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Genetic Variation and Aggressiveness of *Ceratocystis fimbriata* and Resistance of *Tectona grandis* to *Ceratocystis* Wilt

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Thesis submitted to the Plant Pathology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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Co-adviser: Acelino Couto Alfenas

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Assent:

Flávia Sampaio Alexandre
Author

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I dedicate this thesis to the memory of my godparents, Ida and Zé, for the legacy of love, perseverance, and resilience they left behind.

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“Nothing in life is to be feared; it is only to be understood”.
(Marie Curie)

ABSTRACT

ALEXANDRE, Flávia Sampaio, D.Sc., Universidade Federal de Viçosa, November, 2024. **Genetic Variation and Aggressiveness of *Ceratocystis fimbriata* and Resistance of *Tectona grandis* to *Ceratocystis* Wilt**. Adviser: Rafael Ferreira Alfenas. Co-adviser: Acelino Couto Alfenas.

Tectona grandis, commonly known as teak, is a highly valued hardwood species. However, plantations can be affected by Ceratocystis wilt caused by *Ceratocystis fimbriata*. Planting resistant materials is the most effective strategy for disease control. To achieve successful control, it is necessary to understand the genetic variation, aggressiveness, and pathogenicity characteristics of the pathogen and assess the availability of sources of resistance in teak. Therefore, the objectives of this study were: (i) to investigate the genetic variation and aggressiveness of *C. fimbriata* isolates that infect teak, (ii) to assemble the first genome sequence of a *C. fimbriata* isolate from teak and compare it with isolates that infect other plants, identifying their its unique pathogenicity traits, and (iii) to evaluate resistance to Ceratocystis wilt in commercially teak clones and the inheritance of this resistance in progenies from open pollination. Fifty-six *C. fimbriata* isolates from different plantations in Mato Grosso, Brazil were analyzed using 13 microsatellite markers. Nine multilocus genotypes were identified, with one haplotype predominating across the isolates. The aggressiveness of eight *C. fimbriata* isolates, representative of the major haplotypes, were assessed on three different teak clones. Significant variation in isolate-clone interactions was found. The isolates belonging to three closely related haplotypes were the most aggressive. The genome sequence of the teak-infecting *C. fimbriata* isolate LPF2199 was assembled, and a comparative genomic analysis was conducted with isolates that infect eucalyptus, kiwi, and ghaf trees. LPF2199 isolate revealed a size of 31.62 Mb, containing 7,533 genes. The features of this assembly are consistent with those of previously described *Ceratocystis* species. Comparative analysis showed high genomic similarity among isolates, but subtle differences in gene content and unique proteins in LPF2199 that may contribute to its pathogenicity. The results of resistance to Ceratocystis wilt in twelve commercially cultivated teak clones showed five with significant resistance, and the open-pollinated families exhibited segregation for resistance. The results support the interpretation that resistance to Ceratocystis wilt in teak is a quantitative trait with additive gene effects in determining this trait. These findings provide insights into the *Ceratocystis fimbriata*-*Tectona grandis* pathosystem and contribute valuable information for breeding programs to develop teak genotypes

resistant to Ceratocystis wilt.

Keywords: Breeding; Draft genome sequence; Multilocus genotypes; Open pollination; Teak

RESUMO

ALEXANDRE, Flávia Sampaio, D.Sc., Universidade Federal de Viçosa, novembro de 2024. **Variabilidade Genética e Agressividade de *Ceratocystis fimbriata* e Resistência de *Tectona grandis* à Murcha de *Ceratocystis***. Orientador: Rafael Ferreira Alfenas. Coorientador: Acelino Couto Alfenas.

Tectona grandis, comumente conhecida como teca, é uma espécie de madeira nobre muito valorizada. Entretanto, as plantações podem ser afetadas pela murcha-de-ceratocystis causada por *Ceratocystis fimbriata*. O plantio de materiais resistentes é a estratégia mais eficaz para o controle de doenças. Para alcançar um controle bem-sucedido, é necessário entender a variação genética, a agressividade e as características de patogenicidade do patógeno e avaliar a disponibilidade de fontes de resistência na teca. Assim, os objetivos deste estudo foram: (i) investigar a variação genética e agressividade de isolados de *C. fimbriata* que infectam teca, (ii) montar a primeira sequência do genoma de um isolado de *C. fimbriata* de teca e compará-lo com isolados que infectam outras plantas, identificando suas características únicas de patogenicidade e (iii) avaliar a resistência à murcha-de-ceratocystis em clones de teca cultivados comercialmente e a herança dessa resistência em progênies de polinização aberta. Cinquenta e seis isolados de *C. fimbriata* de diferentes plantações em Mato Grosso, Brasil, foram analisados usando 13 marcadores microssatélites. Nove genótipos multilocus foram identificados, com um haplótipo predominando entre os isolados. A agressividade de oito isolados de *C. fimbriata*, representativos dos principais haplótipos, foi avaliada em três diferentes clones de teca. Foi encontrada variação significativa nas interações isolado-clone. Os isolados pertencentes a três haplótipos intimamente relacionados foram os mais agressivos. A sequência do genoma do isolado LPF2199 de *C. fimbriata* que infecta teca foi montada, e uma análise genômica comparativa foi conduzida com isolados que infectam eucalipto, kiwi e ghaf. O isolado LPF2199 revelou um tamanho de 31,62 Mb, contendo 7.533 genes. As características desta montagem são consistentes com aquelas das espécies de *Ceratocystis* descritas anteriormente. A análise comparativa mostrou alta similaridade genômica entre os isolados, mas diferenças sutis no conteúdo genético e proteínas exclusivas no LPF2199 que podem contribuir para sua patogenicidade. Os resultados de resistência à murcha-de-ceratocystis em doze clones de teca cultivados comercialmente mostraram cinco com resistência significativa, e as famílias de polinização aberta exibiram segregação para resistência. Os resultados corroboram a interpretação de que a resistência à murcha-de-

ceratocystis em teca é uma característica quantitativa com efeitos genéticos aditivos na determinação dessa característica. Essas descobertas fornecem informações sobre o patossistema *Ceratocystis fimbriata*-*Tectona grandis* e contribuem com dados valiosos para programas de melhoramento visando o desenvolvimento de genótipos de teca resistentes à murcha-de-ceratocystis.

Palavras-chave: Melhoramento; Sequência genômica de rascunho; Genótipos multilocus; Polinização aberta; Teca

SUMMARY

GENERAL INTRODUCTION

Tectona grandis, commonly known as teak, is a highly valued hardwood species recognized for its exceptional durability and aesthetic qualities, making it a popular choice for high-end furniture and shipbuilding (Kollert and Kleine 2017; Midgley et al. 2015). In Brazil, approximately 76,000 hectares are cultivated with teak, mainly in the states of Mato Grosso and Pará, which play a crucial role in supplying the global teak market, especially in exporting high-quality wood (IBA 2023; Takizawa and Caldeira 2023). However, the teak plantation faces a severe threat from *Ceratocystis fimbriata*, the causal agent of Ceratocystis wilt. First identified in Brazilian teak plantations in 2009 (Firmino et al. 2012). This disease is linked to significant reductions in wood quality and the volumetric growth rates of trees by approximately 30%. In highly susceptible clones and under favorable conditions, it leads to the death of 100% of the trees (F.A. Rafael, unpublished data), posing a serious risk to teak production.

Ceratocystis fimbriata is part of a complex group of fungi that has been the subject of extensive study and frequent revisions over the years. Initially described by Halsted (1890) as the causal agent of black rot in sweet potato (*Ipomoea batatas*) in New Jersey, it is now recognized as a species complex, exhibiting morphological similarities but substantial genetic variability (Fourie et al. 2015; Harrington 2000; Harrington et al. 2011). Isolates infecting *I. batatas* are classified as *C. fimbriata* sensu stricto (s.s.), while those infecting other hosts are designated *C. fimbriata* sensu lato (s.l.) (de Beer et al. 2014; Harrington et al. 2014). This complex is divided into four major phylogenetic clades: the Latin American Clade (LAC), North American Clade (NAC), Asian-Australian Clade (AAC), and African Clade (AFC) (Baker et al. 2003; Ferreira et al. 2010; Fourie et al. 2015; Harrington et al. 2011; Liu et al. 2018). The taxonomic position of the *Ceratocystis* species has undergone significant study and frequent revisions over the years. Despite the recent proposal by Harrington et al. (2023) to reclassify members of the *C. fimbriata* complex of the Latin American Clade (LAC) as *C. manginecans*, it is widely accepted that Ceratocystis wilt in teak is caused by *C. fimbriata*, which is supported by results of previous studies on mating compatibility, phylogenetic, and population composition analyses (Valdetaro 2019; Fernandes et al. 2022). Consequently, this study will adopt the more well-known name *C. fimbriata*.

Ceratocystis fimbriata has a broad host range and affects numerous agronomic and forestry crops worldwide (CABI 2022). In teak plantations, infections by soilborne *Ceratocystis* are frequently observed, but pruning is a common management practice considered one of the main modes of spreading the pathogen in the plantations (Alfenas et al. 2023). The pathogen penetrates and colonizes the plant vascular system, causing mainly wilting and wood darkening. The colonization of the xylem and phloem vessels by the fungus, along with the accumulation of tyloses, blocks the transport of water and nutrients, leading to wilting and eventual death of the infected trees (Alfenas et al. 2023; Silva et al. 2018). With the rising incidence of Ceratocystis wilt, identifying effective management strategies has become essential. Recent studies have revealed that certain teak genotypes resist *C. fimbriata*, highlighting opportunities for breeding programs focused on developing resistant cultivars (Alfenas et al. 2023; Oliveira et al. 2021a). A thorough understanding of the genetic variation and pathogenicity among *C. fimbriata* isolates is crucial for the success of breeding programs.

Studies have demonstrated that *C. fimbriata* populations infecting several host plants are genetically diverse (Baker et al. 2003; Ferreira et al. 2010; Harrington et al. 2011). Such genetic diversity has been assessed using microsatellite (SSR) markers, which has facilitated a better understanding of fungus-host interactions, geographic differentiation, and determining the origin of populations (Fernandes 2024; Ferreira et al. 2010; Oliveira et al. 2015b, 2021b; Valdetaro et al. 2019). Variation in aggressiveness among *C. fimbriata* isolates when inoculated into the same host genotype has also been described (Oliveira et al. 2015a, 2016, 2021b; Valdetaro et al. 2015), adding complexity to the establishment of disease control. Advances in genome sequencing and bioinformatics offer the opportunity to analyze virulence factors such as proteinaceous effectors, CAZymes, and secondary metabolites, providing valuable information into the pathogenicity mechanisms employed by *C. fimbriata* (Fourie et al. 2020) which allow the selection of more aggressive isolates, contributing to the development of effective disease control strategies. Considering these insights, selecting the most aggressive isolates and evaluating a greater number of teak genotypes through inoculations under controlled conditions is an important strategy in the search for disease-resistant genotypes.

The present thesis consists of three chapters. The first chapter, entitled Genetic variation and aggressiveness of *Ceratocystis fimbriata* isolates infecting teak in Mato Grosso state, Brazil, aimed to determine the genetic variation and aggressiveness of *C. fimbriata* isolates obtained from diseased teak plants. The second chapter, entitled Draft genome sequence of a *Ceratocystis fimbriata* isolate causing Ceratocystis wilt in *Tectona grandis* and its unique pathogenicity traits revealed by comparative genomics aimed to assemble the first genome sequence of a *C. fimbriata* obtained from teak in Brazil, identify relevant pathogenesis-associated functions encoded in its genome, and perform a comparative analysis with the genomes of *C. fimbriata* isolates infecting other host plants. Finally, the third chapter, entitled Resistance of *Tectona grandis* to Ceratocystis wilt, caused by *Ceratocystis fimbriata*, aimed to determine the variability in resistance to Ceratocystis wilt in commercial teak clones and to assess the inheritance of resistance in progenies of crosses resulting from open pollination. In summary, to mitigate the impact of *C. fimbriata* on teak plantations, this study employed an integrated approach that combined the analysis of pathogen genetic variation and aggressiveness with the assessment of teak resistance to the disease.

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CHAPTER 1 - GENETIC VARIATION AND AGGRESSIVENESS OF *Ceratocystis fimbriata* ISOLATES INFECTING TEAK IN MATO GROSSO STATE, BRAZIL

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ABSTRACT

Ceratocystis wilt, caused by *Ceratocystis fimbriata*, is a highly destructive disease that negatively impacts teak plantations in South America. The fungus colonizes the vascular tissues, causing internal wood radial darkening, and wilt and death of susceptible genotypes. Planting of resistant genotypes is the best method to control this disease. However, knowledge of the genetic variation and aggressiveness of the *C. fimbriata* populations obtained from teak plantations is presently scarce. This knowledge is useful for breeding programs, mainly for the selection of clones exhibiting higher levels of resistance. Thus, this study investigated the genetic variation of 56 *C. fimbriata* isolates obtained from different teak plantations in Brazil. Additionally, the aggressiveness of eight *C. fimbriata* isolates representative of the major haplotypes was assessed on three different teak clones. Based on 13 microsatellite markers, the isolates were classified into nine distinct multilocus genotypes, with a predominant genotype. The inoculation assays revealed significant variations in aggressiveness among fungal isolates and distinct interactions with different teak clones. Isolates belonging to three closely related multilocus genotypes (H2, H3, and H4) were the most aggressive. This study provides valuable information

on the genetic variation and aggressiveness of the *C. fimbriata* population from teak, which can be used in breeding for resistance.

Keywords: Ceratocystis wilt. Microsatellite markers. Multilocus genotypes. *Tectona grandis*.

1. INTRODUCTION

Tectona grandis (teak) is one of the most valuable hardwoods in the world, highly appreciated for its wood of high-quality and versatile applications (Kollert and Kleine 2017). In Brazil, there are approximately 76,000 ha planted with teak (IBA 2023). The two leading teak-producing states, Mato Grosso and Pará, focus on exporting high-quality wood, primarily to India (Takizawa and Caldeira 2023). However, since the first report of Ceratocystis wilt in 2009 (Firmino et al. 2012) caused by *Ceratocystis fimbriata,* a notable rise in the incidence of the disease has been observed in teak plantations in Brazil. This disease reduces the volumetric growth rate of trees by approximately 30%. In highly susceptible clones, it leads to the death of 100% of the trees (F.A. Rafael, unpublished data), making it the most significant biotic limitation to teak production. *Ceratocystis fimbriata* requires wounds to penetrate the plant vascular tissue. Once inside, it colonizes the xylem and phloem vessels (Ferreira et al. 2006; Silva et al. 2020). The fungal structures, along with the formation of tyloses and phenolic compounds produced by the host as defense mechanisms, obstruct water and nutrient flow, ultimately causing wilting and, eventually, death (Alfenas et al. 2023; Silva et al. 2018). The pathogen can spread via infected seedlings, insect vectors, and mechanically via infested tools employed in practices such as pruning, harvesting, and seedling transplantation (Harrington 2013). Pruning is a frequent management practice in teak forests and is considered one of the main modes of disease dissemination (Alfenas et al. 2023).

Ceratocystis fimbriata was first described in sweet potato (*Ipomoea batatas*) in New Jersey, USA (Halsted 1890). Since then, it has been reported to cause disease in several agronomic and forestry crops worldwide (CABI 2022). *C. fimbriata* is recognized as a cryptic species complex, displaying significant genetic variation despite its morphological and biological similarities (Fourie et al. 2015; Harrington et

al. 2011). Within this complex, the pathogen affecting sweet potato is classified as *C. fimbriata* sensu stricto (s.s.), while pathogens infecting other hosts are designated as *C. fimbriata* sensu lato (s.l.) (de Beer et al. 2014; Fourie et al. 2015; Harrington et al. 2014), comprising four phylogenetic clades that correspond to specific geographic regions due significant genetic variation: the Latin American Clade (LAC), North American Clade (NAC), Asian-Australian Clade (AAC), and African Clade (AFC) (Baker et al. 2003; Ferreira et al. 2010; Fourie et al. 2015; Harrington et al. 2011). The taxonomic position of the *Ceratocystis* species has undergone significant study and frequent revisions over the years. The more widely recognized name *C. fimbriata* will be used in this study. Despite the recent proposal by Harrington et al. (2023) to reclassify members of the *C. fimbriata* complex of the Latin American Clade (LAC) as *C. manginecans*, it is widely accepted that Ceratocystis wilt in teak is caused by *C. fimbriata*. This conclusion is supported by previous studies based on mating compatibility, phylogenetic analyses, and population composition (Valdetaro 2019; Fernandes et al. 2022).

To date, Ceratocystis wilt on teak has been reported only in Brazil (Firmino et al. 2012) and Ecuador (Belezaca-Pinargote et al. 2020). The planting of resistant genotypes is the most effective method of disease control. Hence, understanding genetic and aggressive variation and selecting more aggressive isolates is essential for screening and selecting teak genotypes with higher levels of resistance. Studies have demonstrated that the *C. fimbriata* populations infecting several host plants are genetically diverse (Baker et al. 2003; Ferreira et al. 2010; Harrington et al. 2011). Such genetic diversity has been assessed using microsatellite (SSR) markers, which have facilitated a better understanding of fungus-host interactions, geographic differentiation, and determining the origin of populations (Fernandes 2024; Ferreira et al. 2010; Oliveira et al. 2015b, 2021b; Valdetaro et al. 2019). Some isolates show host specificity (Baker et al. 2003; Valdetaro et al. 2019) while others infect a wide range of species (Fernandes 2024; Harrington et al. 2011). Furthermore, variation in aggressiveness among *C. fimbriata* isolates when inoculated into the same host genotype has also been described (Oliveira et al. 2015a, 2021b), adding complexity to the establishment of disease control.

So far, there is no knowledge about the genetic and aggressiveness variation of *C. fimbriata* isolates infecting teak in Mato Grosso state, Brazil. Furthermore, it is

unknown whether this population is native to the state or was introduced. Thus, this work aimed to determine the genetic and aggressiveness variation of *C. fimbriata* isolates obtained from diseased teak plants in Mato Grosso and to investigate whether this population is native or introduced.

2. MATERIAL AND METHODS

2.1 Plant material

Rooted microcuttings of three teak clones (C5, A3, and C1) exhibiting resistance, moderate resistance, and susceptibility to Ceratocystis wilt, respectively (S.A. Flávia, unpublished data) were transplanted into 3 L pots containing Tropstrato V6 substrate (Vida Verde), supplemented with 6 g of single superphosphate and 6 g of Osmocote® (15-09-12) per 1 kg of the substrate, and kept for 40 days under greenhouse conditions (25 °C ± 5 °C) with irrigation twice a day until inoculation. Weekly fertilizations were carried out with a complete nutrient solution.

2.2 Fungal isolates

Fifty-six isolates of *C. fimbriata* were obtained between 2015 and 2019 from diseased teak trees in commercial plantations located in the municipalities of Nossa Senhora do Livramento (NS, 15°46'30" N 56°20'44" W), Nova Maringá (NM, 13º01'33" N 57º04'26" W) and São José dos Quatro Marcos (QM, 15º37'17" N 58º10'35" W), in Mato Grosso state, Brazil (Figure 1; Supplementary Table S1). These fungal isolates belong to the Culture Collection of the Laboratory of Forest Pathology / Bioagro of the Universidade Federal de Viçosa, Minas Gerais, Brazil. Isolates were grown on PDA culture medium (potato-dextrose-agar) at 25 ± 2 °C with photoperiod 12 h under 20 µmol/m2/s for 15 days. Subsequently, monoascosporic cultures were obtained by spreading ascospore mass in approx. 10 µL of autoclaved water on potato-dextrose-agar (PDA) medium. After the plates were kept at 25 ± 2 ºC for 12 h and the germinated ascospores were transferred to PDA and incubated for 15 days under the same growth conditions. (Alfenas and Mafia 2016).

2.3 DNA extraction and microsatellite (SSR) genotyping

DNA extraction from 15-day-old cultures of single-ascospore isolates was performed as described by Specht et al. (1982) with modifications by Teixeira et al. (2011). Thirteen SSR markers: CfAAG8, CfAAG9, CfCAA9, CfCAA10, CfCAA15, CfCAA38, CfCAA80, CfCAG5, CfCAG15, CfCAT1, CfCAT1200, CfGACA60, and CfGACA650, developed by Steimel et al. (2004) and mapped to the *C. fimbriata* genome by Simpson et al. (2013), were used for genotyping. Forward primers were fluorescently labeled at the 5' end with either HEX, TET, or FAM (Steimel et al. 2004). For PCR amplifications a Veriti 96-well thermal cycler (Applied Biosystems) was used following the protocol previously described by Ferreira et al. (2010). The amplicon sizes were determined by capillary electrophoresis using an ABI Prism 3500-Avant Genetic Analyzer and the GeneMapper v.4.1 software (Applied Biosystems).

2.4 Genetic variation analysis

Genotype accumulation curves with 1000 bootstrap replications were created for the population from each municipality to determine the minimum number of loci necessary to discriminate individuals in the population. Nei's gene diversity index (H) (Nei 1973) was calculated by applying a clone correction to remove bias caused by the over-representation of some clones based on the microsatellite alleles for the populations from each municipality. Multilocus genotypic diversity was estimated using Stoddart and Taylor's index (G) (Stoddart and Taylor 1988). G values were estimated based on the rarefaction curve according to the number of genotypes in the smallest size sampled (= 9). Analysis of molecular variance (AMOVA) was performed, and the significance of genetic variations was determined after 999 replicates (p-value ≤ 0.01). All analyses were implemented using the package “poppr” (Kamvar et al. 2014) in R v. 4.3.2 (R Core Team 2023). The minimum spanning tree analysis (MST) based on the 13 SSR markers was constructed using the software Bionumerics 6.6. (Applied Maths, http://www.applied-maths.com).

2.5 Assessment of fungal aggressiveness

Eight *C. fimbriata* isolates LPF1589, LPF1594, LPF1599, LPF2199, LPF2209, LPF2342, LPF2343, and LPF2373 were chosen as representatives of major teak haplotypes (TH) to be inoculated in three teak clones. Inoculation was performed with mycelium plugs from a 15-day-old fungal culture (Figure 2A). A wound was made at the base of the plant stem using a disinfested 5-mm-diameter cork borer (Figure 2B), and a mycelium plug was placed on it (Figure 2C). The inoculation site was wrapped with a wet cotton plug and parafilm to reduce desiccation and contamination (Figure 2D). The inoculated plants were kept in a greenhouse (28 °C ± 5 °C) with irrigation twice a day in a completely randomized design with ten repetitions per isolate/clone combination. At 90 days after inoculation (DAI), darkening of the internal stem tissue was noticed (Figure 2E, F). Both lesion length and plant height were measured, and disease severity (lesion length/plant height) was calculated and expressed as a percentage. The carrot bait method (Moller and Devay 1968) was used to confirm the infection by *C. fimbriata* in the inoculated plants.

2.6 Statistical analysis

For the analysis of datasets, a two-factor ANOVA was employed to investigate the effects of isolates, clones, and their interaction on the response variables, lesion length, and disease severity. Using the package “easyanova” (Arnhold 2013) in Rbio v. 192 (Bhering 2017) the dataset was subjected to analysis of variance (ANOVA) (p-value ≤ 0.05) and mean comparisons were conducted using the Scott-Knott test (p-value ≤ 0.05).

3. RESULTS

3.1 Genetic variation

Among the 13 SSR loci evaluated, only CfCAA15, CfCAA80, CfCAG15, and CfGACA650 were polymorphic, with 2, 3, 4, and 2 allelic variants, respectively (Supplementary Table S2). Nine multilocus genotypes (MLGs) were identified among the 56 *C. fimbriata* isolates evaluated. The genotype accumulation curve indicated that only three genetic markers were required to effectively differentiate among individuals within the fungal population studied (Supplementary Figure S1).

AMOVA results indicated that 42.77% of the observed diversity was attributable to variations among populations, while 57.23% could be attributed to variations within populations (Supplementary Table S3). The NS population exhibited the highest genetic diversity (H = 0.205) while the QM population displayed the greatest genotypic diversity (G = 3.86) (Table 1). In contrast, the fungal population from NM exhibited clonality (H = 0.000, G = 1.000). The MST analysis, utilizing the alleles of the full set of 13 SSR loci unveiled different numbers of distinct FHs for each population: 6 for QM, 3 for NS, and 1 for NM, with haplotype FH2 being shared between the QM and NM populations. The difference between a specific haplotype and its most similar haplotype was conferred by only 1 or 2 SSR loci (Figure 3).

3.2 Aggressiveness

All eight *C. fimbriata* isolates caused wilt symptoms, including internal darkened lesions in the longitudinal and radial directions of the stem. Significant effects of teak clone and fungal isolate, as well as interaction between these two factors on lesion length and disease severity, were detected (Supplementary Table S4).

The mean lesion length varied from 3.04 to 18.94 cm depending on the *C. fimbriata* isolate-teak clone combination. Overall, isolates LPF1594, LPF2199, and LPF2209 caused the largest lesions on all tested teak clones, except for clone C1 on which LPF2342 caused a lesion significantly larger than the rest of the isolates (Table 2). Conversely, all data considered, isolates LPF1599, LPF2343, LPF1589, and LPF2373, caused the smallest lesions on the teak clones. Significant variation in lesion length among clones was also observed; the mean values varied from 3.04 to 10.54 cm, 9.94 to 18.94 cm, and 3.66 to 8.31 cm for clones A3, C1, and C5, respectively. Comparisons among teak clones revealed that clone C1 exhibited the largest lesion length (Figure 2F). Taken together, the results indicated that the fungal isolate-teak clone combinations that resulted in the largest mean lesion length were when C1 inoculated with isolates LPF2199, LPF2209, and LPF2342. In contrast, the combinations that resulted in the smallest mean lesion length were clones A3 and C5 inoculated with LPF1589 and LPF2373.

As for disease severity, it varied from 13.62 to 75.12% depending on the fungal isolate-teak clone combination. Disease severity varied from 15.78 to 53.96% for clone A3, 38.89 to 63.59% for clone C1, and 13.62 to 30.46% for clone C5. A trend similar to that observed for lesion length was observed. However, in this case, the highest mean severity values were observed when LPF2342 was inoculated in clones C1 and C5, and the smallest mean disease severity was mostly obtained when clone C5 was inoculated with the fungal isolates (Table 2). The pathogen was re-isolated from all inoculated teak plants exhibiting disease symptoms, even those of clone C5, which showed the smallest lesion length (Figure 2G).

4. DISCUSSION

This study presents genetic and aggressiveness variation among *C. fimbriata* isolates that infect teak trees in Mato Grosso state, Brazil. The gene and genotypic diversity observed in this population is comparable to that of native *C. fimbriata* populations in Brazil, suggesting a potential native origin.

Previous studies on Latin American Clade (LAC) members of the *C. fimbriata* complex have distinguished native from introduced populations using SSR markers (Engelbrecht et al. 2004; Ocasio-Morales et al. 2007; van Wyk et al. 2006). Introduced populations typically exhibit genetic bottlenecks, characterized by low diversity and often a single dominant haplotype compared to native populations (Ferreira et al. 2010). For teak, despite this study being conducted in only three municipalities within a single state in Brazil, genetic variation among the isolates is already evident. Similarly, analyses of isolates from other hosts in Brazil using SSR markers have indicated that the fungus appears to be native, evidenced by the high variability detected (Fernandes et al. 2024). However, confirming this hypothesis requires broader comparative analyses, including isolates from teak from other Brazilian states.

The presence of haplotype FH2 in both QM and NM suggests its predominance in Mato Grosso, or even being disseminated through infected and asymptomatic seedlings, as also was reported in kiwifruit (Ferreira et al. 2017). But further sampling across representative teak plantations is needed to confirm this. While *C. fimbriata* is naturally soilborne, contaminated tools used in cultural practices

may also contribute to its spread (Fernandes et al. 2024). Pruning is a common management practice for teak and is considered one of the main pathways for disease dissemination (Alfenas et al. 2023). Due to this, even haplotypes found in the same collection region, such as FH3, suggest a short-distance disease spread, likely through pruning tools.

The isolates exhibited substantial variation in aggressiveness and showed differential interactions with the three teak clones. Similarly, previous research has demonstrated variations in the aggressiveness of *C. fimbriata* isolates on specific host plant genotypes, along with clear differential interactions between the pathogen and the host genotypes inoculated (Fernandes et al. 2024; Harrington et al. 2011; Oliveira et al. 2015a, 2021b). This variation in aggressiveness within the *C. fimbriata* population in Mato Grosso suggests that the pathogen can generate recombinant strains with novel virulence traits, which could compromise the long-term resistance of teak clones in the field, adding complexity to the establishment of disease control. To mitigate this risk, the use of aggressive fungal isolates in selection programs can help better identify resistant teak genotypes, ensuring improved performance under field conditions. In particular, isolates LPF1594, LPF2199, and LPF2209, either individually or in combination, are recommended for use in inoculation trials under controlled environmental conditions in breeding programs aimed at selecting teak genotypes with enhanced resistance.

In addition to the considerable variation in aggressiveness on teak clones documented in this study, it is important to note that *C. fimbriata* isolates obtained from teak have been shown to infect a wide range of host plants, including kiwifruit, sugar-apple, eucalyptus, edible fig, rubber tree, and mango, under artificial inoculation conditions (Fernandes et al. 2024). This highlights the potential risk of selecting crops susceptible to *C. fimbriata* for intercropped plantations with teak in areas where the pathogen is already present. Moreover, breeding programs targeting resistance to *C. fimbriata* in other host species cultivated in Mato Grosso should incorporate isolates aggressive to teak, and vice versa.

There was no clear association between genetic variation and aggressiveness of the *C. fimbriata* isolates. Despite sharing the same haplotype (FH2), isolates LPF1599 (disease severity between 29.07% and 47.14%), LPF2342 (30.39% and 75.12%), and LPF2343 (16.54% and 38.89%) exhibited different levels of

aggressiveness when inoculated onto the teak clones. Consistent with these results, it has previously been shown that haplotypes obtained using the SSR markers employed in this work do not detect variations in aggressiveness (Oliveira et al. 2015a). Hence, the development of markers targeting *C. fimbriata* genomic regions associated with pathogenicity and virulence is highly desirable. Until such markers are developed and validated, it is essential to assess the aggressiveness of isolates through phenotyping assays.

In conclusion, the *C. fimbriata* population infecting teak in Mato Grosso state exhibits genetic and aggressiveness variation and appears to be a native population. However, no association between genetic variation and aggressiveness could be detected using the set of SSR makers employed in this study. Also, differential interaction between the fungal isolates with teak clones was observed, which confirms the existence of different levels of plant resistance against the pathogen. This information is important for the development of strategies to control Ceratocystis wilt by selecting resistant clones upon inoculation with the most aggressive fungal isolates and deploying them to teak production areas.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY

The data that support the findings of this study will be made available upon request from the corresponding author.

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FIGURES

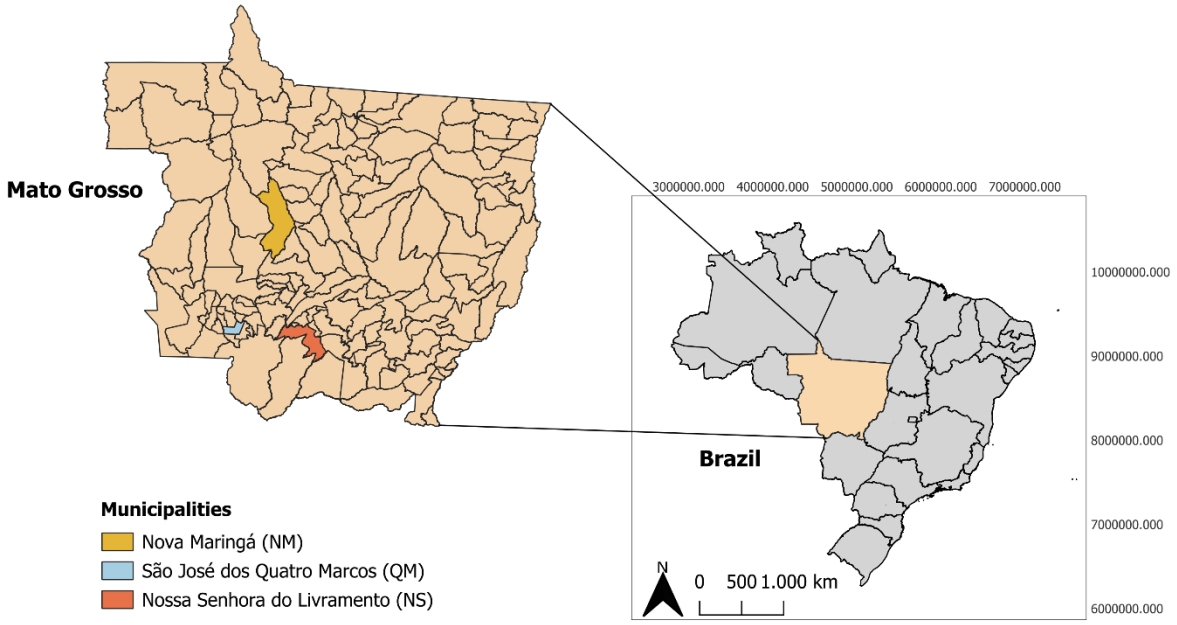

Figure 1. Map of Brazil indicating the municipalities of the Mato Grosso state where *Ceratocystis fimbriata* isolates were collected from diseased teak plants.

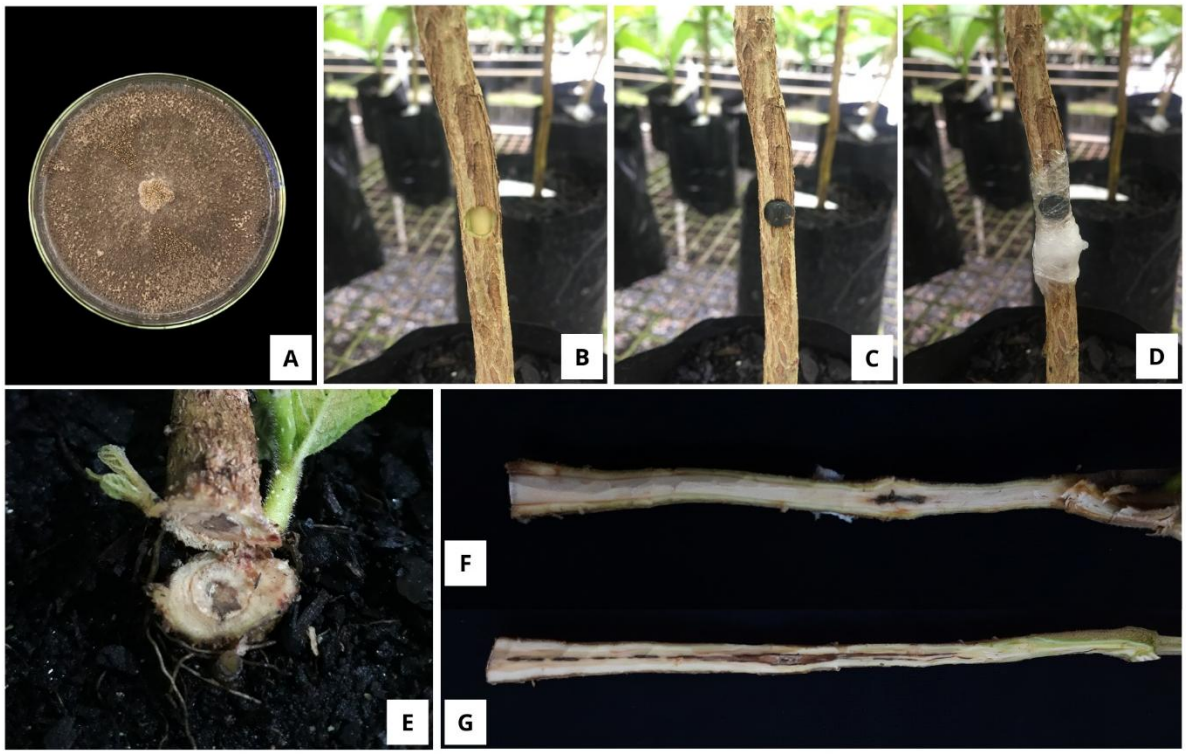

Figure 2. Inoculation of *Ceratocystis fimbriata* on teak. (a) pure culture at 15 days, (b) wounding of the stem, (c) deposition of a mycelium plug in the wound, (d) wrapping of the inoculum with cotton and parafilm, (e) radial darkening of the internal stem tissue, (f) length of the stem lesion in clone C5 inoculated with the LPF2373 isolate, (g) length of the stem lesion in clone C1 inoculated with the LPF2373 isolate.

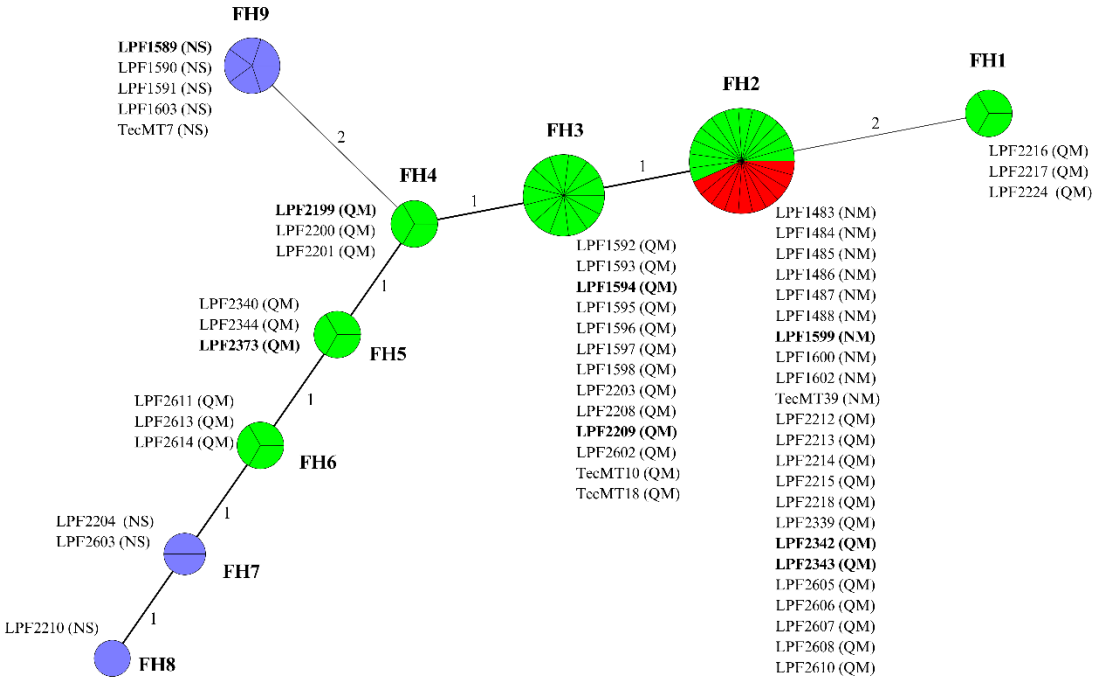

Figure 3. Minimum spanning tree based on alleles of 13 microsatellite (SSR) loci of 56 isolates of the *Ceratocystis fimbriata* populations collected from teak (*Tectona grandis*) in Mato Grosso state. Individual circles represent distinct fungus haplotypes (FH). The number of sections within each circle represents the number of isolates that share the same H. Lines connect haplotypes with numbers that indicate the number of SSR loci that differentiate them. Green color, population from São José dos Quatro Marcos (QM); red color, population from Nova Maringá (NM); and blue color, population from Nossa Senhora do Livramento (NS). Isolates inoculated onto healthy teak plants to determine their aggressiveness are shown in boldface.

TABLES

Table 1. Genetic diversity of *Ceratocystis fimbriata* populations infecting *Tectona grandis* in Mato Grosso state based on 13 microsatellites (SSR) loci

Population[a]	No. of isolates	No. of multilocus genotypes	Nei's gene diversity (H) index		Stoddart & Taylor's genotypic diversity (G) index
			All isolate	Clone-corrected[b]	
QM	38	6	0.120	0.192	3.86
NS	8	3	0.143	0.205	2.13
NM	10	1	0.000	Na	1.00
Total	56	9	0.140	0.199	4.10

[a] Abbreviations for the municipalities in Mato Grosso state, Brazil; QM = São José dos Quatro Marcos; NM = Nova Maringá; NS = Nossa Senhora do Livramento.

[b] Removes bias caused by over-representation of clones based on microsatellite alleles.

Table 2. Lesion length and disease severity on *Tectona grandis* plants at 90 days after inoculation with *Ceratocystis fimbriata* isolates collected in Mato Grosso state, Brazil

Isolates	Haplotype	Clones[a]		
		A3	C1	C5
		Lesion length (cm)		
LPF1589	FH9	4.44 (0.61)[b] Cb	10.71 (0.98) Ca	3.66 (0.80) Bb
LPF1594	FH3	9.65 (0.98) Aa	11.69 (0.91) Ca	8.31 (1.00) Aa
LPF1599	FH2	6.83 (1.13) Bb	10.89 (1.41) Ca	6.71 (1.28) Ab
LPF2199	FH4	10.54 (1.12) Ab	14.14 (1.15) Ba	8.15 (1.45) Ab
LPF2209	FH3	9.10 (1.32) Ab	14.56 (1.03) Ba	8.28 (1.81) Ab
LPF2342	FH2	7.31 (1.06) Bb	18.94 (0.89) Aa	7.88 (1.60) Ab
LPF2343	FH2	6.27 (1.10) Bb	9.94 (1.35) Ca	4.86 (1.70) Bb
LPF2373	FH5	3.04 (0.55) Cb	12.04 (0.92) Ca	4.06 (0.97) Bb
		Disease severity (%)		
LPF1589	FH9	24.64 (3.77) Bb	51.99 (4.44) Ca	13.62 (3.59) Bb
LPF1594	FH3	46.43 (5.01) Aa	53.86 (6.00) Ca	27.56 (2.77) Ab
LPF1599	FH2	39.69 (5.46) Aa	47.14 (4.97) Ca	29.07 (6.19) Ab
LPF2199	FH4	53.96 (5.45) Aa	63.59 (4.99) Ba	30.46 (5.31) Ab
LPF2209	FH3	44.16 (6.06) Ab	58.24 (4.03) Ba	34.86 (6.64) Ab
LPF2342	FH2	30.39 (3.63) Bb	75.12 (3.80) Aa	34.48 (7.42) Ab
LPF2343	FH2	30.64 (4.91) Ba	38.89 (3.73) Ca	16.54 (5.37) Bb
LPF2373	FH5	15.78 (3.80) Bb	40.24 (3.00) Ca	15.37 (3.19) Bb

[a] Values represent the mean lesion length or mean disease severity of ten repetitions. Within columns, means followed by the same uppercase letter are not significantly different from each other, and within a row, means followed by the same lowercase letter are not significantly different from each other according to the Scott-Knott test ($p \leq 0.05$).

[b] Values in parentheses indicate standard errors.

SUPPLEMENTARY MATERIAL

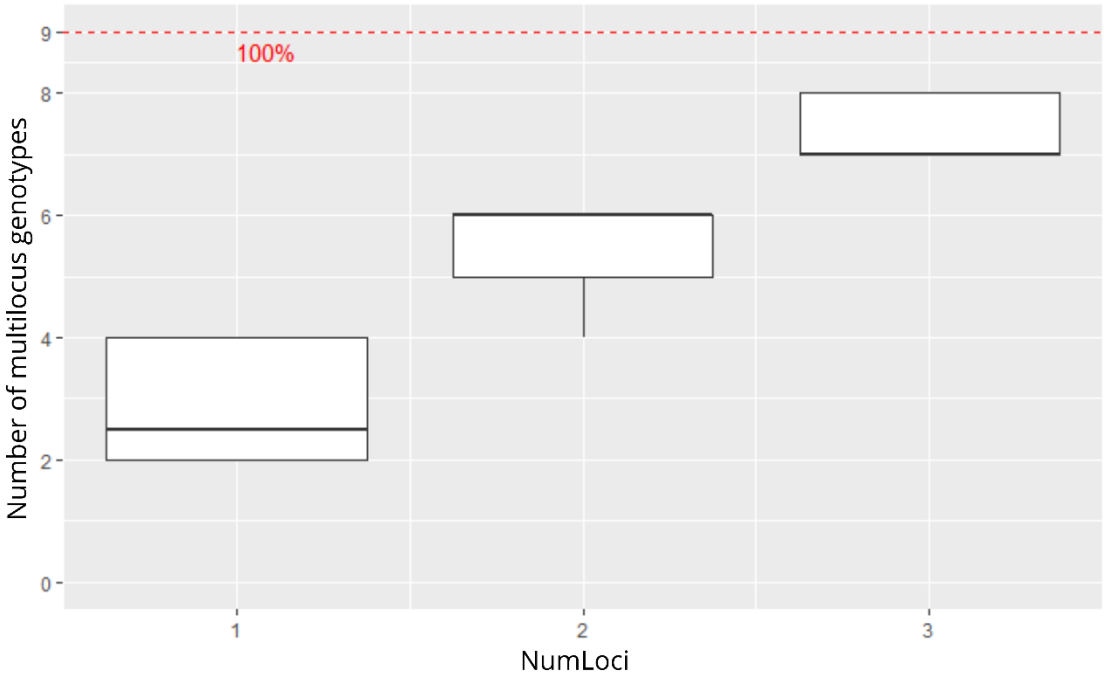

Figure S1. Genotype accumulation curve for the *Ceratocystis fimbriata* populations infecting T*ectona grandis* in Mato Grosso state, Brazil. The dashed line indicates 100% of the number of genotypes identified in each population. The number of loci was randomly sampled 1000 bootstrap.

Table S1. Collection sites of *Ceratocystis fimbriata* isolates from *Tectona grandis* in Mato Grosso state, Brazil

Isolate code[a, b]	Teak genotype	Geographic origin [c]	Year of collection	Collect by
LPF1483	-	NM	2015	Valdetaro, D. C. O. F.
LPF1484	-	NM	2015	Valdetaro, D. C. O. F.
LPF1485	-	NM	2015	Valdetaro, D. C. O. F.
LPF1486	-	NM	2015	Valdetaro, D. C. O. F.
LPF1487	-	NM	2015	Valdetaro, D. C. O. F.
LPF1488	-	NM	2015	Valdetaro, D. C. O. F.
LPF1589	-	NS	2015	Valdetaro, D. C. O. F.
LPF1590	-	NS	2015	Valdetaro, D. C. O. F.
LPF1591	-	NS	2015	Valdetaro, D. C. O. F.
LPF1592	-	QM	2015	Valdetaro, D. C. O. F.
LPF1593	-	QM	2015	Valdetaro, D. C. O. F.
LPF1594	-	QM	2015	Valdetaro, D. C. O. F.
LPF1595	-	QM	2015	Valdetaro, D. C. O. F.
LPF1596	-	QM	2015	Valdetaro, D. C. O. F.
LPF1597	-	QM	2015	Valdetaro, D. C. O. F.
LPF1598	-	QM	2015	Valdetaro, D. C. O. F.
LPF1599	-	NM	2015	Valdetaro, D. C. O. F.
LPF1600	-	NM	2015	Valdetaro, D. C. O. F.
LPF1602	-	NM	2015	Valdetaro, D. C. O. F.
LPF1603	-	NS	2015	Valdetaro, D. C. O. F.
LPF2199	C1	QM	2016	Arenhart, M. L.
LPF2200	C1	QM	2016	Arenhart, M. L.
LPF2201	C1	QM	2016	Arenhart, M. L.
LPF2203	C1	QM	2017	Dorneles, A. I.
LPF2204	Seedling	NS	2017	Dorneles, A. I.
LPF2208	C1	QM	2016	Arenhart, M. L.
LPF2209	C1	QM	2016	Arenhart, M. L.
LPF2210	Seedling	NS	2017	Dorneles, A. I.
LPF2212	E4	QM	2017	Alexandre, F. S.
LPF2213	A1	QM	2018	Alexandre, F. S.
LPF2214	A1	QM	2018	Alexandre, F. S.
LPF2215	A1	QM	2018	Alexandre, F. S.
LPF2216	C1	QM	2018	Alexandre, F. S.
LPF2217	C1	QM	2018	Alexandre, F. S.
LPF2218	C1	QM	2018	Alexandre, F. S.
LPF2224	C1	QM	2018	Alexandre, F. S.
LPF2339	A1	QM	2018	Alexandre, F. S.
LPF2340	A1	QM	2018	Alexandre, F. S.
LPF2342	E4	QM	2018	Alexandre, F. S.
LPF2343	A3	QM	2019	Alexandre, F. S.
LPF2344	A1	QM	2017	Dorneles, A. I.
LPF2373	A1	QM	2018	Alexandre, F. S.
LPF2602	C1	QM	2017	Dorneles, A. I.

Continued...

Table S1 (Cont.)

Isolate code[a, b]	Teak genotype	Geographic origin [c]	Year of collection	Collect by
LPF2603	Seedling	NS	2017	Dorneles, A. I.
LPF2605	E4	QM	2018	Alexandre, F. S.
LPF2606	E4	QM	2018	Alexandre, F. S.
LPF2607	E4	QM	2018	Alexandre, F. S.
LPF2608	E4	QM	2018	Alexandre, F. S.
LPF2610	E4	QM	2018	Alexandre, F. S.
LPF2611	A1	QM	2018	Alexandre, F. S.
LPF2613	A1	QM	2018	Alexandre, F. S.
LPF2614	A1	QM	2018	Alexandre, F. S.
TecMT10	-	QM	2015	Valdetaro, D. C. O. F.
TecMT18	-	QM	2015	Valdetaro, D. C. O. F.
TecMT39	-	NM	2015	Valdetaro, D. C. O. F.
TecMT7	-	NS	2015	Valdetaro, D. C. O. F.

[a] LPF, Laboratório de Patologia Florestal, Universidade Federal de Viçosa, Viçosa, Brazil.

[b] TecMT, *C. fimbriata* isolates from the teak of Mato Grosso state that have not been stored.

[c] Municipalities where the samples were collected. QM: São José dos Quatro Marcos; NM: Nova Maringá; NS: Nossa Senhora do Livramento.

Table S2. Estimated allele sizes of 13 microsatellite (SSR) loci in the *Ceratocystis fimbriata* populations infecting *Tectona grandis* in Mato Grosso state, Brazil

SSR locus	Allele number (Estimated sizes bp)
CfAAG8	1 (171)
CfAAG9	1 (396)
CfCAA9	1 (172)
CfCAA10	1 (136)
CfCAA15	2 (314, 320)
CfCAA38	1 (152)
CfCAA80	3 (311, 317, 356)
CfCAG5	1 (313)
CfCAG15	4 (271, 286, 289, 298)
CfCAT1	1 (252)
CfCAT1200	1 (368)
CfGACA60	1 (183)
CfGACA650	2 (214, 216)

Table S3. Results of analysis of molecular variance (AMOVA) of the *Ceratocystis fimbriata* populations collected from diseased *Tectona grandis* plants in Mato Grosso state, Brazil based on 13 microsatellite (SSR) loci

Source of variation	d.f.[a]	Sum of squares	Mean Squares	Variance components	Percentage of variation (%)
Between populations	2	29.79511	14.897556	0.9944303	42.77
Within populations	53	70.52632	1.330685	1.3306852	57.23
Total	55	100.32143	1.824026	2.3251156	

[a] d.f. = degrees of freedom.

Table S4. Results of analysis of variance (ANOVA) of lesion length and disease severity on *Tectona grandis* plants at 90 days after inoculation with *Ceratocystis fimbriata* isolates collected in Mato Grosso state, Brazil

Factor	d.f.[a]	Sums of Squares	Mean square	F value	p > F[b]
Lesion length					
Clone	2	1966.91	983.46	71.56	<0.001 *
Isolate	7	949.36	135.62	9.87	<0.001 *
Clone x isolate	14	401.50	28.68	2.09	0.0136 *
Residuals	216	2968.65	13.74	-	-
Disease severity					
Clone	2	32977.62	16488.81	69.23	<0.001 *
Isolate	7	19086.41	2726.63	11.45	<0.001 *
Clone x isolate	14	7572.83	540.92	2.27	0.0066 *
Residuals	216	51444.27	238.17	-	-

[a] d.f. = degrees of freedom.

[b] Asterisks indicate statistical significance at $p \leq 0.05$.

CHAPTER 2 - DRAFT GENOME SEQUENCE OF A *Ceratocystis fimbriata* ISOLATE CAUSING CERATOCYSTIS WILT IN *Tectona grandis* AND ITS UNIQUE PATHOGENICITY TRAITS REVEALED BY COMPARATIVE GENOMICS

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ABSTRACT

This study aimed to assemble the genome sequence of the *C. fimbriata* LPF2199 isolate from teak and conduct a comparative genomic analysis with isolates that infect eucalypt, kiwi, and ghaf trees. Genes coding effector proteins, CAzymes, and secondary metabolites, potentially playing significant roles during the interaction with the plant were predicted. The genome of the LPF2199 isolate is 31.62 Mb in size and contains 7,533 genes with a GC content of 47.92%. Of these, 2,542 proteins were assigned functions, including 657 predicted to be effectors. Comparative analysis indicates high genomic similarity among the *C. fimbriata* isolates investigated. However, subtle differences in gene content and the presence of proteins unique to the LPF2199 isolate may contribute to its broader virulence. The findings presented here are the basis for future studies to elucidate how these genomic variations impact pathogenicity, which may be instrumental in developing novel strategies for select resistant teak genotypes.

Keywords: CAZymes. Effectors. Genome assembly. Secondary metabolites. Teak.

1. INTRODUCTION

Ceratocystis wilt on teak, caused by *Ceratocystis fimbriata*, is the most significant disease affecting teak plantations in Brazil. This disease causes substantial negative impacts on wood production and quality, and plant mortality in severe cases. *Ceratocystis* species require wounds or natural openings to penetrate and infect their hosts and are naturally soilborne (Harrington 2013). In teak, pruning is a common management practice considered one of the main modes of dissemination of the disease (Alfenas et al. 2023). After pathogen colonization of the plant vascular system, it promotes the yellowing of the leaves (Figure 1A), cracking and gummosis of the bark (Figure 1B), and epicormic shoots along the tree (Figure 1C) are observed. As the disease progresses, leaves wilt and fall, and internal darkening can be seen when the wood is cut longitudinally (Figure 1D). In transverse sections of the wood, radial darkening of the xylem to the phloem can be noticed in younger plants. In comparison, older plants exhibit discontinuous bluish-gray spots, with greater intensity in the sapwood (Figures 1E-F). Wood darkening occurs due to fungal colonization, tylosis formation, and the accumulation of phenolic compounds, which obstruct water and nutrient transport in the phloem, ultimately leading to tree wilting and death (Alfenas et al. 2023; Silva et al. 2018). The basic density and hardness of teak wood are not affected by *C. fimbriata* infection. However, color changes are evident, particularly in the sapwood (Silva et al. 2021). The main control method for this disease is the planting of resistant genotypes (Guimarães et al. 2021; Oliveira et al. 2021). However, research on teak breeding is still limited, making it crucial to understand the interaction between plant-pathogen and the mechanisms involved in disease occurrence, to develop effective control strategies.

The taxonomic position of the *Ceratocystis* species has been extensively studied and revised over time. Originally described as the causal agent of black rot of sweet potato (*Ipomoea batatas*) in New Jersey, United States (Halsted 1890), *C. fimbriata* has been recognized as a complex of cryptic species that exhibit morphological and biological similarities but considerable genetic variation (Fourie et al. 2015; Harrington et al. 2011). Within this complex, the isolates infecting *I. batatas* are classified as *C. fimbriata* sensu stricto (s.s.), while those infecting other hosts are

considered as *C. fimbriata* sensu lato (s.l.) (de Beer et al. 2014; Fourie et al. 2015). Four phylogenetic clades, corresponding to specific geographic regions, have been identified: Latin American Clade (LAC), North American Clade (NAC), Asian-Australian Clade (AAC), and African Clade (AFC), with Brazilian isolates allocated in the LAC (Fourie et al. 2015; Harrington et al. 2011). Harrington et al. (2023) recently proposed renaming the *C. fimbriata* complex members within the LAC group. Among these, the pathogen causing Ceratocystis wilt in teak was reclassified from *C. fimbriata* to *C. manginecans*. Nonetheless, it is widely accepted that Ceratocystis wilt on teak is caused by *C. fimbriata*, which is supported by results of previous studies on mating compatibility, phylogenetic, and population composition analyses (Valdetaro 2019; Fernandes et al. 2022). Consequently, this study will adopt the more well-known name *C. fimbriata*.

This study was motivated by findings from Fernandes et al. (2024) who suggested that *C. fimbriata* isolates infect teak show physiological specialization, as isolates from other hosts do not infect teak. However, the same study revealed that *C. fimbriata* isolates that infect teak, are also pathogenic to plant species of genera such as *Actinidia*, *Eucalyptus*, and *Mangifera*. Here, it was reasoned that *C. fimbriata* isolates from teak utilize distinct mechanisms to interact with plants compared to isolates from other hosts, which could explain their ability to infect teak and other plant hosts. Advances in genome sequencing and bioinformatics offer the opportunity to address this question by analyzing the biological functions encoded in different isolate genomes (Guttman et al. 2014), determining differences and commonalities among them that could dictate pathogenic behavior (Fourie et al. 2020; Molano et al. 2018; Maguvu et al. 2023; Santos et al. 2020). Identifying virulence factors, such as proteinaceous effectors, CAZymes, and secondary metabolites, provides valuable insights into the pathogenicity mechanisms employed by *C. fimbriata* that may determine host specialization (Fourie et al. 2020) and could support the development of targeted disease control strategies.

Fungi secrete small proteins (so-called effector proteins) that allow them to suppress the plant immune response and/or interfere with its cellular processes to promote infection. The presence/absence of genes coding for effector proteins and/or differences in their amino acid sequences can impact the *Ceratocystis* ability to infect a host plant (Fourie et al. 2020). In addition, carbohydrate-active enzymes (CAZymes) are essential virulence factors of phytopathogens that colonize woody

plants (Kikot et al. 2009), as these enzymes are involved in breaking down plant cell wall components such as cellulose, hemicellulose, lignin, and pectin (Amselem et al. 2011). Other important determinants of fungal pathogenicity/virulence are secondary metabolites, which play significant roles in the ability to infect and damage its host (Maguvu et al. 2023). Thus, this study aimed to assemble the first genome sequence of a *C. fimbriata* obtained from teak in Brazil, identify relevant pathogenesis-associated functions encoded in its genome, and through a comparative analysis with the genomes of *C. fimbriata* isolates from other host plants, gain knowledge on their functional differences and similarities to enhance our understanding of the mechanisms by driving the ability of *C. fimbriata* to infect teak and other hosts.

2. MATERIAL AND METHODS

2.1 Fungal isolate, DNA extraction, and genome sequencing

The *C. fimbriata* LPF2199 isolate was obtained in 2016 from a diseased teak tree in a commercial plantation located in São José dos Quatro Marcos in Mato Grosso state, Brazil (15º37'17" N 58º10'35" W). This fungal isolate belongs to the Culture Collection of the Laboratory of Forest Pathology / Bioagro of the Universidade Federal de Viçosa, Minas Gerais, Brazil. It is a virulent *C. fimbriata* isolate, frequently used in teak resistance screening (Alexandre et al. unpublished). The isolate was grown on a 2% malt extract (ME) medium for at 25°C with agitation at 180 rpm for 48 hours. DNA extraction was performed using the DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quality of the DNA was assessed using 1% agarose gel electrophoresis at 100 V for 40 min, and the quantity was measured with a Qubit 2.0 fluorometer. A DNA library with an insert size of approximately 350 bp was prepared using the NEBNext® Ultra™ II DNA Library Preparation Kit for Illumina (New England Biolabs, USA) following the standard protocol, and sequenced on the Illumina NovaSeq 6000 platform at GenOne Biotech (www.genone.com.br).

2.2 Genome assembly and annotation

The output reads from sequencing were subjected to quality control assessment using FASTQC version 0.11.9 (https://github.com/s-andrews/FastQC). TrimGalore version 0.6.7 (Krueger et al. 2021) was employed to remove adapter sequences using the “auto-detection” setting. Then, paired reads were trimmed and filtered for length using Trimmomatic version 0.39 (Bolger et al. 2014) by selecting the following parameters: HEADCROP:15, CROP:130, SLIDINGWINDOW:4:20, and MINLEN:50. SPAdes version 3.15.3 (Bankevich et al. 2012) was employed for de novo assembly of the genome using the method “isolate” and selecting all odd k-mers between 21 and 127. The descriptive statistics and the sequencing coverage of the scaffolds were obtained with Assembly-stats version 1.0.1 (https://github.com/sanger-pathogens/assembly-stats) and BBMap version 38.76 (https://sourceforge.net/projects/bbmap) respectively.

Genomic prediction was conducted with the Funannotate version 1.8.15 pipeline (Palmer and Stajich 2019). Combined *ab initio* and evidence-based methods, using sequence similarity with known proteins, were utilized for gene prediction. First, Repeat-Modeler version 2.0.1 (Flynn et al. 2020) was used for *ab initio* prediction of transposable elements (TEs) and repetitive elements to produce a library from the scaffolds. Then, a soft-masking of these elements in the scaffold sequences of the predicted library was executed with RepeatMasker version 4.1.1 (Tarailo-Graovac and Chen 2009). After the repeat masking, the tool “predict” of Funnannotate was used to predict the genes by comparison with protein sequences of the *Ceratocystis* genus (NCBI taxonomy ID 5157) available in the UniProt Knowledgebase (UniProtKB; https://www.uniprot.org/; accessed in 2023-10-09). In addition, ribosomal RNA genes were predicted with Barrnap version 0.9 (https://github.com/tseemann/barrnap), selecting the Eukarya kingdom and an E-value threshold of 1e-10.

The predicted gene sets of the *C. fimbriata* LPF2199 isolate were functionally annotated based on similarity with homologous sequences deposited in public databases. BLASTp was used to align the protein sequences by automated searches against the Swiss-Prot database (https://www.uniprot.org/; accessed in 2023-11-13) and using protein sequences of the *Sordariomycetes* class (NCBI taxonomy ID 147550) (UniProtKB; https://www.expasy.org/sprot/; accessed in 2023-11-13). In addition, Reverse Position-Specific BLAST (RPS-BLAST) was used against the

Eukaryotic Orthologous Groups database (KOG; https://ftp.ncbi.nih.gov/; accessed on 2023-11-30).

2.3 Prediction of pathogenesis-related molecules

In order to predict pathogen-associated proteins, searches were performed against the Pathogen-Host Interaction database (PHI; http://www.phi-base.org/; accessed in 2023-11-16). In addition, carbohydrate-active enzymes (CAZymes) were predicted using the Automated Carbohydrate-active Enzyme Annotation database (dbCAN HMM; https://bcb.unl.edu/dbCAN/; accessed in 2023-11-17) (Yin et al. 2012) and the dbCAN2 meta server was employed to group the predicted CAZyme functional modules into six classes: Glycoside Hydrolases (GHs), Polysaccharide Lyases (PLs), Carbohydrate Esterases (CEs), Glycosyl Transferase (GTs), Carbohydrate-Binding Modules (CBMs), and Auxillary Activities (AAs) (http://www.cazy.org) (Lombard et al. 2014). The criteria (identity *coverage ≥ 40%) was used for all comparisons. Additionally, gene clusters coding for biosynthesis of secondary metabolite were predicted using antiSMASH fungal 7.0 (https://fungismash.secondarymetabolites.org) (Blin et al. 2023).

2.4 Comparative genomic analysis

The protein functions encoded in the genome sequence of the *C. fimbriata* LPF2199 isolate were compared to those encoded in other *C. fimbriata* genomes. These included the genome of the isolates LPF1912 obtained from eucalypt (*Eucalyptus* sp.) (Accession: ASM991473v1) (Santos, et al. 2020), and CMW17570 obtained from ghaf tree (*Prosopis cineraria*) (Accession: JJRZ00000000.1) (van der Nest et al. 2014) available in the NCBI Genome database (https://www.ncbi.nlm.nih.gov/genome), and LPF1701 obtained from kiwifruit (*Actinidia* sp.) (this genome is not currently deposited). The isolate CMW17570 was included in the analyses because, despite being originally described as part of an introduced population from Oman, it is closely related to *C. fimbriata* isolates from Brazil (Harrington et al. 2024; Oliveira et al. 2015; Van Wyk et al. 2007). The proteomes, secretomes, effectoromes as well as genes coding for the production of

CAZymes and secondary metabolites were included in the comparisons among the *C. fimbriata* isolates.

To identify orthologous genes shared among the *Ceratocystis* isolates, reciprocal similarity searches were performed using the BLASTp tool from BLAST version 2.13.0 (Altschul et al. 1990), setting an E-value threshold of 1e-10. Subsequently, orthologous genes were identified using OrthoMCL version 2.0.9 (Li et al. 2003) and clustered with the Markov Cluster Algorithm (MCL) version 14-137 (Van Dongen 2008), applying an inflation value of 1.5.

2.5 Prediction of protein secretomes and effectoromes

The secretome of each isolate was predicted through a three-step process. First, proteins containing signal peptides were identified using SignalP version 6.0 (https://services.healthtech.dtu.dk/services/SignalP-6.0/) (Teufel et al. 2022) and TargetP version 2.0 (https://services.healthtech.dtu.dk/services/TargetP-2.0/) (Armenteros et al. 2019). Then, the candidate secreted proteins were analyzed with TMHMM version 2.0 (https://services.healthtech.dtu.dk/services/TMHMM-2.0/) (Krogh et al. 2001) to predict transmembrane domains. Proteins with a PredHel score of 0 (indicating no predicted transmembrane segments) and a predicted N-terminal signal peptide were retained for further analysis. Finally, GPI (glycosylphosphatidylinositol)-anchor proteins, which are surface proteins of the fungus cell, were predicted using PredGPI (http://gpcr.biocomp.unibo.it/predgpi) (Pierleoni et al. 2008) and removed from the final set of secreted proteins. Candidate effectors were predicted by subjecting the secretome to EffectorP 3.0 predictions (https://effectorp.csiro.au/) (Sperschneider and Dodds 2021).

3. RESULTS

3.1 Genome sequencing, assembly, and protein prediction

The genome of *C. fimbriata* LPF2199 isolated from teak was assembled into a 31.62 Mb sequence, comprising 1,231 scaffolds with an N50 of 128,558 bp and GC content of 47.92%. It is predicted to contain 7,051 coding DNA sequences (CDS), 371 transfer RNAs (tRNA) (Table 1; Supplementary Table S1), 109 5S rRNA, one

5.8S rRNA, and one 18S rRNA (Supplementary Table S2). Repetitive sequences account for 18.29% of the genome, including retroelements of the classes small interspersed nuclear elements (SINE), long interspersed nuclear elements (LINEs), and long terminal repeat (LTR), accounting for 0.01, 1.70, and 4.56% of the retroelements, respectively. DNA transposons (3.22%), small RNA (0.03%), simple (1.61%), and low complexity (0.43%) repeat regions and unclassified sequences (6.73%) were also identified in the genome (Table 1).

Among the proteins encoded in the 7,051 predicted CDS, 2,542 were assigned known functions. Of these, 975 were identified as effectors and 25 as CAZymes by PHI-base and dbCAN databases. Moreover, 2,224 proteins were assigned functions by comparison with sequences deposited in the Swiss-Prot, UniProtKB, and KOG databases, some of which had already been identified as effectors or CAZymes (Figure 2A; Supplementary Table S1). Among the proteins classified within the EuKaryotic Orthologous Groups (KOG), the most common assignments in the information storage and processing category were group J (translation, ribosomal structure, and biogenesis) representing 15.69% of the proteins, followed by group A (RNA processing and modification) accounting for 6.93%. For the storage and processing category, the predominant functions were groups O (posttranslational modification, protein turnover, chaperone activity) and U (intracellular trafficking, secretion, and vesicular transport) representing 12.53% and 6.81% of the proteins, respectively. No proteins were allocated in groups N (cell motility) and W (extracellular structures). Assignments into groups C (energy production and conversion) and E (amino acid transport and metabolism) of the metabolism category accounted for 10.58%, and 7.54%, respectively (Figure 2B).

3.2 Comparative proteome and secretome analysis

To better understand the molecular mechanisms underpinning the pathogenicity of *C. fimbriata* LPF2199, a comparative analysis of its genome with those of *C. fimbriata* isolates from eucalypt, kiwi, and ghaf trees was conducted. The quality of the genome assemblies and proportion of repetitive sequences were found to be similar among the *C. fimbriata* genomes compared. The annotation analysis predicted were 7,479, 7,560, and 7,939 total number genes for isolates CMW17570 (31.71 Mb genome size), LPF1701 (30.33 Mb), and LPF1912 (31.57 Mb),

respectively, being similar to the number predicted (7,533) for the teak isolate LPF2199 (31.62 Mb) (Supplementary Table S3).

Proteome ortholog analysis revealed that the total number of proteins varies little across the *C. fimbriata* isolates (6,743 in LPF2199, 6,749 in CMW17570, 6,788 in LPF1701, and 7,011 of LPF1912), 6,432 (95%) of which are shared among them, representing the core proteome for the set of isolates analyzed (Figure 3A). LPF1912 has the highest number of isolate-specific proteins, totaling 334, followed by LPF1701 with 149, CMW17570 with 105, and LPF2199 with 101. The four *C. fimbriata* isolates share a total of 347 proteins predicted to be secreted out of 398 presents in LPF2199, 381 in CMW17570, 405 in LPF1701, and 471 in LPF1912). The number of isolate-specific proteins putatively secreted was 73 for LPF1912, 20 for LPF 1701, 10 for LPF2199, and 5 for CMW17570 (Figure 3B).

3.3 Comparison of predicted pathogenesis-related functions

To identify potential distinctive pathogenicity mechanisms, present in the *C. fimbriata* isolate LPF2199 from teak, its ten unique proteins were further analyzed. Five of these proteins were identified as potential cytoplasmic effectors, while no evidence of being effectors was obtained for the other five proteins (Figure 4A). Despite being subjected to sequence comparisons against different databases (Swiss-Prot, UniProtKB, KOG, PHI, and dCAN), only one of the proteins was predicted to code for a chitinase (by UniProtKB) while none of the others was associated with a known biological function (Figure 4B).

A total of 27, 26, 27, and 25 CAZymes were identified in the CMW17570, LPF1701, LPF1912, and LPF2199 genomes, respectively, distributed in two CAZyme families: auxiliary activities (AA) and carbohydrate-binding modules (CBM) (Figure 5A). Variations in the content of subgroups AA11 (lytic chitin monooxygenase), CBM13 (functions including glycoside hydrolases and glycosyltransferases), and CBM18 (chitin-binding function) among isolates were predicted. It was predicted that isolate LPF1912 contains a larger number of genes only for the CBM18 group when compared to the other isolates. Sequence comparisons with the PHI database revealed few variations in the content of candidate effector proteins among the *C. fimbriata* isolates. The number of genes of the reduced virulence (RV) was the most abundant in all genomes analyzed (Figure 5B).

The total number of gene clusters coding for the biosynthesis of secondary metabolites was identical across all *C. fimbriata* isolates. Each genome contains ten clusters: two for terpenes, two for type I polyketide synthase (T1PKS), one for type III polyketide synthase (T3PKS), one for non-ribosomal peptide synthase (NRPS), one for non-ribosomal peptide metallophones (NRP-metallophone), two for non-ribosomal peptide synthase-like (NRPS-like), and one for ribosomal synthesized and post-translationally modified peptides (fungal-RiPP-like) (Supplementary Table S4).

4. DISCUSSION

This study presents a high-quality draft-genome assembly for the *C. fimbriata* LPF2199 isolate, obtained from a diseased teak tree in Brazil. LPF2199 has a genome consisting of a 31.62 Mb sequence, with 18.29% identified as repetitive sequences. The features of this assembly are consistent with those of *Ceratocystis* species previously described for other isolates, including the *C. fimbriata* type isolate (NCBI accession: APWK00000000) (Wilken et al. 2013), *C. destructans* (NCBI accession: SAMN35663300, and SAMN35663304) (Maguvu et al. 2023), and *C. cacaofunesta* (NCBI accession: PEJQ00000000) (Molano et al. 2018). Among the predicted proteins, we found many classified into KOG groups are associated with pathogenicity, encompassing functions such as protein modification, effector secretion, energy production, and metabolite production essential for nutrient acquisition (Tatusov et al. 2003).

Comparative genomic analysis of *C. fimbriata* isolates from eucalypt, kiwifruit, and ghaf trees with the teak isolate revealed high genomic similarity, including comparable catalogs of CAZymes, effector protein candidates, and gene clusters associated with secondary metabolites production. The CAZymes predicted for the *C. fimbriata* isolates investigated in this study play fundamental roles in pathogenicity. CAZymes AA1, AA3, and AA9 are involved in the degradation of lignin, allowing the pathogen to access vital nutrients and colonize plant tissues. AA2, AA11, and CBM13 contribute to the breakdown of polysaccharides, facilitating the utilization of complex carbohydrates (Levasseur et al. 2013; Bennati-Granier et al. 2015; Boraston et al. 2004). CBM18 acts as a cell wall-bound effector that protects the fungal cell wall from plant-secreted chitinases (Tanaka and Kahmann 2021). These enzymatic activities enhance the pathogen's ability to overcome host

defenses, promoting successful infection. However, CAZyme genes may be present in similar numbers among different isolates or species but expressed differently. For example, studies have shown that necrotrophic and hemibiotrophic fungi have differential expressions of CAZymes based on their interaction with plant cell walls (Zhao et al. 2013). Variations in the expression of CAZymes can influence pathogenicity and the organism's adaptation to diverse environments, affecting its ability to infect hosts and degrade substrates. Therefore, we speculate that the differential expression of CAZymes is the major player in modulating interactions of *Ceratocystis* isolates with their environments and hosts instead of differences in their gene repertoire.

The presence of identical gene clusters for secondary metabolite production across all *C. fimbriata* isolates analyzed here also suggests these molecules play a conserved and crucial role in the pathogen's ability to infect their hosts. These clusters correspond to those previously identified in other *Ceratocystis* species (Fourie et al. 2020; Sayari et al. 2019; Sayari et al. 2018), supporting their conservation and probable important role during plant-fungi interactions.

Comparisons of the proteome and secretome led to the identification of genes that are highly conserved among *C. fimbriata* isolates, and those unique to each isolate. Despite their genetic similarity, *C. fimbriata* isolates from different hosts exhibit variation in pathogenicity toward different plant species and aggressiveness when inoculated in the same host plant (Fernandes et al. 2024). In the case of *C. fimbriata* infecting teak, it does not exhibit physiological specialization as in the case of *C. fimbriata* f. sp. *carapa* (Valdetaro et al. 2019) and *C. cacaofunesta* (Fernandes et al. 2024). Unlike other isolates, non-pathogenic to teak or display low aggressiveness on other hosts, LPF2199 can process a unique set of genes or protein content that may enhance its pathogenicity and virulence. The effector repertoires predicted in the *C. fimbriata* isolates show minimal variation among the different isolates; however, small repertoires of isolate-specific effectors could significantly influence the outcome of the interaction with the plant. Furthermore, genes classified in the PHI database related to reduced virulence were the most abundant in all genomes analyzed, suggesting that mutations in these regions can significantly impair the aggressiveness of the isolates.

LPF2199 showed ten isolate-specific secreted proteins, nine of which have no assigned biological function, and one is a predicted chitinase. Fungal chitinases are

involved in cell wall remodeling, growth, cell division, and interaction with the environment or other organisms. These enzymes can also play a role in competition with other fungi by degrading their cell walls, but their role during interaction with the host plant is still unknown (Langner and Göhre 2016). Given that most proteins predicted to be secreted and exclusive to LPF2199 have unknown biological functions, there is a significant need to explore the roles of these possible uncharacterized effectors, as they may provide insights into the complex interactions between the fungus and its hosts. Based on the comparisons conducted in this study, the genome of isolate LPF2199 bears distinct characteristics that may explain its ability to infect the teak and exhibit pathogenicity toward other hosts. These findings establish a foundation for further research on plant-*Ceratocystis* interactions, paving the way for a more comprehensive understanding of the underlying molecular mechanisms and their implications for managing this disease.

In this study, we present the first high-quality draft genome sequence of the *C. fimbriata* LPF2199 isolate, collected from a teak tree affected by Ceratocystis wilt. By leveraging a suite of bioinformatics tools, we successfully assembled the draft genome sequence and predicted its protein-coding genes. From *in silico* predictions, key putative virulence factors, including CAZymes, gene clusters for the biosynthesis of secondary metabolites, and candidate effector proteins were identified, which may play essential roles during the interactions of *Ceratocystis* isolates with their hosts. Additionally, we conducted an orthologous analysis of the predicted proteins, offering insights into functional similarities and differences among the investigated isolates. The results of this study provide a foundation for future research aimed at unraveling the molecular mechanisms driving the pathogenicity of this important teak pathogen.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY

The raw sequencing data and scaffold sequences of the assembled genome will be deposited in NCBI.

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FIGURES

Figure 1. Symptoms of Ceratocystis wilt in teak trees. (A) wilting and plant defoliation, (B) cracking and gummosis in the bark, (C) epicormic shoots along the tree, (D) longitudinal cuts in the wood showing internal darkening (E) transverse section of the wood with radial darkening of the xylem to the phloem in a young plant, (F) discontinuous, bluish-gray spots (arrow), with greater intensity in the sapwood of an old plant.

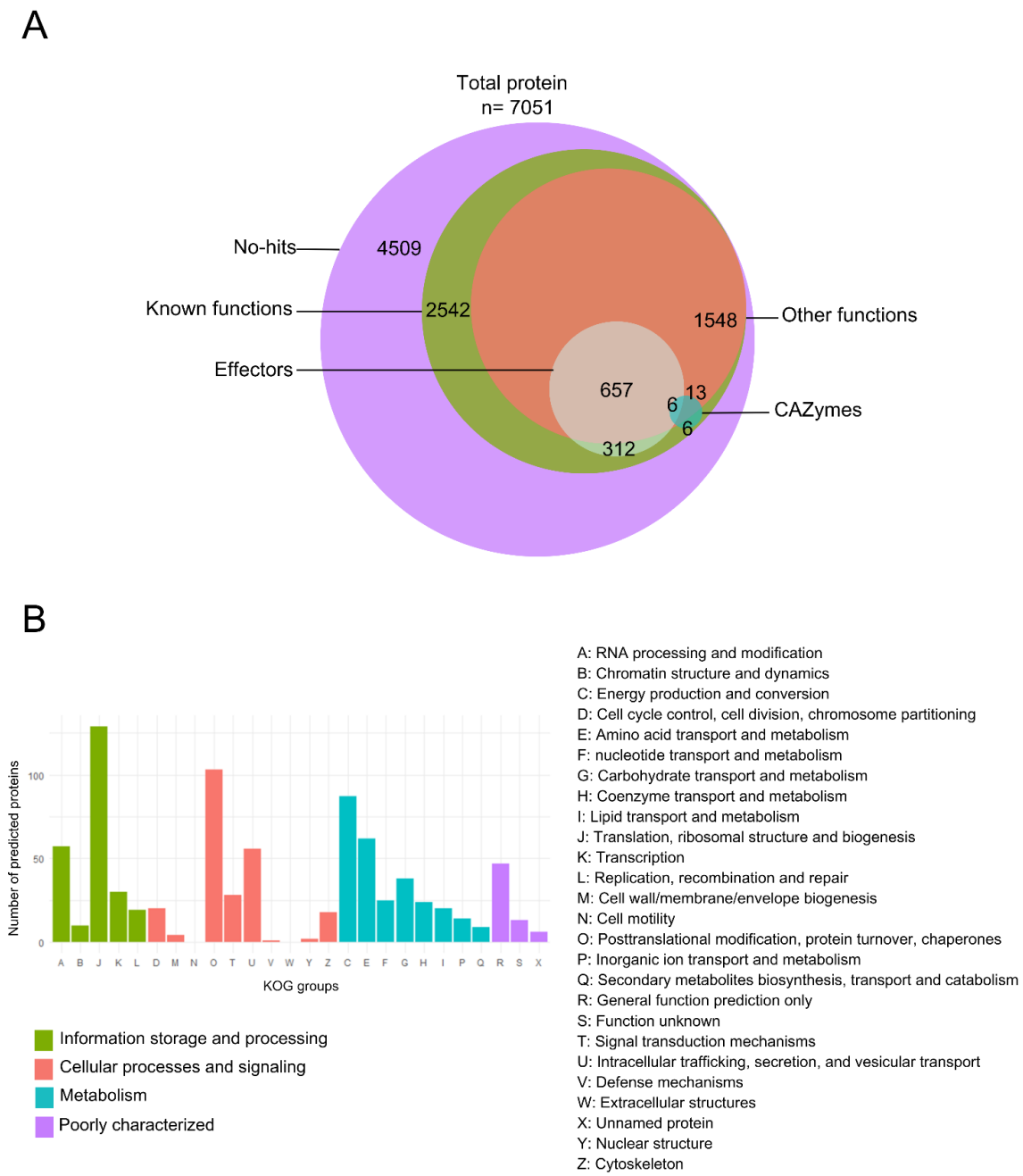


Figure 2. Representation of the *Ceratocystis fimbriata* LPF2199 proteome: (A) Proteins annotated as Effectors (PHI-base), Carbohydrate-active enzyme (dbCAN), playing others functions (Swiss-Prot, UniProtKB, and KOG). (B) Classification by KOG groups.

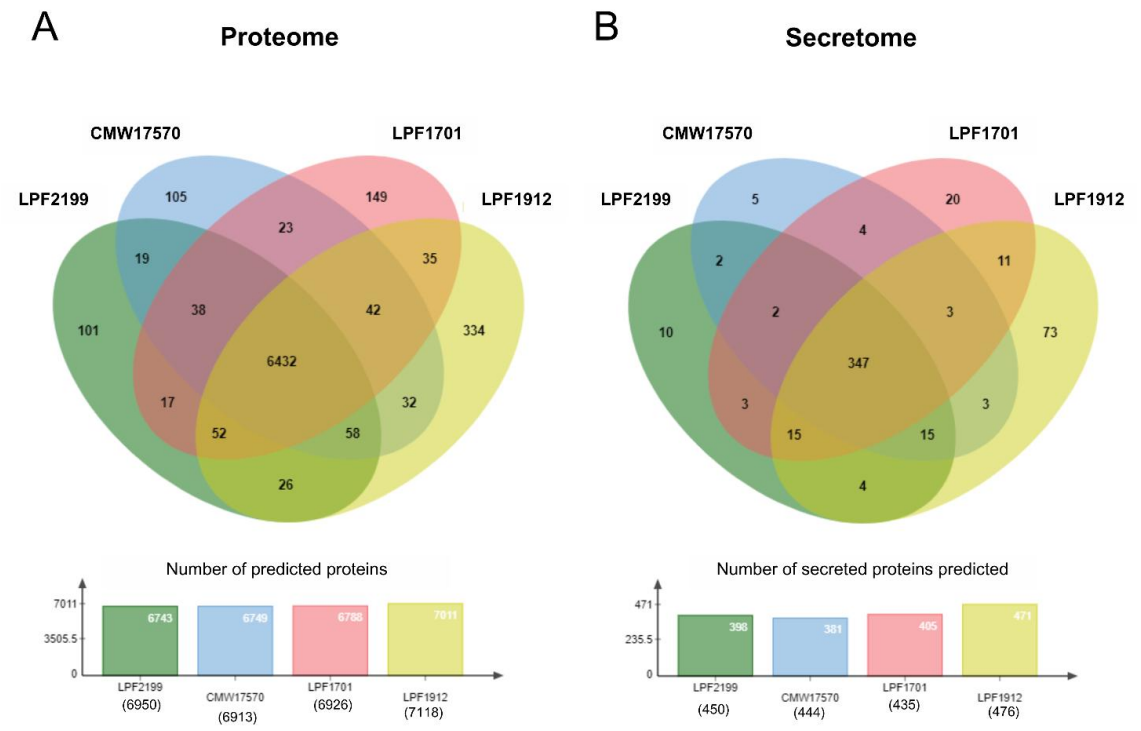
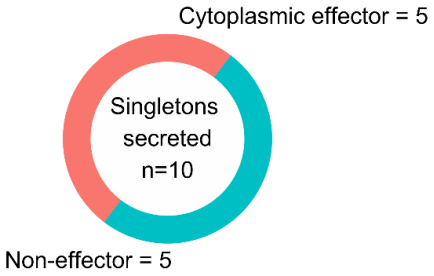


Figure 3. Comparative proteome and secretome content among *Ceratocystis fimbriata* isolates LPF2199, LPF1912, LPF1701, and CMW17570: (A) Venn diagrams illustrating the predicted proteins and; (B) proteins predict to be secreted. Proteins present in each isolate are represented by a distinctive color, with overlapping areas indicating proteins shared by two or more isolates.

A



B

Gene ID	Functional annotation
LPF2199_002668	No hit
LPF2199_006093	No hit
LPF2199_006094	No hit
LPF2199_007361	No hit
LPF2199_007406	No hit
LPF2199_005102	No hit
LPF2199_006276	No hit
LPF2199_006854	No hit
LPF2199_006960	**Chitinase**
LPF2199_007085	No hit

Figure 4. Effectors predicted in the repertoire of proteins exclusive to *Ceratocystis fimbriata* isolate LPF2199 from teak. (A) The number of predicted effectors by EffectorP. (B) Functional annotation of the LPF2199 exclusive proteins predicted to be secreted using Swiss-Prot, UniProtKB, KOG PHI, and dbCAN databases.

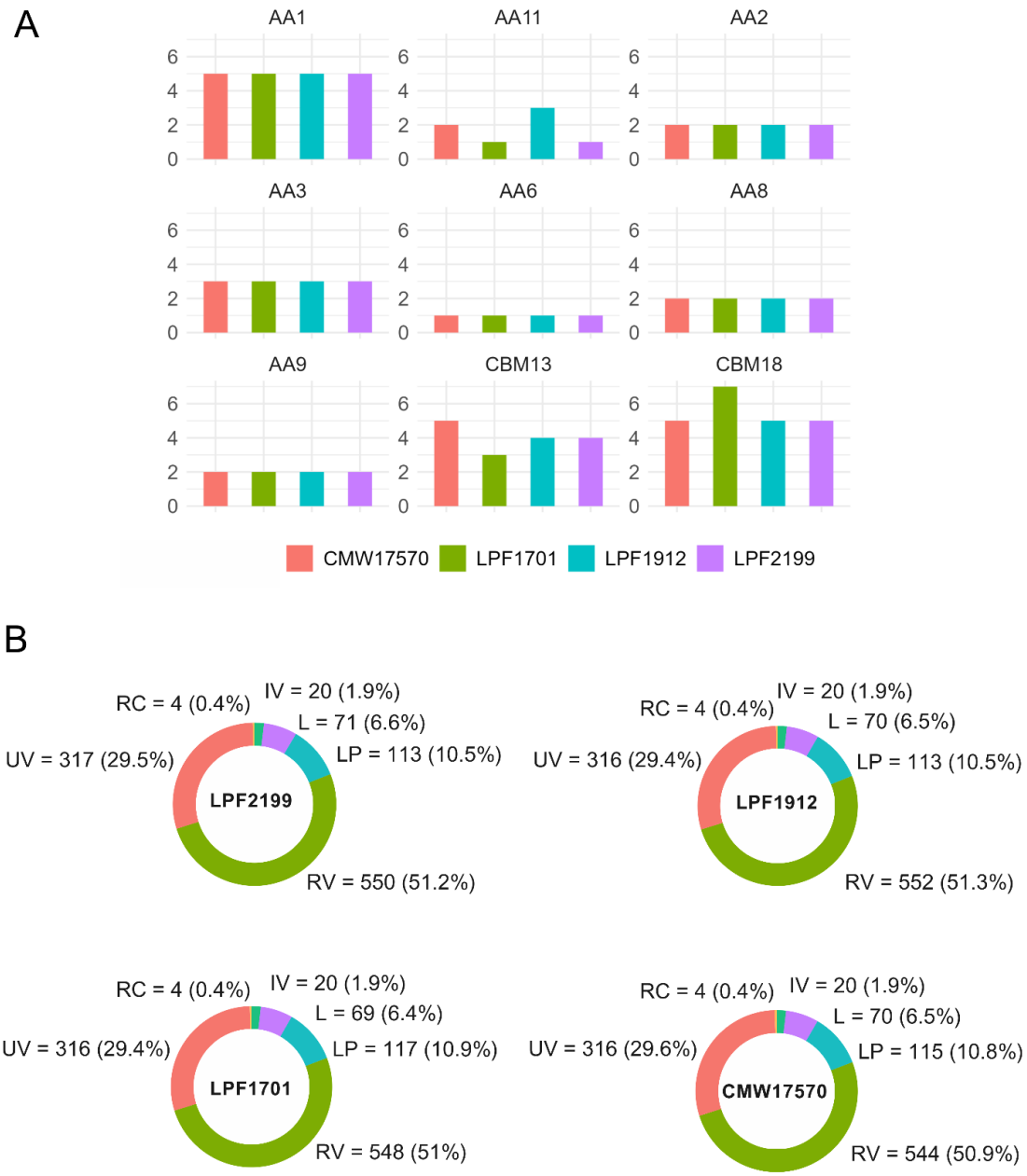


Figure 5. Predicted carbohydrate-active enzyme (CAZymes) and effector candidates of *Ceratocystis fimbriata* isolates LPF2199, LPF1912, LPF1701, and CMW17570. (A) Total number of proteins in each CAZymes family subgroup. (B) Classification of predicted effectors by the Pathogen-Host Interaction (PHI) database: LP: loss of pathogenicity, RV: reduced virulence, UV: unaffected virulence, IV: increased virulence, L: lethal, and RC: resistance chemical.

TABLES

Table 1. Statistics of the *Ceratocystis fimbriata* LPF2199 draft genome sequence

Genome assembly	
Assembled genome size (Mb)	31.62
Number of scaffolds	1,231
Largest scaffolds (bp)	684,015
N50 scaffold length (bp)	128,558
GC content of the draft genome (%)	47.92
Number of genes	7,533
Number of Coding DNA Sequences (CDS)	7,051
Number of transfer RNAs (tRNAs)	371
Number of ribosomal RNAs (rRNAs)	111
Repetitive sequences[1]	
Total repetitive sequences (Mb; %)	5.78; 18.29
Total retroelements (%)	6.27
SINEs (%)	0.01
LINEs (%)	1.70
LTR elements (%)	4.56
DNA transposons (%)	3.22
Small RNA (%)	0.03
Simple repeats (%)	1.61
Low complexity (%)	0.43
Unclassified	6.73

[1] LINE, long interspersed nuclear elements; LTR, long terminal repeat; SINE, small interspersed nuclear element.

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Functional annotation of *Ceratocystis fimbriata* LPF2199 genome, including data from Swiss-Prot, UniProtKB, KOG, PHI, and CAZyDB database, as well as tRNAs genes

Supplementary Table 2. Predicted rRNA genes present in the *Ceratocystis fimbriata* LPF2199 genome

Supplementary Table 3. Genome statistics and annotation of the *Ceratocystis fimbriata* LPF2199, LPF1912, LPF1701, and CMW17570

Supplementary Table 4. Predicted gene clusters for the biosynthesis of secondary metabolites of *Ceratocystis fimbriata* LPF2199, LPF1912, LPF1701, and CMW17570 isolates

CHAPTER 3 - RESISTANCE OF *Tectona grandis* TO CERATOCYSTIS WILT, CAUSED BY *Ceratocystis fimbriata*

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ABSTRACT

Tectona grandis (teak) is a tree species highly appreciated for its high-quality hardwood and versatile industrial applications. Despite clonal teak plantations showing several advantages, such as reduced rotation time and increased wood volume production, the genetic uniformity of plantations makes them more susceptible to diseases. Ceratocystis wilt caused by *Ceratocystis fimbriata* has been the most prevalent disease on teak plantations, reducing tree growth rate, and wood quality and value. Planting resistant genotypes is the most effective measure to mitigate losses caused by the disease. Thus, this work aimed to assess the

resistance to Ceratocystis wilt of twelve teak clones commercially planted in Brazil, and the inheritance of resistance in plants of teak open-pollinated families. Five teak clones exhibited resistance to Ceratocystis wilt, and all investigated open-pollinated teak families segregated resistance. The findings support the interpretation that resistance to Ceratocystis wilt in teak is a quantitative trait with additive gene effects in determining this trait. The information obtained in this work is an important contribution to directing the efforts of teak breeding programs in the attempt to reduce the losses caused by Ceratocystis wilt.

Keywords: Ceratocystis wilt. Inheritance. Segregation. Open pollination. Teak clonal.

1. INTRODUCTION

Tectona grandis (teak) is a wood diploid species of the Lamiaceae family, deciduous, and predominantly cross-pollinated (Kaosa-Ard 1989; Li et al. 2016). Its wood has a golden-brown color that is highly sought after by the industry for manufacturing luxurious furniture, decorative objects (such as doors, frames, and floors), and shipbuilding, making it one of the most valuable hardwoods (Kollert et al. 2024; Midgley et al. 2015). In Brazil, it is estimated that 76,000 ha are planted with teak (IBA 2023). It is an attractive country for teak plantations due to the favorable climate conditions, know-how in forestry management, and large-scale land availability (Takizawa 2022).

The yearly worldwide demand for teak wood is estimated at around 30 million m^3. However, approximately 2.0-2.5 million m^3 of teak roundwood are harvested annually from natural and planted forests (Midgley et al. 2015), resulting in a substantial supply deficit. Such a gap between production and demand highlights the need for expansion in cultivation area and an increase in productivity for this forestry species. Nonetheless, the expansion in planted areas of this species along with factors such as intensive soil preparation, soil heterogeneity in the planting areas, genetic uniformity of planted teak trees, and inadequate implementation of disease management practices in teak plantations, as well as climate fluctuations have favored an increase in the incidence and severity of abiotic and biotic stresses (Alfenas et al. 2023). Among these stresses, Ceratocystis wilt, caused by

Ceratocystis fimbriata is the most significant biotic disease affecting teak plantations, in Brazil.

The taxonomic position of the *Ceratocystis* species has undergone significant study and frequent revisions over the years. Despite the recent proposal by Harrington et al. (2023) to reclassify members of the *C. fimbriata* complex of the Latin American Clade (LAC) as *C. manginecans*, it is widely accepted that Ceratocystis wilt in teak is caused by *C. fimbriata*. This conclusion is supported by previous studies based on mating compatibility, phylogenetic analyses, and population composition (Valdetaro 2019; Fernandes et al. 2022). Therefore, in this study, the more widely recognized name *C. fimbriata* will be used. Ceratocystis wilt was first reported in teak in 2009 in Brazil (Firmino et al. 2012) and in 2020 in Ecuador (Belezaca-Pinargote et al. 2020). This disease compromises the production and quality of teak wood, reducing the rate of volumetric increase in infected trees by around 30%. In highly susceptible clones and under favorable conditions, it results in the death of all trees (F.A. Rafael, unpublished data). The significant increase in disease incidence prompted the demand for more effective control strategies.

In teak plantations, infections by soilborne *Ceratocystis* are frequently observed, but pruning is a common management practice considered one of the main modes of spreading the pathogen in the plantations (Alfenas et al. 2023). The pathogen penetrates and colonizes the plant vascular system, causing mainly wilting and wood darkening. The colonization of the xylem and phloem vessels by the fungus, along with the accumulation of tyloses, blocks the transport of water and nutrients, leading to wilting and eventual death of the infected trees (Alfenas et al. 2023; Silva et al. 2018). The use of resistant genotypes is the most favorable method to control the disease. Selection of resistant genotypes to Ceratocystis wilt has been conducted for *Eucalyptus* spp. (Guimarães et al. 2010; Oliveira et al. 2015; Zauza et al. 2004), *Mangifera indica* (Arriel et al. 2016; Guimarães et al. 2021), *Actinidia* spp. (Oliveira et al. 2020), *Acacia mangium* (Brawner et al. 2015), and *Gmelina arborea* (Méndez-Álvarez et al. 2023). However, for teak, there are few studies on the resistance of Ceratocystis wilt (Alfenas et al. 2023; Oliveira et al. 2021).

Field observations indicate intraspecific variability of *T. grandis* for resistance to Ceratocystis wilt among seed-originated trees and clonal teak genotypes introduced into Brazil (Alfenas 2017). Therefore, the evaluation of resistance in a larger number of clones and the determination of heritability are essential to

understand the genetic basis of resistance in teak genotypes. Inoculation under controlled conditions serves as a valuable tool in this investigation. It is already an established method for evaluating this disease in forest species (Oliveira et al. 2015; Arriel et al. 2016). Thus, the objective of this work was to assess the resistance of commercial teak clones and assess the inheritance of resistance in open-pollinated families to Ceratocystis wilt through inoculations under controlled conditions.

2. MATERIAL AND METHODS

2.1 Growth and maintenance of commercial teak clones

Twelve commercial teak clones were micropropagated and transplanted into 3.5 L pots containing a substrate with pine bark and carbonized rice husk in a 1:1 ratio supplemented with 3 kg of Basacote Mini 6M (13% N, 6% P_2O_5, 16% K_2O) per m^3 of substrate. They were kept for 60 days in the greenhouse (28 °C ± 5 °C), with irrigation three times a day until inoculation. Daily fertilizations were carried out with a nutrient solution.

2.2 Seed collection of open-pollinated trees and growth of their derived plants

In August 2019, the first fruit collection was done in São José dos Quatro Marcos, Mato Grosso, Brazil (15º37'17" S 58º10'35" W), involving open-pollinated crosses A3 (resistant) and C1 (susceptible) clones. Seeds were collected from A3 (family F1) and mother trees C1 (family F2). Additionally, seeds were collected from a monoclonal plantation of clone A3 (family F3). In July 2020, seeds were collected from trees of clone C5, containing a mixed teak compartment of clones A3 and C5, resulting in seeds from clone mother trees C5 (family F4). Furthermore, in an area with mixed teak plantations of clones A3, C5, and E4, fruits were collected from clone mother trees C5 (family F5) (Table 1). Because the crosses occurred under open-pollination conditions, all individuals within families were considered half-sibs. Microsatellite markers CIRAD3TeakA11, CIRAD3TeakB02, CIRAD1TeakB03, CIRAD2TeakB07, CIRAD2TeakC03, and CIRAD3TeakF01, developed by Verhaegen et al. (2005), were used for genotyping to confirm the identity of the mother trees. The procedures for PCR amplifications, capillary electrophoresis on an automated

sequencer, and loci analysis during genotyping were as described by Queiroz et al. (2023). The molecular profile of the teak mother trees was analyzed and compared with the PROTECA company database to verify their genetic identity.

The collected fruits were sun-dried to facilitate pericarp removal. Subsequently, the pericarp was removed, and the seeds were placed in mesh bags and subjected to running tap water for 24 h to break dormancy. The seeds were then sown on a moistened sand bed for germination. For the first six days, the moistened sand bed was covered with black plastic to increase the temperature and relative humidity while reducing light intensity. Subsequently, after removing the plastic cover, the moistened sand bed was irrigated twice a day for 15 days until the seeds began to germinate. The germinated seeds were transplanted into 55 cm^3 pot cells containing a substrate with pine bark and carbonized rice husk in a 1:1 ratio, supplemented with 3 kg of Basacote Mini 6M per m^3 substrate, and kept for 60 days under greenhouse conditions (28 °C ± 5 °C). Fertilizations were carried out daily with a nutrient solution. Rooted seedlings were transplanted into 3.5 L pots containing the same substrate, fertilized as previously described, and kept for 60 days under greenhouse conditions (28 °C ± 5 °C) with irrigation three times a day until inoculation.

2.3 Pathogen inoculation and resistance assessment

The *C. fimbriata* isolate LPF2199 was obtained in 2016 from a diseased teak tree in a commercial plantation located in the municipality of São José dos Quatro Marcos in Mato Grosso state, Brazil. This fungal isolate belongs to the Culture Collection of the Laboratory of Forest Pathology / Bioagro of the Universidade Federal de Viçosa, Minas Gerais, Brazil. The isolate was grown on potato-dextrose-agar (PDA) medium at 25 ± 2 °C with a 12 h photoperiod under 20 $\mu mol/m^2/s$ for 15 days. The inoculation was performed by making a wound at the base of the plant stem using a disinfested 5-mm-diameter cork borer (Figures 1A and 1B) and placing a mycelium plug from the 15-day-old culture of the fungal isolate on the wound (Figure 1C). The inoculation site was wrapped with a wet cotton plug and parafilm to reduce desiccation and contamination (Figure 1D).

Ten plants of each of the twelve commercial teak clones were inoculated with isolate LPF2199 using the method described above. In addition, a total of 82, 60, 70,

131, and 124 plants of open-pollinated families F1, F2, F3, F4, and F5, respectively, were inoculated with the same isolate, including ten plants of the resistant clone A3 and ten plants of the susceptible clone C1 as controls. The inoculated plants were kept in a greenhouse (28 °C ± 5 °C) in a completely randomized design, applying irrigation three times a day.

Disease assessments of the inoculated plants were conducted weekly for up to 90 days after inoculation (DAI). Lesion size and plant height were measured, and disease severity (lesion size/plant height) was calculated and expressed as a percentage. For wilted plants, disease severity was 100%. The pathogen was re-isolated from the infected plants, and the carrot bait method (Moller and Devay 1968) was used to confirm the infection by *C. fimbriata*.

2.4 Statistical analysis

Statistical analyses were conducted in the R environment v. 4.1.3 (R Core Team, 2023). The Scott-Knott test ($p \leq 0.05$) was used to group the clones and open-pollinated families using the “easyanova” package (Arnhold, 2016). Based on this grouping, the clones were classified as either resistant or susceptible to diseases.

For the open-pollinated families, plants were classified as resistant when their disease severity values were equal to or lower than the mean disease severity observed in the resistant control clone (A3); conversely, plants with severity values higher than this threshold were considered susceptible. Additionally, the “lme4” package (Bates et al. 2015) was used to estimate variance components and genotypic values through a mixed-effects linear regression model, employing Restricted Maximum Likelihood/Best Linear Unbiased Prediction (REML/BLUP) methods (Henderson, 1975; Patterson and Thompson, 1971), according to the following equation 1:

$$y = X\beta + Z\,u + e \qquad (1)$$

In which, y is the vector of observations (severity); β is the vector of unknown fixed effect parameters; u is the vector of unknown random effect parameters; X is the known design incidence matrix, where X relates y to β; and Z is the known design incidence matrix, where Z relates y to u. The mother trees X and Z consist of 1’s and 0’s, whereas e refers to the random effect associated with unobservable errors. The

genetic parameters were estimated according to Resende et al. (2007, 2014), considering the disease severity.

3. RESULTS

3.1 Resistance of commercial teak clones to Ceratocystis wilt

The commercial teak clones were categorized into two distinct groups by the Scott-Knott test based on comparisons of disease severity means (Table 2). Clones A12, C1, C7, C16-1, C16-2, D19, and E4 were classified as susceptible (group a). Lesion size in these clones varied from 15.45 to 27.71 cm, and disease severity ranged from 51.51 to 87.71%. In contrast, clones A3, A8, B1, C5, and T4 were considered resistant (group b), with lesion size varying from 11.91 to 19.06 cm and disease severity from 22.83 to 34.88%. All clones evaluated showed typical Ceratocystis wilt symptoms (Figure 2A), but external wilting symptoms were only readily visible in the most susceptible clones starting at 18 DAI (Figure 2B). Internal dark lesions in the longitudinal and radial direction of the stem were also observed at 90 DAI (Figure 2C-D). By the end of the assay, plant wilting reached up to 80% in susceptible clones (Table 2) while the most resistant clones did not wilt. The pathogen was re-isolated from all inoculated teak clones exhibiting disease symptoms, and its growth on carrot slices (Figure 2E), as well as its perithecia and ascospores (Figure 2F), were typical of *C. fimbriata*.

3.2 Resistance to Ceratocystis wilt in open-pollinated families

The progenies in families obtained by open pollination varied in resistance (Table 3). The families showed significant differences in their response to inoculation with *C. fimbriata* and were separated into four distinct groups by the Scott-Knott test based on disease severity. Clone C1, used as a susceptible control, showed the largest means of lesion size and disease severity (12.65 cm and 17.67%, respectively) and formed group a. The families F1 and F2 formed group b with lesion sizes of 10.38 and 8.54 cm and mean disease severity of 11.36 and 12.54%, respectively. The family F3, together with clone A3 (resistant control), formed group c, with mean lesion sizes of 9.00 and 8.92 cm and disease severity of 8.67 and

8.81%, respectively. Finally, families F4 and F5 formed group d with the lowest mean lesion sizes (5.40 and 4.53 cm, respectively) and mean disease severity (6.33 and 4.75%, respectively). In general, the means of lesion size and disease severity decreased from F1 to F5. Wilting was observed only in families F1 and F2, being 1 and 3% of the total plants evaluated, respectively. Re-isolation of the pathogen and its growth characteristics on carrot baits confirmed the infection by *C. fimbriata*.

3.3 Inheritance of resistance in open-pollinated families

All families exhibited segregation for resistance, with a high prevalence of resistant genotypes, especially in families F4 and F5 (Figure 3). Genetic parameters and variance components of teak resistance to *C. fimbriata* were estimated for open-pollinated families (Table 4). The results indicated values of total genetic variance among half-sib families (*Vg*), additive genetic effects (*Va*), environmental variance (*Ve*), and individual phenotypic variance (*Vp*) of 6.05, 24.21, 19.59, and 25.64, respectively. The resistance trait showed high heritability (*ha2* = 94.40). However, there was considerable environmental variability (*CVe* = 63.32) relative to genotype variation (*CVg* = 35.19) with a relative coefficient of variation (*CVg/CVe*) of 0.56, suggesting environmental factors significantly influenced the expression of resistance. The accuracy of genotype selection was high (0.99), indicating the reliability of the selection processes.

4. DISCUSSION

This study aimed to elucidate the resistance to Ceratocystis wilt in commercial teak clones and the inheritance of resistance in open-pollinated families. The results of the present study revealed that five clones planted in Brazil exhibit resistance to Ceratocystis wilt. Among these, one clone was Brazilian provenanced, whereas the others were from the Solomon Islands, Malaysia, and Tanzania; notably, no wilting was observed in these clones at 90 days after inoculation (DAI). The different geographic origins of these clones suggest the existence of genetic variability among them. Similarly, Oliveira et al. (2021) reported that resistance to this disease was identified in only a few of the evaluated teak clones. Due to the limited number of resistant genotypes, the introduction of new genetic materials into teak plantations is

crucial to broadening the genetic base of this species in Brazil. Such a strategy is essential to mitigate the risks associated with establishing genetically uniform populations, in which susceptible clones could dominate. This concern has already been addressed in a study by Queiroz et al. (2023), who, upon evaluating teak clones planted in Brazil, demonstrated that despite the high genetic diversity detected through microsatellite markers, a significant proportion of these clones originated from trees with recent shared ancestry. These findings indicate that the selection of elite clones in breeding programs frequently occurs within the same families, thereby contributing to the narrowing of genetic diversity.

It is important to highlight the inherent challenges associated with performing controlled crosses in teak. The reproductive biology of the species, characterized by a high dependence on natural cross-pollination, asynchronous flowering among individuals, a long vegetative period before flowering, and low seed production per tree (Kaosa-Ard et al. 1998), hinders the implementation of controlled pollination protocols. Due to these limitations, genetic studies and breeding programs in teak often rely on open-pollinated progenies or use clonal tests as a substitute for progeny tests. In this context, the present study investigated plants from families derived through natural open-pollinated crosses, which revealed segregation for resistance to Ceratocystis wilt among the progenies. Notably, the F4 and F5 families, derived from clone C5 as mother trees, exhibited the highest levels of resistance. These findings not only highlight clone C5 as a valuable parent in breeding programs but also reinforce its potential for commercial cultivation due to its disease resistance.

The high narrow-sense heritability observed in the progenies indicates a strong additive genetic component. However, the considerable environmental variance suggests that resistance expression is still heavily influenced by environmental factors (Resende 2007). Together, these findings support the interpretation that resistance to Ceratocystis wilt in teak is a quantitative trait, as previously reported in other pathosystems involving *Ceratocystis* and various plant species, such as *Eucalyptus* spp. (Rosado et al. 2010), *Acacia mangium* (Brawner et al. 2015), *Mangifera indica* (Arriel et al. 2016), and *Actinidia deliciosa* (Oliveira et al. 2020).

The high additive genetic variance reinforces the role of additive gene effects in determining this trait, aligning with Rosado et al. (2010), who reported strong additive genetic control of disease resistance in *Eucalyptus* spp. In the case of

Eucalyptus, the use of families derived from interspecific crosses contributed to greater genetic diversity, increasing the frequency of resistance alleles and resulting in higher heritability estimates. In contrast, for teak, the assumption of true half-sib relatedness among individuals in open-pollinated families is likely inaccurate, potentially inflating estimates of additive variance. Nonetheless, the results indicate that selection among mother trees can still lead to substantial genetic gains in resistance, demonstrating the effectiveness of current screening methods for identifying superior genotypes.

The relatively low broad-sense heritability observed suggests a limited total genetic contribution to resistance, likely due to environmental effects or a narrow genetic base in the studied population. Therefore, further studies under diverse environmental conditions are necessary to validate the stability of this inheritance pattern and to confirm the robustness of resistance to Ceratocystis wilt in teak.

Some level of specificity has been observed in the interactions between *Ceratocystis* isolates and distinct genotypes of the same host (Alexandre et al. unpublished; Oliveira et al. 2020; Guimarães et al. 2021). This level of specificity of the interactions can negatively impact the durability of resistance and increase the risk of the fungus evolving to produce additional virulence factors in specific pathogen populations (Cowger et al. 2000). Hence, the resistance of the teak genotypes evaluated may depend on the physiological specialization of the pathogen population tested. In this study, only one virulent *C. fimbriata* isolate was inoculated into healthy teak plants, limiting to draw clear conclusions on the effectiveness of the resistance against naturally occurring populations. To select teak genotypes likely exhibiting more durable resistance under field conditions, it is necessary to investigate larger and more genetically diverse panels of both teak plants and fungal isolates. Despite this limitation, the findings of this study represent an important step toward strengthening teak breeding efforts. Through rigorous screening, the study revealed genetic variability and heritability for resistance among clones and open-pollinated progenies, enabling the selection of promising resistant genotypes for incorporation into breeding programs and their adoption in large-scale commercial plantations.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY

The data that support the findings of this study will be made available upon request from the corresponding author.

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FIGURES

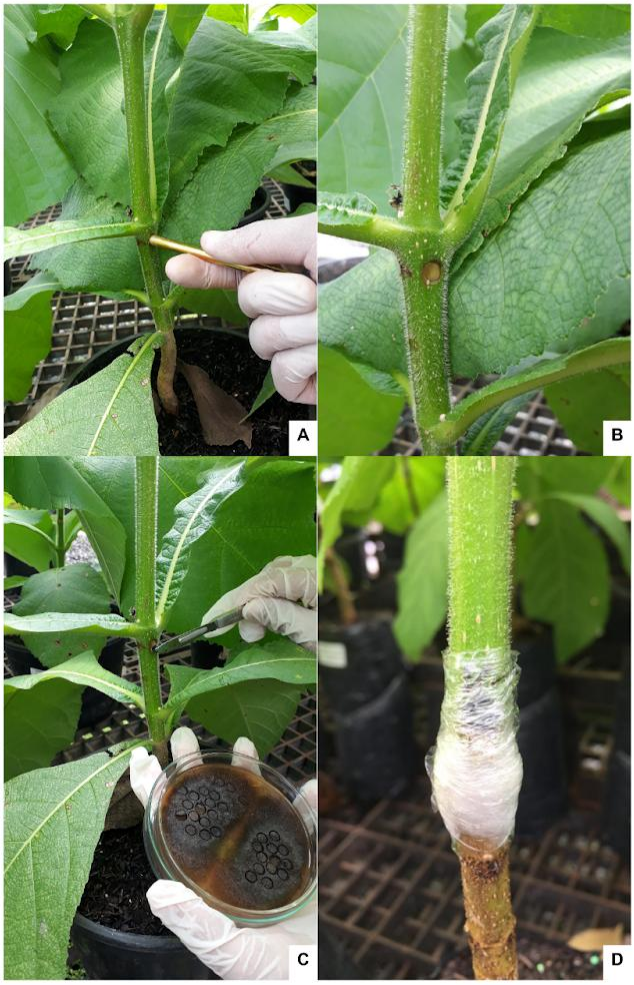
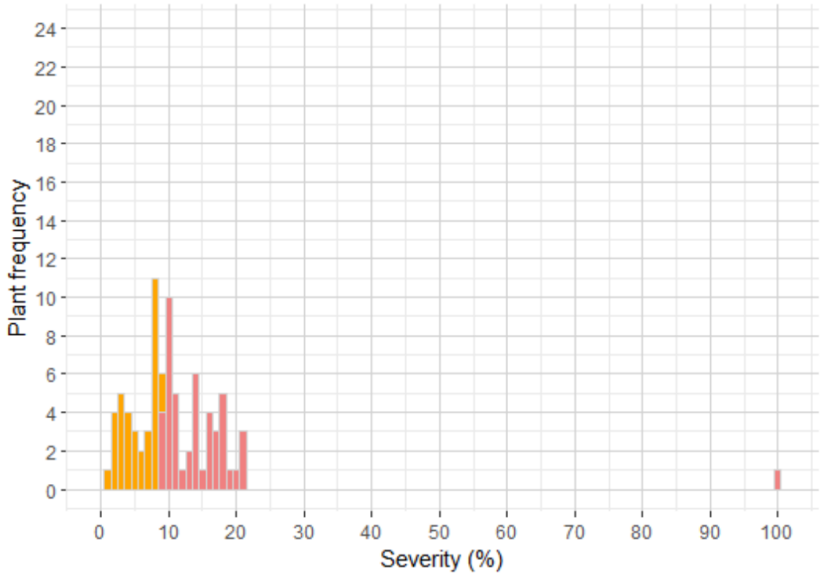


Figure 1. Inoculation of *Ceratocystis fimbriata* on teak. (A, B) wounding of the stem, (C) deposition of a mycelium plug in the wound, (D) wrapping of the inoculum with cotton and parafilm.

Figure 2. Symptoms of Ceratocystis wilt in susceptible teak clones inoculated under controlled conditions and re-isolation of the pathogen. (A) wilted plant, (B) darkening of the outer stem tissue, (C) radial darkening of the internal stem tissue, (D) internal darkening of the stem in a longitudinal section, (E) pathogen recovered from infected tissue growing on carrot discs, (F) perithecia and ascospore mass typical of *Ceratocystis fimbriata*.

F1


Plant frequency
Severity (%)
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2
4
6
8
10
12
14
16
18
20
22
24
0
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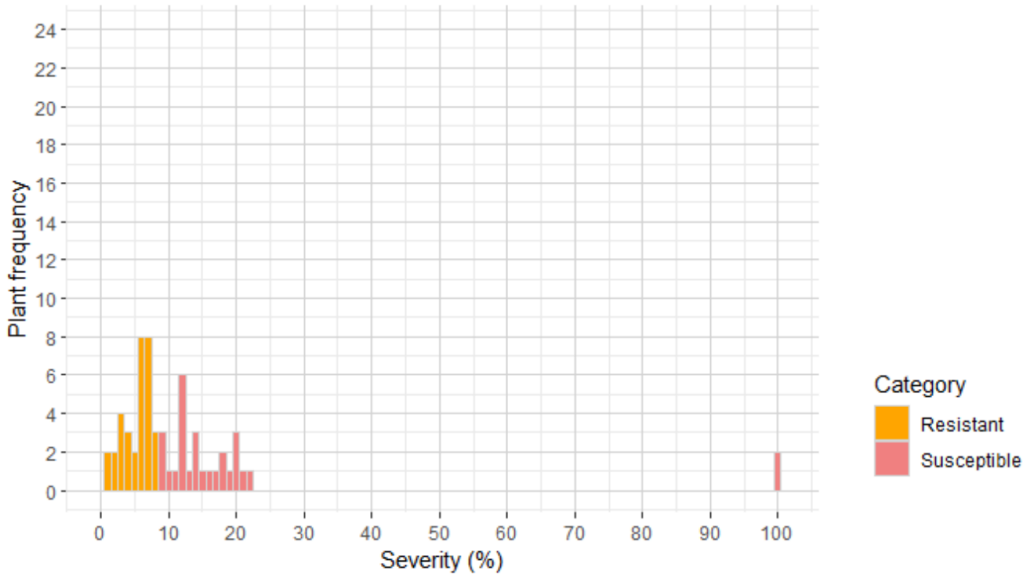
20
30
40
50
60
70
80
90
100

F2


Plant frequency
Severity (%)
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4
6
8
10
12
14
16
18
20
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24
0
10
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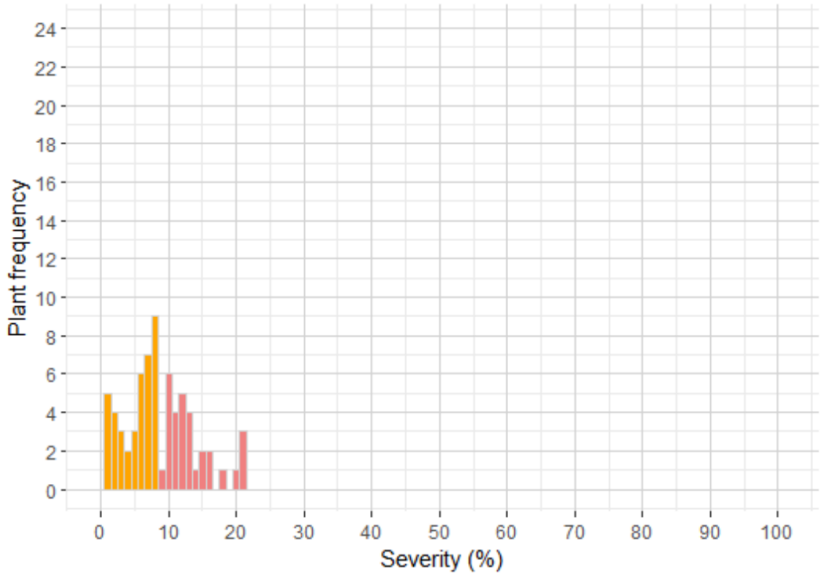
30
40
50
60
70
80
90
100
Category
Resistant
Susceptible

F3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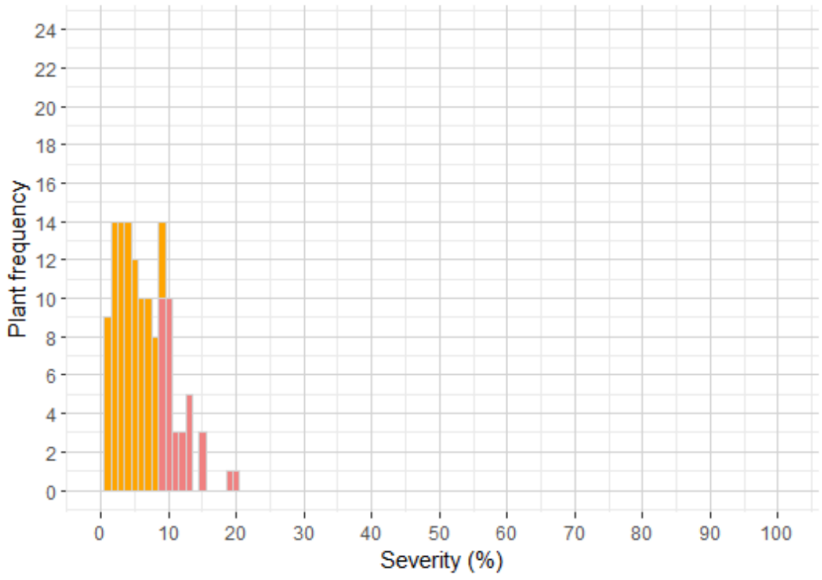
F4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Severity (%)
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Category
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Susceptible

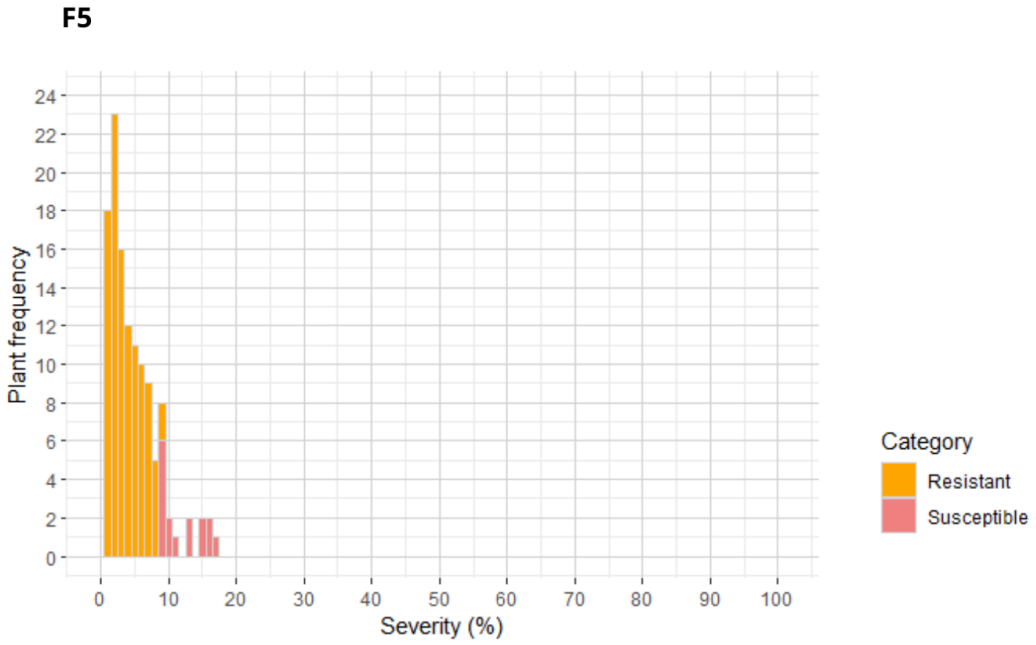

Figure 3. Frequency distribution of disease severity (lesion size/plant height) in plants of five *Tectona grandis* families (F1, F2, F3, F4, and F5) obtained by open pollination at 90 days after inoculation with *Ceratocystis fimbriata*. Genotypes showing disease severity values equal to or less than the mean value of the resistant control (8.81) of the resistant control (clone A3) were classified as resistant (orange), while those with values above this threshold were classified as susceptible (light pink).

TABLES

Table 1. Origin of *Tectona grandis* seed families from open-pollinated crosses

Family	Mother trees	Crossing environment	Possible pollen sources
F1	A3	Open-pollinated (A3 x C1)	C1
F2	C1	Open-pollinated (C1 x A3)	A3
F3	A3	Monoclonal (A3 x A3)	A3 (self or cross with A3)
F4	C5	Open-pollinated (C5 x A3)	A3
F5	C5	Mixed stand (C5 x A3, E4)	A3 or E4

Table 2. Response to *Ceratocystis fimbriata* of commercial *Tectona grandis* clones at 90 days after inoculation

Clone	Provenance	Wilted plants (%)	Lesion size (cm)	Disease severity (%)[a]	Category[c]
A3	Solomon Island	0	15.97 (3.24)	34.88 (10.43)[b] b	R
A8	Solomon Island	0	18.71 (3.06)	22.83 (9.84) b	R
A12	Solomon Island	30	24.75 (2.90)	51.51 (9.33) a	S
B1	Brazil	0	16.76 (2.90)	28.58 (9.33) b	R
C1	Malaysia	60	26.63 (2.90)	72.01 (9.33) a	S
C5	Malaysia	0	11.91 (3.06)	25.34 (9.84) b	R
C7	Malaysia	60	15.45 (2.90)	69.83 (9.33) a	S
C16-1	Malaysia	80	27.71 (2.90)	87.71 (9.33) a	S
C16-2	Malaysia	70	17.22 (3.06)	81.02 (9.84) a	S
D19	India	40	18.78 (3.06)	61.20 (9.84) a	S
E4	Indonesia	40	21.23 (2.90)	55.38 (9.33) a	S
T4	Tanzania	0	19.06 (2.90)	29.42 (9.33) b	R

[a] Means with the same letter within the column are not significantly different, at a 5% level by the Scott-Knott test.

[b] Values in parentheses indicate standard errors.

[c] R, resistant; S, susceptible.

Table 3. Number of *Tectona grandis* genotypes in families obtained by open pollination and their reactions to *Ceratocystis fimbriata* at 90 days after inoculation

Family	Crossing (♀ x ♂) [a]	No. of genotypes	Wilted plants (%)	Lesion size (cm)	Disease severity (%) [b]
F1	A3 x C1	82	1	10.38 (0.51)	11.36 (0.94) [c] b
F2	C1 x A3	60	3	8.54 (0.60)	12.54 (1.10) b
F3	A3 x A3	70	0	9.00 (0.55)	8.67 (1.02) c
F4	C5 x A3	131	0	5.40 (0.40)	6.33 (0.74) d
F5	C5 x A3, E4	124	0	4.53 (0.42)	4.75 (0.76) d
Control					
A3			0	8.92 (1.03)	8.81 (1.90) c
C1			0	12.65 (1.03)	17.67 (1.90) a

[a] The open-pollinated families were formed by crossing the mother trees (♀) with likely fathers (♂).

[b] Means with the same letter within the column are not significantly different, at a 5% level by the Scott-Knott test.

[c] Values in parentheses indicate standard errors.

Table 4. Estimates of genetic parameters and variance components for the resistance of open-pollinated *Tectona grandis* families to *Ceratocystis fimbriata* based on disease severity

Genetic parameters	**Values**
Total genetic variance among half-sib families (*Vg*)	6.05
Additive genetic variance (*Va*)	24.21
Environmental variance (*Ve*)	19.59
Individual phenotypic variance (*Vp*)	25.64
Heritability in the broad-sense (*hg2*%)	23.60
Heritability in the narrow-sense (*ha2%*)	94.40
Coefficient of genotype variation (*CVg*%)	35.19
Coefficient of environmental variation (*CVe*%)	63.32
Coefficient of relative variation (*CVg/CVe*)	0.56
Accuracy	0.99
Mean	6.99
SE: Standard error of prediction	0.32