UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OF TRICHODERMA SPECIES ISOLATED FROM SOIL AND EFFICACY OF TRICHODERMA ASPERELLUM AS BIOCONTROL AGENT OF FUSARIUM WILT DISEASE OF BANANA

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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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Chair : Nur Ain Izzati Mohd Zainudin, PhD
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Chemical pesticides are commonly used for managing Fusarium wilt disease of banana that is caused by *Fusarium oxysporum* f. sp. *cubense*. However, the uses of chemical will lead to environmental disruptions and are ineffective in some conditions and disease stage. In order to initiate of application of an eco-friendly cure of the disease, this study was focused on biological control agent against the pathogen of Fusarium wilt of banana. *Trichoderma* species is one of the microorganisms which having antagonistic properties. The objectives of this study are to isolate and identify *Trichoderma* species isolated from various soil samples using phenotypic and molecular characterization, to screen antagonistic *Trichoderma* species against pathogen of Fusarium wilt disease under *in-vitro* condition, and to examine the efficacy of *Trichoderma asperellum* as a biocontrol agent of Fusarium wilt disease of banana under plant house condition. The soil samples were collected around eleven sites from different states of Malaysia and sixty-nine of the fungal isolates were obtained from Laboratory of Mycology, Department of Biology, Faculty of Science, UPM. The fungal isolation was completed using soil dilution technique and cultured on Rose Bengal Agar (RBA) to obtain a single colony-forming unit (CFU). The CFU was transferred onto Potato Dextrose Agar (PDA) and identification was carried out based on phenotypic and molecular characterization. About 326 isolates were classified into eight *Trichoderma* species, which are *Trichoderma asperellum*, *T. hamatum, T. harzianum*, *T. koningiopsis*, *Hypocrea rodmanii, T. spirale, T. viride* and *T. virens*. All the isolates were further used for *in vitro* study, the finding showed three high percentage inhibition of radial growth (PIRG) were observed in dual culture plates of *Trichoderma* isolates B1902 (84.85%), T2007 (77.78%) and C1667 (75.76%) were selected and further used for *in vivo* study. Based on morphological, internal transcribed spacers (ITS) and translation elongation factor 1 alpha (*TEF-1α*) sequences analysis, those three selected isolates were

identified as *T. asperellum.* In plant house condition, *T. asperellum* B1902, T2007, and C1667 were successfully inhibited the growth of *Fusarium oxysporum* f. sp. *cubense* isolate 9888 at 10 weeks and 20 weeks after inoculation. The best candidate was *T. asperellum* B1902 with Disease Severity Index (DSI) value of 0.2 compared to the inoculated control with DSI at 3.6. As a conclusion, *T. asperellum* can be used as an alternative treatment in managing Fusarium wilt disease. Hence, the future study should be focusing more in applying *T. asperellum* as biological agent in the field and controlling other plant diseases in agricultural plantation.

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

PENCIRIAN SPESIS *TRICHODERMA* YANG DIPENCIL DARIPADA TANAH DAN KEBERKESANAN *TRICHODERMA ASPERELLUM* SEBAGAI EJEN KAWALAN BIOLOGI PENYAKIT LAYU FUSARIUM PADA TANAMAN PISANG

Oleh

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Racun perosak kimia merupakan cara yang digunakan untuk merawat penyakit layu Fusarium pada pisang yang disebabkan oleh *Fusarium oxysporum* f. sp. *cubense* (*Foc*). Walau bagaimanapun, penggunaan racun kimia secara berlarutan dan berlebihan akan menyebabkan kemusnahan alam sekitar dan menjadi tidak berkesan dalam sesetengah keadaan dan peringkat penyakit. Untuk memastikan rawatan penyakit yang mesra alam, kajian ini menumpukan pada penyaringan agen kawalan biologi terhadap patogen Fusarium layu pisang. *Trichoderma* merupakan salah satu spesis mikroorganisma yang memiliki ciri-ciri antagonistik. Objektif untuk kajian ini adalah untuk memencilkan dan mengenalpasti spesis *Trichoderma* yang diisolat daripada pelbagai jenis sampel tanah secara fenotip and pencirian molecular, untuk menyaring isolat-isolat yang antagonik dari spesis *Trichoderma* terhadap pathogen layu Fusarium melalui persekitaran *in-vitro* dan untuk menguji keberkesanan *Trichoderma asperellum* sebagai agen kawalan biologi bagi penyakit Fusarium layu pisang di bawah persekitaran rumah tumbuhan. Sampel tanah diperolehi di sebelas kawasan di Malaysia dan enam puluh sembilan isolat diperolehi daripada Makmal Mikologi, Jabatan Biologi, Fakulti Sains, UPM. Pemencilan kulat telah dilakukan dengan menggunakan teknik pencairan dan dikulturkan di atas Agar Bengal Ros (RBA) untuk menghasilkan unit koloni tunggal (CFU). CFU yang terhasil dipindahkan ke atas Agar Dektrose Kentang (PDA) dan pengenalpastian kulat dilakukan melalui fenotip dan pencirian molekular. Sejumlah 326 isolat telah berjaya dipencilkan dan lapan spesis *Trichoderma* telah dikenalpasti, antaranya ialah *Trichoderma asperellum*, *T. hamatum, T. harzianum*, *T. koningiopsis*, *Hypocrea rodmanii, T. spirale, T. viride*, dan *T. virens.* Kesemua isolat seterusnya digunakan untuk ujikaji secara *in-vitro,* pemerhatian mendapati *percentage inhibition of radial growth* (PIRG) tertinggi dilihat pada plat dwikultur.

Pencilan *Trichoderma* B1902 (84.85%), T2007 (77.78%) dan C1667 (75.76%) yang mana telah dipilih untuk ujikaji *in-vivo*. Berdasarkan ciri morfologi, analisis jujukan *internal transcribed spacers* (ITS) dan *translation elongation factor 1 alpha* (*TEF-1α*), ketiga-tiga isolat tersebut dikenalpasti sebagai *T. asperellum*. Di persekitaran rumah tumbuhan, *T. asperellum* B1902, T2007 dan C1667 telah berjaya merencatkan pertumbuhan *Fusarium oxysporum* f. sp. *cubense* isolat 9888 ketika 10 dan 20 minggu selepas inokulasi. Isolat terbaik adalah *T. asperellum* B1902 dengan nilai indek keseriusan penyakit (DSI) pada 0.2 berbanding dengan pokok kawalan yang diinukalasi dengan nilai DSI 3.6. Kesimpulannya, *T. asperellum* boleh dijadikan sebagai satu kaedah alternatif dalam menguruskan penyakit layu Fusarium. Oleh itu, kajian yang lebih terperinci yang memfokuskan mengenai penggunaan *T. asperellum* sebagai agen kawalan biologi dalam mengawal penyakit tumbuhan yang lain di peringkat ladang perlu ditekankan.

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I certify that a Thesis Examination Committee has met on 6 June 2018 to conduct the final examination of Sharifah Siti Maryam binti Syd Abdul Rahman on her thesis entitled "Characterization of *Trichoderma* Species Isolated from Soil and Efficacy of *Trichoderma asperellum* as Biocontrol Agent of Fusarium Wilt Disease of Banana" 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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This is to confirm that:

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LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Percentage
µl	Microliter
µm	Micrometre
µM	Micromolar
x*g*	Centrifugal force
bp	Base pair
cm	Centimetre
cm^2	centimetre square
dNTP	Deoxynucleotide triphosphate
g	Gram
h	Hour
ha	Hectares
kg	Kilogram
m	Meter
mg	Milligram
min	Minutes
ml	Millilitre
rpm	revolution per minute
s	Second
v/v	Volume per volume
AAGBS	Arshad Ayub Graduate Business School
ANOVA	Analysis of variance
BCA	Biological control agent
BLAST	Basic Local Alignment Search Tool
CGIAR	Consultative Group for International Agricultural Research
CM	Complete medium
CMX	Complete medium xylose
DNA	Deoxyribonucleic acid
DSI	Disease Severity Index
EtBr	Ethidium bromide
FAMA	Federal Agricultural Marketing Authority
FAO	Food and Agricultural Organization of the United Nations
ITS	Internal transcribed spacer
ISTH	International Subcomission on *Trichoderma* and *Hypocrea* Taxonomy
KH_2PO_4	Potassium dihydrogen phosphate
MARDI	Malaysian Agricultural Research and Development Institute
$MgSO_4$	Magnesium sulphate
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PIRG	Percentage Inhibition Radial Growth
RBA	Rose Bengal Agar
TBE	Tris-Boric acid-EDTA
V	Volt

CHAPTER 1

INTRODUCTION

Banana is scientifically known as *Musa acuminate* that is the fourth most important food crop in developing world after rice, wheat, and maize in terms of gross value of production (Crop Trust, Germany, 2014). In Malaysia, banana is mainly cultivated in Johor, Pahang, and Sarawak (Tengku Ab. Malik, Rozieta, Maimun & Umikalsum, 2011). Most of the production are for domestic consumption and have been exported to Singapore, Indonesia, Brunei, Saudi Arabia and Hong Kong (Husain & William, 2011).

Banana plantation having decreasing in worldwide production after had being infected by considered as lethal fungal disease caused by the soil-borne fungus, *Fusarium oxysporum* f. sp. *cubense* (*Foc*) (Ploetz, 2006; Savary, Ficke, Aubertot & Hollier, 2012). Fusarium wilt disease of banana was firstly recorded in 1874 in Australia, where it was observed at Eagle Farm near Brisbane (Bancroft, 1876; Molina *et al.*, 2009). Later, it was introduced in Panama in 1890. The disease had spread widely to Costa Rica and subsequent outbreaks occurred in Suriname (1906), Cuba (1908), Trinidad (1909), Jamaica (1911), Honduras (1916) and Guatemala (1919) within a decade. The disease has since been reported from most all of banana-producing countries in globally (Molina *et al.*, 2009).

The term tropical race 4 (TR4) is used for highly virulence form of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) in Asia to distinguish between the fungal strains that readily cause Fusarium wilt on Cavendish-based banana industries. The strain associated with TR4 was identified in samples from Taiwan in 1967 (Molina *et al*., 2009). The vulnerability of Cavendish cultivars has been detected when newly established plantations were destroyed in Malaysia and Indonesia in the early 1990s (Buddenhagen, 2009). Since then TR4 has been found in the island of Borneo (in both the Malaysian and Indonesian parts of the island) and other Indonesian islands (Kalimantan, Sulawesi and Java). In Malaysia, firstly reported of this lethal disease was in 1992 spreading on banana plantation Nam Heng, Johor. An outbreak of the disease caused a lot of damage and lost (Lee, Teo & Ong, 1999). Based on this, the spreading was increasing throughout Peninsular Malaysia swiftly causes more lost and damage to banana industries.

This phenomenon gives a big impact to banana plantation because the soil-borne fungus difficult to be controlled as it will remain dormant in the soil for more than 30 years (FAOSTAT, 2017). Based on research done by Farquhar in 2012, it was about 5000 hectares of Cavendish plantations developed for export has been totally abandoned caused by this disease. However, about 800 hectares were replanted with resistant Cavendish somaclones from Taiwan. As a result,

less than 10% infection has been observed in these new plantings, which is good but not enough to sustain production for the international market (Molina, Fabregar, Sinohin, Yi, & Viljoen, 2009; Jamaluddin, 2012; FAOSTAT, 2017).

For disease management practices, two major strategies that have successfully used in controlling Fusarium wilt of banana are disease prevention and the use of resistant varieties (Gang *et al*., 2013). Previously, tissue culture plants and phytosanitary practices have been practiced to keep banana fields free from diseases (Gang *et al*., 2013). This option is available to large-scale commercial growers with the financial means to buy such plants, but this option cannot be afforded by small-scale and subsistence growers and they are forced to establish new banana fields by using suckers (the clump formed by the fruit-bearing parent plant), if they want to continue farming bananas (Jamaluddin, 2012). As a solution, they will use chemical pesticides abundantly to control the crops from being infected by disease pathogens (Pérez-Vicente & Dita, 2014).

Chemical pesticides that also known as fungicides are commonly used by small-scale banana growers for managing Fusarium wilt disease of banana. However, the uses of a chemical will lead to environmental disruptions such as ecosystem damaging, pollution to air, water and soils and climate changing (Kaewchai, Soytong & Hyde, 2009). The understanding of the disease protections and treatments for the crops are increasing broadly, as scientists have come with a few solutions by using the eco-friendly cure to the disease. One of the common is by introducing microorganisms that can act as a biological control against the pathogens.

Malaysia also listed as one of the countries that trying to find out new solution in progressive research on biological control to cure Fusarium wilt of banana. Abundance researches have done by using biological organisms as the role model. Based on a preliminary study was conducted by Teng (2016), endogenic earthworm, *Pontoscolex corethrurus*, can act as a potential biocontrol in remediating banana blood disease. Based on Jomduang and Sariah (1995), reported that *Trichoderma harzianum* and *Gliocladium virens* were effective antagonists against *Sclerotium rolfsii* as both can be colonized, penetrated, and sporulated inside the sclerotia of the test pathogen.

Trichoderma species have an ability to control plant pathogens and act as a biological control agent in substituting chemical pesticides. Based on Suhaida and Nur Ain Izzati (2013), the application of *Trichoderma harzianum* T73s has successfully inhibited Fusarium ear rot of maize.

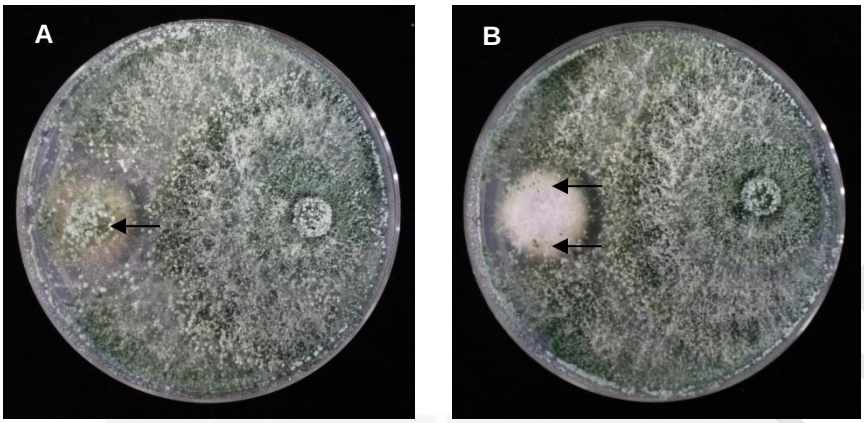

Figure 1.1: Antagonist effect of *Trichoderma harzianum* T73s after nine days of incubation; A: The mycelium of *Trichoderma harzianum* T73s fully covered and sporulated over *Fusarium verticillioides* P202c colony (arrow); B: The mycelium of *Trichoderma harzianum* T73s covered 33% of *Fusarium proliferatum* P202c colony (arrows)

Besides, *Trichoderma harzianum* FA1132 showed its potential in suppressing *Ganoderma boninense* that cause basal stem rot in oil palm (Nur Ain Izzati & Abdullah, 2008). In finding sustainable cure for the most devastating *Ganoderma* disease of oil palm in Malaysia, the result of application of single *Bacillus cereus* was found to be the most effective treatment in suppressing *Ganoderma* disease of oil palm followed by single applications of *Trichoderma harzianum* and mixture of both *T. harzianum* and *B. cereus* (Nusaibah, Saad & Hun, 2017).

In order to ensure an eco-friendly cure of the disease, this study was focusing on screening the biological control agent such as *Trichoderma* species against the pathogen. Most of the research done before were using *Trichoderma harzianum* as biocontrol agent in controlling many pathogens. However, based on this study, *T. asperellum* gave better result compared to *T. harzianum* in inhibiting the growth of *Fusarium oxysporum* f. sp. *cubense* in *in-vitro* condition, and successfully treating Fusarium wilt disease of banana under plant house condition. Perhaps this finding will provide basic information on disease management strategies in preventing the diseases to infect the new banana plants, enhance the plant growth and increasing the yields. The first objective of this study will fulfil the limitation of understanding in soil fungal diversity.

The objectives of this study are:

i. to isolate and identify *Trichoderma* species isolated from various soil samples using phenotypic and molecular characterization,
ii. to screen antagonistic *Trichoderma* species against the pathogen of Fusarium wilt disease under *in-vitro* condition, and
iii. to examine the efficacy of *Trichoderma asperellum* as a biocontrol agent of Fusarium wilt disease of banana under plant house condition.

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