BUKTI KORESPONDENSI

ARTIKEL JURNAL INTERNASIONAL BEREPUTASI (TERINDEKS PADA DATABASE INTERNASIONAL BEREPUTASI DAN BERDAMPAK FAKTOR)

Judul Artikel	: First report of bullet wood (Mimusops elengi) sudden decline disease caused by Ceratocystis manginecans in Indonesia
Jurnal	: Biodiversitas Journal of Biological Diversity. Vol.22 No.5 Hal. 2636-2645, Mei 2021. p-ISSN:1412-033X, e-ISSN:2085-4722
Penulis	: R.Pratama, **Ahmad Muslim*(sebagai Corresponding Author)**, Suwandi, N. Damiri, S. Soleha

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REVIEWER 1

First report of ~~characterisation~~ characterization and pathogenicity of bullet wood (*Mimusops elengi*) sudden decline disease by *Ceratocystis* in Indonesia

Abstract. *Ceratocystis manginecans* causes wilt and death of plants in several important crops and native vegetation in Indonesia. Ceratocystis wilt was recently found to be causing substantial mortality in bullet wood (*Mimusops elengi*) in South Sumatra. The aim of this study was to describe the symptomatology of the new disease and characterise isolates of *C. manginecans* obtained from diseased bullet wood plants. ~~Affected~~ Diseased plants showed substantial discoloration of the woody xylem and wilt-type symptoms of the foliage, with the eventual death of the whole plant. Isolations from infected ~~trees~~ plants yielded ~~a fungus~~ fungi that ~~was~~ were similar morphologically to *C. manginecans*, with typical hat-shaped ascospores and light-coloured perithecial bases. Sequencing of the internal transcribed spacer (ITS) and β-tubulin of the isolates confirmed their identification, grouping them with *C. manginecans* and separating them from all other *Ceratocystis* species. This is the first report of *C. manginecans* in Indonesia causing wilt and death on bullet wood. *C. manginecans* is an important pathogen, and strategies to reduce losses need to be established in Indonesia because the aggressiveness of *C. manginecans* to bullet wood has been shown in inoculation experiments

Keyword: Ceratocystidaceae, Molecular phylogeny, Pathogenicity, Sapotaceae

INTRODUCTION

Bullet wood (*Mimusops elengi*) belongs to the family Sapotaceae, common English names are Asian Bulletwood, Bullet Wood Tree, Indian Medlar, Red Coondoo Spanish Cherry and it is known in Indonesia as Tanjung. The species is native to India, Sri Lanka, the Andaman Islands, Myanmar, Indo-China, Peninsular Malaysia and Vanuatu; it has been introduced and cultivated elsewhere. *M. elengi* can grow in tropical and subtropical climates. This plant thrives in areas with high humidity and seasonal rainfall and seasonal dry periods (Lim, 2012). The bullet wood trees range from small to large, and are found in all parts of Indonesia where bullet wood is cultivated in gardens as an ornamental tree, for medicines and planted along avenues because of its fragrant flowers (Seth, 2003).

M. elengi is widely used for medicine, and various parts of *M. elengi* Linn. (Sapotaceae) have been used widely in traditional Indian medicine for the treatment of pain, inflammation and wounds. *M. elengi* stem bark would be a possible therapeutic candidate having cytotoxic and anti-tumour potential (Kumar et al. 2014); it also has antibacterial and antifungal uses (Ali et al. 2008). At present, *M. elengi* is used as the synthesis of calcium phosphate nanoparticles that is easy, eco-friendly and scalable (Pokale et al. 2014).

Several types of pathogenic fungi have been identified to cause disease in *M. elengi* plants. *Curvularia lunata* caused die-back in India (Khatun et al. 2011); *Pestalotiopsis clavispora* caused leaf blight (Lokesh et al. 2017). *Ceratocystis* was first isolated from a single tree of bullet wood showing sudden decline in Thailand. Symptoms displayed by the diseased trees include gum exudation from the trunks and wilting and loss of the dark green foliage with a corresponding browning of leaves on single branches. In this study they did not confirm the pathogen with a Koch postulates test detail (Pornsuriya and Sunpapao, 2015). Recently we have observed many bullet wood trees showing similar symptoms with *C. manginecans* decline in many locations in South Sumatra, Indonesia.

C. manginecans includes many economically important plant pathogens. This pathogen has caused a sudden decline and has led to the death of thousands of *Mangifera indica* trees in Oman with *Hypocryphalus mangifera* vector (Al Adawi et al. 2013). In Indonesia *C. manginecans* caused die-back on *Acacia mangium* and *A. crassicarpa* plantations in Riau (Tarigan et al. 2010), whereas in Vietnam recently, *C. manginecans* caused wilt disease in *Dalbergia tonkinensis* and *Chukrasia tabularis* (Chi et al. 2019a; Chi et al. 2020); in Pakistan this pathogen also causes wilt disease in *Albizia lebbeck* (Razzaq et al. 2020). Commonly *C. manginecans* causes yellowing of leaves and rapid wilting of leaves was observed on individual branches in affected trees that ultimately spread to the canopy followed by the death of the whole tree. Dark brown to black tissue discoloration was observed in the woody xylem tissues of infected trees.

This study aimed to identify the cause of a new outbreak of wilt disease causing a sudden decline to the trees, wilted canopies and tree death in *M. elengi* in South Sumatera, Indonesia. This study was also conducted to describe the characteristics of the pathogen and confirm Koch's postulates test.

Commented [A1]: Suggested title: "First report of bullet wood (*Mimusops elengi*) sudden decline disease caused by *Ceratocystis manginecans* in Indonesia"

Characterization and pathogenicity test are the tool to reveal that bullet wood sudden decline is caused by *C. manginecans*. Characterization and pathogenicity test themselves are not first report.

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Commented [A3]: Kumar et al 2014 or Kumar et al 2016 as written in references?

MATERIALS AND METHODS

Disease symptoms and specimen collection

The distribution and impact of the *C. manginecans* disease on *M. elengi* were determined from roadside trees planting in Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) and the agricultural field of Sriwijaya University in Indralaya (Ogan Ilir), South Sumatra, Indonesia. Symptoms of wilt diseases were evaluated as follows: the extent of lesion development from discoloration of bark and wood, the extent of foliar wilting or loss and tree death.

Samples of diseased trunks were collected from two to six-year-old trees in September 2019 to April 2020. Wood samples were taken from lesions of wilted trees using a knife sterilised in 70% ethanol. The wood samples collected from *M. elengi* showed brown to black streaking in the woody xylem. Each sample was wrapped in tissue paper and placed in a coolbox. The same day, the wood samples (1–20 mm length, 1–2 mm thick) were sandwiched between two slices of fresh carrot and placed on sterile dry paper in plastic boxes at 25 °C following the method of Moller and DeVay (1968). After 5–10 days, hat-shaped spores of putative *Ceratocystis* pathogens were placed on 2% (w/v) malt extract agar (MEA) (Merck, Germany), and incubated at 25 °C in a laboratory. When cultures had grown to several cm in diameter, hyphal tips were sub-cultured onto new MEA and potato dextrose agar (PDA) (Merck, Germany) plates and incubated at 25–28 °C. Morphological traits of fruiting bodies and spores were observed under an optical Olympus CX33 microscope.

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Genomic DNA extraction, PCR amplification, and sequencing

DNA isolation used YeaStar Genomic DNA Kit (Zymo Research Corporation, California, USA). To extract genomic DNA, cultures were incubated for five days to allow sufficient mycelial growth in potato dextrose broth (PDB) (Merck, Germany). Mycelium was purified with sterile filter paper (Whatman) and transferred to 1.5 mL Eppendorf tubes. The quantity and quality of DNA extracted was evaluated with a spectrophotometer (NanoDrop ND-1000; Thermo Fisher, Waltham, MA, USA) to calibrate the concentration and purity of DNA as PCR templates.

The PCR amplification reactions were conducted on a T-100 thermal cycler (Bio-Rad, Hercules, CA, USA). Amplifications were carried out in 50 µl reactions containing 20 µl DreamTaq Green PCR Master Mix (Eppendorf, Germany) (DreamTaq DNA Polymerase, 2X DreamTaq Green buffer, dNTPs, and 4 mM $MgCl_2$), 1,5 µl of each forward and reverse primer, 4 µl of DNA template and 23 µl sterilised water. The PCRs were performed with a C1000 Touch™ thermal cycler (Bio-Rad, USA). The PCR cycling parameters were as follows: initial denaturation for 5 min at 95 °C, followed by 35 cycles at 95 °C for 30 s, 56 °C for 45 s and 72 °C for 1 min. Amplification was completed at 72 °C for 10 min and the PCR product was stored at 10 °C.

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PCR amplifications were made for two gene regions, including part of the b-tubulin (BT) using primers βt1a (TTCCCCCGTCTCCACTTCTTCATG) and βt1b (GACGAGATCGTTCA TGTTGAACTC) (Glass and Donaldson 1995), and the ITS using ITS1 and ITS4. The resulting PCR products were submitted to 1st BASE (Malaysia) for forward and reverse sequencing reactions. Raw sequence data were assembled, examined, and manually edited using Genestudio 2.1.1.5 (Genestudio, Suwanee, Georgia) and BioEdit software (Hall 1999). The DNA sequences were compared to the GenBank database via the nucleotide-nucleotide BLAST search interface located at the National Center for Biotechnology Information, Bethesda, USA. Relevant sequences were transferred with NoteTab Light v7.2.

Phylogenetic analyses

The sequences of *Ceratocystis* spp. closely related to the one from *Mimusops elengi* were retrieved from GenBank. Phylogenetic sequences from different gene regions were aligned using Mesquite v3.5 (Maddison and Maddison 2018) (http://mesquiteproject.org) and corrected manually. Phylogenetic trees based on a concatenated data set of the ITS and βt were computed and analysed as a single dataset. Maximum Parsimony (MP) analyses were performed in MEGA v. 10 (Kumar et al. 2016; Paul et al. 2018) with 1000 bootstrap replications.

Pathogenicity tests

Pathogenicity studies were conducted on two agroforestry plants, *A. mangium* and *M. elengi*. Plants had stem diameters of 2–3 cm and heights <1 m. The pathogenic potential of isolates was evaluated by the under bark inoculation method described by O'Gara et al. (1997). Bark was wounded to expose the cambium using a 4 mm cork borer, and discs of agar bearing mycelium taken from the margins of actively growing, 2-week-old cultures on 2% MEA (Tarigan et al. 2010; Tarigan et al. 2011; Chi et al. 2019a, Chi et al. 2020), *Ceratocystis* isolates were placed with the mycelium facing the cambium. Ten plants of each tree species were inoculated with sterile MEA plugs to serve as controls. All inoculation points and the ends of the logs were covered with masking tape and polyethylene films, respectively, to prevent desiccation of the inoculum and cambium, and to reduce contamination.

The nursery trial evaluated seven *Ceratocystis* strains isolated from *M. elengi* (CAME30813, CAME30814, CAME30815, CAME30816, CAME30817, CAME30818 and CAME30819) and one strain (CAW30814) from *A. mangium*. There were ten replicate plants per treatment in each row plot in each of the blocks. After 45 days, lesion (L) length and foliar symptoms severity were recorded. Representative wood samples were taken from within lesions outside the inoculation area, and the pathogen reisolated and sequenced for Koch's postulates test.

Foliar symptoms severity (FS) was assessed using a scale of 0 to 4: 0 = healthy; 1 = lower leaves yellow; 2 = slight wilting; 3 = severe wilting; 4 = dead (Muslim et al. 2003; Chi et al. 2019a).

The pathogenicity test data were analysed using SAS university edition software package. Analysis of variance (ANOVA) and Tukey's honestly significant difference (Tukey's HSD) test were used to determine whether there were significant differences in comparisons of means of different treatments.

RESULT AND DISCUSSION

Symptoms of *Mimusops elengi* wilt disease

We observed disease symptoms from September 2019 to April 2020, in various places that are widely planted in the Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) areas, South Sumatra, Indonesia. The disease was found scattered throughout the planted area, with symptoms of partially dead trees, foliar wilting or loss and tree death (Fig. 1a). Initially, the leaves of infected plants lost turgor and brightness, with yellowing symptoms in the older leaves, followed by wilting and death of the plant. Symptomatic plant stems showed xylem discoloration (Fig. 1b), infections generally started in the roots and then moved upwards in the stem ultimately reaching the upper branches of the entire plant, and the plants ultimately died (Fig. 1c). Death of adjacent plants indicates transmission of root infection because these pathogens are also known as soil-borne pathogens. The severity of the infection is also caused by pruning the branches using tools previously used to cut the infected plants.

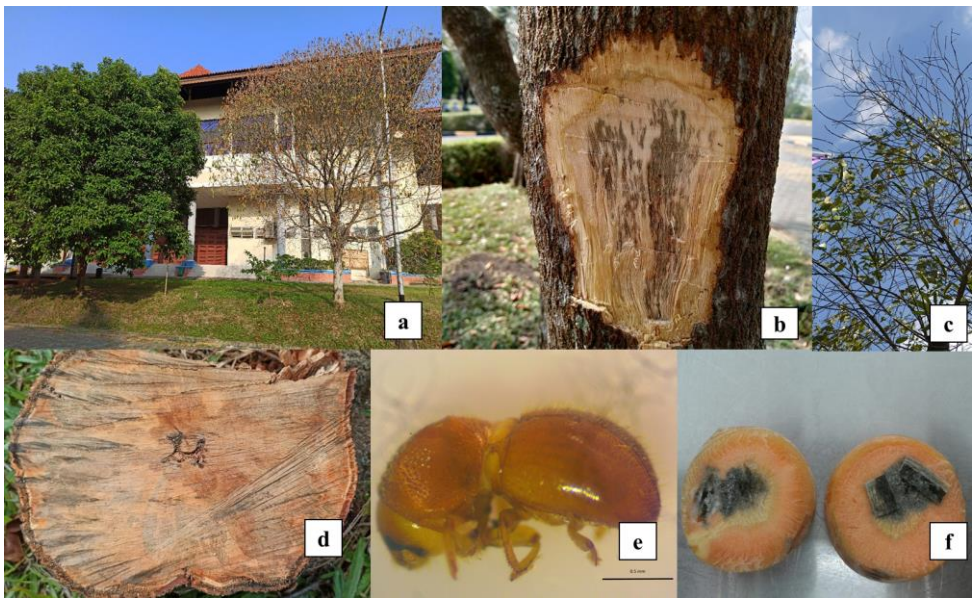

Figure 1. Symptoms of *Ceratocystis manginecans* wilt disease in bullet wood*:* **a**. tree death of *M. elengi*: b. sap stain mould on bullet wood, c. wilted leaves of bullet wood, d. sap stain mould on bullet wood, e. The bark beetle vector of *C. manginecans, Hypocryphalus mangiferae*, f. isolation of the fungus from discoloured xylem showing dark mycelium and sporulation on the carrot slices

Observation of diseased plant xylem tissue in cross-sections of the stem showed dark brown lesion formation in the cambium (inner bark region) towards vascular tissue (Fig. 1d). In the initial stage of plant infection, the foliar symptoms, wilt and the fruits appeared normal, but as the infection progressed, the fruits of affected plants were smaller, shrivelled, wrinkled and dry. Many of the bark beetle vectors of *C. manginecans*, *Hypocryphalus mangiferae* were found around bullet wood diseases (Fig. 1e). Testing by the Moller and DeVay method showed that *Ceratocystis* had grown on the carrots, and ascomata of *C. manginecans* with necks supporting sticky masses of ascospores on the carrot slices (Fig. 1f).

Sampling and isolation

Seven isolates of *C. manginecans* were collected from diseased bullet wood (*M. elengi*) (Fig. 2). There were three isolates (CAME30815, CAME30816 and CAME30817) from Ogan Ilir (Indralaya); two isolates (CAME30818 and

CAME30814) from Jakabaring (Palembang); and two isolates (CAME30819 and CAME30813) from Kayuagung (Ogan Komering Ilir). We also isolated one isolate (CAW30814) from diseased *acacia*, *A. mangium* in the agricultural field of Sriwijaya University, Indralaya.

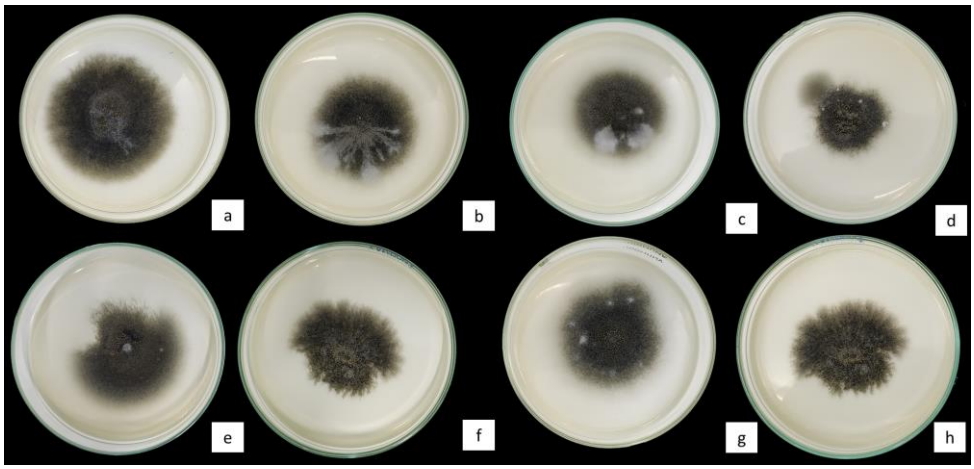

Figure 2. Isolates of *Ceratocystis manginecans* and related species grown on malt extract agar (MEA) for 7 d at 25 ºC. **a, b, c**: *Ceratocystis* CAME30815, CAME30816 and CAME30817, from *Mimusops elengi* in Sriwijaya University, Indralaya. **d, e, f:** *Ceratocystis* CAME30819, CAME30813 and CAME30814 from *Mimusops elengi* in Jakabaring, Palembang. **g**: *Ceratocystis* CAME30818, from *Mimusops elengi* in Kayuagung, Ogan Komering Ilir. **h**: *Ceratocystis* CAW30814, from *Acacia mangium* in Indralaya

Fungal morphology

Seven isolates were morphologically indistinguishable (Table 2). At 7–14 days of incubation at 25 ºC on MEA, cultures were pale brown to dark brown and produced a banana-like odour. Mycelium on MEA was grey, and the reverse side of the colony olivaceous grey; submerged mycelium darkened as the ascomata developed, forming fine, radiating fibrils. Ascomata developing within seven days and mature within ten days, superficially or partly embedded in the agar, dark brown to black (Fig. 3a). Ascomatal bases were submerged or on the agar surface, dark bases dark brown to black, base subglobes to globes, (134.58-) 169.12 - 276.29 (-310.83) µm long and (122.91-) 161.89-244.14 (-283.13) µm wide in diameter (Fig. 3a). Ascomata necks were erect, occasionally curved, black at the base becoming subhyaline towards the apex, smooth to crenulate, (346.51-) 454.94-720.16 (-828.59) µm long including ostiolar hyphae (Fig.3b). Ascospores were hat-shaped, (3.61-) 5.64-6.23 (-6.93) µm length and (2.06-) 2.279-3.67 (-3.85) µm width (Fig.3f). Barrel conidia (8.62-) 8.85-12.79 (-13.25) µm length and (5.89-) 4.12x6.87 (-8.67) µm width. Bacilliform conidia (9.05-) 10.82-22.32 (-35.97) µm length and (2.01-) 2.83-5.71 (-8.87) µm width (Fig.3c). Chlamydospores oval, thick-walled, smooth, (8.21-) 9.15-16.21 (-18.50) µm length and (4.92-) 6.46-15.81 (14.65) µm width (Fig.3e).

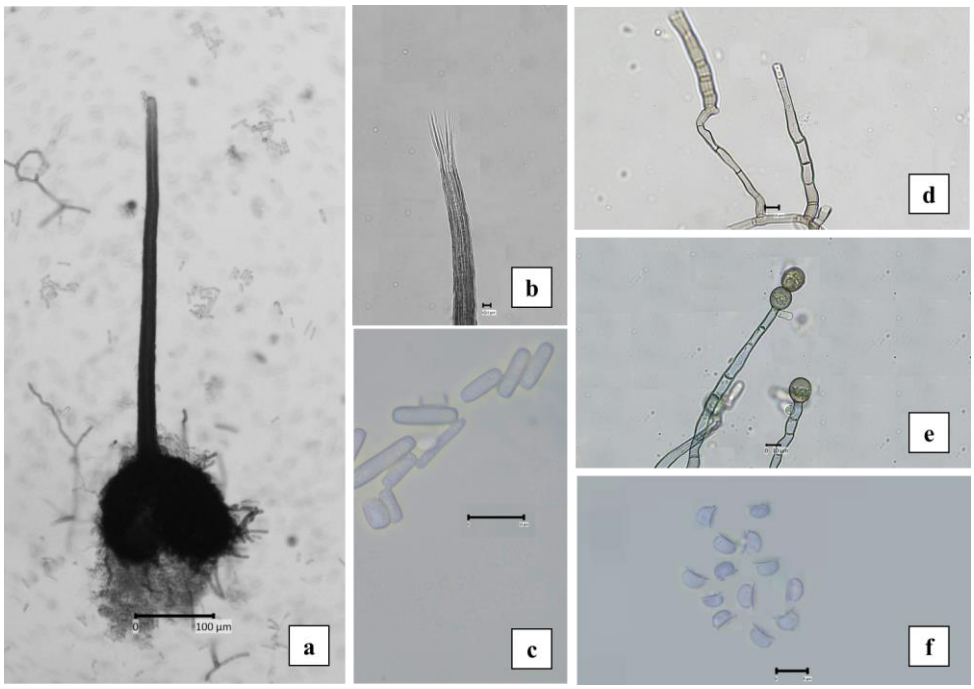

Figure 3. Morphological characteristics of *Ceratocystis* isolated from *M. elengi* stem lesion: a. globose ascomata with long neck, b. divergent ostiolar hyphae, c. barrel-shaped conidia, d. conidiophore/phialide, e. chlamydospores, f. hat-shaped ascospores. Scale bars: a = 100 µm; b,c,d,e = 10 µm; f = 5 µm

Sequence analysis

To confirm the identify of the wilt pathogen, the ITS and β-tubulin 1 gene sequences of two isolates from bullet wood (*M. elengi*) were compared to reference sequences downloaded from the gene bank database (NCBI GenBank) (table 1) and indicated that isolates from Indonesia were grouped within the *C. fimbriata*. s.l species complex and were most closely related to *C. manginecans*. PCR amplification resulted in fragments of ~550 base pairs (bp) in size which had 100% homology with *C. manginecans* (Fig. 4). Bootstrap values were equal to or greater than 50%, derived from 1000 iterations.

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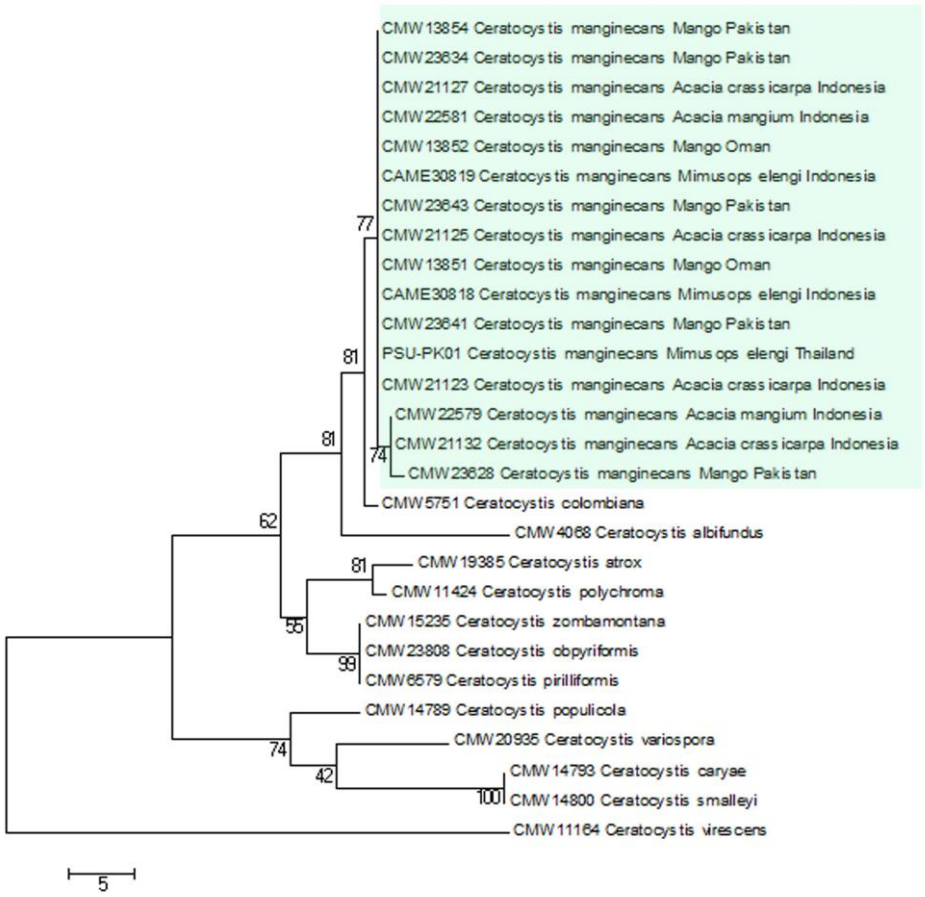

Figure 4. Phylogenetic tree constructed by MEGA with Maximum Parsimony (MP). The tree was built using combined sequence data of the ITS region and b-tubulin gene and their related species from GenBank. Consistency (CI), retention (RI), and composite indexes (CoI) are 0.714286, 0.837209, and 0.664843 for all sites and parsimony-informative sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Bootstrap values >50% are indicated above the branches. The analysis involved 28 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 493 positions in the final dataset

Table 1. *Ceratocystis* isolates considered in the phylogenetic analyses

Isolate no	Identify	Host	Geographic origin	Collector	Gene region/GeneBank accession no	
					ITS	BT
CAME30819	*C.manginecans*	*Mimusops elengi*	Indonesia	**R.Pratama**	MT373423	Submitted
CAME30818	*C.manginecans*	*Mimusops elengi*	Indonesia	**R.Pratama**	MT373424	Submitted
CMW13851	*C.manginecans*	*Mangifera indica*	Oman	M. Deadman	AY953383	EF433308
CMW23643	*C.manginecans*	*Mangifera indica*	Pakistan	A.Al-Adawi	EF433304	EF433313
CMW23641	*C.manginecans*	*Mangifera indica*	Pakistan	A.Al-Adawi	EF433305	EF433314
CMW23634	*C.manginecans*	*Mangifera indica*	Pakistan	A. Al-Adawi	EF433302	EF433311
CMW21125	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588663	EU588642
CMW21123	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588662	EU588641
CMW22581	*C.manginecans*	*Acacia mangium*	Indonesia	M. Tarigan	EU588659	EU604671
CMW21132	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588665	EU588644
CMW22579	*C.manginecans*	*Acacia mangium*	Indonesia	M. Tarigan	EU588658	EU588638
CMW23628	*C.manginecans*	*Hypocryphalus mangifera*	Pakistan	A. Al-Adawi	EF433303	EF433312
CMW13854	*C.manginecans*	*Mangifera indica*	Oman	M. Deadman	AY953385	EF433310
CMW13852	*C.manginecans*	*Hypocryphalus mangifera*	Oman	M. Deadman	AY953384	EF433309
CMW4068	*C.albifundus*	*A. mearnsii*	RSA	J. Roux	DQ520638	EF070429
CMW14793	*C.caryae*	*C. cordiformis*	U.S.A	J. Johnson	EF070424	EF070439
CMW14800 CBS114724	*C.smalleyi*	*C. cordiformis*	U.S.A	G. Smalley	EF070420	EF070436
CMW14789	*C. populicola*	*Populus sp.*	Poland	J. Gremmen	EF070418	EF070434
CMW19385 CBS115778	*C.atrox*	*E. grandis*	Australia	M.J. Wingfield	EF070415	EF070431
CMW11424	*C. polycroma*	*Syzygium aromaticum*	Indonesia	M.J. Wingfield	AY528970	AY528966
CMW23808	*C. obpyriformis*	*A. mearnsii*	South Africa	R.N. Heath	EU245003	EU244975
CMW6579	*C.pirilliformis*	*E. nitens*	Australia	M.J. Wingfield	AF427105	DQ371653

Table 2. Morphological comparisons of *C.manginecans* and other phylogenetically closely related species

Commented [A7]: *Ceratocystis manginecans*

Character	*Ceratocystis manginecans* (from *M. elengi*)	*Ceratocystis acaciivora* (from *A. mangium*)	*Ceratocystis manginecans* (from *A. mangium*)
Ascomata base	(134.58-) 169.12 - 276.29 (-310.83) x (122.91-) 161.89-244.14 (-283.13)[a]	(105-) 131-175 (-206) x (107-) 125-167 (-188)	(132.1-) 175.3 (−233.2)
Ascomata base average	220.01x211.63[b]		
Ascomata neck	(346.51-) 454.94-720.16 (-828.59)	(301-) 348-448 (-522)	(327.3-) 452.7 (−556)
Ascomata neck average	568.41		
Ascospores	(3.61-) 5.64-6.23 (-6.93) x (2.06-) 2.279-3.67 (-3.85)	5-7 x 3-4	(3.1-) 4.2 (−4.7) x (4.1-) 5.5 (−6.8)
Ascospores average	5.62 x 3.93		
Bacilliform conidia	(9.05-) 10.82-22.32 (-35.97) x (2.01-) 2.83-5.71 (-8.87)	(11-) 14-22 (-29) x 3-5	(14.6-) 18.6 (−30.7)x (3.0-) 4.6 (−5.4)
Bacilliform conidia average	16.56x4.27		
Barrel-shaped conidia	(8.62-) 8.85-12.79 (-13.25) x (5.89-) 4.12x6.87 (-8.67)	(8-) 9-11 (-13) x 4-6	(6.8) 8.1 (−10.6)
Barrel-shaped conidia average	11.497 x 15.82		
Clamydospore	(8.21-) 9.15-16.21 (-18.50) x (4.92-) 6.46-15.81 (14.65)	(10-) 12-14 (-15) x (7-) 8-12 (-14)	(10.1-) 13.1 (−15.5) x (6.1-) 9.2 (−11.1)
Clamydospore average	11.13 x 14.18		
Reference	This Study	M. Tarigan et al. 2010	Chi et al. 2019

All measurements are in µm

[a] Measurements are presented in the format [(minimum-) (average-standard deviation) - (average-standard deviation) (-maximum)]

[b] Measurements are presented in the format minimum x maximum

Pathogenicity

The results of the pathogenicity tests of seven isolates (CAME30813, CAME30814, CAME30815, CAME30816, CAME30817, CAME30818, and CAME30819) on *M. elengi* and one isolate (CAW30814) from *A. mangium* are shown in Table 3. All isolates tested showed a varying reaction to lesion and foliar symptoms (Fig. 5). For the pathogenicity test on *M. elengi*, seven isolates from *M. elengi* as well as one isolate from *A. mangium* strongly infected the wood and produced significant lesion length ranging from 3.99 to 10.47 cm and showed significant differences from the control. Three isolates from *M. elengi* (CAME30815; CAME30819; CAME30818) as well as one isolate from *A. mangium* (CAW30814) showed high pathogenicity on the foliar symptom (with foliar severity index of 2.3-3.6 and lesion length 6.52-10.47 cm), while the other isolates (CAME30817; CAME30816; CAME30814, CAME30813) showed moderate pathogenicity to *M. elengi* (foliar severity index of 1.4-2.0 and lesion length 3.99-6.02 cm). When the isolates were tested for their pathogenicity on *A. mangium* as the primary host of the pathogen, two isolates (CAME30815; CAME30819) showed strong pathogenicity on lesion length (13.76-11.89 cm) and also provided high pathogenicity on foliar severity index (4), where all plants tested were dead. Three isolates (CAW30814; CAME30818; CAME30817) showed moderate pathogenicity on lesion length 8.71-10.14 cm and foliar severity index 2.8-3.2, while the other isolates (CAME30816, CAME30814, CAME30813) showed low pathogenicity on lesion length 7.19-8.63 cm and foliar severity index 1.9-2.4.

Table 3. Pathogenicity of *Ceratocystis* isolates on *M. elengi* and *A. mangium* under nursery condition

Isolates	Host test	*M. elengi*		*A. mangium*	
		Lesion Length (cm)	Foliar symptoms	Lesion Length (cm)	Foliar symptoms
CAME30815	10	10.47e	3.6	13.77d	4
CAME30819	10	8.29de	3.1	11.89cd	4
CAW30814	10	7.35cd	2.8	10.14bcd	3.2
CAME30818	10	6.52bcd	2.3	9.92bcd	3.1
CAME30817	10	6.02bcd	2	8.72bc	2.8
CAME30816	10	5.27bc	1.8	8.47bc	2.4
CAME30814	10	4.93bc	1.5	8.64bc	2
CAME30813	10	3.99b	1.4	7.19b	1.9
Control (MEA)	10	0.1a	0	0.1a	0
Fpr		<0.001		<0.001	

Values followed by the same letters in a column are not different among isolates at P=0.05 according to Tukey's HSD multiple range test.

There was a strong linear correlation between foliar symptom severity and lesion length for *M. elengi* and *A. mangium* (Fig. 6). The Koch's postulates test was conducted by reisolating the pathogen, and its identity confirmed to be the same as *C. manginecans*.

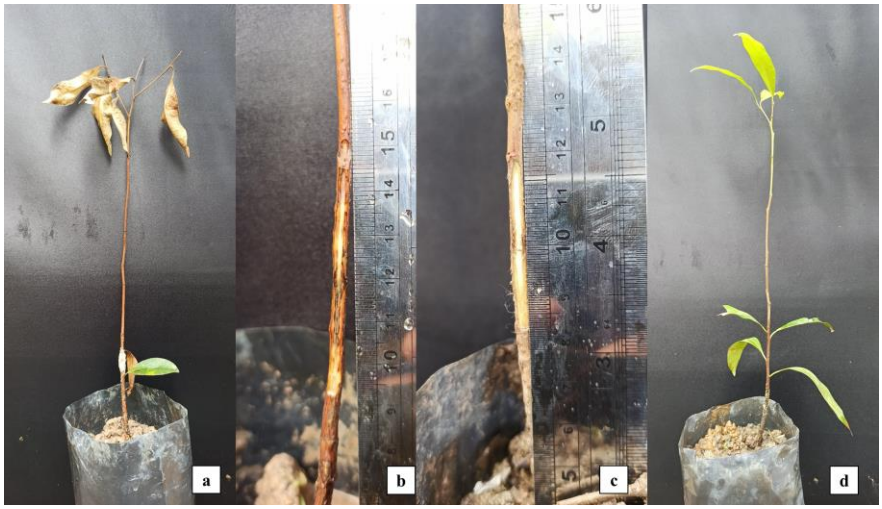

Figure 5. Response after 45 days of *M. elengi* seedlings to under-bark inoculation with mycelium of *Ceratocystis manginecans.* **a.** wilting of seedlings; **b**. lesions on the stem; c. no lesions on the stem; d. normal foliage on the control seedling which received MEA only and appeared healthy. Yellow arrow indicates the point of inoculation, red arrows show the lesion boundary

Commented [A8]: *Mimusops elengi and Acacia mangium*

Commented [A9]: Mimusops elengi

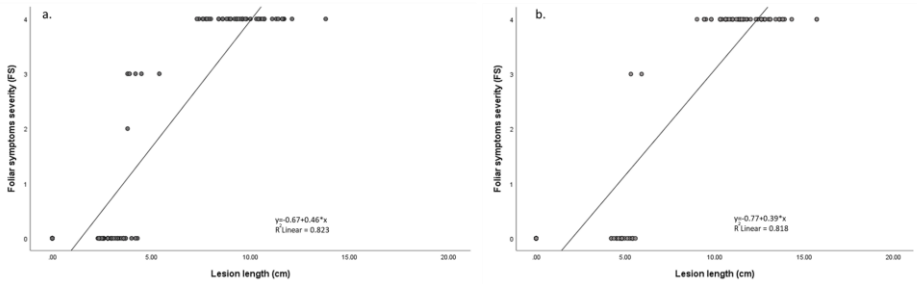

Figure 6. Correlation between foliar symptoms severity (FS) and lesion length (cm): **a.** *Mimusops elengi*, **b.** *Acacia mangium*

Discussion

The results of this study show clearly that a *C. manginecans* was responsible for the wilt outbreak disease that has recently appeared on the *M. elengi* agricultural field in Indralaya (Ogan Ilir) and roadside trees in Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir), South Sumatra. This is the first report of a *Ceratocystis* wilt disease on *M. elengi* in Indonesia. Formerly the disease was reported in Thailand (Pornsuriya and Sunpapao, 2015), however, they just observed a single tree and they did not conduct pathogenicity test and observe morphological characteristics. In Indonesia Tarigan et al. (2010; 2011) reported *C. manginecans* caused wilt and die-back disease in *A. mangium* plantations in Riau.

Mimusops elengi infected with *Ceratocystis* has wilted foliage symptoms, sudden wilt, decline and branch drying, and progressive loss of the canopy resulting in tree death. *M. elengi* trees showed typical symptoms of infection by the *Ceratocystis* fungus; the same was true of a serious wilt pathogen of *A. mangium* and *A. crassicarpa* in Indonesia and Vietnam (Tarigan et al. 2011; Thu et al. 2012; Thu et al. 2016). Vascular wilt, wood stain and stem cankers are the most characteristic symptoms of infection by *C. manginecans* in woody trees (Harrington 2013). Rot in roots or stems, vascular wilt, sapwood discoloration and cankers are symptomatic of plants infected by *C. manginecans* species (Kile 1993).

The comparison of morphological characteristics and gene sequences (β-tubulin and ITS) of the isolates examined in this study were similar to those in descriptions given for *C. manginecans* isolated from diseased *Acacia* trees which form part of the *C. fimbriata* s. l. complex, which is typified by *C. fimbriata* sensu stricto (Engelbrecht and Harrington 2005; Tarigan et al. 2011). The two isolates from Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) have 100% identical sequences of ITS and Beta Tubulin representing a single clone of the pathogen. This supports the view that *M. elengi* wilt disease in Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) emerged from a single introduction of a single haplotype and the population has expanded clonally to infect *M. elengi* trees in many parts of all three regions.

Ceratocystis manginecans was reported in Indonesia associated with wilt and die-back disease on *Acacia* spp. (Tarigan et al. 2011). It was found that many acacia plants were attacked by *Ceratocystis* disease and *Hypocryphalus mangifera* insects in the field raised the suspicion that *M. elengi* were infected by *Ceratocystis* in acacia plants. *H. mangifera* is a vector insect for the spread of *Ceratocystis* in the world (Rossetto et al. 1980; Al-Adawi et al. 2013; Van Wyk et al. 2007; Masood et al. 2008). Pruning *M. elengi* branches using equipment that has previously been infected with *Ceratocystis* also increases the severity of the disease; this can be seen by the number of plants that are attacked after pruning with tools used to prune *Acacia* plants that are attacked by *Ceratocystis*. Cankers of *Ceratocystis* were associated with branch wounds from pruning, pruning time and pruning technique on plants (Chi et al. 2019b). In this study, infected bullet wood was observed surrounding infected acacia by *C. manginecans* with abundant *H. mangifera*. This might be caused by spore contamination on cutting tools after pruning diseased acacia and spore spreading by insect vector *H. mangifera*.

The pathogenicity of *C. manginecans* to these hosts was demonstrated in inoculation trials and it is clear that the fungus is responsible for the widespread death of these trees. It was also possible to show that isolates of the pathogen from these trees are able to infect and kill other plants. The eight *C. manginecans* isolates all formed lesions on the stems of *M. elengi* seedlings when inoculated under the bark in nursery cloches. The most pathogenic was CAME30815 and CAME30819 isolate from *M. elengi* and the CAW30814 isolate from *A. mangium* resulted in foliar symptoms with severity index 3.6, 3.1 and 2.8 after 45 days from inoculation with pathogen. When the isolates were tested on seedling *A. mangium*, the seedlings were infected and mostly dead as they were the main host plant and susceptible to *C. manginecans* (Tarigan et al. 2011).

Previous researchers of *C. manginecans* on *M. elengi* in Thailand have reported molecular identification *C. manginecans* by primers combination the ITS, β-tubulin (βt) and transcribed elongation factor 1-α (TEF1-α) gene regions with one isolate. In our research, we tested two isolates for sequencing, seven isolates for pathogenicity, and Koch's postulates assay using three isolates (CAME30815, CAME30816 and CAME30817) from Ogan Ilir (Indralaya); two isolates (CAME30818 and CAME30814) from Jakabaring (Palembang); and two isolates (CAME30819 and CAME30813) from Kayuagung (Ogan Komering Ilir) and one isolate (CAW30814) from diseased *Acacia*, *A. mangium*.

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All isolates showed the ability to infect both bullet wood and *Acacia*, and all isolates can be reisolated confirming Koch's postulates.

The wilt disease of *M. elengi* appears to be serious and it is clearly a new host tree or pathogen association that has apparently occurred due to a host shift. This category of diseases is increasing in importance in plants, and they can devastate native trees that have not previously encountered them (Roy 2001; Anderson et al. 2004; Slippers et al. 2005; Woolhouse et al. 2005; Desprez-Loustau et al. 2007; Wingfield et al. 2010). In this regard, the wilt disease of *M. elengi* can impact seriously on the natural biodiversity of Indonesia, and studies should be instituted to understand them better. The findings of this study will give knowledge of the characteristics of the symptoms of the disease, their causes and help to minimise the spread of wilt disease in *M. elengi* in plantations or roadside trees and to consider its possible pathogenicity.

This study presents the first report of *Ceratocystis* wilt or sudden decline disease of bullet wood in Indonesia and the discovery of a fungus that has been identified as *C. manginecans.* The disease of bullet wood that gave rise to this study is serious and management options to reduce its incidence are required *C. manginecans* is an aggressive pathogen and a deeper understanding of its role in tree death will be important in the future.

ACKNOWLEDGEMENT

This research was funded by a PMDSU scholarship in the fiscal year of 2019-2021 according to the Director of Research and Community Service, Directorate of Research and Community Service (DRPM), Directorate General for Research and Development, Ministry of Research, Technology, and Higher Education, Indonesia Number: 068/SP2H/AMD/LT/DRPM/2020 chaired by Ahmad Muslim.

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REVIEWER 2

First report of characterisation and pathogenicity of bullet wood (*Mimusops elengi*) sudden decline disease by *Ceratocystis* in Indonesia

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Abstract. *Ceratocystis manginecans* causes wilt and death of plants in several important crops and native vegetation in Indonesia. Ceratocystis wilt was recently found to be causing substantial mortality in bullet wood (*Mimusops elengi*) in South Sumatra. The aim of this study was to describe the symptomatology of the new disease and characterise isolates of *C. manginecans* obtained from bullet wood plants. Affected plants showed substantial discoloration of the woody xylem and wilt-type symptoms of the foliage, with the eventual death of the whole plant. Isolations from infected trees yielded a fungus that was similar morphologically to *C. manginecans*, with typical hat-shaped ascospores and light-coloured perithecial bases. Sequencing of the internal transcribed spacer (ITS) and β-tubulin of isolates confirmed their identification, grouping them with *C. manginecans* and separating them from all other *Ceratocystis* species. This is the first report of *C. manginecans* in Indonesia causing wilt and death on bullet wood. *C. manginecans* is an important pathogen, and strategies to reduce losses need to be established in Indonesia because the aggressiveness of *C. manginecans* to bullet wood has been shown in inoculation experiments

Keyword: Ceratocystidaceae, Molecular phylogeny, Pathogenicity, Sapotaceae

INTRODUCTION

Bullet wood (*Mimusops elengi*) belongs to the family Sapotaceae, common English names are Asian Bulletwood, Bullet Wood Tree, Indian Medlar, Red Coondoo Spanish Cherry and it is known in Indonesia as Tanjung. The species is native to India, Sri Lanka, the Andaman Islands, Myanmar, Indo-China, Peninsular Malaysia and Vanuatu; it has been introduced and cultivated elsewhere. *M. elengi* can grow in tropical and subtropical climates. This plant thrives in areas with high humidity and seasonal rainfall and seasonal dry periods (Lim, 2012). The bullet wood trees range from small to large, and are found in all parts of Indonesia where bullet wood is cultivated in gardens as an ornamental tree, for medicines and planted along avenues because of its fragrant flowers (Seth, 2003).

M. elengi is widely used for medicine, and various parts of *M. elengi* Linn. (Sapotaceae) have been used widely in traditional Indian medicine for the treatment of pain, inflammation and wounds. *M. elengi* stem bark would be a possible therapeutic candidate having cytotoxic and anti-tumour potential (Kumar et al. 2014); it also has antibacterial and antifungal uses (Ali et al. 2008). At present, *M. elengi* is used as the synthesis of calcium phosphate nanoparticles that is easy, eco-friendly and scalable (Pokale et al. 2014).

Several types of pathogenic fungi have been identified to cause disease in *M. elengi* plants. *Curvularia lunata* caused die-back in India (Khatun et al. 2011); *Pestalotiopsis clavispora* caused leaf blight (Lokesh et al. 2017). *Ceratocystis* was first isolated from a single tree of bullet wood showing sudden decline in Thailand. Symptoms displayed by the diseased trees include gum exudation from the trunks and wilting and loss of the dark green foliage with a corresponding browning of leaves on single branches. In this study they did not confirm the pathogen with a Koch postulates test detail (Pornsuriya and Sunpapao, 2015). Recently we have observed many bullet wood trees showing similar symptoms with *C. manginecans* decline in many locations in South Sumatra.

C. manginecans includes many economically important plant pathogens. This pathogen has caused a sudden decline and has led to the death of thousands of *Mangifera indica* trees in Oman with *Hypocryphalus mangifera* vector (Al Adawi et al. 2013). In Indonesia *C. manginecans* caused die-back on *Acacia mangium* and *A. crassicarpa* plantations in Riau (Tarigan et al. 2010), whereas in Vietnam recently, *C. manginecans* caused wilt disease in *Dalbergia tonkinensis* and *Chukrasia tabularis* (Chi et al. 2019a; Chi et al. 2020); in Pakistan this pathogen also causes wilt disease in *Albizia lebbeck* (Razzaq et al. 2020). Commonly *C. manginecans* causes yellowing of leaves and rapid wilting of leaves was observed on individual branches in affected trees that ultimately spread to the canopy followed by the death of the whole tree. Dark brown to black tissue discoloration was observed in the woody xylem tissues of infected trees.

This study aimed to identify the cause of a new outbreak of wilt disease causing a sudden decline to the trees, wilted canopies and tree death in *M. elengi* in South Sumatera, Indonesia. This study was also conducted to describe the characteristics of the pathogen and confirm Koch's postulates test.

MATERIALS AND METHODS

Disease symptoms and specimen collection

The distribution and impact of the *C. manginecans* disease on *M. elengi* were determined from roadside trees planting in Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) and the agricultural field of Sriwijaya University in Indralaya (Ogan Ilir). Symptoms of wilt diseases were evaluated as follows: the extent of lesion development from discoloration of bark and wood, the extent of foliar wilting or loss and tree death.

Samples of diseased trunks were collected from two to six-year-old trees in September 2019 to April 2020. Wood samples were taken from lesions of wilted trees using a knife sterilised in 70% ethanol. The wood samples collected from *M. elengi* showed brown to black streaking in the woody xylem. Each sample was wrapped in tissue paper and placed in a coolbox. The same day, the wood samples (1–20 mm length, 1–2 mm thick) were sandwiched between two slices of fresh carrot and placed on sterile dry paper in plastic boxes at 25 °C following the method of Moller and DeVay (1968). After 5–10 days, hat-shaped spores of putative *Ceratocystis* pathogens were placed on 2% (w/v) malt extract agar (MEA) (Merck, Germany), and incubated at 25 °C in a laboratory. When cultures had grown to several cm in diameter, hyphal tips were sub-cultured onto new MEA and potato dextrose agar (PDA) (Merck, Germany) plates and incubated at 25–28 °C. Morphological traits of fruiting bodies and spores were observed under an optical Olympus CX33 microscope.

Genomic DNA extraction, PCR amplification, and sequencing

DNA isolation used YeaStar Genomic DNA Kit (Zymo Research Corporation, California, USA). To extract genomic DNA, cultures were incubated for five days to allow sufficient mycelial growth in potato dextrose broth (PDB) (Merck, Germany). Mycelium was purified with sterile filter paper (Whatman) and transferred to 1.5 mL Eppendorf tubes. The quantity and quality of DNA extracted was evaluated with a spectrophotometer (NanoDrop ND-1000; Thermo Fisher, Waltham, MA, USA) to calibrate the concentration and purity of DNA as PCR templates.

The PCR amplification reactions were conducted on a T-100 thermal cycler (Bio-Rad, Hercules, CA, USA). Amplifications were carried out in 50 µl reactions containing 20 µl DreamTaq Green PCR Master Mix (Eppendorf, Germany) (DreamTaq DNA Polymerase, 2X DreamTaq Green buffer, dNTPs, and 4 mM $MgCl_2$), 1,5 µl of each forward and reverse primer, 4 µl of DNA template and 23 µl sterilised water. The PCRs were performed with a C1000 Touch™ thermal cycler (Bio-Rad, USA). The PCR cycling parameters were as follows: initial denaturation for 5 min at 95 °C, followed by 35 cycles at 95 °C for 30 s, 56 °C for 45 s and 72 °C for 1 min. Amplification was completed at 72 °C for 10 min and the PCR product was stored at 10 °C.

PCR amplifications were made for two gene regions, including part of the b-tubulin (BT) using primers βt1a (TTCCCCCGTCTCCACTTCTTCATG) and βt1b (GACGAGATCGTTCA TGTTGAACTC) (Glass and Donaldson 1995), and the ITS using ITS1 and ITS4. The resulting PCR products were submitted to 1st BASE (Malaysia) for forward and reverse sequencing reactions. Raw sequence data were assembled, examined, and manually edited using Genestudio 2.1.1.5 (Genestudio, Suwanee, Georgia) and BioEdit software (Hall 1999). The DNA sequences were compared to the GenBank database via the nucleotide-nucleotide BLAST search interface located at the National Center for Biotechnology Information, Bethesda, USA. Relevant sequences were transferred with NoteTab Light v7.2.

Phylogenetic analyses

The sequences of *Ceratocystis* spp. closely related to the one from *Mimusops elengi* were retrieved from GenBank. Phylogenetic sequences from different gene regions were aligned using Mesquite v3.5 (Maddison and Maddison 2018) (http://mesquiteproject.org) and corrected manually. Phylogenetic trees based on a concatenated data set of the ITS and βt were computed and analysed as a single dataset. Maximum Parsimony (MP) analyses were performed in MEGA v. 10 (Kumar et al. 2016; Paul et al. 2018) with 1000 bootstrap replications.

Pathogenicity tests

Pathogenicity studies were conducted on two agroforestry plants, *A. mangium* and *M. elengi*. Plants had stem diameters of 2–3 cm and heights <1 m. The pathogenic potential of isolates was evaluated by the under bark inoculation method described by O'Gara et al. (1997). Bark was wounded to expose the cambium using a 4 mm cork borer, and discs of agar bearing mycelium taken from the margins of actively growing, 2-week-old cultures on 2% MEA (Tarigan et al. 2010; Tarigan et al. 2011; Chi et al. 2019a, Chi et al. 2020), *Ceratocystis* isolates were placed with the mycelium facing the cambium. Ten plants of each tree species were inoculated with sterile MEA plugs to serve as controls. All inoculation points and the ends of the logs were covered with masking tape and polyethylene films, respectively, to prevent desiccation of the inoculum and cambium, and to reduce contamination.

The nursery trial evaluated seven *Ceratocystis* strains isolated from *M. elengi* (CAME30813, CAME30814, CAME30815, CAME30816, CAME30817, CAME30818 and CAME30819) and one strain (CAW30814) from *A. mangium*. There were ten replicate plants per treatment in each row plot in each of the blocks. After 45 days, lesion (L) length and foliar symptoms severity were recorded. Representative wood samples were taken from within lesions outside the inoculation area, and the pathogen reisolated and sequenced for Koch's postulates test.

Foliar symptoms severity (FS) was assessed using a scale of 0 to 4: 0 = healthy; 1 = lower leaves yellow; 2 = slight wilting; 3 = severe wilting; 4 = dead (Muslim et al. 2003; Chi et al. 2019a).

The pathogenicity test data were analysed using SAS university edition software package. Analysis of variance (ANOVA) and Tukey's honestly significant difference (Tukey's HSD) test were used to determine whether there were significant differences in comparisons of means of different treatments.

RESULT AND DISCUSSION

Symptoms of *Mimusops elengi* wilt disease

We observed disease symptoms from September 2019 to April 2020, in various places that are widely planted in the Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) areas. The disease was found scattered throughout the planted area, with symptoms of partially dead trees, foliar wilting or loss and tree death (Fig. 1a). Initially, the leaves of infected plants lost turgor and brightness, with yellowing symptoms in the older leaves, followed by wilting and death of the plant. Symptomatic plant stems showed xylem discoloration (Fig. 1b), infections generally started in the roots and then moved upwards in the stem ultimately reaching the upper branches of the entire plant, and the plants ultimately died (Fig. 1c). Death of adjacent plants indicates transmission of root infection because these pathogens are also known as soil-borne pathogens. The severity of the infection is also caused by pruning the branches using tools previously used to cut the infected plants.

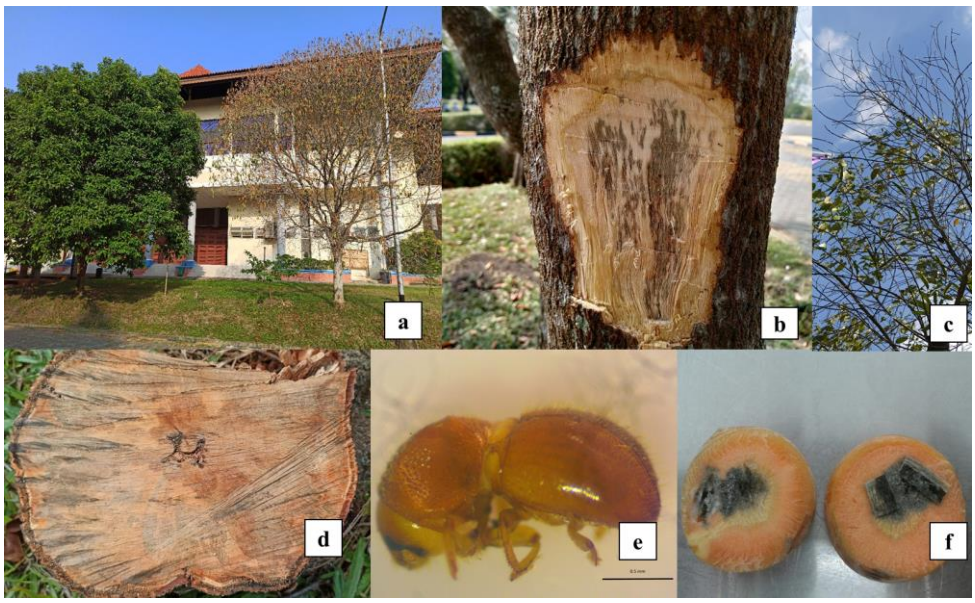

Figure 1. Symptoms of *Ceratocystis manginecans* wilt disease in bullet wood*:* **a**. tree death of *M. elengi*: b. sap stain mould on bullet wood, c. wilted leaves of bullet wood, d. sap stain mould on bullet wood, e. The bark beetle vector of *C. manginecans, Hypocryphalus mangiferae*, f. isolation of the fungus from discoloured xylem showing dark mycelium and sporulation on the carrot slices

Observation of diseased plant xylem tissue in cross-sections of the stem showed dark brown lesion formation in the cambium (inner bark region) towards vascular tissue (Fig. 1d). In the initial stage of plant infection, the foliar symptoms, wilt and the fruits appeared normal, but as the infection progressed, the fruits of affected plants were smaller, shrivelled, wrinkled and dry. Many of the bark beetle vectors of *C. manginecans*, *Hypocryphalus mangiferae* were found around bullet wood diseases (Fig. 1e). Testing by the Moller and DeVay method showed that *Ceratocystis* had grown on the carrots, and ascomata of *C. manginecans* with necks supporting sticky masses of ascospores on the carrot slices (Fig. 1f).

Sampling and isolation

Seven isolates of *C. manginecans* were collected from diseased bullet wood (*M. elengi*) (Fig. 2). There were three isolates (CAME30815, CAME30816 and CAME30817) from Ogan Ilir (Indralaya); two isolates (CAME30818 and

CAME30814) from Jakabaring (Palembang); and two isolates (CAME30819 and CAME30813) from Kayuagung (Ogan Komering Ilir). We also isolated one isolate (CAW30814) from diseased *acacia*, *A. mangium* in the agricultural field of Sriwijaya University, Indralaya.

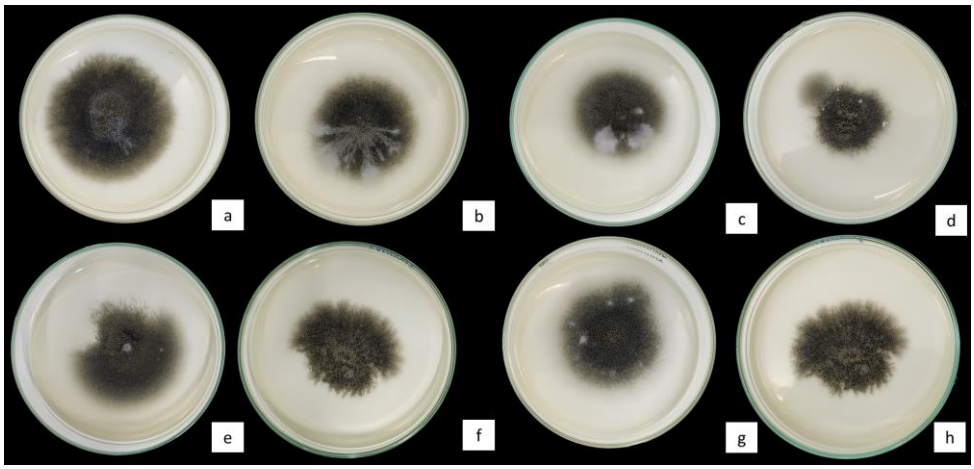

Figure 2. Isolates of *Ceratocystis manginecans* and related species grown on malt extract agar (MEA) for 7 d at 25 ºC. **a, b, c**: *Ceratocystis* CAME30815, CAME30816 and CAME30817, from *Mimusops elengi* in Sriwijaya University, Indralaya. **d, e, f:** *Ceratocystis* CAME30819, CAME30813 and CAME30814 from *Mimusops elengi* in Jakabaring, Palembang. **g**: *Ceratocystis* CAME30818, from *Mimusops elengi* in Kayuagung, Ogan Komering Ilir. **h**: *Ceratocystis* CAW30814, from *Acacia mangium* in Indralaya

Fungal morphology

Seven isolates were morphologically indistinguishable (Table 2). At 7–14 days of incubation at 25 ºC on MEA, cultures were pale brown to dark brown and produced a banana-like odour. Mycelium on MEA was grey, and the reverse side of the colony olivaceous grey; submerged mycelium darkened as the ascomata developed, forming fine, radiating fibrils. Ascomata developing within seven days and mature within ten days, superficially or partly embedded in the agar, dark brown to black (Fig. 3a). Ascomatal bases were submerged or on the agar surface, dark bases dark brown to black, base subglobes to globes, (134.58-) 169.12 - 276.29 (-310.83) µm long and (122.91-) 161.89-244.14 (-283.13) µm wide in diameter (Fig. 3a). Ascomata necks were erect, occasionally curved, black at the base becoming subhyaline towards the apex, smooth to crenulate, (346.51-) 454.94-720.16 (-828.59) µm long including ostiolar hyphae (Fig.3b). Ascospores were hat-shaped, (3.61-) 5.64-6.23 (-6.93) µm length and (2.06-) 2.279-3.67 (-3.85) µm width (Fig.3f). Barrel conidia (8.62-) 8.85-12.79 (-13.25) µm length and (5.89-) 4.12x6.87 (-8.67) µm width. Bacilliform conidia (9.05-) 10.82-22.32 (-35.97) µm length and (2.01-) 2.83-5.71 (-8.87) µm width (Fig.3c). Chlamydospores oval, thick-walled, smooth, (8.21-) 9.15-16.21 (-18.50) µm length and (4.92-) 6.46-15.81 (14.65) µm width (Fig.3e).

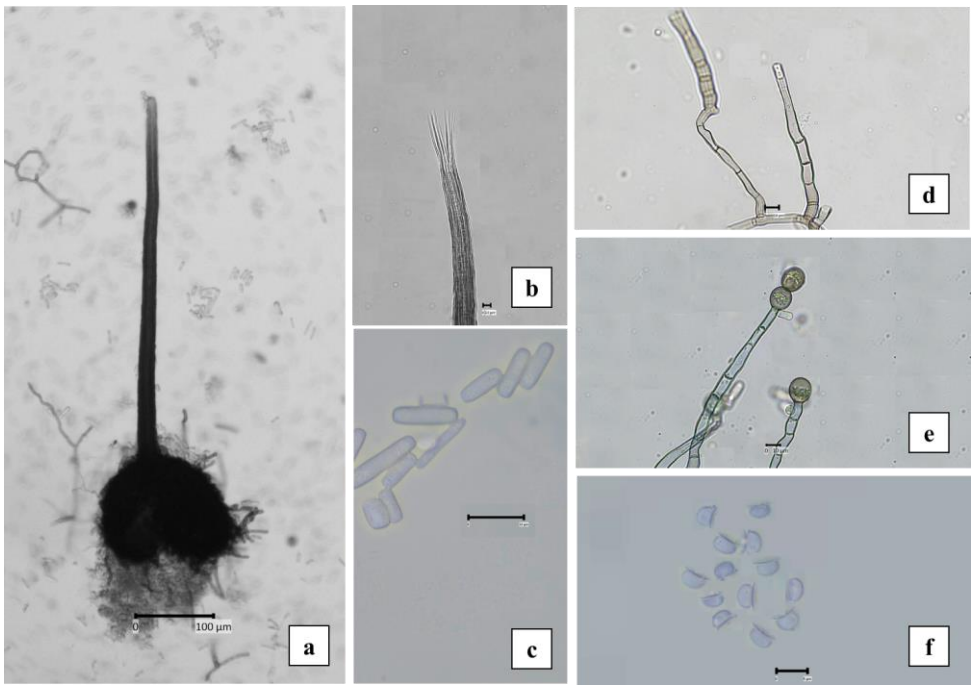

Figure 3. Morphological characteristics of *Ceratocystis* isolated from *M. elengi* stem lesion: a. globose ascomata with long neck, b. divergent ostiolar hyphae, c. barrel-shaped conidia, d. conidiophore/phialide, e. chlamydospores, f. hat-shaped ascospores. Scale bars: a = 100 µm; b,c,d,e = 10 µm; f = 5 µm

Sequence analysis

To confirm the identify of the wilt pathogen, the ITS and β-tubulin 1 gene sequences of two isolates from bullet wood (*M. elengi*) were compared to reference sequences downloaded from the gene bank database (NCBI GenBank) (table 1) and indicated that isolates from Indonesia were grouped within the *C. fimbriata*. s.l species complex and were most closely related to *C. manginecans*. PCR amplification resulted in fragments of ~550 base pairs (bp) in size which had 100% homology with *C. manginecans* (Fig. 4). Bootstrap values were equal to or greater than 50%, derived from 1000 iterations.

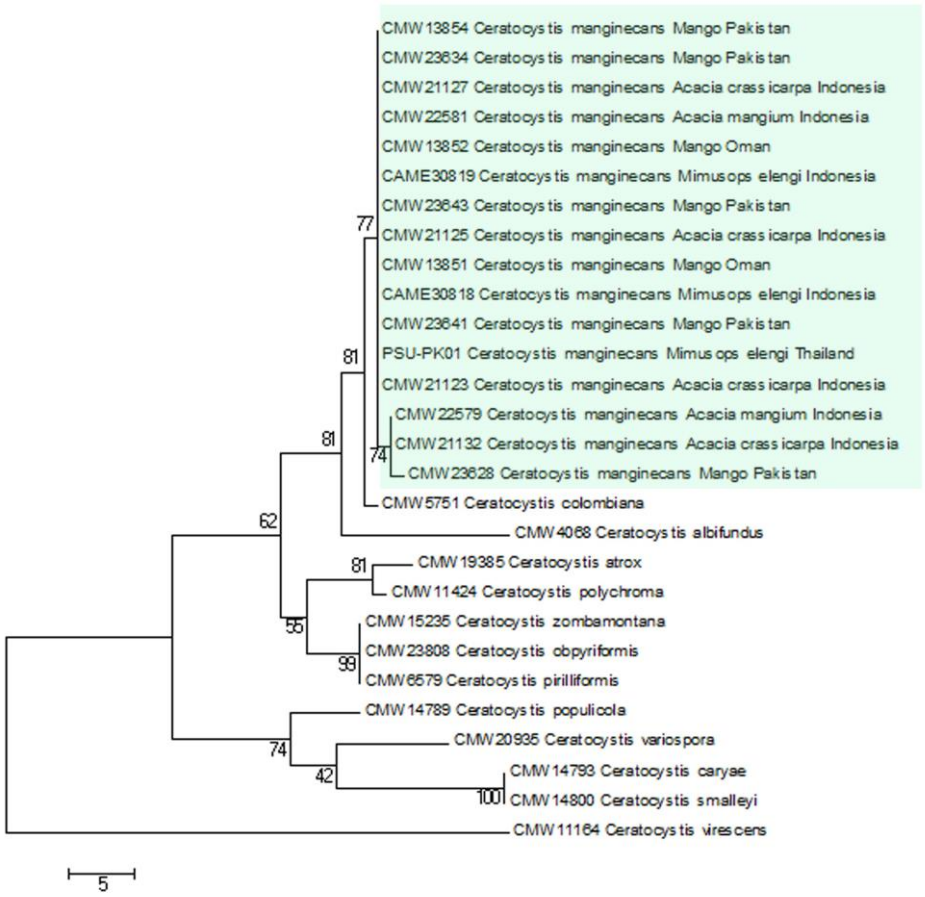

Figure 4. Phylogenetic tree constructed by MEGA with Maximum Parsimony (MP). The tree was built using combined sequence data of the ITS region and b-tubulin gene and their related species from GenBank. Consistency (CI), retention (RI), and composite indexes (CoI) are 0.714286, 0.837209, and 0.664843 for all sites and parsimony-informative sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Bootstrap values >50% are indicated above the branches. The analysis involved 28 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 493 positions in the final dataset

175 **Table 1.** *Ceratocystis* isolates considered in the phylogenetic analyses

Isolate no	Identify	Host	Geographic origin	Collector	Gene region/GeneBank accession no	
					ITS	BT
CAME30819	*C.manginecans*	*Mimusops elengi*	Indonesia	**R.Pratama**	MT373423	Submitted
CAME30818	*C.manginecans*	*Mimusops elengi*	Indonesia	**R.Pratama**	MT373424	Submitted
CMW13851	*C.manginecans*	*Mangifera indica*	Oman	M. Deadman	AY953383	EF433308
CMW23643	*C.manginecans*	*Mangifera indica*	Pakistan	A.Al-Adawi	EF433304	EF433313
CMW23641	*C.manginecans*	*Mangifera indica*	Pakistan	A.Al-Adawi	EF433305	EF433314
CMW23634	*C.manginecans*	*Mangifera indica*	Pakistan	A. Al-Adawi	EF433302	EF433311
CMW21125	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588663	EU588642
CMW21123	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588662	EU588641
CMW22581	*C.manginecans*	*Acacia mangium*	Indonesia	M. Tarigan	EU588659	EU604671
CMW21132	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588665	EU588644
CMW22579	*C.manginecans*	*Acacia mangium*	Indonesia	M. Tarigan	EU588658	EU588638
CMW23628	*C.manginecans*	*Hypocryphalus mangifera*	Pakistan	A. Al-Adawi	EF433303	EF433312
CMW13854	*C.manginecans*	*Mangifera indica*	Oman	M. Deadman	AY953385	EF433310
CMW13852	*C.manginecans*	*Hypocryphalus mangifera*	Oman	M. Deadman	AY953384	EF433309
CMW4068	*C.albifundus*	*A. mearnsii*	RSA	J. Roux	DQ520638	EF070429
CMW14793	*C.caryae*	*C. cordiformis*	U.S.A	J. Johnson	EF070424	EF070439
CMW14800 CBS114724	*C.smalleyi*	*C. cordiformis*	U.S.A	G. Smalley	EF070420	EF070436
CMW14789	*C. populicola*	*Populus sp.*	Poland	J. Gremmen	EF070418	EF070434
CMW19385 CBS115778	*C.atrox*	*E. grandis*	Australia	M.J. Wingfield	EF070415	EF070431
CMW11424	*C. polycroma*	*Syzygium aromaticum*	Indonesia	M.J. Wingfield	AY528970	AY528966
CMW23808	*C. obpyriformis*	*A. mearnsii*	South Africa	R.N. Heath	EU245003	EU244975
CMW6579	*C.pirilliformis*	*E. nitens*	Australia	M.J. Wingfield	AF427105	DQ371653

176 **Table 2.** Morphological comparisons of *C.manginecans* and other phylogenetically closely related species

Character	*Ceratocystis manginecans* (from *M. elengi*)	*Ceratocystis acaciivora* (from *A. mangium*)	*Ceratocystis manginecans* (from *A. mangium*)
Ascomata base	(134.58-) 169.12 - 276.29 (-310.83) x (122.91-) 161.89-244.14 (-283.13)[a]	(105-) 131-175 (-206) x (107-) 125-167 (-188)	(132.1-) 175.3 (−233.2)
Ascomata base average	220.01x211.63[b]		
Ascomata neck	(346.51-) 454.94-720.16 (-828.59)	(301-) 348-448 (-522)	(327.3-) 452.7 (−556)
Ascomata neck average	568.41		
Ascospores	(3.61-) 5.64-6.23 (-6.93) x (2.06-) 2.279-3.67 (-3.85)	5-7 x 3-4	(3.1-) 4.2 (−4.7) x (4.1-) 5.5 (−6.8)
Ascospores average	5.62 x 3.93		
Bacilliform conidia	(9.05-) 10.82-22.32 (-35.97) x (2.01-) 2.83-5.71 (-8.87)	(11-) 14-22 (-29) x 3-5	(14.6-) 18.6 (−30.7)x (3.0-) 4.6 (−5.4)
Bacilliform conidia average	16.56x4.27		
Barrel-shaped conidia	(8.62-) 8.85-12.79 (-13.25) x (5.89-) 4.12x6.87 (-8.67)	(8-) 9-11 (-13) x 4-6	(6.8) 8.1 (−10.6)
Barrel-shaped conidia average	11.497 x 15.82		
Clamydospore	(8.21-) 9.15-16.21 (-18.50) x (4.92-) 6.46-15.81 (14.65)	(10-) 12-14 (-15) x (7-) 8-12 (-14)	(10.1-) 13.1 (−15.5) x (6.1-) 9.2 (−11.1)
Clamydospore average	11.13 x 14.18		
Reference	This Study	M. Tarigan et al. 2010	Chi et al. 2019

177 All measurements are in µm
178 [a] Measurements are presented in the format [(minimum-) (average-standard deviation) - (average-standard deviation) (-maximum)]
179 [b] Measurements are presented in the format minimum x maximum

Pathogenicity

The results of the pathogenicity tests of seven isolates (CAME30813, CAME30814, CAME30815, CAME30816, CAME30817, CAME30818, and CAME30819) on *M. elengi* and one isolate (CAW30814) from *A. mangium* are shown in Table 3. All isolates tested showed a varying reaction to lesion and foliar symptoms (Fig. 5). For the pathogenicity test on *M. elengi*, seven isolates from *M. elengi* as well as one isolate from *A. mangium* strongly infected the wood and produced significant lesion length ranging from 3.99 to 10.47 cm and showed significant differences from the control. Three isolates from *M. elengi* (CAME30815; CAME30819; CAME30818) as well as one isolate from *A. mangium* (CAW30814) showed high pathogenicity on the foliar symptom (with foliar severity index of 2.3-3.6 and lesion length 6.52-10.47 cm), while the other isolates (CAME30817; CAME30816; CAME30814, CAME30813) showed moderate pathogenicity to *M. elengi* (foliar severity index of 1.4-2.0 and lesion length 3.99-6.02 cm). When the isolates were tested for their pathogenicity on *A. mangium* as the primary host of the pathogen, two isolates (CAME30815; CAME30819) showed strong pathogenicity on lesion length (13.76-11.89 cm) and also provided high pathogenicity on foliar severity index (4), where all plants tested were dead. Three isolates (CAW30814; CAME30818; CAME30817) showed moderate pathogenicity on lesion length 8.71-10.14 cm and foliar severity index 2.8-3.2, while the other isolates (CAME30816, CAME30814, CAME30813) showed low pathogenicity on lesion length 7.19-8.63 cm and foliar severity index 1.9-2.4.

Table 3. Pathogenicity of *Ceratocystis* isolates on *M. elengi* and *A. mangium* under nursery condition

Isolates	Host test	*M. elengi*		*A. mangium*	
		Lesion Length (cm)	Foliar symptoms	Lesion Length (cm)	Foliar symptoms
CAME30815	10	10.47e	3.6	13.77d	4
CAME30819	10	8.29de	3.1	11.89cd	4
CAW30814	10	7.35cd	2.8	10.14bcd	3.2
CAME30818	10	6.52bcd	2.3	9.92bcd	3.1
CAME30817	10	6.02bcd	2	8.72bc	2.8
CAME30816	10	5.27bc	1.8	8.47bc	2.4
CAME30814	10	4.93bc	1.5	8.64bc	2
CAME30813	10	3.99b	1.4	7.19b	1.9
Control (MEA)	10	0.1a	0	0.1a	0
Fpr		<0.001		<0.001	

Values followed by the same letters in a column are not different among isolates at P=0.05 according to Tukey's HSD multiple range test.

There was a strong linear correlation between foliar symptom severity and lesion length for *M. elengi* and *A. mangium* (Fig. 6). The Koch's postulates test was conducted by reisolating the pathogen, and its identity confirmed to be the same as *C. manginecans*.

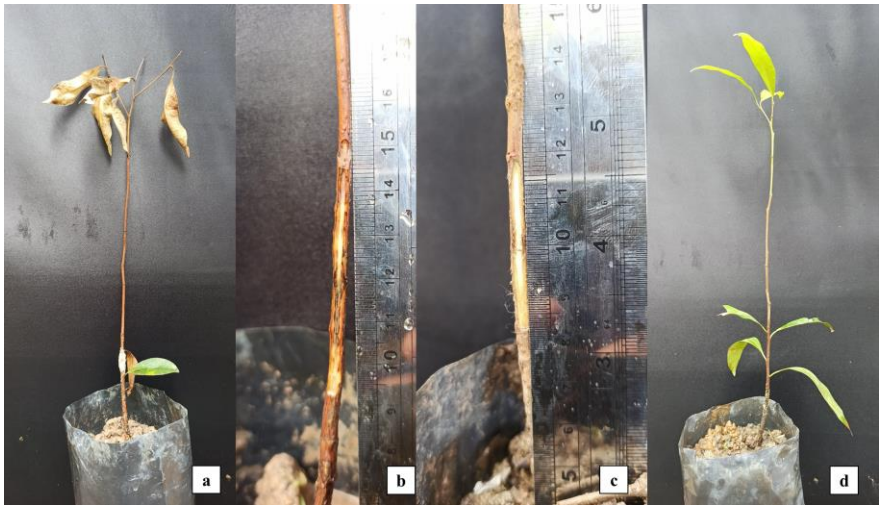

Figure 5. Response after 45 days of *M. elengi* seedlings to under-bark inoculation with mycelium of *Ceratocystis manginecans.* **a.** wilting of seedlings; **b**. lesions on the stem; c. no lesions on the stem; d. normal foliage on the control seedling which received MEA only and appeared healthy. Yellow arrow indicates the point of inoculation, red arrows show the lesion boundary

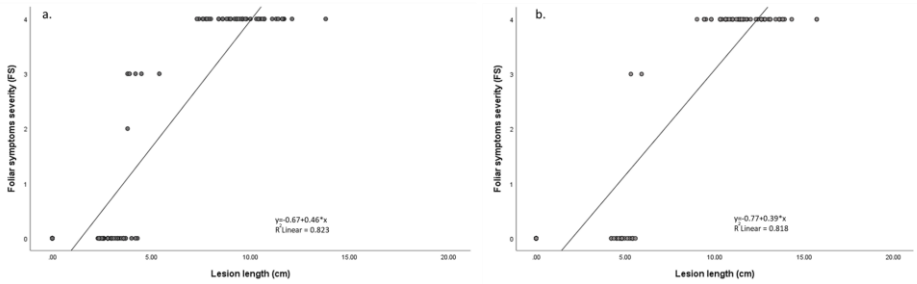

Figure 6. Correlation between foliar symptoms severity (FS) and lesion length (cm): **a.** *Mimusops elengi*, **b.** *Acacia mangium*

Discussion

The results of this study show clearly that a *C. manginecans* was responsible for the wilt outbreak disease that has recently appeared on the *M. elengi* agricultural field in Indralaya (Ogan Ilir) and roadside trees in Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir), South Sumatra. This is the first report of a *Ceratocystis* wilt disease on *M. elengi* in Indonesia. Formerly the disease was reported in Thailand (Pornsuriya and Sunpapao, 2015), however, they just observed a single tree and they did not conduct pathogenicity test and observe morphological characteristics. In Indonesia Tarigan et al. (2010; 2011) reported *C. manginecans* caused wilt and die-back disease in *A. mangium* plantations in Riau.

Mimusops elengi infected with *Ceratocystis* has wilted foliage symptoms, sudden wilt, decline and branch drying, and progressive loss of the canopy resulting in tree death. *M. elengi* trees showed typical symptoms of infection by the *Ceratocystis* fungus; the same was true of a serious wilt pathogen of *A. mangium* and *A. crassicarpa* in Indonesia and Vietnam (Tarigan et al. 2011; Thu et al. 2012; Thu et al. 2016). Vascular wilt, wood stain and stem cankers are the most characteristic symptoms of infection by *C. manginecans* in woody trees (Harrington 2013). Rot in roots or stems, vascular wilt, sapwood discoloration and cankers are symptomatic of plants infected by *C. manginecans* species (Kile 1993).

The comparison of morphological characteristics and gene sequences (β-tubulin and ITS) of the isolates examined in this study were similar to those in descriptions given for *C. manginecans* isolated from diseased *Acacia* trees which form part of the *C. fimbriata* s. l. complex, which is typified by *C. fimbriata* sensu stricto (Engelbrecht and Harrington 2005; Tarigan et al. 2011). The two isolates from Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) have 100% identical sequences of ITS and Beta Tubulin representing a single clone of the pathogen. This supports the view that *M. elengi* wilt disease in Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) emerged from a single introduction of a single haplotype and the population has expanded clonally to infect *M. elengi* trees in many parts of all three regions.

Ceratocystis manginecans was reported in Indonesia associated with wilt and die-back disease on *Acacia* spp. (Tarigan et al. 2011). It was found that many acacia plants were attacked by *Ceratocystis* disease and *Hypocryphalus mangifera* insects in the field raised the suspicion that *M. elengi* were infected by *Ceratocystis* in acacia plants. *H. mangifera* is a vector insect for the spread of *Ceratocystis* in the world (Rossetto et al. 1980; Al-Adawi et al. 2013; Van Wyk et al. 2007; Masood et al. 2008). Pruning *M. elengi* branches using equipment that has previously been infected with *Ceratocystis* also increases the severity of the disease; this can be seen by the number of plants that are attacked after pruning with tools used to prune *Acacia* plants that are attacked by *Ceratocystis*. Cankers of *Ceratocystis* were associated with branch wounds from pruning, pruning time and pruning technique on plants (Chi et al. 2019b). In this study, infected bullet wood was observed surrounding infected acacia by *C. manginecans* with abundant *H. mangifera*. This might be caused by spore contamination on cutting tools after pruning diseased acacia and spore spreading by insect vector *H. mangifera*.

The pathogenicity of *C. manginecans* to these hosts was demonstrated in inoculation trials and it is clear that the fungus is responsible for the widespread death of these trees. It was also possible to show that isolates of the pathogen from these trees are able to infect and kill other plants. The eight *C. manginecans* isolates all formed lesions on the stems of *M. elengi* seedlings when inoculated under the bark in nursery cloches. The most pathogenic was CAME30815 and CAME30819 isolate from *M. elengi* and the CAW30814 isolate from *A. mangium* resulted in foliar symptoms with severity index 3.6, 3.1 and 2.8 after 45 days from inoculation with pathogen. When the isolates were tested on seedling *A. mangium*, the seedlings were infected and mostly dead as they were the main host plant and susceptible to *C. manginecans* (Tarigan et al. 2011).

Previous researchers of *C. manginecans* on *M. elengi* in Thailand have reported molecular identification *C. manginecans* by primers combination the ITS, β-tubulin (βt) and transcribed elongation factor 1-α (TEF1-α) gene regions with one isolate. In our research, we tested two isolates for sequencing, seven isolates for pathogenicity, and Koch's postulates assay using three isolates (CAME30815, CAME30816 and CAME30817) from Ogan Ilir (Indralaya); two isolates (CAME30818 and CAME30814) from Jakabaring (Palembang); and two isolates (CAME30819 and CAME30813) from Kayuagung (Ogan Komering Ilir) and one isolate (CAW30814) from diseased *Acacia*, *A. mangium*.

All isolates showed the ability to infect both bullet wood and *Acacia*, and all isolates can be reisolated confirming Koch's postulates.

The wilt disease of *M. elengi* appears to be serious and it is clearly a new host tree or pathogen association that has apparently occurred due to a host shift. This category of diseases is increasing in importance in plants, and they can devastate native trees that have not previously encountered them (Roy 2001; Anderson et al. 2004; Slippers et al. 2005; Woolhouse et al. 2005; Desprez-Loustau et al. 2007; Wingfield et al. 2010). In this regard, the wilt disease of *M. elengi* can impact seriously on the natural biodiversity of Indonesia, and studies should be instituted to understand them better. The findings of this study will give knowledge of the characteristics of the symptoms of the disease, their causes and help to minimise the spread of wilt disease in *M. elengi* in plantations or roadside trees and to consider its possible pathogenicity.

This study presents the first report of *Ceratocystis* wilt or sudden decline disease of bullet wood in Indonesia and the discovery of a fungus that has been identified as *C. manginecans.* The disease of bullet wood that gave rise to this study is serious and management options to reduce its incidence are required *C. manginecans* is an aggressive pathogen and a deeper understanding of its role in tree death will be important in the future.

ACKNOWLEDGEMENT

This research was funded by a PMDSU scholarship in the fiscal year of 2019-2021 according to the Director of Research and Community Service, Directorate of Research and Community Service (DRPM), Directorate General for Research and Development, Ministry of Research, Technology, and Higher Education, Number: 068/SP2H/AMD/LT/DRPM/2020 chaired by Ahmad Muslim.

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DRAFT PERBAIKAN

First report of bullet wood (*Mimusops elengi*) sudden decline disease caused by *Ceratocystis manginecans* in Indonesia

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Abstract. *Ceratocystis manginecans* causes wilt and death of plants in several important crops and native vegetation in Indonesia. Ceratocystis wilt was recently found to be causing substantial mortality in bullet wood (*Mimusops elengi*) in South Sumatra. The aim of this study was to describe the symptomatology of the new disease and characterize isolates of *C. manginecans* obtained from diseased bullet wood plants. Diseased plants showed substantial discoloration of the woody xylem and wilt-type symptoms of the foliage, with the eventual death of the whole plant. Isolations from infected plants yielded fungi that were similar morphologically to *C. manginecans*, with typical hat-shaped ascospores and light-coloured perithecial bases. Sequencing of the internal transcribed spacer (ITS) and β-tubulin of the isolates confirmed their identification, grouping them with *C. manginecans* and separating them from all other *Ceratocystis* species. This is the first report of *C. manginecans* in Indonesia causing wilt and death on bullet wood. *C. manginecans* is an important pathogen, and strategies to reduce losses need to be established in Indonesia because the aggressiveness of *C. manginecans* to bullet wood has been shown in inoculation experiments

Keyword: Ceratocystidaceae, Molecular phylogeny, Pathogenicity, Sapotaceae

INTRODUCTION

Bullet wood (*Mimusops elengi*) belongs to the family Sapotaceae, common English names are Asian Bulletwood, Bullet Wood Tree, Indian Medlar, Red Coondoo Spanish Cherry and it is known in Indonesia as Tanjung. The species is native to India, Sri Lanka, the Andaman Islands, Myanmar, Indo-China, Peninsular Malaysia and Vanuatu; it has been introduced and cultivated elsewhere. *M. elengi* can grow in tropical and subtropical climates. This plant thrives in areas with high humidity and seasonal rainfall and seasonal dry periods (Lim, 2012). The bullet wood trees range from small to large, and are found in all parts of Indonesia where bullet wood is cultivated in gardens as an ornamental tree, for medicines and planted along avenues because of its fragrant flowers (Seth, 2003).

M. elengi is widely used for medicine, and various parts of *M. elengi* Linn. (Sapotaceae) have been used widely in traditional Indian medicine for the treatment of pain, inflammation and wounds. *M. elengi* stem bark would be a possible therapeutic candidate having cytotoxic and anti-tumour potential (Kumar et al. 2016); it also has antibacterial and antifungal uses (Ali et al. 2008). At present, *M. elengi* is used as the synthesis of calcium phosphate nanoparticles that is easy, eco-friendly and scalable (Pokale et al. 2014).

Several types of pathogenic fungi have been identified to cause disease in *M. elengi* plants. *Curvularia lunata* caused die-back in India (Khatun et al. 2011); *Pestalotiopsis clavispora* caused leaf blight (Lokesh et al. 2017). *Ceratocystis* was first isolated from a single tree of bullet wood showing sudden decline in Thailand. Symptoms displayed by the diseased trees include gum exudation from the trunks and wilting and loss of the dark green foliage with a corresponding browning of leaves on single branches. In this study they did not confirm the pathogen with a Koch postulates test detail (Pornsuriya and Sunpapao, 2015). Recently we have observed many bullet wood trees showing similar symptoms with *C. manginecans* decline in many locations in South Sumatra, Indonesia.

C. manginecans includes many economically important plant pathogens. This pathogen has caused a sudden decline and has led to the death of thousands of *Mangifera indica* trees in Oman with *Hypocryphalus mangifera* vector (Al Adawi et al. 2013). In Indonesia *C. manginecans* caused die-back on *Acacia mangium* and *A. crassicarpa* plantations in Riau (Tarigan et al. 2010), whereas in Vietnam recently, *C. manginecans* caused wilt disease in *Dalbergia tonkinensis* and *Chukrasia tabularis* (Chi et al. 2019a; Chi et al. 2020); in Pakistan this pathogen also causes wilt disease in *Albizia lebbeck* (Razzaq et al. 2020). Commonly *C. manginecans* causes yellowing of leaves and rapid wilting of leaves was observed on individual branches in affected trees that ultimately spread to the canopy followed by the death of the whole tree. Dark brown to black tissue discoloration was observed in the woody xylem tissues of infected trees.

This study aimed to identify the cause of a new outbreak of wilt disease causing a sudden decline to the trees, wilted canopies and tree death in *M. elengi* in South Sumatera, Indonesia. This study was also conducted to describe the characteristics of the pathogen and confirm Koch's postulates test.

MATERIALS AND METHODS

Disease symptoms and specimen collection

The distribution and impact of the *C. manginecans* disease on *M. elengi* were determined from roadside trees planting in Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) and the agricultural field of Sriwijaya University in Indralaya (Ogan Ilir), South Sumatra, Indonesia. Symptoms of wilt diseases were evaluated as follows: the extent of lesion development from discoloration of bark and wood, the extent of foliar wilting or loss and tree death.

Samples of diseased trunks were collected from two to six-year-old trees in September 2019 to April 2020. Wood samples were taken from lesions of wilted trees using a knife sterilised in 70% ethanol. The wood samples collected from *M. elengi* showed brown to black streaking in the woody xylem. Each sample was wrapped in tissue paper and placed in a coolbox. The same day, the wood samples (1–20 mm length, 1–2 mm thick) were sandwiched between two slices of fresh carrot and placed on sterile dry paper in plastic boxes at 25 °C following the method of Li et al. (2014). After 5–10 days, hat-shaped spores of putative *Ceratocystis* pathogens were placed on 2% (w/v) malt extract agar (MEA) (Merck, Germany), and incubated at 25 °C in a laboratory. When cultures had grown to several cm in diameter, hyphal tips were sub-cultured onto new MEA and potato dextrose agar (PDA) (Merck, Germany) plates and incubated at 25–28 °C. Morphological traits of fruiting bodies and spores were observed under an optical Olympus CX33 microscope (Olympus Corporation, Japan).

Genomic DNA extraction, PCR amplification, and sequencing

DNA isolation used YeaStar Genomic DNA Kit (Zymo Research Corporation, California, USA). To extract genomic DNA, cultures were incubated for five days to allow sufficient mycelial growth in potato dextrose broth (PDB) (Merck, Germany). Mycelium was purified with sterile filter paper (Whatman) and transferred to 1.5 mL Eppendorf tubes. The quantity and quality of DNA extracted was evaluated with a spectrophotometer (NanoDrop ND-1000; Thermo Fisher, Waltham, MA, USA) to calibrate the concentration and purity of DNA as PCR templates.

The PCR amplification reactions were conducted on a T-100 thermal cycler (Bio-Rad, Hercules, CA, USA). Amplifications were carried out in 50 µl reactions containing 20 µl DreamTaq Green PCR Master Mix (Eppendorf, Germany) (DreamTaq DNA Polymerase, 2X DreamTaq Green buffer, dNTPs, and 4 mM $MgCl_2$), 1,5 µl of each forward and reverse primer, 4 µl of DNA template and 23 µl sterilised water. The PCRs were performed with a C1000 Touch™ thermal cycler (Bio-Rad, USA). The PCR cycling parameters were as follows: initial denaturation for 5 min at 95 °C, followed by 35 cycles at 95 °C for 30 s, 56 °C for 45 s and 72 °C for 1 min. Amplification was completed at 72 °C for 10 min and the PCR product was stored at 10 °C (Chi et al. 2020).

PCR amplifications were made for two gene regions, including part of the b-tubulin (BT) using primers βt1a (TTCCCCCGTCTCCACTTCTTCATG) and βt1b (GACGAGATCGTTCATGTTGAACTC) (Oliveira et al. 2015), and the ITS using ITS1 and ITS4. The resulting PCR products were submitted to 1st BASE (Malaysia) for forward and reverse sequencing reactions. Raw sequence data were assembled, examined, and manually edited using Genestudio 2.1.1.5 (Genestudio, Suwanee, Georgia) and BioEdit software (van der Nest et al. 2019). The DNA sequences were compared to the GenBank database via the nucleotide-nucleotide BLAST search interface located at the National Center for Biotechnology Information, Bethesda, USA. Relevant sequences were transferred with NoteTab Light v7.2.

Phylogenetic analyses

The sequences of *Ceratocystis* spp. closely related to the one from *Mimusops elengi* were retrieved from GenBank. Phylogenetic sequences from different gene regions were aligned using Mesquite v3.5 (Maddison and Maddison 2018) (http://mesquiteproject.org) and corrected manually. Phylogenetic trees based on a concatenated data set of the ITS and βt were computed and analysed as a single dataset. Maximum Parsimony (MP) analyses were performed in MEGA v. 10 (Kumar et al. 2016; Paul et al. 2018) with 1000 bootstrap replications.

Pathogenicity tests

Pathogenicity studies were conducted on two agroforestry plants, *A. mangium* and *M. elengi*. Plants had stem diameters of 2–3 cm and heights <1 m. The pathogenic potential of isolates was evaluated by the under bark inoculation method described by Deidda et al. (2016). Bark was wounded to expose the cambium using a 4 mm cork borer, and discs of agar bearing mycelium taken from the margins of actively growing, 2-week-old cultures on 2% MEA (Tarigan et al. 2010; Tarigan et al. 2011; Chi et al. 2019a, Chi et al. 2020), *Ceratocystis* isolates were placed with the mycelium facing the cambium. Ten plants of each tree species were inoculated with sterile MEA plugs to serve as controls. All inoculation points and the ends of the logs were covered with masking tape and polyethylene films, respectively, to prevent desiccation of the inoculum and cambium, and to reduce contamination.

The nursery trial evaluated seven *Ceratocystis* strains isolated from *M. elengi* (CAME30813, CAME30814, CAME30815, CAME30816, CAME30817, CAME30818 and CAME30819) and one strain (CAW30814) from *A. mangium*. There were ten replicate plants per treatment in each row plot in each of the blocks. After 45 days, lesion (L) length and foliar symptoms severity were recorded. Representative wood samples were taken from within lesions outside the inoculation area, and the pathogen reisolated and sequenced for Koch's postulates test.

Foliar symptoms severity (FS) was assessed using a scale of 0 to 4: 0 = healthy; 1 = lower leaves yellow; 2 = slight wilting; 3 = severe wilting; 4 = dead (Muslim et al. 2003; Chi et al. 2019a; Muslim et al. 2019).

The pathogenicity test data were analysed using SAS university edition software package. Analysis of variance (ANOVA) and Tukey's honestly significant difference (Tukey's HSD) test were used to determine whether there were significant differences in comparisons of means of different treatments.

RESULT AND DISCUSSION

Symptoms of *Mimusops elengi* wilt disease

We observed disease symptoms from September 2019 to April 2020, in various places that are widely planted in the Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) areas, South Sumatra, Indonesia. The disease was found scattered throughout the planted area, with symptoms of partially dead trees, foliar wilting or loss and tree death (Fig. 1a). Initially, the leaves of infected plants lost turgor and brightness, with yellowing symptoms in the older leaves, followed by wilting and death of the plant. Symptomatic plant stems showed xylem discoloration (Fig. 1b), infections generally started in the roots and then moved upwards in the stem ultimately reaching the upper branches of the entire plant, and the plants ultimately died (Fig. 1c). Death of adjacent plants indicates transmission of root infection because these pathogens are also known as soil-borne pathogens. The severity of the infection is also caused by pruning the branches using tools previously used to cut the infected plants.

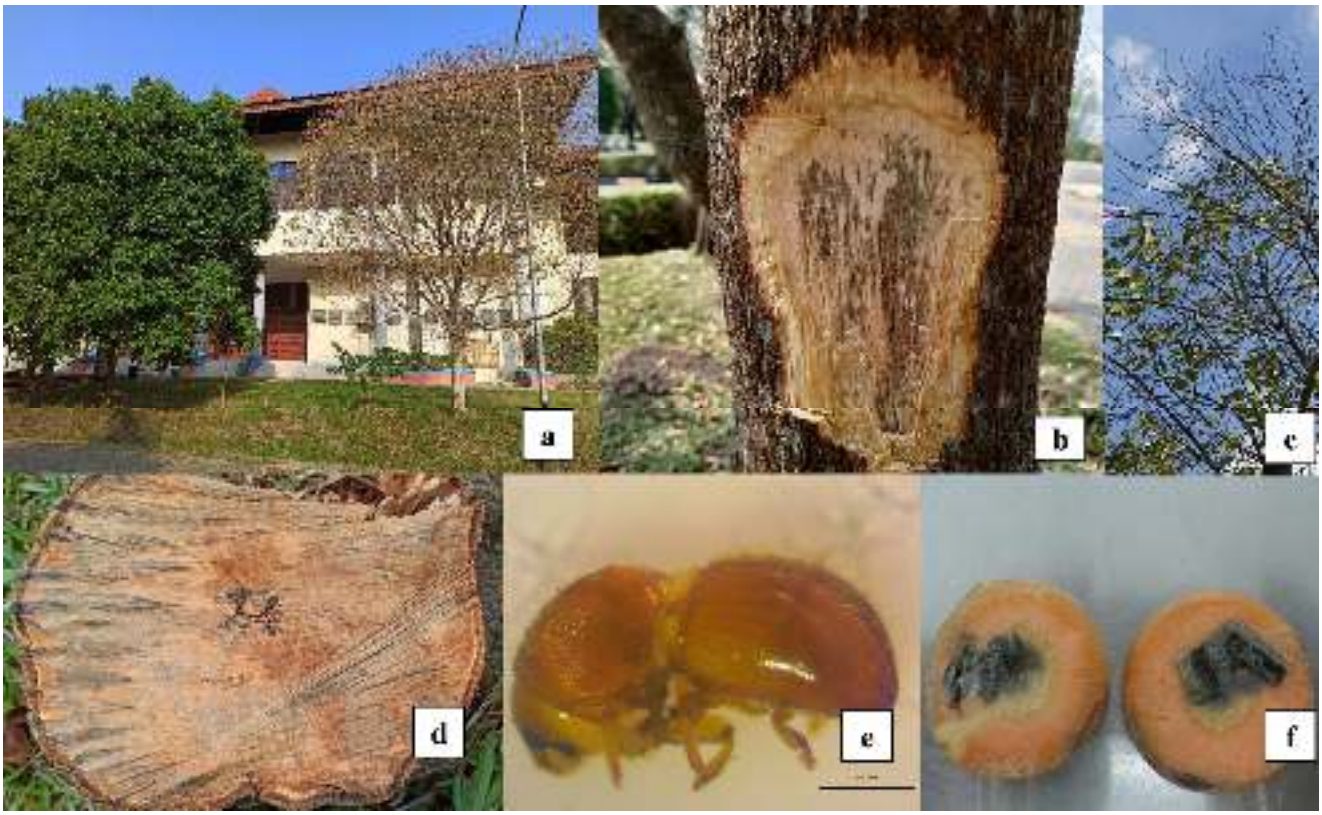

Figure 1. Symptoms of *Ceratocystis manginecans* wilt disease in bullet wood*:* **a**. tree death of *M. elengi*: b. sap stain mould on bullet wood, c. wilted leaves of bullet wood, d. sap stain mould on bullet wood, e. The bark beetle vector of *C. manginecans*, *Hypocryphalus mangiferae,* f. isolation of the fungus from discoloured xylem showing dark mycelium and sporulation on the carrot slices

Observation of diseased plant xylem tissue in cross-sections of the stem showed dark brown lesion formation in the cambium (inner bark region) towards vascular tissue (Fig. 1d). In the initial stage of plant infection, the foliar symptoms, wilt and the fruits appeared normal, but as the infection progressed, the fruits of affected plants were smaller, shrivelled, wrinkled and dry. Many of the bark beetle vectors of *C. manginecans, Hypocryphalus mangiferae* were found around bullet wood diseases (Fig. 1e). Testing by the Li et al. (2014) method showed that *Ceratocystis* had grown on the carrots, and ascomata of *C. manginecans* with necks supporting sticky masses of ascospores on the carrot slices (Fig. 1f).

Sampling and isolation

Seven isolates of *C. manginecans* were collected from diseased bullet wood (*M. elengi*) (Fig. 2). There were three isolates (CAME30815, CAME30816 and CAME30817) from Ogan Ilir (Indralaya); two isolates (CAME30818 and

CAME30814) from Jakabaring (Palembang); and two isolates (CAME30819 and CAME30813) from Kayuagung (Ogan Komering Ilir). We also isolated one isolate (CAW30814) from diseased *acacia*, *A. mangium* in the agricultural field of Sriwijaya University, Indralaya.

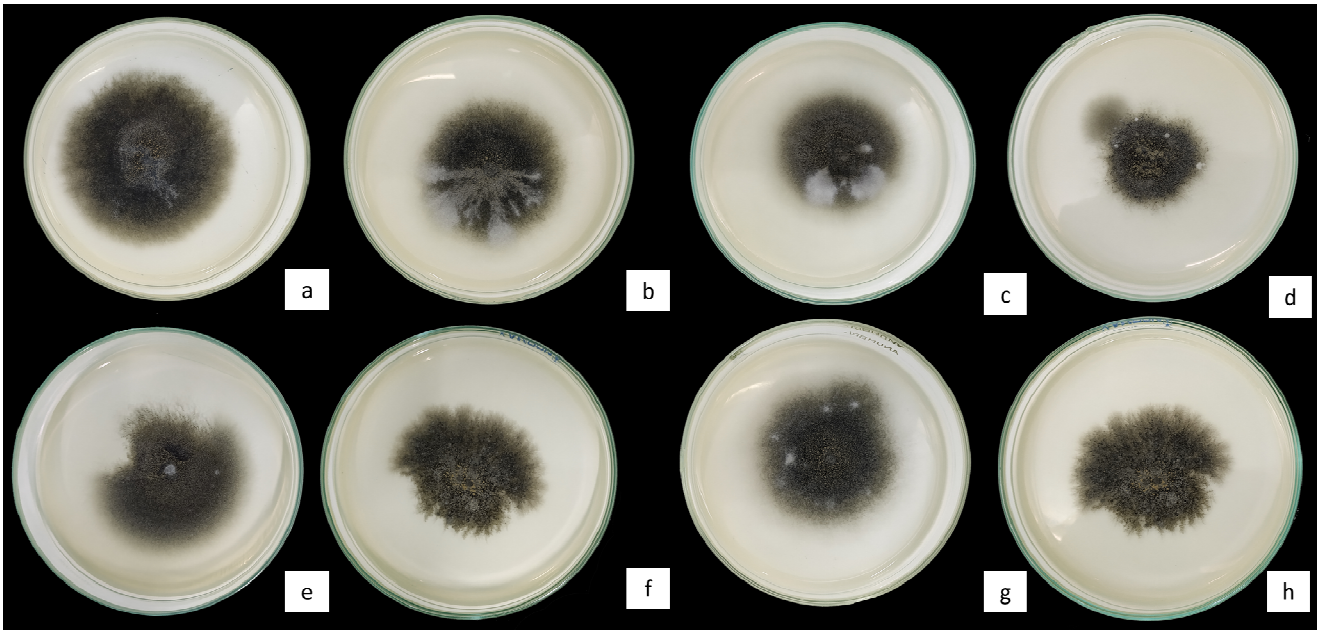

Figure 2. Isolates of *Ceratocystis manginecans* and related species grown on malt extract agar (MEA) for 7 d at 25 ºC. **a, b, c**: *Ceratocystis* CAME30815, CAME30816 and CAME30817, from *Mimusops elengi* in Sriwijaya University, Indralaya. **d, e, f:** *Ceratocystis* CAME30819, CAME30813 and CAME30814 from *Mimusops elengi* in Jakabaring, Palembang. **g**: *Ceratocystis* CAME30818, from *Mimusops elengi* in Kayuagung, Ogan Komering Ilir. **h**: *Ceratocystis* CAW30814, from *Acacia mangium* in Indralaya

Fungal morphology

Seven isolates were morphologically indistinguishable (Table 2). At 7–14 days of incubation at 25 ºC on MEA, cultures were pale brown to dark brown and produced a banana-like odour. Mycelium on MEA was grey, and the reverse side of the colony olivaceous grey; submerged mycelium darkened as the ascomata developed, forming fine, radiating fibrils. Ascomata developing within seven days and mature within ten days, superficially or partly embedded in the agar, dark brown to black (Fig. 3a). Ascomatal bases were submerged or on the agar surface, dark bases dark brown to black, base subglobes to globes, (134.58-) 169.12 - 276.29 (-310.83) µm long and (122.91-) 161.89-244.14 (-283.13) µm wide in diameter (Fig. 3a). Ascomata necks were erect, occasionally curved, black at the base becoming subhyaline towards the apex, smooth to crenulate, (346.51-) 454.94-720.16 (-828.59) µm long including ostiolar hyphae (Fig.3b). Ascospores were hat-shaped, (3.61-) 5.64-6.23 (-6.93) µm length and (2.06-) 2.279-3.67 (-3.85) µm width (Fig.3f). Barrel conidia (8.62-) 8.85-12.79 (-13.25) µm length and (5.89-) 4.12x6.87 (-8.67) µm width. Bacilliform conidia (9.05-) 10.82-22.32 (-35.97) µm length and (2.01-) 2.83-5.71 (-8.87) µm width (Fig.3c). Chlamydospores oval, thick-walled, smooth, (8.21-) 9.15-16.21 (-18.50) µm length and (4.92-) 6.46-15.81 (14.65) µm width (Fig.3e).

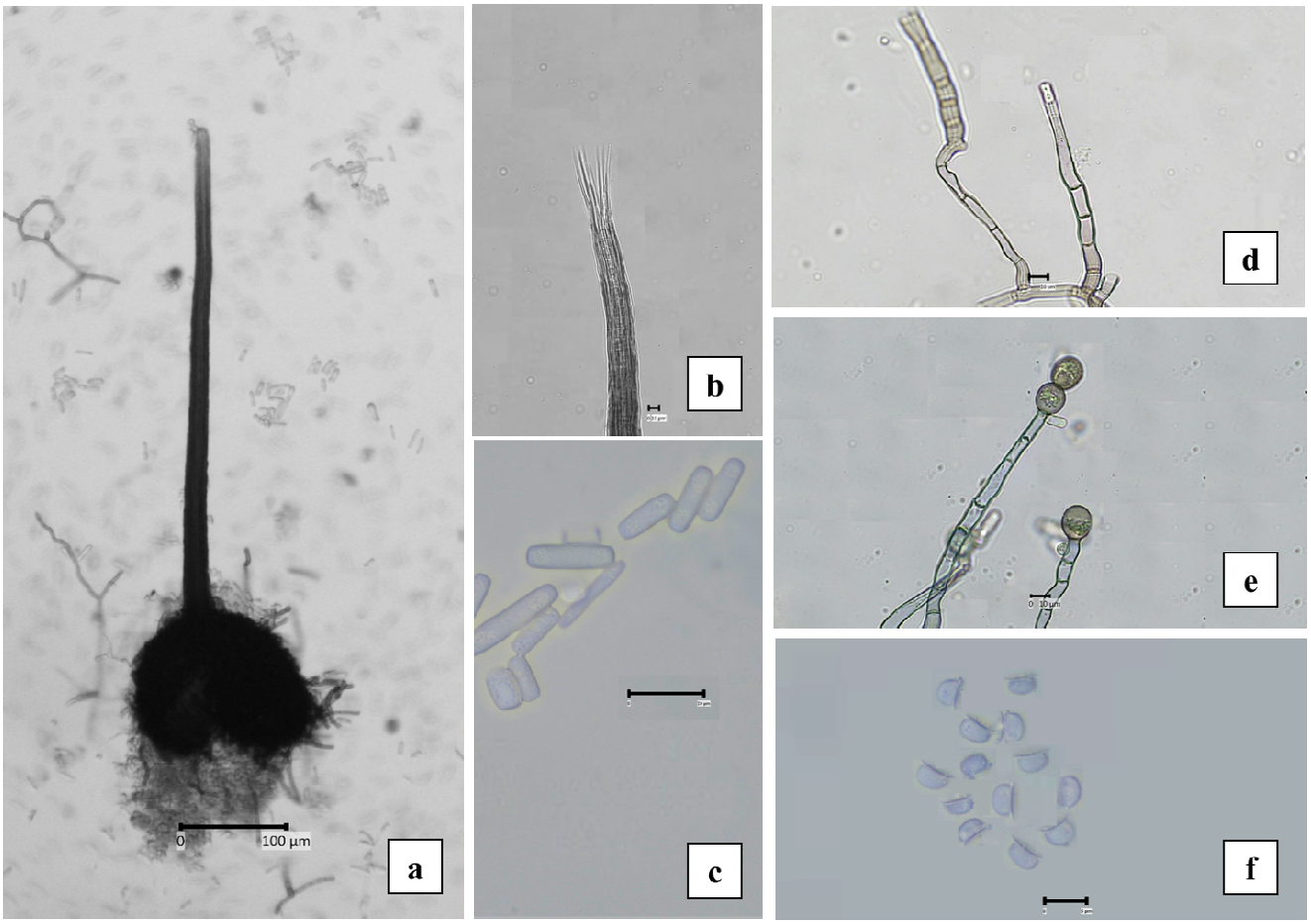

Figure 3. Morphological characteristics of *Ceratocystis* isolated from *M. elengi* stem lesion: a. globose ascomata with long neck, b. divergent ostiolar hyphae, c. barrel-shaped conidia, d. conidiophore/phialide, e. chlamydospores, f. hat-shaped ascospores. Scale bars: a = 100 µm; b,c,d,e = 10 µm; f = 5 µm

Sequence analysis

To confirm the identify of the wilt pathogen, the ITS and β-tubulin 1 gene sequences of two isolates from bullet wood (*M. elengi*) were compared to reference sequences downloaded from the gene bank database (NCBI GenBank) (Table 1) and indicated that isolates from Indonesia were grouped within the *C. fimbriata*. s.l species complex and were most closely related to *C. manginecans*. PCR amplification resulted in fragments of ~550 base pairs (bp) in size which had 100% homology with *C. manginecans* (Fig. 4). Bootstrap values were equal to or greater than 50%, derived from 1000 iterations.

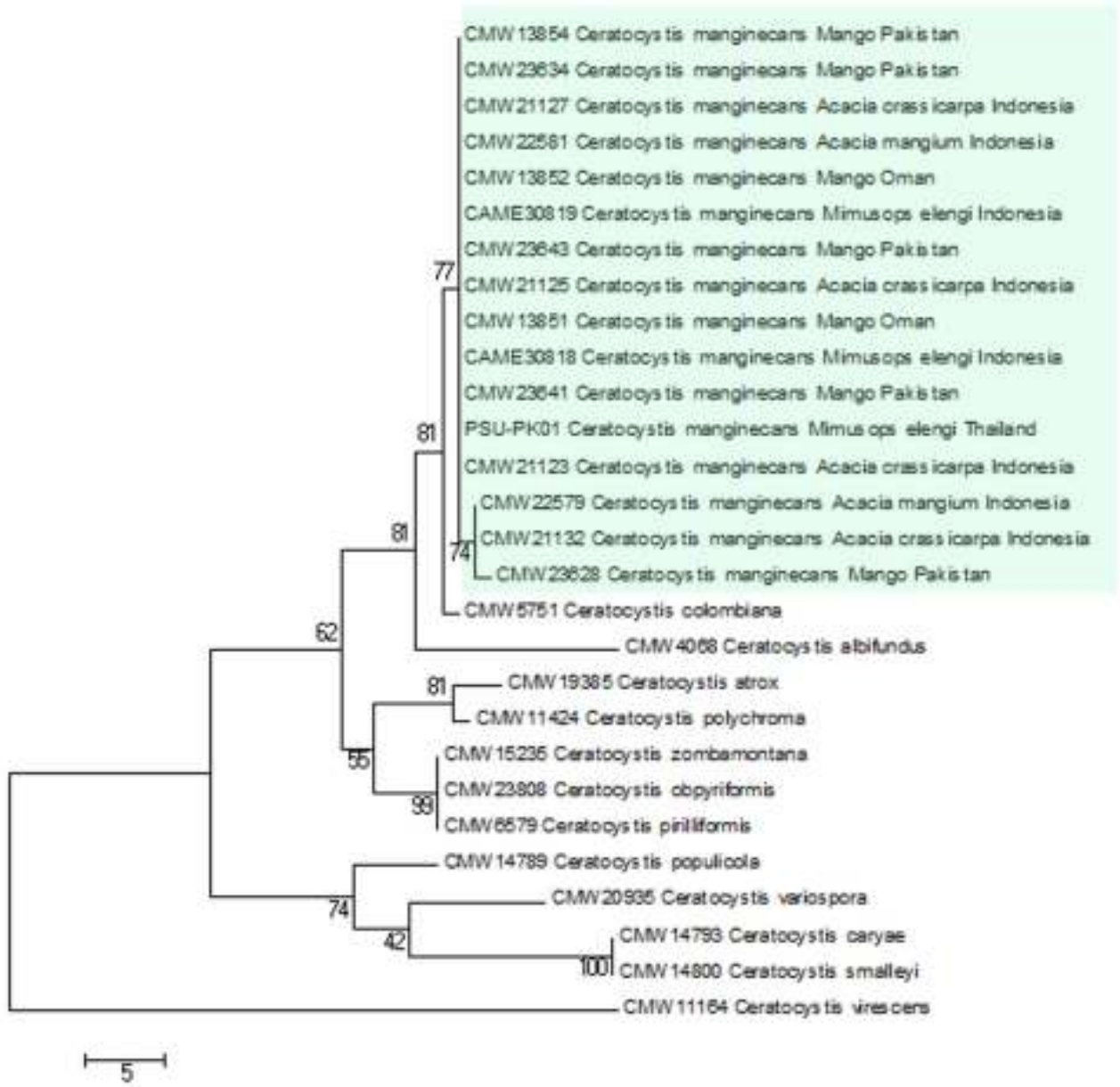

Figure 4. Phylogenetic tree constructed by MEGA with Maximum Parsimony (MP). The tree was built using combined sequence data of the ITS region and b-tubulin gene and their related species from GenBank. Consistency (CI), retention (RI), and composite indexes (CoI) are 0.714286, 0.837209, and 0.664843 for all sites and parsimony-informative sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Bootstrap values >50% are indicated above the branches. The analysis involved 28 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 493 positions in the final dataset

176 **Table 1.** *Ceratocystis* isolates considered in the phylogenetic analyses

Isolate no	Identify	Host	Geographic origin	Collector	Gene region/GeneBank accession no	
					ITS	BT
CAME30819	*C.manginecans*	*Mimusops elengi*	Indonesia	**R.Pratama**	MT373423	Submitted
CAME30818	*C.manginecans*	*Mimusops elengi*	Indonesia	**R.Pratama**	MT373424	Submitted
CMW13851	*C.manginecans*	*Mangifera indica*	Oman	M. Deadman	AY953383	EF433308
CMW23643	*C.manginecans*	*Mangifera indica*	Pakistan	A.Al-Adawi	EF433304	EF433313
CMW23641	*C.manginecans*	*Mangifera indica*	Pakistan	A.Al-Adawi	EF433305	EF433314
CMW23634	*C.manginecans*	*Mangifera indica*	Pakistan	A. Al-Adawi	EF433302	EF433311
CMW21125	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588663	EU588642
CMW21123	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588662	EU588641
CMW22581	*C.manginecans*	*Acacia mangium*	Indonesia	M. Tarigan	EU588659	EU604671
CMW21132	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588665	EU588644
CMW22579	*C.manginecans*	*Acacia mangium*	Indonesia	M. Tarigan	EU588658	EU588638
CMW23628	*C.manginecans*	*Hypocryphalus mangifera*	Pakistan	A. Al-Adawi	EF433303	EF433312
CMW13854	*C.manginecans*	*Mangifera indica*	Oman	M. Deadman	AY953385	EF433310
CMW13852	*C.manginecans*	*Hypocryphalus mangifera*	Oman	M. Deadman	AY953384	EF433309
CMW4068	*C.albifundus*	*A. mearnsii*	RSA	J. Roux	DQ520638	EF070429
CMW14793	*C.caryae*	*C. cordiformis*	U.S.A	J. Johnson	EF070424	EF070439
CMW14800 CBS114724	*C.smalleyi*	*C. cordiformis*	U.S.A	G. Smalley	EF070420	EF070436
CMW14789	*C. populicola*	*Populus sp.*	Poland	J. Gremmen	EF070418	EF070434
CMW19385 CBS115778	*C.atrox*	*E. grandis*	Australia	M.J. Wingfield	EF070415	EF070431
CMW11424	*C. polycroma*	*Syzygium aromaticum*	Indonesia	M.J. Wingfield	AY528970	AY528966
CMW23808	*C. obpyriformis*	*A. mearnsii*	South Africa	R.N. Heath	EU245003	EU244975
CMW6579	*C.pirilliformis*	*E. nitens*	Australia	M.J. Wingfield	AF427105	DQ371653

177 **Table 2.** Morphological comparisons of *Ceratocystis manginecans* and other phylogenetically closely related species

Character	*Ceratocystis manginecans* (from *M. elengi)*	*Ceratocystis acaciivora* (from *A. mangium*)	*Ceratocystis manginecans* (from *A. mangium*)
Ascomata base	(134.58-) 169.12 - 276.29 (-310.83) x (122.91-) 161.89-244.14 (-283.13)[a]	(105-) 131-175 (-206) x (107-) 125-167 (-188)	(132.1-) 175.3 (−233.2)
Ascomata base average	220.01x211.63[b]		
Ascomata neck	(346.51-) 454.94-720.16 (-828.59)	(301-) 348-448 (-522)	(327.3-) 452.7 (−556)
Ascomata neck average	568.41		
Ascospores	(3.61-) 5.64-6.23 (-6.93) x (2.06-) 2.279-3.67 (-3.85)	5-7 x 3-4	(3.1-) 4.2 (−4.7) x (4.1-) 5.5 (−6.8)
Ascospores average	5.62 x 3.93		
Bacilliform conidia	(9.05-) 10.82-22.32 (-35.97) x (2.01-) 2.83-5.71 (-8.87)	(11-) 14-22 (-29) x 3-5	(14.6-) 18.6 (−30.7)x (3.0-) 4.6 (−5.4)
Bacilliform conidia average	16.56x4.27		
Barrel-shaped conidia	(8.62-) 8.85-12.79 (-13.25) x (5.89-) 4.12x6.87 (-8.67)	(8-) 9-11 (-13) x 4-6	(6.8) 8.1 (−10.6)
Barrel-shaped conidia average	11.497 x 15.82		
Clamydospore	(8.21-) 9.15-16.21 (-18.50) x (4.92-) 6.46-15.81 (14.65)	(10-) 12-14 (-15) x (7-) 8-12 (-14)	(10.1-) 13.1 (−15.5) x (6.1-) 9.2 (−11.1)
Clamydospore average	11.13 x 14.18		
Reference	This Study	M. Tarigan et al. 2010	Chi et al. 2019

178 All measurements are in µm
179 [a] Measurements are presented in the format [(minimum-) (average-standard deviation) - (average-standard deviation) (-maximum)]
180 [b] Measurements are presented in the format minimum x maximum

Pathogenicity

The results of the pathogenicity tests of seven isolates (CAME30813, CAME30814, CAME30815, CAME30816, CAME30817, CAME30818, and CAME30819) on *M. elengi* and one isolate (CAW30814) from *A. mangium* are shown in Table 3. All isolates tested showed a varying reaction to lesion and foliar symptoms (Fig. 5). For the pathogenicity test on *M. elengi*, seven isolates from *M. elengi* as well as one isolate from *A. mangium* strongly infected the wood and produced significant lesion length ranging from 3.99 to 10.47 cm and showed significant differences from the control. Three isolates from *M. elengi* (CAME30815; CAME30819; CAME30818) as well as one isolate from *A. mangium* (CAW30814) showed high pathogenicity on the foliar symptom (with foliar severity index of 2.3-3.6 and lesion length 6.52-10.47 cm), while the other isolates (CAME30817; CAME30816; CAME30814, CAME30813) showed moderate pathogenicity to *M. elengi* (foliar severity index of 1.4-2.0 and lesion length 3.99-6.02 cm). When the isolates were tested for their pathogenicity on *A. mangium* as the primary host of the pathogen, two isolates (CAME30815; CAME30819) showed strong pathogenicity on lesion length (13.76-11.89 cm) and also provided high pathogenicity on foliar severity index (4), where all plants tested were dead. Three isolates (CAW30814; CAME30818; CAME30817) showed moderate pathogenicity on lesion length 8.71-10.14 cm and foliar severity index 2.8-3.2, while the other isolates (CAME30816, CAME30814, CAME30813) showed low pathogenicity on lesion length 7.19-8.63 cm and foliar severity index 1.9-2.4.

Table 3. Pathogenicity of *Ceratocystis* isolates on *Mimusops elengi* and *Acacia mangium* under nursery condition

Isolates	Host test	*M. elengi*		*A. mangium*	
		Lesion Length (cm)	Foliar symptoms	Lesion Length (cm)	Foliar symptoms
CAME30815	10	10.47e	3.6	13.77d	4
CAME30819	10	8.29de	3.1	11.89cd	4
CAW30814	10	7.35cd	2.8	10.14bcd	3.2
CAME30818	10	6.52bcd	2.3	9.92bcd	3.1
CAME30817	10	6.02bcd	2	8.72bc	2.8
CAME30816	10	5.27bc	1.8	8.47bc	2.4
CAME30814	10	4.93bc	1.5	8.64bc	2
CAME30813	10	3.99b	1.4	7.19b	1.9
Control (MEA)	10	0.1a	0	0.1a	0
Fpr		<0.001		<0.001	

Values followed by the same letters in a column are not different among isolates at P=0.05 according to Tukey's HSD multiple range test.

There was a strong linear correlation between foliar symptom severity and lesion length for *M. elengi* and *A. mangium* (Fig. 6). The Koch's postulates test was conducted by reisolating the pathogen, and its identity confirmed to be the same as *C. manginecans*.

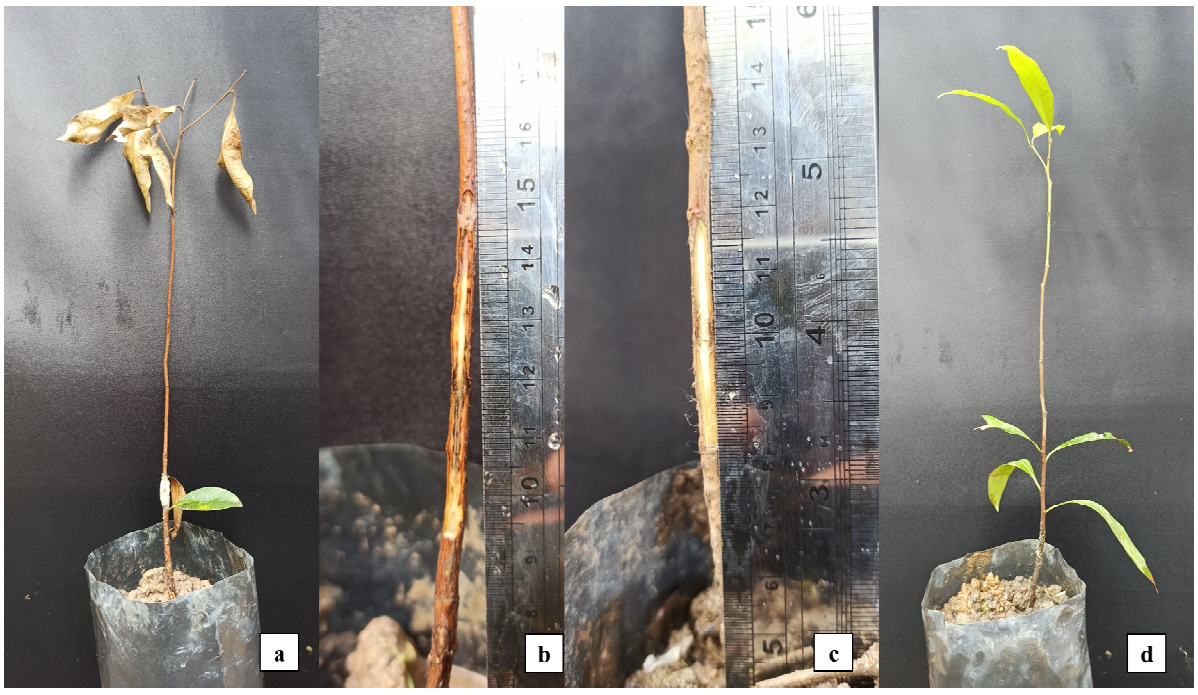

Figure 5. Response after 45 days of *Mimusops elengi* seedlings to under-bark inoculation with mycelium of *Ceratocystis manginecans*. **a.** wilting of seedlings; **b**. lesions on the stem; c. no lesions on the stem; d. normal foliage on the control seedling which received MEA only and appeared healthy. Yellow arrow indicates the point of inoculation, red arrows show the lesion boundary

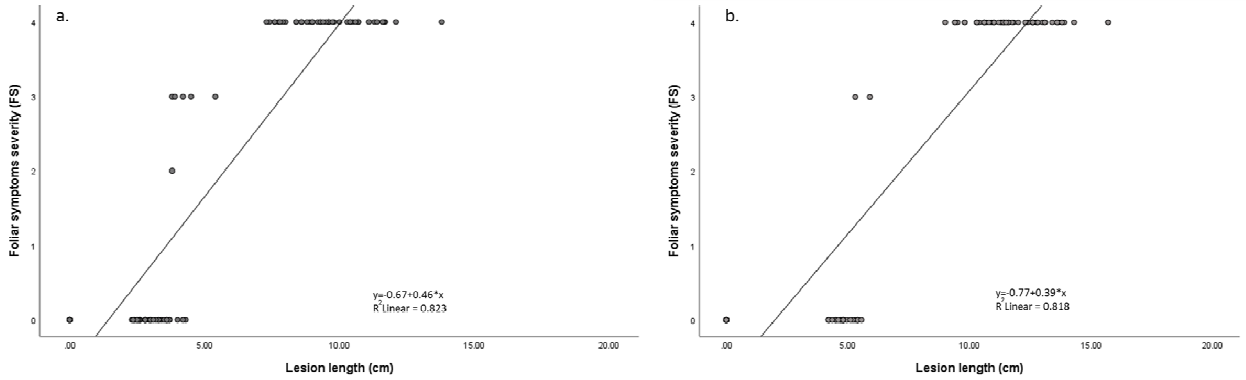

Figure 6. Correlation between foliar symptoms severity (FS) and lesion length (cm): **a.** *Mimusops elengi,* **b.** *Acacia mangium*

Discussion

The results of this study show clearly that a *C. manginecans* was responsible for the wilt outbreak disease that has recently appeared on the *M. elengi* agricultural field in Indralaya (Ogan Ilir) and roadside trees in Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir), South Sumatra. This is the first report of a *Ceratocystis* wilt disease on *M. elengi* in Indonesia. Formerly the disease was reported in Thailand (Pornsuriya and Sunpapao, 2015), however, they just observed a single tree and they did not conduct pathogenicity test and observe morphological characteristics. In Indonesia Tarigan et al. (2010; 2011) reported *C. manginecans* caused wilt and die-back disease in *A. mangium* plantations in Riau.

Mimusops elengi infected with *Ceratocystis* has wilted foliage symptoms, sudden wilt, decline and branch drying, and progressive loss of the canopy resulting in tree death. *M. elengi* trees showed typical symptoms of infection by the *Ceratocystis* fungus; the same was true of a serious wilt pathogen of *A. mangium* and *A. crassicarpa* in Indonesia and Vietnam (Tarigan et al. 2011; Thu et al. 2012; Thu et al. 2016). Vascular wilt, wood stain and stem cankers are the most characteristic symptoms of infection by *C. manginecans* in woody trees (Harrington 2013). Rot in roots or stems, vascular wilt, sapwood discoloration and cankers are symptomatic of plants infected by *C. manginecans* species (Oliveira et al. 2015).

The comparison of morphological characteristics and gene sequences (β-tubulin and ITS) of the isolates examined in this study were similar to those in descriptions given for *C. manginecans* isolated from diseased *Acacia* trees which form part of the *C. fimbriata* s. l. complex, which is typified by *C. fimbriata* sensu stricto (Engelbrecht and Harrington 2005; Tarigan et al. 2011). The two isolates from Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) have 100% identical sequences of ITS and Beta Tubulin representing a single clone of the pathogen. This supports the view that *M. elengi* wilt disease in Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) emerged from a single introduction of a single haplotype and the population has expanded clonally to infect *M. elengi* trees in many parts of all three regions.

Ceratocystis manginecans was reported in Indonesia associated with wilt and die-back disease on *Acacia* spp. (Tarigan et al. 2011). It was found that many acacia plants were attacked by *Ceratocystis* disease and *Hypocryphalus mangifera* insects in the field raised the suspicion that *M. elengi* were infected by *Ceratocystis* in acacia plants. *H. mangifera* is a vector insect for the spread of *Ceratocystis* in the world (Fourie et al. 2016; Al-Adawi et al. 2013; Van Wyk et al. 2007; Masood et al. 2008). Pruning *M. elengi* branches using equipment that has previously been infected with *Ceratocystis* also increases the severity of the disease; this can be seen by the number of plants that are attacked after pruning with tools used to prune *Acacia* plants that are attacked by *Ceratocystis*. Cankers of *Ceratocystis* were associated with branch wounds from pruning, pruning time and pruning technique on plants (Chi et al. 2019b). In this study, infected bullet wood was observed surrounding infected acacia by *C. manginecans* with abundant *H. mangifera.* This might be caused by spore contamination on cutting tools after pruning diseased acacia and spore spreading by insect vector *H. mangifera*.

The pathogenicity of *C. manginecans* to these hosts was demonstrated in inoculation trials and it is clear that the fungus is responsible for the widespread death of these trees. It was also possible to show that isolates of the pathogen from these trees are able to infect and kill other plants. The eight *C. manginecans* isolates all formed lesions on the stems of *M. elengi* seedlings when inoculated under the bark in nursery cloches. The most pathogenic was CAME30815 and CAME30819 isolate from *M. elengi* and the CAW30814 isolate from *A. mangium* resulted in foliar symptoms with severity index 3.6, 3.1 and 2.8 after 45 days from inoculation with pathogen. When the isolates were tested on seedling *A. mangium*, the seedlings were infected and mostly dead as they were the main host plant and susceptible to *C. manginecans* (Tarigan et al. 2011).

Previous researchers of *C. manginecans* on *M. elengi* in Thailand have reported molecular identification *C. manginecans* by primers combination the ITS, β-tubulin (βt) and transcribed elongation factor 1-α (TEF1-α) gene regions with one isolate. In our research, we tested two isolates for sequencing, seven isolates for pathogenicity, and Koch's postulates assay using three isolates (CAME30815, CAME30816 and CAME30817) from Ogan Ilir (Indralaya); two isolates (CAME30818 and CAME30814) from Jakabaring (Palembang); and two isolates (CAME30819 and

CAME30813) from Kayuagung (Ogan Komering Ilir) and one isolate (CAW30814) from diseased *Acacia*, *A. mangium*. All isolates showed the ability to infect both bullet wood and *Acacia,* and all isolates can be reisolated confirming Koch's postulates.

The wilt disease of *M. elengi* appears to be serious and it is clearly a new host tree or pathogen association that has apparently occurred due to a host shift. This category of diseases is increasing in importance in plants, and they can devastate native trees that have not previously encountered them (Roy 2001; Anderson et al. 2004; Slippers et al. 2005; Woolhouse et al. 2005; Desprez-Loustau et al. 2007; Wingfield et al. 2010). In this regard, the wilt disease of *M. elengi* can impact seriously on the natural biodiversity of Indonesia, and studies should be instituted to understand them better. The findings of this study will give knowledge of the characteristics of the symptoms of the disease, their causes and help to minimise the spread of wilt disease in *M. elengi* in plantations or roadside trees and to consider its possible pathogenicity.

This study presents the first report of *Ceratocystis* wilt or sudden decline disease of bullet wood in Indonesia and the discovery of a fungus that has been identified as *C. manginecans.* The disease of bullet wood that gave rise to this study is serious and management options to reduce its incidence are required *C. manginecans* is an aggressive pathogen and a deeper understanding of its role in tree death will be important in the future.

ACKNOWLEDGEMENT

This research was funded by a PMDSU scholarship in the fiscal year of 2019-2021 according to the Director of Research and Community Service, Directorate of Research and Community Service (DRPM), Directorate General for Research and Development, Ministry of Research, Technology, and Higher Education Indonesia, Number: 068/SP2H/AMD/LT/DRPM/2020 chaired by Ahmad Muslim.

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BIODIVERSITAS
Volume 22, Number 5, May 2021
Pages: xxxx

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d2205xx

First report of bullet wood (*Mimusops elengi*) sudden decline disease caused by *Ceratocystis manginecans* in Indonesia

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Manuscript received: xxx. Revision accepted: xxx April 2021.

Abstract. *Pratama R, Muslim A, Suwandi S, Damiri N, Soleha S. 2021. First report of bullet wood (*Mimusops elengi*) sudden decline disease caused by* Ceratocystis manginecans *in Indonesia. Biodiversitas 22: xxxx. Ceratocystis manginecans* causes wilt and death of plants in several important crops and native vegetation in Indonesia. Ceratocystis wilt was recently found to be causing substantial mortality in bullet wood (*Mimusops elengi*) in South Sumatra. The aim of this study was to describe the symptomatology of the new disease and characterize isolates of *C. manginecans* obtained from diseased bullet wood plants. Diseased plants showed substantial discoloration of the woody xylem and wilt-type symptoms of the foliage, with the eventual death of the whole plant. Isolations from infected plants yielded fungi that were similar morphologically to *C. manginecans*, with typical hat-shaped ascospores and light-coloured perithecial bases. Sequencing of the internal transcribed spacer (ITS) and β-tubulin of the isolates confirmed their identification, grouping them with *C. manginecans* and separating them from all other *Ceratocystis* species. This is the first report of *C. manginecans* in Indonesia causing wilt and death on bullet wood. *C. manginecans* is an important pathogen, and strategies to reduce losses need to be established in Indonesia because the aggressiveness of *C. manginecans* to bullet wood has been shown in inoculation experiments

Keywords: Ceratocystidaceae, Molecular phylogeny, Pathogenicity, Sapotaceae

INTRODUCTION

Bullet wood (*Mimusops elengi*) belongs to the family Sapotaceae, common English names are Asian Bulletwood, Bullet Wood Tree, Indian Medlar, Red Coondoo Spanish Cherry and it is known in Indonesia as Tanjung. The species is native to India, Sri Lanka, the Andaman Islands, Myanmar, Indo-China, Peninsular Malaysia and Vanuatu; it has been introduced and cultivated elsewhere. *M. elengi* can grow in tropical and subtropical climates. This plant thrives in areas with high humidity and seasonal rainfall and seasonal dry periods (Lim 2012). The bullet wood trees range from small to large, and are found in all parts of Indonesia where bullet wood is cultivated in gardens as an ornamental tree, for medicines and planted along avenues because of its fragrant flowers (Seth 2003).

M. elengi is widely used for medicine, and various parts of *M. elengi* Linn. (Sapotaceae) have been used widely in traditional Indian medicine for the treatment of pain, inflammation and wounds. *M. elengi* stem bark would be a possible therapeutic candidate having cytotoxic and anti-tumour potential (Kumar et al. 2016); it also has antibacterial and antifungal uses (Ali et al. 2008). At present, *M. elengi* is used as the synthesis of calcium phosphate nanoparticles that is easy, eco-friendly and scalable (Pokale et al. 2014).

Several types of pathogenic fungi have been identified to cause disease in *M. elengi* plants. *Curvularia lunata* caused die-back in India (Khatun et al. 2011); *Pestalotiopsis clavispora* caused leaf blight (Lokesh et al. 2017). *Ceratocystis* was first isolated from a single tree of bullet wood showing sudden decline in Thailand. Symptoms displayed by the diseased trees include gum exudation from the trunks and wilting and loss of the dark green foliage with a corresponding browning of leaves on single branches. In this study they did not confirm the pathogen with a Koch postulates test detail (Pornsuriya and Sunpapao 2015). Recently we have observed many bullet wood trees showing similar symptoms with *C. manginecans* decline in many locations in South Sumatra, Indonesia.

C. manginecans includes many economically important plant pathogens. This pathogen has caused a sudden decline and has led to the death of thousands of *Mangifera indica* trees in Oman with *Hypocryphalus mangifera* vector (Al Adawi et al. 2013). In Indonesia *C. manginecans* caused die-back on *Acacia mangium* and *A. crassicarpa* plantations in Riau (Tarigan et al. 2010), whereas in Vietnam recently, *C. manginecans* caused wilt disease in *Dalbergia tonkinensis* and *Chukrasia tabularis* (Chi et al. 2019a; Chi et al. 2020); in Pakistan this pathogen also causes wilt disease in *Albizia lebbeck* (Razzaq et al. 2020). Commonly *C. manginecans* causes yellowing of leaves and rapid wilting of leaves was observed on individual branches in affected trees that ultimately spread to the canopy followed by the death of the whole tree. Dark brown to black tissue discoloration was observed in the woody xylem tissues of infected trees.

This study aimed to identify the cause of a new outbreak of wilt disease causing a sudden decline to the trees, wilted canopies and tree death in *M. elengi* in South Sumatera, Indonesia. This study was also conducted to describe the characteristics of the pathogen and confirm Koch's postulates test.

MATERIALS AND METHODS

Disease symptoms and specimen collection

The distribution and impact of the *C. manginecans* disease on *M. elengi* were determined from roadside trees planting in Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) and the agricultural field of Sriwijaya University in Indralaya (Ogan Ilir), South Sumatra, Indonesia. Symptoms of wilt diseases were evaluated as follows: the extent of lesion development from discoloration of bark and wood, the extent of foliar wilting or loss and tree death.

Samples of diseased trunks were collected from two to six-year-old trees in September 2019 to April 2020. Wood samples were taken from lesions of wilted trees using a knife sterilised in 70% ethanol. The wood samples collected from *M. elengi* showed brown to black streaking in the woody xylem. Each sample was wrapped in tissue paper and placed in a coolbox. The same day, the wood samples (1–20 mm length, 1–2 mm thick) were sandwiched between two slices of fresh carrot and placed on sterile dry paper in plastic boxes at 25 °C following the method of Li et al. (2014). After 5-10 days, hat-shaped spores of putative *Ceratocystis* pathogens were placed on 2% (w/v) malt extract agar (MEA) (Merck, Germany), and incubated at 25 °C in a laboratory. When cultures had grown to several cm in diameter, hyphal tips were sub-cultured onto new MEA and potato dextrose agar (PDA) (Merck, Germany) plates and incubated at 25-28 °C. Morphological traits of fruiting bodies and spores were observed under an optical Olympus CX33 microscope (Olympus Corporation, Japan).

Genomic DNA extraction, PCR amplification, and sequencing

DNA isolation used YeaStar Genomic DNA Kit (Zymo Research Corporation, California, USA). To extract genomic DNA, cultures were incubated for five days to allow sufficient mycelial growth in potato dextrose broth (PDB) (Merck, Germany). Mycelium was purified with sterile filter paper (Whatman) and transferred to 1.5 mL Eppendorf tubes. The quantity and quality of DNA extracted was evaluated with a spectrophotometer (NanoDrop ND-1000; Thermo Fisher, Waltham, MA, USA) to calibrate the concentration and purity of DNA as PCR templates.

The PCR amplification reactions were conducted on a T-100 thermal cycler (Bio-Rad, Hercules, CA, USA). Amplifications were carried out in 50 µl reactions containing 20 µl DreamTaq Green PCR Master Mix (Eppendorf, Germany) (DreamTaq DNA Polymerase, 2X DreamTaq Green buffer, dNTPs, and 4 mM $MgCl_2$), 1,5 µl of each forward and reverse primer, 4 µl of DNA template and 23 µl sterilised water. The PCRs were performed with a C1000 Touch™ thermal cycler (Bio-Rad, USA). The PCR cycling parameters were as follows: initial denaturation for 5 min at 95 °C, followed by 35 cycles at 95 °C for 30 s, 56 °C for 45 s and 72 °C for 1 min. Amplification was completed at 72 °C for 10 min and the PCR product was stored at 10 °C (Chi et al. 2020).

PCR amplifications were made for two gene regions, including part of the b-tubulin (BT) using primers βt1a (TTCCCCCGTCTCCACTTCTTCATG) and βt1b (GACGAGATCGTTCATGTTGAACTC) (Oliveira et al. 2015), and the ITS using ITS1 and ITS4. The resulting PCR products were submitted to 1st BASE (Malaysia) for forward and reverse sequencing reactions. Raw sequence data were assembled, examined, and manually edited using Genestudio 2.1.1.5 (Genestudio, Suwanee, Georgia) and BioEdit software (van der Nest et al. 2019). The DNA sequences were compared to the GenBank database via the nucleotide-nucleotide BLAST search interface located at the National Center for Biotechnology Information, Bethesda, USA. Relevant sequences were transferred with NoteTab Light v7.2.

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Phylogenetic analyses

The sequences of *Ceratocystis* spp. closely related to the one from *Mimusops elengi* were retrieved from GenBank. Phylogenetic sequences from different gene regions were aligned using Mesquite v3.5 (Maddison and Maddison 2018) (http://mesquiteproject.org) and corrected manually. Phylogenetic trees based on a concatenated data set of the ITS and βt were computed and analysed as a single dataset. Maximum Parsimony (MP) analyses were performed in MEGA v. 10 (Kumar et al. 2016; Paul et al. 2018) with 1000 bootstrap replications.

Pathogenicity tests

Pathogenicity studies were conducted on two agroforestry plants, *A. mangium* and *M. elengi*. Plants had stem diameters of 2–3 cm and heights <1 m. The pathogenic potential of isolates was evaluated by the under bark inoculation method described by Deidda et al. (2016). Bark was wounded to expose the cambium using a 4 mm cork borer, and discs of agar bearing mycelium taken from the margins of actively growing, 2-week-old cultures on 2% MEA (Tarigan et al. 2010; Tarigan et al. 2011; Chi et al. 2019a, Chi et al. 2020), *Ceratocystis* isolates were placed with the mycelium facing the cambium. Ten plants of each tree species were inoculated with sterile MEA plugs to serve as controls. All inoculation points and the ends of the logs were covered with masking tape and polyethylene films, respectively, to prevent desiccation of the inoculum and cambium, and to reduce contamination.

The nursery trial evaluated seven *Ceratocystis* strains isolated from *M. elengi* (CAME30813, CAME30814, CAME30815, CAME30816, CAME30817, CAME30818 and CAME30819) and one strain (CAW30814) from *A. mangium*. There were ten replicate plants per treatment in each row plot in each of the blocks. After 45 days, lesion (L) length and foliar symptoms severity were recorded. Representative wood samples were taken from within

lesions outside the inoculation area, and the pathogen reisolated and sequenced for Koch's postulates test.

Foliar symptoms severity (FS) was assessed using a scale of 0 to 4: 0 = healthy; 1 = lower leaves yellow; 2 = slight wilting; 3 = severe wilting; 4 = dead (Muslim et al. 2003; Chi et al. 2019a; Muslim et al. 2019).

The pathogenicity test data were analysed using SAS university edition software package. Analysis of variance (ANOVA) and Tukey's honestly significant difference (Tukey's HSD) test were used to determine whether there were significant differences in comparisons of means of different treatments.

RESULT AND DISCUSSION

Symptoms of *Mimusops elengi* wilt disease

We observed disease symptoms from September 2019 to April 2020, in various places that are widely planted in the Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) areas, South Sumatra, Indonesia. The disease was found scattered throughout the planted area, with symptoms of partially dead trees, foliar wilting or loss and tree death (Figure 1a). Initially, the leaves of infected plants lost turgor and brightness, with yellowing symptoms in the older leaves, followed by wilting and death of the plant. Symptomatic plant stems showed xylem discoloration (Figure 1b), infections generally started in the roots and then moved upwards in the stem ultimately reaching the upper branches of the entire plant, and the plants ultimately died (Figure 1c). Death of adjacent plants indicates transmission of root infection because these pathogens are also known as soil-borne pathogens. The severity of the infection is also caused by pruning the branches using tools previously used to cut the infected plants.

Observation of diseased plant xylem tissue in cross-sections of the stem showed dark brown lesion formation in the cambium (inner bark region) towards vascular tissue (Figure 1d). In the initial stage of plant infection, the foliar symptoms, wilt and the fruits appeared normal, but as the infection progressed, the fruits of affected plants were smaller, shrivelled, wrinkled and dry. Many of the bark beetle vectors of *C. manginecans, Hypocryphalus mangiferae* were found around bullet wood diseases (Figure 1e). Testing by the Li et al. (2014) method showed that *Ceratocystis* had grown on the carrots, and ascomata of *C. manginecans* with necks supporting sticky masses of ascospores on the carrot slices (Figure 1f).

Sampling and isolation

Seven isolates of *C. manginecans* were collected from diseased bullet wood (*M. elengi*) (Figure 2). There were three isolates (CAME30815, CAME30816 and CAME30817) from Ogan Ilir (Indralaya); two isolates (CAME30818 and CAME30814) from Jakabaring (Palembang); and two isolates (CAME30819 and CAME30813) from Kayuagung (Ogan Komering Ilir). We also isolated one isolate (CAW30814) from diseased *acacia*, *A. mangium* in the agricultural field of Sriwijaya University, Indralaya.

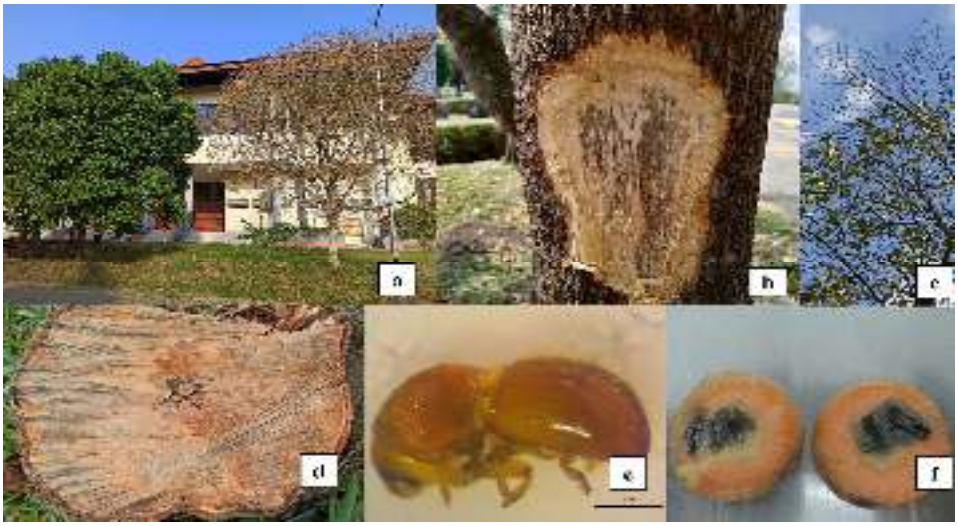

Figure 1. Symptoms of *Ceratocystis manginecans* wilt disease in bullet wood*:* **a**. tree death of *M. elengi*: b. sap stain mould on bullet wood, c. wilted leaves of bullet wood, d. sap stain mould on bullet wood, e. The bark beetle vector of *C. manginecans*, *Hypocryphalus mangiferae,* f. isolation of the fungus from discoloured xylem showing dark mycelium and sporulation on the carrot slices

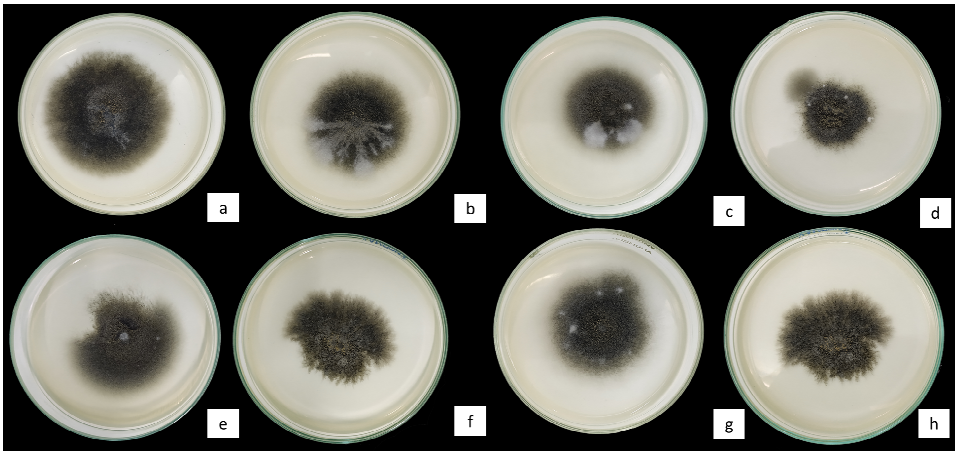

Figure 2. Isolates of *Ceratocystis manginecans* and related species grown on malt extract agar (MEA) for 7 d at 25 °C. **a, b, c**: *Ceratocystis* CAME30815, CAME30816 and CAME30817, from *Mimusops elengi* in Sriwijaya University, Indralaya. **d, e, f:** *Ceratocystis* CAME30819, CAME30813 and CAME30814 from *Mimusops elengi* in Jakabaring, Palembang. **g**: *Ceratocystis* CAME30818, from *Mimusops elengi* in Kayuagung, Ogan Komering Ilir. **h**: *Ceratocystis* CAW30814, from *Acacia mangium* in Indralaya

Fungal morphology

Seven isolates were morphologically indistinguishable (Table 2). At 7–14 days of incubation at 25 °C on MEA, cultures were pale brown to dark brown and produced a banana-like odour. Mycelium on MEA was grey, and the reverse side of the colony olivaceous grey; submerged mycelium darkened as the ascomata developed, forming fine, radiating fibrils. Ascomata developing within seven days and mature within ten days, superficially or partly embedded in the agar, dark brown to black (Figure 3a). Ascomatal bases were submerged or on the agar surface, dark bases dark brown to black, base subglobes to globes, (134.58-) 169.12 - 276.29 (-310.83) µm long and (122.91-) 161.89-244.14 (-283.13) µm wide in diameter (Figure 3a). Ascomata necks were erect, occasionally curved, black at the base becoming subhyaline towards the apex, smooth to crenulate, (346.51-) 454.94-720.16 (-828.59) µm long including ostiolar hyphae (Figure3b). Ascospores were hat-shaped, (3.61-) 5.64-6.23 (-6.93) µm length and (2.06-) 2.279-3.67 (-3.85) µm width (Figure 3f). Barrel conidia (8.62-) 8.85-12.79 (-13.25) µm length and (5.89-) 4.12x6.87 (-8.67) µm width. Bacilliform conidia (9.05-) 10.82-22.32 (-35.97) µm length and (2.01-) 2.83-5.71 (-8.87) µm width (Figure 3c). Chlamydospores oval, thick-walled, smooth, (8.21-) 9.15-16.21 (-18.50) µm length and (4.92-) 6.46-15.81 (14.65) µm width (Figure 3e).

Sequence analysis

To confirm the identify of the wilt pathogen, the ITS and β-tubulin 1 gene sequences of two isolates from bullet wood (*M. elengi*) were compared to reference sequences downloaded from the gene bank database (NCBI GenBank) (Table 1) and indicated that isolates from Indonesia were grouped within the *C. fimbriata*. s.l species complex and were most closely related to *C. manginecans*. PCR amplification resulted in fragments of ~550 base pairs (bp) in size which had 100% homology with *C. manginecans* (Figure 4). Bootstrap values were equal to or greater than 50%, derived from 1000 iterations.

Pathogenicity

The results of the pathogenicity tests of seven isolates (CAME30813, CAME30814, CAME30815, CAME30816, CAME30817, CAME30818, and CAME30819) on *M. elengi* and one isolate (CAW30814) from *A. mangium* are shown in Table 3. All isolates tested showed a varying reaction to lesion and foliar symptoms (Figure 5). For the pathogenicity test on *M. elengi*, seven isolates from *M. elengi* as well as one isolate from *A. mangium* strongly infected the wood and produced significant lesion length ranging from 3.99 to 10.47 cm and showed significant differences from the control. Three isolates from *M. elengi* (CAME30815; CAME30819; CAME30818) as well as one isolate from *A. mangium* (CAW30814) showed high pathogenicity on the foliar symptom (with foliar severity index of 2.3-3.6 and lesion length 6.52-10.47 cm), while the other isolates (CAME30817; CAME30816; CAME30814, CAME30813) showed moderate pathogenicity to *M. elengi* (foliar severity index of 1.4-2.0 and lesion length 3.99-6.02 cm). When the isolates were tested for their pathogenicity on *A. mangium* as the primary host of the pathogen, two isolates (CAME30815; CAME30819) showed strong pathogenicity on lesion length (13.76-11.89 cm) and also provided high pathogenicity on foliar severity index (4), where all plants tested were dead. Three isolates (CAW30814;

CAME30818; CAME30817) showed moderate pathogenicity on lesion length 8.71-10.14 cm and foliar severity index 2.8-3.2, while the other isolates (CAME30816, CAME30814, CAME30813) showed low pathogenicity on lesion length 7.19-8.63 cm and foliar severity index 1.9-2.4.

Table 1. *Ceratocystis* isolates considered in the phylogenetic analyses

Isolate no	Identify	Host	Geographic origin	Collector	Gene region/GeneBank accession no	
					ITS	BT
CAME30819	*C.manginecans*	*Mimusops elengi*	Indonesia	R.Pratama	MT373423	Submitted
CAME30818	*C.manginecans*	*Mimusops elengi*	Indonesia	R.Pratama	MT373424	Submitted
CMW13851	*C.manginecans*	*Mangifera indica*	Oman	M. Deadman	AY953383	EF433308
CMW23643	*C.manginecans*	*Mangifera indica*	Pakistan	A.Al-Adawi	EF433304	EF433313
CMW23641	*C.manginecans*	*Mangifera indica*	Pakistan	A.Al-Adawi	EF433305	EF433314
CMW23634	*C.manginecans*	*Mangifera indica*	Pakistan	A. Al-Adawi	EF433302	EF433311
CMW21125	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588663	EU588642
CMW21123	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588662	EU588641
CMW22581	*C.manginecans*	*Acacia mangium*	Indonesia	M. Tarigan	EU588659	EU604671
CMW21132	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588665	EU588644
CMW22579	*C.manginecans*	*Acacia mangium*	Indonesia	M. Tarigan	EU588658	EU588638
CMW23628	*C.manginecans*	*Hypocryphalus mangifera*	Pakistan	A. Al-Adawi	EF433303	EF433312
CMW13854	*C.manginecans*	*Mangifera indica*	Oman	M. Deadman	AY953385	EF433310
CMW13852	*C.manginecans*	*Hypocryphalus mangifera*	Oman	M. Deadman	AY953384	EF433309
CMW4068	*C.albifundus*	*A. mearnsii*	RSA	J. Roux	DQ520638	EF070429
CMW14793	*C.caryae*	*C. cordiformis*	U.S.A	J. Johnson	EF070424	EF070439
CMW14800	*C.smalleyi*	*C. cordiformis*	U.S.A	G. Smalley	EF070420	EF070436
CBS114724						
CMW14789	*C. populicola*	*Populus* sp.	Poland	J. Gremmen	EF070418	EF070434
CMW19385	*C.atrox*	*E. grandis*	Australia	M.J. Wingfield	EF070415	EF070431
CBS115778						
CMW11424	*C. polycroma*	*Syzygium aromaticum*	Indonesia	M.J. Wingfield	AY528970	AY528966
CMW23808	*C. obpyriformis*	*A. mearnsii*	South Africa	R.N. Heath	EU245003	EU244975
CMW6579	*C.pirilliformis*	*E. nitens*	Australia	M.J. Wingfield	AF427105	DQ371653

Table 2. Morphological comparisons of *Ceratocystis manginecans* and other phylogenetically closely related species

Character	***Ceratocystis manginecans* (from *M. elengi*)**	***Ceratocystis acaciivora* (from *A. mangium*)**	***Ceratocystis manginecans* (from *A. mangium*)**
Ascomata base	(134.58-) 169.12 - 276.29 (-310.83) x (122.91-) 161.89-244.14 (-283.13)[a]	(105-) 131-175 (-206) x (107-) 125-167 (-188)	(132.1-) 175.3 (−233.2)
Ascomata base average	220.01x211.63[b]		
Ascomata neck	(346.51-) 454.94-720.16 (-828.59)	(301-) 348-448 (-522)	(327.3-) 452.7 (−556)
Ascomata neck average	568.41		
Ascospores	(3.61-) 5.64-6.23 (-6.93) x (2.06-) 2.279-3.67 (-3.85)	5-7 x 3-4	(3.1-) 4.2 (−4.7) x (4.1-) 5.5 (−6.8)
Ascospores average	5.62 x 3.93		
Bacilliform conidia	(9.05-) 10.82-22.32 (-35.97) x (2.01-) 2.83-5.71 (-8.87)	(11-) 14-22 (-29) x 3-5	(14.6-) 18.6 (−30.7)x (3.0-) 4.6 (−5.4)
Bacilliform conidia average	16.56x4.27		
Barrel-shaped conidia	(8.62-) 8.85-12.79 (-13.25) x (5.89-) 4.12x6.87 (-8.67)	(8-) 9-11 (-13) x 4-6	(6.8) 8.1 (−10.6)
Barrel-shaped conidia average	11.497 x 15.82		
Clamydospore	(8.21-) 9.15-16.21 (-18.50) x (4.92-) 6.46-15.81 (14.65)	(10-) 12-14 (-15) x (7-) 8-12 (-14)	(10.1-) 13.1 (−15.5) x (6.1-) 9.2 (−11.1)
Clamydospore average	11.13 x 14.18		
Reference	This study	M. Tarigan et al. 2010	Chi et al. 2019

Note: All measurements are in µm. [a] Measurements are presented in the format [(minimum-) (average-standard deviation) - (average-standard deviation) (-maximum)]. [b] Measurements are presented in the format minimum x maximum

Table 3. Pathogenicity of *Ceratocystis* isolates on *Mimusops elengi* and *Acacia mangium* under nursery condition

Isolates	Host test	*M. elengi*		*A. mangium*	
		Lesion length (cm)	Foliar symptoms	Lesion Length (cm)	Foliar symptoms
CAME30815	10	10.47e	3.6	13.77d	4
CAME30819	10	8.29de	3.1	11.89cd	4
CAW30814	10	7.35cd	2.8	10.14bcd	3.2
CAME30818	10	6.52bcd	2.3	9.92bcd	3.1
CAME30817	10	6.02bcd	2	8.72bc	2.8
CAME30816	10	5.27bc	1.8	8.47bc	2.4
CAME30814	10	4.93bc	1.5	8.64bc	2
CAME30813	10	3.99b	1.4	7.19b	1.9
Control (MEA)	10	0.1a	0	0.1a	0
Fpr		<0.001		<0.001	

Note: Values followed by the same letters in a column are not different among isolates at P=0.05 according to Tukey's HSD multiple range test.

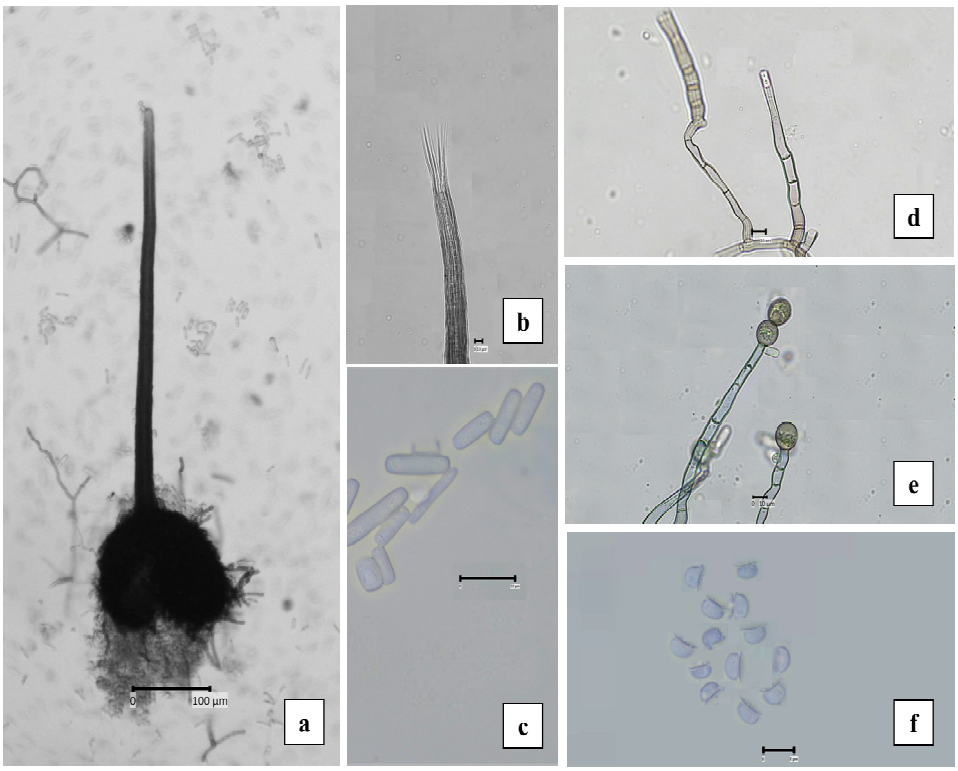

Figure 3. Morphological characteristics of *Ceratocystis* isolated from *M. elengi* stem lesion: a. globose ascomata with long neck, b. divergent ostiolar hyphae, c. barrel-shaped conidia, d. conidiophore/phialide, e. chlamydospores, f. hat-shaped ascospores. Scale bars: a = 100 µm; b,c,d,e = 10 µm; f = 5 µm

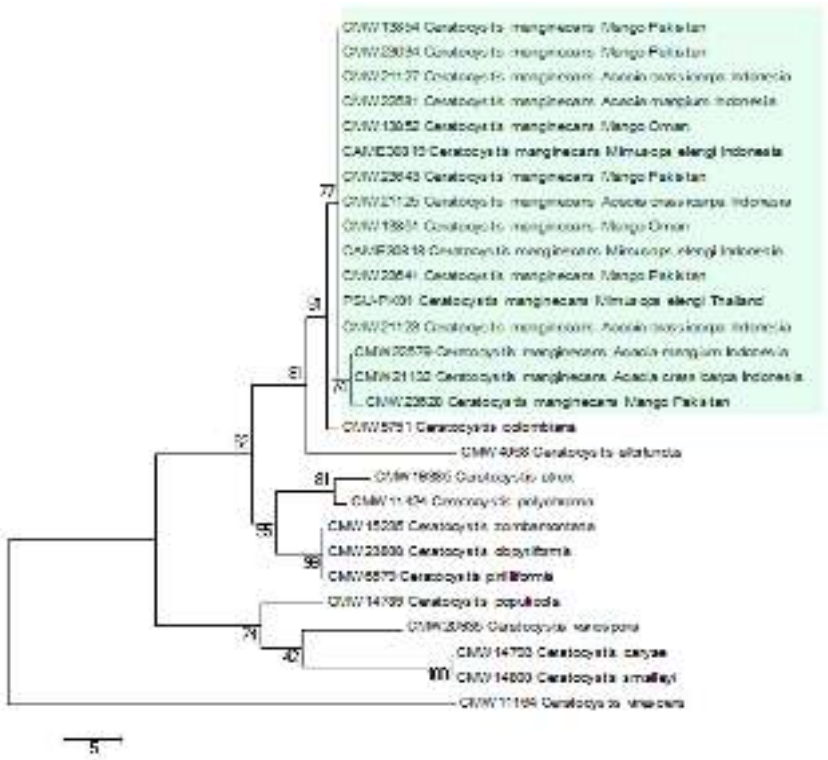

Figure 4. Phylogenetic tree constructed by MEGA with Maximum Parsimony (MP). The tree was built using combined sequence data of the ITS region and b-tubulin gene and their related species from GenBank. Consistency (CI), retention (RI), and composite indexes (CoI) are 0.714286, 0.837209, and 0.664843 for all sites and parsimony-informative sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Bootstrap values >50% are indicated above the branches. The analysis involved 28 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 493 positions in the final dataset

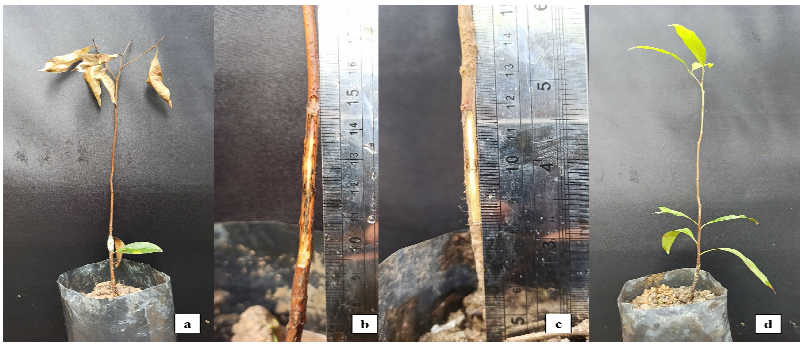

Figure 5. Response after 45 days of *Mimusops elengi* seedlings to under-bark inoculation with mycelium of *Ceratocystis manginecans.* **a.** wilting of seedlings; **b**. lesions on the stem; c. no lesions on the stem; d. normal foliage on the control seedling which received MEA only and appeared healthy. Yellow arrow indicates the point of inoculation, red arrows show the lesion boundary

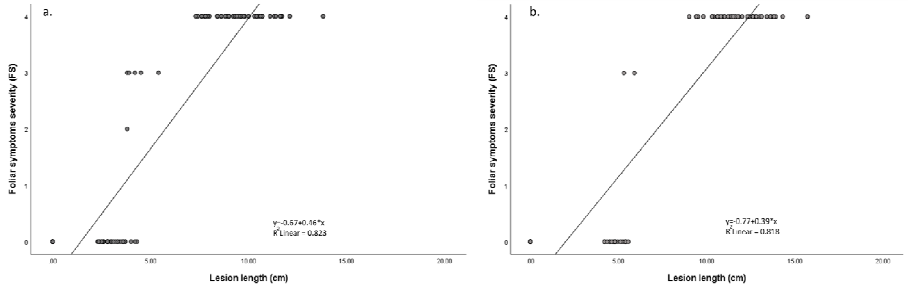

Figure 6. Correlation between foliar symptoms severity (FS) and lesion length (cm): **a.** *Mimusops elengi,* **b.** *Acacia mangium*

There was a strong linear correlation between foliar symptom severity and lesion length for *M. elengi* and *A. mangium* (Figure 6). The Koch's postulates test was conducted by reisolating the pathogen, and its identity confirmed to be the same as *C. manginecans*.

Discussion

The results of this study show clearly that a *C. manginecans* was responsible for the wilt outbreak disease that has recently appeared on the *M. elengi* agricultural field in Indralaya (Ogan Ilir) and roadside trees in Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir), South Sumatra. This is the first report of a *Ceratocystis* wilt disease on *M. elengi* in Indonesia. Formerly the disease was reported in Thailand (Pornsuriya and Sunpapao 2015), however, they just observed a single tree and they did not conduct pathogenicity test and observe morphological characteristics. In Indonesia Tarigan et al. (2010; 2011) reported *C. manginecans* caused wilt and die-back disease in *A. mangium* plantations in Riau.

Mimusops elengi infected with *Ceratocystis* has wilted foliage symptoms, sudden wilt, decline and branch drying, and progressive loss of the canopy resulting in tree death. *M. elengi* trees showed typical symptoms of infection by the *Ceratocystis* fungus; the same was true of a serious wilt pathogen of *A. mangium* and *A. crassicarpa* in Indonesia and Vietnam (Tarigan et al. 2011; Thu et al. 2012; Thu et al. 2016). Vascular wilt, wood stain and stem cankers are the most characteristic symptoms of infection by *C. manginecans* in woody trees (Harrington 2013). Rot in roots or stems, vascular wilt, sapwood discoloration and cankers are symptomatic of plants infected by *C. manginecans* species (Oliveira et al. 2015).

The comparison of morphological characteristics and gene sequences (β-tubulin and ITS) of the isolates examined in this study were similar to those in descriptions given for *C. manginecans* isolated from diseased *Acacia* trees which form part of the *C. fimbriata* s. l. complex, which is typified by *C. fimbriata* sensu stricto (Engelbrecht and Harrington 2005; Tarigan et al. 2011). The two isolates from Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) have 100% identical sequences of ITS and Beta Tubulin representing a single clone of the pathogen. This supports the view that *M. elengi* wilt disease in Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) emerged from a single introduction of a single haplotype and the population has expanded clonally to infect *M. elengi* trees in many parts of all three regions.

Ceratocystis manginecans was reported in Indonesia associated with wilt and die-back disease on *Acacia* spp. (Tarigan et al. 2011). It was found that many acacia plants were attacked by *Ceratocystis* disease and *Hypocryphalus mangifera* insects in the field raised the suspicion that *M. elengi* were infected by *Ceratocystis* in acacia plants. *H. mangifera* is a vector insect for the spread of *Ceratocystis* in the world (Van Wyk et al. 2007; Masood et al. 2008; Al-Adawi et al. 2013; Fourie et al. 2016). Pruning *M. elengi* branches using equipment that has previously been infected with *Ceratocystis* also increases the severity of the disease; this can be seen by the number of plants that are attacked after pruning with tools used to prune *Acacia* plants that are attacked by *Ceratocystis*. Cankers of *Ceratocystis* were associated with branch wounds from pruning, pruning time and pruning technique on plants (Chi et al. 2019b). In this study, infected bullet wood was observed surrounding infected acacia by *C. manginecans* with abundant *H. mangifera.* This might be caused by spore contamination on cutting tools after pruning diseased acacia and spore spreading by insect vector *H. mangifera*.

The pathogenicity of *C. manginecans* to these hosts was demonstrated in inoculation trials and it is clear that the fungus is responsible for the widespread death of these trees. It was also possible to show that isolates of the pathogen from these trees are able to infect and kill other plants. The eight *C. manginecans* isolates all formed lesions on the stems of *M. elengi* seedlings when inoculated under the bark in nursery cloches. The most pathogenic was CAME30815 and CAME30819 isolate from *M. elengi* and the CAW30814 isolate from *A. mangium* resulted in foliar symptoms with severity index 3.6, 3.1 and 2.8 after 45 days from inoculation with pathogen. When the isolates were tested on seedling *A. mangium*, the seedlings were

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infected and mostly dead as they were the main host plant and susceptible to *C. manginecans* (Tarigan et al. 2011).

Previous researchers of *C. manginecans* on *M. elengi* in Thailand have reported molecular identification *C. manginecans* by primers combination the ITS, β-tubulin (βt) and transcribed elongation factor 1-α (TEF1-α) gene regions with one isolate. In our research, we tested two isolates for sequencing, seven isolates for pathogenicity, and Koch's postulates assay using three isolates (CAME30815, CAME30816 and CAME30817) from Ogan Ilir (Indralaya); two isolates (CAME30818 and CAME30814) from Jakabaring (Palembang); and two isolates (CAME30819 and CAME30813) from Kayuagung (Ogan Komering Ilir) and one isolate (CAW30814) from diseased *Acacia*, *A. mangium*. All isolates showed the ability to infect both bullet wood and *Acacia,* and all isolates can be reisolated confirming Koch's postulates.

The wilt disease of *M. elengi* appears to be serious and it is clearly a new host tree or pathogen association that has apparently occurred due to a host shift. This category of diseases is increasing in importance in plants, and they can devastate native trees that have not previously encountered them (Roy 2001; Anderson et al. 2004; Slippers et al. 2005; Woolhouse et al. 2005; Desprez-Loustau et al. 2007; Wingfield et al. 2010). In this regard, the wilt disease of *M. elengi* can impact seriously on the natural biodiversity of Indonesia, and studies should be instituted to understand them better. The findings of this study will give knowledge of the characteristics of the symptoms of the disease, their causes and help to minimise the spread of wilt disease in *M. elengi* in plantations or roadside trees and to consider its possible pathogenicity.

This study presents the first report of *Ceratocystis* wilt or sudden decline disease of bullet wood in Indonesia and the discovery of a fungus that has been identified as *C. manginecans.* The disease of bullet wood that gave rise to this study is serious and management options to reduce its incidence are required *C. manginecans* is an aggressive pathogen and a deeper understanding of its role in tree death will be important in the future.

ACKNOWLEDGEMENTS

This research was funded by a PMDSU scholarship in the fiscal year of 2019-2021 according to the Director of Research and Community Service, Directorate of Research and Community Service (DRPM), Directorate General for Research and Development, Ministry of Research, Technology, and Higher Education Indonesia, Number: 068/SP2H/AMD/LT/DRPM/2020 chaired by Ahmad Muslim.

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BIODIVERSITAS
Volume 22, Number 5, May 2021
Pages: xxxx

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d2205xx

First report of bullet wood (*Mimusops elengi*) sudden decline disease caused by *Ceratocystis manginecans* in Indonesia

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Manuscript received: xxx. Revision accepted: xxx April 2021.

Abstract. *Pratama R, Muslim A, Suwandi S, Damiri N, Soleha S. 2021. First report of bullet wood (*Mimusops elengi*) sudden decline disease caused by* Ceratocystis manginecans *in Indonesia. Biodiversitas 22: xxxx. Ceratocystis manginecans* causes wilt and death of plants in several important crops and native vegetation in Indonesia. Ceratocystis wilt was recently found to be causing substantial mortality in bullet wood (*Mimusops elengi*) in South Sumatra. The aim of this study was to describe the symptomatology of the new disease and characterize isolates of *C. manginecans* obtained from diseased bullet wood plants. Diseased plants showed substantial discoloration of the woody xylem and wilt-type symptoms of the foliage, with the eventual death of the whole plant. Isolations from infected plants yielded fungi that were similar morphologically to *C. manginecans*, with typical hat-shaped ascospores and light-coloured perithecial bases. Sequencing of the internal transcribed spacer (ITS) and β-tubulin of the isolates confirmed their identification, grouping them with *C. manginecans* and separating them from all other *Ceratocystis* species. This is the first report of *C. manginecans* in Indonesia causing wilt and death on bullet wood. *C. manginecans* is an important pathogen, and strategies to reduce losses need to be established in Indonesia because the aggressiveness of *C. manginecans* to bullet wood has been shown in inoculation experiments

Keywords: Ceratocystidaceae, Molecular phylogeny, Pathogenicity, Sapotaceae

INTRODUCTION

Bullet wood (*Mimusops elengi*) belongs to the family Sapotaceae, common English names are Asian Bulletwood, Bullet Wood Tree, Indian Medlar, Red Coondoo Spanish Cherry and it is known in Indonesia as Tanjung. The species is native to India, Sri Lanka, the Andaman Islands, Myanmar, Indo-China, Peninsular Malaysia and Vanuatu; it has been introduced and cultivated elsewhere. *M. elengi* can grow in tropical and subtropical climates. This plant thrives in areas with high humidity and seasonal rainfall and seasonal dry periods (Lim 2012). The bullet wood trees range from small to large, and are found in all parts of Indonesia where bullet wood is cultivated in gardens as an ornamental tree, for medicines and planted along avenues because of its fragrant flowers (Seth 2003).

M. elengi is widely used for medicine, and various parts of *M. elengi* Linn. (Sapotaceae) have been used widely in traditional Indian medicine for the treatment of pain, inflammation and wounds. *M. elengi* stem bark would be a possible therapeutic candidate having cytotoxic and anti-tumour potential (Kumar et al. 2016); it also has antibacterial and antifungal uses (Ali et al. 2008). At present, *M. elengi* is used as the synthesis of calcium phosphate nanoparticles that is easy, eco-friendly and scalable (Pokale et al. 2014).

Several types of pathogenic fungi have been identified to cause disease in *M. elengi* plants. *Curvularia lunata* caused die-back in India (Khatun et al. 2011); *Pestalotiopsis clavispora* caused leaf blight (Lokesh et al. 2017). *Ceratocystis* was first isolated from a single tree of bullet wood showing sudden decline in Thailand. Symptoms displayed by the diseased trees include gum exudation from the trunks and wilting and loss of the dark green foliage with a corresponding browning of leaves on single branches. In this study they did not confirm the pathogen with a Koch postulates test detail (Pornsuriya and Sunpapao 2015). Recently we have observed many bullet wood trees showing similar symptoms with *C. manginecans* decline in many locations in South Sumatra, Indonesia.

C. manginecans includes many economically important plant pathogens. This pathogen has caused a sudden decline and has led to the death of thousands of *Mangifera indica* trees in Oman with *Hypocryphalus mangifera* vector (Al Adawi et al. 2013). In Indonesia *C. manginecans* caused die-back on *Acacia mangium* and *A. crassicarpa* plantations in Riau (Tarigan et al. 2010), whereas in Vietnam recently, *C. manginecans* caused wilt disease in *Dalbergia tonkinensis* and *Chukrasia tabularis* (Chi et al. 2019a; Chi et al. 2020); in Pakistan this pathogen also causes wilt disease in *Albizia lebbeck* (Razzaq et al. 2020). Commonly *C. manginecans* causes yellowing of leaves and rapid wilting of leaves was observed on individual branches in affected trees that ultimately spread to the canopy followed by the death of the whole tree. Dark brown to black tissue discoloration was observed in the woody xylem tissues of infected trees.

This study aimed to identify the cause of a new outbreak of wilt disease causing a sudden decline to the trees, wilted canopies and tree death in *M. elengi* in South Sumatera, Indonesia. This study was also conducted to describe the characteristics of the pathogen and confirm Koch's postulates test.

MATERIALS AND METHODS

Disease symptoms and specimen collection

The distribution and impact of the *C. manginecans* disease on *M. elengi* were determined from roadside trees planting in Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) and the agricultural field of Sriwijaya University in Indralaya (Ogan Ilir), South Sumatra, Indonesia. Symptoms of wilt diseases were evaluated as follows: the extent of lesion development from discoloration of bark and wood, the extent of foliar wilting or loss and tree death.

Samples of diseased trunks were collected from two to six-year-old trees in September 2019 to April 2020. Wood samples were taken from lesions of wilted trees using a knife sterilised in 70% ethanol. The wood samples collected from *M. elengi* showed brown to black streaking in the woody xylem. Each sample was wrapped in tissue paper and placed in a coolbox. The same day, the wood samples (1–20 mm length, 1–2 mm thick) were sandwiched between two slices of fresh carrot and placed on sterile dry paper in plastic boxes at 25 °C following the method of Li et al. (2014). After 5-10 days, hat-shaped spores of putative *Ceratocystis* pathogens were placed on 2% (w/v) malt extract agar (MEA) (Merck, Germany), and incubated at 25 °C in a laboratory. When cultures had grown to several cm in diameter, hyphal tips were sub-cultured onto new MEA and potato dextrose agar (PDA) (Merck, Germany) plates and incubated at 25-28 °C. Morphological traits of fruiting bodies and spores were observed under an optical Olympus CX33 microscope (Olympus Corporation, Japan).

Genomic DNA extraction, PCR amplification, and sequencing

DNA isolation used YeaStar Genomic DNA Kit (Zymo Research Corporation, California, USA). To extract genomic DNA, cultures were incubated for five days to allow sufficient mycelial growth in potato dextrose broth (PDB) (Merck, Germany). Mycelium was purified with sterile filter paper (Whatman) and transferred to 1.5 mL Eppendorf tubes. The quantity and quality of DNA extracted was evaluated with a spectrophotometer (NanoDrop ND-1000; Thermo Fisher, Waltham, MA, USA) to calibrate the concentration and purity of DNA as PCR templates.

The PCR amplification reactions were conducted on a T-100 thermal cycler (Bio-Rad, Hercules, CA, USA). Amplifications were carried out in 50 µl reactions containing 20 µl DreamTaq Green PCR Master Mix (Eppendorf, Germany) (DreamTaq DNA Polymerase, 2X DreamTaq Green buffer, dNTPs, and 4 mM $MgCl_2$), 1,5 µl of each forward and reverse primer, 4 µl of DNA template and 23 µl sterilised water. The PCRs were performed with a C1000 Touch™ thermal cycler (Bio-Rad, USA). The PCR cycling parameters were as follows: initial denaturation for 5 min at 95 °C, followed by 35 cycles at 95 °C for 30 s, 56 °C for 45 s and 72 °C for 1 min. Amplification was completed at 72 °C for 10 min and the PCR product was stored at 10 °C (Chi et al. 2020).

PCR amplifications were made for two gene regions, including part of the b-tubulin (BT) using primers βt1a (TTCCCCCGTCTCCACTTCTTCATG) and βt1b (GACGAGATCGTTCATGTTGAACTC) (~~Oliveira et al. 2015~~Oliveira et al. 2015a), and the ITS using ITS1 and ITS4. The resulting PCR products were submitted to 1st BASE (Malaysia) for forward and reverse sequencing reactions. Raw sequence data were assembled, examined, and manually edited using Genestudio 2.1.1.5 (Genestudio, Suwanee, Georgia) and BioEdit software (van der Nest et al. 2019). The DNA sequences were compared to the GenBank database via the nucleotide-nucleotide BLAST search interface located at the National Center for Biotechnology Information, Bethesda, USA. Relevant sequences were transferred with NoteTab Light v7.2.

Commented [Gdn1]: 2015 a or 2015 b ?

Phylogenetic analyses

The sequences of *Ceratocystis* spp. closely related to the one from *Mimusops elengi* were retrieved from GenBank. Phylogenetic sequences from different gene regions were aligned using Mesquite v3.5 (Maddison and Maddison 2018) (http://mesquiteproject.org) and corrected manually. Phylogenetic trees based on a concatenated data set of the ITS and βt were computed and analysed as a single dataset. Maximum Parsimony (MP) analyses were performed in MEGA v. 10 (Kumar et al. 2016; Paul et al. 2018) with 1000 bootstrap replications.

Pathogenicity tests

Pathogenicity studies were conducted on two agroforestry plants, *A. mangium* and *M. elengi*. Plants had stem diameters of 2–3 cm and heights <1 m. The pathogenic potential of isolates was evaluated by the under bark inoculation method described by Deidda et al. (2016). Bark was wounded to expose the cambium using a 4 mm cork borer, and discs of agar bearing mycelium taken from the margins of actively growing, 2-week-old cultures on 2% MEA (Tarigan et al. 2010; Tarigan et al. 2011; Chi et al. 2019a, Chi et al. 2020), *Ceratocystis* isolates were placed with the mycelium facing the cambium. Ten plants of each tree species were inoculated with sterile MEA plugs to serve as controls. All inoculation points and the ends of the logs were covered with masking tape and polyethylene films, respectively, to prevent desiccation of the inoculum and cambium, and to reduce contamination.

The nursery trial evaluated seven *Ceratocystis* strains isolated from *M. elengi* (CAME30813, CAME30814, CAME30815, CAME30816, CAME30817, CAME30818 and CAME30819) and one strain (CAW30814) from *A. mangium*. There were ten replicate plants per treatment in each row plot in each of the blocks. After 45 days, lesion (L) length and foliar symptoms severity were recorded. Representative wood samples were taken from within

lesions outside the inoculation area, and the pathogen reisolated and sequenced for Koch's postulates test.

Foliar symptoms severity (FS) was assessed using a scale of 0 to 4: 0 = healthy; 1 = lower leaves yellow; 2 = slight wilting; 3 = severe wilting; 4 = dead (Muslim et al. 2003; Chi et al. 2019a; Muslim et al. 2019).

The pathogenicity test data were analysed using SAS university edition software package. Analysis of variance (ANOVA) and Tukey's honestly significant difference (Tukey's HSD) test were used to determine whether there were significant differences in comparisons of means of different treatments.

RESULT AND DISCUSSION

Symptoms of *Mimusops elengi* wilt disease

We observed disease symptoms from September 2019 to April 2020, in various places that are widely planted in the Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) areas, South Sumatra, Indonesia. The disease was found scattered throughout the planted area, with symptoms of partially dead trees, foliar wilting or loss and tree death (Figure 1a). Initially, the leaves of infected plants lost turgor and brightness, with yellowing symptoms in the older leaves, followed by wilting and death of the plant. Symptomatic plant stems showed xylem discoloration (Figure 1b), infections generally started in the roots and then moved upwards in the stem ultimately reaching the upper branches of the entire plant, and the plants ultimately died (Figure 1c). Death of adjacent plants indicates transmission of root infection because these pathogens are also known as soil-borne pathogens. The severity of the infection is also caused by pruning the branches using tools previously used to cut the infected plants.

Observation of diseased plant xylem tissue in cross-sections of the stem showed dark brown lesion formation in the cambium (inner bark region) towards vascular tissue (Figure 1d). In the initial stage of plant infection, the foliar symptoms, wilt and the fruits appeared normal, but as the infection progressed, the fruits of affected plants were smaller, shrivelled, wrinkled and dry. Many of the bark beetle vectors of *C. manginecans, Hypocryphalus mangiferae* were found around bullet wood diseases (Figure 1e). Testing by the Li et al. (2014) method showed that *Ceratocystis* had grown on the carrots, and ascomata of *C. manginecans* with necks supporting sticky masses of ascospores on the carrot slices (Figure 1f).

Sampling and isolation

Seven isolates of *C. manginecans* were collected from diseased bullet wood (*M. elengi*) (Figure 2). There were three isolates (CAME30815, CAME30816 and CAME30817) from Ogan Ilir (Indralaya); two isolates (CAME30818 and CAME30814) from Jakabaring (Palembang); and two isolates (CAME30819 and CAME30813) from Kayuagung (Ogan Komering Ilir). We also isolated one isolate (CAW30814) from diseased *acacia*, *A. mangium* in the agricultural field of Sriwijaya University, Indralaya.

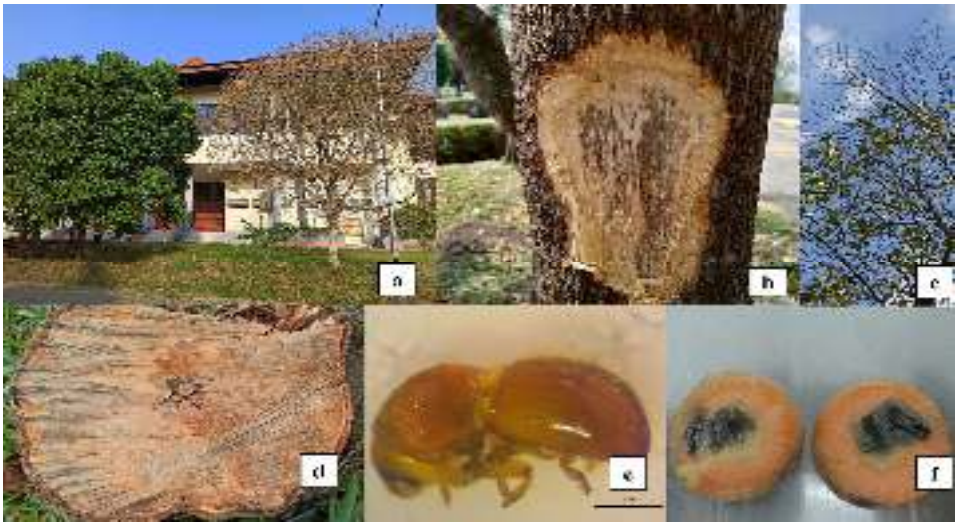

Figure 1. Symptoms of *Ceratocystis manginecans* wilt disease in bullet wood*:* **a**. tree death of *M. elengi*: b. sap stain mould on bullet wood, c. wilted leaves of bullet wood, d. sap stain mould on bullet wood, e. The bark beetle vector of *C. manginecans*, *Hypocryphalus mangiferae,* f. isolation of the fungus from discoloured xylem showing dark mycelium and sporulation on the carrot slices

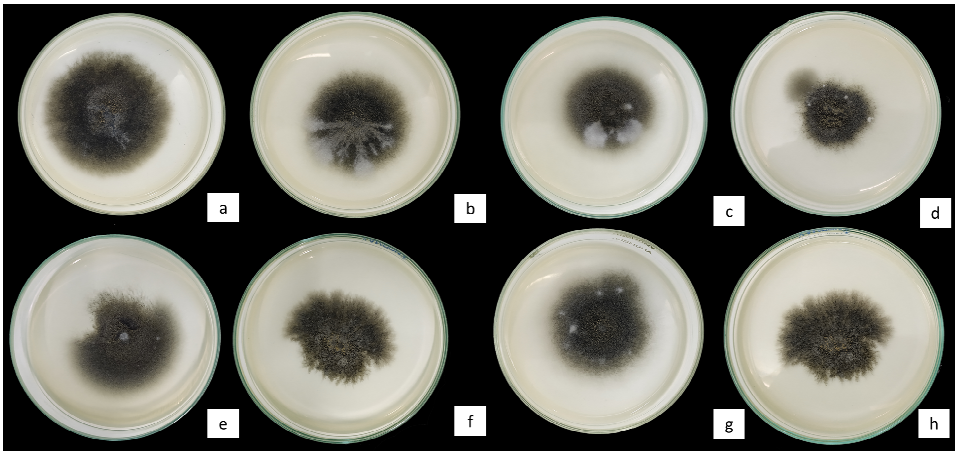

Figure 2. Isolates of *Ceratocystis manginecans* and related species grown on malt extract agar (MEA) for 7 d at 25 °C. **a, b, c**: *Ceratocystis* CAME30815, CAME30816 and CAME30817, from *Mimusops elengi* in Sriwijaya University, Indralaya. **d, e, f:** *Ceratocystis* CAME30819, CAME30813 and CAME30814 from *Mimusops elengi* in Jakabaring, Palembang. **g**: *Ceratocystis* CAME30818, from *Mimusops elengi* in Kayuagung, Ogan Komering Ilir. **h**: *Ceratocystis* CAW30814, from *Acacia mangium* in Indralaya

Fungal morphology

Seven isolates were morphologically indistinguishable (Table 2). At 7–14 days of incubation at 25 °C on MEA, cultures were pale brown to dark brown and produced a banana-like odour. Mycelium on MEA was grey, and the reverse side of the colony olivaceous grey; submerged mycelium darkened as the ascomata developed, forming fine, radiating fibrils. Ascomata developing within seven days and mature within ten days, superficially or partly embedded in the agar, dark brown to black (Figure 3a). Ascomatal bases were submerged or on the agar surface, dark bases dark brown to black, base subglobes to globes, (134.58-) 169.12 - 276.29 (-310.83) µm long and (122.91-) 161.89-244.14 (-283.13) µm wide in diameter (Figure 3a). Ascomata necks were erect, occasionally curved, black at the base becoming subhyaline towards the apex, smooth to crenulate, (346.51-) 454.94-720.16 (-828.59) µm long including ostiolar hyphae (Figure3b). Ascospores were hat-shaped, (3.61-) 5.64-6.23 (-6.93) µm length and (2.06-) 2.279-3.67 (-3.85) µm width (Figure 3f). Barrel conidia (8.62-) 8.85-12.79 (-13.25) µm length and (5.89-) 4.12x6.87 (-8.67) µm width. Bacilliform conidia (9.05-) 10.82-22.32 (-35.97) µm length and (2.01-) 2.83-5.71 (-8.87) µm width (Figure 3c). Chlamydospores oval, thick-walled, smooth, (8.21-) 9.15-16.21 (-18.50) µm length and (4.92-) 6.46-15.81 (14.65) µm width (Figure 3e).

Sequence analysis

To confirm the identify of the wilt pathogen, the ITS and β-tubulin 1 gene sequences of two isolates from bullet wood (*M. elengi*) were compared to reference sequences downloaded from the gene bank database (NCBI GenBank) (Table 1) and indicated that isolates from Indonesia were grouped within the *C. fimbriata*. s.l species complex and were most closely related to *C. manginecans*. PCR amplification resulted in fragments of ~550 base pairs (bp) in size which had 100% homology with *C. manginecans* (Figure 4). Bootstrap values were equal to or greater than 50%, derived from 1000 iterations.

Pathogenicity

The results of the pathogenicity tests of seven isolates (CAME30813, CAME30814, CAME30815, CAME30816, CAME30817, CAME30818, and CAME30819) on *M. elengi* and one isolate (CAW30814) from *A. mangium* are shown in Table 3. All isolates tested showed a varying reaction to lesion and foliar symptoms (Figure 5). For the pathogenicity test on *M. elengi*, seven isolates from *M. elengi* as well as one isolate from *A. mangium* strongly infected the wood and produced significant lesion length ranging from 3.99 to 10.47 cm and showed significant differences from the control. Three isolates from *M. elengi* (CAME30815; CAME30819; CAME30818) as well as one isolate from *A. mangium* (CAW30814) showed high pathogenicity on the foliar symptom (with foliar severity index of 2.3-3.6 and lesion length 6.52-10.47 cm), while the other isolates (CAME30817; CAME30816; CAME30814, CAME30813) showed moderate pathogenicity to *M. elengi* (foliar severity index of 1.4-2.0 and lesion length 3.99-6.02 cm). When the isolates were tested for their pathogenicity on *A. mangium* as the primary host of the pathogen, two isolates (CAME30815; CAME30819) showed strong pathogenicity on lesion length (13.76-11.89 cm) and also provided high pathogenicity on foliar severity index (4), where all plants tested were dead. Three isolates (CAW30814;

CAME30818; CAME30817) showed moderate pathogenicity on lesion length 8.71-10.14 cm and foliar severity index 2.8-3.2, while the other isolates (CAME30816, CAME30814, CAME30813) showed low pathogenicity on lesion length 7.19-8.63 cm and foliar severity index 1.9-2.4.

Table 1. *Ceratocystis* isolates considered in the phylogenetic analyses

Isolate no	Identify	Host	Geographic origin	Collector	Gene region/GeneBank accession no	
					ITS	BT
CAME30819	*C.manginecans*	*Mimusops elengi*	Indonesia	R.Pratama	MT373423	Submitted
CAME30818	*C.manginecans*	*Mimusops elengi*	Indonesia	R.Pratama	MT373424	Submitted
CMW13851	*C.manginecans*	*Mangifera indica*	Oman	M. Deadman	AY953383	EF433308
CMW23643	*C.manginecans*	*Mangifera indica*	Pakistan	A.Al-Adawi	EF433304	EF433313
CMW23641	*C.manginecans*	*Mangifera indica*	Pakistan	A.Al-Adawi	EF433305	EF433314
CMW23634	*C.manginecans*	*Mangifera indica*	Pakistan	A. Al-Adawi	EF433302	EF433311
CMW21125	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588663	EU588642
CMW21123	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588662	EU588641
CMW22581	*C.manginecans*	*Acacia mangium*	Indonesia	M. Tarigan	EU588659	EU604671
CMW21132	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588665	EU588644
CMW22579	*C.manginecans*	*Acacia mangium*	Indonesia	M. Tarigan	EU588658	EU588638
CMW23628	*C.manginecans*	*Hypocryphalus mangifera*	Pakistan	A. Al-Adawi	EF433303	EF433312
CMW13854	*C.manginecans*	*Mangifera indica*	Oman	M. Deadman	AY953385	EF433310
CMW13852	*C.manginecans*	*Hypocryphalus mangifera*	Oman	M. Deadman	AY953384	EF433309
CMW4068	*C.albifundus*	*A. mearnsii*	RSA	J. Roux	DQ520638	EF070429
CMW14793	*C.caryae*	*C. cordiformis*	U.S.A	J. Johnson	EF070424	EF070439
CMW14800	*C.smalleyi*	*C. cordiformis*	U.S.A	G. Smalley	EF070420	EF070436
CBS114724						
CMW14789	*C. populicola*	*Populus* sp.	Poland	J. Gremmen	EF070418	EF070434
CMW19385	*C.atrox*	*E. grandis*	Australia	M.J. Wingfield	EF070415	EF070431
CBS115778						
CMW11424	*C. polycroma*	*Syzygium aromaticum*	Indonesia	M.J. Wingfield	AY528970	AY528966
CMW23808	*C. obpyriformis*	*A. mearnsii*	South Africa	R.N. Heath	EU245003	EU244975
CMW6579	*C.pirilliformis*	*E. nitens*	Australia	M.J. Wingfield	AF427105	DQ371653

Table 2. Morphological comparisons of *Ceratocystis manginecans* and other phylogenetically closely related species

Character	***Ceratocystis manginecans* (from *M. elengi*)**	***Ceratocystis acaciivora* (from *A. mangium*)**	***Ceratocystis manginecans* (from *A. mangium*)**
Ascomata base	(134.58-) 169.12 - 276.29 (-310.83) x (122.91-) 161.89-244.14 (-283.13)[a]	(105-) 131-175 (-206) x (107-) 125-167 (-188)	(132.1-) 175.3 (−233.2)
Ascomata base average	220.01x211.63[b]		
Ascomata neck	(346.51-) 454.94-720.16 (-828.59)	(301-) 348-448 (-522)	(327.3-) 452.7 (−556)
Ascomata neck average	568.41		
Ascospores	(3.61-) 5.64-6.23 (-6.93) x (2.06-) 2.279-3.67 (-3.85)	5-7 x 3-4	(3.1-) 4.2 (−4.7) x (4.1-) 5.5 (−6.8)
Ascospores average	5.62 x 3.93		
Bacilliform conidia	(9.05-) 10.82-22.32 (-35.97) x (2.01-) 2.83-5.71 (-8.87)	(11-) 14-22 (-29) x 3-5	(14.6-) 18.6 (−30.7)x (3.0-) 4.6 (−5.4)
Bacilliform conidia average	16.56x4.27		
Barrel-shaped conidia	(8.62-) 8.85-12.79 (-13.25) x (5.89-) 4.12x6.87 (-8.67)	(8-) 9-11 (-13) x 4-6	(6.8) 8.1 (−10.6)
Barrel-shaped conidia average	11.497 x 15.82		
Clamydospore	(8.21-) 9.15-16.21 (-18.50) x (4.92-) 6.46-15.81 (14.65)	(10-) 12-14 (-15) x (7-) 8-12 (-14)	(10.1-) 13.1 (−15.5) x (6.1-) 9.2 (−11.1)
Clamydospore average	11.13 x 14.18		
Reference	This study	M. Tarigan et al. 2010	Chi et al. 2019

Note: All measurements are in µm. [a] Measurements are presented in the format [(minimum-) (average-standard deviation) - (average-standard deviation) (-maximum)]. [b] Measurements are presented in the format minimum x maximum

Table 3. Pathogenicity of *Ceratocystis* isolates on *Mimusops elengi* and *Acacia mangium* under nursery condition

Isolates	Host test	*M. elengi*		*A. mangium*	
		Lesion length (cm)	Foliar symptoms	Lesion Length (cm)	Foliar symptoms
CAME30815	10	10.47e	3.6	13.77d	4
CAME30819	10	8.29de	3.1	11.89cd	4
CAW30814	10	7.35cd	2.8	10.14bcd	3.2
CAME30818	10	6.52bcd	2.3	9.92bcd	3.1
CAME30817	10	6.02bcd	2	8.72bc	2.8
CAME30816	10	5.27bc	1.8	8.47bc	2.4
CAME30814	10	4.93bc	1.5	8.64bc	2
CAME30813	10	3.99b	1.4	7.19b	1.9
Control (MEA)	10	0.1a	0	0.1a	0
Fpr		<0.001		<0.001	

Note: Values followed by the same letters in a column are not different among isolates at P=0.05 according to Tukey's HSD multiple range test.

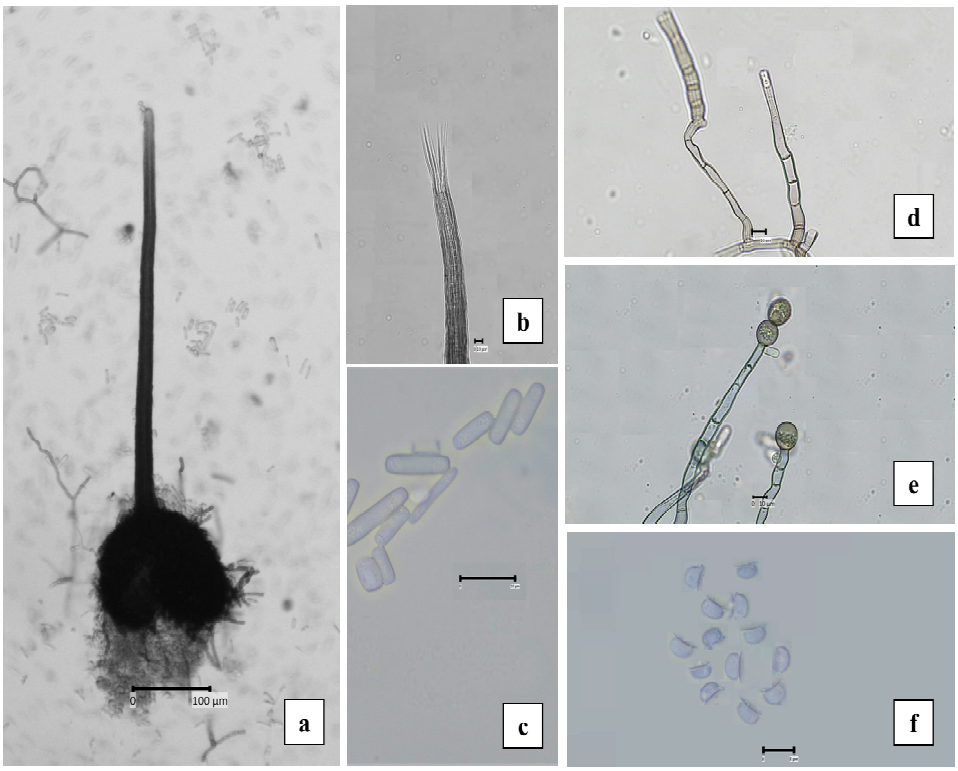

Figure 3. Morphological characteristics of *Ceratocystis* isolated from *M. elengi* stem lesion: a. globose ascomata with long neck, b. divergent ostiolar hyphae, c. barrel-shaped conidia, d. conidiophore/phialide, e. chlamydospores, f. hat-shaped ascospores. Scale bars: a = 100 µm; b,c,d,e = 10 µm; f = 5 µm

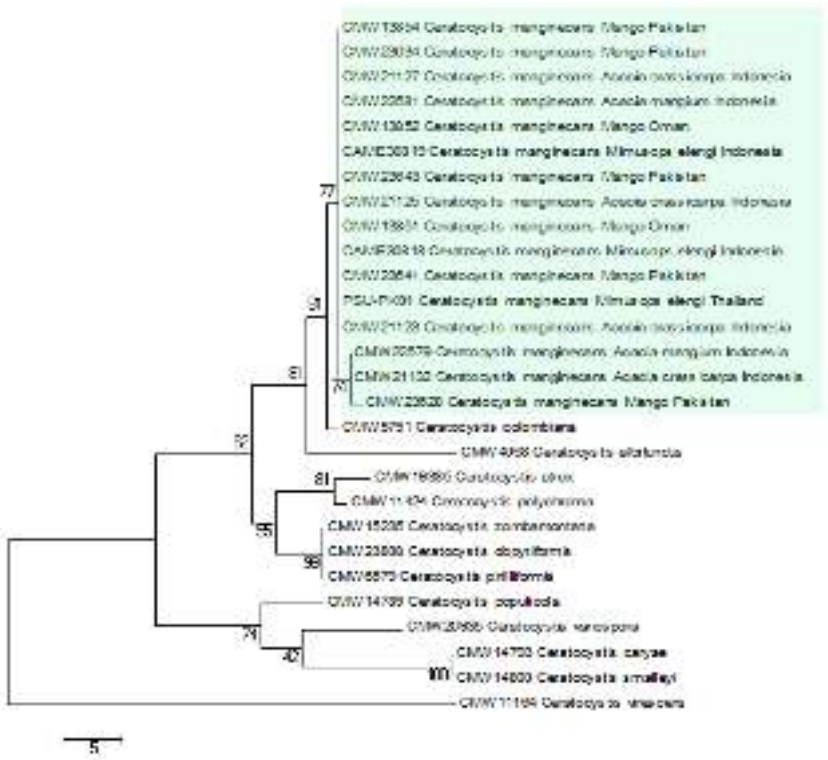

Figure 4. Phylogenetic tree constructed by MEGA with Maximum Parsimony (MP). The tree was built using combined sequence data of the ITS region and b-tubulin gene and their related species from GenBank. Consistency (CI), retention (RI), and composite indexes (CoI) are 0.714286, 0.837209, and 0.664843 for all sites and parsimony-informative sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Bootstrap values >50% are indicated above the branches. The analysis involved 28 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 493 positions in the final dataset

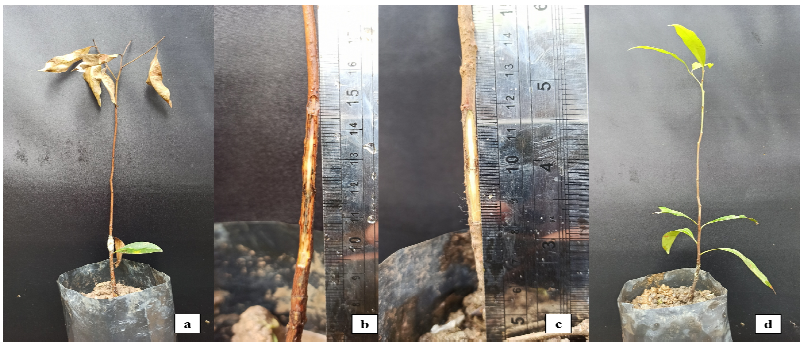

Figure 5. Response after 45 days of *Mimusops elengi* seedlings to under-bark inoculation with mycelium of *Ceratocystis manginecans.* **a.** wilting of seedlings; **b**. lesions on the stem; c. no lesions on the stem; d. normal foliage on the control seedling which received MEA only and appeared healthy. Yellow arrow indicates the point of inoculation, red arrows show the lesion boundary

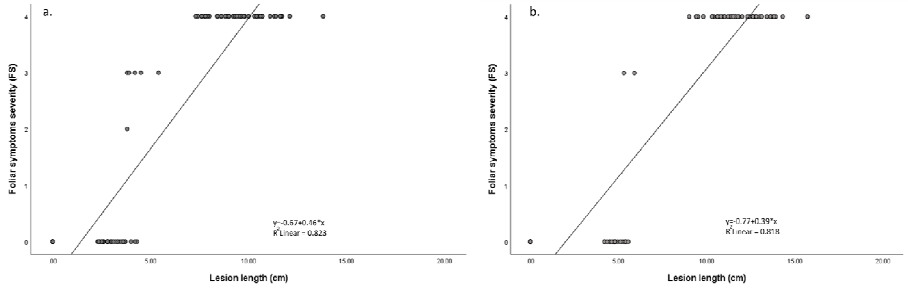

Figure 6. Correlation between foliar symptoms severity (FS) and lesion length (cm): **a.** *Mimusops elengi,* **b.** *Acacia mangium*

There was a strong linear correlation between foliar symptom severity and lesion length for *M. elengi* and *A. mangium* (Figure 6). The Koch's postulates test was conducted by reisolating the pathogen, and its identity confirmed to be the same as *C. manginecans*.

Discussion

The results of this study show clearly that a *C. manginecans* was responsible for the wilt outbreak disease that has recently appeared on the *M. elengi* agricultural field in Indralaya (Ogan Ilir) and roadside trees in Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir), South Sumatra. This is the first report of a *Ceratocystis* wilt disease on *M. elengi* in Indonesia. Formerly the disease was reported in Thailand (Pornsuriya and Sunpapao 2015), however, they just observed a single tree and they did not conduct pathogenicity test and observe morphological characteristics. In Indonesia Tarigan et al. (2010; 2011) reported *C. manginecans* caused wilt and die-back disease in *A. mangium* plantations in Riau and Suwandi et al. (2021) have reported infection of this disease on *Lansium domesticum* tree.

Mimusops elengi infected with *Ceratocystis* has wilted foliage symptoms, sudden wilt, decline and branch drying, and progressive loss of the canopy resulting in tree death. *M. elengi* trees showed typical symptoms of infection by the *Ceratocystis* fungus; the same was true of a serious wilt pathogen of *A. mangium* and *A. crassicarpa* in Indonesia and Vietnam (Tarigan et al. 2011; Thu et al. 2012; Thu et al. 2016). Vascular wilt, wood stain and stem cankers are the most characteristic symptoms of infection by *C. manginecans* in woody trees (Harrington 2013). Rot in roots or stems, vascular wilt, sapwood discoloration and cankers are symptomatic of plants infected by *C. manginecans* species (~~Oliveira et al. 2015~~Oliveira et al. 2015b).

The comparison of morphological characteristics and gene sequences (β-tubulin and ITS) of the isolates examined in this study were similar to those in descriptions given for *C. manginecans* isolated from diseased *Acacia* trees which form part of the *C. fimbriata* s. l. complex, which is typified by *C. fimbriata* sensu stricto (Engelbrecht and Harrington 2005; Tarigan et al. 2011). The two isolates from Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) have 100% identical sequences of ITS and Beta Tubulin representing a single clone of the pathogen. This supports the view that *M. elengi* wilt disease in Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) emerged from a single introduction of a single haplotype and the population has expanded clonally to infect *M. elengi* trees in many parts of all three regions.

Ceratocystis manginecans was reported in Indonesia associated with wilt and die-back disease on *Acacia* spp. (Tarigan et al. 2011). It was found that many acacia plants were attacked by *Ceratocystis* disease and *Hypocryphalus mangifera* insects in the field raised the suspicion that *M. elengi* were infected by *Ceratocystis* in acacia plants. *H. mangifera* is a vector insect for the spread of *Ceratocystis* in the world (Van Wyk et al. 2007; Masood et al. 2008; Al-Adawi et al. 2013; Fourie et al. 2016). Pruning *M. elengi* branches using equipment that has previously been infected with *Ceratocystis* also increases the severity of the disease; this can be seen by the number of plants that are attacked after pruning with tools used to prune *Acacia* plants that are attacked by *Ceratocystis*. Cankers of *Ceratocystis* were associated with branch wounds from pruning, pruning time and pruning technique on plants (Chi et al. 2019b). In this study, infected bullet wood was observed surrounding infected acacia by *C. manginecans* with abundant *H. mangifera.* This might be caused by spore contamination on cutting tools after pruning diseased acacia and spore spreading by insect vector *H. mangifera*.

The pathogenicity of *C. manginecans* to these hosts was demonstrated in inoculation trials and it is clear that the fungus is responsible for the widespread death of these trees. It was also possible to show that isolates of the pathogen from these trees are able to infect and kill other plants. The eight *C. manginecans* isolates all formed lesions on the stems of *M. elengi* seedlings when inoculated under the bark in nursery cloches. The most pathogenic was CAME30815 and CAME30819 isolate from *M. elengi* and the CAW30814 isolate from *A. mangium* resulted in foliar symptoms with severity index 3.6, 3.1 and 2.8 after

Commented [Gdn2]: 2015 a or 2015b ?

45 days from inoculation with pathogen. When the isolates were tested on seedling *A. mangium*, the seedlings were infected and mostly dead as they were the main host plant and susceptible to *C. manginecans* (Tarigan et al. 2011).

Previous researchers of *C. manginecans* on *M. elengi* in Thailand have reported molecular identification *C. manginecans* by primers combination the ITS, β-tubulin (βt) and transcribed elongation factor 1-α (TEF1-α) gene regions with one isolate. In our research, we tested two isolates for sequencing, seven isolates for pathogenicity, and Koch's postulates assay using three isolates (CAME30815, CAME30816 and CAME30817) from Ogan Ilir (Indralaya); two isolates (CAME30818 and CAME30814) from Jakabaring (Palembang); and two isolates (CAME30819 and CAME30813) from Kayuagung (Ogan Komering Ilir) and one isolate (CAW30814) from diseased *Acacia*, *A. mangium*. All isolates showed the ability to infect both bullet wood and *Acacia,* and all isolates can be reisolated confirming Koch's postulates.

The wilt disease of *M. elengi* appears to be serious and it is clearly a new host tree or pathogen association that has apparently occurred due to a host shift. This category of diseases is increasing in importance in plants, and they can devastate native trees that have not previously encountered them (Roy 2001; Anderson et al. 2004; Slippers et al. 2005; Woolhouse et al. 2005; Desprez-Loustau et al. 2007; Wingfield et al. 2010). In this regard, the wilt disease of *M. elengi* can impact seriously on the natural biodiversity of Indonesia, and studies should be instituted to understand them better. The findings of this study will give knowledge of the characteristics of the symptoms of the disease, their causes and help to minimise the spread of wilt disease in *M. elengi* in plantations or roadside trees and to consider its possible pathogenicity.

This study presents the first report of *Ceratocystis* wilt or sudden decline disease of bullet wood in Indonesia and the discovery of a fungus that has been identified as *C. manginecans.* The disease of bullet wood that gave rise to this study is serious and management options to reduce its incidence are required *C. manginecans* is an aggressive pathogen and a deeper understanding of its role in tree death will be important in the future.

ACKNOWLEDGEMENTS

This research was funded by a PMDSU scholarship in the fiscal year of 2019-2021 according to the Director of Research and Community Service, Directorate of Research and Community Service (DRPM), Directorate General for Research and Development, Ministry of Research, Technology, and Higher Education Indonesia, Number: 068/SP2H/AMD/LT/DRPM/2020 chaired by Ahmad Muslim.

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BIODIVERSITAS
Volume 22, Number 5, May 2021
Pages: 2636-2645

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d220522

First report of bullet wood (*Mimusops elengi*) sudden decline disease caused by *Ceratocystis manginecans* in Indonesia

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Manuscript received: 14 January 2021. Revision accepted: 18 April 2021.

Abstract. *Pratama R, Muslim A, Suwandi S, Damiri N, Soleha S. 2021. First report of bullet wood (*Mimusops elengi*) sudden decline disease caused by* Ceratocystis manginecans *in Indonesia. Biodiversitas 22: 2636-2645. Ceratocystis manginecans* cause wilt and death of plants in several important crops and native vegetation in Indonesia. Ceratocystis wilt was recently found to be causing substantial mortality in bullet wood (*Mimusops elengi*) in South Sumatra. The aim of this study was to describe the symptomatology of the new disease and characterize isolates of *C. manginecans* obtained from diseased bullet wood plants. Diseased plants showed substantial discoloration of the woody xylem and wilt-type symptoms of the foliage, with the eventual death of the whole plant. Isolations from infected plants yielded fungi that were similar morphologically to *C. manginecans*, with typical hat-shaped ascospores and light-colored perithecial bases. Sequencing of the internal transcribed spacer (ITS) and β-tubulin of the isolates confirmed their identification, grouping them with *C. manginecans* and separating them from all other *Ceratocystis* species. This is the first report of *C. manginecans* in Indonesia causing wilt and death on bullet wood. *C. manginecans* is an important pathogen, and strategies to reduce losses need to be established in Indonesia because the aggressiveness of *C. manginecans* to bullet wood has been shown in inoculation experiments

Keywords: Ceratocystidaceae, molecular phylogeny, pathogenicity, Sapotaceae

INTRODUCTION

Bullet wood (*Mimusops elengi*) belongs to the family Sapotaceae, common English names are Asian Bulletwood, Bullet Wood Tree, Indian Medlar, Red Coondoo Spanish Cherry and it is known in Indonesia as Tanjung. The species is native to India, Sri Lanka, the Andaman Islands, Myanmar, Indo-China, Peninsular Malaysia and Vanuatu; it has been introduced and cultivated elsewhere. *M. elengi* can grow in tropical and subtropical climates. This plant thrives in areas with high humidity and seasonal rainfall and seasonal dry periods (Lim 2012). The bullet wood trees range from small to large, and are found in all parts of Indonesia where bullet wood is cultivated in gardens as an ornamental tree, for medicines and planted along avenues because of its fragrant flowers (Seth 2003).

M. elengi is widely used for medicine, and various parts of *M. elengi* Linn. (Sapotaceae) have been used widely in traditional Indian medicine for the treatment of pain, inflammation and wounds. *M. elengi* stem bark would be a possible therapeutic candidate having cytotoxic and anti-tumor potential (Kumar et al. 2016); it also has antibacterial and antifungal uses (Ali et al. 2008). At present, *M. elengi* is used as the synthesis of calcium phosphate nanoparticles that is easy, eco-friendly and scalable (Pokale et al. 2014).

Several types of pathogenic fungi have been identified to cause disease in *M. elengi* plants. *Curvularia lunata* caused die-back in India (Khatun et al. 2011); *Pestalotiopsis clavispora* caused leaf blight (Lokesh et al. 2017). *Ceratocystis* was first isolated from a single tree of bullet wood showing sudden decline in Thailand. Symptoms displayed by the diseased trees include gum exudation from the trunks and wilting and loss of the dark green foliage with a corresponding browning of leaves on single branches. In this study they did not confirm the pathogen with a Koch postulates test detail (Pornsuriya and Sunpapao 2015). Recently we have observed many bullet wood trees showing similar symptoms with *C. manginecans* decline in many locations in South Sumatra, Indonesia.

Ceratocystis manginecans include many economically important plant pathogens. This pathogen has caused a sudden decline and has led to the death of thousands of *Mangifera indica* trees in Oman with *Hypocryphalus mangifera* vector (Al Adawi et al. 2013). In Indonesia, *C. manginecans* caused die-back on *Acacia mangium* and *A. crassicarpa* plantations in Riau (Tarigan et al. 2010), whereas in Vietnam recently, *C. manginecans* caused wilt disease in *Dalbergia tonkinensis* and *Chukrasia tabularis* (Chi et al. 2019a; Chi et al. 2020); in Pakistan, this pathogen also causes wilt disease in *Albizia lebbeck* (Razzaq et al. 2020). Commonly *C. manginecans* cause yellowing of leaves and rapid wilting of leaves was observed on individual branches in affected trees that ultimately spread to the canopy followed by the death of the whole tree. Dark brown to black tissue discoloration was observed in the woody xylem tissues of infected trees.

This study aimed to identify the cause of a new outbreak of wilt disease causing a sudden decline to the trees, wilted canopies, and tree death in *M. elengi* in South Sumatera, Indonesia. This study was also conducted to describe the characteristics of the pathogen and confirm Koch's postulates test.

MATERIALS AND METHODS

Disease symptoms and specimen collection

The distribution and impact of the *C. manginecans* disease on *M. elengi* were determined from roadside trees planting in Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) and the agricultural field of Sriwijaya University in Indralaya (Ogan Ilir), South Sumatra, Indonesia. Symptoms of wilt diseases were evaluated as follows: the extent of lesion development from discoloration of bark and wood, the extent of foliar wilting or loss and tree death.

Samples of diseased trunks were collected from two to six-year-old trees from September 2019 to April 2020. Wood samples were taken from lesions of wilted trees using a knife sterilized in 70% ethanol. The wood samples collected from *M. elengi* showed brown to black streaking in the woody xylem. Each sample was wrapped in tissue paper and placed in a coolbox. The same day, the wood samples (1–20 mm length, 1–2 mm thick) were sandwiched between two slices of fresh carrot and placed on sterile dry paper in plastic boxes at 25°C following the method of Li et al. (2014). After 5-10 days, hat-shaped spores of putative *Ceratocystis* pathogens were placed on 2% (w/v) malt extract agar (MEA) (Merck, Germany), and incubated at 25 °C in a laboratory. When cultures had grown to several cm in diameter, hyphal tips were sub-cultured onto new MEA and potato dextrose agar (PDA) (Merck, Germany) plates and incubated at 25-28 °C. Morphological traits of fruiting bodies and spores were observed under an optical Olympus CX33 microscope (Olympus Corporation, Japan).

Genomic DNA extraction, PCR amplification, and sequencing

DNA isolation used YeaStar Genomic DNA Kit (Zymo Research Corporation, California, USA). To extract genomic DNA, cultures were incubated for five days to allow sufficient mycelial growth in potato dextrose broth (PDB) (Merck, Germany). Mycelium was purified with sterile filter paper (Whatman) and transferred to 1.5 mL Eppendorf tubes. The quantity and quality of DNA extracted were evaluated with a spectrophotometer (NanoDrop ND-1000; Thermo Fisher, Waltham, MA, USA) to calibrate the concentration and purity of DNA as PCR templates.

The PCR amplification reactions were conducted on a T-100 thermal cycler (Bio-Rad, Hercules, CA, USA). Amplifications were carried out in 50 µl reactions containing 20 µl DreamTaq Green PCR Master Mix (Eppendorf, Germany) (DreamTaq DNA Polymerase, 2X DreamTaq Green buffer, dNTPs, and 4 mM $MgCl_2$), 1,5 µl of each forward and reverse primer, 4 µl of DNA template and 23 µl sterilized water. The PCRs were performed with a C1000 Touch™ thermal cycler (Bio-Rad, USA). The PCR cycling parameters were as follows: initial denaturation for 5 min at 95°C, followed by 35 cycles at 95°C for 30 s, 56°C for 45s and 72°C for 1 min. Amplification was completed at 72°C for 10 min and the PCR product was stored at 10°C (Chi et al. 2020).

PCR amplifications were made for two gene regions, including part of the b-tubulin (BT) using primers βt1a (TTCCCCCGTCTCCACTTCTTCATG) and βt1b (GACGAGATCGTTCATGTTGAACTC) (Oliveira et al. 2015a), and the ITS using ITS1 and ITS4. The resulting PCR products were submitted to 1st BASE (Malaysia) for forward and reverse sequencing reactions. Raw sequence data were assembled, examined, and manually edited using Genestudio 2.1.1.5 (Genestudio, Suwanee, Georgia) and BioEdit software (van der Nest et al. 2019). The DNA sequences were compared to the GenBank database via the nucleotide-nucleotide BLAST search interface located at the National Center for Biotechnology Information, Bethesda, USA. Relevant sequences were transferred with NoteTab Light v7.2.

Phylogenetic analyses

The sequences of *Ceratocystis* spp. closely related to the one from *Mimusops elengi* were retrieved from GenBank. Phylogenetic sequences from different gene regions were aligned using Mesquite v3.5 (Maddison and Maddison 2018) (http://mesquiteproject.org) and corrected manually. Phylogenetic trees based on a concatenated data set of the ITS and βt were computed and analyzed as a single dataset. Maximum Parsimony (MP) analyses were performed in MEGA v. 10 (Kumar et al. 2016; Paul et al. 2018) with 1000 bootstrap replications.

Pathogenicity tests

Pathogenicity studies were conducted on two agroforestry plants, *A. mangium* and *M. elengi*. Plants had stem diameters of 2–3 cm and heights <1 m. The pathogenic potential of isolates was evaluated by the under bark inoculation method described by Deidda et al. (2016). Bark was wounded to expose the cambium using a 4 mm cork borer, and discs of agar bearing mycelium taken from the margins of actively growing, 2-week-old cultures on 2% MEA (Tarigan et al. 2010; Tarigan et al. 2011; Chi et al. 2019a, Chi et al. 2020), *Ceratocystis* isolates were placed with the mycelium facing the cambium. Ten plants of each tree species were inoculated with sterile MEA plugs to serve as controls. All inoculation points and the ends of the logs were covered with masking tape and polyethylene films, respectively, to prevent desiccation of the inoculum and cambium, and to reduce contamination.

The nursery trial evaluated seven *Ceratocystis* strains isolated from *M. elengi* (CAME30813, CAME30814, CAME30815, CAME30816, CAME30817, CAME30818 and CAME30819) and one strain (CAW30814) from *A. mangium*. There were ten replicate plants per treatment in each row plot in each of the blocks. After 45 days, lesion (L) length and foliar symptoms severity were recorded. Representative wood samples were taken from within

lesions outside the inoculation area, and the pathogen reisolated and sequenced for Koch's postulates test.

Foliar symptoms severity (FS) was assessed using a scale of 0 to 4: 0 = healthy; 1 = lower leaves yellow; 2 = slight wilting; 3 = severe wilting; 4 = dead (Muslim et al. 2003; Chi et al. 2019a; Muslim et al. 2019).

The pathogenicity test data were analyzed using SAS university edition software package. Analysis of variance (ANOVA) and Tukey's honestly significant difference (Tukey's HSD) test were used to determine whether there were significant differences in comparisons of means of different treatments.

RESULT AND DISCUSSION

Symptoms of *Mimusops elengi* wilt disease

We observed disease symptoms from September 2019 to April 2020, in various places that are widely planted in the Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) areas, South Sumatra, Indonesia. The disease was found scattered throughout the planted area, with symptoms of partially dead trees, foliar wilting or loss and tree death (Figure 1a). Initially, the leaves of infected plants lost turgor and brightness, with yellowing symptoms in the older leaves, followed by wilting and death of the plant. Symptomatic plant stems showed xylem discoloration (Figure 1b), infections generally started in the roots and then moved upwards in the stem ultimately reaching the upper branches of the entire plant, and the plants ultimately died (Figure 1c). Death of adjacent plants indicates transmission of root infection because these pathogens are also known as soil-borne pathogens. The severity of the infection is also caused by pruning the branches using tools previously used to cut the infected plants.

Observation of diseased plant xylem tissue in cross-sections of the stem showed dark brown lesion formation in the cambium (inner bark region) towards vascular tissue (Figure 1d). In the initial stage of plant infection, the foliar symptoms, wilt and the fruits appeared normal, but as the infection progressed, the fruits of affected plants were smaller, shriveled, wrinkled and dry. Many of the bark beetle vectors of *C. manginecans, Hypocryphalus mangiferae* were found around bullet wood diseases (Figure 1e). Testing by the Li et al. (2014) method showed that *Ceratocystis* had grown on the carrots, and ascomata of *C. manginecans* with necks supporting sticky masses of ascospores on the carrot slices (Figure 1.F).

Sampling and isolation

Seven isolates of *C. manginecans* were collected from diseased bullet wood (*M. elengi*) (Figure 2). There were three isolates (CAME30815, CAME30816 and CAME30817) from Ogan Ilir (Indralaya); two isolates (CAME30818 and CAME30814) from Jakabaring (Palembang); and two isolates (CAME30819 and CAME30813) from Kayuagung (Ogan Komering Ilir). We also isolated one isolate (CAW30814) from diseased *acacia*, *A. mangium* in the agricultural field of Sriwijaya University, Indralaya.

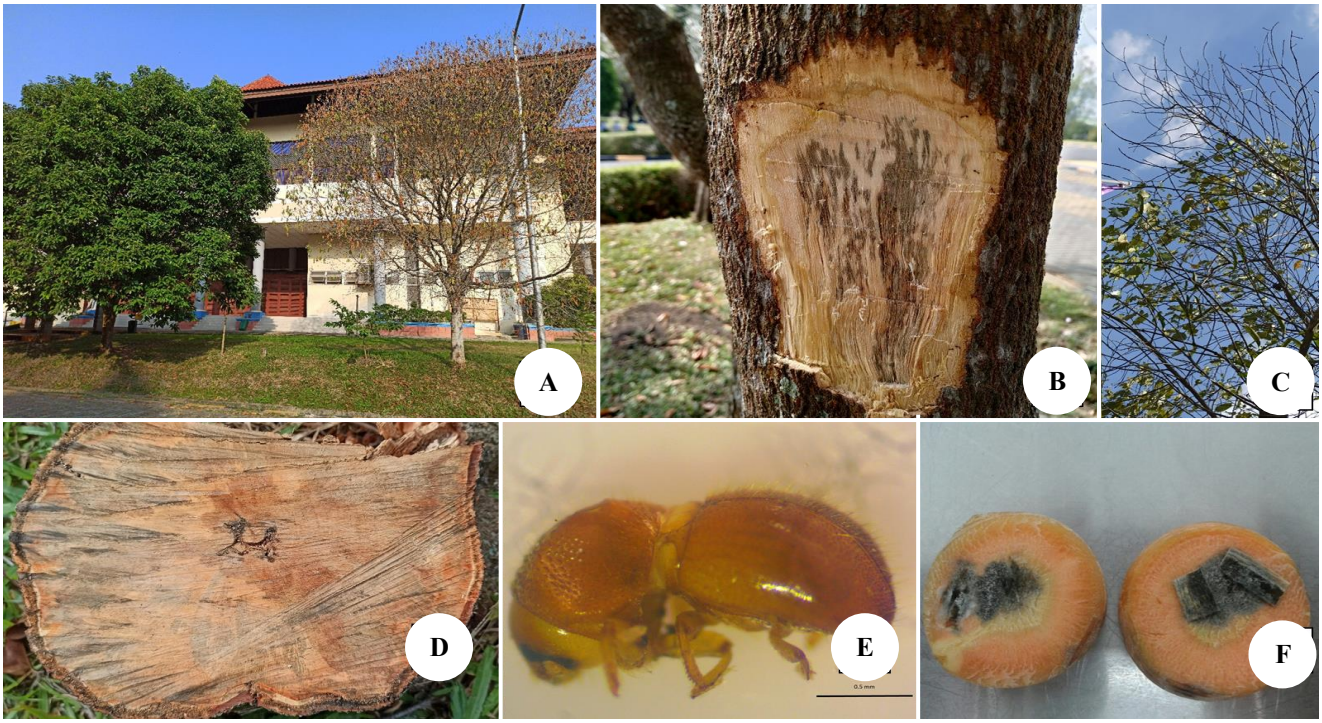

Figure 1. Symptoms of *Ceratocystis manginecans* wilt disease in bullet wood*:* **a**. tree death of *M. elengi*: b. sap stain mold on bullet wood, c. wilted leaves of bullet wood, d. sap stain mold on bullet wood, e. The bark beetle vector of *C. manginecans*, *Hypocryphalus mangiferae,* f. isolation of the fungus from discolored xylem showing dark mycelium and sporulation on the carrot slices

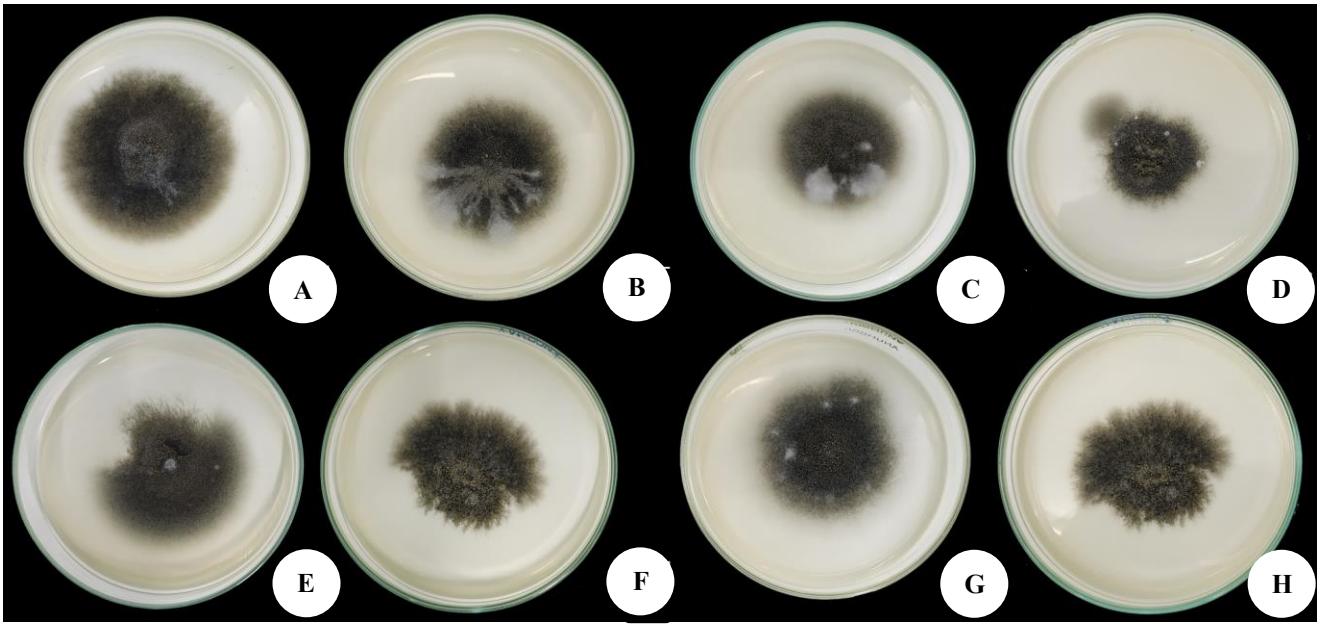

Figure 2. Isolates of *Ceratocystis manginecans* and related species grew on malt extract agar (MEA) for 7 d at 25 °C. A, B, C: *Ceratocystis* CAME30815, CAME30816 and CAME30817, from *Mimusops elengi* in Sriwijaya University, Indralaya. D, E, F: *Ceratocystis* CAME30819, CAME30813 and CAME30814 from *Mimusops elengi* in Jakabaring, Palembang. G: *Ceratocystis* CAME30818, from *Mimusops elengi* in Kayuagung, Ogan Komering Ilir. H: *Ceratocystis* CAW30814, from *Acacia mangium* in Indralaya

Fungal morphology

Seven isolates were morphologically indistinguishable (Table 2). At 7–14 days of incubation at 25 °C on MEA, cultures were pale brown to dark brown and produced a banana-like odor. Mycelium on MEA was grey, and the reverse side of the colony olivaceous grey; submerged mycelium darkened as the ascomata developed, forming fine, radiating fibrils. Ascomata developing within seven days and mature within ten days, superficially or partly embedded in the agar, dark brown to black (Figure 3a). Ascomatal bases were submerged or on the agar surface, dark bases dark brown to black, base subglobose to globes, (134.58-) 169.12 - 276.29 (-310.83) µm long and (122.91-) 161.89-244.14 (-283.13) µm wide in diameter (Figure 3a). Ascomata necks were erect, occasionally curved, black at the base becoming subhyaline towards the apex, smooth to crenulate, (346.51-) 454.94-720.16 (-828.59) µm long including ostiolar hyphae (Figure3b). Ascospores were hat-shaped, (3.61-) 5.64-6.23 (-6.93) µm length and (2.06-) 2.279-3.67 (-3.85) µm width (Figure 3f). Barrel conidia (8.62-) 8.85-12.79 (-13.25) µm length and (5.89-) 4.12x6.87 (-8.67) µm width. Bacilliform conidia (9.05-) 10.82-22.32 (-35.97) µm length and (2.01-) 2.83-5.71 (-8.87) µm width (Figure 3c). Chlamydospores oval, thick-walled, smooth, (8.21-) 9.15-16.21 (-18.50) µm length and (4.92-) 6.46-15.81 (14.65) µm width (Figure 3e).

Sequence analysis

To confirm the identity of the wilt pathogen, the ITS and β-tubulin 1 gene sequences of two isolates from bullet wood (*M. elengi*) were compared to reference sequences downloaded from the gene bank database (NCBI GenBank) (Table 1) and indicated that isolates from Indonesia were grouped within the *C. fimbriata*. s.l species complex and were most closely related to *C. manginecans*. PCR amplification resulted in fragments of ~550 base pairs (bp) in size which had 100% homology with *C. manginecans* (Figure 4). Bootstrap values were equal to or greater than 50%, derived from 1000 iterations.

Pathogenicity

The results of the pathogenicity tests of seven isolates (CAME30813, CAME30814, CAME30815, CAME30816, CAME30817, CAME30818, and CAME30819) on *M. elengi* and one isolate (CAW30814) from *A. mangium* are shown in Table 3. All isolates tested showed a varying reaction to lesion and foliar symptoms (Figure 5). For the pathogenicity test on *M. elengi*, seven isolates from *M. elengi* as well as one isolate from *A. mangium* strongly infected the wood and produced significant lesion lengths ranging from 3.99 to 10.47 cm and showed significant differences from the control. Three isolates from *M. elengi* (CAME30815; CAME30819; CAME30818) as well as one isolate from *A. mangium* (CAW30814) showed high pathogenicity on the foliar symptom (with foliar severity index of 2.3-3.6 and lesion length 6.52-10.47 cm), while the other isolates (CAME30817; CAME30816; CAME30814, CAME30813) showed moderate pathogenicity to *M. elengi* (foliar severity index of 1.4-2.0 and lesion length 3.99-6.02 cm). When the isolates were tested for their pathogenicity on *A. mangium* as the primary host of the pathogen, two isolates (CAME30815; CAME30819) showed strong pathogenicity on lesion length (13.76-11.89 cm) and also provided high pathogenicity on foliar severity index (4), where all plants tested were dead. Three isolates (CAW30814;

CAME30818; CAME30817) showed moderate pathogenicity on lesion length 8.71-10.14 cm and foliar severity index 2.8-3.2, while the other isolates (CAME30816, CAME30814, CAME30813) showed low pathogenicity on lesion length 7.19-8.63 cm and foliar severity index 1.9-2.4.

Table 1. *Ceratocystis* isolates considered in the phylogenetic analyses

Isolate no	Identify	Host	Geographic origin	Collector	Gene region/GeneBank accession no	
					ITS	BT
CAME30819	*C.manginecans*	*Mimusops elengi*	Indonesia	R.Pratama	MT373423	Submitted
CAME30818	*C.manginecans*	*Mimusops elengi*	Indonesia	R.Pratama	MT373424	Submitted
CMW13851	*C.manginecans*	*Mangifera indica*	Oman	M. Deadman	AY953383	EF433308
CMW23643	*C.manginecans*	*Mangifera indica*	Pakistan	A.Al-Adawi	EF433304	EF433313
CMW23641	*C.manginecans*	*Mangifera indica*	Pakistan	A.Al-Adawi	EF433305	EF433314
CMW23634	*C.manginecans*	*Mangifera indica*	Pakistan	A. Al-Adawi	EF433302	EF433311
CMW21125	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588663	EU588642
CMW21123	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588662	EU588641
CMW22581	*C.manginecans*	*Acacia mangium*	Indonesia	M. Tarigan	EU588659	EU604671
CMW21132	*C.manginecans*	*A. crassicarpa*	Indonesia	M. Tarigan	EU588665	EU588644
CMW22579	*C.manginecans*	*Acacia mangium*	Indonesia	M. Tarigan	EU588658	EU588638
CMW23628	*C.manginecans*	*Hypocryphalus mangifera*	Pakistan	A. Al-Adawi	EF433303	EF433312
CMW13854	*C.manginecans*	*Mangifera indica*	Oman	M. Deadman	AY953385	EF433310
CMW13852	*C.manginecans*	*Hypocryphalus mangifera*	Oman	M. Deadman	AY953384	EF433309
CMW4068	*C.albifundus*	*A. mearnsii*	RSA	J. Roux	DQ520638	EF070429
CMW14793	*C.caryae*	*C. cordiformis*	U.S.A	J. Johnson	EF070424	EF070439
CMW14800	*C.smalleyi*	*C. cordiformis*	U.S.A	G. Smalley	EF070420	EF070436
CBS114724						
CMW14789	*C. populicola*	*Populus* sp.	Poland	J. Gremmen	EF070418	EF070434
CMW19385	*C.atrox*	*E. grandis*	Australia	M.J. Wingfield	EF070415	EF070431
CBS115778						
CMW11424	*C. polycroma*	*Syzygium aromaticum*	Indonesia	M.J. Wingfield	AY528970	AY528966
CMW23808	*C. obpyriformis*	*A. mearnsii*	South Africa	R.N. Heath	EU245003	EU244975
CMW6579	*C.pirilliformis*	*E. nitens*	Australia	M.J. Wingfield	AF427105	DQ371653

Table 2. Morphological comparisons of *Ceratocystis manginecans* and other phylogenetically closely related species

Character	*Ceratocystis manginecans* (from *M. elengi)*	*Ceratocystis acaciivora* (from *A. mangium*)	*Ceratocystis manginecans* (from *A. mangium*)
Ascomata base	(134.58-) 169.12 - 276.29 (-310.83) x (122.91-) 161.89-244.14 (-283.13)[a]	(105-) 131-175 (-206) x (107-) 125-167 (-188)	(132.1-) 175.3 (−233.2)
Ascomata base average	220.01x211.63[b]		
Ascomata neck	(346.51-) 454.94-720.16 (-828.59)	(301-) 348-448 (-522)	(327.3-) 452.7 (−556)
Ascomata neck average	568.41		
Ascospores	(3.61-) 5.64-6.23 (-6.93) x (2.06-) 2.279-3.67 (-3.85)	5-7 x 3-4	(3.1-) 4.2 (−4.7) x (4.1-) 5.5 (−6.8)
Ascospores average	5.62 x 3.93		
Bacilliform conidia	(9.05-) 10.82-22.32 (-35.97) x (2.01-) 2.83-5.71 (-8.87)	(11-) 14-22 (-29) x 3-5	(14.6-) 18.6 (−30.7)x (3.0-) 4.6 (−5.4)
Bacilliform conidia average	16.56x4.27		
Barrel-shaped conidia	(8.62-) 8.85-12.79 (-13.25) x (5.89-) 4.12x6.87 (-8.67)	(8-) 9-11 (-13) x 4-6	(6.8) 8.1 (−10.6)
Barrel-shaped conidia average	11.497 x 15.82		
Clamydospore	(8.21-) 9.15-16.21 (-18.50) x (4.92-) 6.46-15.81 (14.65)	(10-) 12-14 (-15) x (7-) 8-12 (-14)	(10.1-) 13.1 (−15.5) x (6.1-) 9.2 (−11.1)
Clamydospore average	11.13 x 14.18		
Reference	*This study*	M. Tarigan et al. (2010)	Chi et al. (2019)

Note: All measurements are in µm. [a] Measurements are presented in the format [(minimum-) (average-standard deviation) - (average-standard deviation) (-maximum)]. [b] Measurements are presented in the format minimum x maximum

Table 3. Pathogenicity of *Ceratocystis* isolates on *Mimusops elengi* and *Acacia mangium* under nursery condition

Isolates	Host test	*M. elengi*		*A. mangium*	
		Lesion length (cm)	Foliar symptoms	Lesion Length (cm)	Foliar symptoms
CAME30815	10	10.47e	3.6	13.77d	4
CAME30819	10	8.29de	3.1	11.89cd	4
CAW30814	10	7.35cd	2.8	10.14bcd	3.2
CAME30818	10	6.52bcd	2.3	9.92bcd	3.1
CAME30817	10	6.02bcd	2	8.72bc	2.8
CAME30816	10	5.27bc	1.8	8.47bc	2.4
CAME30814	10	4.93bc	1.5	8.64bc	2
CAME30813	10	3.99b	1.4	7.19b	1.9
Control (MEA)	10	0.1a	0	0.1a	0
Fpr		<0.001		<0.001	

Note: Values followed by the same letters in a column are not different among isolates at P=0.05 according to Tukey's HSD multiple range test.

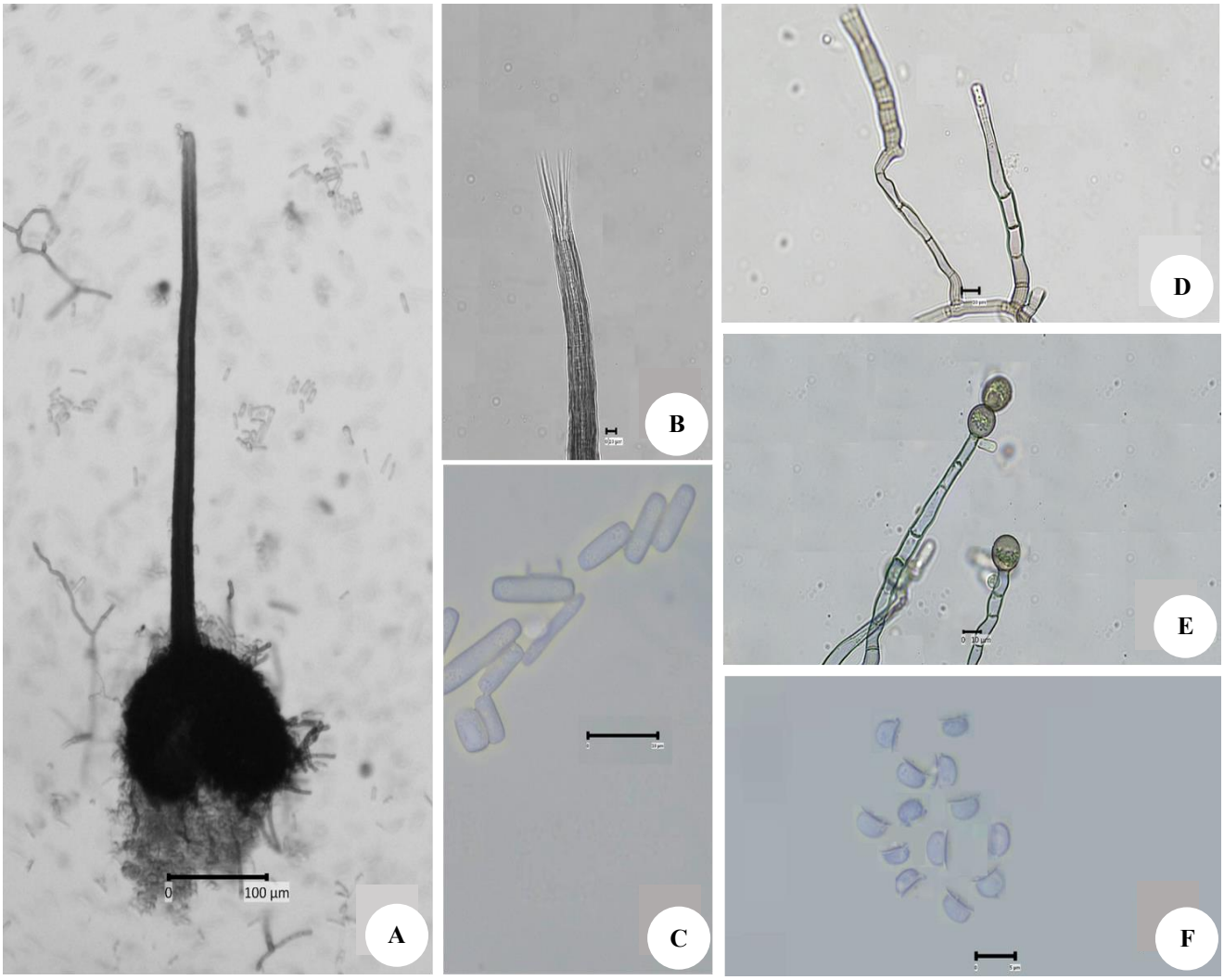

Figure 3. Morphological characteristics of *Ceratocystis* isolated from *M. elengi* stem lesion: A. Globose ascomata with long neck, B. Divergent ostiolar hyphae, C. Barrel-shaped conidia, D. Conidiophore/phialide, E. Chlamydospores, F. Hat-shaped ascospores. Scale bars: A = 100 µm; B, C, D, E = 10 µm;F = 5 µm

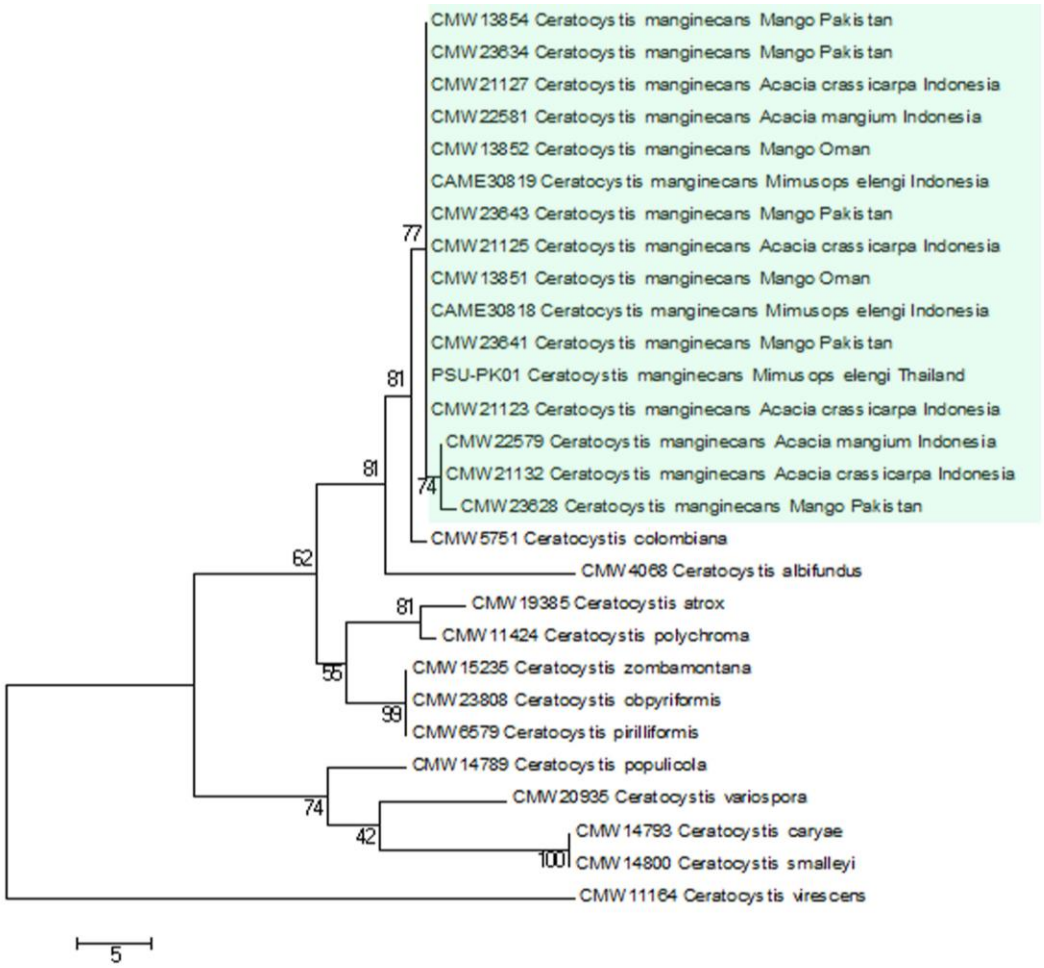

Figure 4. Phylogenetic tree constructed by MEGA with Maximum Parsimony (MP). The tree was built using combined sequence data of the ITS region and b-tubulin gene and their related species from GenBank. Consistency (CI), retention (RI), and composite indexes (CoI) are 0.714286, 0.837209, and 0.664843 for all sites and parsimony-informative sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Bootstrap values >50% are indicated above the branches. The analysis involved 28 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 493 positions in the final dataset

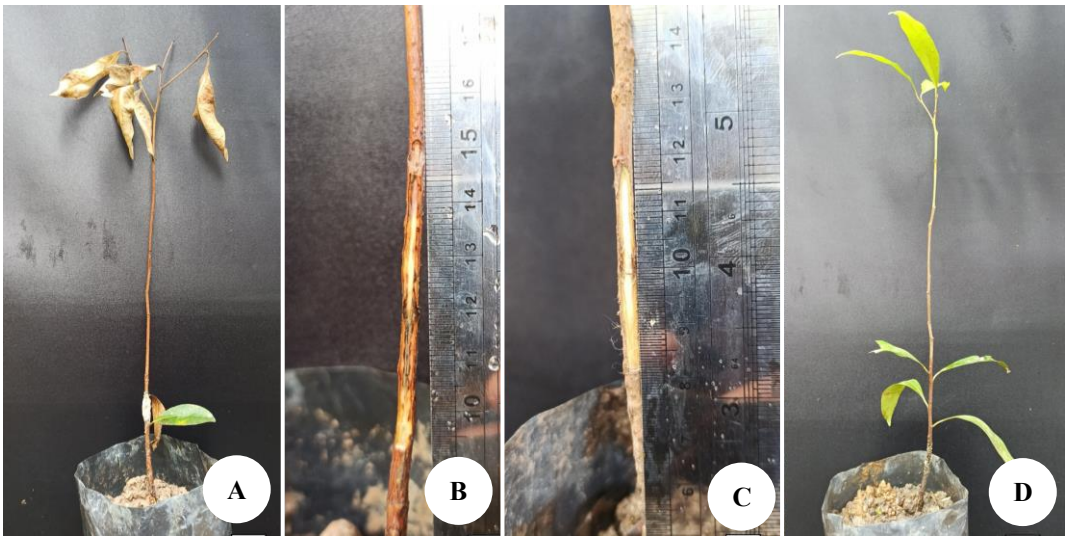

Figure 5. Response after 45 days of *Mimusops elengi* seedlings to under-bark inoculation with mycelium of *Ceratocystis manginecans*. A. Wilting of seedlings; B. Lesions on the stem; C. No lesions on the stem; D. Normal foliage on the control seedling which received MEA only and appeared healthy. Yellow arrow indicates the point of inoculation, red arrows show the lesion boundary

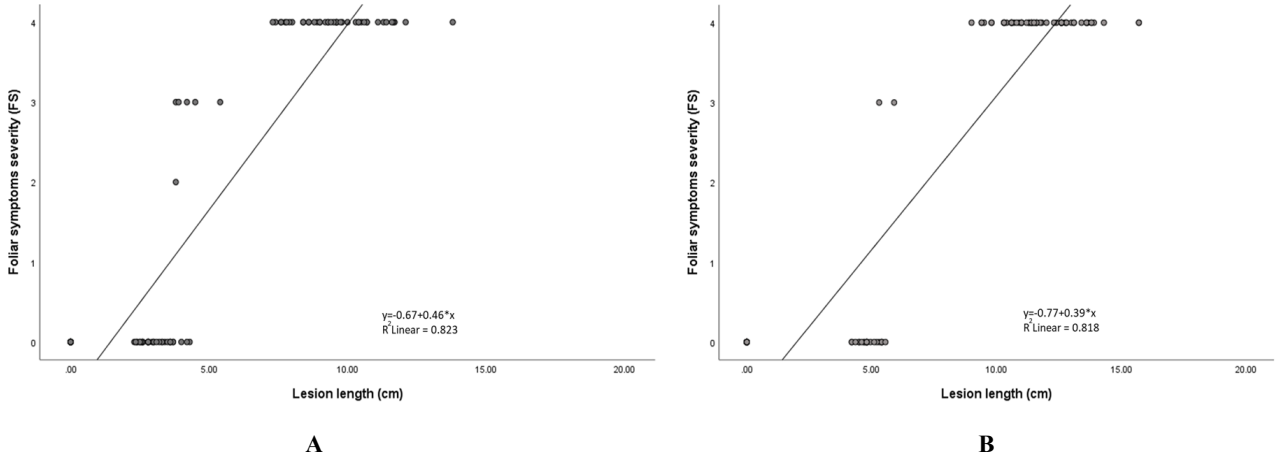

Figure 6. Correlation between foliar symptoms severity (FS) and lesion length (cm): A. *Mimusops elengi,* B. *Acacia mangium*

There was a strong linear correlation between foliar symptom severity and lesion length for *M. elengi* and *A. mangium* (Figure 6). The Koch's postulates test was conducted by reisolating the pathogen, and its identity confirmed to be the same as *C. manginecans*.

Discussion

The results of this study show clearly that *C. manginecans* was responsible for the wilt outbreak disease that has recently appeared on the *M. elengi* agricultural field in Indralaya (Ogan Ilir) and roadside trees in Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir), South Sumatra. This is the first report of a *Ceratocystis* wilt disease on *M. elengi* in Indonesia. Formerly the disease was reported in Thailand (Pornsuriya and Sunpapao 2015), however, they just observed a single tree and they did not conduct pathogenicity tests and observe morphological characteristics. In Indonesia Tarigan et al. (2010; 2011) reported *C. manginecans* caused wilt and die-back disease in *A. mangium* plantations in Riau and Suwandi et al. (2021) have reported infection of this disease on *Lansium domesticum* tree.

Mimusops elengi infected with *Ceratocystis* has wilted foliage symptoms, sudden wilt, decline and branch drying, and progressive loss of the canopy resulting in tree death. *M. elengi* trees showed typical symptoms of infection by the *Ceratocystis* fungus; the same was true of a serious wilt pathogen of *A. mangium* and *A. crassicarpa* in Indonesia and Vietnam (Tarigan et al. 2011; Thu et al. 2012; Thu et al. 2016). Vascular wilt, wood stain and stem cankers are the most characteristic symptoms of infection by *C. manginecans* in woody trees (Harrington 2013). Rot in roots or stems, vascular wilt, sapwood discoloration and cankers are symptomatic of plants infected by *C. manginecans* species (Oliveira et al. 2015b).

The comparison of morphological characteristics and gene sequences (β-tubulin and ITS) of the isolates examined in this study were similar to those in descriptions given for *C. manginecans* isolated from diseased *Acacia* trees which form part of the *C. fimbriata* s. l. complex, which is typified by *C. fimbriata* sensu stricto (Engelbrecht and Harrington 2005; Tarigan et al. 2011). The two isolates from Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) have 100% identical sequences of ITS and Beta Tubulin representing a single clone of the pathogen. This supports the view that *M. elengi* wilt disease in Indralaya (Ogan Ilir), Jakabaring (Palembang) and Kayuagung (Ogan Komering Ilir) emerged from a single introduction of a single haplotype and the population has expanded clonally to infect *M. elengi* trees in many parts of all three regions.

Ceratocystis manginecans was reported in Indonesia associated with wilt and die-back disease on *Acacia* spp. (Tarigan et al. 2011). It was found that many acacia plants were attacked by *Ceratocystis* disease and *Hypocryphalus mangifera* insects in the field raised the suspicion that *M. elengi* were infected by *Ceratocystis* in acacia plants. *H. mangifera* is a vector insect for the spread of *Ceratocystis* in the world (Van Wyk et al. 2007; Masood et al. 2008; Al-Adawi et al. 2013; Fourie et al. 2016). Pruning *M. elengi* branches using equipment that has previously been infected with *Ceratocystis* also increases the severity of the disease; this can be seen by the number of plants that are attacked after pruning with tools used to prune *Acacia* plants that are attacked by *Ceratocystis*. Cankers of *Ceratocystis* were associated with branch wounds from pruning, pruning time and pruning technique on plants (Chi et al. 2019b). In this study, infected bullet wood was observed surrounding infected acacia by *C. manginecans* with abundant *H. mangifera.* This might be caused by spore contamination on cutting tools after pruning diseased acacia and spore spreading by insect vector *H. mangifera*.

The pathogenicity of *C. manginecans* to these hosts was demonstrated in inoculation trials and it is clear that the fungus is responsible for the widespread death of these trees. It was also possible to show that isolates of the pathogen from these trees are able to infect and kill other plants. The eight *C. manginecans* isolates all formed lesions on the stems of *M. elengi* seedlings when inoculated under the bark in nursery cloches. The most pathogenic was CAME30815 and CAME30819 isolate from *M. elengi* and the CAW30814 isolate from *A. mangium* resulted in

foliar symptoms with severity index 3.6, 3.1, and 2.8 after 45 days from inoculation with pathogen. When the isolates were tested on seedling *A. mangium*, the seedlings were infected and mostly dead as they were the main host plant and susceptible to *C. manginecans* (Tarigan et al. 2011).

Previous researchers of *C. manginecans* on *M. elengi* in Thailand have reported molecular identification *C. manginecans* by primers combination the ITS, β-tubulin (βt) and transcribed elongation factor 1-α (TEF1-α) gene regions with one isolate. In our research, we tested two isolates for sequencing, seven isolates for pathogenicity, and Koch's postulates assay using three isolates (CAME30815, CAME30816 and CAME30817) from Ogan Ilir (Indralaya); two isolates (CAME30818 and CAME30814) from Jakabaring (Palembang); and two isolates (CAME30819 and CAME30813) from Kayuagung (Ogan Komering Ilir) and one isolate (CAW30814) from diseased *Acacia*, *A. mangium*. All isolates showed the ability to infect both bullet wood and *Acacia,* and all isolates can be reisolated confirming Koch's postulates.

The wilt disease of *M. elengi* appears to be serious and it is clearly a new host tree or pathogen association that has apparently occurred due to a host shift. This category of diseases is increasing in importance in plants, and they can devastate native trees that have not previously encountered them (Roy 2001; Anderson et al. 2004; Slippers et al. 2005; Woolhouse et al. 2005; Desprez-Loustau et al. 2007; Wingfield et al. 2010). In this regard, the wilt disease of *M. elengi* can impact seriously on the natural biodiversity of Indonesia, and studies should be instituted to understand them better. The findings of this study will give knowledge of the characteristics of the symptoms of the disease, their causes and help to minimize the spread of wilt disease in *M. elengi* in plantations or roadside trees and to consider its possible pathogenicity.

This study presents the first report of *Ceratocystis* wilt or sudden decline disease of bullet wood in Indonesia and the discovery of a fungus that has been identified as *C. manginecans.* The disease of bullet wood that gave rise to this study is serious and management options to reduce its incidence are required *C. manginecans* is an aggressive pathogen and a deeper understanding of its role in tree death will be important in the future.

ACKNOWLEDGEMENTS

This research was funded by a PMDSU scholarship in the fiscal year of 2019-2021 according to the Director of Research and Community Service, Directorate of Research and Community Service (DRPM), Directorate General for Research and Development, Ministry of Research, Technology, and Higher Education Indonesia, Number: 068/SP2H/AMD/LT/DRPM/2020 chaired by Ahmad Muslim.

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