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Characterisation of *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis*, the Stem Canker Diseases of *Eucalyptus* spp. in a Forest Plantation in Malaysia

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Abstract: Commercial plantations of *Eucalyptus* species have been established in Malaysia, especially during the past 10 years, with the aim of sustaining the supply of wood and timber products for industrial use in Malaysia. As part of an assessment of fungal diseases affecting *Eucalyptus* species in four regions in Malaysia, including Kelantan, Pahang, Sabah, and Selangor, stem canker disease was discovered to be a widespread disease infecting *Eucalyptus* species in Malaysia. This study aimed to identify the fungus-causing stem canker disease, test its pathogenicity in Eucalyptus, and determine the mating type of isolates from the infected trees. The fungi were identified based on morphology and through comparisons of DNA sequence data from the *ITS*, *β-tubulin 2* gene, and *TEF-1α* gene regions. Phylogenetic analyses showed that the causal agent of the stem canker was *Chrysoporthe cubensis* infecting *Eucalyptus* plantations in Pahang and *Chrysoporthe deuterocubensis* infecting *Eucalyptus* plantations in Kelantan, Sabah, and Selangor. We believe this is the first report of *Chrysoporthe cubensis*-infected *Eucalyptus* in Malaysia and Southeast Asia, while *Chrysoporthe deuterocubensis* is the first-reported species infecting *Eucalyptus pellita* in Malaysia. Moreover, the fact that the mating-type *MAT1-1* and *MAT1-2* genes and the pheromone genes *ppg1*, *ppg2*, *pre1*, and *pre2* were identified in all isolates indicates that *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis* are homothallic mating systems. Pathogenicity was tested on a 3-year-old standing tree, 1-year-old seedling, and detached healthy leaves, which were re-isolated for fulfilling Koch's postulates. In pathogenicity trials, both *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis* gave rise to lesions on wounded *Eucalyptus*. Both *Chrysoporthe* spp. were equally pathogenic to *Eucalyptus urograndis* and *Eucalyptus pellita* and should be regarded as a biosecurity concern in Malaysia's forest plantation industry.

Keywords: *Chrysoporthe cubensis*; *Chrysoporthe deuterocubensis*; pathogenic; eucalyptus pellita; eucalyptus urograndis; non host; dipterocarp

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1. Introduction

The genus *Eucalyptus* L.'Her., *Corymbia* K.D.Hill and L.A.S. Johnson and *Angophora* Cav., a unique tree group, represents more than 900 species [1–3]. Due to the fast-growing and desirable wood properties, *Eucalyptus* was chosen as a species for forest plantation. Over the past 30 years, the establishment of *Eucalyptus* plantations was recorded across the world with about 22.57 million hectares established [4].

The establishment of *Eucalyptus* plantations significantly increases the emergence of pathogens worldwide. Important diseases in *Eucalyptus* plantations include stem canker caused by species of *Chryphonecteriaceae* [5,6] and *Botryosphaericeae* [6–8]; leaf spots or blight caused *Mycosphaerellaceae* and *Teratosphariaceae* species [9–11]; and bacterial wilt caused by *Burkholderiaceae* [12,13]. Stem canker caused by *Chryphonecteriaceae* is considered to be one

of the most important diseases in *Eucalyptus* plantations. *Chrysoporthe deuterocubensis* and its sibling species, *Chrysoporthe cubensis*, known as *Chrysoporthe* canker, are common in many tropical and subtropical parts of the world. *Chrysoporthe deuterocubensis* commonly present in South East Asia [13] and *Chrysoporthe cubensis* is commonly present in South Africa. Both diseases are known to kill significant numbers of trees, particularly those in young plantations. As reported, the genus *Chrysoporthe* is an important plant pathogen infecting more than 335 plant species, distributed by nearly 100 plant families of *Chryphonecteriaceae* [5].

The study by the authors of [14] showed that *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis* have a homothallic mating system and can complete the sexual cycle without a compatible mate. Since the species has a homothallic mating system, both *MAT1-1* and *MAT1-2* idiomorphs are present in the same nucleus, allowing the ability to complete the sexual cycle without a mate. The sexual reproduction of *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis* plays a role in the disease under natural conditions [15].

Previous research results indicated that a relatively large number of *Chrysoporthe* species are distributed in *Eucalyptus* plantations worldwide. However, the presence of *Chrysoporthe* spp. in *Eucalyptus* plantations in Malaysia is poorly described. Therefore, the understanding of *Chrysoporthe* stem canker diseases in *Eucalyptus* plantations in Malaysia is limited and results in poor disease management. The objectives were (i) to identify fungi associated with canker diseases in *Eucalyptus* plantations in four regions in Malaysia, (ii) to analyse and develop a phylogenetic tree based on a multi-gene locus, (iii) to characterize the mating system of *Chrysoporthe* isolates in this study, (iv) to confirm the pathogenicity of *Chrysoporthe* canker on leaves, seedlings and standing *Eucalyptus* trees for both *Eucalyptus urograndis* and *Eucalyptus pellita* trees, and (v) to test the pathogenicity of *Chrysoporthe* canker of a non-host plant.

2. Materials and Methods

2.1. Study Sites and Sampling

The disease survey was conducted on 11-year-old planted *Eucalyptus urophylla* x *Eucalyptus grandis* hybrid genotypes (known as *Eucalyptus urograndis*) in a forest plantation in Sabah (E117°42′58.7″ N4°33′16.3″), and 3-year-old *Eucalyptus urograndis* planted in Pahang (E101°39′7.8102″ N2°59′10.51152″), Kelantan (E101°69476″ N4°86494″) and in Selangor (E102°7′18.04404″ N3°20′36.86064″). In Selangor, *Eucalyptus pellita* was included in the disease survey.

Diseased outer and inner barks with a conidiomata structure present on infected trees were sampled, placed in zip lock plastic bags, and brought to the laboratory for isolation and morphological examination. The conidia masses were collected, transferred onto 2% (*v*/*v*) malt extract agar (MEA) (Thermo Scientific Oxoid, UK) with sterile needles, and incubated for 7 days. Pure cultures were obtained via a single hyphal tip method isolated from each culture, transferred onto 2% MEA plates, and incubated at room temperature for 14 days. The pure cultures were kept in stock culture in the culture collection in Plant Pathology Laboratory, University Putra Malaysia (UPM), Serdang, Malaysia, for further analysis.

2.2. DNA Extraction, PCR Amplification, and Molecular Phylogeny

DNA was extracted from 7-day-old cultures and mycelia were collected using a sterilised scalpel and transferred to 2 mL Eppendorf tubes. Total genomic DNA was exacted following the Favorgreen Fungi Kits protocol. DNA concentration was measured with Nano-Drop 2000 Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

PCRs were carried out in a volume of 50 µL, with 5 µLv of the DNA template, 25 µL of exTEN PCR MasterMix, 1 µL of each primer (10 µM), and a top up of 18 µL of nuclease-free water. PCR products were amplified using BioRad MyCycler Thermal Cycler (Marshall Scientific LLC Hampton, NH, USA). The internal transcribed spacer regions and intervening 5.8S rRNA gene (ITS) were amplified using the primer of the ITS1/ITS4 region [16], a part of the translation elongation factor 1-alpha (*tef1*) gene was amplified using the primers EF1-728F/EF1-986R [17], and one region of the β-tubulin (*tub2*) gene was amplified with primers

BT2a and BT2b [18]. Specific primers targeted genes encoded at the mating-type locus (*MAT1-1-1*, *MAT1-1-2*, *MAT1-1-3*, and *MAT1-2-1*) gene were amplified using the primers mat1QL/mat1QR, mat2QL/mat2QR, mat112QL/mat112QR, and mat113QL/mat113QR, as well as the specifically targeted primers the genes encoded at the pheromone (*ppg1* and *ppg2-1*) and pheromone receptor (*pre-1* and *pre-2*) genes [19–21] (Table 1).

Table 1. Oligonucleotide sequences as primers used to amplify translation elongation factor 1-alpha, (*tef1*), *ß-tubulin* (*tub2*), and internal transcribed spacer (ITS) regions ITS1 and ITS4, and mating-type, pheromone, and pheromone receptor genes.

Primer	Primer Sequence (5′-3′)	Annealing Temp. (°C)	Region Amplified	Amplicon Size	Reference
ITS1	TCCGTAGGTGAACCTCGCG	59.5	5.8S nrRNA	~600	[16]
ITS4	TCCTCCGCTTATTGATATGC	52.1			
Bt2aF	GGTAACCAAATCGGTGCTGCTTTC	58.8	Bt2a	~400	[18]
Bt2bR	ACCCTCAGTGTAGTGACCCTTGGC	62.5	Bt2b		
EF1-728F	CATCGAGAAGTTCGAGAAGG	52.6	EF1-728F	300–400	[17]
EF1-986R	TACTTGAAGGAACCCTTACC	51.3	EF1-986R		
acdmat111F	CGGGTGTGGACGTTTATC	53.2	MAT1-1-1	700–800	[19]
acdmat111R	CGGGTGTGGACGTTTATC	53.6			
acdmat112F	TTGAAAGCAACMCTGACCGA	55.9	MAT1-1-2	800–900	[19]
acdmat112R	GCCGTGGAGAATATGCAGAA	55.1			
mat113qF	TTCATCATTGCACGTACCGA	53.2	MAT1-1-3	400–700	[19]
acdmat113R	GTACTTTGCTTGGTGTTGAT	53.6			
acdmat121F	AACCGTCTTCTTGTTGGTC	52.6	MAT1-2-1	500–700	[20]
acdmat121R	GTGGTAGTCTTCTTGGAACG	52.8			
pre1Q1_L	GCTCTTGAACATCCGTCTC	53.1	pre1	~200	[20]
pre1Q1_R	TAGTCTCCTTGGTGGTGGT	55.1			
pre2Q1_L	GACAATGACACCGAAGACC	53.3	pre2	100–200	[20] [21]
pre2Q1_R	CCAGGAGGAGTTGAAGTAGAC	54.3			
cappg1Q1L	CCGAGATCTCCAACATGCG	55.8	ppg1	100–200	[21]
cappg1Q1R	CCGAACTTGGACAGGATGG	55.6			
ppg2Q1_L	TCTTCCTCCTCATCCACGTC	56.0	ppg2	~200	[21]
ppg2Q1_R	CTGCAGAGCTGCAAAGAGG	56.4			

Cycling conditions consisted of initial denaturation at 94 °C (5 m), followed by 32 cycles of denaturation at 94 °C (15 s), annealing at 56 °C (15 s), and extension at 72 °C (40 s) for primer sets ITS1/4. Standard PCR conditions were used for all reactions with the annealing temperature for each specific primer pair obtained from primer synthesis reports. PCR products were separated using 1% agarose gel in a 1× TAE buffer (90 mM Tris-acetate and 2 nM EDTA, pH8), stained with Florosafe and documented using FluroChem™ (Alpha Innotech, USA). PCR products were sent for sequencing using MyCyclerTM (Bio-Rad, USA) at the First Base Laboratory Sdn. Bhd., Selangor, Malaysia. Forward and reverse sequences of ITS, *tef1*, *β-tub*, MAT locus, pheromone, and pheromone receptor gene regions were deposited in GenBank (https://www.ncbi.nlm.nih.gov (accessed on 31 March 2023).

All published species' sequences were retrieved from GenBank after a BLASTN search and were used for sequence comparisons and phylogenetic analyses. The datasets of [22] were used as templates for analyses. The alignments were visualised in MEGA 7 [23]. Phylogenetic trees were viewed using MEGA 7, and the sequence data of *Endothia gyrosa* was used as outgroups.

2.3. Pathogenicity Tests

Chrysoporthe cubensis isolated from Pahang and *Chrysoporthe deuterocubensis* isolated from Kelantan, Selangor, and Sabah, respectively, were used for determining pathogenicity and Koch's postulates. Pathogenicity tests were conducted on a 3-year-old standing tree, 1-year-old seedling, and detached leaves, as described below.

2.3.1. Three-Years-Old Standing Tree

A portable electric drill (5 mm diam) was used to drill and wound a 3-year-old standing tree stand approximately 1.5 m above the ground. Agar plugs taken from the edges of actively growing cultures were placed into the wounds with the mycelial surface facing the cambium. Cambium surfaces were sprayed with sterile distilled water to allow the optimum growth of the mycelium of the test pathogen. The inoculated stems were wrapped with parafilm to reduce contamination and desiccation. In total, 20 isolates of *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis* isolated from four different regions were used for the pathogenicity test. The setup is shown in Table 2. In total, 3 technical replicates of each isolate were used for each individual inoculated tree. Non-inoculated malt extract agar (MEA) was used as a control. However, only one strain of *Chrysoporthe cubensis* (NHASUL1) and one strain of *Chrysoporthe deuterocubensis* (NHATWU9, the most virulent strain) was plugged to the tree stand of *Eucalyptus deglupta* and *Aqualaria sinensis*, respectively. The data from the inoculation were subjected to a one-way analysis of variance (ANOVA) and Tukey's test to determine if there was a significant difference in the pathogenicity of the isolates on the different hosts. The analysis was performed using Statistical Analysis Systems (SAS) software version 9.2 (SAS Institute Inc., Cary, NC, USA). Figure 1 shows the replication of the inoculated tree. Lesion development was measured using a digital caliper 8 weeks after inoculation.

Table 2. Pathogenicity test of 3-year-old seedling of *Eucalyptus urograndis* and *Eucalyptus pellita*.

No.	Isolate	Number of Tested Isolates	Origin of Isolate	Number of Tested Trees	
				Eucalyptus urograndis	***Eucalyptus pellita***
1	*Chrysoporthe cubensis*	20	Pahang	20	20
2	*Chrysoporthe deuterocubensis*	20	Kelantan	20	20
3	*Chrysoporthe deuterocubensis*	20	Sabah	20	20
4	*Chrysoporthe deuterocubensis*	20	Selangor	20	20

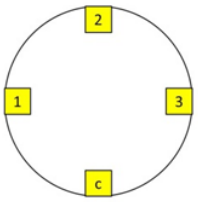

Figure 1. Inoculated stand tree. (1) Isolation for replicate no. 1 and 2; isolation for replicate no. 2 and 3; isolation for replicate no. 3; and (C) control.

2.3.2. Seedling

On seedling trial test, 1-year-old potted *Eucalyptus urograndis* (approximately in 100 cm tall and grown in 20 cm diameter plastic pot) was used for the pathogenicity. The most virulent isolate based on field trial result were applied on seedlings test. The isolate NHASUL1:OQ581890 of *Chrysoporthe cubensis* and NHATWU9:OQ581908 of *Chrysoporthe deuterocubensis* were used for the pathogenicity test (Table 3). A blank agar was used as control.

Table 3. Pathogenicity test of 1-year-old seedling of *Eucalyptus urograndis*.

No.	Isolate	Number of Tested Isolates	Origin of Isolate	Number of Tested Trees ***Eucalyptus urograndis***
1	*Chrysoporthe cubensis*	2	Pahang	2
2	*Chrysoporthe deuterocubensis*	2	Kelantan	2
3	*Chrysoporthe deuterocubensis*	2	Sabah	2
4	*Chrysoporthe deuterocubensis*	2	Selangor	2

2.3.3. Pathogenicity Testing Using Detached Leaves

Leaves obtained from 17 selected tree species were used for the pathogenicity test on *Chrysoporthe deuterocubensis.* Detached leaves for conducting the pathogenicity test were from tree species listed as follows. (1) *Eucalyptus* spp. tree: *Eucalyptus urophylla*, *Eucalyptus camaldulensis*, *Eucalyptus urograndis*, *Eucalyptus deglupta*, *Eucalyptus grandis* and *Eucalyptus pellita;* (2) crop plants: *Syzygium aromaticum*, *Nephelium lappaceum*, *Durio zibenthinus* and *Mangifera indica*; (3) wild herbs: *Melastoma marianum* and *Melastoma malabathricum*; (4) dipterocarp trees: *Aquilaria sinensis*, *Dyera costulata*, *Neobalanorpus heimii* and *Hopea odorata*, which were were tested to conduct the study.

The leaf surface was washed with sterilised water and wounded before inoculation with *Chrysoporthe deuterocubensis* isolates from Sabah (NHATWU9:OQ581908). Inoculations were conducted with a 5 mm diameter mycelia plug from 7-day-old seedling. Mycelia plugs of the isolate were inoculated on ten leaves and each test sample was placed upside down on the abaxial surface of the leaflets. To allow sufficient humidity, the test leaves were kept in plastic boxes (length and height: 20 cm; width: 10 cm) in stable climatic conditions (temperature, 24–26 °C; humidity, 60%–70%). The mycelial plug inoculations were monitored each day, and the length of lesions produced was measured. Leaf disease severity was assessed by estimating the percentage of the lesion area on each leaf with a scale from 0 to 5, where 0 indicated no lesions, 1 indicated that 1 to 10% of the area of the leaf was lesioned, 2 indicated a lesion, 4 indicated that 51 to 75% of the area of the leaf was lesioned, and 5 indicated that 76 to 100% of the area of the leaf was lesioned. For re-isolations, small pieces of the discoloured leaf (approximately 0.04 cm^2) from the edges of the resultant lesions were cut and placed on 2% MEA at room temperature.

3. Results

3.1. Fungal Isolations

A total of 212 isolates belonging to *Chrysoporthe deuterocubensis* were isolated from the bark covering cankers on *Eucalyptus urograndis* and 43 isolates of *Chrysoporthe deuterocubensis* were isolated from *Eucalyptus pellita*. In total, 45 isolates were collected at Pahang and are known as *Chrysoporthe cubensis*. The results are summarised and recorded in the following Table 4.

Table 4. Planting area of *Eucalyptus* spp.

Species	Planted (ha)	Spacing (m × m)	Planted (year)	Number of Infected Trees	Pathogen
Eucalyptus urograndis (Sabah)	13.63	3.0 × 3.0 (1111 stem/ha)	2015	52	*Chrysoporthe deuterocubensis*
Eucalyptus urograndis (Sabah)	23.98	3.0 × 3.0 (1111 stem/ha)	2013	58	*Chrysoporthe deuterocubensis*
Eucalyptus urograndis (Sabah)	11.37	3.0 × 3.0 (1111 stem/ha)	2008	54	*Chrysoporthe deuterocubensis*
Eucalyptus urograndis (Kelantan)	10.38	3.0 × 3.0 (1111 stem/ha)	2018	36	*Chrysoporthe deuterocubensis*
Eucalyptus urograndis (Pahang)	47.20	3.0 × 3.0 (1111 stem/ha)	2016	45	*Chrysoporthe cubensis*
Eucalyptus urograndis (Selangor)	0.86	3.0 × 3.0 (1111 stem/ha)	2018	12	*Chrysoporthe deuterocubensis*
Eucalyptus pellita (Selangor)	1.92	3.0 × 3.0 (1111 stem/ha)	2018	43	*Chrysoporthe deuterocubensis*

3.2. Disease Symptoms and Morphology

3.2.1. *Chrysoporthe deuterocubensis*

Stem cankers caused by *Chrysoporthe deuterocubensis* were localised on dead, cracked and sunken areas of the trunk of infected trees, which may have caused tree fall (die back)

(Figure 2A). Massive *Chrysoporthe deuterocubensis* fruiting bodies were observed to have developed on the outer layer of the bark. The tissues underneath the depressed bark were brown and apparently dead. The bark later split around the infected area and gummosis was generally observed on cankers, as is usually associated with older cankers (Figure 2B,C). The area was infected via the formation of a callus around the site of infection, leading to the bulging of the outer layer of the bark (Figure 2D). A cross-section through the infected trunk showing the gummosis (kino bleeding) caused by *Chrysoporthe deuterocubensis* can be clearly seen (Figure 2E).

Figure 2. (**A**) *Chrysoporthe deuterocubensis* affected on *Eucalyptus urograndis* in plantation. (**B**) Canker caused by *Chrysoporthe deuterocubensis* on trunk fall down. (**C**) Trunk canker showing gummosis. (**D**) Swollen/callus leading to bulging of outer layer of bark. (**E**) Section through trunk canker showing gummosis (kino bleeding). (**F**) Coppices develop on the stem of *Eucalyptus urograndis*. (**G**) Conidiomata of *Chrysoporthe deuterocubensis* on bark surface. (**H**) Long golden-coloured cirrhi fruiting bodies of *Chrysoporthe deuterocubensis*. (**I**) *Chrysoporthe deuterocubensis* grown on MEA agar. (**J**) Fruiting body developed on MEA agar.

The layer was eventually shed resulting in a canker. On certain trees, an infected outer bark may be sloughed off before the cambium is killed. On others, typical cankers are produced as the cambium is killed. Multiple cankers are occasionally found on trunks and become confluent to form long cankerous areas. The cankers usually develop above ground level but occasionally at the base. Large above-ground and basal cankers are responsible for the mortality of trees due to the complete girdling of the phloem. On diseased stumps, fewer sprouted clumps and multiple copy shoots develop. Shoots remained stunted and weak in comparison to those on healthy stumps (Figure 2F). The yellowish conidia on the black long conidiomata of *Chrysoporthe deuterocubensis* developed around the bark surface (Figure 2G) and long golden-coloured asexual fruiting structures (Figure 2H). The culture that grew on MEA is usually white when young and becomes pale yellow-brown in the centre with age with the optimal temperature for growth being 30 °C. Figure 2I shows the living culture of *Chrysoporthe deuterocubensis* after growing for 14 days on MEA at room temperature (Figure 2J) with sexual fruiting structures of *Chrysoporthe deuterocubensis* in culture.

3.2.2. *Chrysoporthe cubensis*

The severe cases of stem canker disease caused by *Chrysoporthe cubensis* can result in tree death. The pathogen kills the cambium, which is cracked and sunken on the trunk of infected trees (Figure 3A). The cankers are usually found at the base or on the lower stems of trees and bark cracking and swelling can be seen in Figure 3B. Conidiomata of *Chrysoporthe cubensis* around bark surface Figure 3C). Fresh and dried gummosis with yellowish conidia of *Chrysoporthe cubensis* was developed around the bark surface (Figure 3D). (Figure 3E) The pyriform conidiomata and extending perithecia necks were covered in dark tissue (Figure 3F). The pulvinate conidiomata and perithecia necks extending from the stromatal surface were covered in orange tissue (Figure 3G). Isolations from single conidia were made from the fruiting structures using malt extract agar MEA and *Chrysoporthe cubensis* grew optimally at 30 °C. Figure 3H shows the culture colour and development of the conidiomata.

3.3. Phylogenetic Analysis

A total of 17 isolates of *Eucalyptus urograndis* from four region; Kelantan, Pahang, Sabah and Selangor, respectively were subjected to DNA sequence analysis of ITS sequence. BLAST analysis of Genbank showed all 17 isolates from Pahang had 100% similarity with the reference sequence of *Chrysoporthe cubensis* MH858337. While all 17 isolates from Kelantan, Pahang, and Sabah showed 100% similarity with the reference sequence of *Chrysoporthe deuterocubensis* MH465621. Supplementary Figure S1 showed polymorphism of *Chrysoporthe cubensis* obtained from Pahang and *Chyrsoporthe deuterocubensis* obtained from Kelantan, Sabah and Selangor of ITS sequence with reference sequence of *Chrysoporthe cubensis* MH858337 and *Chrysoporthe deuterocubensis* MH465621, respectively.

For further analysis *β-tubulin 2* and *TEF-1α* gene regions were used. All 17 isolates of *Chrysoporthe cubensis* from Pahang and 17 isolates of *Chrysoporthe deuterocubensis* from Kelantan, Sabah and Selangor showed 100% similarity with the reference sequence of *TEF-1α* of *Chrysoporthe cubensis* Q290141 and *Chrysoporthe deuterocubensis* CQ290148, respectively. For *β-tubulin 2* all representative isolates showed 100% similarity with the reference sequences of *Chrysoporthe cubensis* HM142129 and *Chrysoporthe deuterocubensis* MH550155. Sequences from isolates were submitted to the Genbank database with ID numbers OQ581870-OQ581919 for ITS, OR096050-OR096059 (*Chrysoporthe cubensis*) and OR120063-OR120102 (*Chrysoporthe deuterocubensis*) for *β-tubulin 2*, and OR096060-OR096069 (*Chrysoporthe cubensis*) and OR096010-OR096049 (*Chrysoporthe deuterocubensis*) for *TEF-1α* gene regions. Supplementary Figure S2A showed molecular phylogenetic analysis of the combined data set ITS, *β-tubulin 2* and *TEF-1α* confirmed the distinction of *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis*. For single sequence molecular phylogenetic tree, all data set showed that *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis* in different clade (Supplementary Figure S2B,C) except for data set sequence of *TEF-1α* (Supplementary Figure S2D).

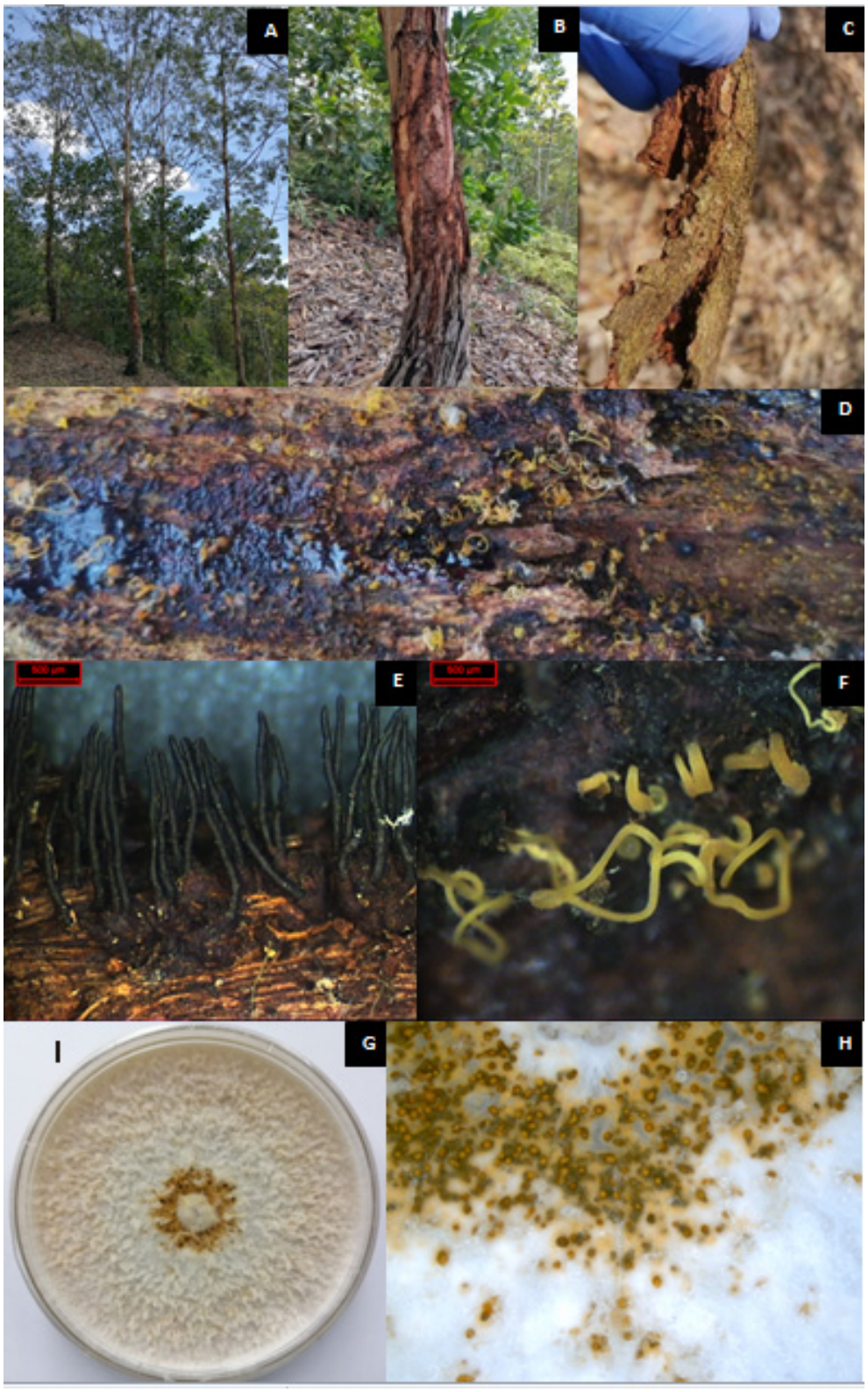

Figure 3. (**A**) *Chrysoporthe cubensis* affecting *Eucalyptus urograndis* in plantation. (**B**) Bark cracking and swelling. (**C**) Conidiomata of *Chrysoporthe cubensis* around bark surface. (**D**) Asexual fruiting structures in form of long golden-coloured cirrhi. (**E**) Conidiomata of *Chrysoporthe cubensis* on bark surface. (**F**) Long golden-coloured cirrhi fruiting bodies of *Chrysoporthe cubensis.* (**G**) Living culture of *Chrysoporthe cubensis* after growing for 14 days on MEA at room temperature. (**H**) Asexual fruiting structures of *Chrysoporthe deuterocubensis* in culture.

3.4. MAT Gene Amplification and Mating Types Assignment

The mating type idiomorphs were successfully amplified in all *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis* isolates. Each isolate was positive amplification by *MAT1-1* locus consists of *MAT1-1-1* gene on 824 bp, *MAT1-1-2* gene on 912 bp and *MAT1-1-3* gene on 467 bp while *MAT1-2-1* on 536 bp gene, respectively. All amplifies isolates of *Chrysoporthe. cubensis* and *Chrysoporthe deuterocubensis* had both the *MAT1-1* and *MAT1-2* mating types, confirming that they are homothallic mating species system. Supplementary Figure S3 shows of *MAT1-1-1*, *MAT1-1-2*, *MAT1-1-3* and *MAT1-2-1* amplicons in selected isolates. In addition the pheromone (*ppg1* and *ppg2-1*) and pheromone receptor (*pre-1* and *pre-2*) were successfully amplified in both *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis*.

3.5. Pathogenicity Tests

3.5.1. 3 Years Old Standing Tree

A total of 20 isolates representing *Chrysoporthe cubensis* and 20 isolates representing *Chrysoporthe deuterocubensis* isolated from Kelantan, Sabah and Selangor were selected and inoculated on 3 years old standing trees of two *Eucalyptus urograndis* and *Eucalyptus pellita* using mycelia plugs (Figure 4). The mycelia plugs of all tested isolates produced lesions on bark surface while no lesions were observed on the negative control. The longest and the shortest average lesion were 2.9 and 6.9 cm, respectively (Figure 5). *Chrysoporthe cubensis* (NHASUL1:OQ581890) induced an average lesion length of 3.5–5.5 cm, while *Chrysoporthe deuterocubensis* induced an average lesion length of 4.9–6.9 cm. *Chrysoporthe deuterocubensis* from Sabah (NHATWU9:OQ581908) was most aggressive on the tested stands of *Eucalyptus urograndis* followed by *Chrysoporthe deuterocubensis* from Selangor (NHAPCH2:OQ581881) with an average of 2.9–6.2 cm and *Chrysoporthe deuterocubensis* from Kelantan (NHAUMW2:OQ581911), with an average of 5.2–5.9 cm, with a value of <0.001 at the 95% confidence level. The *Chrysopothe cubensis* and *Chrysoporthe deuterocubensis* with the same characteristics as those of the originally inoculated fungi were successfully re-isolated from diseased bark, but none were isolated from the negative control, thus fulfilling the requirements of Koch's postulates. Furthermore, *Chrysoporthe cubensis* (NHASUL1:OQ581890) and *Chrysoporthe deuterocubensis* (NHATWU9:OQ581908) were tested on *Eucalyptus deglupta* and the non-host tree *Aqualaria sinensis.* Lesions developed on *Eucalyptus deglupta* infected by *Chrysoporthe cubensis* (NHASUL1:OQ581890) and did not develop for *Chrysoporthe deuterocubensis* (NHATWU9:OQ581908). No lesions developed on *Aquilaria sinensis* for both *Chrysoporthe cubensis* (NHASUL1:OQ581890) and *Chrysoporthe deuterocubensis* (NHATWU9:OQ581908), respectively. Figure 6 shows 3-year-old standing tree stands after 8 weeks of inoculation.

3.5.2. One-Year-Old Seedling

The isolates of *Chrysoporthe cubensis* (NHASUL1:OQ581890) and *Chrysoporthe deuterocubensis* (NHATWU9:OQ581908, NHAPCH2:OQ581881 and NHAUMW1:OQ581910) were tested on a 1-year-old potted seedling of *Eucalyptus urograndis.* After four weeks, all leaves on the upper part and at inoculant points wilted and dried (Figure 7). A seedling also produced few coppices as a response to the death area resulting from infection (Figure 7B). A significant difference was found between both isolates of two *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis* tested ($p < 0.0001$). No necroses or lesions were caused on the control plants. The isolate *Chrysoporthe deuterocubensis* from Sabah (NHATWU9:OQ581908) showed longer a lesion length on seedlings (0.6–1.1 cm). Conidiomata were collected to be re-isolated to fulfil the requirements of Koch's postulates. Figure 8 shows a fruiting structure that developed on the infected area.

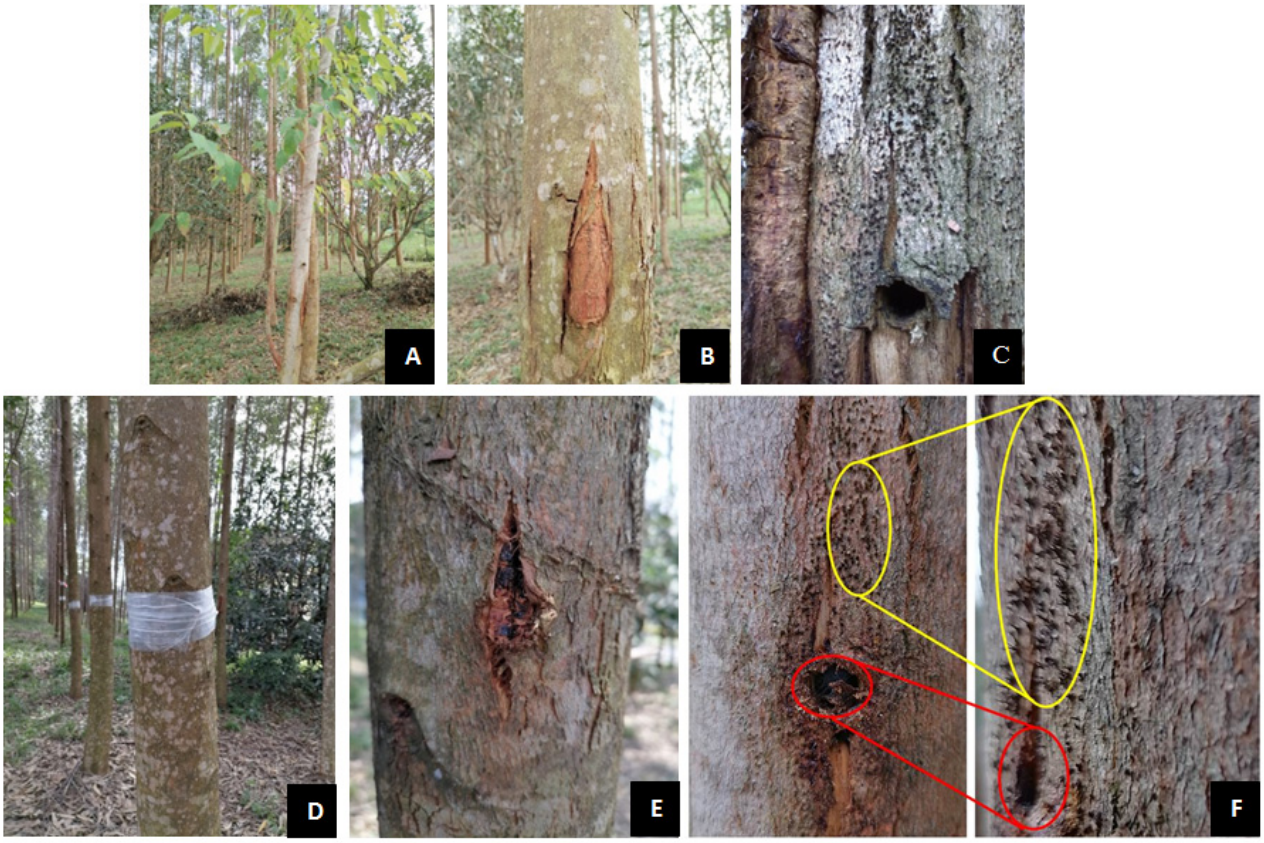

Figure 4. (**A**) Standing tree of *Eucalyptus urograndis* with copies appears below tested area after 8 months. (**B**) Cracks and sunken appearance. (**C**) Perithecia on outer bark. (**D**) Inserted inoculum area wrapped by parafilm. (**E**) Fresh and dried kino/gummosis. (**F**) Perithecia (yellow mark), a drilled area for agar plugs (red mark).

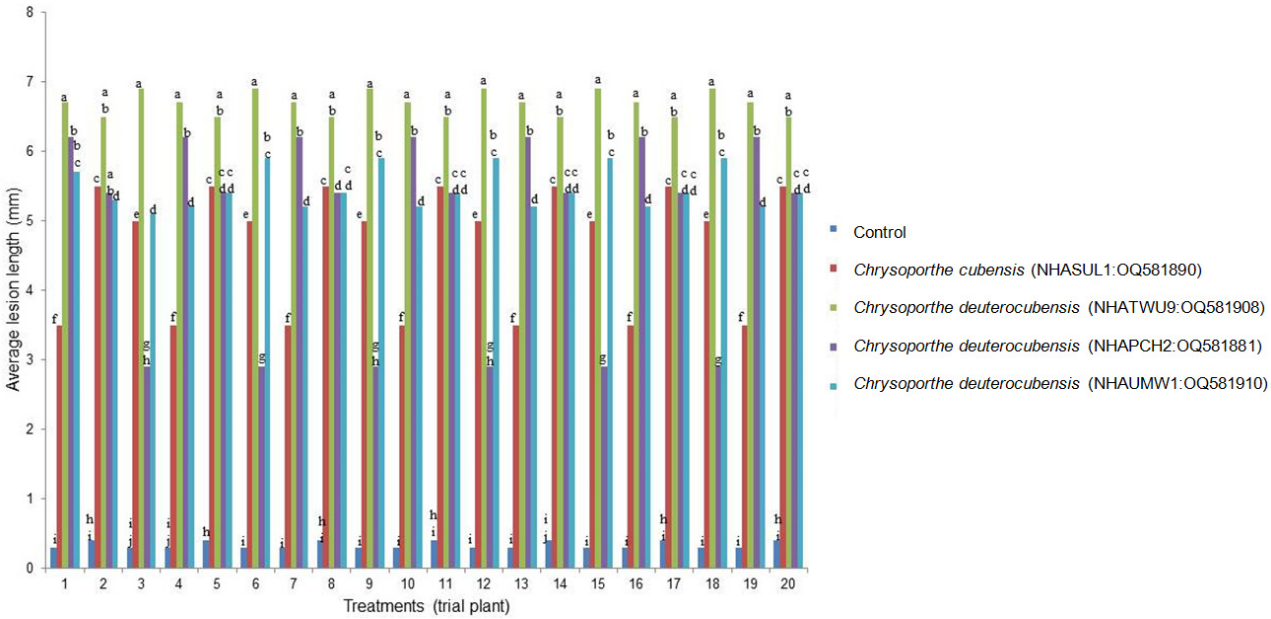

Figure 5. Column chart indicating the lesion length (mm) in the stand trial. The mycelia plug inoculation trials of two *Eucalyptus urograndis* was tested with selected pathogen strains of *Chrysoporthe* isolates and the controls. Bars topped by different letters indicate treatment means that are significantly different ($p = 0.05$).

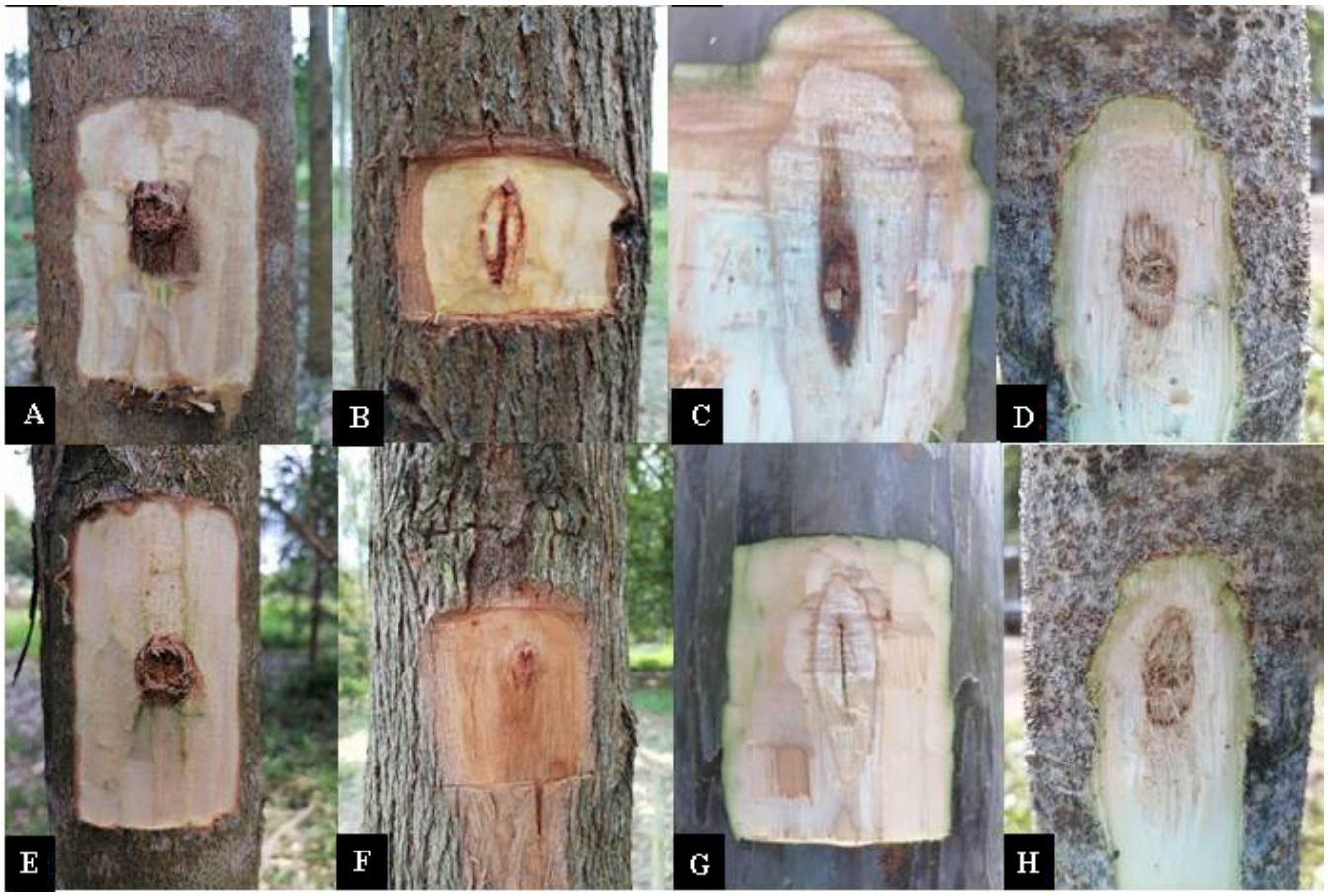

Figure 6. Lesions resulting from inoculations. (**A**) *Eucalyptus urograndis*. (**B**) *Eucalyptus pellita*. (**C**) *Eucalyptus deglupta*. (**D**) *Aqualaria sinensis* after 8 weeks inoculations of *Chrysoporthe deuterocubensis*. (**E**) *Eucalyptus urograndis*. (**F**) *Eucalyptus pellita*. (**G**) *Eucalyptus deglupta*. (**H**) *Aqualaria sinensis* 8 weeks after inoculation affected by *Chrysoporthe cubensis*.

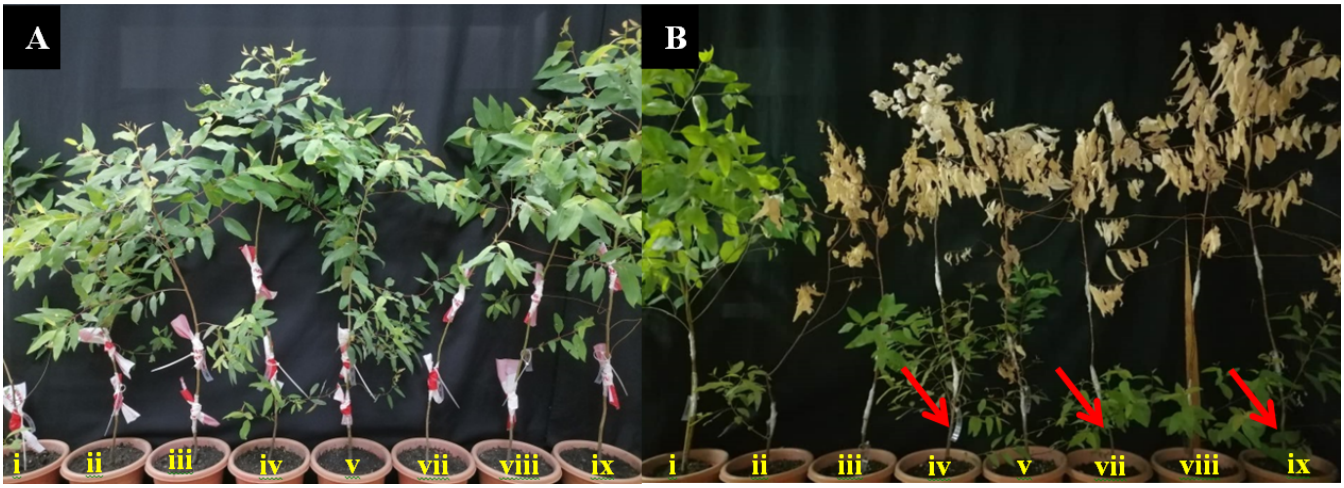

Figure 7. (**A**) Innoculation test from four isolates conducted on *Eucalyptus urograndis* one-year-old seedlings. (**B**) Positive symptoms shown after for 4 weeks of application. (i) Control, (ii,iii) NHATWU9:OQ581908, (iv,v) NHAPCH2:OQ581881, (vi,vii) NHAUMW1:OQ581910, and (viii,ix) NHASUL1:OQ581890. Arrow shows copies.

Figure 8. (**A**) Seedling after 4 weeks inoculation test with *Chrysoporthe deuterocubensis* (NHATWU9:OQ581908) (**B**) Conidiomata developed on seedling stem (**C**,**D**) Positive symptomatic shown development of conidiomata on inoculation site.

The pathogenicity test was also carried out on a 1-year-old seedling of a non-host tree. The tested trees were *Hopea odorata* (Merawan Siput Jantan), *Shorea leprosula* (Meranti Tembaga), *Neobalanocarpus heimii* (Cengal), *Gonystylus bancanus* (Ramin), *Casuarina equisetifolia* (Rhu Pantai), and *Dipterocarpus elongatus* (Keruing). The tested isolates were *Chrysoporthe cubensis* (NHASUL1:OQ581890), and *Chrysoporthe deuterocubensis* (NHATWU9:OQ581908, NHAPCH2:OQ581881, and NHAUMW1:OQ581910). After four weeks, only the *Neobalanocarpus heimii* (Cengal) seedling responded to the infection. All leaves above the inoculant points wilted and dried (Figure 9B) and the seedling also produced a coppice as a response to the death area caused by the infection (Figure 9B). Perithecia on crack and sunken (developed on inoculation area) (Figure 9C) were collected to fulfil the requirements of Koch's postulates. No necrosis or lesions were produced on control plants.

3.5.3. Detached Leaves

Chrysoporthe deuterocubensis isolated from Sabah (NHATWU9:OQ581908) was selected and inoculated on detached leaves. Lesions developed on the leaf surface after 7 days from inoculation (Figure 10) except for *Dyera costulata*, *Neobalanorpus heimii*, and *Hopea odorata*, for which no symptoms were recorded. No lesions were observed on the negative control. *Chrysoporthe deuterocubensis* with the same morphological characteristics as those of the originally inoculated fungi was successfully re-isolated from diseased tissues on the inoculated leaves, but never from the negative control, thus fulfilling the requirements of Koch's postulates. For this test, experiments were repeated twice and showed that lesions were produced. Three (3) dipterocarp leaves (Figure 10N–P) showed that no lesions were developed on the leaves' surface.

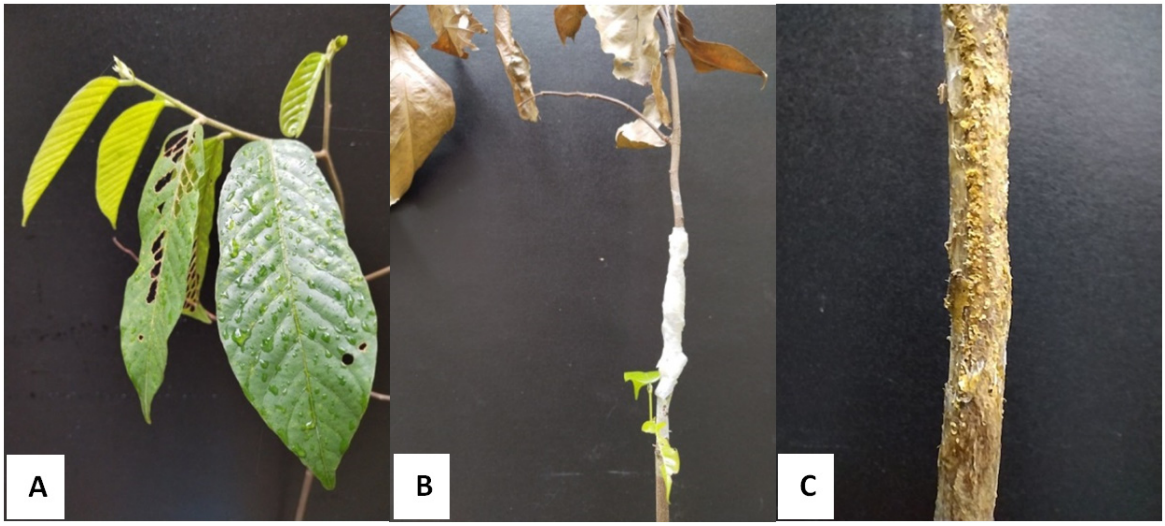

Figure 9. (**A**) Seedling of *Neobalanocarpus heimii*. (**B**) Coppices develop below inoculation area, after 4 weeks of inoculation with *Chrysoporthe deuterocubensis* (NHATWU9:OQ581908). (**C**) Conidiomata developed on stem showing positive symptoms.

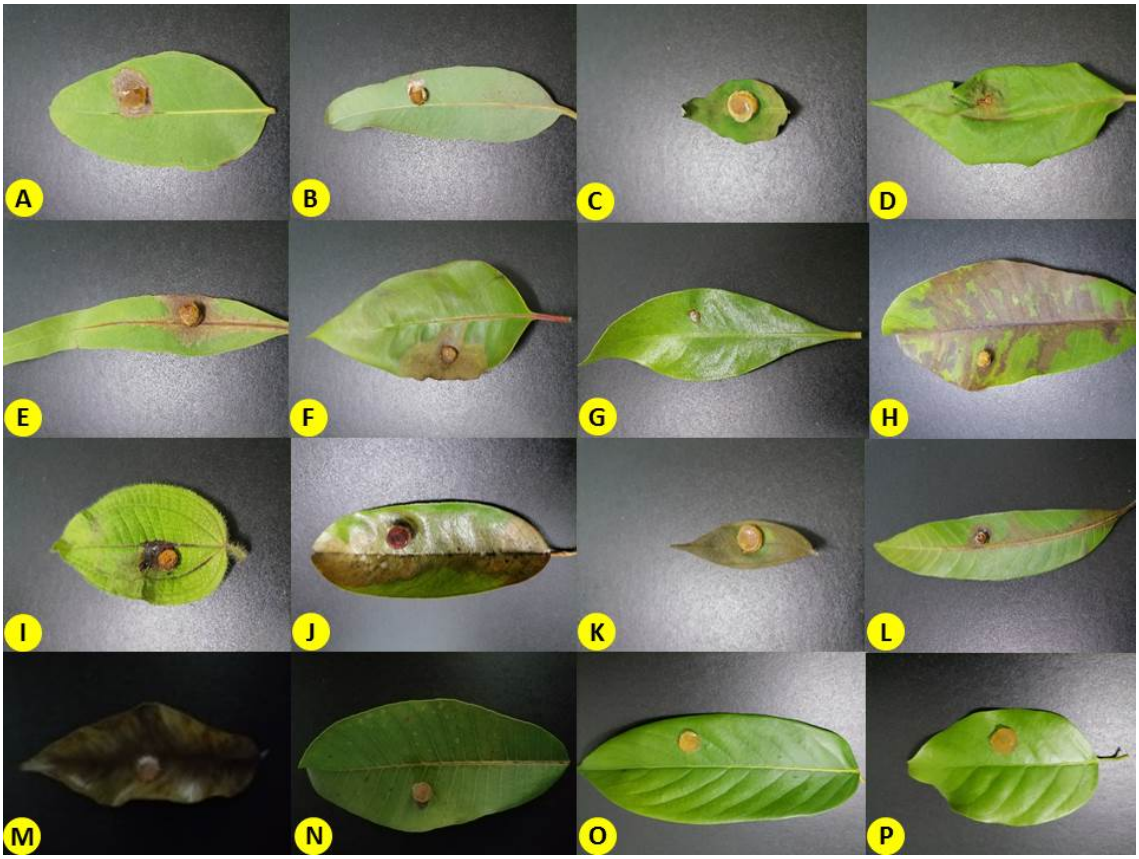

Figure 10. Pathogenicity on leaf under control condition of (**A**) *Eucalyptus urophylla*, (**B**) *Eucalyptus camaldulensis*, (**C**) *Eucalyptus urograndis*, (**D**) *Eucalyptus. deglupta*, (**E**) *Eucalyptus grandis*, (**F**) *Eucalyptus pellita*, (**G**) *Syzygium aromaticum*, (**H**) *Nephelium lappaceum*, (**I**) *Melastoma marianum*, (**J**) *Durio zibenthinus*, (**K**) *Melastoma malabathricum*, (**L**) *Mangifera indica*, (**M**) *Aquilaria sinensis*, (**N**) *Dyera costulata*, (**O**) *Neobalanorpus heimii*, and (**P**) *Hopea odorata*. Pictures show positive symptoms of leaves after days 7 inoculated by *Chrysoporthe deuterocubensis*, NHATWU9:OQ581908 (most virulent strain), for except N, O and P, for which no symptoms were recorded.

4. Discussions

The emergence of *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis* pathogens is a threat to the vitality of *Eucalyptus* spp. plantations in Malaysia. These pathogens are notorious pathogens that have been identified in many countries growing *Eucalyptus* trees for forest plantation, including Australia, Cameroon, Tanzania, Democratic Republic of Congo, Southeast Asia, South, Central and North America, Africa, and Hawaii [6,24,25]. In combination with the results from a previous study, this species was isolated from a Eucalyptus hybrid, namely *Eucalyptus urograndis*, in Malaysia [22], and this is the first recorded case of *Chrysoporthe cubensis* infecting Eucalyptus spp. in Malaysia and in the tropics. Stem disease with the typical symptoms caused by *Chrysoporthe* species was observed in four regions of *Eucalyptus* spp. plantations in Sabah, Pahang, Kelantan, and Selangor. A relatively large number of *Chrysoporthe* isolates were isolated from the diseased bark of two *Eucalyptus* genotypes sampled from trees in the plantation.

The isolates obtained in this study were identified mainly based on DNA sequence comparisons of single and combined *ITS*, *β-tubulin 2* gene and *TEF-1α* gene regions. The sequences of the three genes have been widely used to clearly distinguish between the intra- and inter-specific divergence of the *Chryphonectriaceae* genus [26]. Recently, Ref. [6] conducted a comprehensive phylogenetic analysis of the *Chryphonectriaceae* genus based on DNA sequences of eight gene regions; the results showed that *tef1*, and *tub2* sequences had the strongest ability to correctly identify species. Results confirmed the importance of multi-gene sequence phylogeny in species clarification and identification in *Chryphonectriaceae*.

The whole-genome *MAT1-1* and *MAT1-2* mating system in *Chrysoporthe deuterocubensis* and *Chrysoporthe cubensis* was analysed by the authors of [14], providing first description of mating type gene. In this study, the homothallism in *Chrysoporthe deuterocubensis* and *Chrysoporthe cubensis* was confirmed by the ability to detect the *MAT1-1* and *MAT1-2* locus amplicion isolation of individual isolates from single-hypha culture. A homothallic fungus such as *Chrysoporthe deuterocubensis* might have evolved in a similar pattern to that of asexual organisms with the appearance of clonal meiotic reproduction events that do not lead to the recombination of new alleles. The development and formation of a fruiting body is predominant in all cankers caused by *Chrysoporthe deuterocubensis* and *Chrysoporthe cubensis*, irrespective of the host [27]. Similarly to other homothallic microorganisms from the same phylum (Ascomycetes), the mating type locus of *Chrysoporthe deuterocubensis* contains Sordariomycete MAT genes *MAT1-1* and *MAT1-2* [19,28,29]. These genes were shown to be involved in sexual development [29].

The results of the pathogenicity tests based on mycelial plug inoculations in this study showed that all tested isolates were pathogenic for the two tested *Eucalyptus* species. In comparing both *Chrysoporthe* species, *Chrysoporthe deuterocubensis* has more virulence compared to *Chrysoporthe cubensis.* This was unsurprising for *Chrysoporthe deuterocubensis*, since inoculations in previous studies by the authors of [22] indicated that *Chrysoporthe deuterocubensis* is highly pathogenic in tested *Eucalyptus* species and is considered to be an important pathogen in *Eucalyptus* as well as many other plants [26]. The study conducted by the authors of [5] also showed that *Chrysoporthe deuterocubensis* is the most aggressive and highly pathogenic species among the different eight species in the family of *Cryphonecteriaceae* identified in China. As shown in this study, the severity rates of disease differ between trees, especially according to tree age. In the early stage, the disease could be detected on *Eucalyptus urograndis* three years after it was planted. Factors that influence different severity rates of *Chrysoporthe deuterocubensis* remain unknown. Disease severity could be caused by environmental factors or virulence genes such as CAZymes expressed via the degradation of plant cell walls [30] by the fungus during infection. The weak pathogenicity of other fungi from the *Cryphonectriaceae* family may be facilitated by environmental conditions, such as abiotic stress on the host or disturbance of the host microbiome [30]. Base on field observations, both *Chrysoporthe* spp discovered in this study enter the tree through natural openings and wounds, leading to infection in the inner bark and cambium layers.

This study conducted the first pathogenicity test for *Eucalytus deglupta* and *Aquilaria sinensis* with both *Chrysoporthe cubensis* and *Chrysoporthe cubensis*. *Chrysoporthe deuterocubensis* was pathogenic to all tested plants, which is a cause for concern due to its potential outbreak in another dipterocarp. Various canker pathogens of *Chrysoporthe* are known to undergo host jumps and may be considered latent pathogens that are similar to some endophyte jumps [31,32] between families and genera in *Myrtales* (*Myrtaceae* and *Melastomataceae*). In Colombia, *Miconia rubiginosa* (*Melastomataceae*) caused disease in exotic species of *Eucalyptus* that grew alongside each other. The origin of *Chrysoporthe cubensis* is not known but could have originated in native *Melastomataceae* or *Myrtaceae* in Pahang. These plants are common in the area and could represent a ready source of potential fungal pathogens capable of host jumps.

5. Conclusions

This study expanded our understanding of the species diversity, host range, mating strategy, genetic diversity, and pathogenicity of *Chrysoporthe* in *Eucalyptus* spp. plantations. Further studies are necessary to increase the knowledge of fungi's ecology, the propagation pathway for these species, and the pathogenesis of the species. The inoculation results further indicated that the tolerance of different *Eucalyptus* genotypes is different, which highlights the importance of selecting disease-resistant *Eucalyptus* genotypes in the future.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f14081660/s1, Figure S1: Alignment of a region of partial ITS sequence single polymorphism between *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis*; Figure S2: (A) Phylogram from a maximum likehood of six *Chrysoporthe* spp. and *Endothia gyrosa* as outgroup based on the combined *ITS*, *β-tubulin 2* gene and *TEF-1α*. (B) Phylogram from a maximum likehood of six *Chrysoporthe* spp. and Endothia gyrosa as outgroup based on the *ITS* regions. (C) Phylogram from a maximum likehood search of six *Chrysoporthe* spp. and *Endothia gyrosa* as outgroup based on the *β-tubulin 2* region. (D) Phylogram from a maximum likehood of six *Chrysoporthe* spp. and *Endothia gyrosa* as outgroup based on the *TEF-1α*; Figure S3: Amplicon of *MAT1-1* locus genes and *MAT1-2* locus gene. a. *MAT1-1-1* 842 bp, b *MAT1-1-2* 912 bp, c. *MAT1-1-3* 467 bp and d. *MAT1-2-1* 536 bp.

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