BIOCHEMICAL ANALYSIS ON THE METABOLITES FROM OIL PALM ROOTS RELATED TO *Ganoderma boninense* PATHOGENESIS

ROZLIANAH FITRI BINTI SAID

FACULTY OF SCIENCE AND NATURAL RESOURCES
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ROZLIANAH FITRI BINTI SAID
PS20119014

(Tandatangan Pustakawan)

Tarikh : 30 Ogos 2023

(Prof. Ts. Dr. Chong Khim Phin)
Penyelia Utama

DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, equations, summaries, and references, which have been duly acknowledged.

19 January 2023

Rozlianah Fitri binti Said
PS20119014

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NAME	:	**ROZLIANAH FITRI BINTI SAID**
MATRIC NO.	:	**PS20119014**
TITTLE	:	**BIOCHEMICAL ANALYSIS ON THE METABOLITES FROM OIL PALM ROOTS RELATED TO *Ganoderma boninense* PATHOGENESIS**
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FIELD	:	**BIOTECHNOLOGY**
VIVA DATE	:	**19 JANUARY 2023**

CERTIFIED BY;

Signature

1. **MAIN SUPERVISOR**
Professor Ts. Dr. Chong Khim Phin

____

2. **CO-SUPERVISOR**
Professor Dr. Jualang Azlan Gansau

____

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Rozlianah Fitri binti Said
19 January 2023

ABSTRACT

Basal Stem Rot (BSR) disease caused by *Ganoderma boninense* is the most severe disease in Malaysia and other South East Asia oil palm plantations. This disease contributes to significant yield losses and impacts the oil palm industry. To date, there is no promising remedy or reliable detection technique for this disease is confidently reported. Therefore, this study aims to investigate metabolites that may contribute to the partial resistance or susceptibility of oil palm to *G. boninense***.** Six-month-old oil palm seedlings' roots were elicited with copper sulphate ($CuSO_4$) to investigate the presence of their phytochemical constituents. Oil palm roots were extracted with six different solvents. Among the solvents were methanol, ethanol, acetone, ethyl acetate, chloroform and petroleum ether. The distribution of phytochemicals in six-month-old oil palm roots extracted with different solvents was assessed and compared. The findings suggest that methanol was the best solvent extraction for $CuSO_4$-elicited oil palm roots. It possessed the ability to extract various phytochemical constituents and exhibited the greatest antifungal activities significantly among all the investigated solvents extraction. Meanwhile, a fieldwork study has been conducted to evaluate the level of partial resistance and susceptibility among different commercial oil palm progenies against basal stem rot (BSR) caused by *G. boninense***.** Sixteen-month-old of four different progenies (with different genetic backgrounds) of oil palm seedlings consisting of non-inoculated seedlings served as control and inoculated seedlings with *G. boninense* colonized rubber wood blocks were screened for the differences. Disease development was observed and recorded as disease incidence (DI) percentage at monthly intervals for the period of nine months. Then, the oil palm roots were harvested at nine months after inoculation for ergosterol content analysis and re-isolation of inoculated and non-inoculated root segments on *Ganoderma* Selective Medium (GSM) for further confirmation. Results showed that Johor Labis Deli *dura* x AVROS *pisifera* (P4) and (Nigerian x Deli) *dura* x AVROS *pisifera* (P5) were tolerant progenies. Meanwhile, Banting Deli *dura* x AVROS *pisifera* (P1) and (Ulu Remis x Heze) Deli *dura* x AVROS *pisifera* (P3) were susceptible progenies. DI was observed as early as four months after inoculation for P1 and P3 compared to P4 and P5 that showed DI at five months after inoculation. DI was gradually increased to 100% for

P1 and P3 progenies at nine months after inoculation. Disease severity values for the internal symptoms of bole tissues and accumulation of ergosterol were significantly different and much higher for P1 and P3 in comparison to P4 and P5. The inoculated and non-inoculated crude methanolic extracts of oil palm roots were screened *in vitro* for antifungal activities using agar dilution technique. The results of antifungal tests among all non-inoculated oil palm progenies crude methanolic extracts showed no significant difference. In contrast, results obtained for inoculated oil palm progenies crude extracts of P4 (23.70%) and P5 (22.96%) gave a significantly higher percent of inhibition in comparison to P1 and P3 that gave significantly weak antifungal activities with a lower percent of inhibition, which is 16.66% and 14.42% respectively. GC-MS analysis of the oil palm roots crude extracts revealed the presence of eight phytocompounds and most with higher abundances of several fatty acids and only two compounds from the phenol group. The compounds are benzoic acid, methyl ester; phenol, 2,6-dimethoxy, 1,4-benzenedicarboxylic acid, dimethyl ester; phenol, 2,4-bis (1,1-dimethylethyl); hexadecanoic acid, methyl ester; 9,12-octadecadienoic acid (Z,Z)-, methyl ester; 9-octadecenoic acid (Z)-, methyl ester and octadecanoic acid, methyl ester. The activities of three defense enzymes including peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) were also evaluated. Results showed that at nine months after inoculation the PO, PPO and PAL activity of oil palm roots from the inoculated progenies was higher than that from the non-inoculated. In the inoculated progenies, the PO, PPO and PAL activity of the tolerant progenies was the highest for P4 followed by P5, and that of the susceptible progenies was the lowest for P3 and P1. The PO, PPO and PAL acted as plant active defense mechanisms against the attack of *G. boninense*. This study has discovered new metabolites that can aid future research efforts related to BSR disease. The compounds produced by the oil palm seedlings could be used as biomarkers to assess the resistance level to *G. boninense*, which may provide important insights for the early detection of BSR disease in seedling management.

ABSTRAK

ANALISIS BIOKIMIA PADA METABOLIT AKAR KELAPA SAWIT YANG BERKAITAN DENGAN PATOGENESIS Ganoderma boninense

Penyakit reput pangkal batang (BSR) yang disebabkan oleh Ganoderma boninense adalah penyakit paling teruk di ladang-ladang kelapa sawit di Malaysia dan Asia Tenggara yang lain. Penyakit ini menyumbang kepada kerugian hasil yang ketara dan memberi kesan kepada industri kelapa sawit. Sehingga kini, tidak ada pelaporan yang meyakinkan berhubung rawatan atau teknik pengesanan yang boleh dipercayai untuk penyakit ini. Oleh itu, kajian ini adalah bertujuan untuk menyiasat metabolit yang boleh menyumbang kepada rintangan separa atau kerentanan kelapa sawit kepada G. boninense. Akar anak pokok kelapa sawit berusia enam bulan telah dielisitasi dengan kuprum sulfat ($CuSO_4$) untuk mengenal pasti kehadiran juzuk fitokimia. Akar kelapa sawit diekstrak dengan enam pelarut berbeza. Antara pelarut tersebut ialah metanol, etanol, aseton, etil asetat, kloroform dan petroleum eter. Taburan fitokimia dalam akar kelapa sawit berusia enam bulan yang diekstrak dengan pelarut yang berbeza ini telah dinilai dan dibandingkan. Penemuan menunjukkan bahawa metanol adalah merupakan pengekstrak pelarut terbaik untuk akar kelapa sawit yang dielisitasi oleh $CuSO_4$. Ia mempunyai keupayaan untuk mengekstrak pelbagai juzuk fitokimia dan mempamerkan aktiviti antikulat yang paling signifikan berbanding pengekstrakan oleh pelarut-pelarut yang lain. Sementara itu, kajian kerja lapangan telah dijalankan untuk menilai tahap rintangan separa dan kerentanan dalam kalangan progeni kelapa sawit komersial yang berbeza terhadap penyakit reput pangkal batang (BSR) yang disebabkan oleh G. boninense. Anak benih kelapa sawit berumur enam belas bulan daripada empat progeni berbeza (dengan latar belakang genetik berbeza) yang terdiri daripada anak benih tidak diinokulasi dijadikan sebagai kawalan dan anak benih yang diinokulasi dengan blok kayu getah dikoloni G. boninense telah disaring untuk perbezaannya. Perkembangan penyakit diperhatikan dan direkodkan sebagai peratusan kejadian penyakit (DI) pada selang bulanan untuk tempoh sembilan bulan. Kemudian, akar kelapa sawit dituai dalam tempoh sembilan bulan selepas inokulasi untuk analisis kandungan ergosterol dan pengasingan semula segmen akar yang diinokulasi dan tidak diinokulasi pada Ganoderma Selective Medium (GSM) untuk pengesahan lanjut. Keputusan *menunjukkan Johor Labis Deli dura x AVROS pisifera (P4) dan (Nigeria x Deli) dura x AVROS pisifera (P5) adalah merupakan progeni yang toleran. Sementara itu, Banting Deli dura x AVROS pisifera (P1) dan (Ulu Remis x Heze) Deli dura x AVROS pisifera (P3) adalah progeni yang lemah. DI diperhatikan seawal empat bulan selepas inokulasi untuk P1 dan P3 berbanding P4 dan P5 yang menunjukkan DI pada lima bulan selepas inokulasi. DI meningkat secara beransur-ansur kepada 100% untuk progeni P1 dan P3 pada sembilan bulan selepas inokulasi. Nilai keterukan penyakit untuk simptom dalaman tisu dan pengumpulan ergosterol adalah berbeza dengan ketara dan lebih tinggi untuk P1 dan P3 berbanding P4 dan P5. Ekstrak metanol kasar akar kelapa sawit yang diinokulasi dan tidak diinokulasi telah disaring secara in vitro untuk aktiviti antikulat menggunakan teknik pencairan agar. Keputusan ujian antikulat untuk semua ekstrak metanol kasar progeni kelapa sawit tidak diinokulasi menunjukkan tiada perbezaan yang ketara. Sebaliknya, keputusan yang diperoleh untuk ekstrak kasar progeni kelapa sawit yang diinokulasi iaitu P4 (23.70%) dan P5 (22.96%) memberikan peratus perencatan yang lebih tinggi berbanding P1 dan P3 yang memberikan aktiviti antikulat yang lemah dengan peratus perencatan yang lebih rendah, iaitu masing-masing 16.66% dan 14.42%. Analisis GC-MS bagi ekstrak kasar akar kelapa sawit mendedahkan kehadiran lapan fitosebatian dan kebanyakannya dengan kelimpahan beberapa asid lemak yang lebih tinggi dan hanya dua sebatian daripada kumpulan fenol. Fitosebatian tersebut adalah benzoic acid, methyl ester; phenol, 2,6-dimethoxy, 1,4-benzenedicarboxylic acid, dimethyl ester; phenol, 2,4-bis (1,1-dimethylethyl); hexadecanoic acid, methyl ester; 9,12-octadecadienoic acid (Z,Z)-, methyl ester; 9-octadecenoic acid (Z)-, methyl ester dan octadecanoic acid, methyl ester. Aktiviti tiga enzim pertahanan termasuk peroksidase (PO), polyphenol oxidase (PPO) dan phenylalanine ammonia lyase (PAL) turut dinilai. Keputusan menunjukkan bahawa pada sembilan bulan selepas inokulasi, aktiviti PO, PPO dan PAL bagi akar kelapa sawit daripada progeni yang diinokulasi adalah lebih tinggi daripada yang tidak diinokulasi. Bagi progeni yang diinokulasi, aktiviti PO, PPO dan PAL untuk toleran progeni adalah yang tertinggi untuk P4 diikuti oleh P5, dan progeni yang lemah menunjukkan aktiviti enzim yang paling rendah iaitu P3 dan P1. PO, PPO dan PAL bertindak sebagai mekanisme pertahanan aktif tumbuhan terhadap serangan G. boninense. Kajian ini telah menemui metabolit baharu yang boleh membantu dalam usaha penyelidikan pada masa depan berkaitan penyakit BSR.*

Sebatian yang dihasilkan oleh anak benih kelapa sawit boleh digunakan sebagai biomarker untuk menilai tahap rintangan kepada G. boninense, yang mungkin memberikan pandangan penting untuk pengesanan awal penyakit BSR dalam pengurusan anak benih.

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The values represent the means of three technical replicates.

LIST OF ABBREVIATIONS

BSR	-	Basal Stem Rot
CWDE	-	Cell Wall Degrading Enzyme
CPO	-	Crude Palm Oil
$CuSO_4$	-	Copper Sulphate
GC-MS	-	Gas Chromatography-Mass Spectrometry
GSM	-	*Ganoderma* Selective Medium
PAL	-	Phenylalanine Ammonia-Lyase
PO	-	Peroxidase
PPO	-	Polyphenol Oxidase
PR	-	Pathogen-Related
ROS	-	Reactive Oxygen Species
RWB	-	Rubber Wood Blocks

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CHAPTER 1

INTRODUCTION

1.1 Introduction to Oil Palm Plantation in Malaysia

Oil palm (*Elaeis guineensis* Jacq.) has emerged as the most important estate crop in Malaysia. It is the world's largest edible oil and is even used for biofuels and some manufactured products. Malaysia and Indonesia contribute 85% of the global palm oil supply (Beringer *et al.*, 2023), which accounted for 34% of world vegetable oils consumption in 2018 (Kushairi *et al.*, 2019). They are currently the world's largest oil palm production in South-East Asia. The world production of palm oil has hit around 73.8 million metric tons from the year 2021 to 2022 (Shahbandeh, 2023). Malaysia is still the world's largest exporter of palm oil, although it is the second-largest palm oil producer after Indonesia (Ayanda *et al.*, 2020). In 1920, Malaysia had 400 ha under palm and this area increased to 0.6 million hectares in 1975 followed by a further increase to 5.67 million hectares in 2022 (Malaysian Palm Oil Board, 2022).

The palm oil sector in Malaysia faced numerous obstacles in the year 2020. Overall, the industry's performance that year was fairly inconsistent. Compared to 2019, crude palm oil (CPO) output, palm oil stocks, palm oil exports, and palm oil imports decreased. However, overall export earnings of oil palm products increased despite reporting a lower export volume. Total CPO production in 2020 was 19.14 million tonnes, 0.72 million tonnes or 3.6% lower than 2019's 19.86 million MT due to labour constraints and disruptions in FFB harvesting and milling processes on plantations induced by the government's movement control order (MCO) due to Covid-19 pandemic. In 2019, fresh fruit bunches (FFB) yield decreased to 16.73 tonnes/hectare from 17.19 tonnes/hectare, while oil extraction rates (OER) decreased by 1.4% to 19.92% from 20.21%. In 2020, Malaysian palm oil exports amounted to 17,368 million tonnes, down 1.103 million tonnes, or 5.97%, from 2019's total of 18,471 million tonnes. The reduction is mainly attributable to lower exports to India, which has traditionally been the largest market for Malaysian palm oil. As a result of the Covid-19 pandemic, travel and movement restrictions to prevent outbreaks and supply chain delays reduced palm oil demand. Due to insufficient demand, Malaysian palm oil exports to India, Pakistan, Turkey, and a few other countries have decreased. In 2020, China was the top importer of Malaysian palm oil, importing 2.73 million tonnes. India came in second with 2.727 million tonnes, followed by the Netherlands with 1.073 million tonnes, Pakistan with 1.004 million tonnes, the Philippines with 0.693 million tonnes, and Turkey with 0.616 million tonnes. According to MPOB, the average price of CPO in 2020 was RM 2685.50 per tonne, up from RM 2079.00 per tonne in 2019. In 2020, the Malaysian palm oil business effectively navigated the obstacles, adapted to the new standards, and maintained its outstanding performance despite facing several domestic and international problems (Iskahar *et al.*, 2021).

Nevertheless, like other crops, oil palm is also prone to a number of diseases attack and one of the most important is basal stem rot (BSR) caused by *Ganoderma boninense*. BSR has been reported as the most devastating disease of oil palm in South East Asia particularly in Malaysia and Indonesia (Olaniyi & Szulczyk, 2020; Siddiqui *et al.*, 2021). Previously, BSR was reported to occur only on old palms during the first cycle but recently the disease was found to attack young palms during the second planting cycle (Utomo & Niepold, 2000). Furthermore, the disease does not show early infection when it progresses from the palm base and visible symptoms appear at a very late stage of infection, when the fungus has killed more than half of the basal stem tissue. To date, there is no reliable technique to detect early infection of oil palm caused by *G. boninense*. Among a few detection techniques that have been carried out are polymerase chain reaction (PCR) based on specific deoxyribose nucleic acid (DNA) sequences of the pathogen and polyclonal antibody techniques, electrochemical DNA biosensor and enzyme-linked immunosorbent assay (ELISA) (Hilmi *et al.*, 2022; Lo *et al.*, 2023; Midot *et al.*, 2019) which required sophisticated processing procedures, time-consuming and possibilities of crossreaction with other saprophytic fungi. Several studies have been carried out for the detection of this disease in oil palm plantations based on the development of a few technologies for instance, electronic nose, tomography, hyperspectral remote sensing and hyperspectral imaging (Ahmadi *et al.*, 2022; Anuar *et al.*, 2021).

In spite of the availability of these techniques, there is a demand for fast, accurate and reliable detection of BSR in oil palm. Today, metabolomics approaches are considered to be a complementary tool facilitating our understanding of plant biology and physiology, which has been utilized in a great number of plant research studies, for instance, in understanding plant stress responses and determining plant health traits for crop improvements (Razzaq *et al.*, 2022; Silva *et al.*, 2023). Hence, the metabolomics approach to detect specific volatile and non-volatile metabolites compounds can be further utilized as a detection tool for the incidence of BSR in oil palm plantations as well as biomarkers to distinguish levels of resistance in oil palm seedlings against *G. boninense*. When there is a compatible plant-pathogen interaction, the infected plants release secondary metabolites as defensive compounds, which respond to the impacts of the chemical molecules released by the pathogen to harm and also infect the plant tissues. Secondary metabolites produced are in response to wounding, infection or stress, and these compounds serve as active and potent defensive mechanisms in plants. As discussed by Castro-moretti *et al.* (2020), metabolomics could help to advance our understanding of metabolites that may functionally contribute to plant susceptibility and resistance to pathogens. Therefore, metabolomics can discover beneficial applications to get rid off or else devastating plant disease will be triggered by this fungus.

1.2 Problem Statement

The oil palm industry is currently facing significant losses due to BSR, a major issue that requires early detection for effective management. However, the current methods for detecting BSR such as electrochemical DNA biosensors, ELISA, and PCR are time-consuming and may result in inaccurate data. Although agricultural technology has introduced several device systems for BSR detection, they have limitations in detecting the early stages of infection and specific patterns. Moreover, artificially inducing *G. boninense*-infected rubber wood blocks for testing oil palm progenies is both costly and time-consuming. It is usually conducted at the nursery level. Early detection is still difficult due to the fact that infected oil palms may appear symptomless during the initial stages of infection. This is the greatest obstacle in BSR disease management. To overcome these challenges, there is a pressing need for more rapid and reliable detection methods that can complement the existing techniques. Additionally, identifying specific compounds produced by oil palm seedlings could serve as biomarkers to distinguish levels of resistance against *G. boninense*, providing valuable insights for seedling management. Such measures can help to identify early signs of BSR and prevent significant losses in the oil palm industry.

1.3 Significance of Study

This study is to provide a new rapid and reliable technology for detecting specific metabolite compounds that can be used to indicate the presence of BSR in oil palm plantations and for the selection of partial resistant among different oil palm progenies. The availability of such diagnostic techniques for early detection of *G. boninense* would complement other existing techniques and benefit decision-making for appropriate disease control strategies to prevent the development and the spread of BSR.

1.4 Research Objectives

This study is, therefore, undertaken with the following objectives:

1. To identify different solvent systems for oil palm root extraction that yield the highest extraction efficiency, focusing on their ability to extract various phytochemical constituents and exhibit the most significant antifungal activities.