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New Reports of Fungi on Mahogany (*Swietenia macrophylla* King, Meliaceae) Leaves and Seeds in Brazil

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Abstract

Mahogany (*Swietenia macrophylla* King, Meliaceae) is a forest species of high commercial value and is considered noble in many countries. Fungi are the main plant pathology agents, and can attack roots, leaves, flowers, fruits, woods, and seeds. Studies on diseases caused by fungi that affect *S. macrophylla* are incipient. There is little information about foliar diseases and pathogens that are transmitted by seeds. Because of the scarcity of this type of study in the above referenced species, this study aimed to identify and characterize the fungi found in leaves and seeds of mahogany collected in a small urban forest located in Brasilia, Federal District, Brazil, as well as to evaluate the pathogenicity of potentially pathogenic fungi. In our study, a foliar fungus, not yet reported for mahogany in Brazil, was found, *Phomopsis* sp. In seeds, this paper represents the first report of the occurrence of *Fusarium oxysporum* causing reduction in seed germination and death of mahogany seedlings. In addition, the fungi *Aspergillus*, *Curvularia*, *Penicillium* and *Rhizopus* also were found on mahogany seeds.

Keywords: foliar fungus, forest pathology, *Fusarium oxysporum*, *Phomopsis*, seed fungi

1. Introduction

Brazilian mahogany (*Swietenia macrophylla* King, Meliaceae) is a forest species of high commercial value and is considered noble in many countries (Carvalho, 2007). Due its exceptional growth in tropical regions, the commercial planting and the urban arborization are recommended and stimulated. So, the production of mahogany seedlings is essential to supply such demands.

The production of mahogany seedlings is done by seed, but due to the small quantity of seeds on the market, their value is extremely high. Propagation is via seed and initiation of fruiting in the trees begins at the age of eight (Yared & Carpanezzi, 1981). A mature tree can produce up to 600 fruits or 30,000 seeds per year, which are dispersed by wind up to 80 meters from the tree trunk, mainly in the direction of the strongest winds and during the late dry season (Gullison et al., 1996).

Fungi are the main plant pathology agents, and can attack roots, leaves, flowers, fruits, woods, and seeds, using different strategies to colonize plants (Doehlemann et al., 2017). Studies related to the occurrence and identification of fungi in forest seeds and their transmission to seedlings in Brazil are scarce. Most of the works are based on seed detection tests, without the concern to verify the pathogenicity in seedlings. In general, research on the dissemination of pathogens in forest species is concentrated in India, Canada, the United States and Africa, with conifers being the most studied (Santos et al., 2011).

Considering the low supply of mahogany seeds and its high commercial value, the full use of seeds is desirable. The losses caused by phytopathogenic fungi associated with seeds can be minimized through seed treatment,

however, in order to delineate phytopathogen control strategies, it is necessary to know them. There is little information about fungi that are transmitted by mahogany seeds.

Among the foliar diseases, the main disease reported in *S. macrophylla* in Brazil is *Cylindrocladium* blight, caused by the fungus *Cylindrocladium* sp. (= *Callonectria*), detected in leaves of mahogany in forest plantings to recover degraded areas in the Amazon (Gasparotto et al., 2014). In addition to this, several species have been reported that cause leaf spots, including the genus *Meliola* (Assis et al., 2010), *Cylindrocladium floridanum* Sobers & Seym (Mendes et al., 1998), *Colletotrichum gloeosporioides* (Penz.) Sacc. (Trindade et al., 2004), *Erythricium salmonicolor* (Berk. &Broome) Burds. (Hadi et al., 1993) and *Gloeosporium* sp. (Suharti & Irianto, 1992). In addition to these fungi, the plant-parasitic algae *Cephaleuros virescens* Kunze was found causing leaf lesions in mahogany in the State of Goiás (Pereira et al., 2020). There are no studies on fungi causing leaf diseases in mahogany in central Brazil.

This study aimed to identify and characterize the fungi found in mahogany leaves and seeds collected in a small urban forest located in Brasilia, Brazil, as well as to evaluate the pathogenicity of potentially pathogenic fungi.

2. Method

2.1 Detection of Fungi on S. macrophylla Leaves

Leaf spots were observed in trees used in urban forestry in Brasilia, Brazil and also in the production of species seedlings in a greenhouse at Embrapa Genetic Resources and Biotechnology. The samples were analyzed preliminarily with the aid of a stereoscopic microscope. Fungi were removed with a sterile needle, placed on glass slides with cotton blue and viewed with an optical microscope. To isolate the pathogen, regions of leaves containing fungal signs and symptoms were disinfected superficially (70% alcohol for 1 min, sodium hypochlorite (2.5% Cl^-) for 3 min, ethanol for 30 s, and finally 3 rinses in sterile, distilled water) and placed on PAD medium for growth and sporulation in a germination chamber (BOD) at 25±0.5 °C for 10 days. A pure culture was grown from dilution of conidial mass in distilled water and a grown fungal colony was transferred to another plate with PDA medium for growth and sporulation.

2.2 Test of Pathogenicity of Phomopsis

To confirm that the fungus found on the leaves caused the damage, Koch's postulate was performed. To this end 80 two-month-old mahogany seedlings were used, 40 of which were inoculated with the fungus and 40 served as control without inoculation. Spore suspensions (10^8 conidia/mL) obtained from pure fungal culture served as a source of inoculum. The suspension was mixed with distilled water, and then sprayed on mahogany leaves and the material was kept in a moist chamber for 72 hours. The evaluation began three days after inoculation and lasted until the thirtieth day, until the full development of the disease, and then, the pathogen was reisolated from leaves with symptoms.

2.3 Origin of Mahogany Seeds

The mahogany seeds were collected in Brasilia, Brazil, during the months from October to December 2013 and stored at room temperature until the conduct of sanitary testing. Sanitary testing of the seeds was performed in the Entomopathogenic Bacteria Laboratory of Embrapa Genetic Resources and Biotechnology, located in Brasilia, Brazil. The method of symptoms in seedlings was conducted in a greenhouse without humidity and temperature control during the months of January to March 2014.

2.4 Blotter Test

One hundred ninety-two (192) non-disinfected mahogany seeds without the wing were divided into 12 replicates of 16 seeds each, arranged on autoclaved paper towel moistened with autoclaved distilled water and packed in gerbox boxes previously disinfected with 70% alcohol. The material then was placed in the B.O.D. at a temperature of 25±2 °C and a photoperiod of 12 hours of light and 12 hours dark, for seven days. After that, each seed was analyzed, and fungi found were grown in culture medium (PDA) for subsequent identification at the genus or species level.

2.5 Method of Symptoms in Seedlings

One hundred twenty (120) mahogany seeds without the wing were divided into two blocks with 60 seeds each. The substrate used was autoclaved BioPlant® Silver HT [pine bark, manure, sawdust, coconut fiber, vermiculite, rice husk, ash, calcium carbonate, gypsum, magnesium, magnesium thermophosphate, fertilizer and additives (Yoorin Fertilizantes, Minas Gerais, Brazil)]. The seeds were sown in a horizontal position, at about 1 cm in depth, to facilitate and accelerate germination. The evaluation of the occurrence of pathogens was made by observing the emergence of the seedlings, looking for disease symptoms and signs of pathogens in the seedlings

and non-germinated seeds, consistent with Santos et al. (2011). The non-germinated seeds without fungal signs were placed in a humidity chamber for seven days to confirm the absence of fungi. The fungi found were visualized under stereoscopic and light microscope and preserved in PDA for later identification.

2.6 Morphological Identification

Fungi found by the two methodologies were isolated on PDA medium and described in detail as to their growth through visual analysis of the mycelium, color and form of growth. The fungal structures were observed by stereoscopic (loop) and light microscope, by dying the slides with lactoglycerol/cotton blue (Cotton-Blue) or glycerol-KOH/basic phloxine and sealing with two layers of commercial nail enamel. The morphological descriptions were based on attributes of the conidiophore, conidiogenous cell and conidium, for anamorphic fungi. To identify each genus or species, specific keys according to its anamorphic characteristics were used (Barnett & Hunter, 1972; Santos et al., 2011).

2.7 Molecular Identification of Fusarium oxysporum

The purified isolate grew in PDA medium, in a germination chamber (BOD) at 25±0.5 °C for 10 days. Three discs (10 mm) were cut from the culture medium containing the fungus and inoculated into a 200 mL Erlenmeyer flask with liquid SDY medium (4% dextrose, 1% yeast extract and 1% peptone). After 72 hours of incubation on a rotary shaker at 25±0.5 °C and 250 rpm, the mycelium was collected on filter paper under vacuum filtration and used for DNA extraction following the method described by Raeder and Broda (1985). The intergenic Bloc region was amplified and sequenced according Renher et al. (2011). The obtained sequence was edited by baser DNA program (DNABaser Sequence Assembler 3, Heracle Biosoft, Pitesti, Romania).

2.8 Phylogenetic Analysis of Fusarium oxysporum Isolate

The 13 sequences with the highest identity with the isolate obtained here using blastn and 36 outliners were downloaded from the GenBank (Benson et al., 2017) (Table A1) and the multiple sequence alignement were prepared using MUSLCE (Edgar, 2004). Bayesian inference was used to construct phylogenetic tree performed with MrBayes (Ronquist & Huelsenbeck, 2003) using 20,000 generations and excluding the first 1,000 generations. The best nucleotide substitution model was select using MrModeltest (Nylander, 2004) in the Akaike Information Criterion (GAMMA). Trees was visualized and edited using iTol (Letunic & Bork, 2019).

2.9 Pathenogenicity of Fusarium oxysporum in Mahogany Seeds and Seedlings

The isolate used for this test was obtained from the Blotter test. Forty mahogany seeds were used, divided into two lots with 20 seeds each. One lot was inoculated with fungus by direct contact with the mycelium and the other lot used as control. Seeds were sterilized in 70% alcohol for 30 seconds and 1% sodium hypochlorite for two minutes and washed with sterile distilled water following Santos et al. (2011). The seeds were placed on autoclaved paper for drying and subsequently placed in contact with the fungi cultures grown on PDA for 72 hours and then sowed in autoclaved BioPlant® Silver HT substrate. Thirty (30) days after sowing the number of seedlings with symptoms, the number of healthy seedlings and the number of non-germinated seeds were counted. Symptomatic seedlings and non-germinated seeds were placed in a humidity chamber for seven days, for identification and reisolation of the fungus.

3. Results

3.1 Foliar Fungus Found on S. macrophylla

The fungus found on *S macrophylla* leaves was identified as *Phomopsis* sp., presenting blackened conidiomata and conidia type alpha and beta, characteristic of the genus (Sutton, 1980; Hanlin & Menezes, 1996) (Figure 1D). The fungal culture in PDA after 10 days of incubation showed white coloration with blackened conidiomata (Figure 1B), with formation of abundant conidial masses (Figure 1C). The symptoms were leaf spot surrounded by amphigenous stromal area dark brown to black with black spots (conidiomata) distributed in a light-brown inner area (Figure 1A). Causal agent: hyaline mycelium, septate, immersed in the host tissue; amphigenous pseudostroma with internal hyaline mycelium, incorporating brown degraded cells, surrounded by blackened melanotic tissue; clypeate conidiomata, amphigenous, immersed, mesophilic, stromal, dark brown to black; phialidic enteroblastic conidiogenous cells, cylindrical, hyaline; conidia alpha 6.5 − 9.75 × 2.5 µm hyaline, guttulate, oblong-ellipsoid, aseptate; conidia beta 14 − 28.5 × 1.0 µm hyaline, filiform, apically uncinate or straight (Figure 1D).

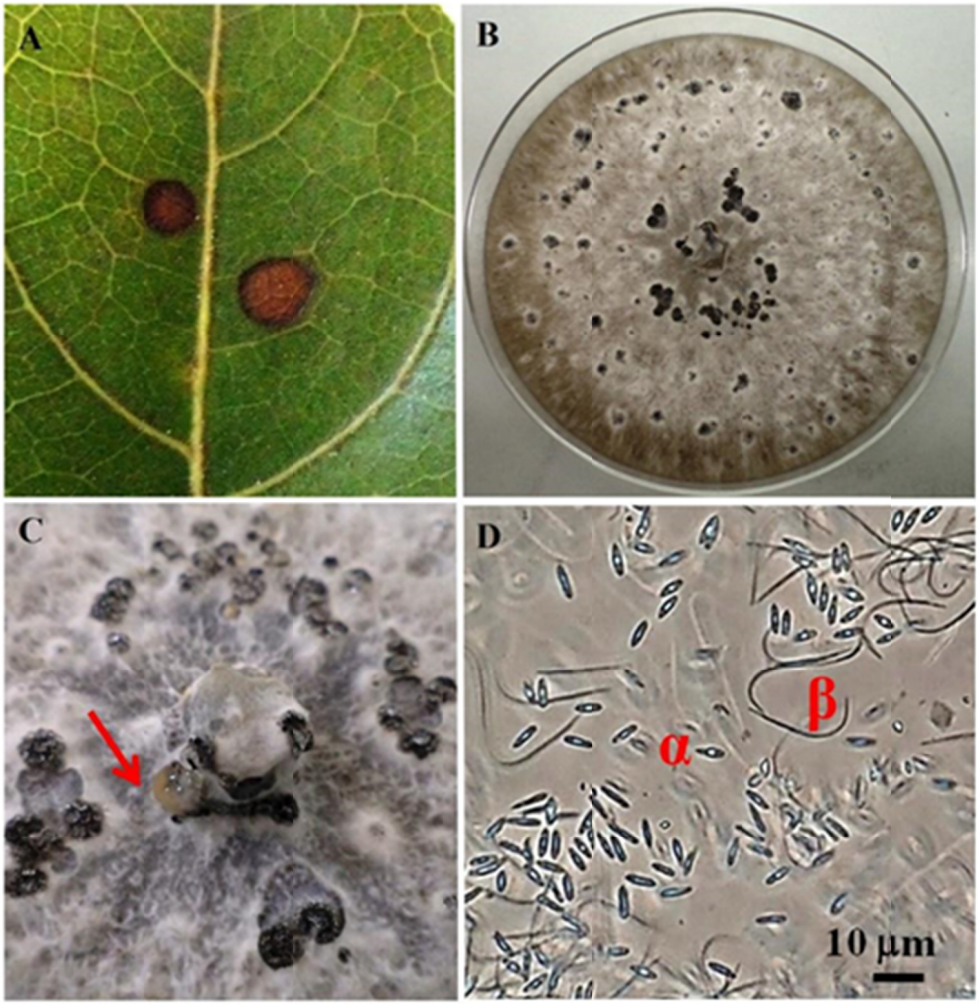

Figure 1. *Phomopsis* sp. infesting mahogany leaves. (A) Leaf spot caused by the fungus. (B) Fungal culture in PDA. (C) Conidial masses in PDA. (D) Alpha and beta conidia

3.2 Test of Pathogenicity of Phomopsis

The pathogenicity test showed that the *Phomopsis* sp. isolated from leaves is pathogenic, since 28 (70%) of inoculated seedlings reintroduced the symptoms and the reisolated pathogen showed the same characteristics as the fungus isolated from the collected lesion, closing the postulate (Figure 2).

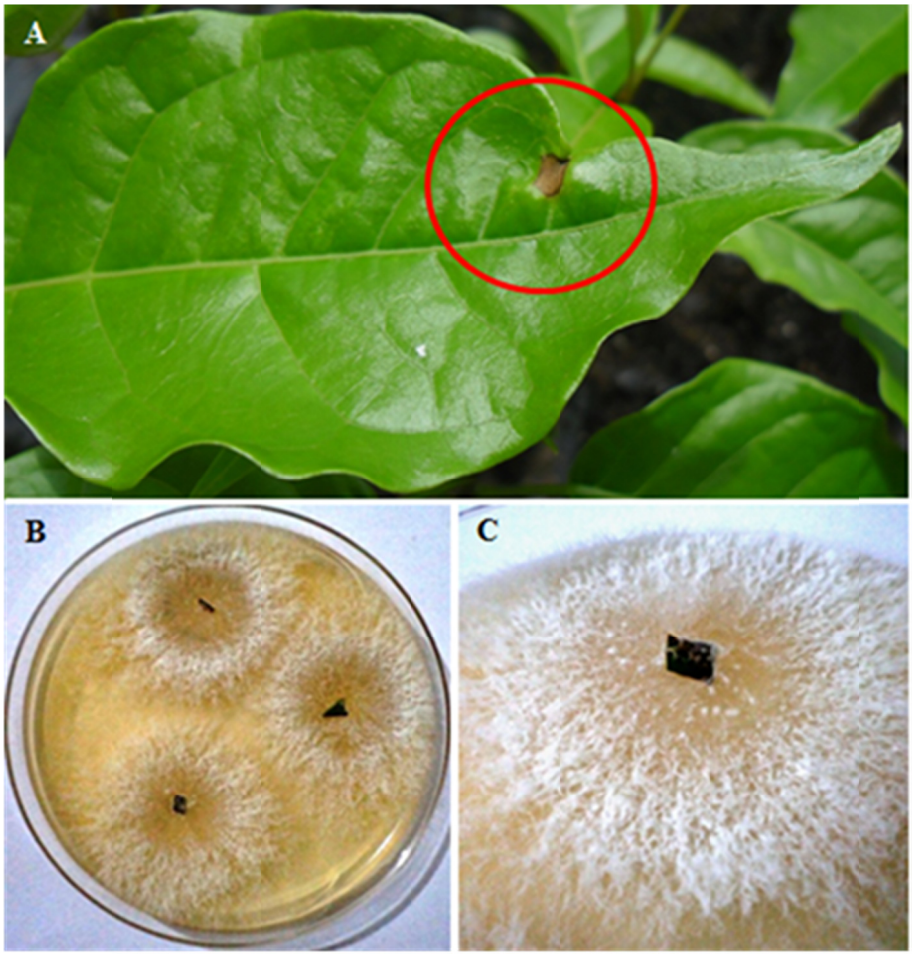

Figure 2. Reinoculation of *Phomopsis* sp. in PDA by symptomatic plants. (A) Mahogany seedling with leaf spot after inoculation. (B) PDA with pieces symptomatic leaves. (C) Detail of *Phomopsis* sp. growing from symptomatic leaves in PDA

3.3 Blotter Test

As for the Blotter test analysis, of the 192 seeds analyzed (13,54%) showed signs of fungi (Figure 3), as shown in Table 1. Many seeds had more than one fungus on their surface.

Fungi were mostly saprophytes (81.5%). Among the saprophytic fungi, those of the genera *Aspergillus* and *Penicillium* were the most abundant; *Rhizopus* fungus was observed less frequently. The potentially plant pathogenic fungus of the species *Fusarium oxysporum* Schltdl (99.7% of similarity - GenBank/99.8% of similarity - Fusarium ID) was associated with 7.5% of the seeds that presented fungal signs.

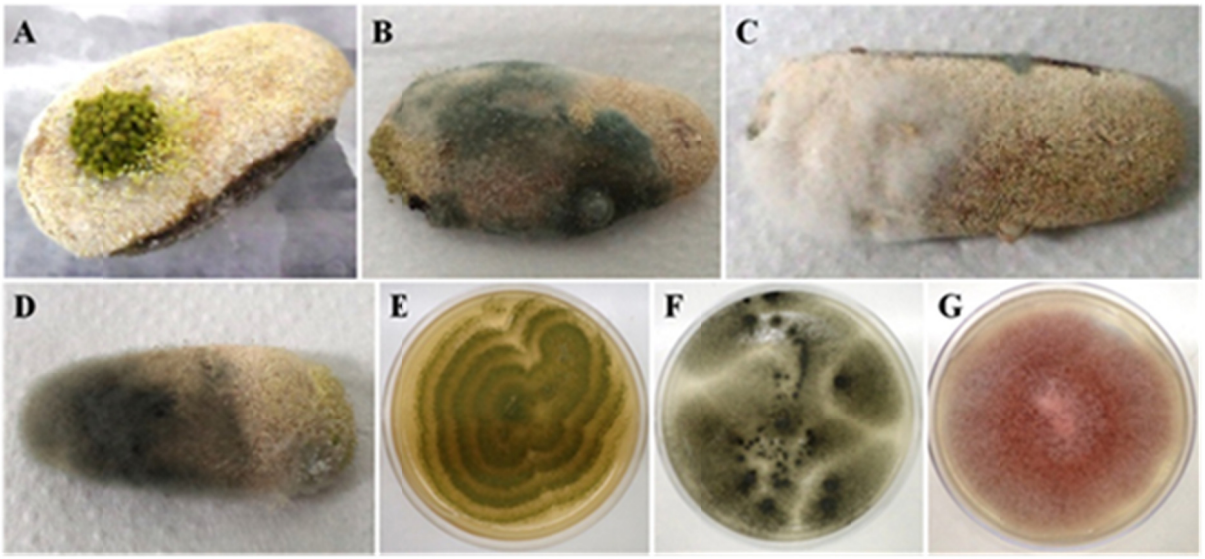

Figure 3. Some fungi found in mahogany seeds from the Blotter test. (A) *Aspergillus flavus*. (B) *Penicillium* sp. (C) *Fusarium oxysporum*. (D) *Curvularia* sp. (E) *Asperillus flavus* on PDA. (F) *Aspergillus niger* on PDA. (G) *Fusarium oxysporum* on PDA

Table 1. Fungi found in mahogany seeds from the Blotter test

Fungal genus	Number of seeds (%)	Habit
Penicillium	4.68	Saprophyte
Aspergillus	4.16	Saprophyte
Rhizopus	2.60	Saprophyte
Curvularia	1.56	Phytopathogenic
Fusarium	0.52	Phytopathogenic

3.4 Method of Symptoms in Seedlings

In the method of symptoms in seedlings, of 120 seeds planted, 85 germinated (71%) and 35 did not germinate (29%). Among those that failed to germinate, 14 (11.6%) had signs and symptoms of fungi on the seeds. Three distinct species of different fungi were found, *Aspergillus flavus* Link, *Aspergillus niger* Tiegh. and *F. oxysporum*, as described in Table 2. No seedling developed symptoms.

Table 2. Fungi found in mahogany seeds by the method of symptoms in seedlings*

Fungal genus	Number of seeds (%)	Habit
Aspergillus	10.00	Saprophyte
Fusarium	1.60	Phytopathogenic

Note. * Fungi were found in non-germinated seeds, no seedling developed symptoms.

3.5 Phylogenetic Analysis of Fusarium oxysporum Isolate

Using Bayesian Phylogeny approach, it was possible to verify the formation of two well-structured branches, one formed exclusively by *F. oxysporum*, in which the sequence obtained in this study is inserted (*F. oxysporum* isolate) and other formed by outliners (Figure 4). Using the phylogeny, we were able to confirm that the sequenced isolate belongs to the species *F. oxysporum*.

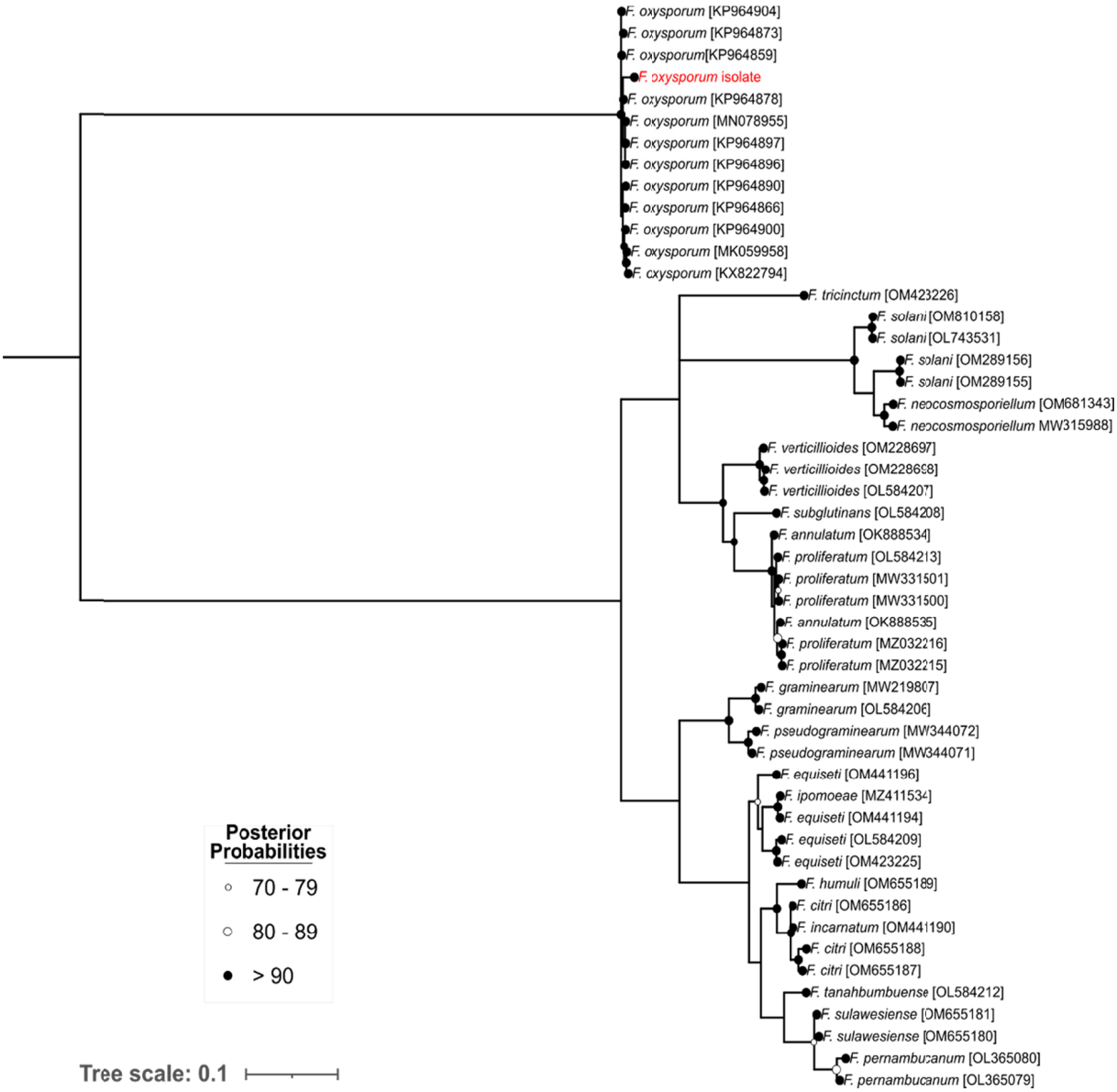

Figure 4. Phylogenetic analysis of *Fusarium oxysporum* isolate

3.6 Pathenogenicity of Fusarium oxysporum in Mahogany Seeds and Seedlings

In determining the pathogenicity of the isolate of *F. oxysporum* from the mahogany seeds, it was observed that, of 20 seeds planted that were inoculated with the fungus, only five germinated, the other fifteen did not germinate and showed signs and symptoms of the fungus. Symptoms caused by *F. oxysporum* in the five seeds that germinated and were inoculated with the pathogen were root rot, plant crown damage, cotyledon rot of the seedling and subsequent death, which indicates that the isolate is pathogenic. The controls did not show symptoms.

4. Discussion

In our study, we found a potential pathogenic fungus in mahogany leaves, identified as *Phomopsis* sp. The *Phomopsis* genus has more than 1,000 described species, 29 varieties and 16 formae speciales (Kirk et al., 2001). This fungal taxonomy has been based in a combination of molecular, morphological, cultural and phytopathological factors (Undayanga et al., 2011). Molecular studies should be conducted to determine whether the fungus found associated with the mahogany is a likely new species within the genus or not.

Phomopsis species are commonly found as pathogens and endophytes of plants (Undayanga et al., 2011). As pathogens they are responsible for several diseases in many plant species, such as browning of the leaves of

"aroeira" (*Myracrodruon urundeuva* Fr. All.) and "jatobá" (*Hymenaea courbaril* L.) (Anjos et al., 2001). Walker et al. (2013) found a *Phomopsis* sp. strain on "angico-vermelho" seeds (*Parapiptadenia rigida* Benth.), that caused seeds wrinkle and leaf spot. Santos et al. (2018) isolated *Phomopsis* sp. de from *Tectona grandis* Linn. F. in Sergipe state, Brazil, causing leaf spot on young leaves. Similar symptoms were observed in this study, suggesting that this fungus can cause loss in mahogany seedling production.

The two methods used in this study to investigate the occurrence of fungi in mahogany seeds, show that the Blotter test is more effective when compared with the method of symptoms in seedlings. Most fungi found in seeds belong to the subdivision Deuteromycotina, like *Aspergillus*, *Curvularia, Fusarium* and *Trichoderma* (Carvalho & Muchovej, 1991; Santos et al., 2011).

Previously Stein et al. (1997) examined the occurrence of fungi in mahogany seeds originated from the Amazon. At the time, the authors detected fungi *Botryodiplodia* sp., *Aspergillus* spp. and *Penicillium* spp. In the present study, only *Botryodiplodia* sp. was not found, however, three different fungi were observed, which demonstrates that the occurrence of fungi associated with seeds of the species varies by region. These fungi are considered as potential fungi of storage, since the incidence may increase in the post-harvest period (Cherobini et al., 2008). Carneiro (1990) recommends the control of these genera as to the incidence in seeds, since a high percentage of infestation tends to reduce viability and interfere with storage conditions, accounting for reductions in the viability and longevity of seeds.

The presence of *F. oxysporum* was first reported in association with mahogany seeds in Brazil. Fungi of the genus *Fusarium* are known to behave as pathogens in forest seeds. *Fusarium* species complex can affect a large hosts number, including forest species (O'Donnell et al., 2010; Borges et al., 2018; Mazarotto et al., 2020). In Brazil, this genus was described associated with more than 100 forest species (Santos et al., 2011). Benetti et al. (2009) reported that isolates of *Fusarium* and *Pestalotia* in cedar seeds (*Cedrella fissilis* Vell.) reduced germination after inoculation. *Fusarium oxysporum* can prevent seed germination, or even can be transmitted to plants via seeds, causing root problems and damping-off of seedlings, as observed by Lazarotto et al. (2012) in cedar seedlings.

In conifers of North America, including pine, most fungi transmitted by seeds to seedlings belong to the genus *Fusarium* (Cram & Fraedrich, 2010). According Dhingra et al. (1980) and Machado (1988), *Fusarium* contamination occurs during the formation or ripening of the fruit. The pathogens present in the seeds, both internally and externally, become active as soon as the seeds are sown in moist soil. These seeds rot before they germinate, or the pathogen may not attack the seed but infect the seedling (Dhingra et al., 1980).

This study highlights the importance of studies to identify pathogens associated with the seeds and leaves of forest species, which are essential for the design of programs for their effective control and consequently to avoid potential problems in the production of mahogany seedlings.

5. Conclusion

Phomopsis sp. was found in mahogany leaves, causing leaf spots. When inoculated in the seedlings, this fungus caused disease and is reported for the first time in mahogany. The Blotter test is more efficient to detect the occurrence of fungi in mahogany seeds. *Fusarium oxysporum* was isolated from mahogany seed and presented potential damage in seeds and seedlings. In addition, the fungi *Aspergillus, Curvularia, Penicillium* and *Rhizopus* also were found on mahogany seeds.

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Appendix A

Table A1. Nucleotide sequences from GenBank used in this study

Acession Code*	Organism	Gene region
OK888534	*Fusarium annulatum*	translation elongation factor 1-alpha
OK888535	*Fusarium annulatum*	translation elongation factor 1-alpha
OM655186	*Fusarium citri*	translation elongation factor 1-alpha
OM655188	*Fusarium citri*	translation elongation factor 1-alpha
OM655187	*Fusarium citri*	translation elongation factor 1-alpha
OM441196	*Fusarium equiseti*	translation elongation factor 1-alpha
OM441194	*Fusarium equiseti*	translation elongation factor 1-alpha
OL584209	*Fusarium equiseti*	translation elongation factor 1-alpha
OM423225	*Fusarium equiseti*	translation elongation factor 1-alpha
MW219807	*Fusarium graminearum*	translation elongation factor 1-alpha
OL584206	*Fusarium graminearum*	translation elongation factor 1-alpha
OM655189	*Fusarium humuli*	translation elongation factor 1-alpha
OM441190	*Fusarium incarnatum*	translation elongation factor 1-alpha
MZ411534	*Fusarium ipomoeae*	translation elongation factor 1-alpha
OM681343	*Fusarium neocosmosporiellum*	translation elongation factor 1-alpha
MW315988	*Fusarium neocosmosporiellum*	translation elongation factor 1-alpha
KP964859	*Fusarium oxysporum*	translation elongation factor 1-alpha
KP964878	*Fusarium oxysporum*	translation elongation factor 1-alpha
KP964866	*Fusarium oxysporum*	translation elongation factor 1-alpha
KP964900	*Fusarium oxysporum*	translation elongation factor 1-alpha
KX822794	*Fusarium oxysporum*	translation elongation factor 1-alpha
KP964904	*Fusarium oxysporum* f. sp. cepae	translation elongation factor 1-alpha
KP964873	*Fusarium oxysporum* f. sp. cepae	translation elongation factor 1-alpha
KP964896	*Fusarium oxysporum* f. sp. dianthi	translation elongation factor 1-alpha
MK059958	*Fusarium oxysporum* f. sp. lactucae	translation elongation factor 1-alpha
MN078955	*Fusarium oxysporum* f. sp. narcissi	translation elongation factor 1-alpha
KP964897	*Fusarium oxysporum* f. sp. narcissi	translation elongation factor 1-alpha
KP964890	*Fusarium oxysporum* f. sp. phaseoli	translation elongation factor 1-alpha
OL365080	*Fusarium pernambucanum*	translation elongation factor 1-alpha
OL365079	*Fusarium pernambucanum*	translation elongation factor 1-alpha
OL584213	*Fusarium proliferatum*	translation elongation factor 1-alpha
MW331501	*Fusarium proliferatum*	translation elongation factor 1-alpha
MW331500	*Fusarium proliferatum*	translation elongation factor 1-alpha
MZ032216	*Fusarium proliferatum*	translation elongation factor 1-alpha
MZ032215	*Fusarium proliferatum*	translation elongation factor 1-alpha
MW344072	*Fusarium pseudograminearum*	translation elongation factor 1-alpha

MW344071	*Fusarium pseudograminearum*	translation elongation factor 1-alpha
OM810158	*Fusarium solani*	translation elongation factor 1-alpha
OL743531	*Fusarium solani*	translation elongation factor 1-alpha
OM289156	*Fusarium solani*	translation elongation factor 1-alpha
OM289155	*Fusarium solani*	translation elongation factor 1-alpha
OL584208	*Fusarium subglutinans*	translation elongation factor 1-alpha
OM655181	*Fusarium sulawesiense*	translation elongation factor 1-alpha
OM655180	*Fusarium sulawesiense*	translation elongation factor 1-alpha
OL584212	*Fusarium tanahbumbuense*	translation elongation factor 1-alpha
OM423226	*Fusarium tricinctum*	translation elongation factor 1-alpha
OM228697	*Fusarium verticillioides*	translation elongation factor 1-alpha
OM228698	*Fusarium verticillioides*	translation elongation factor 1-alpha
OL584207	*Fusarium verticillioides*	translation elongation factor 1-alpha

Note. * GenBank Acession code.