UNIVERSITI PUTRA MALAYSIA

ROOT COLONIZATION OF OIL PALM (Elaeis guineensis Jacq.) USING GFP-EXPRESSING Ganoderma boninense AND EFFECTS OF LIGNIN ON DISEASE PROGRESSION

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By

NISHA A/P THOPLA GOVENDER

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

January 2016

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DEDICATION

Special dedication to:

My late grandfather, Mr. Kandasamy Krishnan PJK.

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

ROOT COLONIZATION OF OIL PALM (*Elaeis guineensis* Jacq.) USING GFP-EXPRESSING *Ganoderma boninense* AND EFFECTS OF LIGNIN ON DISEASE PROGRESSION

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January 2016

Chair : Associate Professor Wong Mui Yun, PhD
Faculty : Institute of Tropical Agriculture

Oil palm is the world's most efficient oil-bearing tree. Major diseases impeding the oil palm productivity have been caused by fungi, particularly *Ganoderma boninense*, the causal agent of basal stem rot (BSR). Visible symptoms can only be observed nearing the plant death stage while early penetration and infection strategy remain cryptic due to the fungal hyaline nature. The underlying principles on how *Ganoderma* penetrates and infects oil palm roots are unknown. Therefore, a tagged *G. boninense* harbouring GUS-GFP fusion gene would ideally serve as a tool to unravel early pathogenesis of *G. boninense*. Lignin, a heterogeneous complex polymer is poorly understood during BSR development and thus, assessments of lignin content and composition in different planting materials and during the disease development were performed. In addition, enzyme activity and gene expression of key components in the phenylpropanoid pathway were investigated. Lignin content and composition were screened in oil palm lines with differential tolerance to BSR. Both parameters were associated to growth factors (height, weight and girth) and micronutrients depositions were measured using X-Ray Fluorescence (XRF). Efficient *Agrobacterium*-mediated transformation protocol was established via optimization of several parameters; *Agrobacterium* strain (LBA4404, GV3101, EHA101 and EHA105), explants (mycelia, spore and protoplast), *vir* gene induction period and modification of binary vector. The transformant was utilized to discern early stage colonization of BSR using the confocal microscopy. Glass-house trial on BSR development was performed to evaluate enzyme activities and gene expression of the following defence genes; phenylalanine ammonia lyase (PAL), cinnamyl alcohol dehydrogenase (CAD) and peroxidase (POD) using enzyme assay and quantitative real-time PCR respectively. Lignin content and composition were significantly different among the oil palm seedlings with different tolerance to BSR. The susceptible and intermediate lines showed significantly higher lignin content in comparison to the tolerant line, while lignin composition denoted as S/G ratio was higher in tolerant line in comparison to both susceptible and intermediate lines. Apparent lignin accumulation was supported by micronutrients deposition which comprised copper, silicon, titanium and sulphur. A successful transformation system was developed for *G. boninense* using protoplast and *Agrobacterium* strain LBA 4404. The binary vector pCAMBIA 1304, modified to harbour GPD fungal promoter from

plasmid p416 improved the expression of GUS-GFP fusion protein. Colonization pattern was initiated with active differentiation of the tagged *G. boninense* into micro-hyphae. The needle-like structure was able to penetrate the epidermis layer randomly and progressed longitudinally into exodermis and cortex region. Induced lignification during defense showed great participation from both PAL and CAD genes and enzymes. The S/G ratio increased significantly in the induced lignin as compared to constitutive lignin indicated alterations employed by host as part of their defense strategy during *Ganoderma* infection. Low lignin content supported growth without compromising oil palm biomass while creating an avenue for greater proportion of induced lignin which consists of S monomer during *G. boninense* infection. The findings can be adopted in oil palm breeding strategies aimed to produce resistant planting materials.

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

KOLONISASI AKAR POKOK KELAPA SAWIT (*Elaeis guineensis* Jacq) MENGGUNAKAN *Ganoderma boninense* BEREKSPRES-GFP DAN KESAN LIGNIN SEMASA PERKEMBANGAN PENYAKIT

Oleh

NISHA A/P THOPLA GOVENDER

Januari 2016

Pengerusi : Profesor Madya Wong Mui Yun, PhD
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Kelapa sawit merupakan pokok paling efisen di dunia bagi penghasilan minyak sayuran. Kebanyakan penyakit yang membantutkan produktiviti kelapa sawit adalah berpunca dari kulat khususnya *Ganoderma boninense*. Kulat ini merupakan agen penyebab penyakit reput pangkal batang. Simptom-simptom penyakit ini hanya jelas kelihatan pada hujung peringkat kematian. Strategi penembusan kulat ini untuk melancarkan infeksi masih kurang diketahui memandangkan kulat ini bersifat halus. Oleh itu, *G. boninense* bertag yang mempunyai gen GUS-GFP mampu mengenalpasti corak patogenisiti penyakit reput pangkal batang. Lignin, polimer heterogenus kompleks, menjalankan peranan pertahanan ketika perkembangan penyakit reput pangkal batang dan mekanisma ini tidak banyak diketengahkan. Dengan itu, pemahaman lignin boleh diadaptasi sebagai faktor ketahanan terhadap penyakit reput pangkal batang. Kandungan dan komposisi lignin dari tisu akar pokok kelapa sawit yang terdiri daripada kumpulan pelbagai toleran terhadap penyakit reput pangkal batang dikenalpasti. Kedua-dua parameter ini dikaitkan dengan faktor tumbesaran (tinggi, berat dan diameter) sementara peratus mikronutrien ditentukan dengan menggunakan X-Ray Fluorescence (XRF). Sebuah protokol yang efisen berasaskan penggunaan *Agrobacterium* telah dibina bagi tujuan transformasi kulat *G. boninense*. Pengoptimuman beberapa parameter seperti berikut dilakukan: Isolat *Agrobacterium* (LBA4404, GV3103, EHA101 dan EHA105), explan (miselium, spora dan protoplas), tempoh induksi gen *vir* dan modifikasi vektor binari. Transforman digunakan bagi tujuan mengenalpasti corak kolonisasi penyakit reput pangkal batang pada peringkat awal penyakit dengan menggunakan mikroskop konfokal. Satu eksperimen rumah kaca mengkaji interaksi *Ganoderma*-perumah dijalankan untuk menentukan aktiviti enzim dan ekspresi gen (PAL, CAD dan POD) masing-masing dengan menggunakan esei enzim dan tindak balas rantaian. Kandungan lignin dan komposisi menunjukkan perbezaan yang signifikan di kalangan anak pokok kelapa sawit dari kumpulan pelbagai toleran terhadap penyakit batang pangkal reput. Kumpulan yang rendah dan sederhana toleran, masing-masing menunjukkan kandungan lignin yang lebih tinggi berbanding kumpulan toleran manakala komposisi lignin yang terdiri daripada nisbah S/G menunjukkan nisbah yang tinggi di kalangan kumpulan yang toleran berbanding kumpulan yang kurang toleran dan kumpulan tidak toleran. Pembentukan lignin

disokong bersama pemendapan mikronutrien yang terdiri daripada kuprum, silikon, titanium dan sulfur. Sistem transformasi berjaya dibangunkan untuk *G. boninense* dengan menggunakan protoplas sebagai sumber eksplan dan *Agrobacterium* stren LBA4404. Vektor binari pCAMBIA1304 diubahsuai dengan menambah promoter kulat GPD dari plasmid p416. Modifikasi ini berjaya meningkatkan lagi ekspresi gen gabungan bersama GUS-GFP. Induksi proses lignifikasi ketika interaksi *Ganoderma*-perumah disokong kuat oleh kedua-dua gen dan enzim CAD. Nisbah S/G meningkat secara signifikan di dalam lignin induksi berbanding dengan lignin konstitutif. Perubahan komposisi lignin yang ketara menjelaskan strategi pertahanan perumah, kelapa sawit ketika infeksi *Ganoderma*. Keputusan ini didapati seiring dengan kumpulan anak pokok kelapa sawit yang bertoleran tinggi terhadap penyakit reput pangkal batang, yang menunjukkan nisbah S/G yang tinggi. Kandungan lignin yang rendah boleh menyokong proses tumbesaran tanpa sebarang kesan signifikan ke atas biomas dan menyediakan ruang untuk pembentukan lignin induksi pada kadar yang lebih tinggi. Hasil kajian ini boleh diadaptasi dalam usaha pembiakbakaan kelapa sawit yang bertoleran terhadap penyakit reput reput pangkal batang.

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I certify that a Thesis Examination Committee has met on 19 January 2016 to conduct the final examination of Nisha a/p Thopla Govender on her thesis entitled "Root Colonization of Oil Palm (*Elaeis guineensis* Jacq.) using GFP-Expressing *Ganoderma boninense* and Effects of Lignin on Disease Progression" 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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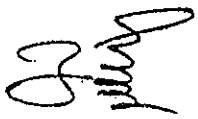

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Name of Member of Supervisory Committee: Idris Abu Seman, PhD

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LIST OF ABBREVIATIONS

A	Absorbance
ANOVA	Analysis of variance
AAR	Applied Agricultural Resources
AMT	Agrobacterium-mediated transformation
β-Actin	Beta-Actin
BH	bole height
BLAST	Basic Local Alignment Search Tool
BSA	N,O-bis(trimethylsilyl)acetamide
BSR	basal stem rot
CAD	cinnamyl alcohol dehydrogenase
CCoAOMT	Caffeoyl-CoA 3-O-Methyltransferase
cDNA	complementary deoxyribonucleic acid
CRD	Completely Randomized Desigh
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotides
DNase	deoxyribonuclease
DI	disease incidence
DTT	dithiothreitol
EDTA	Ethylenediaminetetracetic acid
FAOSTAT	Food and Agriculture
F5H	Ferulate 5-hydroxylase
GAPDH	Glyceraldehyde phosphate dehydrogenase
GCM	Ganoderma Complete Medium
GC-MS	Gas Chromatography-Mass Spectrometer
GFP	green fluorescent protein
GPD	Glyceraldehydes 3-phosphate dehydrogenase
GUS	β-glucuronidase
HCl	hydrochloric acid
HPLC	High Performance Liquid Chromatography
IM	induction medium
LB	Luria-Bertani
M	Molar
MAI	month after inoculation
MCS	multiple cloning site
MEA	Malt Extract Agar
MES	2-(N-morpholino)-ethanesulfonic acid
MM	minimal medium
min	minute
mL	mililiter
mM	miliMolar
µM	microMolar
µg	microgram
MPOB	Malaysian Palm Oil Board
MPOC	Malaysian P alm Oil Council
ng	nano gram
N_A	Avogadro's number
NAA	Naphtaleneacetic acid

OD	optical density
OMT	O-methyltranferase
PAL	phenyl ammonia lyase
pM	picoMolar
POD	peroxidase
PCR	Polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
RNA	ribonucleic acid
RSPO	Round-table for Sustainable Palm Oil
RWB	rubber wood block
STDV	standard deviation
SYBR	syber
Taq	*Thermus aquaticus*
T-DNA	transfer DNA
TFA	Trifluroacetic acid
TGA	Thioglycolic acid
Ti	tumor inducing
UPM	Universiti Putra Malaysia
USA	United States of America
Vir	virulence
XRF	X-ray Fluorescence

CHAPTER I

INTRODUCTION

Oil palm (*Elaeis guineensis*) is the world's most efficient oil bearing tree with an average yield of 3-4 tonnes of oil per hectare per year (Basiron, 2015; Wahid et al., 2005). Palm oil input to output ratio exceeds 10 times greater than its competitors, which includes soybean, rapeseed and sunflower oils. At present, the crop is widely cultivated nearing the equatorial belts (tropical regions) of the world such as Malaysia, Indonesia, Thailand, Zaire, Nigeria, Columbia and Cameroon, which collectively accounts about 9.3-10.2% of total world's permanent agricultural land (FAOSTAT, 2013). The crop thrives well under hot and humid weather, which endeavours successful growth and subsequently results to good productivity. In Malaysia, the palm oil industry has been the nation's utmost important key economic driver since its introduction in 1917, in which Tennamaram Estate in Selangor became the first oil palm plantation (Hartley, 1988). Over the years, the industry has graduated steadily to generate more income needed to beef up the nation's development while creating employment opportunities amongst its people.

The basal stem rot (BSR) disease has been recognized as the most destructive disease infecting oil palm trees in South East Asia. The disease manifests a decrease in palm stands and yield which subsequently renders economic losses (Susanto et al., 2005; Idris, 2009). To date, the status of the disease never changed, since methods to address the disease effectively remain scant. No genetic resistance to the disease has been described while control strategies remain unsatisfactory (Soepena et al., 2000). Basal stem rot (BSR) is caused by white rot fungi, *Ganoderma boninense* Pat. May. The pathogen is a saprophytic soil inhabitant, borne with ability to initiate pathogenesis at every stage of the plant growth followed by progressive death of the host. The BSR disease utilizes reproducible infected roots for pathogenesis. Rapid progression throughout the root is initiated by biotrophic phase in root cortex and lower stem via lignin degradation followed by rapid starch depletion and simultaneous cell wall breakdown (Rees et al., 2009; Flood et al., 2011).

Reports have documented an up to 500 million USD loss per year solely attributed by fungal diseases (Ommelna et al., 2012; Arif et al., 2011) in the oil palm industry. In a study conducted at oil palm plantation in Johor (Roslan and Idris, 2012), *Ganoderma* attack was shown to reduce fresh fruit bunch (FFB) production between 0.04 tonnes to 4.34 tonnes per hectare on 10 years and 22 years of planting periods respectively. The study had also estimated a total of 400 thousand hectares of oil palm plantation area in Malaysia could be affected with *Ganoderma* by 2020.

Transgenic fungal pathogens are latest advancements in genetic engineering. Since the first successful yeast *Saccharomyces cerevisiae* (Bundock et al., 1999) transformation, genetic transfer has been widely employed to explore molecular insights of fungal

pathogenicity. The underlying principle involves transferring foreign genes into organism of interest from both related and non-related organisms. *Ganoderma boninense* has never been shown amenable to transformation system. A transgenic *G. boninense* would create an avenue of understandings during BSR development. Thus, assigning function of genes and their products relevant to pathogenicity could be deduced. Biochemical changes of fungal cell wall composition during disease development can be learnt in the quest for BSR resistance. *Agrobacterium*-mediated transformation utilizes *Agrobacterium* species as the vector for its ability to transfer a discrete segment of its DNA into the fungal cells. The transferred DNA (T-DNA) is transported to fungal cell where it is integrated into the fungal genomic DNA. This is based on the presence of a large tumor inducing (Ti) plasmid in *Agrobacterium*, which contains a set of genes (the virulence genes) that can mobilize the T-DNA. *Agrobacterium*-mediated transformation is highly preferred compared to other gene transfer method due to its feasibility in preparation and higher transformation frequencies. Different kinds of intact host cells such as conidia, mycelia, sexual spores and fruiting body tissues from fungi can be relatively used through this method (Michielse et al., 2005).

Lignin supports the vascular system for transportation of water and nutrients and first line defense mechanism through lignifications in plants (Maeda et al. 2011). *Ganoderma boninense*, degrades polysaccharides and lignin components of host tissue during development of BSR. Thus, host's degree of lignifications may explain differential tolerance to BSR. Lignin content and composition selective to *G. boninense* degradation would provide information on defense strategy employed by host through lignification. The complex biochemical grid of lignin biosynthesis results from accumulative participation of numerous enzymes, each serves as confounding unit to one another to produce the end product, lignin polymer. Lignin biosynthesis is supported by a variable degree of regulation of each individual enzymes participating in the phenylpropanoid pathway. Regulatory functions of key enzyme in the phenylpropanoid pathway during defense mechanism vary in response to growth and defense (Higuchi and Kyoto, 1990). Therefore, understanding the phenylpropanoid pathway alterations would provide insights on defense lignin accumulation during BSR development.

In this study, the fungus *G. boninense* was tagged with GUS-GFP fusion gene via *Agrobacterium*-mediated transformation to discern early stage colonization pattern of *G. boninense*. Characterization of oil palm lignin in association to growth and defense was deduced. The expression of several lignin biosynthetic genes and enzymes were evaluated to understand defense responses of oil palm seedlings during pathogenesis of BSR disease and its subsequent implications on the phenylpropanoid pathway. Thus, the objectives of this study were as followings:-

i) To determine lignin content and composition of oil palm seedlings with different tolerance to BSR and its association to growth and micronutrient content.

ii) To develop an efficient *Agrobacterium*-mediated transformation protocol for *G. boninense* harbouring GUS-GFP fusion gene.

iii) To establish early stage infection pattern of basal stem rot using transgenic *G. boninense*.

iv) To determine the lignin content, lignin composition, expression and activity of lignin biosynthetic genes and enzymes (PAL, CAD and POD) during BSR development.

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