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In vitro suppression of fungal root pathogens in *Annona muricata* L. by *Trichoderma* strains and conventional fungicides

Supresión *in vitro* de patógenos fúngicos de raíz en *Annona muricata* L. por cepas de *Trichoderma* y fungicidas convencionales

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ABSTRACT

Fusarium falciforme and *Lasiodiplodia theobromae* are the main root and stem fungal pathogens in soursop trees (*Annona muricata* L.) in Nayarit, Mexico. Regional fruit producers do not have effective strategies to control this pathology. Therefore, the main objective of the research was to evaluate the *in vitro* biocontrol potential of strains of *Trichoderma* antagonists and the efficacy of conventional fungicides. Thirteen *Trichoderma* strains were evaluated *in vitro* against *F. falciforme* and *L. theobromae*. Also, the *in vitro* sensitivity of both fungal pathogens to eight commercial fungicides was evaluated. The strains of *T. hamatum* and *T. asperellum* inhibited the *in vitro* mycelial growth of *F. falciforme* up to 75.31%. On the other hand, Mancozeb was the only fungicide that completely inhibited the *in vitro* mycelial growth of both phytopathogens. Obtained data suggest the potential use of both control strategies for the suppression of root and stem diseases in soursop. It is recommended to conduct additional studies on the possible synergistic effects of the combination of both strategies in preventing and controlling this fungal disease in *A. muricata*.

KEY WORDS: Antagonism, inhibition, molecules, soursop, *Trichoderma*.

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ABSTRACT

Fusarium falciforme y *Lasiodiplodia theobromae*, son los principales patógenos fúngicos en raíz y tallo en árboles de guanábana (*Annona muricata* L.) en Nayarit, México. A la fecha los productores de este frutal en la región no cuentan con estrategias efectivas para el control de esa patología. Por lo que, el objetivo de la investigación fue evaluar el potencial de biocontrol *in vitro* de cepas de antagonistas de *Trichoderma* y la eficacia de fungicidas convencionales. Se evaluaron *in vitro* 13 cepas de *Trichoderma* contra *F. falciforme* y *L. theobromae*. Así mismo, se evaluó la sensibilidad *in vitro* de ambos patógenos fúngicos a ocho fungicidas comerciales. Las cepas de *T. hamatum* y *T. asperellum* inhibieron el crecimiento micelial *in vitro* de *F. falciforme* hasta un 75.31 %. Por otra parte, Mancozeb fue el único fungicida que inhibió por completo el crecimiento micelial *in vitro* de ambos fitopatógenos. Los resultados sugieren el potencial uso de ambas estrategias de control para la supresión de estas enfermedades radiculares y del tallo en guanábana. Se sugiere conducir estudios adicionales relacionados con los posibles efectos sinérgicos de la combinación de ambas estrategias en la prevención y control de esta enfermedad fúngica en *A. muricata*.

KEY WORDS: Antagonismo, inhibición, moléculas químicas, guanábana, *Trichoderma*.

Introduction

In Mexico, up to 30 790 tons of soursop (*Annona muricata* L.) (Annonaceae) are produced and there is a total area of 3 612 ha established, distributed in 10 states, wherein Nayarit is positioned as the main producer, with an area of 2 456 ha and 23 230 t (SIAP, 2019). Due to its bittersweet, pleasant, and aromatic flavor, the *A. muricata* fruits are used in the food industry for fresh consumption, in fruits combinations, or industrialization (Sosa *et al*., 2022), likewise, this fruit tree has benefits for human health, as it can favor digestion and has preventive and curative effects for obesity, hypertension, cancer, and heart disease (Clement *et al*., 2016; Leiva *et al.,* 2018).

However, the quality and size of *A. muricata* fruits are affected by diverse diseases caused by phytopathogens such as *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. and *C. theobromicola* Declar. which cause anthracnose in inflorescences, leaves, and stems (Álvarez *et al*., 2004; Betancourt *et al*., 2019), and some strains of *Colletotrichum* spp., *L. pseudotheobromae, Pestalotiopsis* sp. and *Rhizopus oryzae*, which cause soursop fruit rot (Álvarez *et al*., 2004; Cambero et *al*., 2019). These affectations are in addition to those caused by other fungal pathogens such as the fungi *Fusarium falciforme* (Hypocreales, Nectriaceae) (belonging to the *F. solani*

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species complex) and *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl. (Botryosphaeriales, Botryosphaeriaceae) have recently been identified as root and stem pathogens causing necrosis, individually or as a complex, in soursop crops in Compostela and San Blas, Nayarit, Mexico (Cambero *et al*., unpublished data); trees affected by these pathogens, as a consequence, decrease its production, unfortunately, the affectations by these fungal pathogens have not yet been estimated in Nayarit.

One of the first actions implemented for the control of fungal diseases in fruit crops, including soursop, is the use of chemical fungicides (commonly broad spectrum). In commercial soursop plantations in Nayarit state, growers commonly use methyl thiophanate, captan, Mancozeb, or copper hydroxide to reduce the effects of fungal diseases, however, available data on the use of fungicides for the suppression of phytopathogenic fungi in soursop is limited, and there are no available records at COFEPRIS for its use (COFEPRIS, 2023).

Based on the negative impacts of fungal pathogens on soursop in Nayarit and the low effectiveness of chemical fungicides commonly used for their control, it is of utmost importance to look for strategies to reduce the losses caused by such pathogens in this fruit tree. Therefore, using microbial biocontrol agents such as *Trichoderma* and new chemical fungicides could be a promising alternative for effectively controlling *F. falciforme* and *L. theobromae.*

The antifungal capacity of *Trichoderma* has been widely documented against plant pathogenic fungi, including members of the genera *Fusarium* (*F. oxysporum, F. nygamai, F. oxysporum* f. sp. *ciceri,* among others) (Martinez *et al*., 2018; Michel *et al*., 2018; Michel *et al*., 2019) and *Lasiodiplodia* (*L.* pseudo theobromae, *L. theobromae, L. citricola,* among others) (Valle *et al*., 2019; Cambero *et al*., 2020). Thus, in search of viable alternatives for the management of root diseases of soursop crops in Nayarit, this study aimed to evaluate the *in vitro* sensitivity of *Fusarium falciforme* and *Lasiodiplodia theobromae* to conventional fungicides and the inhibition of its mycelial growth by *Trichoderma* strains.

Material and Methods

Reagents, microbial strains, and fungicides

All used reagents (analytical grade) were purchased from Sigma Aldrich Corp. (St. Louis, MO, USA). *Trichoderma* isolates were obtained from soil near the root zone of healthy soursop trees in Compostela and San Blas, Nayarit, Mexico. *Trichoderma* sp., *T. longibrachiatum*, *T. asperellum,* and *T. harzianum,* provided by the Centro de Investigación en Alimentación y Desarrollo, A.C. (CIAD, A.C.), Unidad Cuauhtémoc, Chihuahua, were also included. The chemical fungicides evaluated were obtained from local commercial stores.

Strains of *Lasiodiplodia theobromae* and *Fusarium falciforme* were isolated and morphologically and molecularly characterized [Cambero *et al*. (unpublished data)], from root tissues of diseased soursop trees from San Blas and Compostela, Nayarit, Mexico.

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Trichoderma isolation and identification

Antagonistic microorganisms were isolated from soil near the rhizosphere of healthy-appearing soursop trees. In July 2020, in six commercial soursop orchards (Table 1), soil samples (500 g per tree) were collected from five trees located at the cardinal points and the center, subsequently, homogenized, and finally 200 g per orchard were recovered, and placed in transparent plastic bags (25 × 35 cm) for processing at the Laboratorio de Parasitología Agrícola of the Centro Multidisciplinario de Investigación Científica 03 (CEMIC-03) of the Universidad Autónoma de Nayarit (UAN).

Table 1. Sites for collection of rhizospheric soil samples from *Annona muricata* trees in Nayarit, Mexico.

MUNICIPALITY	ORCHARD	COORDINATES	ALTITUDE
Compostela	Capomo I	N 21°07'08" W 105°10'02"	36
	Divisadero I	N 21°07'18" W 105°11'33"	94
	Tonino I	N 21°04'02" W 105°12'48"	335
San Blas	Infiernillo I	N 21°32'21" W 105°11'06"	175
	La Palma I	N 21°31'00" W 105°11'57"	9
	La Palma II	N 21°32'00" W 105°10'43"	213

Trichoderma isolation was done by serial dilutions (1:10) of the soil. Aliquots of 50 µL of the dilutions 10^{-4} to 10^{-6} were sown in Petri dishes (90 × 15 mm) with Potato-Dextrose-Agar (PDA) culture medium. The fungal microorganisms that showed antagonistic activity were purified using monohyphal cultures (Moreno & Albarracín, 2012; Pérez *et al*., 2015; Méndez *et al*., 2017). All fungi with purified antagonistic qualities were preliminarily tested *in vitro* for their antifungal activity against *F. falciforme* and *L. theobromae*. Isolates that did not show antifungal properties were not considered in subsequent *in vitro* tests (Mendoza *et al*., 2020).

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In vitro biocontrol tests on *Trichoderma* strains

The ability of *Trichoderma* strains to suppress *in vitro* the fungal pathogens *F. falciforme* and *L. theobromae* was evaluated through an *in vitro* Dual Confrontation Assay. Both microorganisms (antagonists and pathogens) were confronted in Petri dishes (90 × 15 mm) containing solidified PDA medium. In the *Trichoderma* confrontation assays, explants (PDA with mycelium and conidia with 7 d of growth) of 6 mm in diameter of both the antagonist and the pathogen were used. Explants of both groups of microorganisms were simultaneously inoculated into the ends of Petri dishes containing PDA culture medium (Bell *et al*., 1982). In these confrontations, the level of antagonism was determined according to the scale proposed by Bell *et al*. (1982) and the type of antagonism (antibiosis, mycoparasitism, and/or competition) according to Infante *et al*. (2009).

Experimental Petri dishes were incubated at 24 ± 2 °C in darkness until the control pathogen filled the Petri dish. Mycelial growth of pathogenic fungi (alone and confronted) was measured daily until the control pathogen filled the Petri dish (Rios *et al*., 2016).

In vitro susceptibility testing of *F. falciforme* and *L. theobromae* to conventional fungicides

The poisoned medium technique was used according to Alburqueque & Gusqui (2018), on solidified PDA + fungicide at the dose recommended by the manufacturer (Table 2) in Petri dishes (90 × 15 mm), 6 mm diameter explants (with surface growth of mycelium and conidia of 7 d of growth) of the pathogenic fungus were placed. Petri dishes with PDA without fungicide were used as controls. The experimental Petri dishes were incubated at 24 ± 2 °C in the dark until the control filled the Petri dish.

Molecular identification of antifungal *Trichoderma* isolates

Only the strains (T62, T1, and T10) of *Trichoderma* that inhibited mycelial growth of fungal pathogens by more than 60 % were molecularly characterized. *Trichoderma* genomic DNA (gDNA) was extracted according to the methodologies described by Allers & Litchen (2000) and Bobadilla *et al*. (2020), from fungal mycelium with 3 d of growth grown in liquid medium (broth) PDB (Potato Dextrose Broth). DNA quality was visualized by electrophoresis on a 1% agarose gel.

DNA was used to amplify the internal transcribed spacer (ITS) of rDNA, with the universal primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCTCCTCCGCTTATTGATATATGC-3'), an initial step at 94 °C/5 min, 30 cycles at 94 °C/30 s, 60 °C/30 s, 72 °C/10 min and final elongation at 72 °C/4 min were employed (White *et al*., 1990; Ruíz *et al*., 2017). Detection of PCR products was performed by 1.5 % agarose gel electrophoresis for 110 min at 80V. The 100 bp molecular weight marker (Invitrogen by Thermo Fisher Scientific®) was used. PCR products were sequenced by Macrogen company in Beotkkot-

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ro, Geumcheon-gu, Seoul (Gasan-dong, World Meridian I), South Korea. The sequences obtained were compared with those available in the National Center for Biotechnology Information (NCBI, 2022) database by BLAST algorithm, and finally deposited in the NCBI database.

Table 2. Active ingredients of conventional fungicides used in the *in vitro* assays against *Lasiodiplodia theobromae* and *Fusarium falciforme*, both root and stem fungal pathogens on *Annona muricata*.

ACTIVE INGREDIENT	RECOMMENDED DOSAGE	CHEMICAL GROUP
Mancozeb	12.5 g/L	Dithiocarbamate
Pidiflumethofen+Difenoconazole	5 ml/L	Triazole, Carboxamides
Chlorothalonil	15 ml/L	Chloronitrile
Carbendazim	0.5 g/L	Benzimidazole
Cyprodinil+Fludioxonil	0.75 g/L	Fenilpiroles, AP
Captan	25 g/L	Phthalimide
Mancozeb+Azoxystrobin	25 g/L	Carbamates, Strobilurin
Boscalid+Pyraclostrobin	4 g/L	Pyridinecarboxamides, Methoxy-carbamates

Statistical analysis

In the *in vitro* confrontations (*Trichoderma* and fungicides vs. *F. falciforme* and *L. theobromae*), nine Petri dishes were used in each confrontation, and a control group (culture of the phytopathogens in the absence of the antagonists-fungicides). In both fungal pathogens, Mycelial Growth Inhibition (MCI) by *Trichoderma* was evaluated, with the formula MCI= (R1-R2) /R1 ×100, where R1 is the radius of the control pathogen (*F.* falciforme-L*. theobromae*) and R2 is the radius of the pathogen in the confrontation (Rios *et al*., 2016). All experiments were conducted in triplicate independently. MSI data were subjected to analysis of variance (ANOVA) with Statistical Analysis System version 9.0, 2002 (SAS, 2002), and means were separated with Tukey's range test (p= 0.05).

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Results and Discussion

Isolation and *in vitro* confrontation of antagonistic microorganisms

A total of 12 fungi of the genus *Trichoderma* were isolated, 10 isolated from Compostela and two from San Blas, of which, in preliminary *in vitro* tests, only nine showed antagonistic activity against *Fusarium falciforme* and *Lasiodiplodia theobromae*. Different species of antagonists may exist, since the rhizosphere is considered a complex ecosystem, with a great diversity of microorganisms (Sokolova, 2015), where climatic or soil conditions are determinants for the survival of microbial communities (Ordóñez *et al*., 2020).

Inhibition of mycelial growth *in vitro* of *Fusarium falciforme* and *Lasiodiplodia theobromae* by *Trichoderma*

The strains evaluated showed antifungal potential against *F. falciforme* and *L. theobromae,* after 13 and two days of incubation, respectively (Figure 1). The mycelial growth inhibition values by the *Trichoderma* strains ranged from 0 to 21.72 % for *L. theobromae* and 32.13 to 75.31 % for *F. falciforme. Trichoderma* isolates T1, T10, and T62 native to soils near the root zone of soursop trees inhibited the growth of *F. falciforme* by more than 64.05 %, but against *L. theobromae* the growth inhibition of these three *Trichoderma* strains was less than 11.84 %. Isolate T62 was the most effective in inhibiting up to 75.31 % of the mycelial growth of *F. falciforme* (Figure 1b). The inhibition values of *Trichoderma* on *F. falciforme* in our study are among the ranges reported on other *Fusarium* species, Rios *et al*. (2016) reported mycelial growth inhibitions on *F. oxysporum* from 43.3 to 52.3 %, by *T. asperellum* strains. On the other hand, Cambero *et al*. (2020) recorded mycelial growth inhibitions of 49.5 % to 57.9 % against *L. pseudotheobromae* with *Trichoderma* strains (*T. longibrachiatum, T. harzianum,* and *T. asperellum*) provided by CIAD, A.C., Unidad Cuauhtémoc, Chihuahua.

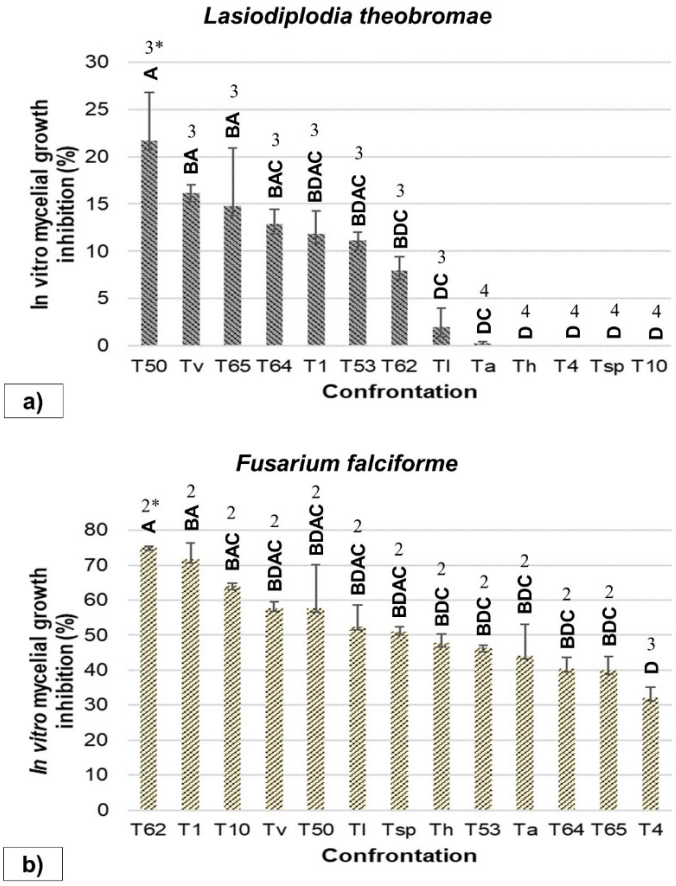

Figure 1. *Trichoderma* isolates inhibit the mycelial growth of pathogenic fungi *Lasiodiplodia theobromae* and *Fusarium falciforme.*

Equal literals within the same column show statistical equality according to Tukey's test (p = 0.05). 2*, 3*, and 4*= Level on the Bell *et al*. (1982) scale.

Similarly, Bhadra *et al*. (2014) reported mycelial growth inhibitions of *L. theobromae of* up to 80 % with *T. koningii* and *T. viridae* attributing the antifungal capacity of these strains to mycoparasitism and its rapid growth, allowing them to compete for space and available nutrients. The mycelial growth inhibition values of *L. theobromae* by *Trichoderma* were lower (Figure 1a) than those documented against *F. falciforme,* this is possible since in this study *L. theobromae* could grow rapidly and fill the Petri dish in only two days after sowing, having a faster growth than *Trichoderma* spp. In the same way, the isolates of native *Trichoderma* presented higher percentages of mycelial growth inhibition than those evidenced by the strains provided by CIAD A.C., suggesting that they are more likely to adapt to the climatological conditions of the producing zones of *A. muricata* in Nayarit.

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Trichoderma suppresses fungal pathogens through different mechanisms, such as mycoparasitism, antibiosis, as well as, space, light, and nutrient competition, as well as plant growth stimulation (Martínez *et al*., 2013). Competition for space and available nutrients were the biocontrol mechanism evidenced in our *Trichoderma* strains on the pathogens *L. theobromae* and *F. falciforme*. Fungi of the genus *Trichoderma* have been reported as successful microbial biocontrol agents of phytopathogenic *Fusarium* species (Sánchez *et al*., 2017), such as *Fusarium oxysporum* f. sp. *cubense, F. roseum (*Infante *et al*., 2009; Schuster & Schmoll, 2010) and *Lasiodiplodia* such as *L. pseudotheobromae* (Cambero *et al*., 2020).

In vitro sensitivity of *F. falciforme* and *L. theobromae* to conventional fungicides

In vitro, the sensitivity of pathogens to the fungicides tested is shown in Figure 2. Both pathogens showed sensitivity to the eight fungicides tested, with mycelial growth inhibitions of over 74.55 %. *Fusarium falciforme* and *L. theobromae* were completely inhibited (100 %) only with the fungicide Mancozeb, possibly as it belongs to the dithiocarbamate group, capable of inhibiting motor proteins and multisite activity (Medina *et al*., 2022). The fungicides Cyprodinil+Fludioxonil and Mancozeb+Azoxystrobin inhibited the mycelial growth of *L.* theobromae by 100 %. *Fusarium falciforme* was less sensitive to the fungicides Boscalid+Pyraclostrobin and Chlorothalonil (Figure 2).

Piñeros *et al*. (2019) evidenced high sensitivity (100 % MGI) in *F. subglutinans* and *F. graminearum* to the fungicides Prochloraz+Difenoconazole, Carboxin+Captan and Tebuconazole+Trifloxystrobin. Michael *et al*. (2018), obtained MGI of 76.67 % on *Fusarium oxysporum* f. sp. *gladioli* with the fungicide Metalaxyl+Chlorothalonil. Tovar *et al*. (2013) proposed the use of Cyprodinil+Fludioxonil, Pyraclostrobin+Boscalid, Prochloraz, Tebuconazole, and Iprodine as the most effective fungicides for *in vitro* control of *L. theobromae* on mamey. Fungicides belong to different chemical groups, therefore, they have different mechanisms of action, they can inhibit mycelial growth, conidial germination, and respiration, and alter the cell membrane, among others (FRAC, 2020), however, in response to the pressure exerted by fungicides, plant pathogenic fungi can acquire resistance or lose sensitivity to them (Li *et al*., 2020). To prevent pathogens from developing resistance to fungicides, it is suggested to use systemic and contact fungicides in rotation or combination (Denman *et al*., 2004).

Both fungal pathogens (*F. falciforme* and *L. theobromae*) showed mycelial growth 10 d after being treated with the fungicides, evidencing only fungistatic action by Mancozeb+Azoxystrobin, Boscalid+Pyraclostrobin, Pidiflumethofen+ Difenoconazole and Chlorothalonil, possibly these fungicides do not completely inhibit conidia germination of these causal agents, which is essential for successful chemical control of pathogenic fungi in plants (Shin *et al*., 2014), the development of resistance to fungicides may be one of the main reasons (Moreira *et al*., 2021).

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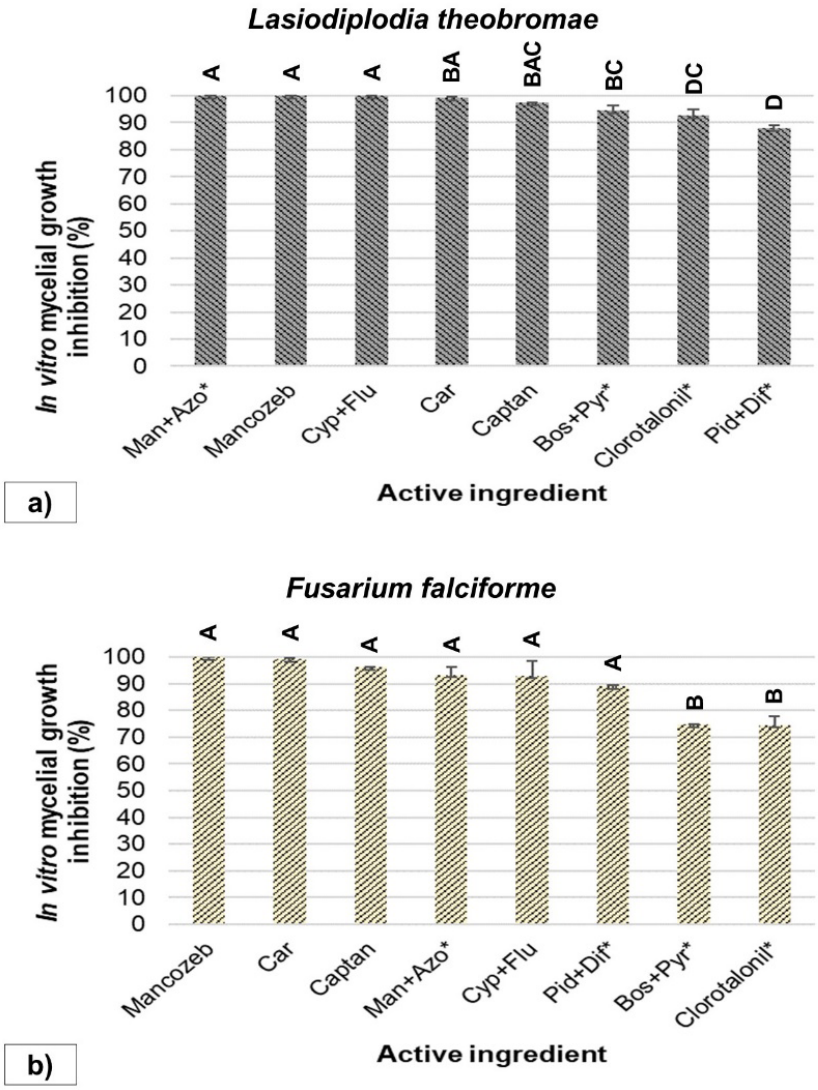

Figure 2. *In vitro* sensitivity of *Lasiodiplodia theobromae* and *Fusarium falciforme* to conventional fungicides.

Means (± standard error) with equal literals indicate statistical equality according to Tukey's range test ($p = 0.05$). Man+Azo = Mancozeb+Azoxystrobin; Cip+Flu = Cyprodinil+Fludioxonil; Bos+Pir = Boscalid+Piraclostrobin; Pid+Dif = Pidiflumetofen+Diphenoconazole; Car = Carbendazim; *The microorganism evaluated against this fungicide continued with very slow growth (no measurement).

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These control alternatives can be considered in integrated disease management and a timely diagnosis will allow us to have better results. On the other hand, the use of microorganisms can be applied preventively, and fungicides can be used only in cases where the problem begins to exceed the economic threshold.

Molecularly identified native antifungal *Trichoderma* isolates

Nine *Trichoderma* isolates were obtained from the municipalities of Compostela and San Blas, Nayarit, Mexico, only the isolates (T62, T1, T10) were identified as inhibiting >60 % mycelial growth of *Fusarium falciforme*. According to its molecular features and when compared with NCBI database available sequences, 100% similarity of isolates T62 with *T. hamatum,* T1 with *T. asperellum,* and isolate T10 with *T. asperellum* was observed. Sequences of these isolates were registered in GenBank under the following accession numbers (OQ696060, OQ696061, and OQ696062, respectively). It is worth mentioning that this is the first record of these antagonists in commercial soursop orchards in Nayarit, Mexico. Strains of *T. hamatum,* have been identified in Catamarca, Argentina, with antagonistic capacity against *Verticillium dahliae* KLEB (Gonzáles *et al*., 2021), on the other hand, *T. asperellum* has been identified in Peru in avocado (*Persea americana* Mill.) orchards with biocontrol potential against *Phytophthora cinnamomi (*Morales *et al*., 2020), although there is a wide diversity of *Trichoderma* species such as *T. asperellum, T. atroviridae, T. koningiopsis, T. piluliferum, T. viridae* among others (Sánchez *et al*., 2018).

Conclusions

Trichoderma spp. isolates successfully inhibited *F. falciforme. Trichoderma hamatum* (T62) inhibited the mycelial growth of *F. falciforme* to *a* greater extent. *Lasiodiplodia theobromae* was less susceptible to *Trichoderma* strains. On the other hand, both fungal pathogens were inhibited 100 % by Mancozeb, while *Lasiodiplodia theobromae* was also completely inhibited by Cyprodinil+Fludioxonil. The *in vitro* antifungal effects on *F. falciforme* and *L. theobromae* of both *Trichoderma* and the evaluated fungicides are promising. Therefore, it is suggested to conduct more antifungal *in situ* or field experiments to demonstrate the same antifungal efficacy.

Contribution of the authors

C. A. C. B. Methodology development, data management; **R. V. C.** Experimental validation; **L. E. G.** Experimental validation; **L. G. G. G.** Methodology development; **E. V. M. O.** Conceptualization of the work, experimental validation; **C. C. O. J.** Fundraising, project manager, experimental validation.

“All authors of this manuscript have read and accepted the published version of it”.

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Interest conflict

“The authors declare no conflict of interest”.

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