UNIVERSITI PUTRA MALAYSIA

EFFECTS OF NATURALLY OCCURING PHENOLIC COMPOUND ON CELL WALL DEGRADING ENZYMES AND SUPPRESSION OF Ganoderma boninense INFECTION IN OIL PALM SEEDLINGS

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IPTSM 2018 4

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

January 2018

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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January 2018

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Palm oil is one of the major sources of edible oil in the world with 85% of it being produced by Malaysia and Indonesia. However, the production is greatly hindered by the basal stem rot (BSR) disease. The causal pathogen of BSR disease is *Ganoderma* sp. *Ganoderma boninense*, the causal pathogen of BSR in oil palm is white rot basidiomycetes. This pathogen infects oil palm primarily via roots by degrading the lignin and cellulose components. Therefore, understanding the mode of infection of *G. boninense* in oil palm would be advantageous. An alternative solution to control the emergence of BSR is to inhibit the lignolytic and the cellulolytic enzymes of *G. boninense*. The phenolic compounds present naturally in the plants play a critical role in the pathogen elimination, signaling, increasing the resistance and the lignin biosynthesis. Hence, ten naturally occurring phenolic compounds namely, benzoic acid, coumaric acid, 2,6-dimethoxyl benzoic acid, 2,6-dimethoxyl phenol, guaiacol, ferulic acid, pyrocatechol, salicylic acid, syringic acid, and vanillic acid were selected to evaluate their potential to inhibit *G. boninense*. In this study, the phenolic compounds were tested for their ability to inhibit the growth of *G. boninense* and their inhibitory effect towards the production of lignolytic and cellulolytic enzymes. Further, their efficacy was tested to suppress the BSR infection in oil palm seedlings. The ten selected phenolic compounds were able to inhibit the growth of *G. boninense* with different degrees depending on their concentrations. Microscopic observations revealed that mycelia growing on media containing phenolic compounds showed deterioration. A significant ($p \leq 0.05$) decrease in the production of lignolytic enzymes, as well as celluase, amylase and xylanase of about 40-100% was identified. Except benzoic acid all the other phenolic compounds increased the secretion of lignolytic and cellulolytic enzymes at 1 mM concentration. However, as the concentrations increased more than 1 mM the inhibition

increased. The enzyme inhibitions have been further quantified and the type of inhibition was analysed along with their physicochemical properties. Most of the selected phenolic compounds inhibited the enzymes as uncompetitive or noncompetitive inhibitors. The lignolytic enzymes were active over a wide range of temperature from 40-80°C but sensitive to pH at 6. The cellulolytic enzymes are more stable in a wide range of pH from 3 to 8 and temperature 40-80°C when compared to the lignolytic enzymes. The wood degradation assay suggested that the *G. boninense* is a sequential degrader of lignin and cellulose components. The selected phenolic compounds significantly reduced the degradation rate upto 100% when compared to the control. This was due to their ability to inhibit the lignolytic and cellulolytic enzymes of *G. boninense*. Significant reductions in the disease progression upto 100% were observed in the oil palm seedlings treated with benzoic and salicylic acid. The oil palm seedling treated with benzoic and salicylic acid increased the growth parameters such as height, diameter of the stem, chlorophyll content, root and shoot weight. In addition, the oil palm seedlings treated with phenolic compounds showed increased lignification of four percentages. This was due to the increased activity of phenylaline amino- lyases, peroxidase and polyphenol oxidase by 1.5 folds. These enzymes are known to be involved in lignin biosynthesis pathway. The benzoic acid and salicylic acid tested are the processors for the synthesis of lignin. The findings of this study could be useful for developing new strategies in controlling the spread of disease, which may reduce the BSR disease severity in oil palm areas of production.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN-KESAN SEBATIAN SEMULAJADI FENOLIK PADA ENZIM YANG MENGURAIKAN DINDING SEL DAN PENINDASAN TERHADAP JANGKITAN *Ganoderma boninense* DALAM BENIH KELAPA SAWIT

Oleh

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Minyak sawit ialah salah satu sumber utama minyak makan di dunia dan 85% di antaranya dihasilkan oleh Malaysia dan Indonesia. Walau bagaimanapun, pengeluaranya amat terjejas akibat penyakit reput pangkal batang (BSR) sawit yang disebabkan oleh *Ganoderma* sp. *Ganoderma boninense* adalah patogen penyebab penyakit BSR dalam sawit dan ia merupakan sejenis basidiomycetes reput putih. Patogen ini menjangkiti sawit terutamanya melalui akar dengan menguraikan komponen lignin dan selulosa. Oleh kerana itu, memahami mod jangkitan *G. boninense* dalam kelapa sawit adalah agak penting. Antara penyelesaian alternatif untuk mengawal emergen BSR adalah dengan menyekat enzim lignolitik dan selulolisis *G. boninense*. Sebatian fenol hadir secara semulajadi dalam tumbuhan memainkan peranan kritikal dalam penyingkiran patogen, pengisyaratan, menambahkan rintangan dan juga lignin biosintesis. Maka sepuluh sebatian fenol yang berlaku secara semulajadi iaitu asid benzoik, asid kumarik, asid benzoik 2,6-dimethoxyl, fenol 2,6-dimethoxyl, guaiakol, asid ferulik, pirokatekol, asid salisilik, asid syringik dan asid vanillik telah terpilih untuk menilai kecekapan mereka dalam mengawal *G. boninense.* Dalam kajian ini, sebatian fenol diuji untuk kemampuan mereka menghalang pertumbuhan *G. boninense* dan kesan bertentangan mereka ke arah pengeluaran lignolitik dan enzim selulolisis. Tambahan, keberkesanan mereka dalam menghalang jangkitan BSR di dalam anak benih sawit. Sepuluh sebatian fenol terpilih mampu menyekat pertumbuhan *G. boninense* dengan efikasi berbeza bergantung pada kepekatan mereka. Pemerhatian-pemerhatian mikroskopik mendedahkan bahawa mycelia tumbuh di atas media mengandungi sebatian fenol menunjukkan kemerosotan. Satu penurunan penting ($p \leq 0.05$) dalam pengeluaran enzim-enzim lignolitik, serta selulosa, amilase dan xilanase telah dikenal pasti. Kecuali asid benzoik semua sebatian fenol yang lain menambah rembesan lignolitik dan enzim selulolisis di

kepekatan 1 mM. Bagaimanapun, apabila kepekatan menambah mereka bertindak sebagai satu perencat. Perencatan enzim selanjutnya dijumlahkan dan jenis perencatan dianalisis bersama dengan sifat fisiko kimia mereka. Kebanyakan daripada sebatian fenol terpilih menghalang enzim sebagai perencat tiada tandingan atau tidak melibatkan persaingan. Enzim-enzim lignolitik aktif atas julat suhu 40-80ºC luas tetapi sensitif kepada pH 6. Enzim selulolisis lebih stabil dalam julat pH yang lebih luas 3-8 dan suhu 40-80ºC apabila dibandingkan dengan enzim- enzim lignolitik. Cerakinan degradasi kayu mencadangkan, *G. boninense* ialah degradasi berjujukan komponen lignin dan selulosa. Sebatian fenol terpilih mengurangkan kadar penguraian sehingga 100% apabila dibandingkan dengan kawalan. Ini adalah disebabkan kemampuan mereka menghalang lignolitik dan enzim selulolisis *G. boninense*. Pengurangan signifikan dalam kemaraan penyakit telah diperhatikan dalam anak-anak benih pokok kelapa sawit dirawat dengan asid benzoik dan salisilik. Anak benih sawit dirawat dengan asid benzoik dan salisilik menambah parameter pertumbuhan seperti ketinggian, garis pusat batang, kandungan klorofil, berat akar dan pucuk. Selain parameter pertumbuhan, anak-anak benih pokok kelapa sawit dirawat dengan sebatian fenol menunjukkan pengligninan bertambah. Peningkatan dalam pengligninan adalah disebabkan aktiviti phenylaline amino-lyases, peroxidase dan polyphenol oxidase yang meningkat dengan 1.5 kali ganda. Enzim-enzim ini terkenal dalam penglibatan laluan biosintesis lignin. Asid benzoik dan salisilik yang diuji ialah pemproses untuk sintesis lignin. Penemuan kajian ini boleh digunakan untuk membangunkan strategi baru dalam mengawal penyebaran penyakit yang boleh mengurangkan penyakit BSR dalam estet kelapa sawit.

ACKNOWLEDGEMENT

At the outset, let me express my deep sense of indebtedness and gratitude to my supervisor Dr.Yasmeen Siddiqui for her valuable suggestions, constructive criticism, constant encouragement, abiding interest, unstinted help and well wishes during the entire period of my research work. She not only guided me to bring out this thesis but also honed my skills which would, I hope, of great help in days to come in my life and carrier.

I also acknowledge the great help extended by my supervisory committee, Dr. Nusaibah Syd Ali, Prof. Sivakumar Manickam and Prof. Halimi Mohd Saud for their valuable suggestions and critical appraisal in completing up this thesis.

I would also like to place my heartfelt gratitude to the support of some very special individuals. They helped me immensely by giving me encouragement and friendship. Firstly Mr. Mahashanon for his valuable advice on FT-IR and TGA and secondly MS. Hasfar Syafiqah for her kind help in greenhouse. They both have exhorted me to complete this work on time.

I place my thanks to the members of the staff, Institute of Tropical Agriculture and Food Security, UPM. Who have all helped me and goaded me to approach them for any help in completing this thesis. Heartfelt gratitude to Mrs Ummi Kalthum (Science officer) for always approving my application to work overtime and also to Mr. Mohd Faizol Azral bin Sulaiman (Assistant Agriculture officer) who accompanied me at my overtime work. Special thanks to the authorities of MOSTI for funding this project and giving me the sponsorship through special graduate research assistantship.

I would like thank University Putra Malaysia (UPM) for giving me an opportunity to peruse doctorate studies in plant pathology and supporting me to attend various conferences and also to publish my research work. Thank you.

I would also like to thank few of my friends Ms.Vidhya Varatharajan, Ms.Gowthami, Mr. Kamlesh Salve and Late Sujitha Vinoth for their moral support during my PhD period. Special thanks to my friend Kamlesh Salve for his great support.

My deep and sincere gratitude to my family for their continuous and unparalleled love, help and support. I am grateful to my Appa, Amma, Meena and Ludu kutty for always being there for me as a pillar of support. I am forever indebted to my parents for giving me the opportunities and experiences that have made me who I am. They selflessly encouraged me to explore new directions in life and seek my own destiny. This journey would not have been possible if not for them, and I dedicate this milestone to them.

Finally, I would like to express my gratitude to the almighty Lord Shiva for his blessings.

I certify that a Thesis Examination Committee has met on 11 January 2018 to conduct the final examination of Arthy Surendran on her thesis entitled "Effect of Naturally Occurring Phenolic Compounds on Cell Wall Degrading Enzymes and Suppression of Ganoderma *boninense*" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

scale of 0-4.

LIST OF ABBREVIATIONS

Ø	Diameter
%	Percentage
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonicacid) diammonium salt
AIL	Acid insoluble lignin
ANOVA	Analysis of variance
ASL	Acid soluble lignin
AUDPC	Area under disease progress curve
BSR	Basal stem rot
Cfu	Colony forming unit
Cm	Centimeter
CMC	Carboxymethyl cellulose
DR	Disease reduction
DS	Disease severity
EDTA	Ethylenediaminetetraacetic acid
FT-IR	Fourier transform infrared spectroscopy
g	Gram
h	Hours
K_m	Substrate concentration
L	liter
LB	Luria-Bertani broth
M	Molar
MAI	Moth after inoculation
MEA	Malt extract agar
mg	Milligram
Min	minutes
mm	Millimeter
mM	millimolar
MPOB	Malaysian Palm Oil Board
OD	Optical density
PAL	Phenyl ammonia lyase
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PIRG	Percentage Inhibition of Radial Growth
POD	peroxidase
PPO	polyphenol oxidase
RBBR	Remazol Brilliant Blue R
RPM	Revolution per minute
SEM	Scanning electron microscope
SDS	Sodium dodecyl sulfate
TGA	Thermogravimetry analysis
USR	Upper stem rot
V_{max}	Velocity of the reaction
$_w^{-1}$	Volume per volume
$_{wv}^{-1}$	Weight per volume

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background

Oil palm (*Elaeis guineensis* Jacq.) is an important cash crop of Malaysia, which bolsters the economy of the country. Oil palm is a native crop to Africa and it was introduced to Malaysia by the British in 1870 as an ornamental plant (Corley and Teo, 1976). It has been first commercially planted at Selangor state in 1970 (Hartley, 1967). Palm oil is gaining fast recognition as it contributes 30% of world's edible oil demand. The oil palm input to output outpaces other oil bearing crops such as soybean, rapeseed and sunflower (MPOC 2015). The palm oil contributes about 8% of total income of Malaysia and hence it is called as the "golden crop" of Malaysia.

The statistics from the past indicates that the strongest period of growth was in 1989-2008. During this period, the production was raised by 4% every year. In the last decade, an unexpected break in the growth rate was observed with 2% decremented yield loss annually (MPOB, 2015), due to various factors such as adverse climatic condition, labour cost, tree age, low yielding crop variety and diseases. Hence, Malaysia is compelled to increase the production of palm oil to meet the world's oil demand. The improvement in palm oil is not only governed by implementing new plantation, using high yield varieties and replanting the old trees with new ones or by increasing the total plantation area but also a more efficient pest and disease control measures to fill the production gap (Jalani et al., 2002).

Many pests and diseases from the seed hinder the growth of oil palm. Some of the oil palm pests and diseases are brown germ, bagworm, upper stem rot disease (USR) and basal stem rot disease (BSR). However, the palm oil production is majorly affected by the BSR disease.

1.2 Problem statement

The BSR disease in oil palm is caused by a white rot basidiomycete fungus *Ganoderma boninense* and is considered as the most virulent strain (Rees et al., 2009). Basal stem rot disease was first identified by Thomson in 1931. This disease causes serious economic impact on Malaysia's oil palm industry today. The disease incidence increases/accumulates over the successive planting cycles (Susanto et al., 2005). The high incidence of BSR results in the drastic reduction in weight and number of bunches produced, they can reach at times to zero percentage yield (Chong et al., 2012). Initially, the *Ganoderma* reported

to infect only aged plants but in the last decade infection was observed even in seedlings less than six months old (Rees et al., 2007). The most accepted mode of infection is considered when there is a contact of infected roots with healthy oil palm roots (Rees et al., 2009; Singh et al., 1991). Another mode of infection is by the dispersal of basidiospore (Paterson, 2007).

To date, the status of disease management remains the same, since the method to address the disease effectively is scanty. Various methods have been introduced to the industry to prolong the life span of the tree and to increase the yield of the infected oil palm. These include soil mounding, trunk injection with fungicide, clean and clear practices. So far the hexaconazol applied via trunk injection is effective in controlling the BSR disease in oil palm to up to 70% (Idris et al., 2004 a). However the chemical control method is found to be cost forbidding.

The salient feature of white rot fungus (WRF) is the complete degradation of lignin and cellulose components in the plant. White rot fungus spends energy to degrade lignin in order to get access for itself in cellulose components. The degradation of lignin is to be the rate limiting step in the infection process (Paterson et al., 2009). Nevertheless, the pattern of lignin degradation of *Ganoderma* has not been explored till date. Two types of patterns were observed in the WRF degradation, the first one is the degradation of lignin followed by cellulose whereas second type is the concurrent degradation of both lignin and cellulose (Hatakka, 1994). To degrade the cellular structure WRF employs lignolytic and cellulolytic enzymes. Hence, the lignocellulolytic enzymes may also play an important role in the BSR disease progression.

Therefore, it is desirable to find a biomolecule that could inhibit the lignolytic and the cellulolytic enzymes of *Ganoderma boninense* to control the BSR disease in oil palm. The biomolecule should be of small in size so that it can travel towards the site of infection and is resistant to auto-oxidation. If this compound can induce resistance and there by leverage the oil palm seedlings over the BSR disease.

Phenolic compounds are the compounds which can address the above demand. The phenolic compounds are naturally present in plants at normal conditions. It gets elevated during biotic and abiotic stresses. Although, these compounds are involved in the signal transduction pathway in the stress mode, they primarily function in production lignin and facilitate cell wall synthesis. They are tightly bound to the lignin structure and play an important role in protecting and strengthening the cell wall structure (Hammerschmidt, 2005).

1.3 Hypothesis

Inhibition of lignocellulolytic enzymes (the key enzymes in the infection process) utilizing phenolic compounds could be an effective method to control the BSR disease of oil palm.

1.4 Research objectives

The general objective is to examine the effect of phenolic compounds in inhibiting BSR disease in oil palm. The specific objectives of the study are as follow:

1. To determine antagonistic effect of phenolic compounds on the growth and secretion of lignolytic and cellulolytic enzymes of *G. boninense*.

2. To characterize and determine the inhibition of lignolytic and cellulolytic enzyme secretion by *G. boninense* utilizing naturally occurring phenolic compounds.

3. To characterize and assess the pattern of oil palm wood degradation by *G. boninense* in the presence of phenolic compounds.

4. To determine the effect of benzoic and salicylic acid in the immunization of oil palm seedlings- challenged by *G. boninense.*

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