UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION OF SUPPRESSION MECHANISMS AND ANTIFUNGAL COMPOUNDS FROM Trichoderma virens AGAINST Ganoderma boninense

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FBSB 2016 4

IDENTIFICATION OF SUPPRESSION MECHANISMS AND ANTIFUNGAL COMPOUNDS FROM *Trichoderma virens* AGAINST *Ganoderma boninense*

By

LEE PEI LEE ANGEL

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

July 2016

DEDICATION

This thesis is dedicated to my family.

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

IDENTIFICATION OF SUPPRESSION MECHANISMS AND ANTIFUNGAL COMPOUNDS FROM *Trichoderma virens* AGAINST *Ganoderma boninense*

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Chairman : Mohd Termizi bin Yusof, PhD
Faculty : Biotechnology and Biomolecular Sciences

Ganoderma boninense has been identified as the pathogen that causes basal stem rot (BSR), a devastating disease affecting the oil palm tree. *Trichoderma* has long been recognized as a potential antagonistic biological control agent (BCA) against pathogenic fungi that are responsible for several plant diseases. The success of *Trichoderma* isolates as potent BCAs is due to the antagonistic characteristics such as mycoparasitism and antibiosis. Therefore, this study attempts to identify the mechanisms involved in the suppression of *G. boninense* and the antifungal compounds released by endophytic *Trichoderma*. Two potential endophytic *Trichoderma* isolates, namely *Trichoderma virens* 7b and *Trichoderma virens* 159c were assessed through *in vitro* assays. *Trichoderma* able to produce siderophore and able to chelate the iron under iron starvation. The culture filtrates of these *Trichoderma* isolates were then extracted using hexane, ethyl acetate (EtOAc) and butanol (BuOH), respectively. The resulting extracts were tested against *G. boninense* PER 71 for their antifungal activity through a modified well diffusion assay. The active fraction was further fractionated with chromatographic methods and the composition of the active fraction was analyzed with gas chromatography-mass spectrometry detector (GC/MSD). Both *Trichoderma* strains were highly active in producing siderophores according to the qualitative CAS reaction observation. Hexane extract of *T. virens* 7b and ethyl acetate extract of *T. virens* 159c were found to cause the highest inhibition against *G. boninense* PER 71 in inhibition radial growth (PIRG) (62.60% ±6.41 and 78.39% ±5.40, respectively). The activity of each extract was further demonstrated under the scanning electron microscope (SEM) where severe deformation of *G. boninense* PER 71 mycelia was observed at the inhibition region. The active fractions of *T. virens* 7b constituted of compounds such as ketones, alcohols, aldehydes, lactones, sesquiterpenes, monoterpenes, sulphides and free fatty acids whereas *T. virens* 159c produced ketones, aldehydes, acetamide,

alcohol, lactones and free fatty acids. Secretion of phenylethyl alcohol was found to be produced by both *T. virens* isolates. The findings of this study contribute to the understanding of the potential mechanisms involved in the suppression of *G. boninense* by endophytic *Trichoderma*.

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

MENGENALPASTI MEKANISME PENINDASAN DAN KOMPAUN ANTIKULAT DARIPADA *Trichoderma virens* UNTUK KAWALAN *Ganoderma boninense*

Oleh

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Pengerusi : Mohd Termizi bin Yusof, PhD
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Ganoderma boninense telah dikenal pasti sebagai patogen yang menyebabkan reput basal batang (BSR), suatu penyakit yang dapat memusnahkan pokok kelapa sawit. *Trichoderma* telah lama diketahui berpotensi sebagai agen kawalan biologi (BCA) antagonis terhadap kulat patogen yang bertanggungjawab kepada beberapa penyakit tumbuhan. Kejayaan pencilan *Trichoderma* yang berkesan sebagai agen kawalan biologi adalah kerana ciri-ciri antagonisnya seperti mikoparasitisme dan antibiosis. Oleh itu, kajian ini cuba untuk mengenal pasti mekanisme yang terlibat dalam penindasan *G. boninense* dan sebatian antikulat yang dikeluarkan oleh endofitik *Trichoderma*. Dua endofitik pencilan *Trichoderma* yang berpotensi adalah *T. virens* 7b dan *T. virens*159c yang dinilai melalui asai *in vitro*. *Trichoderma* mempunyai keupayaan untuk menghasilkan siderofor dan mampu untuk kelat besi dalam keadaan kekurangan besi. Turasan kultur pencilan *Trichoderma* ini masing-masing telah diekstrak menggunakan heksana, etil asetat (EtOAc) dan butanol (BuOH). Ekstrak yang terhasil telah diuji menggunakan *G. boninense* PER 71 untuk mengkaji aktiviti antikulat melalui resapan telaga asai yang diubahsuai. Pecahan aktif selanjutnya dipecahkan menggunakan kaedah kromatografi dan komposisi pecahan aktif dianalisis dengan pengesan spektrometri kromatografi jisim gas (GC/MSD). Kedua-dua strain *Trichoderma* mengeluarkan siderofor berdasarkan pemerhatian reaksi CAS kualitatif dengan aktif. Ekstrak heksana *T. virens* 7b dan ekstrak etil asetat *T. virens* 159c telah didapati menyebabkan perencatan paling tinggi terhadap *G. boninense* PER 71 dengan merencatkan pertumbuhan jejari (PIRG) (masing-masing 62.60% ± 6.41 dan 78.39% ± 5.40). Aktiviti setiap ekstrak seterusnya telah dibuktikan di bawah mikroskop elektron imbasan (SEM) dan didapati pencacatbentukan *G. boninense* PER 71 miselium pada kawasan perencatan. Pecahan aktif *T. virens* 7b terdiri daripada sebatian seperti keton, alkohol, aldehida, lakton, seskuiterpen, monoterpen, sulfida dan asid lemak bebas manakala *T. virens* 159c menghasilkan keton, aldehida, asetamida, alkohol, lakton dan asid lemak bebas. Alkohol feniletil dirembeskan oleh kedua-dua pencilan *T. virens*. Hasil kajian ini menyumbang

kepada pemahaman tentang mekanisme yang berpotensi yang terlibat dalam penindasan *G. boninense* oleh endofitik *Trichoderma.*

ACKNOWLEDGEMENTS

First and foremost, I express my sincere gratitude to all my supervisory committee members whom have assisted me throughout the research project and made it a worthwhile experience. I would like to show my greatest appreciation to my Master Supervisor in UPM, Dr. Mohd Termizi bin Yusof for his guidance and encouragement throughout my Master studies. He has been always been there to listen and to give advices. I would also like to express my deepest gratitude to my co-supervisor, Dr. Shamala Sundram who gave me the opportunity to continue my postgraduate studies. I have been fortunate to have an advisor who gives me the opportunity to explore more than a Master student and for her patience and assistance in overcoming many difficult situations. I am truly grateful to Ms. Bonnie Tay Yen Ping who guided me throughout my research, helping me in developing my chemistry background and most importantly providing me the equipments for my research. I would also like to thank Dr. Intan Safinar Ismail who willingly helped and gave good research suggestions. Thank you for the assistance and guidance from MPOB staffs, especially Hasliza, Shazwana, Intan, Diyana, Erhan, Hafiz and Khomsatun who made everything possible throughout my study. Special thanks to Ms Lee Yim Leng, Raimathy, Haslina and Farah Amira who has always been there for me throughout my Master research in MPOB. I would like to convey my sincere appreciation to MPOB for providing the financial support throughout my Master research programme. An honorable message goes to my mother and Lim Yong Hui. I am indebted to my wonderful family and my friends for their understanding, endless patience and encouragement throughout my study.

I certify that a Thesis Examination Committee has met on 21 July 2016 to conduct the final examination of Lee Pei Lee Angel on her thesis entitled "Identification of Suppression Mechanisms and Antifungal Compounds from *Trichoderma virens* 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

BSR	Basal stem rot
BuOH	Butanol
B	Band
CAS	Chrome azurol S
CPD	Critical point dryer
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
Cm	Centimeter
ºC	Celsius
EtOAc	Ethyl acetate
EtOH	Ethanol
F	Fraction
$FeCl_3$. $6H_2O$	Iron (III) chloride hexahydrate
G	gram
G. boninense	*Ganoderma boninense*
GC/MSD	Gas chromatography mass spectrometry detector
H	Hour
HCl	Hydrochloric acid
HDTMA	Hexadecyltrimethylammonium bromide
L	Liter
MEA	Malt extract agar
mM	Milli molar
M	Molar
Mg	Milligram
Mm	Millimeter
Ml	Milliliter
MeOH	Methanol
Min	Minute
NaOH	Sodium hydroxide
Na_2SO_4	Sodium sulphate
NCBI	National Center of Biotechnology Information
NIST	National Institute of Standard and Technology
Nm	Nanometer
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PER 71	*Ganoderma boninense*
PEA	Phenylethyl alcohol
PIRG	Percentage inhibition of radial growth
PIPES	Piperazinediethanesulfonic acid
pKa	Acid dissociation constant
P-TLC	Preparative thin layer chromatography
R	Radial
R_f	Retention factor
%	Percent
SEM	Scanning electron microscope
Spp.	Species

T. virens	*Trichoderma virens*
TLC	Thin layer chromatography
UV	Ultra violet
µL	Micro liter
Mm	Micro meter
VOC	Volatile organic compounds
v:v:v	Volume ratio
v/v	Volume per volume
w/w	Weight per weight

CHAPTER 1

INTRODUCTION

The world's most produced and consumed oil today is none other than palm oil. The oil is widely used in food, oleochemicals such as cosmetic products, household cleaning products and biofuel (Official Palm Oil Information Source, n.d.). Malaysia is currently the world's largest exporter of palm oil and the second-largest producer of palm oil (MPOB Statistic, 2014). According to the Malaysian Palm Oil Board (MPOB) Statistics (2014), the cultivation of oil palm in Malaysia is more than 14% (5.3 million hectares) in 2014 since it was introduced to Malaya in 1870. The oil palm is highly productive and commercially profitable at large scales. Over the years, the demand for palm oil is steadily rising (Sheil *et al*., 2009). Profits from the oil palm industry offer wealth and development to the country, which made oil palm one of the important commodity crops in Malaysia.

Unfortunately, the oil palm is prone to various diseases caused by pathogenic fungi which lead to a major decline in the productivity of the oil palms and eventually affecting the oil palm industry adversely (Hushiarian *et al*., 2013). Basal stem rot (BSR) disease is currently a major threat to the oil palm industry in Malaysia (Fee, 2011). The pathogen that is responsible for BSR infection in the oil palm is a white rot fungus from the genus *Ganoderma*. As a matter of fact, there are several species of *Ganoderma* involved in BSR infection but *Ganoderma boninense* was reported to be the most aggressive compared to the others (Wong *et al*., 2012). The attack of *G. boninense* targets the aging palms, as well as the younger palms (Corley and Tinker, 2007). Despite the main cause of BSR in oil palm having been identified, an effective strategy for controlling *G. boninense* is still far from being discovered.

The use of biological control agent (BCA) as a control strategy is widely being investigated as it is considered a more natural and environmentally friendly alternative to the existing chemical treatment methods (Butt *et al.*, 2001; Eziashi *et al*., 2006). *Trichoderma* spp. are the most common saprophytic fungi that are present in soil and root ecosystem (Barnett and Binder, 1973). The ability of *Trichoderma* as a BCA to control various plant diseases has been reported to be closely related with many aspects of a typical endophytic association (Evans *et al*., 2003; Harman *et al*., 2004). Various mechanisms such as competing for nutrients and space, modifying the environmental conditions, antibiosis and mycroparasitism, make *Trichoderma* spp. a potential BCA (Benítez *et al*., 2004; Harman *et al*., 2004; Yedidia *et al*., 1999). Recently, fungal endophytes have been considered as important resources for BCA in order to suppress plant pathogens due to their abilities to colonize and provide nutrients to the host plants and establish buffers from external environmental stresses and microbial competitions (Schulz and Boyle, 2005).

Endophytic *Trichoderma* was successfully isolated from oil palm roots by Sundram (2013) and this was the first attempt of using endophytic *Trichoderma* for the suppression of *G. boninense*. The aim of this study was to decipher the mechanisms and identify the antifungal compounds released from active endophytic *Trichoderma* for the suppression of *G. boninense*. *Trichoderma* spp. secretes different types of secondary metabolites ranging from non-polar to polar compounds that adversely affect the growth of different fungi (Morath *et al*., 2012; Reino *et a*l., 2007; Siddiquee *et al*., 2012). The compounds responsible for growth suppression of *G. boninense* were assessed by solvent separation and chromatographic methods. The physical mechanism of action on *G. boninense*'s hyphae by antifungal compounds was shown using scanning electron microscopy (SEM). Iron is crucial for the survival of microorganisms and they release the iron chelator (siderophore) for iron acquisition (Symeonidis and Marangos, 2012). The rate of iron chelator (siderophore) release by endophytic *Trichoderma* and *G. boninense* were detected with chrome azurol S (CAS) agar (Schwyn and Neilands, 1987).

Hence, this study aimed to contribute to the understanding of the physical and chemical mechanisms and competition for nutrient (iron) by the potential endophytic *Trichoderma* isolates during the growth suppression of *G. boninense*. In addition, the application of a natural source to develop antagonistic activity against harmful plant pathogens is highly recommended in sustainable agriculture as well as to replace the use of chemical pesticides which are harmful to the environment and humans. It also has a potential use as a prophylactic and suppressive treatment for standing oil palms, which in return prolongs the productivity of the palms in the field.

The objectives of the study were to:

1. To screen potentially useful isolates of *Trichoderma in vitro*.
2. To identify the mechanisms involved in the suppression of *G. boninense* PER 71 growth.
3. To identify the antifungal compounds released from endophytic *Trichoderma* isolates that were responsible for *G. boninense* PER 71 growth suppression.

Hypothesis

Endophytic *Trichoderma* has superior production of siderophores and releases antifungal compounds that are responsible for the growth suppression of *G. boninense* PER 71.

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