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DIFFERENTIAL ANALYSES OF LEAF PROTEOMES IN OIL PALM SEEDLINGS INOCULATED WITH PATHOGENIC AND NONPATHOGENIC SPECIES OF GANODERMA

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May 2017

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Basal stem rot (BSR) is a destructive disease of oil palm caused by the basiodiomycete fungus known as *Ganoderma* spp. Basal stem rot disease is considered the most serious disease affecting commercial oil palm plantations in South East Asia, especially in Malaysia and Indonesia. The disease reduces the productivity and yield of infected oil palm by disrupting water and nutrient movement from the roots to the other parts of the plant. Until now, there is no protein biomarker available to detect BSR disease at early stage of infection due to the insufficient information of *Ganoderma* spp. and most of the analyses related to the interaction between oil palm and *Ganoderma* spp. were conducted using roots tissues which require destructive sampling. Therefore, the objective of this study is to search for specific responsive protein candidates for early and non-destructive protein-based disease detection method by using leaf sample. Proteomic analysis of oil palm leaf was conducted on samples collected 72 hours following introduction of oil palm seedlings onto the mycelium of *G. boninense* and *G.tornatum* in flask. It is hypothesized that specific responsive proteins related to defence mechanism will be differently expressed by the leaf protein from oil palm seedling inoculated with *G. boninese* compared to the un-inoculated control and *G.tornatum*-inoculated seedlings.

A total of 82 proteins were resolved by two-dimensional gel electrophoresis with significant differences in the spot abundance. From there, 24 differentially expressed proteins in response to *Ganoderma* spp. inoculations were successfully identified by mass spectrophotometry (MALDI TOF/TOF). The proteins are mainly involved in photosynthesis and energy metabolism, RNA and protein metabolism as well as stress/defence mechanism. Proteins related to photosynthesis and energy production such as ATP synthase and ribulose bisphosphate carboxylase (Rubisco) were down-regulated while proteins such as 70kDa heat shock protein and cyclophilin which

involves in protein metabolism were up-regulated in comparison to un-inoculated sample. Defence related proteins, WAK proteins was up-regulated while hyrdroxyproline-rich glycoprotein like protein (HRGP) and mannose-binding lectin were down-regulated during the mycelium attachment process. Based on predicted cellular function classification, this study managed to identify several specific responsive proteins that can be used as possible candidates for biomarker or biological indicator for BSR early detection in oil palm which is important to properly manage the disease.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Sarjana Sains

ANALISIS PERBEZAAN KE ATAS PROTEOM DAUN ANAK BENIH POKOK KELAPA SAWIT YANG DIINOKULASI DENGAN SPESIS GANODERMA PATOGENIK DAN TIDAK PATOGENIK

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Penyakit Reput Pangkal Batang (BSR) merupakan penyakit merosak kelapa sawit yang disebabkan oleh kulat yang dikenali sebagai *Ganoderma*. Penyakit BSR dianggap sebagai penyakit yang paling serius yang menjejaskan perladangan kelapa sawit komersil di Asia Tenggara, terutamanya di Malaysia dan Indonesia. Penyakit ini mengurangkan produktiviti dan hasil kelapa sawit yang dijangkiti dengan mengganggu pergerakan air dan nutrien dari akar ke bahagian lain tumbuhan. Sehingga kini, tidak ada biomarker protein yang tersedia untuk mengesan penyakit BSR pada peringkat awal jangkitan kerana maklumat yang tidak mencukupi tentang *Ganoderma* spp. dan sebahagian besar analisis yang berkaitan dengan interaksi antara kelapa sawit dan *Ganoderma* spp dijalankan menggunakan tisu akar yang memerlukan pensampelan yang merosakkan pokok. Oleh itu, objektif kajian ini adalah untuk mencari protein responsif yang spesifik bagi kaedah pengesanan awal tanpa merosakkan pokok dengan menggunakan sampel daun. Analisis proteomik daun kelapa sawit dilakukan pada sampel yang dikumpul setelah 72 jam diinokulasi dengan miselium *G. boninense* dan *G.tornatum* dalam kelalang. Ia dihipotesiskan bahawa spesifik responsif protein berkaitan dengan mekanisme pertahanan akan dizahirkan secara berbeza oleh daun yang diperoleh dari anak benih kelapa sawit yang diinokulasi dengan *G. boninense* berbanding dengan daripada anak benih yang tidak diinokulasi dan yang diinokulasi dengan miselium *G. tornatum*.

Sebanyak 82 protein telah diasingkan oleh elektroforesis gel dua dimensi dengan perbezaan ketara dalam jumlah yang banyak. Daripada analisa, 24 protein yang menunjukkan perbezaan signifikan sebagai tindak balas kepada inokulasi *Ganoderma* spp. telah berjaya dikenalpasti oleh spektrofotometri massa (MALDI TOF / TOF). Protein terutamanya yang terlibat dalam fotosintesis dan metabolisme tenaga, metabolism RNA dan protein serta mekanisme tekanan /pertahanan telah dikenalpasti. Protein yang berkaitan dengan fotosintesis dan pengeluaran tenaga seperti ATP

synthase dan ribulosa bisphosfat karboksilase (Rubisco) menunjukkan peningkatan di dalam pengekspresan protein manakala protein seperti protein kejutan haba 70kDa dan cyclophilin yang terlibat di dalam metabolisme protein menunjukkan penurunan pengekpresan protein berbanding dengan sampel yang tidak diinokulasi. Protein yang berkaitan dengan pertahanan, protein WAK menunjukkan peningkatan di dalam pengekspresan protein manakala glycoprotein yang kaya dengan hidroksiproline (HRGP) dan mannose-binding lektin menunjukkan penurunan pengekpresan semasa proses perlekatan miselia. Berdasarkan klasifikasi fungsi selular yang diramalkan, kajian ini dapat mengenal pasti beberapa protein responsif tertentu yang boleh digunakan sebagai calon yang mungkin untuk penanda bio atau penunjuk biologi untuk pengesanan awal BSR dalam kelapa sawit yang penting untuk mengurus penyakit dengan betul.

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I certify that a Thesis Examination Committee has met on 31 May 2017 to conduct the final examination of Norasfaliza binti Rahmad on her thesis entitled "Differential Analyses of Leaf Proteomes in Oil Palm Seedlings Inoculated with Pathogenic and Nonpathogenic Species of Ganoderma" 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 Master of Science.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2DE	two dimensional electrophoresis
ABC	ammonium bicarbonate
APS	ammonium persulfate
BSA	bovine serum albumin
BSR	basal stem rot
COX II PS17	cytochrome C oxidase subunit II PS17
CWDEs	cell wall degrading enzymes
CyPs	cyclophilins
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ETI	effector triggered immunity
GSM	Ganoderma selective medium
hpi	hours post inoculation
HR	hypersensitive response
HRGP	hydroxyproline-rich glycoprotein like protein
Hsp70s	heat shock protein 70s
IAA	iodoacetamide
IEF	isoelectric focusing
IMP	integral membrane protein
IPG	immobiline drystrips gel
$K_3Fe(CN)_6$	potassium ferricyanide
MALDI	matrix assisted laser desorption ionization
MBL	mannose-binding lectin
MDH	malate dehydrogenase
MS	mass spectrometry
Na_2SO_3	sodium thiosulfate
OEE1	oxygen enhancing enzymes 1
PAMPs	pathogen associated molecular patterns
PDA	potato dextrose agar
PMF	peptide mass fingerprint
PPIases	peptidylprolyl cis-trans isomerase
PRRs	protein recognition receptors
PTI	PAMP-triggered immunity
PTM	post trans modification
ROS	reactive oxygen species
Rubisco	ribulose bisphosphate carboxylase

RWD	rubber wood block
SAR	systemic acquired resistance
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrphoresis
SEM	scanning electron microscope
TCA	tricholoacetic acid
TFA	trifluoacetic acid
TEMED	tetramethylethylenediamine
TOF	time-of-flight

CHAPTER 1

INTRODUCTION

Oil palm (*Elaeis guineensis*) is the most productive and profitable oil-bearing crop that produces a substantial percentage of the global vegetable oils (Naher *et al.,* 2013). It is widely cultivated in tropical countries such as Brazil, Indonesia, Malaysia and Africa. Unfortunately, the oil palm plantations in Southeast Asia, especially Malaysia and Indonesia are threatened by a devastating disease known as basal stem rot (BSR) or wilt infection.

BSR disease in oil palm causes severe economic losses by reducing the oil yield of the infected plants, loss of stands and shortening the cycle to replant the new palms (Ho *et al*., 2016). The disease is caused by a white rot fungus known as *Ganoderma* spp. and the most aggressive species is *Ganoderma boninense*, which can destroy up to 80% of palms over repeated planting cycles (Alizadeh*et al*., 2011). In addition, two other species, *Ganoderma zonatum* and *Ganoderma miniatotinctum* also have been documented as causal agents for BSR in oil palms in Malaysia (Wong *et al*., 2012; Rakib *et al*., 2014). Despite being classified as a minor pathogen in other parts of the world (Pilotti, 2005), *Ganoderma tornatum* is considered as non-pathogenic locally and only infects dead trunk of oil palm (Wong *et al*., 2012).

Basic understanding of molecular aspects during the interaction between oil palm and *Ganoderma* spp are important for effective disease control especially in biomarker identification for early detection and disease diagnosis. There are several methods for disease detection using molecular approaches such as DNA-based detection and enzyme-linked immunosorbent assay (ELISA). Basal stem rot are commonly diagnosed by detecting the presence of *G. boninense* via DNA-based molecular techniques and ELISA. However, the accuracy and efficiency of those techniques are largely hampered by lack of sufficient information about *Ganoderma* species, leading to false positive result (Hushiarian *et al*. 2013). In this case, proteins or DNA from plant that is only expressed in response to *G. boninense* infection pose as a good alternative in diagnosing BSR. Recently, protein-based biomarker are gaining more attention compared to DNA-based biomarker due to the fact that proteins are abundant in organism and are more stable than DNA and metabolites (Liu *et al*., 2014).

In recent years, many proteomic studies were carried out to gain a better understanding on oil palm-*Ganoderma* interaction (Daim *et al*., 2015, Al-Obaidi *et al*., 2014, Syahanim *et al*., 2013, Tony *et al*., 2013). Proteins such as ATP synthase, ribulose-1,5-bisphosphate carboxylase/oxygenase, ascorbate peroxidase, beta 1,3-glucanase, heat shock protein and cyclophilin are among the commonly affected proteins identified during the interaction. In addition, transcriptomics and metabolomics studies also have been done, providing more clear and holistic information related to the BSR disease development in palm trees (Nusaibah *et al*., 2016, Ho *et al*., 2016, Naher *et al*., 2012). However, most of the analyses were conducted using roots tissues which require

destructive sampling and the samples were taken at a late stage following the appearance of disease symptoms.

Therefore, this proteomic study was carried out to search for significantly regulated oil palm protein(s) in response to *G. boninense* as potential protein candidates for early and non-destructive protein-based disease detection method using leaf sample. Changes in oil palm leaf protein profile in response to the pathogenic *G. boninense* were compared to the un-inoculated oil palm seedlings and non-pathogenic *G.tornatum. G. tortanum* was included in this study to validate the specificity of the identified proteins thus avoiding any false positive during the detection of BSR. Due to the nature of the interaction between both *Ganoderma* species and oil palm, it is hypothesized that specific responsive proteins related to defence mechanism will be differently expressed by the leaf protein from oil palm seedling inoculated with *G. boninese* compared to the un-inoculated control and *G.tornatum*-inoculated seedlings. Significantly regulated proteins identified as early as 72 hours post-inoculation can be the candidates for protein-based biomarkers in the early detection of BSR disease and for monitoring the plant health.

The specific objectives of this study are as follows:

1. To identify differently expressed protein inoculated with pathogenic and non-pathogenic species of *Ganoderma*.
2. To classify the identified proteins based on their predicted cellular function.

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