



UNIVERSITI PUTRA MALAYSIA

***ISOLATION AND IN VITRO ANTAGONISTIC SCREENINGS OF OIL
PALM (*Elaeis guineensis* Jacq.) FUNGAL ENDOPHYTES AS
BIOCONTROL AGENTS AGAINST *Ganoderma boninense* Pat.***

ALLICIA ANAK JACK

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AGAINST *Ganoderma boninense* Pat.**

By

ALLICIA ANAK JACK

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

May 2014



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DEDICATION

This thesis is dedicated to my beloved parents, sisters, family, great friends & my late supervisor Allahyarhamah Professor Dr. Faridah Abdullah



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

ISOLATION AND *IN VITRO* ANTAGONISTIC SCREENINGS OF OIL PALM (*Elaeis guineensis* Jacq.) FUNGAL ENDOPHYTES AS BIOCONTROL AGENTS AGAINST *Ganoderma boninense* Pat.

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May 2014

Chairman : Professor Umi Kalsom Yusuf, PhD

Faculty : Science

Basal stem rot (BSR) disease is the principal disease of oil palm. The main aim of present studies is to screen the efficacy of oil palm root fungal endophytes as new potential biocontrol agents of BSR disease. Oil palm seedlings were subjected to five treatments: (i) *Trichoderma harzianum*-infused mulch (TC); (ii) *Ganoderma boninense* and simultaneous application of *T. harzianum* mulch (GB + TC); (iii) *G. boninense* (GB); (iv) *Glomus* sp. (arbuscular mycorrhizal fungi) (AM) and, (v) no treatment as control. The results revealed that the highest number of fungal endophytes were obtained from the TC treatment with 75 isolates and the lowest, 20 isolates were from the AM treatment. However, treatment with the highest diversity index was represented by AM treatment with 14 species of endophytic fungi isolated. Overall, 225 isolates of endophytic fungi were obtained in this study and seven species were recorded as dominant namely *Chaetomium trilaterale*, *Chaetomium gracile*, *Chaetomium cupreum*, *Penicillium janthinellum*, *Penicillium aculeatum*, *Eupenicillium javanicum* and *Paecilomyces* sp. All isolates were screened *in vitro* against *G. boninense* (PER 71). The dual culture bioassay showed that isolates AM26, GB03 and AM23 displayed the strongest antifungal activity against PER 71 with mean PIRG value of 98.9%, 97.8% and 97.5% respectively. Only 11 isolates recorded mean percentage inhibition of radial growth (PIRG) values of > 80% and retested for *in vitro* antagonistic volatile organic compounds (VOCs) screening. Results from VOCs screening showed that isolates AM26, AM23, GB03 and AM17 produced inhibitory effects with average PIRG values of > 50%. AM26 and AM23 produced VOCs that retarded the growth of PER 71 after the second days of incubation with mean PIRG value of 67.5% and 64.6% respectively. Fungal molecular identification based on internal transcribed spacer (ITS) regions has confirmed that AM26, AM23 and GB03 were *Daldinia eschscholzii*. Present study demonstrated that *D. eschscholzii* had shown great antimicrobial characteristics against *G. boninense* *in vitro*. The VOCs produced by this species offers very good prospects for integrated management of BSR disease as it has the potential to be developed as an

alternative to chemical fumigation. Thus, further *in vivo* studies should be carried out to confirm its efficacy as pathogen biocontrol agent.



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

**PEMENCILAN DAN SARINGAN ANTAGONISTIK *IN VITRO* KULAT
ENDOFIT KELAPA SAWIT (*Elaeis guineensis* Jacq.) SEBAGAI AGEN
KAWALAN BIOLOGI KE ATAS *Ganoderma boninense* Pat.**

Oleh

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Penyakit reput pangkal batang (RPB) merupakan penyakit prinsipal kelapa sawit. Tujuan utama kajian ini adalah untuk menyaring keberkesanan kulat endofit akar kelapa sawit sebagai agen kawalan biologi baru berpotensi untuk penyakit RPB. Anak kelapa sawit telah diberi lima rawatan: (i) *Trichoderma harzianum* terapan sungkupan (TC); (ii) *Ganoderma boninense* dan aplikasi berturutan *T. harzianum* terapan sungkupan (GB + TC); (iii) *G. boninense* (GB); (iv) *Glomus* sp. (kulat mikoriza arbuskul) (MA) dan, (v) tiada rawatan sebagai kawalan. Keputusan menunjukkan bilangan kulat endofit yang tertinggi diperolehi dari rawatan TC dengan jumlah 75 isolat dan bilangan terendah 20 isolat diperolehi daripada rawatan MA. Walau bagaimanapun rawatan MA mencatatkan indeks kepelbagaian tertinggi dengan 14 spesies kulat terpen cil. Secara keseluruhan, 225 isolat kulat endofit telah diperolehi dan tujuh spesies dominan telah direkod iaitu *Chaetomium trilaterale*, *Chaetomium gracile*, *Chaetomium cupreum*, *Penicillium janthinellum*, *Penicillium aculeatum*, *Eupenicillium javanicum* dan *Paecilomyces* sp. Kesemua isolat disaring secara *in vitro* ke atas *G. boninense* (PER 71). Bioesei dua kultur menunjukkan isolat AM26, GB03 dan AM23 mempamerkan aktiviti antikulat terkuat ke atas PER 71 dengan nilai min PPPR masing-masing iaitu 98.9%, 97.8% dan 97.5%. Hanya sebelas isolat mencatatkan min peratus perencatan pertumbuhan radius (PPPR) > 80% dan diuji semula untuk saringan antagonistik *in vitro* sebatian organik meruap (SOM). Keputusan saringan SOM menunjukkan isolat AM26, AM23, GB03 dan AM17 telah menghasilkan kesan perencatan dengan purata nilai PPPR > 50%. AM26 dan AM23 menghasilkan SOM yang merencatkan pertumbuhan PER 71 selepas hari kedua inkubasi dengan nilai min PPPR 67.5% dan 64.6%. Pengecaman molekul kulat berdasarkan kawasan penjarak tertranskripsi dalaman (PTD) mengesahkan bahawa AM26, AM23 dan GB03 adalah *Daldinia eschscholzii*. Kajian ini menunjukkan *D. eschscholzii* mempamerkan ciri-ciri antimikrob ke atas *G. boninense* secara *in vitro*. SOM yang dihasilkan oleh spesies ini menawarkan prospek yang sangat baik untuk kawalan bersepadu penyakit RPB dan mempunyai potensi untuk dibangunkan sebagai alternatif kepada fumigasi kimia. Oleh

itu, ujikaji lanjut secara *in vivo* seharusnya dilakukan untuk memastikan keberkesanannya sebagai agen kawalan biologi patogen.



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I certify that a Thesis Examination Committee has met on 21 May 2014 to conduct the final examination of Alicia anak Jack on her thesis entitled "Isolation and *In Vitro* Antagonistic Screenings of Oil Palm (*Elaeis guineensis* Jacq.) Fungal Endophytes as Biocontrol Agents Against *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 Master of Science.

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LIST OF ABBREVIATIONS

>	More than
<	Less than
μL	Microliter
AM	Arbuscular mycorrhiza fungi treatment
AMF	Arbuscular mycorrhiza fungi
BLAST	Basic Local Alignment Search Tool
BSR	Basal stem rot
BCA	Biocontrol agent
bp	Base pair
°C	Celsius
CF	Colonization frequency
CPO	Crude palm oil
D	Simpson's index
DMRT	Duncan's Multiple Range Test
DNA	Deoxyribonucleic acid
ddH ₂ O	Double distilled water
dNTP	Deoxyribose triphosphate
EB	Ethidium bromide
EDTA	Ethylenediaminetetraacetic acid
FB	Fruit bunch
FEB	Fresh fruit bunches
GB	<i>Ganoderma boninense</i>
GB+TC	<i>Ganoderma boninense</i> with simultaneous application of <i>Trichoderma</i> -infused mulch
<i>H'</i>	Shannon-Wiener diversity index
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
IOPRI	Indonesian Oil Palm Research Institute
ITS	Internal transcribed spacer
MgCl ₂	Magnesium chloride
MITI	Ministry of International Trade and Industry
mM	Milimolar

MMT	Million metric tons
MPOB	Malaysian Palm Oil Board
NaCl	Sodium chloride
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
p_i	Number of individual within species divided by the total fungal isolates
psi	Pound per square
PIRG	Percentage inhibition of radial growth
PKC	Palm kernel cake
POME	Palm oil mill effluent
rDNA	Ribosomal deoxyribonucleic acid
RPB1	RNA (Ribonucleic Acid) Polymerase II
rpm	Rotation per minute
SAS	Statistical Analysis System
SDS	Sodium dodecyl sulphate
SE	Standard error
TAE	Tris-acetate-EDTA
<i>Taq</i>	<i>Thermus aquaticus</i>
TC	<i>Trichoderma harzianum</i> -infused mulch
TFC	Total flavonoid content
TPC	Total phenolic content
USDA	United States Department of Agriculture
UV	Ultraviolet
VOCs	Volatile organic compounds



CHAPTER 1

INTRODUCTION

Oil palm (*Elaies guineensis* Jacq.) is the world highest yielding oil producing crop. Palm oil production constituted 53.83 million metric tons (MMT) out of the total 157.76 MMT world vegetable oil production. Followed by soybean at 43.18 MMT and rapeseed (23.80 MMT) (Anonymous, 2013c). Today, there has been rapid expansion of oil palms cultivated areas in Malaysia. Oil palm plantation development include not only in the Peninsular, but also in the East Malaysia. Today more than five million hectares of land in Malaysia are under oil palm cultivation (Anonymous, 2013b). Expansion process has involved in large scale conversion of forest land and replanting of the existing plantation. Intense monoculture of oil palms after few decades has resulted in environmental imbalance. Although monoculture practices produce much food or yields for international commerce, single crops are also vulnerable to disease and pest problems. These in turn lead to the increasing applications of more potent pesticides and chemical fertilizers to the lands to fight diseases and weeds, causing pollution, soil degradation and persistent occurrence of pesticides resistant disease in which eventually affecting the quality of the crop yields and productions.

The oil palm industry is one of the major revenue earners of Malaysia. However, this lucrative industry may face a bleak future with the constant attack by the Basal Stem Rot (BSR) disease which was reported to be major constraint of oil palm plantations in Southeast Asia, causing severe economic loss especially in Malaysia and Indonesia (Wong *et al.*, 2012). BSR disease of oil palm is caused by the white-rot fungus *Ganoderma boninense*. The white-rot mode of attack involved the degradation of lignin component of the wood leaving the white cellulose exposed. Symptoms of disease include water stress, one-sided mottling of the canopy or yellowing of the fronds followed by necrosis, multiple unopened spears and production of basidiocarps (fruiting body) on the lower stem. Once oil palm showed visible symptoms, at least 50% of the basal stem tissues have been colonized by *G. boninense* and the infection was already firmly established (Laila *et al.*, 2012b).

Although the BSR disease has been long known, there are no current effective method of early detection and controlling the disease. Numerous agronomic practises were suggested in the past to control the disease such as trench digging and soil mounding around diseased palms, collecting fruiting body from palm base, fungicidal treatment by trunk injection in affected palm and stump treatment. However, none was able to generate satisfactory results due to lack of understanding of this disease aetiology. They were either too time consuming, costly, labour intensive and detrimental to environment and human health. *Ganoderma* has various resting stages which were the factors that add to the ineffectiveness of fungicides due to degradation in the soil before they could reach the targeted area. Therefore, integrated biological control and the breeding of resistant oil palm progenies have been suggested as a sustainable solution for the constraints. The use of biocontrol agent (BCA) is more preferable since it can be developed over shorter time scale compared to resistant progenies breeding process.

The use of biological control approaches have become popular alternatives in managing several crop diseases. There were increasing reports on the use of BCA such as beneficial bacteria, fungi and plant endophytes in combating diseases in commercial crops such as soybean, wheat, fruits and vegetables (Mercier *et al.*, 2004; Mercier *et al.*, 2005; Lacey *et al.*, 2006; Ownley *et al.*, 2008; Chatterton *et al.*, 2008). As a result, they were extensively explored and proven to be able of achieving promising result. Biological control aims at inhibiting the growth of the pathogen and subsequently its entry into the host plant. Control can be achieved through treatment of susceptible host plants with antagonistic microorganisms. The BCAs are mainly fungi and bacteria from soil rizhosphere, or endophytic microorganisms which associated with internal tissues of plants.

Hypothetically, microbes that originally isolated from any part of the host plant itself may be better adapted to that specific plant species and give better control of diseases than microbes isolated from other plant species or resources. As they experience less competition and soil fungistasis from resident soil microbes, the chances of survival for endophytes in host plants are also enhanced. Therefore endophytic fungi of root parts are chosen as the antagonist candidates since evidences showed that the main mode of infection by *G. boninense* is by roots invasion. They are also expected to be able to compete with the pathogen as both share similar niche, source of nutrients and establishment sites which is the vascular tissues limiting *G. boninense* for both nutrients and space during its proliferation. In addition to this study, the diversity and abundancy of oil palm roots endophytic fungi were also observed.

To date, the information on fungal endophytes dynamics and mechanism of interaction among the endophytic fungi themselves or with the host plants and are still understudy. Most reports described the diversity of fungi isolated from healthy plant or of other resources in natural environment. This study is driven by the intriguing factor on how fungal endophytes population in oil palm roots were altered when the seedlings were subjected to the infections of different fungal treatments in a glass house condition. With the presence of inoculums, the occurrence of certain fungal endophytes was predicted to be correlate with other fungi. The presence of some common fungi would be negatively correlated with the presence of others because they may suppress the growth of other fungi by secondary metabolite production, parasitism, or competition for resources.

In the present study, the effects of different fungal inoculum to the diversity of the oil palm seedlings root fungal endophytes were observed and compared. The oil palm seedlings were artificially infected with the pathogen (*G. boninense*) itself, treated with the BCA (*T. harzianum*), *G. boninense* with simultaneously application *T. harzianum* and arbuscular mycorrhizal fungi with non-inoculated seedlings as control. The ability of the fungal endophytes obtained from both diseased and healthy plants to produce antimicrobial metabolites were determined by *in vitro* screening against *G. boninense*.

1.2 Objectives

1.2.1 General objective

The aim of this study was to develop a biocontrol against BSR by using endophytic fungi isolated from roots of oil palm seedlings.

1.2.2 Specific objectives

1. To determine the diversity and abundance of root fungal endophytes of oil palm seedlings subjected to different fungal inoculums.
2. To screen the efficacy of the isolated fungal endophytes against *G. boninense* in *in vitro* study.
3. To identify and select potential endophytic fungi by morphological characterization and phylogenetics.

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