CHARACTERIZATION OF Fusarium Species ASSOCIATED WITH WILT DISEASE OF CUCURBITS AND EFFECTIVENESS OF Trichoderma asperellum AS BIOLOGICAL ENHANCER

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By

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Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Chairman: Assoc. Prof. Nur Ain Izzati Mohd Zainudin, PhD
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Fusarium wilt disease caused by *Fusarium* species is a serious soilborne fungal disease that threatens the production of cucurbits in Malaysia. Despite multiple controls and measures, this economically important pathogenic genus continues intruding the crops. Therefore, this study intends to identify the pathogenicity of *Fusarium* species from five infected hosts namely cucumber, pumpkin, luffa, gourd and rock melon. Infected cucurbits fruit and leaf obtained from the fields were cultured onto pentachloronitrobenzene agar (PCNB). All isolated fungi were purified using a hyphal tip technique and sub-cultured onto potato dextrose agar (PDA) and carnation leaf agar (CLA) for observing morphological characteristics such as macroconidia, microconidia, formation of chlamydospore, colony features and pigmentation. Ninety-four isolates were successfully recovered from 12 locations throughout Peninsular Malaysia. Isolated fungi were also identified molecularly using translation elongation factor 1-alpha (*tef1α*) and beta-tubulin (BT) genes sequences analysis. From the findings, the highest number of identified species were *F. incarnatum* (52 isolates) followed by *F. solani* (18 isolates), *F. proliferatum* (17 isolates), *F. oxysporum* (6 isolates) and *F. longipes* (1 isolate). A phylogenetic tree was generated from a combination of *tef1α* and *ß-Tub* genes sequences. To ascertain the virulence of *Fusarium* species, all isolates were tested for pathogenicity test by soaking the seeds into $2x10^6$ conidia/mL of conidial suspension for 12 hours. Sterile distilled water served as control. *Fusarium solani* isolates M1799C and M1800C recorded the highest disease severity of 100% on cucumber, *F. solani* (C2526P) recorded 93.8% disease severity on pumpkin, *F. solani* (D2499L) and *F. proliferatum* (B1777L) recorded 66.75% disease severity on luffa and *F. oxysporum* (B2547M) recorded 23% disease severity on rock melon. However, no symptoms observed on inoculated gourd plants. In order to eco-friendly control the disease and understand the mechanisms; therefore, *Trichoderma asperellum* B1902 was testified as a potential plant biological enhancer in wilt-infected cucumber. Efficacy of *T. asperellum* B1902 was testified by infesting 100 mL/kg inoculum (with concentration 2x10^6 conidia/mL) onto soil containing infected cucumber plants. After 30 days post-inoculation (dpi), *T. asperellum* B1902-treated plants increased length of stem, area of total leaves and number of leaves. Furthermore, *T. asperellum* B1902 also gave rise to the efficiency of photosystem II (PSII) and enhanced the photosynthetic performance of cucumber plants. The information channeled from this study leads a foundational knowledge for better management in controlling Fusarium wilt infection.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENCIRIAN species *Fusarium* BERKAIT DENGAN PENYAKIT LAYU PADA CUCURBIT DAN KEBERKESANAN *Trichoderma asperellum* SEBAGAI PENGGALAK BIOLOGIKAL

Oleh

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Penyakit layu *Fusarium* disebabkan oleh *Fusarium* species adalah penyakit kulat bawaan tanah yang serius serta mengancam pengeluaran *cucurbit* di Malaysia. Walaupun pelbagai kawalan dan langkah dijalankan, genus patogenik yang mempunyai kepentingan ekonomi ini tetap menyerang tanaman *cucurbit*. Oleh itu, kajian ini bertujuan untuk menentukan kepatogenan spesies *Fusarium* daripada lima tanaman iaitu timun, labu, petola, labu air dan tembikai wangi. Buah dan daun *cucurbit* yang dijangkiti diperolehi dari lapangan dikultur di atas agar pentachloronitrobenzene (PCNB). Semua pencilan kulat ditulenkan melalui teknik hujung hifa dan sub-kultur pada media agar kentang dekstrosa (PDA) dan agar daun *carnation* (CLA) bagi pencirian morfologi seperti makrokonidia, mikrokonidia, pembentukan klamidospora, corak koloni dan pigmentasi. Sembilan puluh empat pencilan berjaya diperoleh daripada 12 lokasi seluruh Semenanjung Malaysia. Pencilan kulat juga dikenalpasti secara molekular menggunakan analisis jujukan gen *translation elongation factor 1-alpha* (*tef1α*) dan *beta-tubulin*. Daripada penemuan ini, spesis tertinggi dikenalpasti adalah *F. incarnatum* (52 pencilan), diikuti dengan *F. solani* (18 pencilan), *F. proliferatum* (17 pencilan), *F. oxysporum* (6 pencilan) dan *F. longipes* (1 pencilan). Filogenetik dendogram telah dihasilkan daripada kombinasi jujukan gen *tef1α* dan *ß-Tub*. Bagi menentukan kevirulenan spesis *Fusarium*, kesemua pencilan diuji dengan ujian kepatogenan dengan merendam biji benih ke dalam ampaian konidia dengan kepekatan $2x10^6$ conidia/mL selama 12 jam. Air suling steril digunakan sebagai kawalan. *Fusarium solani* pencilan M1799C dan M1800C mencatatkan keparahan penyakit tertinggi iaitu 100% pada timun, *F. solani* (C2526P) mencatat 93.8% keparahan penyakit pada labu, *F. solani* (D2499L) dan *F. proliferatum* (B1777L) mencatat 66.75% keparahan penyakit pada petola dan *F. oxysporum* (B2547M) mencatat 23% keparahan penyakit pada tembikai wangi. Namun, tiada simptom diperhatikan pada labu air yang diinokulasi. Untuk mengawal penyakit ini secara mesra-ekonomi dan memahami mekanisme tersebut; maka *Trichoderma asperellum* B1902 telah diuji sebagai penggalak biologikal tumbuhan yang berpotensi terhadap timun layu

terinfeksi. Keberkesanan *T. asperellum* B1902 diuji dengan infestasi 100mL/kg inokulum (pada kepekatan $2x10^6$ conidia/mL) ke dalam tanah berisi pokok timun terinfeksi. Setelah 30 hari selepas inokulasi (hsi), panjang tangkai, luas keseluruhan dan bilangan daun meningkat pada pokok timun dirawat dengan *T. asperellum* B1902. Tambahan lagi, *T. asperellum* B1902 juga meningkatkan kecekapan fotosistem II (PSII) dan menggalakkan prestasi fotosintetik pokok timun. Informasi yang disalurkan daripada kajian ini menyumbang pengetahuan berkenaan pengurusan dalam pengawalan penyakit layu Fusarium.

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LIST OF ABBREVIATIONS

%	Percentage
^0C	Degree Celsius
µg $cm^{-2}s^{-1}$	Microgram per centimeter square per second
µL	Microliter
ΦPSII	Relative quantum efficiency of PSII
x g	Relative centrifugal/gravitational force
ABA	Abscisic acid
ANOVA	Analysis of variance
BC	Before century
bp	Base pair
CH_3CN	Acetonitrile
CHOOH	Formic acid
CLA	Carnation leave agar
cm	Centimeter
cm^2	Centimeter square
CRD	Complete randomized design
DNA	Deoxyribonucleic acid
dpi	Day post inoculation
DSI	Disease severity index
Foc	Fusarium oxysporum f. sp. cucumerinum
Fm	Maximum fluorescence
Fv	Variable fluorescence
Fv/fm	Maximum efficiency of photosystem II
Fv/fo	Maximum yield of photosystem II
Fo	Minimal fluorescence
f. sp.	Forma speciales
g	Gram
ha	Hectare
H_2O	Water
hr	Hour
JA	Jasmonic acid
KCl	Potassium chloride
kg	Kilogram
LHCs	Light harvesting complexes
nm	Nano meter
Min	Minute
mg	miligram
mL	Milliliter
mm	milimeter
mM	Milimolar
Mt	Million tonne
O_2	Oxygen
P	Probability
PCNB	Pentachloronitrobenzene agar
PDA	Potato dextrose agar
PDB	Potato dextrose broth

PFD	Photon flux density
PGPF	Plant growth promoting fungi
PI	Performance index
PSII	Photosystem II
RC/ABS	density of reaction centres per PSII antenna chlorophyll
ROS	Reactive oxygen species
s	Second
SA	Salicylic acid
scm^{-1}	Second per centimeter
SD	Standard deviation
TBE	Tris-Borate-EDTA buffer
UFLC	Ultra-fast liquid chromatography
WA	Water agar

CHAPTER 1

INTRODUCTION

1.1 Background of study

Agriculture provides people with food, where it contributes 7.5% total output of Malaysia economy and making it the fourth highest sector in the country. Malaysia recorded 2.9%, the lowest agriculture growth rate in Southeast Asia together with Thailand and the Philippines (Birthal, Joshi, Roy and Pandey, 2019). As an agricultural-based economy country, it is important to look profoundly into any factors that could enhance productivity. United Nation Food and Agriculture Organization (FAO, 2012), reported that the demand for agricultural products is expecting to be up to 1.1% every year until 2050. On the other hand, knowledge is the key to manage good agriculture. Lack of specialized information could lead to an abandonment of farming and inappropriate agriculture and subsequently affect the biodiversity (Ruxandra and Dacinia, 2010).

The consumerism of crops belong to Cucurbitaceae family is increasing together with the world populations. Among all cucurbits, melon is the most popular crop in the family, followed by cucumber, squash, and pumpkin (Bisognin, 2002). Since the past decades, outnumber of studies revealed the advantages of the crops. This soft-vined plant growing by means of crawling on any surfaces supplies multiple utilizability and essentials. The seed especially has a high source of lipid, and proteins (Mladenovic, Berenji, Ognjanov, Ljubojevic and Cuanovic, 2012) frequently being processed to be used as organic cooking oil (McCreight, Staub, Wehner and Dhillon, 2013). Pharmaceutically, the leave, flower and fruit provide essentials in reducing human health risks such as treatment of hemorrhages in the internal organs, epilepsy, nerve disease, high antioxidants and anti-inflammatory properties. The high contents of β-carotene also can lower the risk of heart attack and cancer (Gabriele, Alberto, Sergio, Fernanda and Marco, 2000; Ismail, Chan, Mariod and Ismail, 2010; Avinash and RavishankarRai, 2013).

In Malaysia, cucumber is the leading crop being produce among any other cucurbits cultivated throughout the country. According to Ministry of Agriculture Malaysia (MOA, 2018), the production of cucumber in 2018 was up to 94, 520 Mt followed by pumpkin 25, 290 Mt, luffa 18, 782 Mt, gourd 5, 413 Mt and rockmelon 3,928.4 Mt. From all these, Peninsular Malaysia produces more hectareage and harvested areas comparing to Sabah and Sarawak. As cucurbits are originally from Western Asia (Deyo and O'Malley, 2008) and essentially a group of tropical plants (Pessarakli, 2016), the cultivation of the crop is compatible in almost every state in Malaysia. The cultivation condition favors to tropical and subtropical regions (Norrizah, Hashim, Siti Fasiha and Yaseer, 2012).

However, several factors such as Phytium crown and root rot, powdery mildew and stem blight (Punja, Tirajoh, Collyer and Ni, 2019; Ishii, Fujiwara and Nishimura,

2018) were found to be contributed and destroying the cucurbit plantations. On top of these factors is a fungal infection caused by *Fusarium* species. Among all plant pathogens, fungi and fungi-like organisms contribute more plant diseases compared to any other group of plant pests (Ellis, Boehm and Mitchell, 2008). *Fusarium oxysporum* was listed as the fifth out of the ten most important plant pathogens in the world (Sharma *et al.,* 2016). Among all the diseases, Fusarium wilt has the highest record of infecting most of the economically important crops. It is one of the oldest described diseases and the most economically important disease of many crops worldwide. It occurs in every continent except Antarctica and new races of the pathogen continue to impact production in many areas around the world. Long-term survival of the pathogen in the soil and the evaluation of new races make the management of Fusarium wilt difficult. The fungi enter through the roots and interfere with the water vessels of the plant. As the infection spreads up into the stems and leaves, it restricts water flow causing the foliage to wilt and yellow (Egel and Martyn, 2007).

Fusarium wilt causes various economic losses. It causes an obvious and direct loss in yield because the plant dies and subsequently brings loss to marketable yields (i.e. fruits that is low quality and cannot be sold because they are too small, misshapen, and low in sugar, cracked and sunburned). About $253 million loss in Malaysia due to *F. oxysporum* tropical race 4 (TR4) invasions on banana (Ordonez *et al*., 2015). Fusarium wilt has also been a vast impact on the agriculture production of fruits and vegetables belong to the Cucurbitaceae family. Indirectly, these impacts increase the agricultural waste disposed into landfills annually by up to 1.2 million tonnes (Agamuthu, Hidzir and Hamid, 2009). Alternatively, several efforts and researchers had trying to overcome this disease invasion. This causative pathogen is a soil-borne fungus and chemical application could not control the disease entirely as it has possible secondary threat on the environment, human and animals (Minuto, Spadaro, Garibaldi and Gullino, 2006). Performing crop rotation, soil solarization, chemical fungicide application and fumigation (Tamietti and Valentino, 2006; Arguelles-Arias, Brans, Joris, and Fickers, 2009; Wang *et al*., 2013; Ghadikolaei, Cheung and Yung, 2019) which were strictly emphasized previously could not be succeeded as the conidia of the fungus could persistent and survive in the soil for a long period, in critical conditions and also compatible in a wide host range (Cao *et al.,* 2011).

Thus, current information on Fusarium wilt infection is still unable to well manage to control the disease on cucurbits. Although many studies implied various information and alternative on how to reduce Fusarium wilt infection. But still, the infection pervasive persistently in the crop's soil. In fact, most of the studies have not targeted and aimed at the plant defense system as alternative measures to reduce this infection.

The phenomena and behavioral of *Fusarium* species isolated from cucurbits offer a broad significant to many aspects such as virulence, host specificity, toxin production, and functional characteristics. Hence, this study theorized that not all species isolated from infected cucurbits is the causal pathogen that is responsible for Fusarium wilt infection. Hypothetically, *Fusarium oxysporum*, *F. solani* and *F. proliferatum* are dominantly pathogenic and highly virulent than any other species of the genus that infecting the plant vascular system. The same *Fusarium* species possess different pathogenicity, provided equal environmental condition and nutrient. As a resolution to the Fusarium wilt infection in cucurbits, *Trichoderma* spp. applied as a bio-enhancer onto infected cucumber plants revealed the enhanced photosynthetic performances.

This research study proposes the explanation and discussion on understanding and characterizing the *Fusarium* species. Apart from that, the information on genetic diversity and how the species evolution runs proportionally to time are relatively affected. This study also provides the mechanism and sequential process on how the pathogenesis occurs presenting distinct virulence. Finally, through the advanced technologies on plant physiology, the induction of bio-enhance in cucurbits plants in response to *Fusarium* infection was discovered by analyzing the photosynthetic measurements of cucurbit plants by the application of *T. asperellum*.

Therefore, following with the same mission, this study generally provides updated and additional information related to *Fusarium* species. From the first objective of the study, the findings generally introduced the very basic of all organisms by delivering the picture and visualize how diverse the fungal species associated with cucurbits. The rationale behind this objective is that morphological features are mutually related to a particular function and reactions of the fungus. *"It's one thing to make a picture of what a thing looks like, it's another thing to make a picture of what they are",* quoted by well-known American photographer, Paul Caponigro. Visualize the *Fusarium* characteristics explains us more about the organism. Besides that, molecular identification provides valid and genuine identification of *Fusarium* as it goes directly from DNA, the hereditary materials, instructions, and information of this organism.

The study went deeper into the potential and efficiency of *Fusarium* as a plant pathogen. The determination of *Fusarium* pathogenicity conveys the species virulence. A number of notions reported the pathogenicity of *Fusarium* on cucurbits as a saprophyte, opportunist and parasite. Hence, the pathogenicity test resolves their degree of severity made on the hosts. Over the years, the study on *Trichoderma* as a biocontrol agent progressively being held due to its efficacy to suppress and inhibit the pathogen growth on hosts. This study conveys the application of *T. asperellum* onto infected cucumber plants to enhance the plant growth, defense and induction of phytohormone. This enhancement subsequently affects the photosynthetic efficiency of treated cucumber plants through the analysis of photosystem II efficiency.

1.2 Research objectives

This study was conducted in order to manage a better cultivation of cucurbits; the species diversity and virulence of *Fusarium* species as the pathogen are some of the major factors to be testifying. This study is important in contributing to much further information related to *Fusarium* and understanding plant-pathogen-biocontrol agent interaction. Therefore, the main objectives of this study are:

i. To identify *Fusarium* species associated with wilt disease of cucurbits based on morphological characteristics and molecular approaches.
ii. To ascertain the pathogenicity of *Fusarium* species isolated from bottle gourd, cucumber, luffa, pumpkin and rock melon infected samples.
iii. To examine the biological changes in Fusarium wilt-infected cucumber plants treated with *Trichoderma asperellum*.

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