ISOLATION AND CHARACTERIZATION OF ENTOMOPATHOGENIC FUNGI FROM SELECTED RECREATIONAL FORESTS IN THE KINTA REGION, PERAK

By

CHONG ZHENG YANG

A project report submitted to the Department of Agricultural and Food Science
Faculty of Science
Universiti Tunku Abdul Rahman
in partial fulfillment of the requirements for the degree of
Bachelor of Science (Honours) Agricultural Science
September 2023

ABSTRACT

ISOLATION AND CHARACTERIZATION OF ENTOMOPATHOGENIC FUNGI FROM SELECTED RECREATIONAL FORESTS IN THE KINTA REGION, PERAK

CHONG ZHENG YANG

Entomopathogenic fungi (EPF) are a class of fungi that are pathogenic to insects and they play an essential role as biocontrol agents for agricultural pests. The presence of indigenous EPFs from recreational forests in the Kinta Region, Perak has not been documented. The aim of this study is to bioprospect indigenous EPF from recreational forests in the Kinta region, Perak and to evaluate the pathogenicity of the indigenous EPF isolates. EPF were isolated from three recreational forests in the Kinta region using *Tenebrio molitor* as the insect bait. The isolated EPF were characterized based on morphological characteristics and molecular identification. The morphological keys of each isolate were verified with published taxonomic keys by Humber (2012), Samson (1988) and other publications. A total of 7 fungal isolates were obtained from this study. Isolate SK1(a)I1 and SK3(a)I1 were identified as *Metarhizium anisopliae*. The conidia of M. *anisopliae* are long and cylindrical shaped, central slight narrowing forming a very long and laterally adherent chain with the length ≤ 9 µm long. The conidial size for both isolates were 6.10 ± 0.67 × 2.24 ± 0.38 µm (SK1(a)I1) and 5.67 ± 0.54 × 2.10 ± 0.21 µm (SK3(a)I1). Other isolates were identified as *Clonostachys rogersoniana* and *Trichoderma spirale* and

Trichoderma virens. PCR was performed to identify the isolates and edited sequences were subjected to BLAST homology search. Molecular characterization revealed that the 7 isolates were *M. anisopliae*, *C. rogersoniana*, *T. spirale*, and *T. virens*. The pathogenicity of both *Metarhizium* isolates were evaluated and the mortality rate of *T. molitor* was 100% and 83.33% after 14 days of inoculation, while other isolates showed no mortality from pathogenicity evaluation. In short, different genera of EPFs were identified and characterized from recreational forests in Perak. However, further study of the pathogenicity of *C. rogersoniana* should be evaluated. The presence of EPF of recreational forests from other regions in Perak are required to be investigated in the future.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my utmost gratitude to my supervisor, Dr. Clement Wong Kiing Fook for his guidance and patience throughout the entire process in completing my final year project. This project would not be completed without his guidance and expertise.

Next, I would also like to express my gratitude to my family for giving me encouragement throughout this project. Nonetheless, I would like to thank UTAR lab officers, who always give assistance and guidance whenever I seek for help.

Lastly, I would like to thank my friends for their support and encouragement throughout the period in completing this project.

DECLARATION

I hereby declare that this final year project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

(CHONG ZHENG YANG)

APPROVAL SHEET

This project entitled "**ISOLATION AND CHARACTERIZATION OF ENTOMOPATHOGENIC FUNGI FROM SELECTED RECREATIONAL FORESTS IN THE KINTA REGION, PERAK**" was prepared by CHONG ZHENG YANG and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Agricultural Science at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Dr. Clement Wong Kiing Fook) Date: 1/9/2022

Supervisor

Department of Agricultural and Food Science

Faculty of Science

Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: 1/9/2023

PERMISSION SHEET

I, **CHONG ZHENG YANG** (ID No: **19ADB03982**) hereby certify that I have completed the final year project titled **"ISOLATION AND CHARACTERIZATION OF ENTOMOPATHOGENIC FUNGI FROM SELECTED RECREATIONAL FORESTS IN THE KINTA REGION, PERAK"** under the supervision of Dr Clement Wong Kiing Fook (Supervisor) from the Department of Agricultural and Food Science, Faculty of Science.

I understand that the University may upload the softcopy of my final year project in PDF to the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

_____

(CHONG ZHENG YANG)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis Of Variance
BB	Batu Berangkai, Kampar
BLAST	Basic Local Alignment Search Tool
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic Acid
EPF	Entomopathogenic fungi
HCl	Hydrochloric Acid
ITS	Internal Transcribed Spacer
kb	kilobase
KOH	Potassium Hydroxide
M	Molality
MEGA	Molecular Evolutionary Genetics Analysis
mg	Miligram
$MgCl_2$	Magnesium Chloride
Mins	Minute
mL	Mililitre
nM	Nanomolar
NCBI	National Center for Biotechnology Information
nM	Nanomolar
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
RNA	Ribonucleic Acid

rRNA	Ribosomal Ribonucleic Acid
rpm	Revolutions per minute
s	Second
sp.	Species (Singular)
spp.	Species (Plural)
SS	Hutan Lipur Sungai Salu, Jeram
SK	Kledang Saiong Forest Eco Park, Meru
TBE	Tris/ Borate/ Ethylenediaminetetraacetic Acid
V	Volume
v/v	Volume per volume
w/v	Weight per volume
µM	Micromolar
µL	Microlitre
°C	Celcius

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Entomopathogenic fungi (EPF) are a group of fungi that are pathogenic to insects. It is widely known as a biological control agent to control agricultural pests. According to recent studies on EPF, there are approximately 1000 species and 900 genera of fungi identified to be pathogenic to insects (St. Leger and Wang, 2010). For example, EPF were use as biocontrol agents against whiteflies, such *as Metarhizium anisopliae*, *Beauveria bassiana*, and *Verticillium lecanii* (Malsam, et al., 2002; Javed, et al., 2019, Gebremariam, Chekol and Assefa, 2022). Several reviews showed the common EPF genera discovered in Malaysia were discovered such as *Metarhizium, Beauveria, Aspergillus, Isaria, Hirsutella, Lecanicillium, Paecilimyces*, *Fusarium* (Bamisile, et al., 2021; Wakil, Yasin and Shapiro-Ilan, 2017) EPFs complete their life cycle by spreading their conidia on the cuticle of the insects. As the conidia germinate, the fungus proliferates throughout the body of insects by penetrating the cuticle of arthropods with penetration hypha. The fungus eventually formed a mass of mycelia and spread through the external cuticle of insects (Jaronski, 2023).

Bioprospecting refers to the search for bioresources such as plants, microbes, and animals that are beneficial to human society and commercialization (Oyemitan, 2017). Bioprospecting was widely done in several industries such as the pharmaceutical industry, manufacturing, engineering, construction, and agriculture (Beattie, et al., 2011). Bioprospecting of EPFs is done in this study to develop new sources of EPF as biocontrol agents, and to discover indigenous EPF species that could readily establish themselves in the local environment (Pathan, Patil and Deshpande, 2019). Recreational forests are one of the good sources for EPFs as they can be found naturally in the soils of natural forests which are free from agricultural inputs such as chemical fertilizers and pesticides (Elham, et al., 2018). Besides that, recreational forests consist of secondary forest which has not been widely developed or intervened by human activities such as urbanization or agricultural activities.

EPF applications are well known as one of the sustainable pest control measures. According to Ambethgar (2009), there are above 600 species of phytophagous insect pests developed resistance against synthetic insecticides. For example, Diamondback moth (*Plutella xylostella*) has developed multiple insecticide resistances in Cameron Highlands, Malaysia due to the frequent application of insecticides on selective crops (Ooi, 2015). Hence, EPFs could be an alternative to control these insecticide-resistance pests (Orozco, et al., 2016). Besides that, Ambethgar (2009) also suggested that EPFs can induce the susceptibility of insecticides in target pests when associated with fungal infection. This will help to

minimize human health issues and environmental impact caused by mass insecticide application. Other than that, a recent study showed that EPFs play important ecological roles in promoting plant growth by providing nitrogen to plants during the parasitization of insects (Behie and Bidochka, 2014). They also protect plants from microbial pathogens by suppressing disease-causing agents and increasing plant defense responses (Klieber and Reineke, 2016).

1.2 Problem statements

Although there are many EPF isolation studies throughout Malaysia from different locations such as oil palm plantation in Terengganu, Gomantong Cave in Sabah and Teluk Intan in Perak (Grace, et al., 2017; Kin, et al., 2017; Wasti, et al., 2018). However, up to date, there is only one study conducted in a recreational forest found in Hutan Melintang, Perak (Jessica, et al., 2018). There is no study of the presence of indigenous EPF isolation from recreational forests conducted in the Kinta Region, Perak so far, and the pathogenicity of three indigenous EPFs has not been determined.

1.3 Objectives

Hence this study aims:

1. To isolate EPF from three recreational forests located in the Kinta Region using mealworms baiting method.

2. To identify EPF isolates using morphological and molecular methods.

3. To evaluate the pathogenicity of EPF isolates against mealworms.

CHAPTER II

LITERATURE REVIEW

2.1 Classification of EPF

EPFs are phylogenetically diverse as they can be found in several phyla in the kingdom of Fungi, such as Zygomycota, Ascomycota, Deuteromycota, Oomycota, and Chytridiomycota (Shah and Pell, 2003). Most of the EPF genera are found in the class Entomophthorales from Zygomycota and Hyphomycetes in the Deuteromycota (Shah and Pell, 2003). Humber (2012) proposed a new phylum, Entomophthoromycota, formerly known as one of the phylogenetic lineages of Zygomycota, which was introduced to reclassify the entomophthoroid fungi (Entomophthorales) based on the phylogenetic studies on EPF species. For example, Entomophthorales which was previously an order in the class Zygomycota, is now under the class Entomophthoromycota, named after the genus Entomophthora (Humber, 2012). In other words, the taxonomic classifications of EPF still remain unclear and further research is needed to standardize the EPF nomenclature.

2.2 Diversity of EPF in Malaysia

2.2.1 EPF Genera and Species Found in Malaysia

Different EPF genera and species were discovered in different regions and locations in Malaysia. The discoveries of EPF in Malaysia were summarized in Table 2.1.

Table 2.1: Indigenous EPF found in Malaysia.

Findings	Location	Source
Paecilomyces carneus and *P. farinosus* were isolated from oil palm soil.	MPOB Oil palm plantation, Keratong	(Bakeri, et al., 2009)
I. amoenerosa and *M. anisopliae* were isolated from mineral soil and pest soils.	MPOB Research Station Hulu Paka in Terengganu MPOB Research Station Teluk Intan in Perak.	(Kin, et al, 2017)
Six unknown EPF isolated from red palm weevil (*Rhynchophorus ferrugineus*).	FELDA oil palm plantations and sandy coastal area in Terengganu.	(Grace, et al., 2017)

Table 2.1: Indigenous EPF found in Malaysia (continue)

Findings	Location	Source
Twenty-three species EPF were isolated and identified consisting species from the genera *Aspergilus spp*., *Penicillum spp*. and *Fusarium spp*.	Limestone cave in Gomantong Cave, Sabah.	(Wasti, et al., 2018)
Total 7 isolates of *M. anisopliae* were isolated from BRIS (Beach Ridges Interspersed with Swales) and coastal area.	Island of Taman Tropika Kenyir and BRIS soil of Marang, Terengganu.	(Elham, et al., 2018)
Eleven isolates of of *Isaria fumosorosea* from Hutan Melintang were isolated using *Coptotermes curvignathus* and *C. gestroi* as insect baits.	Recreational forest in Hutan Melintang, Perak.	(Jessica, et al., 2018)
Seven *M. anisopliae* and 2 *P. lilacinum* isolates were isolated and identified.	Oil palm plantation in UPM (Universiti Putra Malaysia).	(Sani, et al., 2022)

Based on the findings shown in Table 2.1, different genera of EPF including *Aspergillus*, *Isaria*, *Metarhizium*, *Beauveria*, *Parcilomyces* and *Penicilium* were isolated and identified in from the coastal area, oil palm plantation, cave and recreational forest throughout Malaysia, which indicates Malaysia is rich in EPF diversity in various locations.

2.2.2 Recreational Forest as the source of EPF

Recreational forests could be a good source for EPF bioprospecting. Besides the example by Jessica, et al, 2017 as shown in Table 1, there are studies showing that EPF was isolated from secondary forests from different countries and regions.

Table 2.2: Isolated EPF from secondary forests throughout different countries.

Findings	Locations	Source
Cordyceps tenuipes was isolated from lepidopteran larvae and pupae.	Forest and butterfly gardens in Quindío, Columbia	(Castillo, et al., 2018)

Table 2.2: Isolated EPF from secondary forests throughout different countries (continue).

Findings	Locations	Source
Batkoa major isolates was isolated.	Hardwood forest in Ithaca, New York.	(Gryganskyi, Goan ad Hajek, 2022)
Six genera EPF (*Beauveria* sp., *Metarhizium* sp., *Fusarium* sp., *Aspergillus* sp., *Trichoderma* sp. and *Ventricillium* sp.) were isolated and identified from forest soils.	Forest from Eastern Ghats of South Indian.	(Vivekanandhan, Bedini and Shivakumar, 2020)
Twenty-nine strains of *Beauveria* spp. and 2 strains of *Paecilomyces* spp. were isolated and identified using *Thecodioplosis japonensis* as bait.	Forest soil in South Korea.	(Seo et al., 1995)
Five EPF genera (*Beauveria* spp., *Metarhizium* spp., *Purpureocillium* spp., *Lecanicillium* spp. and *Paecilomyces* spp.) were identified.	Forest soil in Croatia.	(Kovač, M., Tkaczuk, C. and Pernek, M., 2021)

Table 2.2: Isolated EPF from secondary forests throughout different countries (continue).

Findings	Locations	Source
Pasrairia alba, P. arcta, P. rosea were isolated from the forest soil using *Leptoptera* sp. and *Coleoptera* sp.	Forest soil in China and Thailand.	(Wei, et al., 2021)
B. bassiana and *M. anisopliae* were isolated from Rio de Janeiro and Atlantic Rain Forest.	Forest soils in Rio de Janeiro, Brazil	(Esparza M, Costa Rouws and Fraga, 2016)

Based on Table 2, diversity of EPF are abundant in the forests all over the globe. Different species from different fungal genera such as *Cordyceps*, *Batkoa*, *Metarhizium*, *Fusarium*, *Aspergillus*, *Trichoderma*, *Beauveria*, *Purpureocillium*, *Lecanicillium*, *Paecilomyces* and *Pasrairia* spp. were isolated from different insect hosts across different countries.For Malaysia wise, there is a study reported by Jessica, et al. (2018), EPF were isolated from a recreational forest in Hutan Melintan, Perak. Hence, we can hypothesize that recreational forests in Malaysia are also a potential EPF source as biocontrol agents for agricultural pests.

2.2.3 Life cycle of EPF

EPF can infect many orders of insects in all developmental stages, including egg, larvae, pupae or adult stage (Araújo and Hughes, 2016). Generally, EPF attacks insects and proliferates through several stages, starting from adhesion to germination, followed by hemocoel penetration and hemocoel replication (Mora, Castilho, Fraga, 2017). Firstly, the conidia of pathogenic fungi adhere to the cadaver of susceptible host. The conidia germinate and penetrate through the cuticle of the insects by the formation of appressorium and penetration peg (Shahid, et al., 2012; Singh, Raina and Singh, 2017). The fungal appressoria grow within the hemocoel and tissues of the insect to disrupt the defense mechanism of the insect host. EPF then continues to proliferate within the host while producing conidia at the same time (Vega, et al., 2012). EPF completes their life cycle by forming a mass of mycelia and spreading their conidia the exterior part of the on insect cuticle (Jaronski, 2023). Cadavers of fungal-infected insects will be mummified as the water content were drawn from the cadaver covered with dense mycelia (mycosis) (Evans and Hywel-Jones, 1997; Jaronski and Mascarin, 2016; Mathulwe, et al., 2017).

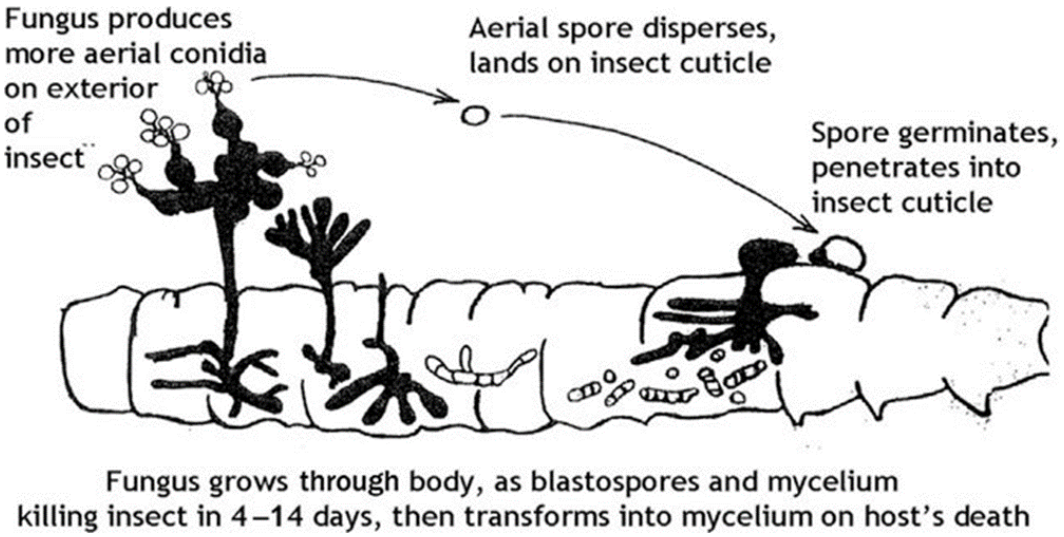

Figure 2.1: Life cycle of EPF.
(Image adapted form Jaronski and Mascarin, 2016)

2.3 Application of EPF as biocontrol agents

The application of EPF as a biocontrol agent in agricultural practice is known as one of the environmentally friendly measures as it can potentially reduce the use of synthetic chemical for agricultural pest control (Lacey, et al., 2001). There are about 170 strains of EPF that were commercialized as mycopesticides as they can cause high mortality rate on targeted insect pests (Bamisile, et al., 2021). According to Ambethgar (2009), the combined use of insecticides with selected insecticide-tolerant EPF can improve the efficacy for controlling pests, minimize environmental impact and delay resistance in insect pests. EPF only target specific insect species and it is safe for nontarget organisms, which helps to reduce the pesticide residue in the natural environment, and the biodiversity will not be disturbed by the application of EPF (Lacey, et al, 2001). Besides that, recent studies

showed that EPF can act as endophytes which help in plant growth and host plant tolerance against pathogens (Shahid, et al., 2012; Mantzoukas, et al., 2022). Several EPF has been reported showing plant growth promoting properties and antagonistic properties to phytopathogens in preventing pathogens attacking the crops (Barelli, Moreira and Bidachka, 2018).

For example, *B. bassiana* and *M. anisopliae* were reported to cause a mortality rate nearly 90% against all stages of fruitfly (*Bactrocera zonata*) (Murtaza, et al., 2022). Besides that, these two EPF were also reported to affect the growth, adult emergence, and weight of red palm weevil (*R. ferrugineus*). According to Pont and colleagues (2017), *M. anisopliae* showed high infecting compatibility (susceptibility) against subterranean termites (*Coptotermes curvignathus*). For instance, the application of *B. bassiana, T. gamsii* and *M. brunneum* also showed significant decrease of green peach aphid (*Myzus persicae*) population on pepper plants (Mantzoukas, et al. 2018).

2.4 Isolation and characterization of EPF

2.4.1 Isolation of EPF from insect bait

There are two species of insect larvae commonly used as insect bait for EPF isolation namely mealworm (*T. molitor*) and greater wax moth (*Galleria mellonella*). Both methods can be use to isolate common EPF such as *Metarhizium* spp.and *Beauveria* spp. (Sharma et al., 2018). The **"**Galleria-bait method"

introduced by Zimmermann in 1986, referred to greater wax moth, was used as a standard insect bait for EPF isolation (Senthilkumar, Amaresan and Sankaranarayanan, 2021). Mealworms is the alternative insect bait for EPF isolation as mealworms are highly susceptible to several EPF genera such as *Metarhizium sp., Clonostachys* spp*., Beauveria* spp*., Fusarium* spp*, Purpureocillium* spp. (Chang, et al., 2021, Sani, et al. 2022). Besides that, Masoudi and colleagues (2020) conducted EPF isolation on forest soils and grassland from Hebei and Sichuan province, has successfully isolated three major EPF families (*Clavicipitaceae*, *Cordycipitaceae* and *Ophiocordycipitaceae*), which including EPF genera from *Metarhizium* spp., *Isaria* spp. and *Beauveria* spp using mealworm as bait. Besides using insect bait to isolate EPF from soil, soil dilution method is an alternative to isolate EPF by spreading diluted soil suspension on various selective agar plates (Angelona and Biodochka, 2018).

2.4.2 Morphological Characterization of EPF

The morphology of fungi can be investigated macroscopically (colony morphology such as color, shape, size and hyphae) and microscopically under a light microscope. Microscopic investigation of fungi can be differentiate through distinct structures such as conidial length and shape, mycelia, phialide and conidiophores (Alsohaili and Bani-Hasan, 2018; Sani, et al., 2022). However, there are limitations if relying on morphological identification at species level due to several reasons such as inaccurate morphological characteristics groupings within an evolutionary framework which is still a challenging task, even for experienced mycologists (Raja,

et al., 2017). Besides that, Raja et al. (2017) mentioned that there are limited number of morphological descriptions for fungal identification that produce both asexual and sexual states that show morphological plasticity characters, making the morphological identification become more difficult (Raja, et al., 2017).

2.4.3 Molecular Identification of EPF

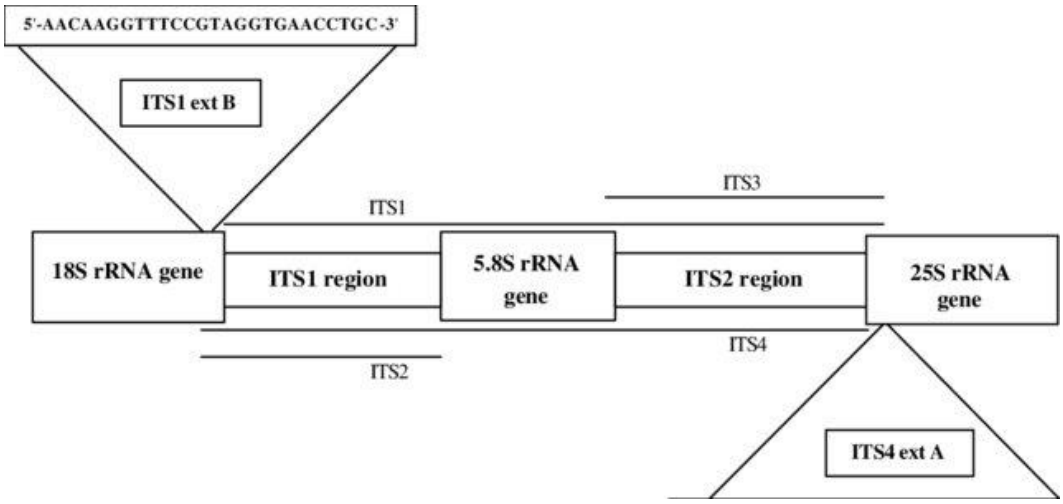

Figure 2.2: Location of the primers ITS1 and ITS4 for nuclear ribosomal DNA amplification in ITS region and 5.8S rRNA gene.

(Source:Yadav, 2008)

ITS primers are the highly promising PCR primers for fungal molecular identification in the preliminary of fungal molecular identification method, which amplify 5.8S ITS region of diverse fungi species including Dikaryomycota, a division of Ascomycota and Basidiomycota (White et al., 1990; Martin and Rygiewicz, 2005). ITS1 and ITS4 primers were widely used in fungal identification as the primer sets were to to amplified a diverse of fungal species due to high

interspecific variation compared to ITS1 and ITS2 sequences (Martin and Rygiewicz, 2005).

2.5 Pathogenicity evaluation of EPF

Generally, conidial suspension of EPF will be prepared with specific conidial concentrations. The insects will be inoculated by fungal conidia through dipping the insects into suspension or through conidial spraying (Hoe et al., 2009; Jessica et al., 2018; Sani, et al., 2018; Safavi, 2010). For example, the dipping method was performed for red palm weevil for 120 seconds in the prepared conidial suspension (Fong, Addis and Azmi, 2018; Mathulwe et al, 2021). The mortality of insects will be recorded to determine the virulence of EPF against the insects. Besides that, conidial spraying method can be done by preparing conidial suspension were also introduced as one of the methods for to evaluate the pathogenicity of EPF (Panyasiri, Attathom and Poehling, 2007). Other than that, Grace, et al (2017) evaluate the pathogenicity of isolated *B. bassiana* against red palm weevil by inoculating insects directly to the fungal culture plate, and the inoculated insects were incubated for 14 days to investigate the virulence of EPF.

CHAPTER 3

METHODOLOGY

3.1 Soil Sampling

The soil samples were collected from three regions in Kinta Region, Perak. The GPS coordinates for each location as shown in Table 3.1.

Table 3.1: Location and GPS coordinates for soil sampling.

Location	GPS coordinates
1) Batu Berangkai, Kampar	(N 4° 18' 30.557" E 101° 10' 19.916")
2) Hutan Lipur Sungai Salu, Jeram	(N 4° 22' 43.609" E 101° 10' 27.284")
3) Kledang Saiong Forest Eco Park, Meru	(N 4° 22' 43.609" E 101° 10' 27.284")

Soil samples were collected at 15 cm of soil depth. Five randomly selected points from each location were selected and the soil sample was collected using a soil corer. Next, the soil was brought back to the laboratory and stored at 4 °C. before EPF isolation was conducted.

3.2 Isolation of EPF using mealworm (*T. molitor*) as bait

Soil samples from each location was filled up into plastic containers up to 1 cm depth. A total of 10 mealworms purchased from Weng Kee Akuarium Sdn. Bhd., Kampar (N 4° 22’ 43.609” E 101° 10’ 27.284”), were introduced into each plastic container containing soil samples. The introduced mealworms were incubated at 25°C for 14 days and the mealworms were observed every 3 days for infection symptoms. The infected mealworms were collected and surfaced sterilized with 70% (v/v) ethanol for 5 s and blot dried with sterile filter paper. The sterilized mealworms were cut into small pieces, and transferred to Potato Dextrose Agar (PDA) for EPF isolation. The EPF-infected mealworms were identified through the hardening of cadavers and obvious mycosis on the surface of mealworms, and no movement when disturbed physically (Mathulwe et al., 2021; Grace et al, 2017). EPF pure cultures were obtained by subculturing the fungi from the mealworm-inoculated PDA using PDA with the antibiotic, chloramphenicol.

3.3 Morphological identification of EPF

The morphology and size of 30 conidia were measured and recorded using a light microscope equipped with Motic Camera (Motic, USA). The average size of conidia for each isolate were calculated. The morphological data of EPF isolates was compared with taxonomic keys from Samson (1998), Humber (2012), and other publications to identify the fungi morphologically (Chaverri, Smuelas and Stewart. 2001; Fernandes, et al., 2010; Jang, et al., 2017; Schroers, 2001).

3.4 Pathogenicity of EPF isolates against mealworm (*Tenebrio molitor*)

3.4.1 Direct Inoculation ofEPF onto *T. molitor* culture.

The fungal pure culture of 14-day-old was prepared for each isolate. A total of 10 mealworms (*T. molitor*) was inoculated to the pure culture for 5 mins. Then, the inoculated mealworms were transferred to a sterile petri dish with oatmeal. Mortality experiments were conducted with 3 replicates (petri dish) for each EPF isolates. The mortality of mealworms was calculated and recorded after 14 days of incubation.

3.4.2 Dripping Method and Conidial Spraying Method

A volume of 10 mL sterile distilled water was added onto sporulating fungal cultures. The spores were dislodged from the mycelia mats by gentle scrapping by using the back of a sterile spatula. The conidia suspension was then filtered through 2 layers of sterile cotton gauze overlaid on a glass funnel. The conidia concentration was determined using a hemocytometer and the following formula. The stock conidia suspension was diluted and adjusted to 1×10^6 conidia/ mL using the following formula.

$$Conidia/mL = \frac{Average\ number\ of\ conidia\ \times dilution\ factor}{10^{-4}mL}$$

$$M_1V_1 = M_2V_2$$

M_1 = Concentration of stock suspension (conidia/ mL)

V_1 = Volume of stock suspension (mL)

M_2 = Conidia concentration for pathogenicity assay (1×10^6 conidia/ mL)

V_2 = Volume of diluted conidia suspension (mL)

A total of 10 mealworms were placed in a petri dish overlaid with a sterile filter paper. The dripping method as conducted by dripping a few drops of conidia suspension were dripped onto the filter paper of the petri dish with mealworms until the filter paper was moisten with conidial suspension. The pathogencity assay was conducted with 3 replicates (petri dish) for each EPF isolates. Besides that, the mortality test was repeated by spraying the conidia suspension with to 1×10^6 conidia/ mL on the mealworms using a sprayer. Mortality rate was calculated for each isolate for both dripping and spraying method. Statistical analysis was conducted by performing one-way ANOVA using SPSS software. Differences among treatment means was determined using Duncan's Multiple Range Test ($p < 0.05$).

$$\text{Mortality rate} = \frac{\text{No. of mealworm infected by EPF}}{\text{Total no. of mealworms inoculated to EPF}} \times 100\%$$

3.5 Molecular identification of Fungal Isolate

3.5.1 DNA Extraction

A 250 mL conical flask filled with 40 mL Potato dextrose broth (PDB). A 10 mm mycelial plug from 7-day-old EPF pure culture was inoculated into 40 mL of sterile PDB using a cork borer. The PDB culture was incubated at 25°C for 7 days. The mycelial mats were harvested, about 100 mg weighed of mycelial mats was blot dried on filter paper. Then, the mycelial mats were macerated using sterile mortar and pestle with 500 µL of DNA extraction buffer (200 mM Tris-HCl, 250 mM, NaCl, 25 mM EDTA, 0.5% (w/v) SDS). The mixture was transferred to a sterile microcentrifuge tube. A volume of 12 µL of β-mercaptoethanol and 120 µL of 20% (w/v) SDS was added in the mixture. The mixture was mixed by inverting the microcentrifuge tube for several times. Then, the mixture was incubated at 65°C water bath for 15 mins. An equal volume of chloroform and isoamyl alcohol (C:I) with the ratio 24:1 was added into the tube and mixed thoroughly for a few seconds. The mixture was centrifuged with 12,000 rpm at 4°C for 10 mins. The supernatant was transferred to a new centrifuge tube. The C:I extraction was repeated for the supernatant. A 0.7 v of isopropanol was added to the supernatant. The mixture was incubated on ice for 10 mins. Next, the supernatant was centrifuged with 12000 rpm 4°C for 4 mins. The supernatant was discarded, and 200 µL of 70% (v/v) ethanol was added into the microcentrifuge tube. The mixture was mixed by inverting the tube several times and centrifuge again at 12000 rpm at 4°C for 2 mins. The ethanol was removed from the tube, the ethanol washing step was repeated to purify the DNA pellet. The DNA pellet was dried using vacuum centrifugal

concentrator (Eppendorf Concentrator plus) for 15 mins. A volume of 100 µL of sterile distilled water was added to resuspend the DNA pellet. The DNA concentration and purity was measured using a Nanodrop spectrophotometer (Thermo Fisher, USA) at the absorbance of 260/280 nm ratio of 1.8 to 2.0. The DNA suspension was stored in the freezer at -20°C.

3.5.2 Polymerase Chain Reaction

The DNA primers, ITS1 and ITS4 were used for PCR (White, et al, 1990). A total volume of 25 µL PCR master mix with 1 µl of 0.4 µM of DNA primer was prepared as shown in Table 3.2.

Table 3.2: PCR Master Mix

Reagent	Final concentration	Volume
[a] 10X *Taq* buffer	1X	[a]2.5 µL
[a]*Taq* DNA polymerase	1.25 U/50 µl	[a]0.2 µL
[a] dNTP mix	0.2 mM	[a]1 µL
[a] 25mM $MgCl_2$	2 mM	[a]2 µL
Forward Primer (ITS1)	0.4 µM	1 µL
Reverse Primer (ITS4)	0.4 µM	1 µL
DNA template (50 ng)	200 pg/ µL	1 µL
Sterile distilled water	-	16.3 µL
	Total volume	25 µL

[a] The components are included in the PCR mix.

The DNA extracted from each EPF isolates was subjected to PCR with the following parameters as shown in Table 3.3 (Vejar-Cota, et al., 2017).

Table 3.3: PCR parameters for thermal cycler

PCR steps	Temperature	Duration	Cycles
Pre-denaturation	94 °C	5 mins	-
Denaturation	94°C	30 s	35 X
Annealing	58°C	60 s	
Extension/ Elongation	72°C	45 s	
Post-extension	72°C	7 mins	-
Chilling	10°C	Hold	Run until collected

3.5.3 Gel Electrophoresis

The PCR amplicons and1kb DNA ladder for size estimation were electrophoresed on 1% (w/v) agarose gel using 1X TBE buffer at 80V for 45 minutes. The gel was placed on the transilluminator, and the image of gel was captured using Gel Documentary Image Analyzer (Biorad, USA).

3.5.4 DNA Sequencing and Homology Search

PCR amplicons were sent to Apical Scientific Sdn. Bhd. (Malaysia) for Sanger Sequencing. The sequences received was processed using MEGAX software to remove noisy sequences from 3' and 5' (Wintachai et al., 2022). The forward and reverse sequence of each sample was aligned for nucleotide insertions and deletions. A homology search was conducted by using BLAST algorithm against the sequences in NCBI database.

3.5.5 Phylogenetic Analysis

A multiple sequence alignment was performed between the EPF sequence and the retrieved sequences from NCBI. Phylogenetic trees were constructed using the neighbour-joining method with a bootstrap test of 1000 times were constructed using the MEGAX software (Wintachai et al., 2022).

CHAPTER 4

RESULTS

4.1 Isolated fungi from recreational forests in the Kinta Region, Perak

A total of seven fungal isolates were isolated from the soils of three recreational forests in the Kinta Region, Perak using *T. molitor*. Two isolates were isolated from Batu Berangkai, Kampar (BB4(a)I1 and BB4(a)I2), two isolates from Sungai Salu Hutan Lipur, Jeram (SS1(a)I2 and SS5(b)I1), and three isolates from Kledang Saiong Eco Park, Meru (SK1(a)I1 and SK3(a)I1), as shown in Table 4.1.

Table 4.1: Isolated fungi from three recreational forests in the Kinta Region, Perak using *T. molitor***.**

Location	Fungal isolates
Batu Berangkai, Kampar	BB4(a)I1
	BB4(a)I2
Sungai Salu Hutan Lipur, Jeram	SS1(a)I2
	SS5(b)I1
Kledang Saiong Eco Park, Meru	SK1(a)I1
	SK3(a)I1
	SK3(b)I1

Note: BB, indicates soil sample from Batu Berangkai; SS, indicates soil sample from Sungai Salu Hutan Lipur, Jeram; SK, indicates soil sample from Kledang Saiong Eco Park, Meru.

4.2 Morphological characterization of fungal isolates

A total of 7 fungi were isolated and identified morphologically from PDA as shown in Figures 4.1-4.4. Each fungal isolates were identified based on taxonomic keys from the respective publications. Fungal colony morphology was observed and recorded based on their colour, structure, and surface texture. Microscopic morphology such as conidia size (Table 4.2), shape, and conidiophore morphology was described and compared with the respective fungal morphological studies (Table 4.3). Fungal colonies were mainly existed in white to green color with cottony to filamentous mycelia. The conidial shape is varied from ovoid, globose, oblong, and cylindrical according to fungal species. The morphology of conidiophore of each fungal isolates are distinct from each isolate. In short, the fungal isolates meet the agreements from taxonomic keys of respective publications (Chaverri, Smuelas and Stewart. 2001; Fernandes, et al., 2010; Jang, et al., 2017; Schroers, 2001).

4.2.1 Isolate BB4(a)I2 (*C. rogersoniana*)

The fungal colony of Isolate BB4(a)I2 was observed white, flat and filamentous structure (Figure 4.1A). The shape of conidia is ovoidal (Figure 4.1B), primary conidiophores are verticillium-like, secondary conidiophores raised from primary branches independently (Figure 4.1C). The adpressed phialides form from the terminal part of the secondary branches. The average conidial size measured from 30 conidia of Isolate BB4(a)I2 is $4.86 \pm 0.55 \times 2.46 \pm 0.41$.

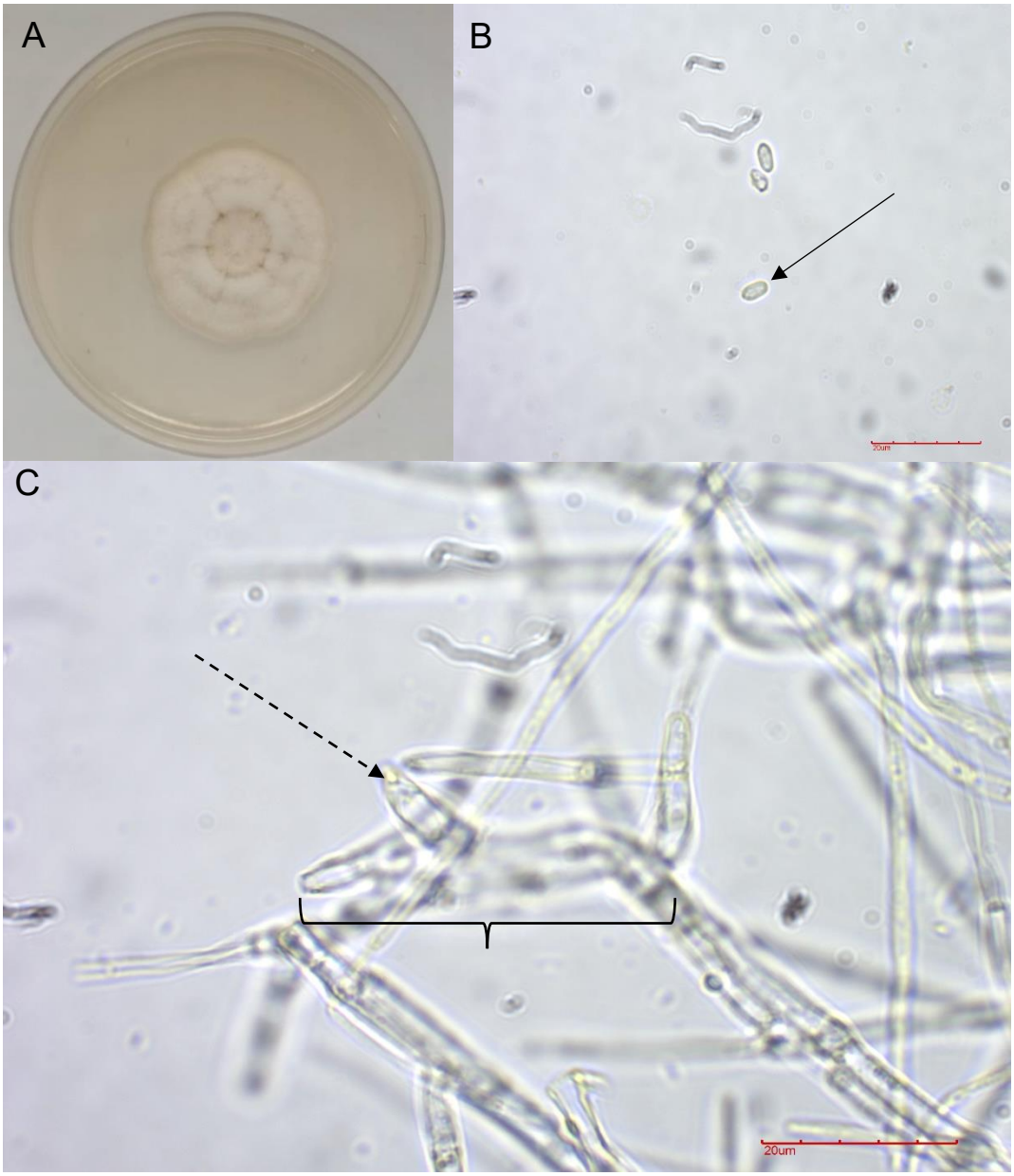

Figure 4.1: Morphology of Isolate BB4(a)I2 (*C. rogersoniana*). **A**, fungal colony at 7-day-old. **B**, conidia. **C**, conidiophore (brace bracket) and phialide (dotted line arrow). Bar represents 20 µm.

4.2.2 Isolate BB4(a)I1, SS1(b)I2 and SS5(b)I1 (*T. spirale*)

Greyish green and cottony mycelial fungal colony of *T. spirale* was observed at 7-day-old fungal culture (Figure 4.2A). The conidia are oblong to ellipsoidal shaped (Figure 4.2B). Primary conidiophores arise from the hyphae base, 1 to 2 secondary conidiophore branches arise from primary conidiophore. Phialides densely clustered and nearly doliiform (Figure 4.2C). The average conidial size of Isolate BB4(a)I1, SS1(b)I2 and SS5(b)I1 are 4.58 ± 0.73 × 2.60 ± 0.46, 4.78 ± 0.84 × 2.61 ± 0.40, and 4.72 ± 0.48 × 2.68 ± 0.38 respectively.

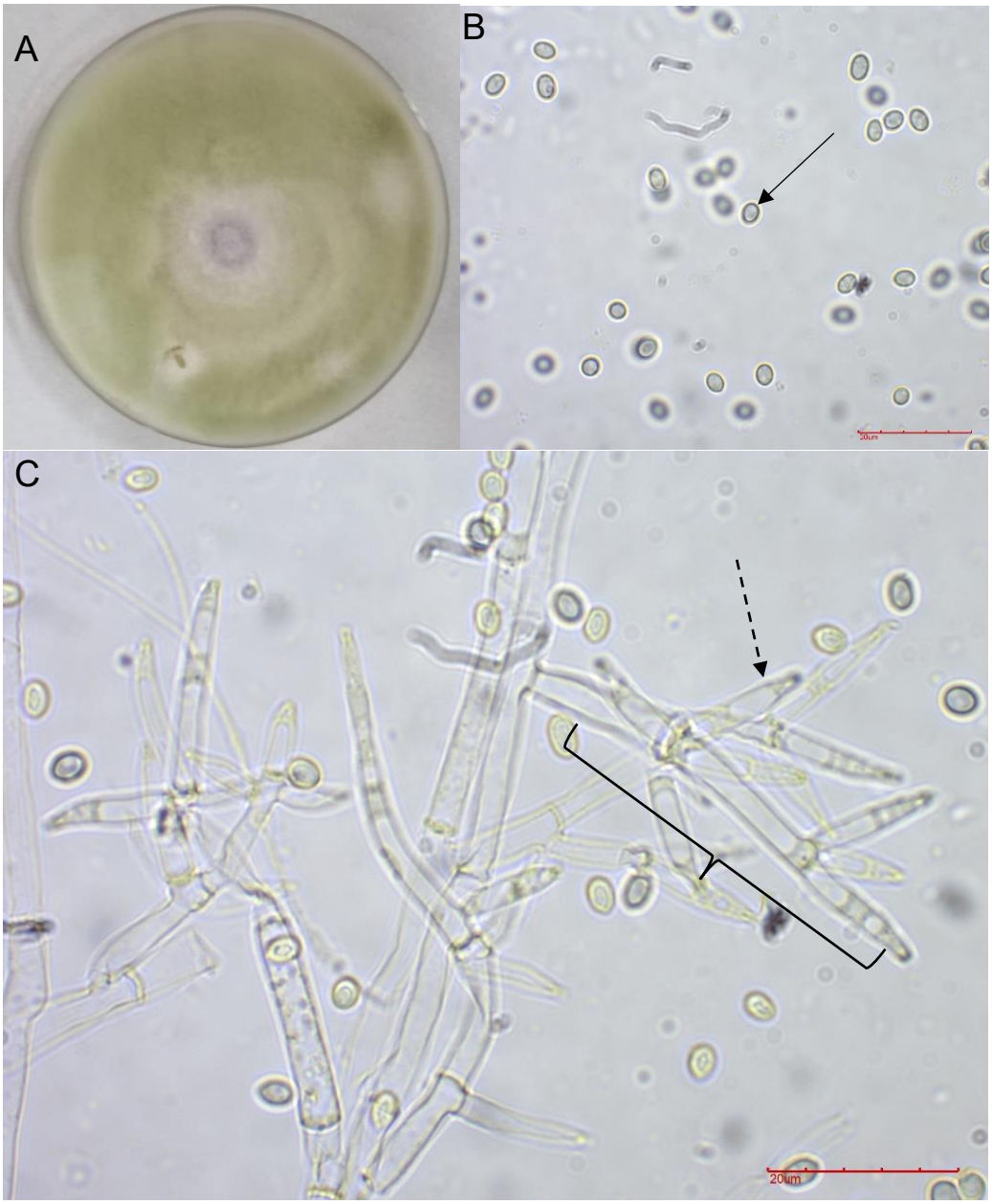

Figure 4.2: Morphology of Isolate BB4(a)I1, SS1(b)I2, SS5(b)I2 (*T. spirale*). **A**, fungal colony at 7-day-old. **B**, conidia. **C**, conidiophore (brace bracket) and phialide (dotted line arrow). Bar represents 20 µm.

4.2.3 Isolate SK1(a)I1 and SK3(a)I1 (*M. anisopliae*)

M. anisopliae fungal colony (Figure 5A) formed dark yellow mycelia at the center of colony, surrounded by young, white cottony mycelia at the edge of the fungal colony (Figure 4.3A). Dark green dark green conidia produced between two mycelia in circular ring. The conidia are cylindrical or ovoid, can be individually formed or attached together in long chains (Figure 4.3B). Conidiophores broadly branched, intermesh tightly, and aggregate in prismatic columns. Phialides arranged in candle-like pattern (Figure 4.3C). The average conidial size of Isolate SK1(a)I1 and SK3(a)I1 are $6.10 \pm 0.67 \times 2.24 \pm 0.38$ and $5.67 \pm 0.54 \times 2.10 \pm 0.21$.

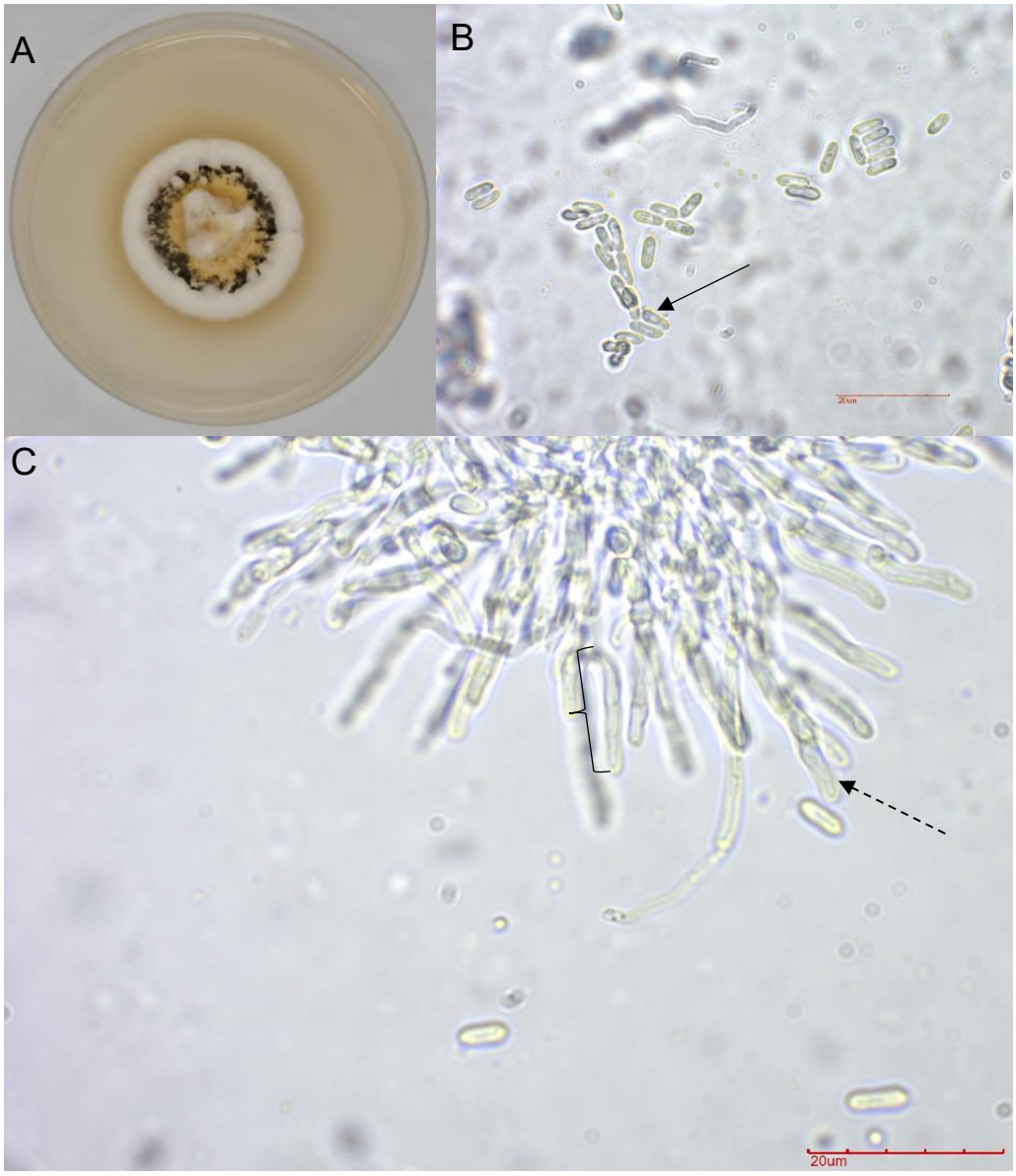

Figure 4.3: Morphology of Isolate BB4(a)I1, SS1(b)I2, SS5(b)I2 (*T. spirale*). **A**, fungal colony at 7-day-old. **B**, conidia. **C**, conidiophore (brace bracket) and phialide (dotted line arrow). Bar represents 20 µm.

4.2.4 Isolate SK3(b)I1 (*T. virens*)

The fungal colony of *T. virens* formed white and cottony mycelia at 2-3 day-old. Dark green colony was observed as the conidia sporulated (Figure 4.4A). The shape of conidia are globose to subglobose (Figure 4.4B). Primary conidiophores are glioladium-like. Secondary branches arise in pairs and not uniformed spaced. The secondary branches form along the main axis, ampulliform-shaped phialide is raised from the terminal of each secondary branch (Figure 4.4C). The average conidial size of Isolate SK3(b)I1 are $4.36 \pm 0.46 \times 4.02 \pm 0.44$.

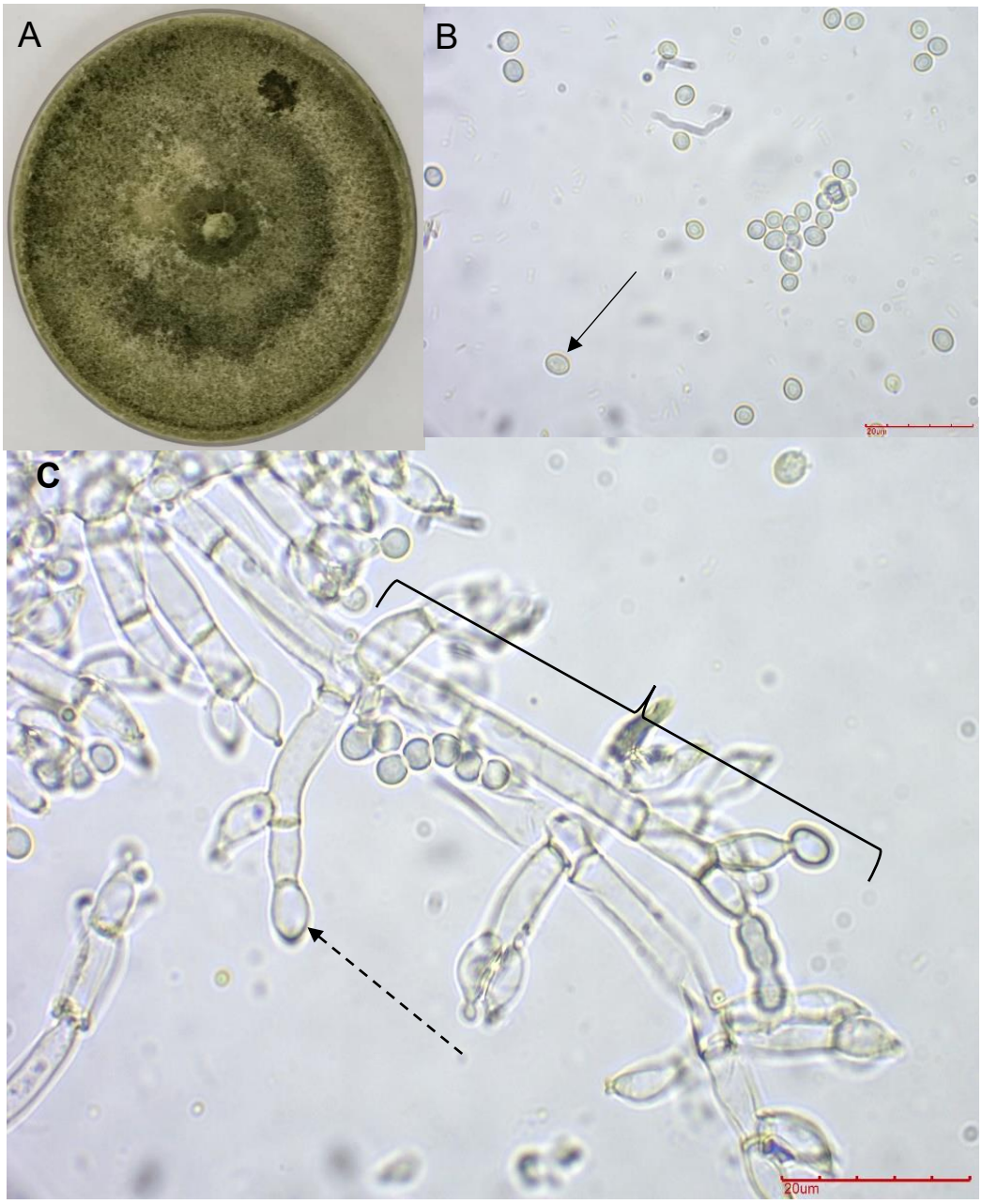

Figure 4.4: Morphology of Isolate SK3(b)I1 (*T. virens*). **A**, fungal colony at 7-day-old. **B**, conidia. **C**, conidiophore (brace bracket) and phialide (dotted line arrow). Bar represents 20 µm.

Table 4.2: Average conidia size of fungal isolates

Isolates	Species	[a]Average conidia size (µm)	Expected average conidia size (µm)
BB4(a)I2	*C. rogersoniana*	4.86 ± 0.55 × 2.46 ± 0.41	4.80–9.60 × 2.20–4.20 (Schroers, 2001)
BB4(a)I1	*T. spirale*	4.58 ± 0.73 × 2.60 ± 0.46	4.1–5.1 × 2.5–3 (Jang, et al., 2017)
SS1(b)I2		4.78 ± 0.84 × 2.61 ± 0.40	
SS5(b)I1		4.72 ± 0.48 × 2.68 ± 0.38	
SK1(a)I1	*M. anisopliae*	6.10 ± 0.67 × 2.24 ± 0.38	5.62-10.57 × 3.98 - 4.74 (Fernandes, et al., 2010; Samson, 1998)
SK3(a)I1		5.67 ± 0.54 × 2.10 ± 0.21	
SK3(b)I1	*T. virens*	4.36 ± 0.46 × 4.02 ± 0.44	4.0-6.50 × 4.00-6,50 (Chaverri, Samuels and Stewart, 2001)

[a] Average conidia size derived from the measurements of 30 conidia per isolate.

Table 4.3: Morphology of isolated fungi from three recreational forests in the Kinta Region, Perak.

Fungal Isolate	Colony colour	Structure	Conidia shape	Conidiophore
BB4(a)I2	White	Flat and filamentous	Ovoidal	Verticillium-like primary conidiophores and secondary conidiophores form independently from the primary branches, Adpressed phialides form the terminal part.
BB4(a)I1 **SS1(b)I2** **SS5(b)I1**	Grayish green	Cottony	Oblong to ellipsoidal	Conidiophores arise from the hyphae base, 1-2 secondary conidiophore branches arsing from primary cells. Phialides clusters densely and nearly doliiform.
SK1(a)I1 **SK3(a)I1**	Dark yellow at center, dark green conidia form circular ring, white mycelia surround the edge of the colony	Cottony	Cylindrical or ovoid in long chains	Broad-branched, intermesh tightly, aggregate in prismatic columns. Phialides arranged in a candle-like pattern.
SK3(b)I1	Dark green	White cottony mycelia (Day 2-3)	Globose to subglobose	Gliocladium-like conidiophore, pair branches from along the main axis ampulliform shaped phialides. Secondary branches arise in pair and not uniformly spaced.

4.3 Pathogenicity Evaluation of EPF

Based on Table 4.4, isolate SK1(a)I1 and SK3(a)I1 showed mean mortality of 100% and 88.33% respectively, and showed mortality differed significantly ($P < 0.05$) using conidia spraying method on Day 3. The rest of the isolates did not cause any mortality rate by the fungal pathogens even after 14 days of incuabation. Besides that, there were no mortality of direct was observed when performing *T. molitor* inoculation to EPF culture and dripping method after 14 days of incubation.

Table 4.4: Mortality of *T. molitor* larvae using conidia spraying method after 14 days of incubation.

Isolates	Replicate	No. of infected larvae	Mortality of larvae (%)	[a]Average mortality (%)
BB4(a)I1	R1	0	0	0[a]
	R2	0	0	
	R3	0	0	
BB4(a)I2	R1	0	0	0[a]
	R2	0	0	
	R3	0	0	
SS1(b)I2	R1	0	0	0[a]
	R2	0	0	
	R3	0	0	
SS5(b)I1	R1	0	0	0[a]
	R2	0	0	
	R3	0	0	
SK1(a)I1	R1	10	100	100.00[b]
	R2	10	100	
	R3	10	100	
SK3(a)I1	R1	9	90	83.33[c]
	R2	8	80	
	R3	8	80	
SK3(b)I1	R1	0	0	0[a]
	R2	0	0	
	R3	0	0	

[a] Different statistical letters indicate significant difference of mortality rate between different EPF isolates ($P < 0.05$).

4.4 Phylogenetic analysis of EPF

The two isolates, SK1(a)I1 and SK3(a)I1, that showed entomopathogenic activity, were subjected to further molecular characterization to validate the morphological characteristics. Figure 4.5 and Figure 4.6 showed the phylogenetic trees of 2 fungal isolates (SK1(a)I1 and SK3(b)I1) with their respective out-group based on neighbour-joining method with 1000 bootstrap replication. The two isolates were identical to other *M. anisopliae* isolates with a bootstrap value of 81% and 72% respectively.

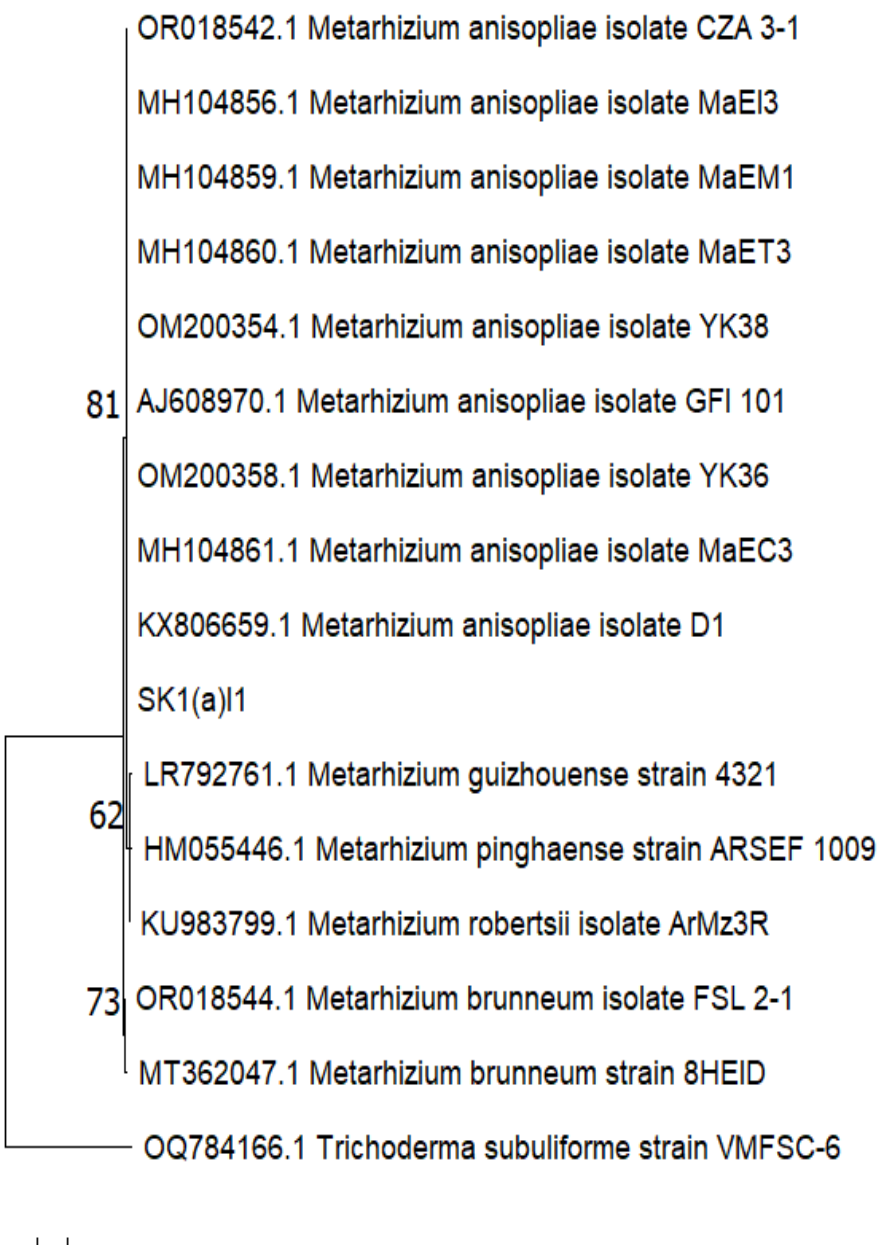

Figure 4.5: Phylogenetic trees of isolate SK1(a)I1 based on neighbour joining analysis with *Trichoderma subuliforme* strain VMFSC-6 as the outgroup. Isolate SK1(a)I1 was clustered together with other *M. anisopliae* isolates with a bootstrap value of 81%. The scale bar corresponds to 0.02 nucleotide substitutions per site.

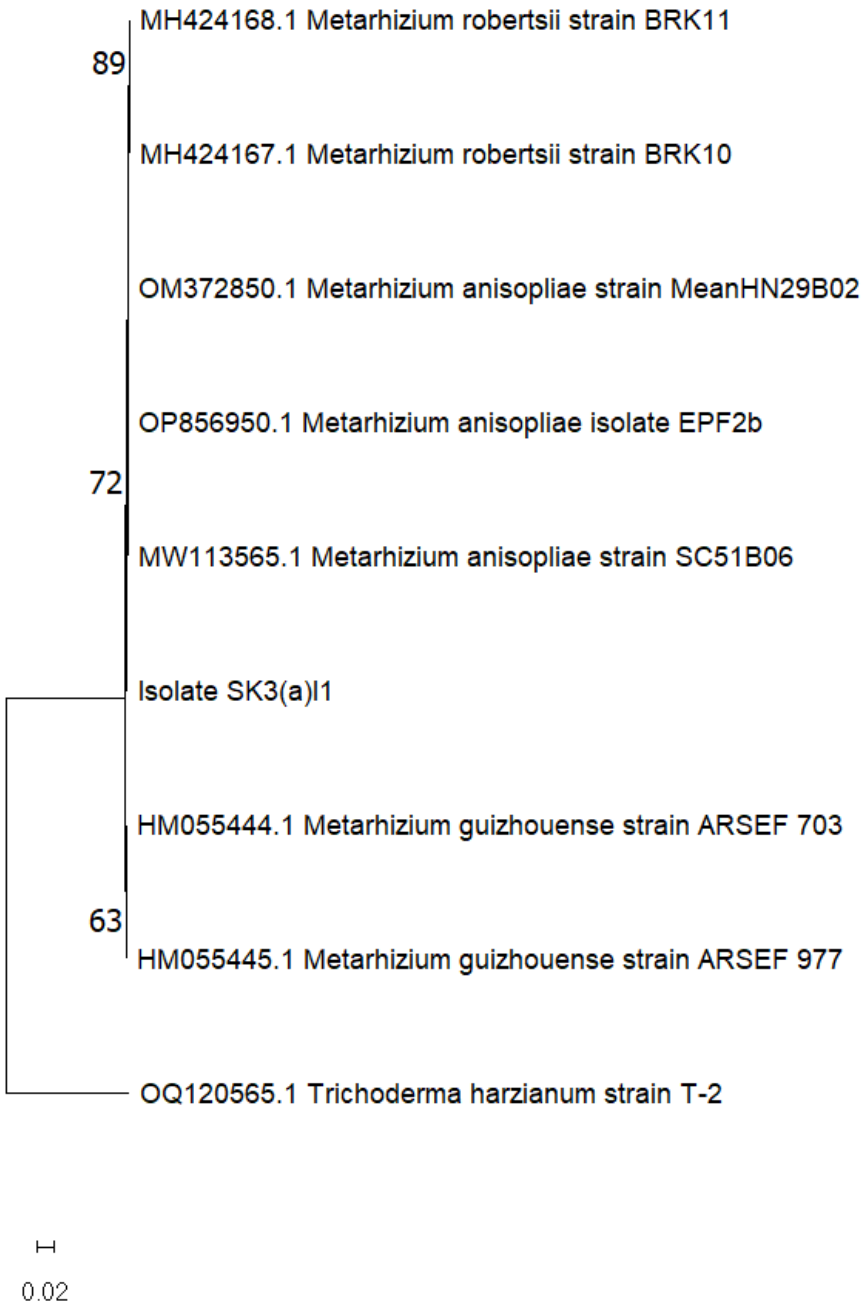

Figure 4.6: Phylogenetic trees of isolate SK3(a)I1 based on neighbour joining analysis with *Trichoderma harzianum* strain T-2 as the outgroup. Isolate SK3(a)I1 was clustered together with other *M. anisopliae* isolates with a bootstrap value of 70%. The scale bar corresponds to 0.02 nucleotide substitutions per site.

CHAPTER 5

DISCUSSION

5.1 EPF Diversity in Recreational Forests in Malaysia

EPF are pervasive and can be found in natural forests with high diversity (Wei, et al., 2021). There are several studies shown that EPF were successfully isolated from secondary forests in different countries across the continents such as Croatia, Columbia, Thailand, China, Brazil, South Korea and India (Castillo, et al, 2018, Mora, Rouws and Fraga, 2016; Seo, et al., 2016; Kovač, M., Tkaczuk, C. and Pernek, M., 2021; Vivekanandhan, Bedini and Shivakumar, 2020,; Wei, et al., 2021). However, most of the studies in Malaysia showed that EPF were isolated from agricultural lands such as oil palm plantations or tropical forests (Grace, et al., 2017; Wasti, et al., 2018; Bakeri, et al., 2009). So far, there is only one study in Malaysia reported that EPF isolation from recreational forests located at Hutan Melintang, Perak (Jessica, et al., 2018). Jessica and colleagues (2018) have suceessfully isolated 11 isolates of *I. fumosorosea* from their research. In this study, only 2 EPF isolates of *M. anisopliae* were isolated from Batu Berangkai, Perak. Therefore, it is difficult to conclude in discovery of the diversity of indigenous EPF from the recreational forests in the Kinta Region.

In this experiment, the soil sample collected from three recreational forests were used for EPF isolation. The soil from recreational forests which are abundant in plants have similar edaphic properties as tropical forests in Malaysia (Khairil, et al., 2015). Tropical forests or secondary forests rich in organic matter content are generally acidic in nature and have higher carbon (C) and nitrogen (N), which as a good nutrient source for fungal growth, resulting in high soil microbial diversity (Perumal, et al., 2015; Van der Wal, et al., 2013). The physiochemical of soils could influence the occurrence of EPF diversity as the fungi have their preference in their living environment. The soil texture is one of the crucial factors that determining the diversity of soil microbes. For example, *B. bassiana* was reported has the highest incidence in soil texture of clay, with relative alkali (pH ≥ 8-8.5) and lower organic content of soils, while *M. anisopliae* is more likely found in coarse texture soil and more adaptable in acid soil (Mora, Rouws and Fraga, 2016; Quesada-Moraga, et al., 2007).

The occurrence of EPF has the most prevalent diversity in different soils from different forest landscapes (Velavan, et al., 2021). Environmental factors like high humidity and high temperature could reduce fungal sporulation rate, leading to the reduction of conidia formation (Meyling and Eilenberg, 2006). Besides that, there are studies that revealed organic matter content of soils is associated with the occurrence of different EPF (Ali-Shtayeh, Mara'I and Jamous, 2003; Medo and Cagan, 2011). EPF diversity is more likely to have a high occurrence in higher organic matter content (Ali-Shtayeh, Mara'I and Jamous, 2003). However, the

physiochemical analysis of the soil samples was not carried out in this experiment using the collected soil sample. Further studies can be conducted investigating the physiochemical properties of soil from recreational forests to evaluate the correlation of soil propertied and EPF diversity.

5.2 Isolation Method of EPF

The occurrence of EPF depends on its habitat types and the insect-baiting methods because different EPF are susceptible to different insects (Sharma, et al., 2018). Insect baiting method is widely used in isolating EPF. Mealworm (*T. molitor*) and greater wax moth larvae (*G. mellonella*) are two common insects used as EPF insect bait (Masoudi, et al., 2020; Sharma, et al., 2018; Tuininga, et al., 2009). There is no significant difference in EPF diversity in isolating EPF using *G. mellonela* or *T. molitor* (Klingen, Eilenberg and Meadow, 2002; Mora, Rouws and Fraga, 2016). However, different EPF species were obtained when different insect baits were utilized for EPF isolation (Chandler, Hay and Reid, 1997; Klingen, Eilenberg and Meadow, 2002). For example, Mora et al. (2016) and Sharma et al. (2018) reported that *B. G. mellonella* isolates were more susceptible to *B. bassiana*, which under Lepidoptera family, while *Metarhizium* species, especially for *M. anisopliae* isolates showed higher occurrence when isolated using *T. molitor* as bait. There are only seven fungal isolates from three genera were isolated in this experiment, which is considered a relatively low number of fungi isolated from the soil. The isolation can be improved by performing exhaustive isolation method.

The exhaustive isolation method refers to the isolation of every possible fungi by diluting soil samples, in which the diluted soil suspension was spread on the culture media plate (Angelona and Biodochka, 2018). Soil dilution plate method has statistically proven effective in determining microbial community and diversity (Nandhini and Josephine, 2013; Yasanthika, et al., 2022). Dilution plate method was reported to be effective in isolates high diversity of fungal species were isolated over soil plate method and alcohol treatment method, from same soil samples (Kumar, et al. 2015; Abdullah and Saadullah, 2013). Therefore, soil baiting using *G. mellonella* and soil dilution plate method is strongly recommended to isolate EPF of different genera from the soil in recreational forests and to study the diversity of EPF in future studies.

5.3 Entomopathogenic Properties of Isolated Fungi

5.3.1 *Clonostachys rogersoniana*

In this study, isolate BB4(a)I2 were isolated from recreational forest at Batu Berangkai, Kampar, and had been morphologically identified as *C. rogersoniana* (Figure 2). The *Clonostachys* genus is grouped under Sordariomycetes family in Ascomycota (Schroers, 2001). *Clonostachys spp*. are well known as biocontrol agents against plant pathogenic fungi and secondary metabolites producers (Zhai, et al., 2016). *C. rogersoniana* is known as metabolite producers for pharmaceutical and agrochemical applications (Han, et al., 2020). For example, *C. rogersoniana*

produces metabolites such as rogesonin, glioadiosin which carries antibacterial properties (Han, et al., 2020). Besides that, a study revealed that *C. rogersoniana* were used to undergo fermentation of *Illigera aromatica* to produce cytotoxic menthane-type monoterpenoid dimers and metabolites for acetylcholinesterase inhibition for medical industries (Dong, et al., 2017; De Oliveira, et al., 2022).

There are studies revealed that *C. rogersoniana* showed insect-pathogenic properties. Pathogenicity of *C. rogersoniana* against cotton leafworm larvae (*Spodoptera littoralis* (Boisd.) was studied. The study showed low mortality (10%) of *S. littoralis* larvae when treated with conidial concentration of 1×10^6 conidia/mL, but the mortality increased as conidial concentration increases (Li, et al., 2017). The mortality up to 75.45% was obtained when treated with 1×10^9 conidia/mL after 14 days of inoculation (Li, et al., 2017). Besides that, *C. rogersoniana* was also reported successfully isolated and caused more than 70% of mortality rate against *T. molitor* (Kim, et al., 2017).

However, no mortality of *T. molitor* treated with Isolate BB4(a)I2 (*C. rogersoniana*) was observed in the pathogenicity test (Figure 4.11). By comparing the results by Li et al (2017), no mortality could be due to low conidial concentration which is insufficient to cause a significant mortality against *T. molitor*. The other reasons could be due to the isolate fungi might lack of crucial pathogenicity gene for insect targeting and induce pathogenicity against insects (Zeilinger, et al., 2005). Study

on the higher conidial concentration of BB4(a)I2 are recommended to prepare against *T. molitor* to verify whether this *C. rogersoniana* isolate are entomopathogenic. In order to overcome low sporulation rate of *Clonostachys* spp, Kapeua-Ndacnou and colleagues (2023) suggested a mass conidial production procedure for *Clonostachys* fungi, by inoculating conidia suspension from oatmeal agar plate (OA) fungal culture to 2% malt extract broth (MEB), and incubate for 10 days of 12 hours daylight at 25 °C. The harvested conidia concentration can be up to 1×10^8 conidia/ mL (Kapeua-Ndacnou et al., 2023). PDB can be used for alternative for MEB as it produced the highest sporulation rate among other five media when applied on *Clonostachys rosea*, one of the mycoparasites from *Clonostachys* genus (Sun, et al., 2014).

5.3.2 *Trichoderma spirale* and *T. virens*

In this study, isolate BB4(a)I1, SS1(b)I2 and SS5(b)I2 were morphologically identified as *T. spirale* while SK3(b)I1 were morphologically identified as *T. virens*. *Trichoderma* spp. are well known as biocontrol agents in the agricultural sector (Poveda, 2021; Vinale, 2008; Zin and Badaluddin, 2020). They play important roles as antagonistic agents against fungal pathogens and diseases, induce plant immunity, and stimulate plant growth (Adnan, et al., 2019; Praprotnik, Lončar and Razinger, 2021; Morán-Diez, et al., 2021; Yao, et al., 2023). For example, *Trichoderma* spp. act as direct control agents for phytopathogens which activates host defense mechanism of the plant against pathogens (Tyśkiewicz, et al., 2022). On the other hand, *Trichoderma spp*. showed insect-pathogenic properties by controlling insect

pests directly or indirectly. *Trichodema* spp. act as direct biocontrol agent by parasitic to insect and produce insecticidal metabolites to kill insects, or undergo midgut disruption by secreting enzymes disrupting the balance of gut enzymatic activities (Poveda, 2021, Tyśkiewicz, et al., 2022). Indirect control of Trichoderma works against insect are induced by activation of plant defense and releasing phytocompounds that are toxic to phytophagous insects, or releasing metabolites to attract natural enemies of insect pests (Poveda, 2021).

T. harzianum, T. longibrachiatum, T, album has been reported to be pathogenic to various insect families such as hemipterans, coleopterans, lepidoptereans, diptereans, orthoptereans and acari (Poveda, 2021). Besides that, several *Trichoderma* species were successfully isolated from *T. molitor* such as *T. harzianum*, *T. atroviride* and *T. gamsii* (Poveda, 2021, Praprotnik, Loncar and Razinger, 2021). For example, *T. harzainum* showed 100% of mortality rate against *T. molitor* after 15 days of infection (Shakeri and Foster, 2007). Based on the pathogenicity result shown in Table 4.3, no mortality of *T. molitor* by *T. spirale* and *T. virens*. To date, there are no papers or research had been reported that these two *Trichoderma* species are pathogenic to insects.

T. spirale plays its role as the common *Trichoderma* species which acted as biocontrol agent for fungal pathogens. *T. spirale* can release antifungal volatile compounds to reduce the damage caused leaf spot fungi (*Corynespora casiicola* or

Curvularia aeria) on lettuce (Baiyee, et al., 2019). *T. virens* has been reported as an aggressive mycoparasite to control *Rhizotonia solani*, *Sclerotium rolfsii*, *Pythium* spp, etc (Mukherjee, et al., 2003; Romão-Dumaresq, et al., 2016; Singh, Raina and Singh, 2017). In other words, the *Trichoderma* isolates from this study might play their role saprophytes that feed on the dead mealworms. Therefore, *T. spirale* and *T. virens* found in soil samples might be saprophytic against EPF, resulting the isolation of *Trichoderma* spp. from insect baits. To avoid the isolation the saprophytes, isolated mealworms should be monitored on daily basis to isolate EPF earlier once mycosis on the insect cadaver is observed before the EPF is colonized by other fungal saprophytes.

5.3.3 *Metarhizium anisopliae*

Two *M. anisopliae* isolate (SK1(a)I1 and SK3(a)I1) showed a significant mortality rate of 100.00% and 83.33% against *T. molitor* in the pathogenicity evaluation (Figure 4.4). Many studies have been reported that *M. anisopliae* showed high virulence against *T. molitor* with mortality rate within 90-100% in 14 days of monitoring (Adatia, Johnson and Entz, 2010; Oreste, et al., 2012; Sahin and Yanar, 2021).

M. anisopliae is undeniably one of the common biocontrol agents in insect pest control, and it has been commercialized in Malaysia to control the rhinoceros beetle (*Oryctes rhinoceros*), and there are more commercialized *M. anisopliae* products

are developing throughout the world (Moslim, et al., 2006; Zimmermann, 2007). Furthermore, *M. anisopliae* has a wide of hosts which targeting many agricultural pests such as rubber termite (*C. curvignathus*), red palm weevil (*R. ferrugineus*) and silverleaf whitefly (*Bemisia tabaci*) (Islam, Omar and Shabanimofrad, 2014; Cheong and Azmi, 2020). Besides the broad range of targeting hosts, high sporulation and high growth rate are also the criteria making *M. anisopliae* a good biocontrol agent for agricultural pest control (Samsuddin, Sajap and Mahamed, 2015). Furthermore, Zimmermann (2007) studied that *M. anisopliae* is safe for agricultural application since it does not cause health issues to human or vertebrate animals, and it has minimal impacts on natural environment.

5.4 Pathogenicity evaluation on EPF

Pathogenicity tests were carried out using three methods: direct inoculation of *T. molitor* to the fungal pure culture, followed by the insect dipping method and conidia spraying method. For the first method introduced by Grace et al. (2017), insects were inoculated directly to the petri dish with EPF pure culture for 5 mins and then incubated the insects in new petri dish supplemented with food source for the insects. However, no mortality or signs of fungal infection on *T. molitor* after 14 days of monitoring when this method was adopted in this study. This could be due to the lack of moisture of fungal growth during the monitoring period as most of EPF have high occurrence in high moisture condition (Mora, Rouws and Fraga, 2016). Therefore, this method is not recommended for this study.

Besides that, Mathulwe and colleagues (2021) suggested that the sterilization of *T. molitor* using 70% (v/v) ethanol 5 s before dipping into the conidial suspension. This is not recommended as well because *T. molitor* could be drowned to death easily after dipping into 70% (v/v) ethanol. The same results were obtained when performing dipping method in this experiment. Dead *T. molitor* is not desired for EPF pathogenicity test because dead insect cadavers might have a high chance of isolating undesired saprophytes instead of EPF. Besides that, the use of surfactants like Tween 20 or Tween 80 in conidial suspension, suggested by Anand and Tiwary (2009) and Krutmuang and Mekchay (2005) should be avoided to improve conidial adhesion on *T. molitor*, because these surfactants can block the respiratory opening of insects which inhibits the respiratory activities of insects. The pathogenicity test was improved and modified by adopting the conidia suspension spraying method on *T. molitor* (Cheong and Azmi, 2020; Han, et al. 2014). In this study, direct inoculation method and dipping method did not cause any mortality on *T. molitor* with fungal isolates. Two fungal isolates, SK1(a)I1 and SK3(a)I1 showed mortality rate of 100% and 83.33% when using conidial spraying method, indicating the spraying method is a more efficient method for pathogenicity evaluation.

Based on results as shown in Figure 4.11, both *Metarhizium* isolates SK1(a)I1 and SK3(a)I1 showed a significant difference in the mortality rate. The virulence of different fungal isolates might show different virulence properties against targeting insects. The virulence could be influenced by the ability of penetration structure of EPF, namely aappressoria and germ tubes, to penetrate insect (Keyhani, 2018).

Virulence of EPF could be affected by the deletion or mutation of virulence gene that targeting specific proteins involved in cuticle invasion by fungal penetrating structure (Ortiz-Urquiza and Keyhani, 2015). To determine whether they are from different strains of *M. anisopliae*, molecular characterization and several chemical enzymatic tests are necessary to carried out to further study the genomic features relating to virulence against insects and their evolutionary relationships (Wang and Wang, 2017).

5.5 Recommendations for Future Studies

The EPF isolation was conducted using *T. molitor* as insect bait in this research. However, other baiting method such as *G. mellonella* as insect bait and fungal isolation method like soil dilution plating method is recommended to isolate a broad diversity of EPF, as different EPF could be susceptible to different insect hosts as mentioned by Klingen, Eilenberg and Meadow (2002). Besides that, different soil depth has various edaphic conditions as the presence of EPF diversity might be varied. Therefore, EPF from different soil horizon can be studied to investigate relationship between the occurrence of EPF in different soil depth and its physiochemical factors in the recreational forests. Furthermore, the pathogenicity of isolate BB4(a)I2 (*C. rogersoniana*) at high concentration should be repeated to confirm its insect-pathogenic properties. The procedure of conidial production for this fungal isolate suggested by Kapeua-Ndacnou et al. (2023) can be referred as conidial production methods for *Clonostachys* spp. Lastly, future research to study

the EPF abundancy of other region of recreational forests in Perak are required to establish a complete diversity profile of the EPF species.

CHAPTER 6

CONCLUSION

A total of 7 fungal isolates from different genera including *Clonostachys* spp., *Trichoderma* spp. and *Metarhizium* spp. were identified and characterized from three recreational forests in the Kinta Region, Perak. Isolate BB4(a)I2 were identified as *C. rogersoniana*, three fungal isolates, BB4(a)I1, SS1(b)I2 and SS5(b)I1 were identified as *T. spirale*, and isolate SK3(b)I1 were identified as *T. virens* using conidial spraying method. The morphological characteristics of all isolates meet the agreements with published taxonomic keys, and the result of molecular identification are tally with the morphological identification. Two *M. anisopliae* isolates, isolate SK1(a)I1 and SK3(a) I1 were isolated and showed a significant mortality rate of 100% and 83% against *T. molitor*, respectively. Further research is required to evaluate the pathogenicity of isolate BB4(a)I2, the *C. rogersoniana* isolate. Besides that, the occurrence of EPF of other regions of recreational forests in Perak are needed to be studied in the future.

REFERENCES

Adatia, A., Johnson, D. and Entz, S., 2010. Pathogenicity of two new isolates of *Metarhizium anisopliae* from Canadian soil to *Melanoplus bivittatus* (Orthoptera: Acrididae) and *Tenebrio molitor* (Coleoptera: Tenebrionidae). *The Canadian Entomologist*, 142(2), pp.128-134.

Adnan, M., Islam, W., Shabbir, A., Khan, K. A., Ghramh, W., Huang, Z., Chen, H. Y. H. and Lu, G., 2019. Plant defense against fungal pathogens by antagonistic fungi with *Trichoderma* in focus. *Microbial Pathogenesis*, 129, pp. 7-18. DOI: 10.1016/j.micpath.2019.01.042

Ali-Shtayeh, M. S., Mara'i, A. B. B. and Jamous, R. M, 2003. Distribution, occurrence and characterization of entomopathogenic fungi in agricultural soil in the Palestinian area. *Mycopathologia*, 156, pp. 235–244. DOI: 10.1023/A:1023339103522

Alsohaili, S.A. and Bani-Hasan, B.M., 2018. Morphological and molecular identification of fungi isolated from different environmental sources in the northern eastern Desert of Jordan. *Jordan Journal of Biological Sciences*, 11(3), pp.329–337.

Anand, R. and Tiwary, B. N., 2009. Pathogenicity of entomopathogenic fungi to eggs and larvae of *Spodoptera litura*, the common cutworm. *Biocontrol Science and Technology*, 19(9), pp. 919-929. DOI: 10.1080/09583150903205069

Angelone, S. and Bidochka, M. J., 2018. Diversity and abundance of entomopathogenic fungi at ant colonies. *Journal of Invertebrate Pathology*, 156(2018), pp. 73-76. DOI: 10.1016/j.jip.2018.07.009

Araújo, J. P., & Hughes, D. P., 2016. Diversity of entomopathogenic fungi: which groups conquered the insect body? *Advances in genetics*, 94, pp. 1–39. DOI: 10.1016/bs.adgen.2016.01.001

Baiyee, B., Pornsuriya, C., Ito, S. and Sunpapao, A., 2019. *Trichoderma spirale* T76-1 displays biocontrol activity against leaf spot on lettuce (*Lactuca sativa* L.) caused by *Conynespora cassiicola* or *Curvularia aeria*. *Biological Control*, 129, pp. 195-200. DOI: 10.1016/j.biocontrol.2018.10.018.

Bakeri, S. A., Ali, S. R. A., Tajuddin, N. S. and Kamaruzzaman, N. E., 2009. Efficacy of entomopathogenic fungi, *Paecilomyces* spp., in controlling the oil palm bagworm, *Pteroma pendula* (Joannis). *Journal of Oil Palm Research*, 21, pp. 693-699.

Bamisile, B. S., Akutse, K. S., Siddiqui, J. A. and Xu, Y., 2021. Model application of entomopathogenic fungi as alternatives to chemical pesticides: prospects, challenges, and insights for next-generation sustainable agriculture. *Frontiers in Plant Science*, 12, 741804. DOI: 10.3389/fpls.2021.741804

Barelli, L., Moreira, C. C. and Bidochka, M. J., Initial stages of endophytic colonization by *Metarhizium* involves rhizoplane colonization. *Microbiology*, 164, pp. 1531-1540. DOI: 10.1099/mic.0.000729

Beattie, A.J., Hay, M., Magnusson, B., de Nys, R, Smeathers, J. and Vincent, J. F. V., 2011. Ecology and bioprospecting. *Austral Ecology*, 36(3), pp. 341-356. DOI: 10.1111/j.1442-9993.2010.02170.x

Behie, S. W., Bidochka, M. J., 2014. Ubiquity of insect-derived nitrogen transfer to plants by endophytic insect-pathogenic fungi: an additional brance of the soil nitrogen cycle. *Applied and Environmental Microbiology*, 8, pp.1553-1560. DOI: 10.1128/AEM.03338-13

Castillo, L. P., Osorio, A., Vargas, N., Sanjuan, T., Grajales, A. and Restrepo, S., 2018. Genetic diversity of the entomopathogenic fungus *Cordyceps tenuipes* in forests and butterfly gardens in Quindío, Colombia. *Fungal Biology*, 122(9), pp. 891-899. DOI: 10.1016/j.funbio.2018.05.003.

Chandler, D., Hay, D. and Reid, A. P., 1997. Sampling and occurrence of entomopathogenic fungi and nematodes in UK soils. *Applied Soil Ecology*, 5, pp. 133-141.

Chang, J.C., Wu, S.S., Liu, Y.C., Yang, Y.H., Tsai, Y.F., Li, Y.H., Tseng, C.T., Tang, L.C. and Nai, Y.S., 2021. Construction and selection of an entomopathogenic fungal library from soil samples for controlling *Spodoptera litura*. *Frontiers in Sustainable Food Systems*, 5(February), pp.1–14.

Cheong, J. L. and Azmi, W. A., 2020. Dataset on the influence of relative humidity on the pathogenicity of *Metarhizium anisopliae* isolates from Thailand and Malaysia against red palm weevil (*Rhynchophorus ferrugineus*, Olivier) adult. *Data in Brief*, 30, 105482. DOI: 10.1016/j.dib.2020.105482

De Oliveira, G. P., Barreto, D. L., C., Silva, M. R., Augusti, R., Marriel, I. E., de Paula Lana, U. G., Takahashi, A., 2022. Biotic stress caused by in vitro co-inoculation enhances the expression of acetylcholinesterase inhibitors by fungi. *Natural Product Research*, 36(16), pp.4266-4270. DOI: 10.1080/14786419.2021.1975701

Elham, M. S. H., Pong, K. K., Grace, L. E. L., Ishak, I. and Azmi, W. A., 2018. Occurrence Of Entomopathogenic fungus, *Metarhizium Anisopliae* isolated from island, BRIS and coastal soils of Terengganu, Malaysia. *Journal of Sustainability Science and Management*, 5, pp. 179-189. doi: 10.1007/s11676-018-0614-9

Mora, E. M. A., Rouws, C. J. R. and Fraga, M. E., 2016. Occurrence of entomopathogenic fungi in Atlantic forest soils. *Microbiology Discovery*, 4(1), pp.1.

Fong, J. H., Addis, S. N. K. and Azmi, W. A., 2018. Virulence of evaluation of entomopathogenic fungi against the red palm weevil, *Rhynchophorus ferrugineus* (Coleoptera: Dryopthoridae). *Malaysian Applied Biology*, 47(5), pp. 25-30.

Gebremariam, A., Chekol, Y., & Assefa, F., 2022. Extracellular enzyme activity of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* and their pathogenicity potential as a bio-control agent against whitefly pests, *Bemisia tabaci* and *Trialeurodes vaporariorum* (Hemiptera: Aleyrodidae). *BMC research notes*, 15(117), pp. 1-6. DOI: 10.1186/s13104-022-06004-4

Grace, L. E. L., Halim, J. M. S., Ahmad, M. F. and Azmi, W. A., 2017. Entomopathogenic fungi isolated from the soil of Terengganu, Malaysia as potential bio-pesticides against the red palm weevil, *Rhynchophorus ferrugineus. Journal of Sustainability Science and Management*, 12(2), pp. 71-79.

Gryganskyi, A. P., Golan, J. and Hajek, A. E., Season-long infection of diverse hosts by the entomopathogenic fungus *Batkoa major*. *PLoS ONE*, 17(5), e0261912. DOI: 10.1371/journal.pone.0261912

Han, J. H., Jin, B. R., Kim, J. J. and Lee, S. Y., 2014. Virulence of entomopathogenic fungi, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* for the Microbial Control of *Spodoptera exigua*, *Mycobiology,* 42(4), pp. 385-390. DOI: 10.5941/MYCO.2014.42.4.385

Han, P., Zhang, X., Xu, D., Zhang, B., Lai, D. and Zhou, L., 2020. Metabolites from *Clonostachys* fungi and their biological activities. *Journal of Fungi*, 6(4), pp. 1-25. DOI: 10.3390/jof6040229

Hoe, P., Bong, C., Jugah, K. and Rajan, A., 2009. Evaluation of *Metarhizium anisopliae* var. anisopliae (Deuteromycotina: Hyphomycete) Isolates and their Effects on Subterranean Termite *Coptotermes curvignathus* (Isoptera: Rhinotermitidae). *American Journal of Agricultural and Biological Sciences*, 4(4), pp. 289-297. DOI: 10.3844/ajabssp.2009.289.297

Humber, R. A., 2012. Identification of entomopathogenic fungi. In L. A. Lacey, ed, 2012. *Manual of Techniques in Invertebrate Pathology*. Academic Press: London. pp. 151-187. DOI: 10.1016/B978-0-12-386899-2.00006-3.

Islam, M.T. and Shabanimofrad, D. O. M., 2014. Molecular identification and virulence of six isolates of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) to *Bemisia tabaci* Q biotype. *Journal of Asia-Pacific Entomology*, 17(3), pp. 237-241. DOI: 10.1016/j.aspen.2014.01.008

Jaronski, S. T., 2023. Mass production of entomopathogenic fungi – state of the art. *Mass production of Beneficial Organisms (Second Edition)*. pp. 317-357. DOI: 10.1016/B978-0-12-822106-8.00017-8

Javed, K., Javed, H., Mukhhtar, T. and Qiu, D., 2019. Efficacy of *Beauveria bassiana* And *Verticillium lecanii* for the management of whitefly and aphid. *Pakistan Journal of Agricultural Science*, 56(3), pp.669-674. DOI: 10.2162/PAKJAS/19.8396

Jessica, J. J. Peng, T. L., Sajap, A. S., Lee, S. H., Syazwan, S. A., 2018. Evaluation of the virulence of entomopathogenic fungus, *Isaria fumosorosea* isolates against subterranean termites *Coptotermes* spp. (Isoptera: Rhinotermitidae).

Journal of Forestry Research, 30(1), pp. 213-218. DOI:10.1007/s11676-018-0614-9

Kapeua-Ndacnou, M., de Abreu, L.M., de Macedo, D.M., da Nóbrega, T.F., Pereira, C.M., Evans, H.C. and Barreto, R.W., 2023. Assessing the biocontrol potential of *Clonostachys* species isolated as endophytes from *Coffea* species and as mycoparasites of *Hemileia* rusts of coffee in Africa. *Journal of Fungi*, 9(2), pp. 1-24.

Keyhani, N. O., 2018. Lipid Biology in fungal stress and virulence: entomopathogenic fungi. *Fungal Biology*, 122(6), pp. 420-429. DOI: 10.1016/j.funbio.2017.07.003

Kin, P. K., Azmi, W. A., Kamarudin, N., Ali, S. R. A. and Moslim, R., 2017. The occurrence of entomopathogenic fungi on mineral and peat soils in peninsular Malaysia. *American Journal of Agricultural and Biological Science*, 12(1), pp. 1-12. DOI: 10.3844/ajabssp.2017.1.12

Klieber, J., Reineke, A., 2016. The entomopathogen *Beauveria bassiana* has epiphytic and endophytic activity against the tomato leaf miner *Tuta absoluta*. *Journal of Applied Entomology*, 140, pp.580-589. DOI: 10.1111/jen.12287.

Klingen, I., Eilenberg, J. and Meadow, R., 2002. Effects of farming system, field margins and bait insect on the occurrence of insect pathogenic fungi in soils. *Agriculture, ecosystems & environment*, 91(1-3), pp.191-198.

Kovač, M., Tkaczuk, C. and Pernek, M., 2021. first report of entomopathogenic fungi occurrence in forest soils in Croatia. *Forests*, 12(12), 1690. DOI: 10.3390/f12121690

Krutmuang, P. and Mekchay, S., 2005. Pathogenicity of entomopathogenic fungi *Metarhizum anisopliae* against termites. *Conference on International Agriculture Research for Development.*

Malsam, O., Kilian, M., Oerke, E., and Dehne, H., 2002. Oils for increased efficacy of *Metarhizium anisopliae* to control whiteflies. *Biocontrol Science and Technology*, 12(3), pp. 337-348. DOI:10.1080/09583150220128121

Mantzoukas, S., Kitsiou, F., Natsiopoulos, D. and Eliopoulos, P.A., 2022. Entomopathogenic fungi: interactions and applications. *Encyclopedia*, 2(2), pp. 646–656.

Martin, K. J. and Rygiewicz, P. T., 2005. Fungal specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC Microbiology*, 5(28), pp. 1-11. DOI: 10.1186/1471-2180/28

Mathulwe, L.L., Jacobs, K., Malan, A.P., Birkhofer, K., Addison, M.F. and Addison, P., 2021. Characterisation of *Metarhizium majus* (Hypocreales: Clavicipitaceae) isolated from the Western Cape Province, South Africa. *PLoS ONE*, 16(2 Febuary), pp.1–11. DOI: 10.1371/journal.pone.0240955.

Meyling, N.V. and Eilenberg, J., 2006 Occurrence and distribution of soil borne entomopathogenic fungi within a single organic agroecosystem. *Agriculture, Ecosystems and Environment*, 113(1-4), pp.336-341. DOI: 10.1016/j.agee.2005.10.011

Monte, E., 2023. The sophisticated evolution of *Trichoderma* to control insect pests. *Applied Biological Science*, 120(12), pp. 1-3. DOI: 10.1073/pnas.2301971120

Mora, M.A. E., Castilho, A. M. C. and Fraga, M. E., 2018. Classification and infection mechanism of entomopathogenic fungi. *Arquivos do Instituto Biológico*, 84(0). DOI:10.1590/1808-1657000552015

Morán-Diez, M. E., de Alba, A. E. M., Rubio, M. B, Hermosa, R. and Montem E., 2021. *Trichoderma* and the plant heritable priming responses. *Journal of Fungi*, 7(318), pp.1-23.

Nandhini, B. and Josephine, R.M., 2013. Original Research Article A study on bacterial and fungal diversity in potted soil. *International Journal of Current Microbiology and Applied Science*, 2(2), pp.1–5.

Ong, S., Ahmad, H., Majid, A. H. A. and Jaal, Z., 2017. Interaction Between *Metarhizium anisopliae* (Met.) and the insecticides used for controlling house fly (Diptera: Muscidae) in poultry farm of Malaysia. *Journal of Medical Entomology*, 54(6), pp. 1626–1632, DOI: 10.1093/jme/tjx128

Oreste, M., Bubici, G., Poliseno, M., Triggiani, O. and Tarasco, E., 2012. Pathogenicity of *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Metarhizium anisopliae* (Metschn.) Sorokin against *Galleria mellonella* L. and *Tenebrio molitor* L. in laboratory assays. *Redia*, *95*, pp.43-48.

Ortiz-Urquiza, A., & Keyhani, N. O., 2015. Stress response signaling and virulence: insights from entomopathogenic fungi. *Current genetics*, 61(3), pp. 239–249. DOI: 10.1007/s00294-014-0439-9

Oyemitan, I. A., 2017. African medicinal spices and genus *Piper*. In: V, Kuete, ed. Medicinal Spices and Vegetables from Africa. Amsterdam: Elsevier, pp. 581-597. DOI: 10.1016/B978-0-12-809286-6.00027-3

Pathan, E, K., Patil, A. and Deshpande, M., 2019. Bioprospecting of entomo- and myco-pathogen. In: T. Satanarayana, S. K. Deshmukh and M. Deshpande, 2019. *Advancing Frontiers in Mycology & Mycotechnology*. Springer Nature Singapore Pte Ltd.

Perumal, M., Wasli, M.E., Ying, H.S., Lat, J. and Sani, H., 1970. Soil morphological and physicochemical properties at reforestation sites after enrichment planting of *Shorea macrophylla* in Sampadi Forest Reserve, Sarawak, Malaysia. *Borneo Journal of Resource Science and Technology*, 5(2), pp.28–43.

Poveda, J., 2021. *Trichoderma* as biocontrol agent against pests: new uses for a mycoparasite. *Biological Control*, 159(104634). DOI: 10.1016/j.biocontrol.2021.104634.

Praprotnik, E., Lončar, J., & Razinger, J., 2021. Testing virulence of different species of insect associated fungi against yellow mealworm (Coleoptera: Tenebrionidae) and their potential growth stimulation to maize. *Plants (Basel, Switzerland)*, *10*(11), 2498. DOI: 10.3390/plants10112498

Quesada-Moraga, E., Navas-Cortés, J. A., Maranhao, E. A. A., Ortiz-Urquiza, A. and Santiago-Álvarez, C., 2007. Factors affecting the occurrence and distribution of entomopathogenic fungi in natural and cultivated soils. *Mycological Research*, 111(8), pp. 947-966. DOI: 10.1016/j.mycres.2007.06.006

Raja, H. A., Miller, A. N., Pearce, C. J. and Oberlies, N. H., 2017. Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community. *Journal of Natural Products*, 80(3), pp.756-770. DOI: 10.1021/acs.jnatprod.6b01085

Romão-Dumaresq, A. S., Dourado, M. N., Fávaro, L. C., Mendes, R., Ferreira, A., & Araújo, W. L., 2016. Diversity of Cultivated Fungi Associated with Conventional and Transgenic Sugarcane and the Interaction between Endophytic *Trichoderma virens* and the Host Plant. *PloS one*, *11*(7), e0158974. DOI: 1371/journal.pone.0158974

Sani, I., Jamian, S., Ismail, S. I., Saad, N., Abdullah, Sumaiyah, A., Hata, E. M., Kamaruddin, M. A. and Jalinas, J., 2022. Identification of entomopathogenic fungi *Metarhizium anisopliae* and *Purpureocillium lilacinum* from oil palm plantation soils in Universiti Putra Malaysia. *Malaysian Journal of Microbiology*, 18(1), pp. 105-112.

Sani, I., Jamian, S., Saad, N., Abdullah, S., A., Hata, E. M., Jalinas, J. and Ismail, S. I., 2023. Identification and virulence of entomopathogenic fungi, *Isaria javanica* and *Purpureocillium lilacinum* isolated from the whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) in Malaysia. *Egyptian Journal of Biological Pest Control*, 33(14), pp. 1-12. https://doi.org/10.1186/s41938-023-00657-4

Shakeri, J. and Foster, H. A., 2007. Proteolytic activity and antibiotic production by *Trichoderma harzianum* relation to pathogenicity to insects. *Enzyme and Microbial Technology*, 40(4), pp. 961-968. DOI: 10.1016/j.enzmitec.2006.07.041.

Senthilkumar, M., Amaresan, N., Sankaranarayanan, A., 2021. *Galleria* bait technique for the isolation of entomopathogenic fungi from soil. In: Plant-Microbe Interactions. *Springer Protocols Handbooks*. Humana, New York, NY. DOI: 10.1007/978-1-0716-1080-0_73

Seo, J., Jin, B., Shin, S., Park, H., Lee, B., Chang, K., Kang., S., 1995. Isolation of entomopthogenic fungi for infection to the opine gall midges, *Thecodiplosis*

japonensis from the forest soil in Korea. *Korean Journal of Applied Entomoplogy*, 34(4), pp. 368-372.

Shah, P. A. and Pell, J. K., 2003. Entomopathogenic fungi as biocontrol agents. *Applied Microbiology and Biotechnology*, 61, pp. 413-423. DOI: 10.1007/s00253-003-1240-8

Shahid, A. A., Rao, A. Q., Bakhsh, A. and Husnain, T., 2012. Entomopathogenic fungi as biological controllers: New Insights into their virulence and pathogenicity. *Archives of Biological Sciences*, 64(1), pp. 21-42. DOI:10.2298/ABS1201021S

Sharma, L., Oliveira, I., Torres, L. and Marques, G., 2018. Entomopathogenic fungi in Portuguese vineyards soils: Suggesting a '*Galleria-Tenebrio*-bait method' as bait-insects galleria and tenebrio significantly underestimate the respective recoveries of *Metarhizium (robertsii*) and *Beauveria (bassiana*). *MycoKeys*, 38, pp.1–23.

Sharma, R., Sharma, P., 2021. Fungal entomopathogens: a systematic review. *Egyptian Journal Biological Pest Control*, 31(57), pp. 1-13. DOI: 10.1186/s41938-021-00404-7

Shen, C., Wang, J., He, J., Yu, F. and Ge, Y., 2021. Plant diversity enhances soil fungal diversity and microbial resistance to plant invasion. *Applied and Environmental Microbiology*, 87(11), e00251-21.

Singh, D., Raina, T. K. and Singh, J., 2017. Entomopathogenic fungi: An effective biocontrol agent for management of insect populations naturally. *Journal of Pharmaceutical Sciences and Research*, *9*(6), pp. 830-839.

St. Leger, R. J. and Wang, C., 2010. Genetic engineering of fungal biocontrol agents to achieve efficacy against insect pests. *Applied Microbiology and Biotechnology*, 85, pp. 901-907.

Sun, M . H., Chen, Y. M., Liu, J. F., Li, S. D. and Ma, G. Z., 2014. Effects of culture conditions on spore types of *Clonostachys rosea* 67-1 in submerged fementation. *Letters in Applied Microbiology*, 58(4), pp. 318-324. DOI: 10.1111/lam.12193

Tuininga, A. R., Miller, J. L., Morath, S. U., Daniels, T.J., et al. 2009. Isolation of entomopathogenic fungi from soils and *Ixodes scapularis* (acari: ixodidae) ticks: prevalence and methods. *Journal of Medical Entomology*, 46, pp. 557–565. DOI: 10.1603/033.046.0321

Tyśkiewicz, R., Nowak, A., Ozimek, E., Jaroszuk-Ściseł, J., 2022. *Trichoderma*: The current status of its application in agriculture for the biocontrol of fungal phytopathogens and stimulation of plant growth. *International Journal of Molecular Sciences*, 23(4), 2329. DOI: 10.3390/ijms23042329

Van der Wal, A., Geydan, T.D., Kuyper, T.W. and De Boer, W., 2013. A thready affair: Linking fungal diversity and community dynamics to terrestrial decomposition processes. *FEMS Microbiology Reviews*, 37(4), pp.477–494.

Vejar-Cota, G., García-Gutiérrez, C., Rosas-García, N. M., Escobedo-Bonilla, C. M. and González-Ocampo, H. A., 2017. Morphological and molecular characterization of entomopathogenic fungi with potential to control sugarcane borers at Sinaloa. *Southwestern Entomologist*, 42(2), pp. 395-400. DOI: 10.3958/059.042.0208

Velavan, V., Rangeshwaran, R., Sivakumar, G., Sasidharan, T.O., Sundararaj, R. and Kandan, A., 2021. Occurrence of *Metarhizium* spp. isolated from forest samples in South India and their potential in biological control of banana stem weevil *Odoiporus longicollis* Oliver. *Egyptian Journal of Biological Pest Control*, 31(1), 1-12. DOI:

Vivekanandhan, P., Bedini, S. and Shivakumar, M. S., 2020. Isolation and identification of entomopathogenic fungus from Eastern Ghats of South Indian forest soil and their efficacy as biopesticide for mosquito control. *Parasitology International*, 76(2020), 102099. DOI: 10.1016/j.parint.2020.102099.

Wakil, W., Yasin, M. and Shapiro-Ilan, D., 2017. Effects of single and combined applications of entomopathogenic fungi and nematodes against *Rhynchophorus ferrugineus* (Olivier). *Scientific Reports*, 7(5971), pp. 1-11. DOI: 10.1038/s41598-017-05615-3

Wang, C. and Wang, S., 2017. Insect pathogenic fungi: Genomics, molecular interactions, and genetic improvements. *Annual Review of Entomology*, 62(2017), pp. 73-90. DOI: 10.1146/annurev-ento-031616-035509

Wasti, I. G., Foo, S.F., Tan, Q.Z.,Cheh, W.M., Mohammad, H.S.K., Mahadimenakbar, M. D., Noor, H.H., Subbiah, V.K., Faisal, A.A.K., Seelan, J.S.S., 2020. Fungi from dead arthropods and bats of Gomantong Cave, northern Borneo, Sabah (Malaysia). *Journal of Cave and Karst Studies*, 82(4), pp.261-275. DOI: 10.4311/2019MB0146

Wei, D.P., Wanasinghe, D.N., Xu, J.C., To-anun, C., Mortimer, P.E., Hyde, K.D., Elgorban, A.M., Madawala, S., Suwannarach, N., Karunarathna, S.C., Tibpromma, S. and Lumyong, S., 2021. Three Novel Entomopathogenic Fungi From China and Thailand. *Frontiers in Microbiology*, 11(January).

White, T. J., Bruns, T., Lee, S. and Taylor, J. W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M. A., Gelfand D. H., Sninsky J.J, White T. J., ed. *PCR Protocols: A Guide to Methods and Applications*. New York: Academic Press Inc; 1990. pp. 315–322.

Wintachai, P., Phaonakrop, N., Roytrakul, S., Naknaen, A., Pomwised, R., Voravuthikunchai, S.P., Surachat, K. and Smith, D.R., 2022. Enhanced antibacterial effect of a novel Friunavirus phage vWU2001 in combination with colistin against carbapenem-resistant *Acinetobacter baumannii*. *Scientific Reports*, 12(1), pp.1–20.

Yao, X., Guo, H., Zhang, M., Ruan, J. and Chen, J., 2023. *Trichoderma* and its role in biological control of plant fungal and nematode disease. *Frontiers in Microbiology*, *14*, pp.1-15. DOI: 10.3389/fmicb.2023.1160551

Yasanthika, W.A.E., Wanasinghe D.N., Mortimer P.E., Monkai, J. and Farias A.R.G., 2022. The importance of culture-based techniques in the genomic era for assessing the taxonomy and diversity of soil fungi. Mycoshere, 13(1), pp. 724-751. DOI: 10.5943/mycosphere/13/1/8.

Zeilinger, S., Reithner, B., Scala, V., Peissl, I., Lorito, M. and Mach, R.L., 2005. Signal transduction by Tga3, a novel G protein α subunit of Trichoderma atroviride. *Applied and environmental microbiology*, *71*(3), pp.1591-1597.

Zhai, M., Qi, F., Li, J., Jiang, C., Hou, Y., Shi, Y., Di, D., Zhang, J. and Wu, Q., 2016. Isolation of secondary metabolites from the soil-derived fungus *Clonostachys rosea* YRS-06, a Biological Control Agent, and Evaluation of Antibacterial Activity. *Journal of Agricultural and Food Chemistry*, 64(11), pp. 2298-2306. DOI: 10.1021/acs.jafc.6b00556

Zimmermann, G., 2007. Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Science and Technology*, 17, pp. 879-920. DOI: 10.1080/09583150701593963

Zin, N. A. and Badaluddin, N. A., 2020. Biological functions of *Trichiderma* spp. for agriculture applications. *Annals of Agricultural Sciences*, 65(2), pp. 168-178. DOI: 10.1016/j.aoas.2020.09.003