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Fusarium solani associated with *Cedrela odorata* and *Swietenia macrophylla* and their sensitivity to conventional fungicides

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ABSTRACT

Background / Objective. In the state of Yucatan, Mexico, 10 million forest plants were produced in the last five years for various conservation and restoration actions. The main limitations in the production of these plants in nursery are disease induced by the genus *Fusarium* spp., that cause stem and root rots and plant production losses of up to 50%. The objective of the work was to identify the causal agent associated with stem and root rot and necrosis of cedar (*Cedrela odorata*) and mahogany (*Swietenia macrophylla*) and their *in vitro* sensitivity to conventional fungicides.

Materials and Methods. *C. odorata* and *S. macrophylla* plants were collected at three and six weeks of germination, respectively, with symptoms of necrosis and rot indicated; from where five fungal isolates were obtained and morphologically and molecularly identified. The Minimum Inhibitory Concentration (MIC) of spores and the Minimum Lethal Concentration (MLC) of six conventional fungicides of recurrent application in the region (Prochloraz, Carbendazim, Benomyl, Fosetyl Al, Captan and Mancozeb) were determined *in vitro* by the microdilution method and validate their effectiveness and viability in the management of this problematic.

Results. The morphology and molecular sequences of the isolates were similar to the reported for *Fusarium solani*. The MIC of *F. solani* spores for Prochloraz, Carbendazim, Benomyl, Captan and Mancozeb were 2.44. 11.38, 14.06, 7.81

and 7.81 ppm, respectively; Fosetyl Al, did not inhibit spore germination normal mycelial growth of the fungus was observed at the concentration evaluated.

Conclusion. Prochloraz and Mancozeb had the lowest MLC with 2.44 and 7.81 ppm, respectively.

Key words: cedar, mahogany, root of rot, nursery, chemical control

Introduction

In Mexico, approximately 1 billion forest plants were produced over the last five years, including species from arid, temperate, and tropical climates. Of this production, the state of Yucatán produced around 10 million plants for various reforestation or restoration efforts. The main species produced were *C. odorata*, *S. macrophylla*, *Tabebuia rosea*, *Brosimun alicastrum*, and *Cordia dodecandra* (SNIGF, 2021). One of the limiting factors in the production process is stem and root rot diseases caused by *Fusarium* spp. (Kisekka, 2022). According to information provided by nursery growers, production losses range from 30 to 50%.

The genus *Fusarium* has approximately 1,779 species with a cosmopolitan distribution (Sun *et al*., 2023). They are ubiquitous in nature, as they can tolerate any environmental condition. They can be found in air, water, soil, plants, and plant debris. A large number of its species are plant pathogens, producers of mycotoxins, and induce a wide range of diseases that affect various crops. The symptoms they cause mainly include root and stem rot, cankers, vascular wilt, and fruit and seed rot (Ekwomadu and Mwanza, 2023).

In nurseries of temperate regions, characterized by a humid or subhumid climate with average temperatures between 12 and 18 °C and annual precipitation between 600 and 1500 mm (GeoEnciclopedia, 2022), the vegetation consists of pine forests, oak forests, and pine-oak forests (Galicia *et al*., 2018). In this context, *F. circinatum* in pine plants causes the main disease known as "pitch canker," characterized by root rot, reducing plant quality and leading to production losses of up to 40% (García-Díaz *et al*., 2017). In tropical regions, where the temperature is above 18 °C and annual precipitation ranges from 800 to 4000 mm (GeoEnciclopedia, 2022), and the vegetation consists of various species of evergreen, sub-evergreen, deciduous, and sub-deciduous types (Secretaría de Medio Ambiente y Recursos Naturales, 2017), *Fusarium* sp. has been identified as responsible for stem rot in *Eucalyptus pellita* (Arsensi and Mardji, 2021) and *F. solani* as the causal agent of root rot in *Tectona grandis* (Kiran *et al*., 2021).

In Mexico, information on the diagnosis and etiology of the causal agents of fungal diseases observed in tropical forest nurseries is scarce, as well as on the

methods used for their control. Currently, the control of these diseases in forest plants growing in nurseries relies on synthetic fungicides due to their rapid response in controlling the pathogen and their ease of application (Kisekka, 2022). However, applications are sometimes unsuccessful because no prior sensitivity studies are conducted *in vitro* and *in vivo* with the available local fungicides.

In plant production in nurseries, good phytosanitary conditions are the foundation of any production or reforestation project, as planting diseased plants in the field lowers their chances of survival and makes them sources of inoculum for healthy plants. To develop an effective control method for the phytosanitary problems affecting nursery plants, identifying the causal agents is essential. This knowledge allows for the design of efficient control strategies and the application of specific products for their management, thereby also reducing environmental contamination. Thus, the objective of this work was to identify the causal agent associated with stem and root rot and necrosis of *C. odorata* and *S. macrophylla* and its *in vitro* sensitivity to conventional fungicides.

Materials and Methods

Sample collection. In 2023, at the Mexico Primero nursery located at 88° 58′48.15¨ W and 19° 59′10.56¨ N, in the municipality of Tzucacab, Yucatan, plants of *C. odorata* and *S. macrophylla* were collected at three- and six-weeks post-germination, respectively, showing symptoms of stem and root base necrosis and rot (Figure 1). Samples were placed in brown paper bags, then transferred to plastic bags, labeled, and transported to the Phytopathology Laboratory of the National Technological Institute of Mexico, Campus Conkal, for isolation and identification of the causal agent(s).

Isolates from Symptoms. The stems and roots of plants showing symptoms of necrosis and rot were rinsed with tap water to remove substrate residues. Approximately 0.5 to 1 cm² cuts were made from the damaged tissue. To eliminate external contaminants, they were disinfested with 2% commercial sodium hypochlorite for 2 minutes. Subsequently, they were rinsed three times with sterile distilled water for 2 minutes each and dried with sterile absorbent paper. Finally, five fragments of approximately 0.5 to 1 cm² were placed in Petri dishes with Potato Dextrose Agar (PDA) medium and incubated at 28±2 °C. After 48 hours, mycelial growth was observed and re-isolated onto PDA culture plates. The re-isolated mycelial growths were purified using hyphal tips and subsequently from spores, monosporic cultures were initiated (Ríos-Hernández *et al*., 2021), yielding three isolates of *C. odorata* and two isolates of *S. macrophylla*.

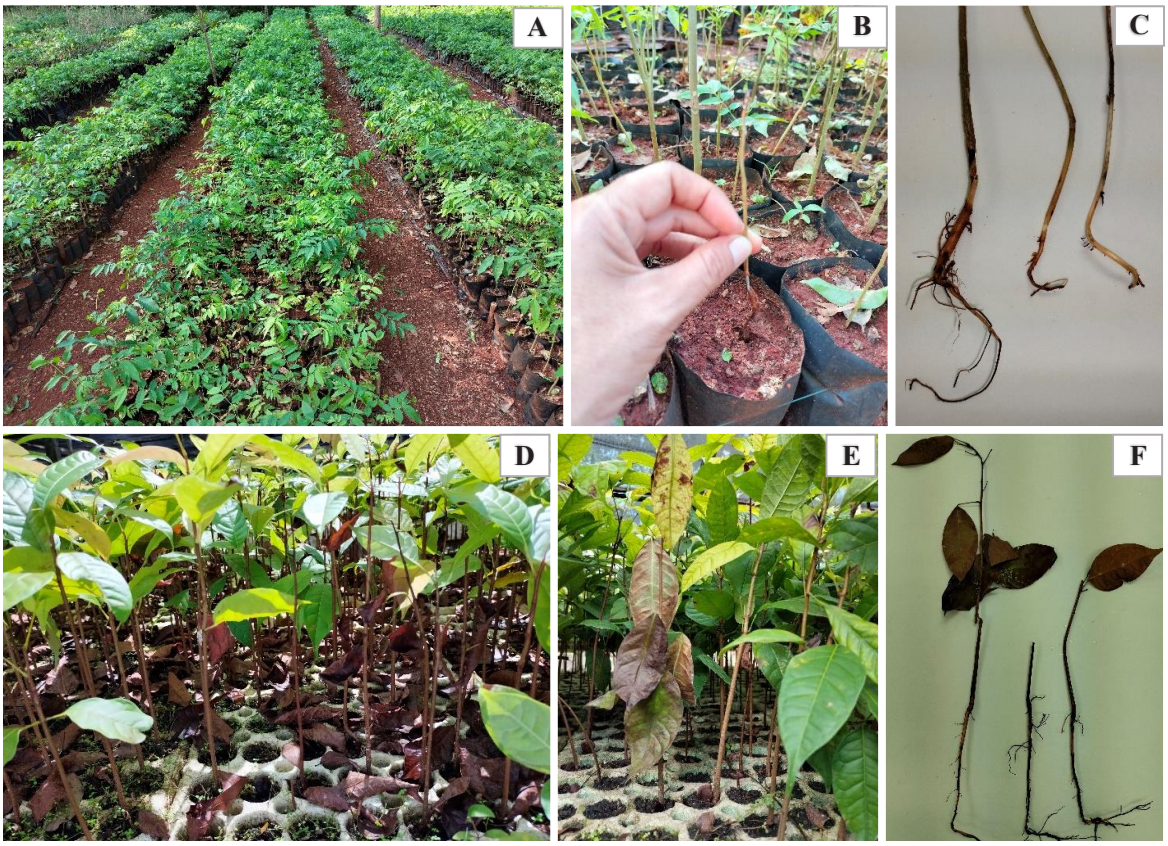

Figure 1. Plants with symptoms of necrosis and rot in the stem and root. **A, B**, and **C)** *Cedrela odorata* plants. **D, E**, and **F)** *Swietenia macrophylla* plants.

Morphological identification. For the morphological identification of fungal isolates at the genus level, sterile samples of approximately 5 mm diameter mycelium were taken with a dissecting needle. These were placed in Petri dishes with PDA medium and incubated at 28±2 °C. They were monitored until reproductive structures appeared, assessing growth characteristics such as mycelial type, isolate color, type and shape of spores, and fruiting bodies (Barnett and Hunter 1998; Leslie *et al*., 2006). Semi-permanent preparations were made and morphological features were observed under a microscope (Axiostar Plus, Carl Zeiss) equipped with an ocular measurement grid, calibrated for each objective as necessary. Length and width measurements (n=100) of macroconidia and microconidia were taken.

Molecular identification. Considering that the five isolates exhibited similar morphological characteristics, one isolate from each host was randomly selected for molecular identification. This involved DNA extraction from each fungus using the DNeasy Kit (QIAGEN). The extracted DNA was used to amplify the Internal

Transcribed Spacer (ITS) region through Polymerase Chain Reaction (PCR), employing the universal primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White *et al*., 1990), and elongation factor-1α with primers EF-1 (5′-ATGGGTAAGGARGACAAGAC-3′) and EF-2 (5′-GGARGTACCAGTSATCATG¬TT-3′) (Ríos-Hernández et al., 2021). PCR amplification conditions were as follows: Initial denaturation at 94 °C for 30 s, followed by 38 cycles (denaturation at 90 °C for 30 s, annealing at 58 °C for 45 s, and extension at 72 °C for 1 min), with a final extension at 72 °C for 5 min (Uc-Várguez *et al*., 2018). Amplification was conducted using an AB thermocycler (Applied Biosystems), and the PCR products were analyzed by 1% agarose gel electrophoresis. Purification and sequencing of the PCR products were performed at Macrogen Genome Center in Seoul, Republic of Korea. The resulting sequences were edited using BioEdit software (version 7.7.1, available online) and subsequently compared with sequences in the National Center for Biotechnology Information (NCBI, 2024) database using the BLAST tool, also available online.

Minimum Inhibitory Concentration for Spore Germination and Minimum Lethal Concentration. To estimate the antifungal activity of six active ingredients from conventional fungicides (Table 1) against fungal isolates of *C. odorata* and *S. macrophylla*, the Minimum Inhibitory Concentration (MIC) for spore germination was determined. The microdilution method was used in sterile 96-well polystyrene microplates with Potato Dextrose Agar at twice its concentration (CLSI, 2017; CLSI, 2020). Each well of the microplate received 100 µL of Potato Dextrose broth, and 100 µL of sterile distilled water was added to wells in the negative control row. Subsequently, each well in the first column received 100 µL of the respective fungicidal solution, mixed thoroughly. 100 µL of the mixture from each well was transferred to the wells of the second column, and so forth, maintaining a 50% concentration of the fungicidal solution from the first well onwards. After dilutions

Table 1. Conventional fungicides evaluated for their effect on spore germination and mycelial growth of *Fusarium solani* isolated from *Cedrela odorata* and *Swietenia macrophylla*.

Fungicide (a. i.)	Fungicide group	Type of fungicide	Maximum initial dose (ppm)
Prochloraz	Imidazole	Systemic	625
Carbendazim	Bencimidazole	Systemic	364
Benomyl	Bencimidazole	Systemic	225
Fosetyl Al	Ethyl phosphonate	Systemic	1500
Captan	Phthalimide	Contact	1000
Mancozeb	Ditihocarbamate	Contact	1000

were complete, 100 µL of spores of the fungus, counted in a Neubauer chamber at a concentration of 1 x 10^5 spores mL^{-1}, were added to each well. Positive control wells contained Potato Dextrose broth and fungus spores at described concentrations, resulting in a final volume of 200 µL per well. The contents of each well were mixed (Harčárová *et al*., 2021; Ríos-Hernández *et al*., 2021). Microplates were incubated at 29°C; the assay followed a completely randomized experimental design with four replicates. Data were analyzed using analysis of variance after data transformation with SAS ver. 9.4 (y=arsin(sqrt(y/100)), and mean comparisons were performed using Tukey's method ($p \leq 0.05$). Spore germination inhibition was evaluated at 24, 48, and 72 hours using resazurin (Sharma et al., 2016; Balouri *et al*., 2016; Kumar *et al*., 2016; Ríos-Hernández *et al*., 2021) as an oxidation-reduction indicator to confirm spore viability by a change from blue (non-fluorescent) to pink-red (fluorescent) staining due to chemical reduction of the growth medium by microbial cell growth (Elshikh *et al*., 2016; Caso *et al*., 2021). The MIC of each fungicide, defined as the lowest concentration preventing fungal mycelial growth, was determined after two hours of incubation (Kowalska-Krochmal and Dudek-Wicher, 2021). Subsequently, the Minimum Lethal Concentration (MLC), defined as the lowest concentration of fungicide at which no fungal growth was observed 24 hours after subculturing in fungicide-free medium (Perczak *et al*., 2019; Harčárová *et al*., 2021; Ríos-Hernández *et al*., 2021), was calculated in triplicate using 5 µL of fungal content inhibited at the MIC and four concentrations above the MIC, incubated at 29 °C. Fungal growth presence or absence was observed at 24 hours; fungistatic activity was noted when fungal growth occurred, and fungicidal activity when no growth was observed (Ríos-Hernández *et al*., 2021).

Results and Discussion

Morphological identification. The morphological characteristics of the five isolates from necrosis, stem rot, and root rot of *C. odorata* and *S. macrophylla* (Figure 2) exhibited septate and smooth mycelium during the first four to five days of growth, becoming cottony with shades ranging from white to pale cream by day 8. Abundant microconidia were produced in long monophialides, hyaline, oval, and fusiform, generally with zero to one septum, measuring 8-25 µm long and 3.5-5 µm wide; irregularly shaped sporodochia of pale cream color and numerous robust, slightly curved, hyaline macroconidia with three to four septa, measuring 24-36 µm long and 4-5 µm wide were observed. Single and double chlamydospores with a globular shape and thick walls were also noted. These isolate characteristics were consistent with those described for *Fusarium solani* (Leslie *et al.,* 2006; Hafizi *et al*., 2013; Omar *et al*., 2018; Kurt *et al*., 2020).

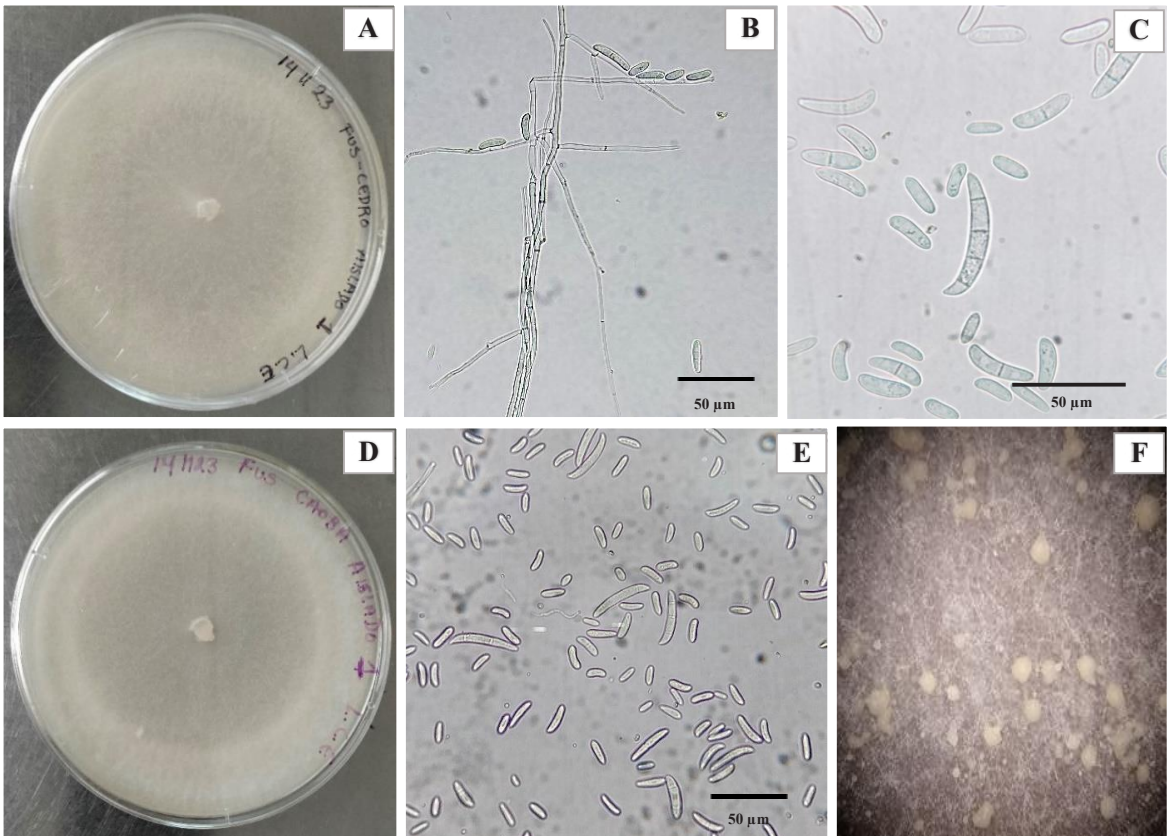

Figure 2. **A** and **D)** View of 14-day-old *Fusarium solani* isolated from *Cedrela odorata* and *Swietenia macrophylla*, **B)** Septate mycelium and microconidia of *F. solani*, **C)** Macroconidia (3-4 septa) and microconidia (0-1 septum) of *F. solani* observed at 100X magnification, **E)** Macroconidia and microconidia observed at 40X magnification, and **F)** Sporodochia.

Molecular identification. The resulting sequences from the amplified fragments of the ITS region (600 bp) of the two isolates from *C. odorata* and *S. macrophylla*, when compared to sequences in the NCBI database, showed similarities of 99.26% and 99.79%, respectively, with sequences corresponding to *F. solani* and *F. falciforme*. Consequently, analysis of the sequences from the fragments of the elongation factor-1α (700 bp) genes of the same isolates revealed similarities of 99.83% and 95.73%, respectively, with sequences of *F. solani* and *F. falciforme*. Considering the morphological characteristics and close molecular similarity of the analyzed sequences, it is concluded that the two isolates from necrosis, stem rot, and root rot of *C. odorata* and *S. macrophylla* correspond to *F. solani*, given that *F. falciforme* is a member of the *F. solani* complex, which encompasses a diversity of genotypes due to its genetic variability and ecological plasticity (Sabahi *et al*., 2023). This constitutes the first report of *F. solani* associated with nursery plant production in *C. odorata* and *S. macrophylla* in southeastern Mexico.

Determination of MIC and MLC of synthetic fungicides against *F. solani*. Prochloraz inhibited spore germination and consequently the growth of *F. solani* at concentrations starting from 2.44 ppm and higher. Treated spores exhibited a blue (non-fluorescent) staining compared to the positive control spores, which changed from blue to pink-red (fluorescent), indicating fungal cell growth. Upon determining the MLC, the fungicidal effect persisted, with fungal samples from spores treated with fungicide concentrations subcultured in fungicide-free PDA medium showing no mycelial growth during the 24-hour evaluation, unlike fungal samples from the positive control that did develop mycelial growth (Figure 3). Based on these results, 2.44 ppm was both the MIC and MLC of Prochloraz (Table 2). This demonstrates that Prochloraz caused spore death by preventing germination and mycelial growth. These findings directly correlate with the fungicide's mode of action, which disrupts sterol biosynthesis (Medina-Osti *et al*., 2022), particularly

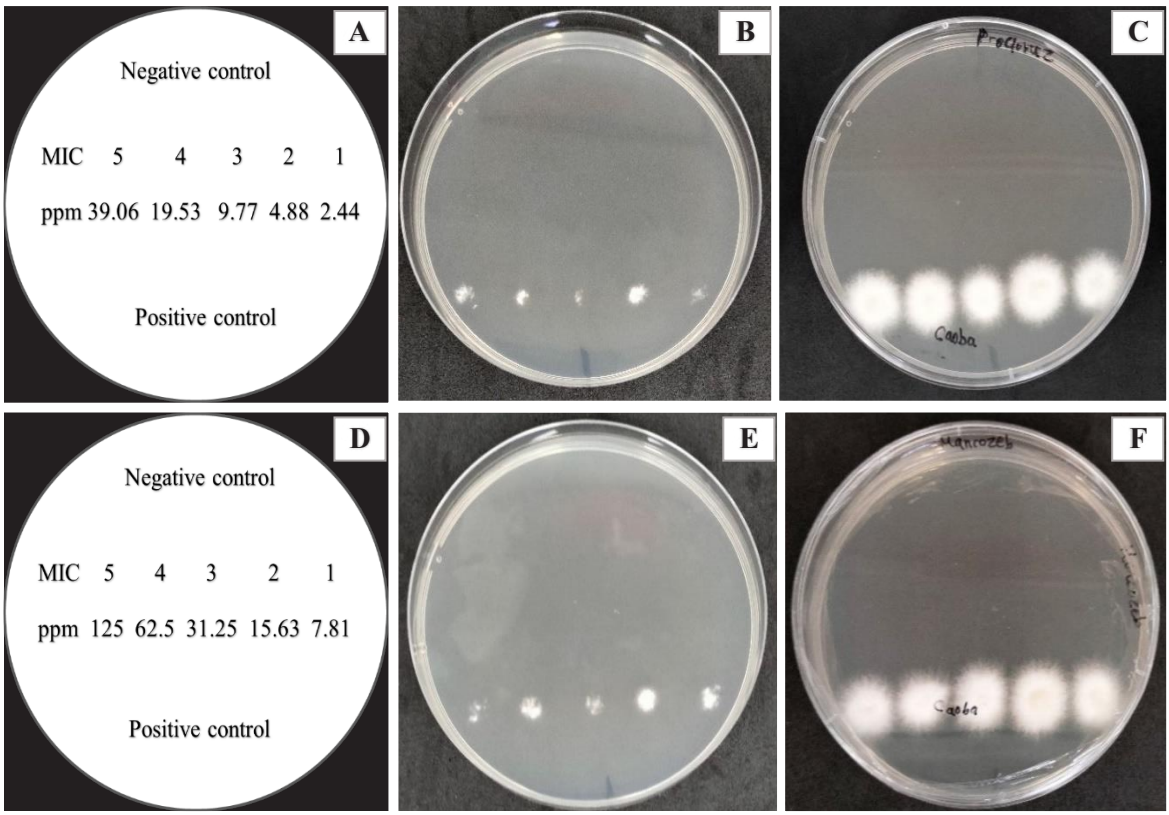

Figure 3. Fungicidal effect of Prochloraz and Mancozeb on the spore solution of *Fusarium solani* obtained from the microdilution plate after two days of evaluation. **A)** Distribution in Petri dishes with PDA medium of five MIC obtained from Prochloraz, **B** and **C)** No mycelial growth of *F. solani* in the MIC of Prochloraz evaluated after 24 and 48 h, **D)** Distribution in Petri dishes with PDA medium of five MIC obtained from Mancozeb, **E** and **F)** No mycelial growth of *F. solani* in the MIC of Mancozeb evaluated after 24 and 48 h.

Table 2. Minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of conventional fungicides against *Fusarium solani* isolated from *Cedrela odorata* and *Swietenia macrophylla*.

Fungicide-treatment (a.i.)	MIC (ppm)	[y] Inhibition of spore germination (%)	MLC (ppm)	[z] Inhibition of mycelial growth (%)
Prochloraz	2.44	100 a	2.44	100 a
Carbendazim	11.38	100 a	11.38	0 b
Benomyl	14.06	100 a	14.6	0 b
Fosetyl-Al	1500	0 b	1500	0 b (-)
Captan	7.81	100 a	7.81	0 b
Mancozeb	7.81	100 a	7.81	100 a
Control	0	0 b	0	0 b (-)

[y] Means with the same letter between columns are equal ($p \leq 0.05$).

[z] 100 %) fungicidal activity, 0 %) fungistatic activity, and 0 (-) no activity.

targeting ergosterol, an essential component of the fungal plasma membrane that maintains rigidity and permeability of spores. Disruption of this component can lead to membrane structure lysis, affecting nutrient transport and chitin synthesis, both crucial for mycelial growth. Studies on *F. oxysporum* and *Colletotrichum acutatum* showed structural damage, resulting in shrunken and wrinkled spores, and consequently fungal inviability, upon ergosterol inhibition (Kim *et al*., 2019). Similar effectiveness was observed in other studies where concentrations of 4, 40, and 400 ppm of Prochloraz inhibited the growth of *F. graminearum*, *F. proliferatum*, and *F. verticillioides*, causing corn ear rot (Massiello *et al*., 2019), and in *Fusarium* sp. causing citrus vascular wilt, with 100 ppm inhibiting mycelial growth by 92% (Ortiz-Martínez *et al*., 2022).

The MIC assay for Carbendazim started at 11.38 ppm (Table 2). However, when evaluating the fungicide's effect and estimating the MLC, mycelial growth occurred when subcultured in fungicide-free PDA medium, suggesting a fungistatic rather than fungicidal effect. Different results were observed in isolates from papaya causing root rot, where this fungicide at 150 ppm inhibited mycelial growth by 93.5 to 95.3% (Kumar *et al*., 2020) and at 750 ppm inhibited 100% of *F. solani* growth causing tomato vascular wilt (Ayvar-Serna *et al*., 2021). For Benomyl, the MIC was 14.06 ppm (Table 2), inhibiting spore germination of *F. solani*. However, like Carbendazim, when evaluating the MLC, spores regained viability, showing mycelial growth, also suggesting a fungistatic effect at these doses. In studies with doses higher than 250 ppm, it inhibited 100% mycelial growth (Coronel *et al*., 2022). But in *F. oxysporum*, responsible for strawberry plant wilt and collapse, mycelial growth was inhibited at concentrations as low as 5 ppm (Vega-López and

Granado-Montero, 2023). These findings suggest that fungicide effectiveness also depends on the species and strain of the phytopathogen. Carbendazim and Benomyl, classified as preventive and curative, inhibit motor proteins and the cytoskeleton, specifically in the assembly of β-tubulin during mitosis, affecting cell division, movement, appressorium formation, and overall mycelial growth (Alburqueque and Gusqui, 2018; FRAC, 2024). Conversely, none of the evaluated concentrations of Fosetyl-Al showed antifungal activity, as fungal growth occurred, confirming the low effectiveness and selectivity of this active ingredient with oomicide effect (Cristóbal-Alejo *et al*., 2013). Although there are few cases of resistance in some phytopathogens, frequent use may reduce its effectiveness in controlling fungal phytopathogens (FRAC, 2024).

For Captan and Mancozeb, the MIC was 7.81 ppm (Table 2). At this concentration, spore germination of the fungus was inhibited up to the higher evaluated concentrations, as indicated by no change in resazurin staining in the microplates compared to the positive controls, where resazurin staining did change. However, in the assay to determine the fungicide's effect and the MLC, only Mancozeb at this concentration showed an MLC effect on *F. solani*. There was no mycelial growth of the fungus at this and higher evaluated concentrations when subcultured in fungicide-free PDA medium from the microplates containing fungicide concentrations (Figure 2). This indicated that Mancozeb not only inhibited spore germination while in contact but also caused spore death, preventing mycelial development.

In contrast, Captan allowed mycelial growth similar to the positive control, suggesting it only prevented spore germination while in contact in the microplates. These results suggest that although both fungicides are contact fungicides with multi-site action affecting spore germination (FRAC, 2024), concentration and exposure time might influence effectiveness. The 48-hour contact period with Captan was insufficient for cell penetration. Recent evaluations showed efficacy against *Fusarium* species. When tested against *F. incarnatum*, a fungus associated with grass seed damage, Captan and Mancozeb at concentrations of 200 and 300 ppm inhibited mycelial growth by 97.8% and 93%, respectively (Zárate-Ramos *et al*., 2021). Against *F. falciforme*, recently identified as a root and stem pathogen of soursop, concentrations of 12,500 and 10,000 ppm inhibited fungal growth by 95 to 100% (Cambero-Ayón *et al*., 2023). Additionally, Captan interferes with the fungal respiration mechanism, hindering germination and mycelial development, while Mancozeb modifies and inactivates redox-sensitive proteins involved in transcription, translation, and oxidative DNA stress (Zárate-Ramos *et al*., 2021) Finally, only Prochloraz and Mancozeb showed fungicidal effects, with no mycelial growth of F. solani observed during the MLC assay after 24 and 48 hours of subculturing in fungicide-free PDA medium, confirming spore death. In contrast,

Carbendazim, Benomyl, and Captan exhibited fungistatic effects, as mycelial growth occurred during the MLC test after 24 and 48 hours of exposure in PDA medium without fungicide. This indicated that these fungicides did not kill the spores but only inhibited germination while in contact, with viability and growth resuming once removed from the fungicide medium (Table 2 and Figure 3).

Conclusions

The morphotaxonomic characteristics and molecular sequence analysis of the isolates from stem and root necrosis and rots of *C. odorata* and *S. macrophylla* corresponded to *F. solani*. This species was thus associated as the probable biotic factor responsible for 30 to 50% of the estimated plant production losses by nurserymen in southeastern Mexico.

Prochloraz and Mancozeb showed the best in vitro antifungal effects against *F. solani*, with average MIC and MLC values of 2.44 and 7.81 ppm, respectively.

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