate for use in the control of different fungal diseases. However, it is necessary to determine which metabolites are produced in the presence of other fungi and which ones produce an inhibitory effect on phytopathogens.

On the other hand, the methanolic extract of *P. nidus-avis* mycelium tested against the four PFs had no effect. In contrast, the *P. nidus-avis* supernatant extract showed an inhibition effect of 16.08 % against *F. oxysporum* ATCC-417 (Figure 3A and 3B). This result is relevant since *F. oxysporum* strain ATCC-417 is responsible for wilting and death of plants characterized by secreting effector proteins in the xylem, called SIX (Secreted in Xylem), which contribute to its virulence. Growth inhibition of *F. oxysporum* (ATCC-417) with the methanolic extract of *P. nidus-avis* culture supernatant shows an interesting potential for biological control of this fungus containing nine of the fourteen *SIX* genes (Gamboa-Becerra *et al.*, 2021).

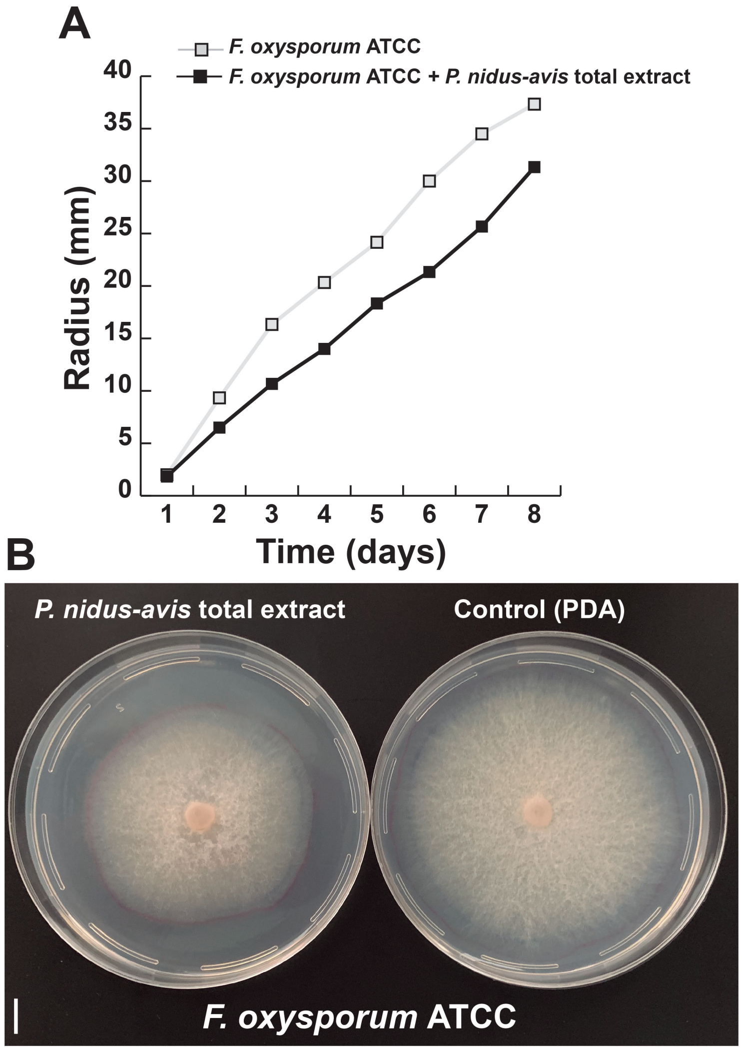


Figure 3. Time course of *F. oxysporum* ATCC-417 growth in the absence or presence of *Pseudomarasmius nidus-avis* supernatant extract. A: Growth inhibition of *F. oxysporum* ATCC-417 in the presence of 100 μL of the crude extract of the supernatant of *P. nidus-avis*; B: Representative photographs of the growth of *F. oxysporum* ATCC-417 in the presence of crude extract of *P. nidus-avis* supernatant.

It is known that fungi can modify the production of secondary metabolites depending on several characteristics, such as the medium in which they grow, incubation conditions, C:N sources and concentrations, and the extraction solvent. Competitive behavior and damage can also affect their secondary metabolic profile (Bertrand *et al*., 2014). The differences observed in this study between the effect of dual confrontations and methanol extractions could be associated with some of these conditions.

CONCLUSIONS

Due to the harmful effects of synthetic pesticides, it is urgent to find new alternatives for pest control agents of biological origin and low environmental impact. The present results suggest that MMF WF species have potential for application in agriculture. In particular, *Pseudomarasmius nidus-avis*, a fungus endemic to Veracruz, has great potential for use in the biological control of pests caused by microorganisms.

P. nidus-avis showed a greater antifungal effect against *Leptosphaerulina australis* and two strains of *Fusarium oxysporum* than the other wild fungi tested. Furthermore, the methanolic extract obtained from the supernatant of *P. nidus-avis* also had an inhibitory effect on the development of strain ATCC-417, which by containing 9 *SIX* genes makes it one of the most pathogenic strains. Therefore, it would be of great interest to investigate the mechanism of action of *P nidus-avis*, in addition to its spectrum on other phytopathogens. The current tests and results proved the bioactivity of *P. nidus-avis*, which could provide some evidence on the advantages of the selection of this fungal species in the interaction with birds of the family Tyrannidae during nesting.

ACKNOWLEDGMENTS

We thank Dr. Roberto Gamboa-Becerra, M. Sc. Nut Liahut-Guin, M. Sc. Zelene Durán-Barradas for her support in the isolation, micro- and macroscopic and molecular characterization of phytopathogenic fungi and to Biol. David Ramos Rendón for technical assistance. This research was supported by grants CB 252431 for the study of tropical fungal species in Veracruz, Mexico, FORDECYT-PRONACES/CF 263959 and PDCPN 2015-882 from CONACyT-Mexico.

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