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PATHOGENICITY ANALYSIS ON *CERATOCYSTIS* STRAINS ISOLATED FROM *GMELINA ARBOREA* IN COSTA RICA

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The appearance of severely diseased trees of *Gmelina arborea* (melina) in Costa Rica with high incidence rates and rapid mortality due to *Ceratocystis* wilt causes concern and raises new studies to determine the best management of the disease. The main reason of this work was to evaluate the severity of different strains and species of *Ceratocystis* present in melina. Isolations obtained from diseased melina trees, with symptoms such as leaf loss, exudations on the trunk and outgrowths on the stem, denoted the presence of three species of fungi of the genus *Ceratocystis*: *C. fimbriata*, *C. mangivora* and *C. fimbriatomima*. Koch's postulates were consistent for all *Ceratocystis* strains investigated and were pathogenic for the melina genotypes. According to the pathogenicity test, two strains (CIF001 and CIF004) showed the highest incidence rates, the elite genotypes were classified according to internal lesion into susceptible (15N and 20), tolerant (57 and 58) and resistant (1). This study constitutes the first pathogenicity test of several *Ceratocystis* species associated with *G. arborea* wilt in Costa Rica, which generates valuable information to determine disease control strategies.

Keywords: Resistance disease, inoculation, melina, genotypes, pathogen

INTRODUCTION

Gmelina arborea Roxb. ex Sm (melina) is a fast-growing tree species, widely planted in tropical countries for production of timber, pallets, plywood, and furniture in general (Dvorak 2004). In Costa Rica this species was introduced from Southeast Asia in the late 1960s and is currently the second most planted tree species (Ávila et al. 2015, INEC 2015). The possibility of producing timber fast and at low cost motivated the development of several regional breeding initiatives (Méndez et al. 2020, Hernández et al. 2021a).

Phytosanitary problems associated with melina were first reported until early 1990s (Wingfield & Robinson 2004, Umaḥa et al. 2015, Arguedas et al. 2018). However, in 1995 the appearance of severely diseased trees with high incidence rates and rapid mortality began to be reported everywhere (Arguedas et al. 1995). Among the main symptoms, an onset with generalised wilting is observed, starting with a descending foliar loss, cortical cankers occasionally with exudation on the trunk, epicormic sprouting, wood stain and finally death after a few months (Salas et al. 2016a, Arguedas et al. 2018, Murillo et al 2016). Méndez et al. (2020) diagnosed *Ceratocystis fimbriata* Ellis & Halst as the pathogen responsible for causing this disease. Recent studies from breeding programs have reported genotypes with a high degree of genetic tolerance to the pathogen (Salas et al. 2016b, Méndez et al. 2021). This has promoted mass clonal propagation of melina based on safe genotypes for plantation establishment, as part of several integrated disease management options (Salas et al. 2016b, Méndez et al. 2021, Hernández et al. 2021b).

Ceratocystis is the largest genus of the family Ceratocystidaceae (Liu et al. 2018), of which more than 40 species have been described (Dharmaraj et al. 2022), characterised by having an ascomatal base, mostly black and globose, with long elongated necks ending in an ostiole, through which they exude sticky hat-like ascospores (Barnes et al. 2018). Species of the genus *Ceratocystis* belonging to the *C.*

fimbriata sensu lato (s.l.) complex are mostly disease-causing pathogens of woody and some herbaceous plants in tropical, subtropical, and temperate regions (Kile 1993, Holland et al. 2019, Valdetaro et al. 2015).

Symptoms caused by these agents include vascular wilt and cankers, leaf dieback, brown spots on xylem rays, decrease in the number of viable fruit and, in most cases, mortality (Van Wyk et al. 2009, Harrington 2013, Ferreira et al. 2017, Nasution et al. 2019). Also, its high host specificity is well known (Méndez et al. 2023, Meneses 2008) suggesting the existence of different species and strains of the fungus, some of which could exhibit high specialisation and different level of aggressiveness (Harrington 2000). Thus, it is necessary to establish trails with different isolates obtained from the specific host (Ferreira et al. 2011). Therefore, the objective of this study was to evaluate the severity of different strains and species of *Ceratocystis* on *Gmelina arborea* in Costa Rica.

MATERIALS AND METHODS

Samples of *Gmelina arborea*

Trees with evident symptoms of leaf loss, trunk exudates and stem sprouts (Salas et al. 2016a) were selected from different regions of the country, where melina is planted on a larger scale. Each selected tree was felled down and two 3 cm thick wood discs were obtained (basal and at 5 m trunk height). They were placed in identified plastic bags, transferred and processed by the Forest Pathology Laboratory at the Forestry Research and Innovation Center, School of Forestry Engineering, Instituto Tecnológico de Costa Rica.

Isolation methods

From the wood discs obtained from each selected tree, wood segments were obtained in the laboratory from the infected areas with canker progression. Wood samples were disinfected with 70% alcohol for 30s, 5% a.i. sodium hypochlorite for 3 min, followed by three consecutive washes in sterile distilled water (SDA). The disinfected segments were placed on carrot baits previously immersed in water with antibiotic (penicillin 0.2g L^{-1}, streptomycin 0.2g L^{-1} and chloramphenicol 0.2g L^{-1}). The samples were incubated at 25 ± 2°C for 8 days with a photoperiod of 12 h of light. Once typical *Ceratocystis* ascocarps were observed, the ascospore masses formed on top of the perithecia were transferred to Petri dishes containing MEYA culture medium with antibiotic (Malta, yeast extract, agar, penicillin 0.2g L^{-1}, streptomycin 0.2g L^{-1} and chloramphenicol 0.2g L^{-1}). They were incubated at 25 °C for seven days, with 12 h photoperiod. Each isolate was stored in the mycotheca at the School of Forest Engineering of the Technological Institute of Costa Rica, under the codification CIF 001, CIF 002, CIF 003, CIF 004, CIF 005, CIF 006 and CIF 012.

Identification of isolates

For characterisation of microscopic fungal structures, perithecia obtained from different positions of the colonies were placed on slides with lactic acid, photographed under a Nikon optical microscope. Structure measurements were made using NisElement software. Thirty measurements of the length and width of conidia and ascospores were made and, the maximum, minimum, mean, standard deviation and 95% confidence intervals were determined.

For each isolate, genomic DNA extraction was performed using the Wizard genomic DNA purification kit (Promega Corporation, WI, USA), followed by polymerase chain reaction (PCR) using DreamTaq Master Mix (MBI Fermentas, Vilnius, Lithuania), following the manufacturer's protocols. For amplification and sequencing of tDNA, primer pairs ITS4 and ITS5 and EF1 and EF2 were used at the Laboratory of Molecular Techniques at the Crop Protection Research Center (CIPROC) of the University of Costa Rica. The sequences were edited and assembled with Sequencher 5.4 (Genes Codes Corporation, Michigan), then the consensus sequences were compared by nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the National Center for Biotechnology Information (NCBI) database. The following comparison parameters were defined E-value 0, score greater than 700, query coverage between 80% and 100%, and percent identity greater than or equal to 95%.

Pathogenicity test

The pathogenicity test was conducted under complete closed greenhouse conditions with a zenithal opening, an average temperature of 28 °C, an average relative humidity of 82.5% and irrigation once a day for 10 minutes at the GENFORES facilities at the Instituto Tecnológico de Costa Rica, campus Santa Clara, San Carlos, Costa Rica (10° 21› 15.78›› N and 84° 30› 58.88). A randomised complete block experimental design with five replicates and seven *Ceratocystis* strains plus a control was established. The replication effect was confirmed by five elite genotypes (clones).

Each strain was inoculated on five plants in each genotype (experimental unit). All plants were four-month-old grown in plastic pots. A control treatment without fungus was also included. All individual trees were inoculated with a 5 mm diameter disc of mycelium placed in a stem wound made at the base of the tree, following procedures according to Méndez et al. (2020). The inoculated area on the stem was covered with parafilm paper. For the negative control (control test), discs of culture medium (MEYA) without fungal colony were placed.

After inoculation, the behavior of the inoculum on the plants was evaluated at 30, 60 and 90 days. As discrete or categorical variables, the incidence of the disease was determined binomially, as healthy, and alive (0) or sick and dead (1). Based on this categorisation, Equation 1 proposed by Cavallini (1998) was used to determine the incidence for each treatment (strain), genotype and in general for the trial in the three evaluations.

$$\text{Incidence} = \frac{\text{Total number of plants affected}}{\text{Total number of plants}} \times 100 \qquad (1)$$

The degree of resistance was determined by evaluating the internal lesion in the vascular tissue of each plant, generated by the advance of the fungus in the plant. After 90 days, the entire trial was cut, the controls were evaluated first, and then the treatments were evaluated. Each plant was labeled according to its genotype and treatment received, the leaves and branches were removed, and longitudinal cuts were made where the vascular tissue was visualised, from the base of the plant to where no internal stain or lesion was observed. Subsequently, the total length of the spot was measured, as described by Oliveira (2010). Based on the total height of everyone, the degree of resistance was calculated using Equation 2:

$$\text{Injury severity} = \frac{\text{Internal lesion length}}{\text{Total plants height}} \times 100 \qquad (2)$$

Based on that result, a classification was made according to this percentage into resistant (≤10 %), tolerant (10 < X ≤ 20 %) and susceptible (>20%).

Statistical analysis

Data was evaluated according to ANDEVA procedures for randomised complete block experimental design (Equation 3), previously analysed for homeostacity and normality arguments.

$$Y = \beta\text{Genotype} + \alpha\text{Treatment} + \varphi\text{Genotype} \times \text{Treatment} + e \qquad (3)$$

where, β = the effect of the i-th Genotype, α = the effect of the j-th Treatment and φ = effect of the interaction of the l-th Genotype × Treatment (experimental unit).

When the ANDEVA showed significant differences in any of the variables, a Duncan test (α = 0.05) was performed to determine the differences between treatments and genotypes. All statistical analyses were performed using InfoStat software version 2019.

RESULTS

All diseased trees sampled for pathological analysis showed wilting symptoms (Figure 1a) and a decrease in leaf size in cortical cankers (Figure 1b). Sometimes exhibiting exudations, epicormic sprouts (Figure 1c), wood stains (Figure 1d) and finally mortality.

From the isolates, three species of the genus *Ceratocystis* causing the disease in *G. arborea* trees were identified as *C. fimbriata, C. mangivora* and *C. fimbriatomima* (Table 1).

Ceratocystis fimbriata

Colonies of this species in MEYA showed slow growth, with irregular edge, gray to olive-green aerial mycelium and white aerial hyphae, the colony has a fruity odor. Ascocarps (perithecia) are produced in clusters and form concentric

Figure 1 Symptoms recorded on *G. arborea* diseased trees: (a) Leaf wilt, (b) cortical cankers, (c) epicormic sprouts, (d). wood stain

Table 1 Provenances of *Ceratocystis* strains isolated from diseased *Gmelina arborea* trees in different regions throughout Costa Rica

Code	Canton	Province	Region	Species
CIF 001	Guácimo	Limón	Caribbean	*C. fimbriata*
CIF 002	San Carlos	Alajuela	Northern Zone	*C. mangivora*
CIF 003	Los Chiles	Alajuela	Northern Zone	*C. fimbriatomima*
CIF 004	Pital	Alajuela	Northern Zone	*C. fimbriatomima*
CIF 005	Pococí	Limón	Caribbean	*C. fimbriata*
CIF 006	Pérez Zeledón	San José	South Pacific	*C. fimbriata*
CIF 012	Nicoya	Guanacaste	North Pacific	*C. fimbriata*

rings (Figure 2a). Perithecial ascocarp black, with globose base and long slender black necks (Figure 2b). Terminal, simple, thick-walled, brown, sub-globose, sub-globose chlamydospores (Figure 2c). Hyaline divergent ostiolar hyphae are formed (Figure 2d). Secondary phialides producing cylindrical conidia in chains (Figure 2e). Ascospores hat-shaped (3,3-)4,0-4,8(-5,2) µm × (5-) 5,3-6,3(-7) µm (Table 2) (Figure 2f). Elongated cylindrical primary conidia (13-)17.1-23.3(-25) µm × (3-)3.6-4.5(-5) µm (Table 1), hyaline and short barrel-shaped secondary conidia (Figure 2g); sometimes these conidia can be observed with a chain-like Figure (Figure 2h).

At the molecular level, the consensus sequence with the ITS marker showed a 99% similarity to *Ceratocystis fimbriata* (AY953386.1). Meanwhile, with the EF marker sequence, it registered a percentage similarity of 95.83% with *Ceratocystis fimbriata* from isolate SSB3 (MN296250.1). Together with morphological characterisation and symptoms description, it was confirmed the identification at species level of isolate CIF001, CIF005, CIF006 and CIF012.

Ceratocystis mangivora

The colonies of this species in MEYA showed a very slow growth, with gray to olive-green aerial mycelium and white aerial hyphae. The colony had a fruity (banana) odor (Figure 3a), as well as perithecial ascarpus brown or black, with globose base and long thin black necks (Figure 3b). Besides, secondary conidiophore, hyaline with emerging chain of barrel-shaped conidia (Figure 3c) and primary conidiophore hyaline (Figure 3d). Chlamydospores light brown, globose to sub-globose with thick walls was also exhibited (Figure 3e). Hyaline divergent ostiolar

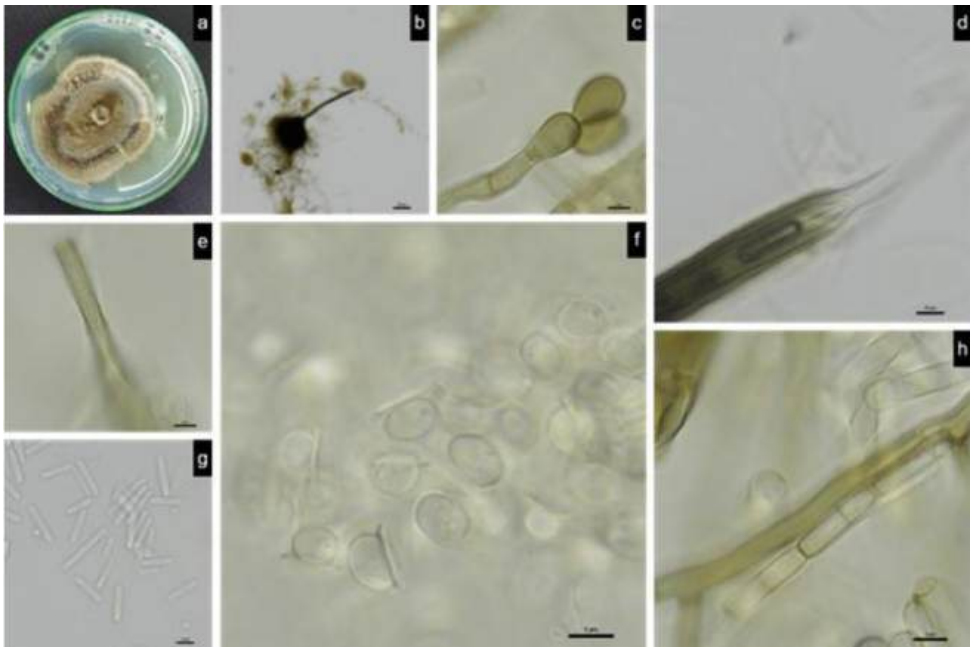

Figure 2 Morphological characteristics of *C. fimbriata*. (a) Morphology of the colony in MEYA. (b) Ascomatal base (perithecium) with mass of ascospores. Scale bar = 100 µm. (c) Subglobose chlamydospores Scale bar= 5 µm. (d) Ostiolar hyphae. Scale bar= 10 µm. (e). Secondary phialides. Scale bar= 5 µm. (f) Hat-shaped ascospores. Scale bar= 5 µm. (g) cylindrical conidia. Scale bar= 10 µm. (h) cylindrical conidia in chains. Scale bar= 5 µm

Figure 3 Morphological characteristics of *C. mangivora*. (a) Colony morphology in MEYA. (b) Ascomatal base (perithecium). Scale bar = 100 µm. (c) Secondary conidiophore. Scale bar = 5 µm. (d) Primary conidiophore. Scale bar = 5 µm. (e) Globose chlamydospores. Scale bar = 10 µm. (f) Ostiolar hyphae. Scale bar = 5 µm. (g) Hat-shaped ascospores. Scale bar = 5 µm. (h) cylindrical conidia. Scale bar = 5 µm

Table 2 Comparative morphological characteristics of *Ceratocystis fimbriata, C. mangivora* and *C. fimbriatomima* isolated from *G. arborea* in Costa Rica

	Cylindrical conidia		Literature		Hat-shaped ascospora		Literature		References
Species	L (µm)	w(µm)	L(µm)	w(µm)	L(µm)	w(µm)	L(µm)	w(µm)	
*C. fimbriat*a	(13-)17, 1-23, 3(-25)	(3-)3,6-4,5(-5)	(15–)18–24(–25)	4-5	(3,3-)4,0-4,8(-5,2)	(5-)5,3-6,3(-7)	3-5	5-7	
			(13–)19–20(–24)	4.-5					(Van Wyk *et al.* 2012)
			(10–)13–17(–18)	3-4					
			(10–)11–15(–18)	2-3					
*C. mangivor*a	(12,6)18, 1-25-(28)	(2-)2,8-4,7(-5)	(12–)16–24(–31)	2-5	(3-)3,8-4,4(-5)	(5-)5,8-7(-8)	3–5	5-8	(Van Wyk *et al.* 2011)
*C. fimbriatomim*a	(14-)17-26 (-31)	(3-)3,7-4,7(-5)	(14–)20–28(–31)	3-5	(2-)2,8-3,8(-4)	(5-)5,4-6,6(-7)	2-4	5-7	(Van Wyk *et al.* 2012)

L = length, w = width

hyphae was observed (Figure 3f) as well as ascospores hat-shaped, sizes (3-)3.8-4.4(-5) µm × (5-)5.8-7(-8) µm (Figure 3g, Table 2). Finally, primary conidia cylindrical elongate, hyaline, size (12.6-)18.1-25-(28) µm × (2-)2.8-4.7(-5) µm were also observed (Table 2).

Regarding the molecular identification of the consensus sequence with the EF marker, it presented a similarity percentage of 96.36% with *Ceratocystis mangivora* (FJ200290.1). Meanwhile, the ITS marker registered a percentage similarity of 95.82% with *Ceratocystis mangivora* (FJ200264.1). Together with the morphological characterisation and symptom description the species identification for isolate CIF002 was confirmed.

Ceratocystis fimbriatomima

The colonies of this species in MEYA showed a slow growth, with irregular edge, aerial mycelium of gray to olive green color and banana odor like the other species. Ascocarps (perithecia) were produced in groups and formed concentric rings (Figure 4a). Perithecial ascocarp black, with globose base and long, slender black necks became lighter in color as they approached the tip (Figure 4b). Besides, terminal, simple, thick-walled, brown, sub-globose, sub-globose chlamydospores were observed (Figure 4c). Hyaline divergent ostiolar hyphae were conformed (Figure 4d). Ascospores (2-)2.8-3.8(-4) µm × (5-)5.4-6.6(-7) µm (Table 2) hat-shaped, hyaline (Figure 4e) was observed, as well as elongated cylindrical primary conidia (14-)17-26(-31) µm × (3-)3.7-4.7(-5) µm (Table 2), hyaline (Figure 4f). Finally, sometimes chain-like barrel-shaped conidia were observed (Figure 4g).

The identification at the molecular level of CIF 003 isolate with the consensus sequences through the ITS marker, presented a percentage similarity of 95.54% with *Ceratocystis fimbriatomima* (FJ200290.1). Meanwhile, the sequence of our isolate CIF 004 with the ITS marker, presented a percentage similarity of 95.8% with *C. fimbriatomima* (NR_166018.1).

For the EF marker, isolates CIF 003 and CIF 004 showed a percentage similarity between 95.96% and 93.28%, respectively, with *Ceratocystis fimbriatomia* (EF190962.1). Meanwhile, isolate CIF 004 registered a similarity percentage below the established parameters, therefore, results based solely on molecular identification can be considered as insufficient. However, based on the other two parameters, molecular characterisation and reported symptomatology, it was possible to confirm the identification of isolate CIF 004 associated to wilting of *G. arborea*.

In Table 2 a comparison of conidia and ascospore size from our isolates with other studies

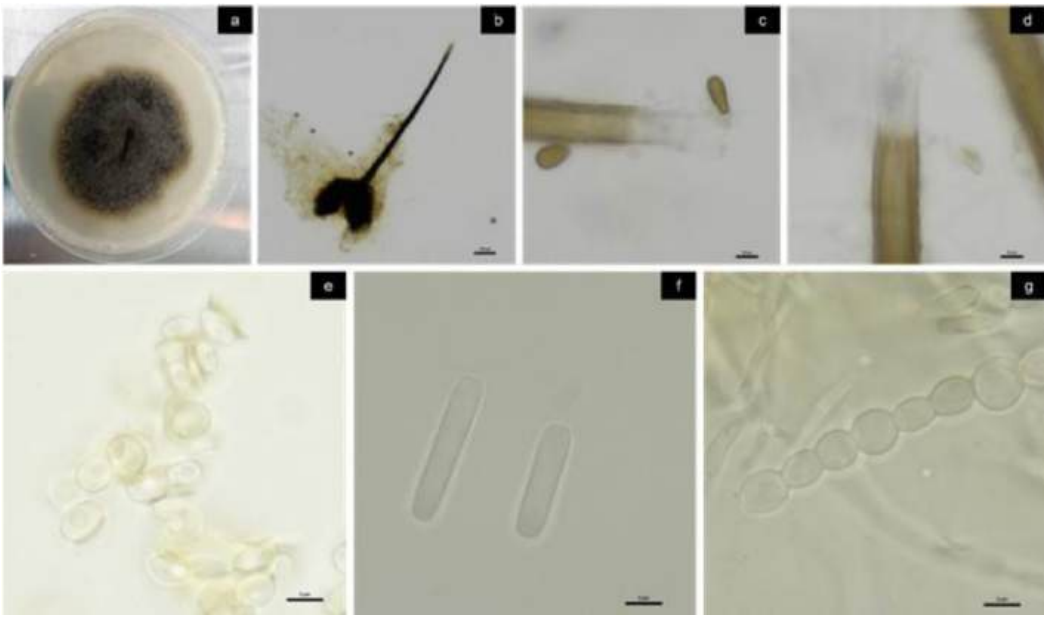

Figure 4 Morphological characteristics of *C. fimbriatomima*. (a) Colony morphology in MEYA. (b) Ascomatal base (perithecium) with mass of ascospores. Scale bar = 100 µm. (c) Subglobose chlamydospores Scale bar = 5 µm. (d) Ostiolar hyphae. Scale bar = 10 µm. (e). Hat-shaped ascospores. Scale bar = 5 µm. (f) cylindrical conidia. Scale bar= 10 µm. (g) Barrel-shaped conidia in chains. Scale bar = 5 µm

Figure 5 Vascular tissue necrosis caused by *Ceratocystis* strains on one elite melina genotype (clone 58), 90 days after trial establishment; (a) and (b) Control, (c) and (d) CIF 001 (*C. fimbriata*), (e) and (f) CIF 002 (*C. mangivora*), (g) and (h) CIF 0003 (*C. fimbriatomima*) and (i) and (j) CIF 004 *(C. fimbriatomima*)

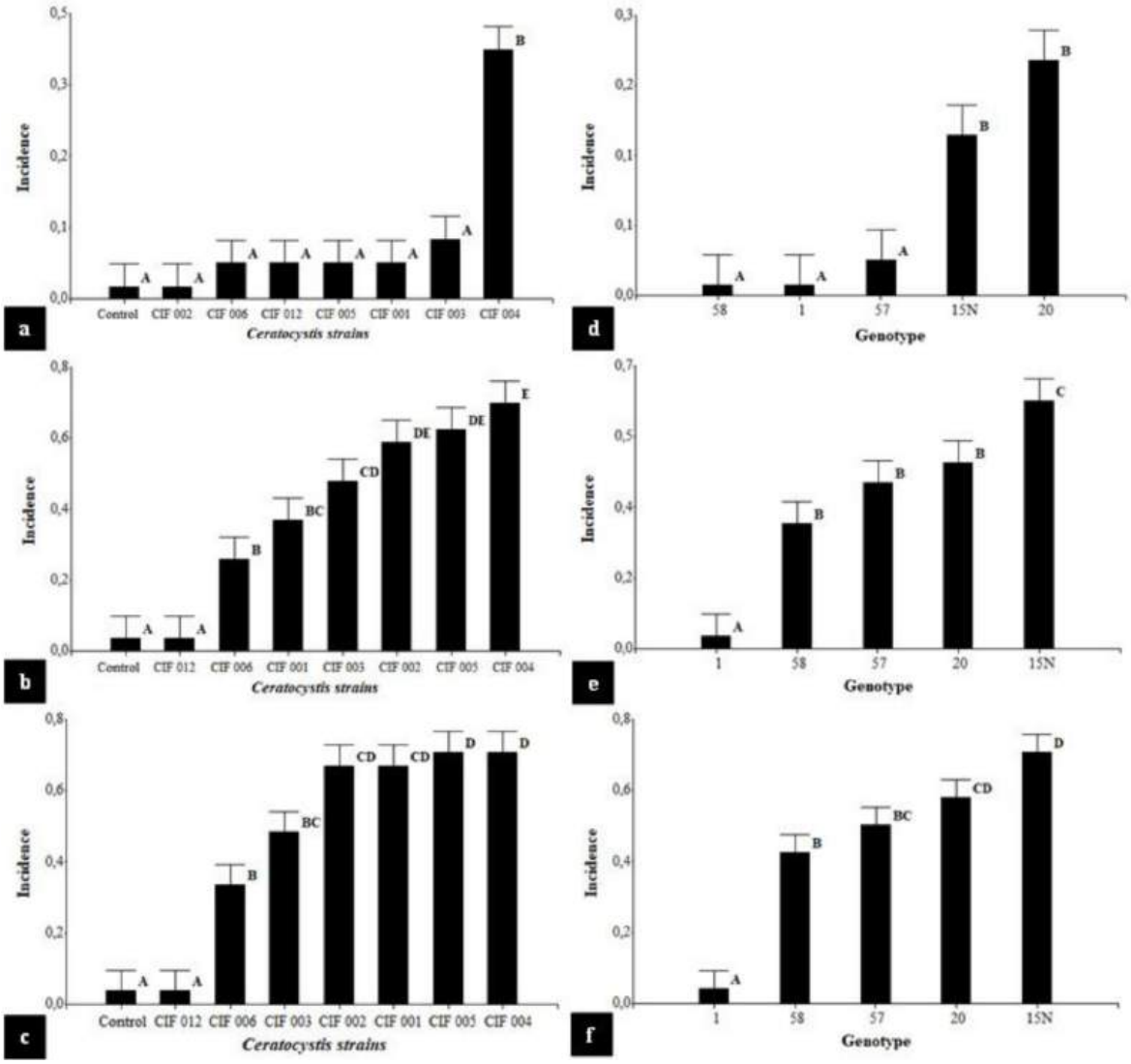

Figure 6 Incidence according to the origin of *Ceratocystis* strains at (a) 30 days, (b) 60 days and (c) 90 days. Incidence of *Ceratocystis* wilt disease evaluated according to genotype at (d) 30 days, (e) 60 days and (f) 90 days

is shown. Data obtained in this investigation falls within the range of values reported in the literature

Pathogenicity test performed on melina elite genotypes induced wilting and necrosis of vascular tissue at 30 days, showing symptoms like those observed in the field (Salas et al. 2016a, Méndez et al. 2020). Figure 5 shows the progression of the external lesion on the inoculation wound, as well as the internal lesion in the vascular tissue caused by different strains of *Ceratocystis*, in the same melina genotype 90 days after inoculation. The overall incidence of the trial at 30 days was 8%, at 60 days it was 38% and at 90 days it was 45%.

In the incidence analysis according to strain origin (Figure 6), it was found that at 30 and 60 days, CIF 004 from Pital site affected several individuals (Figure 6a and 6b). At 60 and 90 days, CIF 012 from Nicoya site showed the same behavior as the controls, with any type of affectation to plants (Figure 6b and 6c). At 90 days, CIF 004 from Pital site and CIF 005 from Pococí site, behaved as the strains with greatest affectation in individuals (Figure 6c).

Regarding clonal evaluation at 30 days, genotypes 15N and 20 exhibited highest number of affected individuals, while genotypes 1, 57 and 58 did not present any diseased individuals (Figure 6d). At 60 and 90 days, genotype 1 exhibited highest resistance compared to the other clones evaluated. While genotype 15N was

the most susceptible one recording the highest incidence percentage (Figure 6e and 6f).

According to the resistance evaluation, Figure 7a shows which genotypes scored statistically significant, with higher affectation rates and more susceptibility. There were two groups of genotypes response, one group with susceptible genotypes (20 and 15N) and, another group with tolerant genotypes (1, 57 and 58). Where genotype 1 can be considered as highly tolerant. Strain analyses registered significant differences between them. Strain CIF 012 behaved similarly as the control, while strain CIF 001 caused more internal lesions (Figure 7b).

DISCUSSION

Morphological and molecular pathogens identification isolated from melina, allowed the determination of three different *Ceratocystis* species in Costa Rica. Based on this identification it was possible to evaluate aggressiveness or virulence of the seven strains, as well as the response of five elite melina genotypes. The greenhouse assay demonstrated the ability of strains CIF001, CIF002, CIF003, CIF004, CIF005 and CIF006 to cause disease by observing internal lesions and symptoms in individuals after 30 days as similarly reported by Nasution et al. (2022), Lapammu et al. (2023) and Nasution et al. (2019) in *Acacia mangium*.

Pathogenicity variation observed among *Ceratocystis* strains resembles reports from Valdetaro et al. (2015), Oliveira et al. (2015), Oliveira et al. (2016), Rosado et al. (2016), dos Santos et al. (2021) and Guimarães et al. (2021); who showed that there is a relationship among level of aggressiveness, geographic region, and genetic diversity of the strains. In this study, strain CIF004 caused the highest incidence rate in melina clones, compared to the other strains, while strain CIF012 did not cause mortality at all (Figure 6).

Likewise, different levels of aggressiveness were observed in strains of the same species, such as with CIF003 and CIF004 and, among CIF001, CIF005, CIF006 and CIF012. A similar result was reported by Brito et al. (2021) in yerba mate 15 days after inoculation. These results confirm that some strains are more virulent and, that pathogen genetic variability, as well as geographical origin, may play a key factor in disease development (Oliveira et al. 2021, Guimarães et al. 2021, Chi et al. 2023).

During evaluation of the degree resistance, strains CIF001 and CIF004 caused more than 20% internal injury in melina genotypes (Figure 7b). Thus, considered as strains with highest disease capacity of melina in Costa Rica. Such results suggest that host genotypes exhibit different levels of susceptibility to *Ceratocystis* sp. strains. Genotype 15N was determined as highly susceptible, while genotype 1 was highly tolerant (Figure 7a).

These results suggests that there is a genetic makeup variation among clones, that explain differences in their ability to resist infection by this pathogen (Méndez et al. 2021, Méndez et al. 2023). Similar results were reported by Shirasawa et al. (2020) in *Ficus*, dos Santos et al. (2021) in cocoa, Oliveira et al. (2021) in kiwifruit and, Oliveira et al. (2015) in *Eucalyptus*.

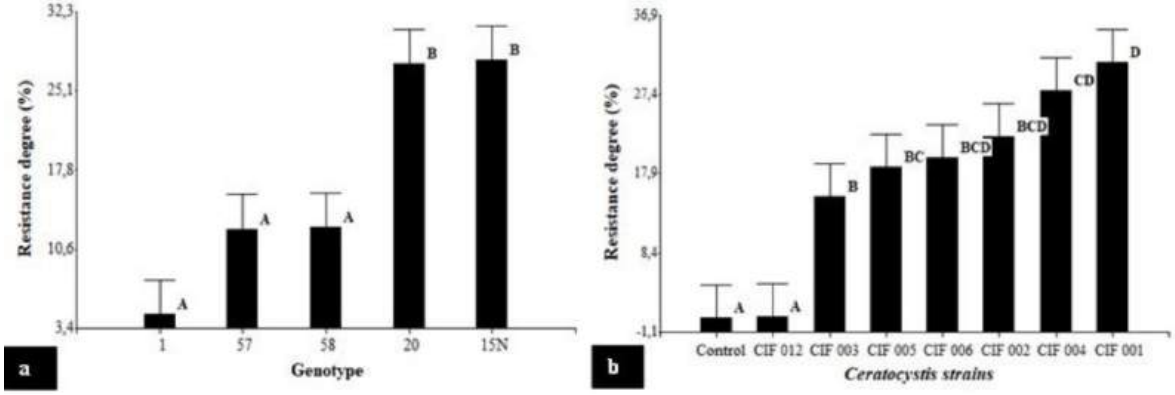

Figure 7 Degree of resistance evaluating the internal lesion, according to (a) genotypes and (b) origin of Ceratocystis strains at 90 days of evaluation

Main results from this study will have direct implications into forest disease approaches. The determination of variability in strains pathogenicity, implies that management strategies should be more effectively tailored. This may include geographic identification of plantation areas, that could be more susceptible to specific strains of this pathogen. More importantly, identifying genotypes resistant or tolerant to specific pathogens, can be a very effective strategy.

CONCLUSIONS

Morphological and molecular isolates identification confirmed the presence of *C. fimbriata, C. mangivora* and *C. fimbriatomima*, associated to wilting of *G. arborea* in Costa Rica. Pathogenicity test showed that all three strains are causal agents of melina wilt. Isolate CIF 004, *C. fimbriatomima*, was considered the most aggressive or virulent strain, which will be used in the screening commercial and elite melina material, to be deployed for operational reforestation and breeding efforts. There is evidence of genetic differences among genotypes in their wilt tolerance caused by *Ceratocystis* strains. To our knowledge, this is the first pathogenicity study with different isolates of *Ceratocystis* sp. on melina, which generates valuable information to determine disease control strategies.

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