UNIVERSITI PUTRA MALAYSIA

EFFICACY OF Trichoderma spp. CONSORTIUM AS POTENTIAL BIOCONTROL AGENTS OF GANODERMA DISEASE OF OIL PALM, AND INVOLVEMENT OF ANTIOXIDANT ENZYMES AND TOTAL PHENOLIC CONTENT

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By

HABU MUSA

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

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DEDICATION

The work in this thesis is dedicated to the memory of loving daughter and son, to my entire family members and my beloved wife, Rashida Umar for their boundless love, understanding, encouragement and patience throughout my study.

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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January 2017

Chairman : Nusaibah binti Syd Ali, PhD
Faculty : Agriculture

Oil palm (*Elaeis guineensis*) is an economically important cash crop, grown primarily for its oil and has become one of the main oil crop in the world. Unfortunately, a major constrain faced by oil palm industry particularly in Malaysia and Indonesia is the devastating disease known as *Ganoderma* disease caused by *Ganoderma* spp. To date, this disease has been causing considerable oil palm yield losses and collapse of oil palm trees, consequently affecting its contribution to the producer's economy. Thus, research on sustainable and eco-friendly remedy to counter *Ganoderma* disease is on the upsurge to avoid the current control measures via synthetic fungicides. *Trichoderma* spp. have been the most studied and valued microbes as biological control agents in an effort to combat a wide range of plant diseases sustainably. In addition, knowledge on *Trichoderma*'s mechanism employed to act as biological control agent, could be useful in improving on their ability as potent biological control agents against *Ganoderma* spp. Therefore, in this current study, the potential of *Trichoderma* spp.(*Trichoderma asperellum*, *Trichoderma harzianum*, and *Trichoderma virens*) as a consortium approach was evaluated as biological control agents against *Ganoderma* disease on oil palm with these objectives: i) To characterize and identify the selected *Trichoderma* spp. via morphological and molecular characterization, ii) To evaluate their bio-inoculant potential as oil palm seedling treatment for the control of *Ganoderma* disease and iii) To study the effects on vegetative growth and suppression of *Ganoderma boninense* infection in oil palm seedlings treated with *Trichoderma* spp. as single or consortium application.

Prior to *in vitro* assessment, all *Trichoderma* isolates studied were subjected to morphological and molecular identification. Antagonistic effects of *Trichoderma* spp. against *G. boninense* growth evaluated via dual culture test and culture filtrate test. The results demonstrated that the percentage inhibition of radial growth (PIRG) of *G. boninense* was of >75% in both in-*vitro* assays. In addition, ability of *Trichoderma* spp. in producing fungal cell wall degrading enzymes such as chitinase, glucanase, β-1, 3-glucanases and protease were also assessed. All the isolates studied demonstrated potential for the production of cell wall degrading enzymes.

However, to measure the potential in *Ganoderma* disease suppression and enhancement of vegetative growth, an *in-vivo* trial of six months duration was conducted on oil palm seedlings via *G. boninense* artificial inoculation treated with single species and consortium of *Trichoderma* spp. in a nursery condition. The consortium of *Trichoderma* spp. applied found to be the most effective treatment in suppressing *Ganoderma* disease with 83.03% and 89.16% from the foliar and bole symptoms respectively. Besides that, the consortium application of *Trichoderma* spp. exhibited tremendous enhancement in the oil palm seedling vegetative growth parameters such as plant height, stem girth, leaf area, frond count, shoot and root (fresh and dried weight) with significant difference at $P<0.05$ compared to all other treatments designed. Nevertheless, to further confirm the ability of *Trichoderma* spp. as an excellent biological control agent in a consortium approach via *in vivo* study, involvement of antioxidant enzymes [peroxidase (PO) and polyphenol oxidase (PPO)] and total phenolic content (TPC) of treated oil palm roots were measured. The results obtained demonstrated, highly induced significant activity of PO, PPO and TPC was recorded in the consortium treatment compared to the control treatment. Disease development was slower in the seedlings treated with consortium of *Trichoderma* spp. compared to the positive control, which exhibited with the highest percentage of disease severity. Therefore, the consortium application of *Trichoderma* spp. to the oil palm seedling roots before transplant (at nursery stage) could provide a promising sustainable approach for the management of *Ganoderma* disease of oil palm. Despite this, there is a need for further study in evaluating the biological control potential as consortium approach of *Trichoderma* spp. under field conditions.

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

KEBERKESANAN TRICHODERMA SPP. KONSORTIUM SEBAGAI EJEN BIOKONTROL POTENSI GANODERMA PENYAKIT DALAM KELAPA SAWIT, DAN PENGLIBATAN ENZIM ANTIOKSIDAN DAN JUMLAH KANDUNGAN FENOLIK

Oleh

HABU MUSA

Januari 2017

Pengerusi : Nusaibah binti Syd Ali, PhD
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Sawit (*Elaeis guineensis*) merupakan tanaman komersil yang penting bagi ekonomi sesebuah negara pengeluar dan ia ditanam terutamanya untuk hasil minyak sayuran. Kini pokok sawit telah menjadi salah satu tanaman minyak sayuran utama di dunia. Namun begitu, masalah utama yang dihadapi oleh industri sawit khasnya di Malaysia dan Indonesia adalah penyakit yang disebabkan oleh kulat yang memberikan kesan yang dahsyat. Penyakit ini dikenali sebagai penyakit *Ganoderma* yang disebabkan oleh kulat *Ganoderma* spp. Sehingga kini, penyakit ini telah menyebabkan kehilangan hasil industri sawit yang agak membimbangkan dan menyebabkan pokok sawit tumbang pada peringkat akhir fasa jangkitan dan seterusnya menjejaskan sumbangannya kepada ekonomi negara pengeluar. Oleh itu, penyelidikan dalam menghasilkan kawalan penyakit *Ganoderma* yang mampan lagi mesra alam sedang giat dan ini adalah untuk mengelakkan penggunaan racun kulat sintetik yang masih digunakan secara meluas.

Trichoderma spp. merupakan mikrob yang paling banyak dikaji sebagai agen kawalan biologi dalam usaha melawan pelbagai penyakit tumbuhan secara mampan. Tambahan pula, maklumat mengenai mekanisme yang digunakan oleh *Trichoderma* untuk bertindak sebagai agen kawalan biologi boleh digunakan bagi memperbaiki keupayaan *Trichoderma* spp. sebagai agen kawalan biologi terhadap penyakit *Ganoderma*. Oleh itu, dalam kajian ini, potensi *Trichoderma* spp. (*Trichoderma asperellum*, *Trichoderma harzianum,* dan *Trichoderma virens*) sebagai agen kawalan biologi terhadap penyakit *Ganoderma* pada anak sawit dalam bentuk gabungan telah dievaluasi berdasarkan objektik-objektif berikut: i) Untuk menjalankan pencirian dan identifikasi *Trichoderma* spp. berdasarkan ciri-riri morfologi dan molikul, ii) Untuk mengenalpasti potensi bio-inokulan *Trichoderma* spp. sebagai kawalan penyakit

Ganoderma pada anak sawit dan iii) Untuk mengkaji kesan pertumbuhan vegetatif dan perencatan jangkitan *Ganoderma boninense* pada anak sawit yang telah dirawat dengan *Trichoderma* spp. secara individu atau pun gabungan.

Sebelum penilaian secara *in vitro* dijalankan, isolat-isolat *Trichoderma* yang dikaji telah diidentifikasi secara morfologi dan molikul. Di dalam kajian secara *in vitro*, kesan-kesan antagonistik *Trichoderma* spp. terhadap pertumbuhan *G. boninense* telah dinilai melalui ujian dwi-kultur dan asai tapisan kultur. Keputusan yang diperolehi telah menunjukkan bahawa peratusan perencatan pertumbuhan jejarian (PIRG) *G. boninense* adalah > 75% dalam kedua-dua ujian *in vitro* tersebut. Di samping itu, keupayaan *Trichoderma* spp. dalam menghasilkan enzim yang mampu mendegradasi dinding sel kulat seperti enzim chitinase, glucanase, β-1, 3-glucanases dan protease juga telah dinilai. Berdasarkan keputusan kajian yang diperolehi, kesemua isolat yang dikaji telah mempamerkan potensi dalam menghasilkan enzim-enzim tersebut.

Walaubagaimanapun, untuk mengukur potensi dalam merencatkan jangkitan penyakit *Ganoderma* serta peningkatan dalam pertumbuhan vegetatif, satu kajian secara *in-vivo* selama enam bulan telah dijalankan ke atas anak sawit melalui kaedah inokulasi *G. boninense* secara artifisial dan dirawat dengan spesies tunggal dan gabungan *Trichoderma* spp. dalam keadaan nurseri yang terkawal. Rawatan *Trichoderma* spp. yang diaplikasi secara gabungan telah dikenalpasti sebagai rawatan yang paling efektif dalam merencatkan jangkitan *Ganoderma* dengan memberikan 83.03% dan 89.16% melalui simptom-simptom pada daun dan akar padat masing-masing. Selain itu, aplikasi *Trichoderma* spp. secara gabungan juga telah mempamerkan peningkatan yang memberangsangkan pada parameter-parameter pertumbuhan vegetatif anak sawit seperti ketinggian pokok, ukur lilit batang, keluasan daun, bilangan daun, pucuk dan akar (berat segar dan berat kering) dengan perbezaan signifikan pada $P<0.05$ berbanding dengan rawatan- rawatan lain yang telah dijalankan.

Disamping itu, bagi mengesahkan lagi keupayaan *Trichoderma* spp. sebagai agen kawalan biologi yang cemerlang dalam pendekatan rawatan secara gabungan melalui kajian *in vivo* ini, penglibatan enzim antioksida [peroxidase (PO) dan polifenol oxidase (PPO)] dan jumlah kandungan fenolik (TPC) di dalam akar sawit yang telah dirawat diukur. Berdasarkan keputusan yang diperolehi, didapati aktiviti PO, PPO dan TPC, teraruh dengan tinggi dalam rawatan gabungan berbanding dengan rawatan kawalan. Pada keseluruhan, perkembangan penyakit didapati lebih perlahan pada anak sawit yang dirawat dengan gabungan *Trichoderma* spp. berbanding dengan kawalan positif, yang telah mempamerkan peratusan tertinggi bagi tahap keterukan penyakit.

Oleh yang demikian, aplikasi *Trichoderma* spp. secara gabungan pada akar anak sawit sebelum di pindahkan ke ladang (pada peringkat nurseri) boleh memberikan pendekatan mapan yang memberangsangkan terhadap pengurusan penyakit *Ganoderma* sawit. Disamping kajian ini, terdapat keperluan untuk kajian lebih lanjut dalam menilai potensi kawalan biologi gabungan *Trichoderma* spp. di ladang.

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I certify that a Thesis Examination Committee has met on 6 January 2017 to conduct the final examination of Habu Musa on his thesis entitled "Efficacy of *Trichoderma* spp. Consortium as Potential Biocontrol Agents of Ganoderma Disease in Oil Palm, and Involvement of Antioxidant Enzymes and Total Phenolic Content" 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.

Members of the Thesis Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment for the requirement for the degree of Master of Science.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACC	1- Carboxylic acid
ANOVA	Analysis of variance
BLAST	Basic local alignment search tool
BSR	Basal stem rot
$CaCl_2$	Calcium Chloride
CDA	Czapex dox agar
CM	Centimetre
CMA	Corn meal agar
$CoCl_2.6H_2O$	Cobalt (II) Chloride Hexahydrate
Cu	Copper
CWDEs	Cell wall degrading enzymes
DI	Disease incidence
DMRT	Duncan multiple range test
DNA	Deoxyribonucleic acid
et al.	And others
ET	Ethylene
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Statistics
Fe	Iron
$FeSO_4.7H_2O$	Iron (II) Sulfate Heptahydrate
Ft	Feet
g	Gram
G-α	G- protein
H_2O	Water
HA	Harzianic acid
HA	Harzianic acid
HCL	Hydrochloric acid
IAA	Indole-3-acetic acid
ITS	Internal transcribed spacer
ITS	Internal transcribed spacer
K	Potassium
KH_2PO_4	Potassium Dihydrogen Phosphate
KOH	Potassium hydroxide
KOH	Potassium hydroxide
L	Litre
M^2	Meter square
MAMPs	Microbe associated molecular patterns
MAP	Mitogen activated protein
MEGA	Molecular evolutionary genetic analysis
Mg	Magnesium
$MgSO_4.7H_2O$	Magnesium Sulfate Heptahydrate
Mj	Megajoule
mL	Millilitre
mm	Millilitre
Mn	Manganese
$MnSO_4.H_2O$	Manganese (II) Sulfate Monohydrate

$(NH4)_2SO_4$	Ammonium Sulphate
N	Nitrogen
P	Phosphorus
PDA	Potato dextrose agar
PDB	Potato dextrose broth
pH	Potential hydrogen
PIRG	Percentage inhibition rate growth
PO	Peroxidase
PPO	Polyphenol oxidase
PR	Pathogenesis Related
PVC	polyvinyl pyrrolidone
RNA	Ribonucleic acid
rpm	revolution per minute
RWB	Rubber wood block
SA	Salicylic acid
SAR	Systemic acquired resistance
SAS	Statistical analysis software
SFS	Severity of foliar symptoms
SMs	Secondary metabolites
TPC	Total phenolic content
UPGMA	unweighted pair group methods with arithmetic
USD	United states dollar
Zn	Zinc
$ZnSO_4.7H_2O$	Zinc Sulfate Heptahydrat

CHAPTER 1

INTRODUCTION

Oil palm (*Elaies guineensis* Jacq.) is an economically important cash crop, grown primarily for its oil and has become one of the main oil crop in the world. It is the world's highest yielding oil producing crop known today. Oil palm produces about five tonnes per hectare of oil per year, which is comparable to 13, eight and seven times higher the yield obtained from soybean, sunflower and rapeseed respectively (Jacquemard, 2012). Palm oil accounts approximately 33% of the world's vegetable cooking oil and 45% of edible oil production worldwide and as the largest source of biofuel (Tye et al., 2011). As important oil producing crop, its annual yield worldwide is estimated to be 56 million tones with the largest producers in Southeast Asia; Indonesia producing 28 million and Malaysia producing 19 million tonnes per year constituting 83.9% of the total world production (FAOSTAT, 2013).

Oil palm cultivation in Indonesia and Malaysia has been an important sector since the 1970s as it contributes significantly to the economy and provides employment to thousands of people in the tropical rural regions (Rist et al., 2010). However, a devastating disease known as Ganoderma disease caused by pathogenic fungi from Ganoderma spp. has been threatening the oil palm industry in the major producing countries such as Indonesia and Malaysia. This pathogenic fungus was previous reported on older palms; however recent studies have found that it could also infect younger palms as early as 12 to 24 months at the field and currently found on younger palms as well (Naher *et al*., 2013). *Ganoderma* spp. has the ability to produce ligninolytic enzymes such as manganase peroxidase, lignin peroxidase and laccase, which are used to degrade the lignin content in the palm cell walls (Ganeson *et al.*, 2014).

The most obvious visible symptom of the disease is the formation of basidiomata that unfortunately appears at the later stages of infection (Mazliham et al., 2007). Even though, the presence of basidomata has been the primary symptom of Ganoderma disease, there are many cases where no appearance of basidiomata detected in collapsed palms due to Ganoderma disease. The presence of Ganoderma basidiomata on a palm may indicate that it has been infected for several years with severe internal decay of roots and stem (Najmie *et al.*, 2011).

Ganoderma disease was reported as the major constrain in many oil palm plantations in Malaysia (Idris et al., 2003; Chong et al., 2011). Besides that, in Malaysia, the disease incidences are higher in oil palm plantations on the coastal soils (Khairudin and Chong, 2008). Chong (2011) reported that Ganoderma disease not only causes reduction of total oil yield but also resulted in direct loss of stand due to palm death and the economic losses in Malaysia alone that amounted between $68 to $455 million USD annually. Generally, the losses due to *Ganoderma* disease on oil palm could be either direct or indirect, whereby the direct losses refers to the collapse or

death of the palm, whereas indirect loss may be referred to the decline in fresh fruit bunches (FFB) weight and quality (Susanto, *et al.,* 2005).

Therefore, various control techniques are being employed for the control of *Ganoderma* disease of oil palm. These include; cultural practices, use of resistant cultivars, mechanical control and chemical control. A wide range of systemic and protectant fungicides have been found to be an effective alternative to prolong the lifespan of palm infected with *Ganoderma* disease. Despite the effectiveness of synthetic chemicals in plant disease management, continuous utilization has resulted in the development of fungi with multiple resistances, which further complicates the management of plant diseases (Boubaker *et al*., 2009). In addition, many fungicides are now becoming ineffective against most important plant pathogenic fungi. Furthermore, the use of these chemicals in plant protection is also being restricted owing to stringent regulations, health hazard, residual toxicity, long degradation period, environmental contamination and growing public concern about the chemical residues in food materials (Tripathi and Dubey, 2003; Palou *et al*., 2008). Therefore, there is an increasing concern and effort to develop a better plant disease management approach that is safe, ecological friendly and economically feasible to the farmers. The most recent studies have shown that biological control approaches has promising potential against many plant pathogenic fungi (Troian *et al*., 2014; Kotasthane *et al.*, 2015; Contreras-Cornejo *et al*., 2016). *Trichoderma* spp have been widely used as biocontrol agents, and they can also stimulate plant growth and suppress plant diseases by one or more different direct and/or indirect mechanisms. The success of *Trichoderma* in the rhizosphere is due to their high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere and strong aggressiveness against plant pathogenic fungi (Benıtez *et al*. 2004; Harman 2006). Thus, based on the promising progresses reported in the literature on the use of beneficial microbes for plant disease control, this present study was conducted to explore the potential of *Trichoderma* spp. as an effective biological control agent (BCA) of *Ganoderma* disease of oil palm as a single and consortium application.

Therefore this research was undertaken with the following objectives:

1. To characterize and identify the selected *Trichoderma* spp. via morphological and molecular characterization;
2. To evaluate *Trichoderma* spp biocontrol potential against *Ganoderma boninense* in an *in-vitro* conditions;
3. To study the effects on vegetative growth and suppression of *Ganoderma boninense* infection in oil palm seedlings treated with *Trichoderma* spp. as single or consortium application and;
4. To determine the involvement of oil palm antioxidant enzymes and total phenolic content in *ganoderma boninense-trichoderma* spp. interactions

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