CHARACTERIZATION, BIOLOGICAL CONTROL OF *CHRYSOPORTHE CUBENSIS* AND *CHRYSOPORTHE DEUTEROCUBENSIS* AND FUNGAL DIVERSITY IN EUCALYPTUS PLANTATIONS IN MALAYSIA

By

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Eucalyptus spp. trees are essential in Malaysia's forest plantation sector due to their fast growth rate, high wood density, and higher economic return. These characteristics make them a valuable source of alternative wood for paper manufacture and construction materials. Long-term plantations with short rotations, extreme human disturbance, and natural allelochemical activities from *Eucalyptus* tissues may reduce the native microbiome diversity in *Eucalyptus* hybrid (*Eucalyptus urophylla* x *Eucalyptus grandis*) plantations. As part of exploring the diversity of fungal groups that exist in the soil and on the leaves of *Eucalyptus*, the study also assesses the *Chrysoporthe* stem canker disease affecting *Eucalyptus* plantations from four regions in Malaysia: Kelantan, Pahang, Sabah, and Selangor. The discovery of these canker diseases led to further action in developing biocontrol agents to eliminate and control the disease. Therefore, these present studies aim to; i) utilise a metagenomic approach to fungal communities in soil and on the leaves of *Eucalyptus* spp., ii) assess the pathogenicity of the fungi causing stem canker disease, and iii) evaluate biocontrol agents of the secondary metabolite from antagonism activity against *Chrysoporthe* stem canker disease. To perform the study, a total of 6 phyla, 32 classes, and 821 genera of fungi were discovered from two different types of samples: soil and leaves in the *Eucalyptus* plantation by shotgun metagenomic sequencing. According to the Chao1 estimator and the Shannon index, fungal species diversity and richness are lower in leaves than in soil samples. The fungi species from classes of *Fusarium*, *Humicola*, *Talaromyces*, and *Trichoderma* were discovered as potential biological controls in two different samples from four different regions. The results will help to better understand fungal interactions and provide information on *Eucalyptus* hybrid (*Eucalyptus urophylla* x *Eucalyptus grandis*) fungal communities, particularly the role of pathogenic fungi, which can be useful in minimising disease-related fungal communities. To reveal the study on the assessment of *Chrysoporthe* stem canker disease, the molecular DNA sequence data of the *ITS*, *β*-tubulin 2, and TEF-*1α* with additional mating type genes *MAT1-1* and *MAT1-2*, pheromone genes (*ppg1*, *ppg2*, *pre1*, and *pre2*) and pathogenicity studies in field trials confirmed the stem canker disease caused by *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis*. Both *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis* gave rise to lesions on the wounded (2.9 – 6.9 cm) and were equally pathogenic to *Eucalyptus* hybrid and *Eucalyptus pellita*. This finding should be regarded as a biosecurity concern in Malaysia's forest plantation industry. Hence, further studies were developed on *Eucalyptus* endophyte fungi for potential sources of biological compounds for producing secondary metabolite products. Of the 88 selected endophyte isolates, only six isolates positive against the *Chrysoporthe* culture were processed to produce crude compounds. The 27 major compounds are present in most potential isolates from the *Botryosphaeria dothidea* strain and may be the reasons this endophyte can go against *Chrysoporthe* spp. with 82.14-78.57% inhibition rates. It includes an α-pyridone derivative, 3-hydroxy-2-methoxy-5-methylpyridin-2(1H)-one, and 3-hydroxy-N-(1-hydroxy-4-methylpentan-2-yl)-5-oxohexanamide. The extract displayed was believed to have potent antifungal and antimicrobial activity with the same mechanism as carbendazim (a nationally licensed fungicide). To conclude, the study reveals that *Eucalyptus* plantations in Malaysia have been identified and confirmed. While the biological compound identified from endophytic fungi can be utilised for commercialization in controlling the *Eucalyptus Chrysoporthe* stem canker disease outbreak in the plantation in Malaysia.

Keywords: *Chrysoporthe deuterocubensis*, *Chrysoporthe cubensis*, Canker disease, *Eucalyptus*, endophyte, biological control

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PENCIRIAN, KAWALAN BIOLOGI *CHRYSOPORTHE CUBENSIS* DAN *CHYSOPORTHE DEUTEROCUBENSIS* DAN KEPELBAGAIAN KULAT LADANG EUCALYPTUS DI MALAYSIA

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Spesis *Eucalyptus* adalah tanaman ladang hutan yang penting dalam sektor perladangan hutan di Malaysia kerana kadar pertumbuhannya yang cepat, kepadatan kayu yang tinggi, dan pulangan ekonomi yang tinggi. Ciri-ciri ini menjadikannya ianya sebagai tanaman sumber kayu alternatif yang berharga sebagai bahan pembuatan kertas dan bahan binaan. Perladangan jangka panjang dengan kadar kitaran yang pendek, aktiviti manusia, dan kehadiran sebatian semulajadi alelokimia dari tisu tanaman *Eucalyptus* boleh mengurangkan populasi kepelbagaian mikrobiom tersedia di dalam tanah di ladang *Eucalyptus* hibrid (*Eucalyptus urophylla* x *Eucalyptus grandis*). Setaraf kepada sebahagian daripada penerokaan kepelbagaian populasi kulat yang wujud tersedia di dalam tanah dan di dalam daun *Eucalyptus*, kajian ini juga menilai keseriusan penyakit *Chrysoporthe* kanker batang yang menjejaskan ladang *Eucalyptus* di empat negeri di Malaysia; Kelantan, Pahang, Sabah, dan Selangor. Penemuan penyakit kanker *Chrysoporthe* ini membawa kepada

tindakan selanjutnya dalam pembangunan agen kawalan biologi dalam usaha penghapusan dan pengawalan kadar sebaran penyakit ini. Oleh itu, kajian bertujuan untuk; i) menggunakan pendekatan metagenomik terhadap populasi kulat yang tersedia di dalam tanah dan dalam sap sel daun tanaman *Eucalyptus* spp.; ii) mengenal pasti ujian patogenik terhadap kulat yang menyebabkan penyakit kanker batang; dan iii) menilai agen kawalan biologi melalui penghasilan metabolit sekunder hasil daripada aktiviti antagonis terhadap penyakit kanker *Chrysoporthe* batang. Melalui kajian ini, sebanyak 6 filum, 32 kelas, dan 821 genera kulat telah ditemui dari dua sampel tanah dan daun di ladang hutan *Eucalyptus* melalui analisa penjujukan metagenomik shotgun. Menurut analisis indek Chao1 dan indek Shannon, kepelbagaian dan kepadatan spesies kulat adalah lebih rendah dalam sap sel daun tanaman *Eucalyptus* berbanding dari sampel tanah. Melalui analisis ini juga mendapati bahawa spesis kulat dari kelas *Fusarium*, *Humicola*, *Talaromyces*, dan *Trichoderma* dikategori sebagai spesis yang berkeupayaan dalam pembangunan kawalan biologi penyakit. Oleh yang demikian, dapatan daripada kajian ini dapat membantu untuk lebih memahami interaksi di antara kulat dan ianya dapat memberikan maklumat mengenai peranan kulat yang menyebabkan patogen. Ini adalah usaha meminimumkan populasi kulat patogen yang menyebabkan penyakit di ladang hutan *Eucalyptus* hibrid (*Eucalyptus urophylla* x *Eucalyptus grandis*). Bagi pengesahan penilaian kajian terhadap penyakit kanker batang *Chrysoporthe*, analisis yang melibatkan data jujukan molekul DNA bagi spesifik gen *ITS*, *β*-tubulin 2, TEF-*1α*, *MAT1-1*, *MAT1-2* (*ppg1*, *ppg2*, *pre1* dan *pre2*) mendapati bahawa kajian patogenik di lapangan mengesahkan penyakit kanker batang adalah disebabkan oleh *Chrysoporthe cubensis* dan *Chrysoporthe deuterocubensis*. Kedua-dua kulat patogen, *Chrysoporthe cubensis* dan *Chrysoporthe deuterocubensis* menyebabkan luka pada permukaan batang (2.9-6.9 cm) dan menjadikannya amat patogenik terhadap *Eucalyptus* hibrid dan spesis *Eucalyptus pellita* di Malaysia. Penemuan ini harus dianggap sebagai kebimbangan biosekuriti dalam industri perladangan hutan di Malaysia. Oleh yang demikian, kajian lanjut dibuat ke atas kulat endofit *Eucalyptus* bagi mengenalpasti sebatian metabolit sekunder yang diperolehi daripada endofit. Daripada 88 strain endofit yang berpotensi, hanya enam strain yang terpilih bagi penghasilan penyulinan sebatian aktif. Melalui analisis GC-MS, terdapat 27 sebatian aktif utama yang terkandung di dalam strain dari spesies *Botryosphaeria dothidea*. Ianya termasuk derivatif *α-pyridone*, *3-hydroxy-2-methoxy-5-methylpyridin-2(1H)-1*, dan *3-hydroxy-N-(1-hydroxy-4-methylpentan-2-yl)-5-oxohexanamide*. Ekstrak yang dihasilkan dipercayai mempunyai aktiviti antikulat dan antimikrob yang berkesan dan mempunyai mekanisma yang sama seperti carbendazim (racun kulat berlesen yang terdapat di pasaran tempatan). Kajian ini merumuskan pendedahan mengenai penyakit utama di ladang *Eucalyptus* di Malaysia oleh serangan kulat *Chrysoporthe* telah dikenalpasti serta disahkan. Sebatian biologi yang dikenal pasti daripada kulat endofit boleh digunakan bagi tujuan pengkomersialan dalam pengawalan penyakit *Chrysoporthe* kanker batang pada *Eucalyptus* di ladang Malaysia.

Kata Kunci: *Chrysoporthe deuterocubensis*, *Chrysoporthe cubensis*, Penyakit kanser batang, *Eucalyptus*, endofit, kawalan biologi

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LIST OF ABBREVIATIONS

BCA	biological control agent
EFs	endophypte fungi
SMs	secondary metabolism
C-group	*clavicipitaceous* group
NC-group	*nonClavicipitaceou*s group
MLD	*Mycosphaerella* leaf disease
MEA	malt extract agar
NGS	metagenomics-based next-generation sequencing
HMBC	heteronuclear multiple bond correlation

CHAPTER 1

INTRODUCTION

1.1 Background and problem statement

The genus *Eucalyptus* L.'Her., *Corymbia* K.D. Hill & L.A.S. Johnson and *Angophora* Cav., common commercial trees planted in Malaysian forest plantations, are native to Australia and belong to the *Myrtaceae* family. They represent more than 900 species and are planted in 95 countries across tropical, subtropical, and temperate regions, accounting for more than 22.57 million hectares worldwide (Zhang and Wang, 2021; Yahya, 2020; Nicolle, 2006; Keane *et al*., 2000; Brooker, 2000). The planting of *Eucalyptus* spp. and its hybrids in Malaysia has been increasing lately and is poised to replace *Acacia* spp. as the main species planted.

One of the important hybrids of *Eucalyptus* spp. planted in Malaysia is an interspecies hybrid type between *Eucalyptus urophylla* × *Eucalyptus grandis* (known as clone 3226, clone 3227), *Eucalyptus grandis* × *Eucalyptus urophylla* (known as clone G09), pure species, *Eucalyptus pellita*, and *Eucalyptus deglupta*. It is ideal for a variety of products from *Eucalyptus* tree utilizations such as solid wood, charcoal, and pulp and paper, with productivity that can exceed an average of >40 m^3 per ha/year in some areas where proper conditions (soil, climate, genetic material, and silvicultural practices) are in place due to potential rapid growth, high resistance to pest and disease attacks, exceptional rooting ability, and wood quality.

In 2021, the report from the Malaysian Timber Industry Board (MTIB) contributed RM13,421 million to for total trade, with RM10,164.49 million in exports and RM3,256.51 mil in imports for wood products to the country (MTIB, 2023). This number clearly indicates that the wood-based industry is facing a shortage of raw timber, which may require sustainable resources for wood. As the wood-based industry contributes significantly to Malaysia’s economy, the readiness of Malaysia towards a sustainable wood-based industry has been considered with the introduction of new species for sustainability in the industries. However, Malaysia remains dependent on other countries for seedling supplies, such as China.

Due to the demands, the Ministry of Plantation Industries and Commodities (MPIC) Malaysia launched a development program for forest plantations as part of aggressive attempts to ease the pressure on native forests as a source of raw materials and to guarantee the survival of the domestic timber industry. Using this strategy, the Malaysian Timber Industry Board promotes the growth of extensive commercial forest plantations (Zaiton *et al*., 2020). Over the past 10 years, the Malaysian forest industry, which aims to produce 75 million m^3 of timber annually to meet the raw material requirement of the Malaysian timber industry, has exported more than MYR 100 billion (USD 24.4 billion) of wood products or 17% of Malaysia’s total exports, is potentially at risk from pest and disease outbreaks in *Eucalyptus* plantations (Yahya, 2020; Zaiton *et al*., 2020).

However, studies have shown that *Eucalyptus* plantations are susceptible to pathogenic fungi that cause diseases such as rust, canker, and wilt disease (Fisher *et al*., 2020). The fungal species associated with the most common diseases include, *Chrysoporthe cubensis* (Bruner) Gryzenh. & M.J. Wingf. (Chen *et al*., 2018; Van *et al*., 2010), *Chrysoporthe deuterocubensis* Gryzenh & M.J. Wingf. (Mausse-Sitoe *et al*., 2016; Pegg *et al*., 2010), *Ralstonia solanacearum* Smith (Gomes *et al*., 2023; Coutinho *et al*., 2000), *Erythricium salmonicolor* (Graziosi *et al.*, 2020), *Teratosphaeria* spp. (Solís *et al*., 2022), *Mycosphaerella* spp. (Barreto *et al*., 2022), *Lasiodiplodia theobromae* (Admasu *et al*., 2023), and *Botryosphaeria dothidea* *(*Deidda *et al*., 2016). 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* is commonly present in South-eastern Asia (Old *et al*., 2003) and *Chrysoporthe cubensis* 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 *Chryphonecteriaceae* (Wang *et al*., 2020).

The study by Kanzi *et al.* (2019) 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 have 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. Sexual reproduction of *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis* plays a role in the disease under natural conditions (Myburg *et al*., 2002). 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.

Therefore, the utilisation of a metagenomic approach to identify fungal communities that exist in the soil and on the leaves of the *Eucalyptus* hybrid (*Eucalyptus urophylla* × *Eucalyptus grandis*), as well as to predict and help to better understand the fungal community that exists on or around a *Eucalyptus* Forest plantation in Malaysia. With this information, we can take steps to minimize the fungal community that is associated with the disease. Recent advances in metagenomics have allowed for the identification of fungal communities in a wide range of environmental samples using next-generation sequencing (NGS) technology (Laudadio *et al*., 2018).

NGS based on metagenomics is a culture-independent approach that provides a direct way to identify microorganism populations, making it an increasingly prominent tool in microbial ecology research (Laudadio *et al*., 2018). Researchers may investigate a vast number of precise taxonomic profiles from samples using NGS technologies such as Illumina sequencing, which can provide useful insights into the makeup and function of fungal communities in varied environments (Jimu *et al*., 2015). A study successfully identified stem canker diseases and leaf spot diseases caused by fungi using DNA sequences up to the species level in *Eucalyptus* tree species. Reference Awing *et al*. (2023) revealed that *Eucalyptus* trees affected with canker diseases experienced less shrinkage than healthy trees, notably volumetric and radial shrinkage. Based on this discovery, we reasoned that there is a correlation between fungal communities in the tree and fungal pathogens that cause this canker disease (Tan *et al*., 2021; Huang *et al*., 2020).

Recently, two species of *Chrysoporthe* have been discovered in Malaysia, identified as *Chrysoporthe cubensis* recorded in Pahang while *Chrysoporthe deuterocubensis* was recorded in Kelantan, Sabah, and Selangor. These species were identified using phylogenetic species hypothesis based on the internal transcribed spacer regions (*ITS*), β-tubulin2, and elongation-α. Pathogenicity tests also showed that both *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis* were pathogenic to *Eucalyptus* hybrids, specifically *Eucalyptus urophylla* × *Eucalyptus grandis* and *Eucalyptus pellita*. Similar to other countries, *Chrysoporthe* disease can be successfully controlled by early recognition of symptoms on three-year-old plants and applying suitable chemical fungicides such as Thiram, Prochloraz Manganese Chloride, Benlate, Cuprofix, Difcor, Halexyl, Lipel SP, Ridomil, and Tazer (Annya *et al*., 2023; Old *et al.*, 2003). However, this approach is neither economical nor practical in large *Eucalyptus* plantations that cover thousands of hectares. Previous reliance on synthetic chemical fungicides has been shown to be temporary and can lead to the loss of beneficial organisms due to chemical run off in soils and water irrigation. Therefore, the proposed study is essential to assess potential diseases in expanding *Eucalyptus* plantations and promote appropriate biological control practices in Malaysia.

As a result, the search for antifungal substances with novel structures and outstanding bioactivity compounds from natural products has become a research focus in forest plantations. Natural products are derived from natural sources such as microorganisms, plants, or animals (Ikram *et al.*, 2019). Endophytic fungi, along with other microorganisms, inhabit the tissues of living plants without causing harm to their host plants (Hamayun *et al.*, 2021; Sharma *et al*., 2020; Ikram *et al*., 2019). Endophytic fungi of plants are known as a valuable source of natural products with diverse bioactivities, including antifungal, antibacterial, larvicidal, antioxidant, anti-inflammatory, and phytotoxic activities (Farid *et al.*, 2022; Nisa *et al.*, 2020; Sharma *et al*., 2020). An increasing number of active substances with unique chemical structures have been isolated from endophytic fungi using a combination of morphological, phylogenetic, and metagenomics analyses. The main antibacterial, antifungal, antiviral and anticancer compounds come from natural sources such as fungi, and most endophytic fungi produce bioactive secondary metabolites such as alkaloids, terpenoids, steroids, quinones, isocaumarin lignans, phenylpropanoids, phenols and lactones (Deshmukh and Gaikwad, 2014; Radić and Strukelj, 2012).

Only a limited number of studies have explored the presence of endophytic fungi in various *Eucalyptu*s species. Therefore, our ongoing investigation focuses on endophytic fungi in *Eucalyptus* species leaf sources, including *Eucalyptus camaldulensis*, *Eucalyptus deglupta, Eucalyptus grandis, Eucalyptus pellita, Eucalyptus urograndis,* and *Eucalyptus urophylla*. These sources are believed to harbor endophytes with antimicrobial activity against *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis* canker as they colonize plant tissues internally, providing protection from abiotic and biotic stresses and fluctuations.

Furthermore, the presence and activity of fungi in these communities might influence the availability of nutrients and other factors that influence pathogen proliferation (Burke *et al*., 2011). Similarly, microorganisms on plant leaves can influence harmful fungus growth with chemicals that hinder the pathogen or modify the plant's defense mechanisms (Vinale *et al*., 2014), producing hormone compounds such as myrcene and p-cymene-8-01 (Du *et al*., 2020; Bai and Tang, 2020; Francke and Vité, 1983), biosynthesizing and acquiring nutrients from plant growth and development, and secreting stress-adaptor metabolites to protect the host plant from the invasion of pathogens with compounds such as α-pyridone (Amer *et al.,* 2021), 3-hydroxy-2-methoxy-5-methylpyridin-2(1H)-one (Schulz *et al*., 2002), chaetoglobosin C (Onocha *et al*., 1995), stemphyperylenol (Qin *et al*., 2009), 2,3-dihydroxypropanal (Xiao, 2014), 3-deoxy-D-mannoic lactone (Zhu *et al*., 2023), alteusin (Banakar and Jayaraj, 2018), γ-terpinene (Zochedh *et al*., 2022), cis-P-menth-2-en-1-ol (Padalia *et al*., 2018), terpinen-4-ol Morcia *et al*., (2012), and cryptone (Elaissi *et al*., 2011) and insect pests such as terpinolene (Menezes *et al.,* 2021), improving the host's adaptability to abiotic stressors. Overall, the composition and activity of fungal communities in soil and on plant leaves can have a substantial impact on the success or failure of fungal diseases or soil-borne pathogens in a given environment.

1.2 Objectives

The objectives of this study were to produce biological control through the application of beneficial endophytes to control *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis* in *Eucalyptus* tree plantations. The following were further determined:

i) fungal communities in the soil and on plants part using a metagenomic approach
ii) pathogenicity studies in trial and control conditions
iii) the effectiveness of beneficial antagonists, biological control, and crude substances in controlling *Chrysoporthe cubensis* and *Chrysoporthe deuterocubensis*

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