POTENTIAL OF ANTAGONIST BACTERIA AGAINST *GANODERMA BONINENSE* AND IDENTIFICATION OF EFFECTIVE KEY METABOLITES RELEASED

By

LAU WAN KOON

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

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in Fulfillment of the requirement for the degree of Master of Science

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By

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April 2021

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Palm oil is the most consumed vegetable oil in the world. However, the oil palm industry in Southeast Asia is facing greatest threat from basal stem rot (BSR) disease caused by *Ganoderma boninense*. It causes severe economical loss to the producing countries. Currently there are no methods to effectively detect the disease at early stages of infection; therefore, there is an urgent need to find a practical and environmental-friendly disease control system. Application of biofertiliser with a consortium of biological control agents (BCAs) is widely used in oil palm industry to control BSR disease. In this research, two potential BCAs which were obtained from ACGT Microbial Culture Collection (AMCC): BCA01 (*Chromobacterium* sp.) and BCA03 (*Lysobacter* sp.) were selected from 21 isolates via dual culture screening which exhibited 51% and 61% of *G. boninense* mycelial growth inhibition, respectively. The antagonistic ability was further supported by the characteristic of nitrogen fixation, protease and chitinase production in both BCA01 and BCA03. In addition, the ability to produce siderophore and cellulase enzyme were also determined in BCA03. Additionally, key metabolites involved in the suppression of *G. boninense* growth were identified to better understand the contributing factor of antagonism. Prominent metabolites detected in BCA01 that could be associated with the biological control activity included mevalonolactone, p-cresol, 4-hydroxycinnamic acid, tiadinil and 13S-hydroperoxy-9Z,11E,15Z-octadecatrienoic acid (13(S)-HpOTrE). These metabolites may be responsible in inhibiting the growth of *G. boninense in vivo*. However, no potential bioactive metabolites were identified from BCA03 in this experiment. Apart from that, efficacy of the selected BCAs in suppression of *G. boninense* in oil palm seedlings was conducted using three months-old seedlings. Despite the positive antagonism result obtained in the laboratory, both selected BCAs were not showing similar effect at nursery trial. Treatments with BCA01 and BCA03 recorded disease incidence (DI) percentage as 95% and 93%, respectively demonstrated non-significant infection rate

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compared to control (90%). Besides that, the expression of cDNAs in oil palm roots at 1, 2, 3, and 4 weeks post inoculation (wpi) with BCA01, BCA03 and *G. boninense* alone were profiled to further understand the role of BCAs in the induction of defense mechanisms. Real time PCR (qPCR) analysis revealed that *cinnamyl alcohol dehydrogenase* (*CAD*), *chitinase* dan *β-1,3-glucanase* genes were up-regulated (more than 2-fold) in BCA01 treated roots at 4 wpi compared to negative control (non-infected roots). The result demonstrated that introduction of BCA01 potentially triggered the defense mechanisms of host plants during the interaction with *G. boninense* at 4 wpi. However, BCA03 did not display any up or down regulations on the three transcripts in this study. From this study, BCA01 was demonstrated as a promising BCA compared to BCA03 in inhibiting the growth of *G. boninense* by inducing the defense mechanisms of the host plants during infection. The two BCAs could be utilized in developing an environmental-friendly bio-formulation to reduce the usage of synthetic fungicides

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

POTENSI BAKTERIA ANTAGONIS DALAM PERENCATAN *GANODERMA BONINENSE* DAN PENGENALPASTIAN METABOLIT ANTIKULAT YANG PENTING

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Minyak sawit merupakan penyumbang terbesar sebagai sumber minyak makanan dunia. Walau bagaimanapun, industri sawit di Asia Tenggara menghadapi ancaman terbesar dari penyakit reput pangkal batang (BSR) yang disebabkan oleh kulat *Ganoderma boninense*. Ini mengakibatkan kerugian ekonomi yang besar kepada negara pengeluaran minyak sawit. Pada masa kini, masih tiada cara yang berkesan untuk mengesan penyakit ini pada peringkat awal jangkitan, oleh itu, ada keperluan segera untuk mencari kawalan penyakit yang praktikal dan alam mesra. Kaedah kawalan biologi yang biasa digunakan untuk mengawal penyakit ini adalah dengan penggunaan baja biologi yang mengandungi pelbagai jenis ejen kawalan biologi (BCA). Dalam kajian ini, dua potensi BCA, BCA01 (*Chromobacterium* sp.) dan BCA03 (*Lysobacter* sp.) yang diperolehi dari ACGT Microbial Culture Collection (AMCC) telah dipilih daripada 21 calon BCA melalui penyaringan kultur berpasangan dengan *G. boninense*, dan mencatatkan keputusan perencatan sebanyak 51% and 61% masing-masing. BCA01 dan BCA03 juga telah dikenalpasti dengan ciri antagonis seperti keupayaan pengikatan nitrogen, pengeluaran enzim protease dan kitinase. Selain itu, BCA03 juga berupaya meghasilkan siderofor serta enzim selulase. Di samping itu, pengenalpastian komponen metabolit utama yang berpotensi terlibat dalam proses kawalan pertumbuhan *G. boninense* juga dijalankan dalam kajian ini untul lebih memahami faktor penyumbang antagonis. Metabolit-metabolit dari isolat BCA01 yang dikaitkan dengan aktiviti kawalan biologi telah dikenalpasti sebagai mevalonolactone, p-cresol, 4-hydroxycinnamic asid, tiadinil dan 13S-hydroperoxy-9Z,11E,15Z-octadecatrienoic aid (13(S)-HpOTrE). Metabolit-metabolit ini yang mungkin berpotensi untuk mengawal pertumbuhan miselium *G. boninense*. Walau bagaimanapun, kajian ini gagal mengenalpasti metabolit aktif yang berpotensi dalam ekstrak BCA03. Selain itu, keupayaan BCA yang dipilih dalam mengawal penyakit BSR juga dikaji dengan

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menggunakan anak sawit yang berusia tiga bulan. Walaupun keputusan antagonis yang positif diperolehi di makmal, rawatan BCA01 dan BCA03 ke atas sawit di nurseri gagal menunjukkan hasil yang sama. Rawatan BCA01, BCA03 dan kawalan masing-masing mencatatkan peratusan insiden penyakit (DI) sebanyak 95%, 93% dan 90%. Selain itu, profil pengekspresan cDNA dalam akar anak sawit yang telah diinokulasi dengan *G. boninense*, serta dirawat dengan isolat-isolat BCA01 dan BCA03 pada minggu satu, dua, tiga dan empat telah direkodkan. Tindak balas berantai polimerase (PCR) secara masa nyata (qPCR) telah menunjukkan peningkatan (lebih dari 2-kali ganda) pada transkrip *CAD*, *chitinase* dan *β-1,3-glucanase* pada minggu ke-4 dalam akar anak sawit yang telah dirawat dengan BCA01 berbanding dengan kawalan negatif (akar yang tidak diinokulasi dengan *G. boninense*). Keputusan ini menyatakan BCA01 berupaya untuk mencetuskan mekanisme pertahanan anak sawit semasa interaksi dengan *G. boninense* pada minggu ke-4. Namun demikian, tiada peningkatan atau penurunan dalam transkrip ketiga-tiga gen pada sampel akar anak sawit yang telah dirawat dengan BCA03 direkodkan. Sebagai kesimpulannya, hasil kajian ini telah menunjukkan bahawa, BCA01 merupakan BCA yang berpotensi berbanding dengan BCA03 kerana berupaya untuk merencatkan pertumbuhan *G. boninense* serta mencetuskan mekanisme pertahanan dalam anak sawit yang dijangkiti oleh *G. boninense*. Kedua-dua BCA ini berpotensi dibangunkan sebagai bio-formulasi yang mesra alam untuk mengawal penyakit BSR supaya dapat mengurangkan penggunaan racun kulat sintetik.

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LIST OF ABBREVIATIONS

GDP	gross domestic product
BSR	basal stem rot disease
FFB	fresh fruit bunch
AMF	arbuscular mycorrhiza fungi
BCA	biological control agent
PAL	phenylalanine ammonia lyase
CAD	*cinnamyl alcohol dehydrogenase*
CPO	crude palm oil
NAD	NAD(P)H dehydrogenase (quinone)
°C	degree Celsius
SEM	scanning electron microscope
AUDPC	area under disease progression curve
PGP	plant growth promoting
LPSN	list of prokaryotic names with standing in nomenclature
LPs	Lipopeptides
2,4-DAPG	2,4-diacetylphloroglucinol
DMDS	dimethyl disulphide
NRPS	non-ribosomal peptide synthetase
PRN	Pyrrolnitrin
HR	hyersensitive response
PTI	patterns-triggered immunity
ETI	effector triggered immunity
PCD	programmed cell death
ROS	reactive oxygen species

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NO	nitric oxide
PR	pathogenesis-related
MAMP	microbe-associated molecular pattern
PAMP	pathogen-associated molecular pattern
FLS2	Flagellin Sensing 2
LRR-RK	Leucine-rich repeat receptor kinase
ISR	induced systemic resistance
SAR	systemic acquired resistance
SA	salicyclic acid
JA	jasmonic acid
ET	Ethylene
wpi	week post inoculation
IRF	interferon regulatory factor
HSP	heat shock protein
POD	Peroxidase
AMCC	ACGT Microbial Culture Collection
NA	nutrient agar
NB	nutrient broth
PDA	potato dextrose agar
PDB	potato dextrose broth
PIRG	percent inhibition of radial growth
PIDG	percentage inhibition diameter growth
PCR	polymerase chain reaction
DNA	deoxyribonucleic acid
rDNA	ribosomal DNA

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RNA	ribonucleic acid
rRNA	ribosomal RNA
dNTPs	deoxyribonucleotide triphosphates
NCBI	National Center for Biotechnology Information
NF	nitrogen-free
CAS	chrome azurol S
HDTMA	hexadecyltrimethylammonium bromide
NBRIP	National Botanical Research Institute's phosphate
BSC	biosafety cabinet
ACC	1-aminocyclopropane-1-carboxylic acid
rpm	Revolutions per minute
YT	yeast tryptone
IAA	indole-3-acetic acid
SMA	skim milk agar
v/v	volume / volume
w//v	weight / volume
LB	Luria broth
CMCA	carboxy-methyl-cellulose agar
CMC	carboxy-methyl-cellulose
NaCl	sodium chloride
CCA	colloidal chitin agar
KOH	potassium hydroxide
K_2HPO_4	dipotassium phosphate
$MgSO_4.7H_2O$	magnesium sulphate
$Ca_3(PO_4)_2$	tricalcium phosphate

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$CaCl_2$	calcium chloride
$FeSO_4.H_2O$	iron (II) sulphate
$NaMoO_4.2H_2O$	sodium molybdate
$MnSO_4.7H_2O$	manganese (II) sulphate
NH_4Cl	ammonium chloride
$MgCl_2.6H_2O$	magnesium chloride
KCl	potassium chloride
$(NH_4)_2SO_4$	ammonium sulphate
$FeCl_3$	iron (III) chloride
$CaCO_3$	calcium carbonate
KH_2PO_4	potassium dihydrogen phosphate
Na_2HPO_4	disodium hydrogen phosphate
H_3BO_3	boric acid
$ZnSO_4.7H_2O$	zinc sulphate
$CuSO_4.5H_2O$	copper sulphate
MoO_3	molybdenum trioxide
NaOH	sodium hydroxide
LiCl	lithium chloride
EDTA	ethylenediaminetetraacetic acid
TAE	tris-acetate-EDTA
bp	base pair
BLAST	Basic Local Alignment Search Tool
LC-MS	liquid chromatography mass spectrometry
HPLC	high performance liquid chromatography
13(S)-HpOTrE	13-hydroperoxy octadecatrienoic acid

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PBZ1	probenazole-inducible protein
RPR1	RNase P ribonucleoprotein *1*
HSAF	heat stable antifungal factor
NRPS	non-ribosomal peptide synthase
PKS	polyketide synthase
RWB	rubber wood blocks
CFU	colony forming unit
RCBD	randomized complete block design
AUDPC	area under the disease progress curve
DI	disease incidence
DSI	disease severity index
DR	disease reduction
ANOVA	analysis of variance
HSD	Tukey's honest significant different
LSD	Fisher least significant different
RNase	Ribonuclease
DNase	Deoxyribonuclease
RIN	RNA integrity number
qPCR	quantitative polymerase chain reaction
RT-qPCR	real time-qPCR
V	voltage
g	gram
mg	milligram
ml	mililitre
l	litre

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µl	microlitre
mm	milimeter
µm	micrometer
nm	nanometer
M	molar

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CHAPTER 1

INTRODUCTION

Oil palm industry in Malaysia has significant contribution to our national economic growth with an export value of RM72.3 billion for palm oil and palm oil-based products in year 2020 (Malaysia Palm Oil Council, MPOC, 2021). Besides, in year 2018, oil palm industry was known as the largest contributor to the gross domestic product (GDP) in Malaysia for agriculture sector which 37.9% (Department of Statistic Malaysia Official Portal, 2020). In short, oil palm industry plays a significant role in both export income and employment which directly or indirectly contributes to the economy growth in Malaysia. With an ever-expanding demand for palm oil derived products, the health of productive oil palm is definitely an industry priority.

Fungus attack by *Ganoderma boninense* on oil palm caused substantial loss of yearly harvest. *G. boninense* is the causal agent of basal stem rot disease (BSR) of oil palm or also known as white rot pathogen by decomposing lignin as well as cellulose and related polysaccharides found in the trunk of oil palm trees (Rees et al., 2009). Fresh fruit bunch (FFB) reduction in BSR infected plantations has surpassed the given 20% economically tolerable threshold and resulted in annual economical loss of up to RM1.5 billion (Hushiarian et al., 2013).

Infection of *G. boninense* was initiated from oil palm roots and gradually spread to the bole of the palm and lastly causing dry rot. Such infection and disruption in oil palm internal structure causes the oil palm to gradually lose their ability to produce fruits and eventually collapse (Ariffin et al., 2000). Detection method through molecular approaches and non-molecular approaches such as detection of ergosterol and tomography imaging system are still immature to develop a reliable species-specific diagnosis system for *G. boninense* (Hushiarian et al., 2013).

As early detection is still under debate, thus, there is an urge to find a practical and successful disease control system. There are a lot practices in controlling the disease, such as soil mounding, surgery, sanitation or removal of diseased materials, planting of legume cover crops, fallowing, ploughing and hallowing, chemical treatments and biological control. Chemical treatment such as trunk injection fungicides is unfavorable as it is less effective and caused environmental concern. Biological control refers to the application of antagonistic microorganisms which are able to control or inhibit the growth of *G. boninense.* This practice is widely used in oil palm industry as it is more sustainable and environmental-friendly. In agriculture, biological control agent (BCA) refers to microorganisms such as bacteria and fungi with potentials to inhibit the growth of plant pathogens.

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To date, the most effective BCAs involve multiple antagonistic mechanisms. For instance, some bacteria secrete a potent enzyme which could destroy other cells by digesting their cell walls and degrade the cellular materials. In some cases, the antagonistic bacteria will secrete certain inhibitory substances such as bioactive molecules which are highly specific in their actions affecting only specific species. For example, *Pseudomonas aeruginosa* is known to produce pyocyanin and phenazine which have been shown to be essential for the biological control activity of certain fungi (DeBritto et al., 2020, Parvin et al., 2016).

In oil palm industry, the most common BCAs which showed potentials to control the BSR infection are arbuscular mycorrhiza fungi (AMF), antagonist fungi such as species of *Trichoderma*, and endophytic bacteria (Idris et al., 2010) such as *Burkholderia cepacia* (Azadeh et al., 2010), *Serratia marcescens* (Sapak et al., 2008), *Streptomyces* spp. (Lim et al., 2018), and *P. aeruginosa* (Sathyapriya et al., 2012). They have been recognised as potential BCAs for inhibition of *G. boninense*. The application of BCAs is not likely to prevent the BSR disease but is expected to prolong the productive years of infected palms (Sarashimatun and Tey, 2009).

Plants naturally have defensive genes and serve as markers for resistance against a particular pathogen (Hushiarian et al., 2013). In higher plant defense mechanism, expression of chitinases is enhanced upon the infection as they have antifungal activities which lyse the hyphal tips (Punja and Zhang, 1993). Gene expression of β-1,3-glucanase by oil palm were suppressed during *G. boninense* infection but regulated in the presence of *Trichoderma harzianum* (Yeoh et al., 2012). Expression of genes that are involved in the oil palm defense mechanism is one of the important molecular approaches to understand the interaction between the BCAs, environmental factors and plants (Kwan et al., 2016).

In short, oil palm industry in Southeast Asia is facing the greatest threat from BSR infection caused by *G. boninense.* There is an urgent need to find a practical, effective and environmental-friendly disease control system to reduce the disease incidences. If the selected BCAs and the identified key metabolites show positive impact during nursery trials, it can then be used for large scale production to complement the existing treatment methods in the market. Therefore, this study was carried out to identify BCAs as a sustainable and environmental-friendly control strategy for combating *G. boninense* disease. The specific objectives of this study were:

i) To determine the antagonistic ability and the antagonist biochemical activity of two selected BCAs.
ii) To identify key metabolites produced by two selected BCAs for suppression of the growth of *G. boninense in vitro*.

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iii) To determine the effectiveness of selected BCAs against *G. boninense* in the nursery trial and the expression level of four oil palm defense genes *phenylalanine ammonia lyase* (*pal*), *cinnamyl alcohol dehydrogenase* (*cad*), *chitinase* and *β-1,3-glucanase* with the application of selected BCAs.

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