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CERATOCYSTIS MANGINECANS AND NOT *C. FIMBRIATA* A THREAT TO PROPAGATED *ACACIA* SPP. IN SABAH, MALAYSIA

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Acacia mangium plantations in Sabah, Malaysia are seriously affected by a vascular wilt and canker disease caused by a species of *Ceratocystis*. A similar and devastating disease occurs in Indonesia and is caused by the fungal pathogen *Ceratocystis manginecans*. A closely related fungus, *Ceratocystis fimbriata sensu stricto*, is a common soil-borne pathogen of sweet potato (*Ipomoea batatas*) in areas of Malaysia where *A. mangium* is grown. This study characterised the species causing wilting on *A. mangium* and compared it with the *I. batatas* pathogen using DNA sequence-based comparisons used artificial inoculations to assess the effect of the *A. mangium* pathogen on *Acacia auriculiformis* and *Acacia crassicarpa* and considered the ability of the sweet potato pathogen to cause disease on the three *Acacia* species. DNA sequence comparisons confirmed that isolates from diseased *A. mangium* were *C. manginecans* and those on sweet potato were *C. fimbriata s.s. Ceratocystis manginecans* was pathogenic on all *Acacia* spp., with *A. mangium* being most susceptible followed by =*A. auriculiformis* and *A. crassicarpa*. Pathogenicity tests showed that *C. fimbriata s.s.* from sweet potato is not able to cause disease on any of the three *Acacia* spp. considered. This study also confirmed that *C. manginecans* is the primary cause of ceratocystis canker and wilt disease in Sabah and that the sweet potato fungus, *C. fimbriata s.s.,* poses no threat to propagated *Acacia* spp.

Keywords: *Acacia mangium*, canker disease, *Ceratocystidaceae*, *C. manginecans*, fungal disease, wilt disease

INTRODUCTION

Acacia mangium Willd. was first introduced in Sabah, Malaysia from Australia in 1966 (Potter et al. 2006). This tree species displayed an ability to adapt easily to the tropical climate and consequently, more than 2 million ha have been established in various countries in South East Asia (SEA) (Harwood et al. 2015). Of these, more than 250,000 ha of *A. mangium* have been planted in Malaysia (Harwood & Nambiar 2014).

Diseases have emerged as a major constraint to the propagation of *A. mangium* in SEA. One of the first problems to emerge has been root rot, primarily caused by *Ganoderma philippii* Bres. & Henn.: Sacc. (Coetzee et al. 2011, Mohammed et al. 2014). More recently, a severe canker and wilt disease caused by *Ceratocystis manginecans* van Wyk, A. Al Adawi & M. J. Wingf., has devastated plantations of *A. mangium* in Indonesia, Malaysia and Vietnam (Tarigan et al. 2011a, Brawner et al. 2015, Trang et al. 2017, Wingfield et al. 2023). This disease is commonly referred to as Ceratocystis Canker and Wilt Disease (CCWD).

Most species of *Ceratocystis* are pathogens of angiosperm trees, although some species cause disease on root crops, such as sweet potato (Kile 1993, Roux & Wingfield 2013, Marin-Felix et al. 2017). The taxonomy of *Ceratocystis* has been subject to different interpretations depending on the species concept followed. More specifically some authors have treated *Ceratocystis fimbriata* Ellis & Halst., which is the type species of the genus, broadly, based on the biological species concept, to include isolates with a wide range of hosts and an extensive geographic distribution (Harrington et al. 2014, Oliveira et al. 2015, Li et al. 2016). In contrast, applying phylogenetic inference based on DNA sequence, 42 species of *Ceratocystis* have been recognised (Li et al. 2017, Marin-Felix et al. 2017, Barnes et al. 2018, Liu et al. 2018, Holland et al. 2019, Cho et al. 2020), where *C. fimbriata* is treated in the strict sense (*C. fimbriata sensu stricto*) and it includes only isolates that infect sweet potato (Marincowitz et al. 2020).

Ceratocystis species predominantly infect their hosts through wounds, either naturally occurring or man-made (Roux & Wingfield 2009, Al Adawi et al. 2013a, Wingfield et al. 2023) or through trichomes (Sun et al. 2020). On trees, these fungi result in a wide variety of symptoms including a streaked discolouration of the vascular tissues, cankers, wilt and ultimately, death (van Wyk et al. 2007, van Wyk et al. 2010, Tarigan et al. 2011a, Barnes et al. 2018). On root crops such as sweet potato (*Ipomoea batatas* (L.) Lam.) and taro (*Colocasia eusculenta* (L.) Schott), various *Ceratocystis* species cause black rot symptoms (Huang et al. 2008, Li et al. 2017, Liu et al. 2018, Marincowitz et al. 2020).

Some species of *Ceratocystis* are highly host-specific while others have wide host ranges; for example, *Ceratocystis platani* Engelbrecht & Harrington is known to infect only *Platanus* spp. (Engelbrecht et al. 2003, Tsopelas et al. 2017) while *Ceratocystis albifundus* M.J. Wingf., De Beer & M.J. Morris has an extensive host range including different genera and families of plants (Roux et al. 2007, Roux & Wingfield 2013, Lee et al. 2016). Pathogenicity tests have also been used to show that different isolates of the same *Ceratocystis* species display different levels of aggressiveness (Tarigan et al. 2011a, Oliveira et al. 2015 & 2016). In the case of *C. manginecans*, Tarigan et al. (2011a) showed intraspecific variation in pathogenicity on *A. mangium* for isolates representing different ITS types. This variation was, however, not observed on inoculated *A. crassicarpa* A. Cunn: Benth. (Tarigan et al. 2011a).

Ceratocystis manginecans is an aggressive pathogen on a diversity of tree hosts, such as mango (*Mangifera indica* L.) and other legume trees in Oman and Pakistan (van Wyk et al. 2007, Al Adawi et al. 2013b), *Punica granatum* L. in China (Chen et al. 2013, Li et al. 2016), and *Acacia* and other host species in SEA, such as Vietnam and Indonesia (Tarigan et al. 2011a, Brawner et al. 2015, Trang et al. 2017, Chi et al. 2019, Chi et al. 2021, Pratama et al. 2021). In a recent study, Trang et al. (2017) considered the susceptibility of *A. auriculiformis* A. Cunn.: Benth. and *A. mangium* and showed that *A. mangium* was highly susceptible, *A. auriculiformis* was most tolerant and that six tested hybrids displayed variable susceptibility to infection. Similarly, Tarigan et al. (2011a & 2011b) reported high levels of susceptibility in *A. mangium* and that *A. crassicarpa* was less susceptible to infection.

Ceratocystis fimbriata s.s. from sweet potato and *C. manginecans* are closely related but can be distinguished morphologically from each other by the presence of barrel-shaped conidia in *C. manginecans* (van Wyk et al. 2007). However, isolates of the two species can be induced to undergo sexual reproduction when crossed under laboratory conditions (Li et al. 2016, Fourie et al. 2018). For this reason, some researchers consider these species to be conspecific (Oliveira et al. 2015). In Malaysia, sweet potato is commonly infected with *C. fimbriata s.s.* and its fields are planted in close proximity to *Acacia* plantation areas. This has raised questions amongst foresters as to whether isolates of *C. fimbriata s.s.* from sweet potato could infect *Acacia* spp.

In this study, we collected isolates of *Ceratocystis* from infected *A. mangium,* and sweet potato in Sabah and identified them using DNA sequence comparisons. The relative pathogenicity of isolates from these two hosts was then tested on *A. mangium*, *A. auriculiformis* and *A. crassicarpa* using artificial inoculation tests.

MATERIALS AND METHODS

Sample collection and isolation

Isolations were made from symptomatic sweet potato tubers (Stokes Purple and Beauregard) displaying black rot (Figure 1a), collected from markets in Keningau (centre of Sabah), Ranau and Telipok (west Sabah), Sandakan (northeast Sabah) and Tawau (southeast Sabah). *Acacia mangium* isolates were collected from trees displaying typical symptoms of CCWD (Figure 1b) in Tawau (southeast Sabah), Sipitang (southwest Sabah), Bintulu (Sarawak) and Mentakab (Pahang) (Figure 2). Moist chambers were used to induce sporulation of the fungus on wood samples showing xylem discolouration or infected tubers. Ascospore masses that developed at the apices of ascomata, typical of *Ceratocystis* spp., were spread onto 2 % MEA (Malt Extract Agar: 20 g l^{-1} malt extract + 20 g l^{-1} agar) supplemented with 100 mg l^{-1} streptomycin sulphate, using 10 µl Soltrol® 130 isoparaffin solvent. After 24 hours, single germinating hyphae were transferred to new MEA plates to obtain monosporic cultures.

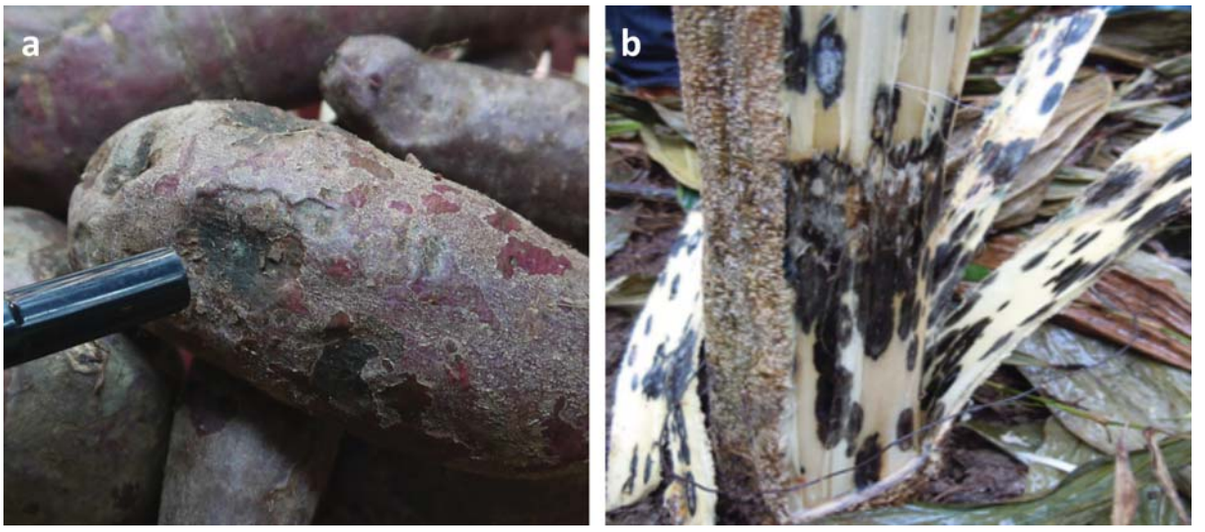

Figure 1 Disease symptoms caused by *Ceratocystis* species on sweet potato and *A. magium* in Sabah, Malaysia; a) blackened and sunken surfaces on *Ipomoea batatas* caused by *C. fimbriata s.s.*, b) kino pockets of black discolouration with white fungal mats of *C. manginecans* in the sapwood of *A. mangium*

DNA extraction, PCR amplification and DNA sequence comparison

Axenic cultures were grown on MEA at room temperature (22 ˚C) for two weeks to harvest mycelium for DNA extraction. The mycelium was scraped off the surface of the agar, transferred to 2.0 ml Eppendorf tubes and freeze dried. DNA extraction was performed using the CTAB (cetyl trimethyl ammonium bromide) method described by Möller et al. (1992). Extracted DNA was visualised under UV light, using gel electrophoresis on a 1 % agarose gel, to confirm DNA yield. Extracted DNA was quantified using a ND-1000 spectrophotometer. The DNA for each sample was diluted to a working concentration of 30 ng μl^{-1}.

Primers ITS1 and ITS4 (White et al. 1990) were used to amplify the Internal Transcribed Spacer 1 and 2 (ITS) and 5.8S region of 51 isolates. A total PCR reaction volume of 25 µl was prepared consisting of 1 µl DNA template, 2.5 µl PCR FastStart Buffer (10 x conc.) containing MgCl (25 mM), 0.3 µl FastStart Taq (5 U μl^{-1}), 2.5 µl of dNTP mix (10 mM), 0.5 µl of each primer ITS1 and ITS4 (10 mM concentration each) and 15 µl Sabax water. Amplification was performed using a programmed thermal cycler with an initial denaturing temperature of 96 ˚C (60 s) followed by 35 cycles of 94 ˚C (30 s), 56 ˚C (60 s) and 72 ˚C (90 s) and a final step at 72 ˚C (10 min). The PCR products were visualised using 2 % agarose gel electrophoresis and purified using Sephadex G-50 columns.

Sequence reactions for forward and reverse sequencing were performed in 12 µl final volumes with the same primer used for the PCR reactions. The mixtures contained 2 µl sequencing buffer, 1 µl of BigDye® Terminator v. 3.1 ready reaction mixture and 1 µl of forward or reverse primer (10 mM) and 3 µl of the cleaned PCR products. The thermal cycling conditions included 25 cycles at 96 ˚C for 10 s, 56 ˚C for 4 s and 60 ˚C for 4 min. Amplicons were purified using Sephadex G-50 columns and dried using an Eppendorf 5301 vacuum concentrator at 60 ˚C for five to ten minutes and run on an ABI PRISM™ 3100 DNA Analyzer to obtain sequences.

Sequence reads were assembled in CLC Main Workbench v. 8 to obtain consensus sequences. In order to identify the fungal species, sequence data were then aligned with authenticated DNA sequences of species, or ex-type species obtained from GenBank (https://www.ncbi.nlm.nih.gov) (Table 4 in Marin-Felix et al. 2017) using Muscle alignment in MEGA 7 (Kumar et al. 2016).

An additional four gene regions were sequenced for an isolate chosen randomly from each host and each location for further species verification using a phylogenetic approach. The gene regions *bt1, ef1, ms204* and *rpb2* were PCR amplified with primers Bt1a and Bt1b (Glass & Donaldson 1995), TEF1F and TEF2R (Jacobs et al. 2004), MS204F.cerato and MS204R.

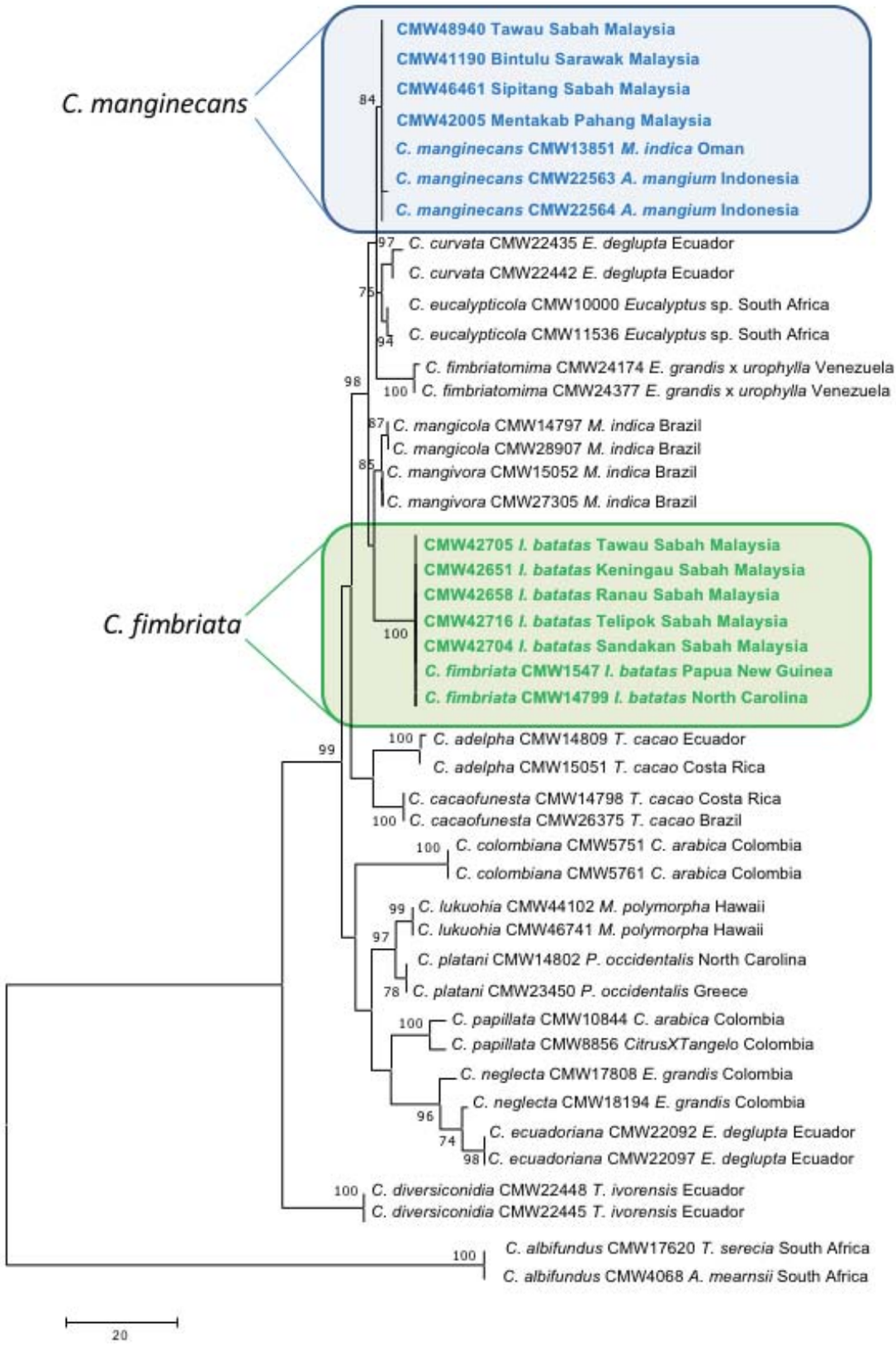

Figure 2 Maximum Parsimony (MP) phylogeny of *Ceratocystis* isolates from Malaysia sequenced in this study based on the combined analyses of the *bt1, ef1, ms204* and *rpb2* gene regions. All isolates from *Acacia mangium* from Sabah, Sarawak and Pahang were identified as *Ceratocystis manginecans* (in blue), while all isolates obtained from *Ipomoea batatas* from five locations in Sabah were of *C. fimbriata s.s.* (in green). MP bootstrap support for branches >60 % are indicated at the nodes. *Ceratocystis albifundus* was used as the outgroup taxon

cerato (Fourie et al. 2015), and RPB2-5Fb and RPB2-7Rb (Fourie et al. 2015) respectively. The PCR amplification, sequencing reactions, and generation of consensus sequences were the same as those described by Fourie et al. (2015).

Phylogenetic analyses

Sequences generated for the four gene regions (*bt1, ef1*, *ms204* and *rpb2*) were aligned with the dataset generated in Barnes et al. (2018) available in TreeBASE (No. S22005) (http://purl.org/phylo/treebase/phylows/study/TB2:S22005) for species in the *Ceratocystis* Latin American Clade (LAC). Maximum parsimony analyses were conducted in MEGA v. 7 (Kumar et al. 2016) using Tree-Bisection-Reconnection (TBR) and where gaps or missing data were treated as a fifth character. Branch support was determined using 500 Bootstrap replications and the Consistency Index (CI) and Retention Index (RI) were calculated.

Pathogenicity tests

Two isolates each, obtained from *I. batatas* (CMW42704 and CMW42705) and *A. mangium* (CMW46461 and CMW48940) that were sequenced in this study, were randomly selected for pathogenicity tests. Inoculations were performed on 1-year-old *A. mangium*, *A. auriculiformis* and *A. crassicarpa* trees growing under field conditions. The trees were grown in a 3 × 3 m spacing and the average temperature in the plantation ranged from 29 ˚C to 32 ˚C with average rainfall 2500 mm per year.

Each isolate was inoculated onto 20 trees for each of the *Acacia* spp. using procedures described by Tarigan et al. (2011a). Tree diameters ranged from 2.0 cm to 10.0 cm and heights from 2.5 m to 5.0 m. An 8 mm diameter wound was made on the tree stems 1 m above ground using a cork borer. Mycelial plugs (8 mm diam.) were cut from the margins of actively growing cultures on MEA and placed, mycelium side facing inwards, into the cork borer wounds. The wounds were covered with plastic wrap to reduce desiccation and contamination from the surrounding environment. For the negative controls, four trees of each species were inoculated with clean discs of MEA.

Lesion lengths on the inoculated tree stems were measured after four weeks by removing the bark to expose the lesions. In order to meet the requirements of Koch's postulates, wood from the leading edges of the lesions were placed in moist chambers to induce sporulation and to confirm that the recovered fungi were the same as those used in the inoculation.

Statistical analyses of lesion lengths were made using analysis of variance (ANOVA) followed by a HSD test using Tukey (alpha = 0.05) test. The combinations of each isolate (four isolates and control) inoculated onto each *Acacia* species (three) were each considered as a different treatment; hence, 15 treatment categories were analysed (i.e., CMW42704 on *A. mangium*). The analyses were conducted using R software (R Core Team, 2020) with the agricolae package version 1.2-4.

RESULTS

Fungal isolations

A total of 51 isolates with morphological characteristics typical of *Ceratocystis* spp., including black ascomatal bases with long necks and ostiolar hyphae, were obtained. Of these, 15 were from diseased *A. mangium* trees and the rest from *Ipomoea batatas* tubers (Table 1). All cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1).

Fungal identifications

The sequence data obtained for the ITS gene region resulted in sequence amplicons of approximately 500 bp in size. The 15 isolates from *A. mangium* were identified as *C. manginecans* having 100 % sequence homology with representative strains of this species displaying either ITS type 1 (EU588657) or type 2 (AY953383) (Al Adawi et al. 2013b). The 35 isolates from sweet potato were 100 % identical to each other and to the ex-epitype reference sequence of *C. fimbriata* (KC493160) (Marincowitz et al. 2020).

Phylogenetic analyses

The sequence data obtained for the *bt1, ef1*, *ms204* and *rpb2* gene region resulted in sequence amplicons of approximately ~610 bp, ~780 bp,

Table 1 Locations and respective hosts of where *Ceratocystis manginecans* and *C. fimbriata sensu stricto* were isolated from in Malaysia

Fungal species		Geographical area		Isolate (CMW)[1] number
		Province/ State	Location(s)	
Ceratocysis manginecans	*Acacia mangium*	Sabah	Tawau	39153, 39162, 39174, 39182, 42003, **48940**[2,3]
			Sipitang	41181, 41182, 42006, **46461**[2]
		Sarawak	Bintulu	41190, 41193, 41194
		Pahang	Mentakab	41202, **42005**
Ceratocystis fimbriata	*Ipomoea batatas*	Sabah	Keningau	42656, 42657, 42650, **42651**, 42653, 42654, 42655
			Ranau	**42658**, 42670, 42675, 42676, 42677, 42678, 42685, 42686, 42688, 42694
			Sandakan	42648, 42703, **42704**[2]
			Tawau	42681, **42705**[2], 42706, 42707, 42708, 42709, 42710, 42712
			Telipok	42683, 42714, 42715, **42716**, 42717, 42718, 42719, 42720

[1]CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, [2]Isolates used in the inoculation study, [3]Isolates in bold were used in the phylogenetic analyses

~970 bp and ~1 200 bp respectively in size. The combined dataset for these analyses included 3264 characters of which 46 were parsimony-informative. The CI and RI were 0.879 and 0.937 respectively. The most parsimonious tree with length of 284 is shown in Figure 2. All isolates from *A. mangium* were grouped in the clade representing *C. manginecans* with 84 % bootstrap support while all the isolates from sweet potato grouped with *C. fimbriata s.s.* with 100 % bootstrap support.

Pathogenicity

Two weeks after inoculation, symptoms were visible on all of the trees inoculated with the two isolates of *C. manginecans* from *A. mangium* (Figure 3). These included sunken wounds and gummosis around the inoculation points. The *C. manginecans* isolates from *A. mangium* gave rise to extensive lesions on all the tested *Acacia* spp. that were significantly different in length (p-value $2.2^{e\text{-}16}$, at a 95 % confidence level) to those of the negative controls on all *Acacia* spp. (Figure 4). None of the *C. fimbriata s.s.* isolates from sweet potato resulted in lesions (Figure 3), and the lesions were not significantly different to those associated with the negative control inoculations (Figure 4). The lesion lengths on *Acacia* spp. inoculated with *C. fimbriata s.s.* from sweet potato and *C. manginecans* were significantly ($p < 0.05$) different to each other (Figure 4).

There were significant differences between the lesion lengths on *A. mangium* compared with *A. auriculiformis* and *A. crassicarpa*. *Acacia auriculiformis* and *A. crassicarpa* did not differ significantly in their susceptibility to infection by *C. manginecans* (Figure 4). The longest lesions (average = 79.2 cm) were observed on *A. mangium* followed by *A. auriculiformis* (average = 52.2 cm) and *A. crassicarpa* (average = 46.6 cm). Re-isolation from infected tissue consistently yielded *Ceratocystis* isolates that were morphologically identical to those used for inoculations. No *Ceratocystis* sp. could be isolated from trees inoculated with *C. fimbriata s.s.* from sweet potato and isolations from the negative control inoculations were also free of pathogenic fungi.

DISCUSSION

The results of this study confirmed the association of *C. manginecans* with a devastating wilt and canker disease of *A. mangium* in Sabah. Isolates from sweet potato were readily collected from tubers and these were confirmed as being *C. fimbriata s.s.*. DNA sequence data also showed that they were distinctly different to *C. manginecans*

Figure 3 Stems of *Acacia mangium* showing lesions after inoculation with a) an isolate of *C. fimbriata s.s.* from sweet potato, b) an isolate of *C. manginecans* from *A. mangium* and c) a clean agar control

from *A. mangium*. Inoculation studies showed that *C. manginecans* was highly pathogenic, with various levels of aggressiveness on *A. mangium*, *A. auriculiformis* and *A. crassicarpa*. Importantly, isolates from sweet potato were not able to infect the inoculated *Acacia* spp., confirming their specificity to that host.

Comparisons of DNA sequence data for multiple gene regions confirmed that *C. manginecans* is the pathogen responsible for the devastation of *Acacia* plantations in Sabah, Malaysia (Wingfield et al. 2023). This is the same species causing CCWD on this tree species in Indonesia and Vietnam (Tarigan et al. 2011a, Trang et al. 2017). The presence of *C. manginecans* on *A. mangium* in Malaysia is, therefore, not surprising as SEA is considered a centre of diversity for this fungus (Fourie et al. 2016). Population genetic studies have shown that the population in Malaysia on *Acacia* is not clonal (Liu et al. 2021), contrary to the populations on mango and leguminous trees in Oman and Pakistan (Al Adawi et al. 2014, Fourie et al. 2016), where rapid spread has been facilitated by the mango bark beetle *Hypocryphalus dilutes* (previously *H. mangiferae*) (Al Adawi et

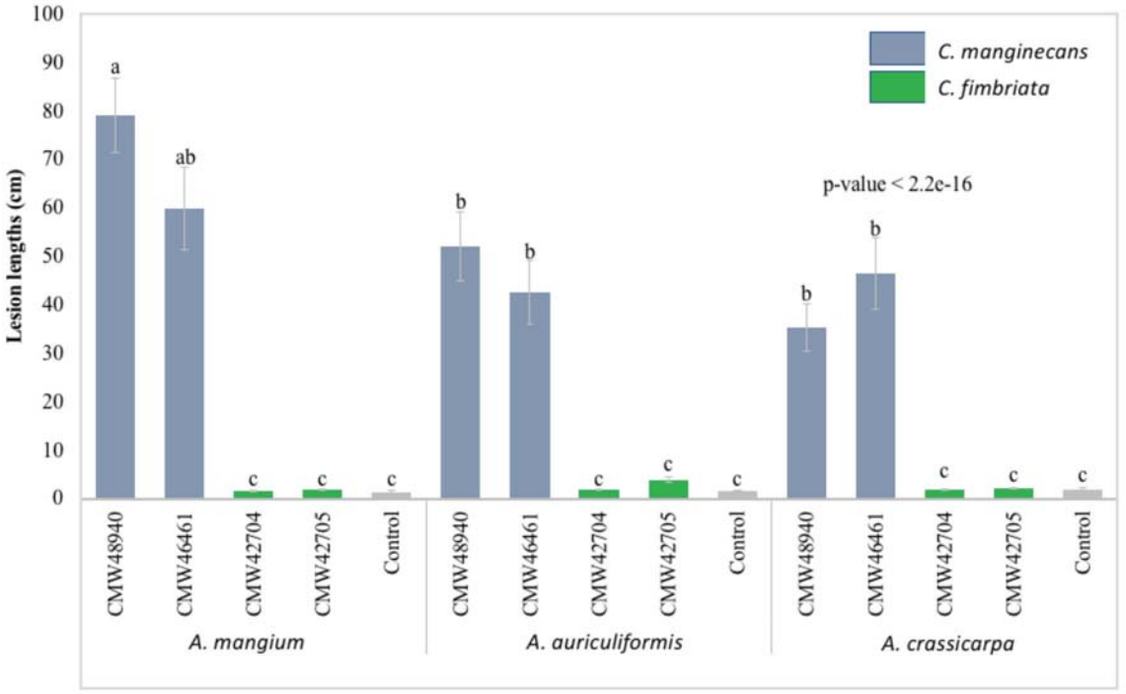

Figure 4 Lesion lengths on *Acacia* spp. inoculated with *Ceratocystis manginecans* isolates (CMW48940 and CMW46461) from *A. mangium* and *Ceratocystis fimbriata s.s.* isolates (CMW42704 and CMW42705) from sweet potato after 4 weeks. Means with standard error (SE) bars annotated with the same letters are not significantly different from each other. Clean agar was used in the control inoculations. CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa

al. 2013a, Johnson et al. 2017). Little is known regarding the epidemiology of *C. manginecans* in Malaysia but there is emerging evidence that frass, associated with the bark beetles *Euwallacea perbrevis* and *Xylosandrus crassiusculus* (Wingfield et al. 2023), contribute to its distribution.

The *C. manginecans* isolates from *A. mangium* in Malaysia were all able to cause significant lesions on *A. mangium*, *A. crassicarpa* and *A. auriculiformis*. However, the levels of susceptibility on the tested *Acacia* spp. differed markedly. In this regard, *A. mangium* was the most susceptible followed by *A. auriculiformis* and *A. crassicarpa*. These results are similar to those of Tarigan et al. (2011a & 2011b) where *A. mangium* was shown to be considerably more susceptible to infection than *A. crassicarpa*. Trang et al. (2017) and Brawner et al. (2020) also found that *A. mangium* was more susceptible than *A. auriculiformis*. The present study therefore, supports the results of Brawner et al. (2020) that *A. auriculiformis* harbours resistance genes to *C. manginecans* and consequently, this species may be an important hybrid partner with *A. mangium* as has been suggested by Lapammu et al. (2023) and Wingfield et al. (2023).

Ceratocystis fimbriata s.s. isolates collected from sweet potato in Sabah were not pathogenic to any of the *Acacia* spp. considered in this study. This is consistent with earlier findings (Engelbrecht et al. 2003, Fourie et al. 2018) showing that isolates of *C. fimbriata s.s.* from sweet potato were pathogenic only to that host and did not cause disease on cacao, sweet potato, sycamore or *A. mangium*. The present study confirmed that *C. fimbriata s.s.*, which is common on sweet potato propagated extensively in Malaysia, poses no threat to *Acacia* spp. in the region.

CONCLUSION

This study confirmed the occurrence and identity of *C. manginecans* on *A. mangium* and *C. fimbriata s.s.* on sweet potato in Malaysia. *Ceratocystis manginecans* from *A. mangium* was able to infect inoculated *Acacia* spp. but *C. fimbriata s.s.* was not pathogenic on these trees. The inoculation trials showed that

Table 2 Summary of the analysis of variance (ANOVA) of the *Acacia* spp. inoculated with *Ceratocystis fimbriata s.s.* and *C. manginecans*

	df*	Sum Sq	Mean Sq	F value	Pr (>F)
Host species - isolates - Lesion	14	191765	13697	21.27	$< 2e^{-16}$ ***
Residuals	237	152591	644		

* = Degree of freedom
*** = Statistically significant (alpha = 0)

C. manginecans is considerably more pathogenic on *A. mangium* than it is on *A. auriculiformis* and *A. crassicarpa*. The latter tree species provide possible options to replace *A. mangium* in plantations devastated by *C. manginecans* in the humid tropics of Southeast Asia.

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