MORPHOLOGICAL CHARACTERIZATION AND SEQUENCE ANALYSIS OF 5.8S-ITS REGION OF *Trichoderma* SPECIES

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MORPHOLOGICAL CHARACTERIZATION AND SEQUENCE ANALYSIS OF 5.8S-ITS REGION OF *Trichoderma* SPECIES

By

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ABSRACT

MORPHOLOGICAL CHARACTERIZATION AND SEQUENCE ANALYSIS OF 5.8S-ITS REGION OF *Trichoderma* SPECIES

Tan Siew Hui

Recently, the use of biological control agents (BCAs) has gained its popularity in agriculture as a way to decrease the application of synthetic pesticides. In the genus *Trichoderma,* a great number of fungal strains have been studied and utilized as BCAs. In the present study, 11 *Trichoderma* isolates were isolated from rhizosphere soils, humus and compost. These isolates were characterized and identified by morphological characterization and sequence analysis of 5.8S-ITS region. The morphological characteristics examined include the colony appearance, growth rate at 28℃ and 35℃ on Potato Dextrose Agar (PDA) and Cornmeal Dextrose Agar (CMD), the shapes and sizes of conidia, the branching patterns of conidiophores and phialides, the production of chlamydospores, the production of sweet coconut odour by colony and the ability of isolate to form pustules on CMD. In molecular approach, the 5.8S-ITS sequences were compared to GenBank and *Trich*OKEY database for species identification. The results from morphological and molecular characterization were found to be in agreement. Among the 11 isolates, five *T. harzianum*, four *T. asperellum*, one *T. virens*

and one *T. strigosum* were identified. The antagonistic effects of these *Trichoderma* isolates were also tested against two pathogenic fungi, *Fusarium oxysporum* and *Fusarium solani*. Dual culture technique was employed and the percentage of inhibition (I%) on the mycelial growth of pathogenic fungi by *Trichoderma* isolates were determined. Isolate PPY1, which was identified as *T. asperellum*, could be a potential BCA as it shows the highest antagonistic effect against both *F. oxysporum* and *F. solani*, with inhibition percentage of 66.67% and 65.52%, respectively.

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Last but not least, I would like to take this opportunity to express my gratitude to my family and friends for their on-going supports and encouragement.

DECLARATION

I hereby declare that the 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 used previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

TAN SIEW HUI

APPROVAL SHEET

This project report entitled "**MORPHOLOGICAL CHARACTERIZATION AND SEQUENCE ANALYSIS OF 5.8S-ITS REGION OF *Trichoderma* SPECIES**" was prepared by TAN SIEW HUI and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) in Biotechnology at Universiti Tunku Abdul Rahman.

Approved by,

_____

(Ms Leong Sau Kueen) Date:…….

Supervisor

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Date: _____

PERMISSION SHEET

It is hereby certified that **TAN SIEW HUI** (ID No: **09ADB03477**) has completed this final year project entitled **"MORPHOLOGICAL CHARACTERIZATION AND SEQUENCE ANALYSIS OF 5.8S-ITS REGION OF *Trichoderma* SPECIES"** under supervision of Ms Leong Sau Kueen from the Department of Biological Science, Faculty of Science.

I hereby give permission to my supervisor to write and prepare manuscripts of these research findings for publication in any form, if I do not prepare it within six (6) months from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisor.

Yours truly,

_____
(TAN SIEW HUI)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
BCA	Biological control agent
BLAST	Basic Local Alignment Search Tool
CMD	Cornmeal Dextrose Agar
CWDEs	Cell wall degrading enzymes
DNA	Deoxyribonucleic acid
ITS	Internal transcribed spacer
IGS	Intergenic spacer
L/W	Length / Width
MEA	Malt Extract Agar
$MgCl_2$	Magnesium Chloride
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
RAPD	Random Amplified Polymorphic DNA
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
RFLPs	Restriction Fragment Length Polymorphisms
spp.	Species
WA	Water Agar

CHAPTER 1

INTRODUCTION

In the past 50 years, global population had increased more than double and it is expected to increase over 9 billion by 2050. The rapidly growing human population needs an increase in agricultural production. However, the emergence of plant diseases raised the difficulty of this challenge (Boyd et al., 2012). Plant diseases had greatly reduced the production of many major crops, including potato, rice, barley, wheat, and soybean (Chakraborty et al., 1999). Abiotic stresses such as climate changes or biotic stresses like fungi, bacteria, viruses, nematodes and phytoplasma contributed to the development of plant diseases (Anderson et al., 2004). Among the plant pathogens, Ellis et al. (2008) reported that fungi and fungal-like organisms cause more plant diseases compared to other groups of plant pests.

Many fungal plant pathogens were reported to cause plant diseases, and greatly reduce the production of economically important crops. For instance, *Fusarium oxysporum* f. sp. *cubense*, a soilborne fungus, was shown to cause Panama disease in banana. Between 1940 and 1960, Panama disease caused 30,000 hectares lost in the Ulua Valley of Honduras (Ploetz 2005). In 2001, the cocoa production worldwide was greatly reduced due to major diseases, including black pot disease, witches' broom and frosty pod rot disease. The estimated annual reduction in potential cocoa production is 810,000 tons, which cost $761 million (Bowers et al., 2001). On the other hand, the

Fusarium dry rot disease that caused by some species of *Fusarium*, such as *F. solani* and *F. oxysporum*, causes potato yield losses in storage, ranged from 6% to 25% annually. In severe cases, the potato yield losses due to Fusarium dry rot disease could be as high as 60% (Al-Mughrabi 2010). Besides that, rice blast disease caused by *Magnaporthe oryzae* is estimated to cost an estimated $66 billion in annual losses worldwide (Wang et al., 2012).

Plant diseases affect plants in the field as well as post-harvested crops. Apart from reducing crop yields, some of the species like *Fusarium, Aspergillus, Penicillum,* and *Alternaria* may produce mycotoxins which downgrade the crops (Medina et al., 2006). Aflatoxin, a mycotoxin which is produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* infects drought-stressed maize and groundnuts in the field. The consumption of aflatoxin-contaminated crops is related to several human diseases, including liver cancer, chronic gastritis, kwashiorkor, and Reye's syndrome (Bhat and Miller 1991). Other than that, *Fusarium* head blight pathogens, *Fusarium graminearum* and *Fusarium culmorum* may produce trichothecenes which contaminate wheat, barley and maize. These mycotoxins are able to cause toxicoses such as dermatitis, vomiting, immunosuppression and hemorrhagic septicemia (Kimura et al., 2006).

Various strategies have been used to control and manage plant diseases, including the application of pesticides (Pedlowski et al., 2011; Valenciano et al., 2006), development of disease-resistant plants (Gururani et al., 2012; Tian et al., 2008) and utilization of biological control agents (Vinale et al., 2007;

Al-Mughrabi, 2010). Although chemical pesticides are effective in increasing crop yields and combating pests and plant diseases, the excessive use of pesticides can lead to contamination of land and water. Moreover, many components of pesticides are recalcitrant and tend to persist in the environment for long period of time (Hai et al., 2012). Besides that, it is not cost effective to use chemical pesticides in the long run. With the price of around $40 billion, about 3 billion kg of pesticides are applied per year worldwide (Pimentel 2009). Another alternative, which is the use of disease-resistant plant, does eliminate the input of pesticides. However, it is a time consuming process for conventional breeding for resistance and the integration of resistance genes from one species to the gene pool of another by repeated backcrossing. Usually, it takes many hybrid generations for backcrossing to occur (Gururani et al., 2012).

Recently, the application of biological control agents (BCAs) in agriculture has gained popularity as a way to reduce or eliminate the use of synthetic pesticides (Vinale et al., 2007). They act against plant pathogens in several ways, either by mycoparasitism, antibiotic-mediated suppression, lytic enzymes and other byproducts production, competition for nutrient, or induction of host resistance (Pal and Gardener 2011). A number of BCAs are now available commercially for discrete usage in disease control, or incorporate with reduced amount of chemical pesticides in the control of plant pathogens. These BCAs include strains belonging to fungal genera such as *Trichoderma, Candida* and *Gliocladium,* and bacterial genera such as *Bacillus*

and *Pseudomonas* (Vinale et al., 2007; Tarantino et al., 2007; Melnick et al., 2008; Validov et al., 2009).

Among the BCAs, *Trichoderma* species are the most intensively studied species (Morgan 2011). They are the most isolated soil-borne fungi commonly found in plant root ecosystem (Vinale et al., 2007). Besides that, these opportunistic, avirulent plant symbionts are antagonistic towards many phytopathogenic fungi. Depending upon the strain, the application of *Trichoderma* is proven to improve root and plant growth, as well as to induce resistance in plants (Harman et al., 2004).

In order to utilize the full potential of *Trichoderma* species in specific applications, precise identification and characterization of these fungi is vital (Lieckfeldt et al., 1999). The present study was carried out to characterize and identify *Trichoderma* species isolated from rhizosphere soils, humus and compost from Kampar and Penang by using morphological characterization and sequence analysis of the internal transcribed spacer (ITS) region. Morphological studies were carried out based on the colony appearance and pigmentation, growth rate, and microscopic features such as branching patterns of conidiophores, the arrangement of phialides, and the shapes and sizes of conidia. The 5.8S-ITS regions of the *Trichoderma* isolates were amplified using primer pair ITS1 and ITS4. Then, the sequences of 5.8S-ITS region were compared to GenBank and a specific database for *Trichoderma* using BLAST and *Trich*OKEY, respectively. Other than that, the antagonistic

effects of *Trichoderma* isolates were tested against *Fusarium oxysporum* and *Fusarium solani*.

CHAPTER 2

LITERATURE REVIEW

2.1 *Trichoderma*

Trichoderma species are green-spored ascomycetes present in nearly all types of temperate and tropical soils. They can often be found in decaying plant material and in the rhizosphere of plants (Schuster and Schmoll 2010). Their diverse metabolic capability and aggressively competitive nature made them as the successful colonizers of their habitats (Gams and Bissett 2002).

Hypocrea, the teleomorphs of *Trichoderma,* are first decribed by Tulasne brothers in 1865 (Gams and Bissett 2002). Now, increasing numbers of *Trichoderma* species have been linked to their teleomorphs. For example, *T. virens* is the anamorph of *H. virens, and T. harzianum* is the anamorph of *H. lixii*. Yet, there are some common species like *T. asperellum* have not been linked to a teleomorph and they may be clonal (Samuels 2006).

2.2 Taxonomy of *Trichoderma*

The taxonomy of *Trichoderma* was first described by Persoon in his classification of fungi in 1794. Unfortunately, his classification of *Trichoderma* was problematic where his observation included other fungi such as *Puccinia, Mucor, Ascobolus* and some slime molds such as *Physarum, Trichia* and *Stemonitis* (Klein and Eveleigh 2002). In 1939, Bisby proposed

that *Trichoderma* consists of a single species, *T. viride*. This concept led to nearly all strains of *Trichoderma* was identified as "*T. viride*" in literatures before 1969. Therefore, most of the taxa determined before 1969 are probably misidentified since *T. viride* is a relatively rare species (Druzhinina and Kubicek 2004).

In 1969, Rifai proposed the concept of "aggregate" species, where *Trichoderma* species are divided into nine "species aggregates", namely *T. aureoviride* Rifai, *T. hamatum* Bain, *T. harzianum* Rifai, *T. koningii* Oudem, *T. longibrachiatum* Rifai, *T. piluliferum* Rifai, *T. polysporum* Rifai, *T. pseudokoningii* Rifai and *T. viride*. However, Rifai admitted that each species aggregate was likely to contain more than one morphologically indistinguishable species (Chaverri and Samuels 2004).

Starting from 1984, Bissett started to revise Rifai's aggregate species. In 1991, Bissett discussed the difficulty to distinguish *Trichoderma* species based on Rifai's species aggregates, since only five of Rifai's aggregates species (*T. harzianum, T. longibrachiatum, T. pseudokoningii, T. piluliferum* and *T. polysporum*) were narrowly defined, while other aggregates were having relatively large number of species (Chaverri and Samuels 2004). In the same year, Bissett subdivided the genus into five sections, which are *Longibrachiatum, Trichoderma, Pachybasium, Saturnisporum* and *Hypocreanum* (Druzhinina and Kubicek 2004).

Later, with the advent of molecular techniques the morphology-based taxonomy of *Trichoderma* was reevaluated. The molecular markers used in the study of *Trichoderma* taxonomy include protein markers (isozyme analysis) and DNA markers. The strategies to identify *Trichoderma* using DNA markers are sequence analysis of internal transcribed spacer (ITS) region, restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), and chromosome and karyotyping analysis (Lieckfeldt et al., 2002). In recent years, the development of *Trich*OKEY and *Tricho*BLAST facilitate the identification of *Trichoderma* and *Hypocrea* based on oligonucleotide DNA barcode. *Trich*OKEY is a program used to identify *Trichoderma* and *Hypocrea* based on several genus-specific hallmarks located within the ITS1 and ITS2 sequences (Druzhinina et al., 2005). *Tricho*BLAST is a database supported by sequence diagnosis and similarity search tools based on those frequently used phylogenetic markers, including ITS1 and 2, intron tef1_int4 and intron tef1_int5 (Kopchinskiy et al., 2005). By 2006, International Submission on *Trichoderma* and *Hypocrea* Taxonomy listed 104 *Hypocrea/Trichoderma* species which have been characterized at molecular level (Druzhinina and Kopchinskiy 2006).

2.3 Morphology of *Trichoderma*

Since 1969, morphological characteristics have been used to characterize and distinguish *Trichoderma* species (Gams and Bissett 2002). Besides that, Samuels et al. (2002a) also provided detailed observations on the morphological characters of defined species in *Trichoderma*.

2.3.1 Macroscopic Features

Certain colony characters like growth rate, pigmentation, pustules formation and odours can be characteristics of a species. However, colony appearance does not provide sufficient information for characterization due to the difficulty to establish a precise description (Gams and Bissett 2002).

According to Samuels et al. (2002a), majority of the *Trichoderma* cultures grow rapidly at 25 ℃ to 30 ℃ and typically not growing at 35 ℃. Yet, some species grow well at 35 ℃. This served as an important distinguishing criterion between morphologically similar species. For example, *T. harzianum* can be distinguished from morphologically similar species such as *T. aggressivum* and *T. atroviride* by growing them at 35 ℃. After 96 hours, neither *T. aggressivum* nor *T. atroviride* can have colony radius more than 5 mm while *T. harzianum* grows well and sporulates at 35 ℃ (Samuels 2004).

Characteristics of mycelia development and pigmentation can be better observed in rich medium like Potato Dextrose Agar (PDA). The colonies are white on rich media such as PDA and transparent on Cornmeal Dextrose Agar (CMD) (Samuels et al., 2002). Scattered blue-green or yellow-green patches become observable when conidia are formed. Occasionally, concentric rings made by these patches can be observed. Reverse of the colonies are pale, tan or yellowish (Rex et al., 2001). The colonies of some *Trichoderma* on PDA are shown in Figure 2.1. Furthermore, some species of *Trichoderma* such as *T. viride*, will produce a characteristic sweet smell resembling ‘coconut’ odour (Gams and Bissett 2002).

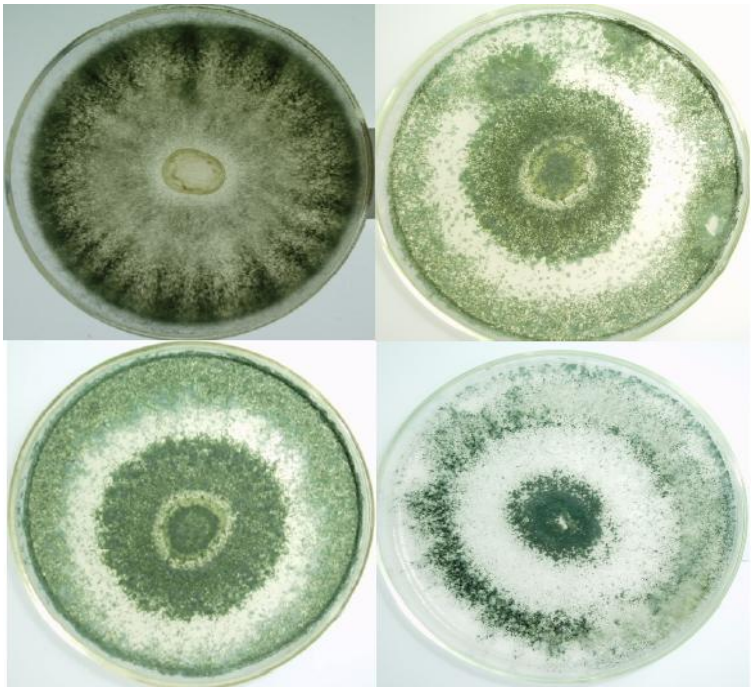

Figure 2.1: Colonies of *Trichoderma* species on PDA (7 days), up left: *T. atroviride*; up right: *T. longibrachiatum*; down left: *T. virens*; down right: *T. harzianum* (Zhang and Wang 2012).

2.3.2 Microscopic Features

Trichoderma species usually form vegetative hyphae which are septated, hyaline and smooth-walled (Rex et al., 2001; Gams and Bissett 2002). Conidiophores (Figure 2.2) are highly branched. Lateral side branches produced from main branches may or may not be paired, and sometimes may rebranch. Normally, the branches will form at or near 90 ° with respect to the main branch. Paired branches will assume a pyramidal structure. The typical conidiophore terminates with one or a few phialides that usually arising directly from the axis near the tip. In some species, however, the main branches are terminated with sterile or fertile elongations (Samuels et al., 2002a).

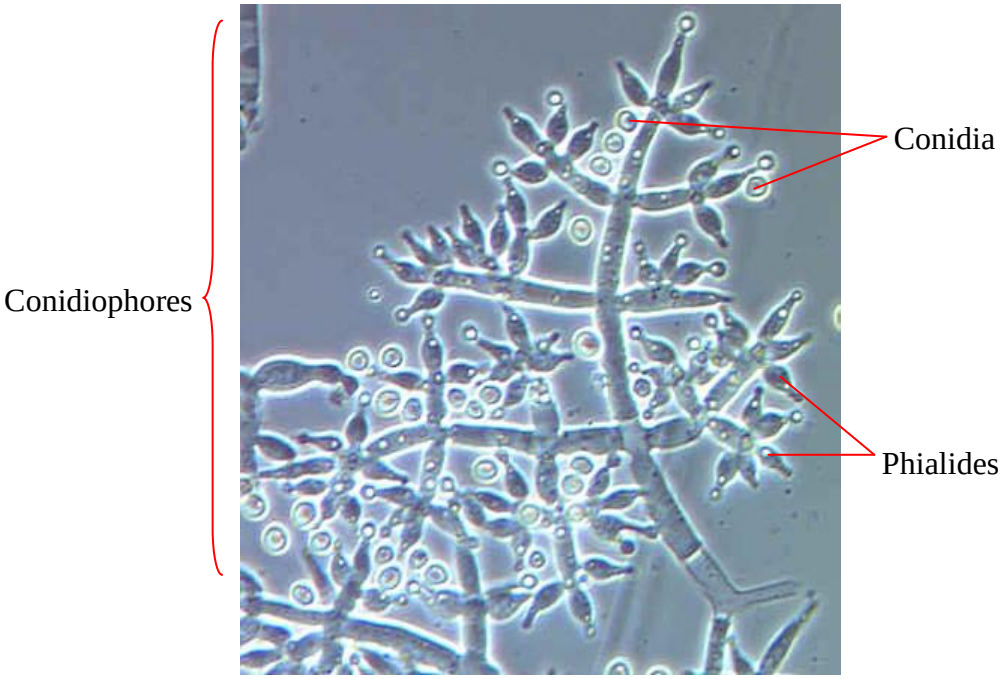

Figure 2.2: Conidiophores of *T. harzianum* (Samuels et al., 2002a).

Phialides, also known as conidiogenous cells, are typically enlarged in the middle like a flask-shape, and may be cylindrical or nearly subglobose. They are held in divergent verticils at the end of the conidiophores, or in whorls beneath septa along the conidiophores and branches. They may be held irregularly, paired, or in solitary (Samuels et al., 2002a; Gams and Bissett 2002; Rex et al., 2001).

Conidia are one-celled, and either ellipsoidal (3-5×2-4 µm, L/W => 1.3) or globose (L/W < 1.3). They are typically green, or sometimes colourless, grayish or brownish. Their surfaces are typically smooth, but roughened conidia can be found in a few species, such as *T. viride* (Samuels et al., 2002a; Gams and Bissett 2002).

Chlamydospores play important role in survival. They are normally found as thick-walled, enlarged vegetative cells with condensed cytoplasm (Lin and Heitman 2005). These unicellular, globose to subglobose chlamydospores are either formed within hyphae or at the hyphal tips. Typically, they are colourless, pale yellowish or greenish (Samuels et al., 2002a; Gams and Bissett 2002).

2.4 Roles of *Trichoderma* as Biological Control Agent (BCA)

2.4.1 *Trichoderma*-pathogen Interactions

Trichoderma species possess several control mechanisms to combat against phytopathogenic organisms. These biocontrol mechanisms include competition with plant pathogens, mycoparasitism, antibiosis, production of lytic enzymes and secretion of secondary metabolites (Vinale et al., 2007).

Trichoderma species are relatively good antagonists against pathogenic fungi. They are able to survive under extreme competitive conditions. They are able to overcome fungistatic effects (Benítez et al., 2004). Moreover, they are resistant against many toxic compounds, including metabolites produced by soil microflora and plants, fungicides, herbicides and antibiotics. These abilities might be due to the presence of ATP-binding cassette (ABC) transporter. The increased expression of these ABC-transporter genes reduces toxicant accumulation in the cells (Harman et al., 2004). Thus, allowing them to survive under extreme conditions and become more competitive compared to other soil fungi.

Other than that, *Trichoderma* species are good in mobilizing and uptaking of nutrients compared to other organisms (Ben ŕez et al., 2004). They compete for nutrients, growth factors and space with plant pathogens (Vinale et al., 2007). Lack of easily accessible nutrients in the soil starved the pathogens and thus controls the growth of pathogens. For example, biological control strains of *Trichoderma* are able to make highly efficient siderophores that chelate iron from other filamentous fungi. Those fungi such as *Pythium*, need iron for survival will be killed (Ben ŕez et al., 2004).

Besides that, *Trichoderma* species can parasitize many other fungi. Under normal conditions, *Trichoderma* species always secret low level of cell wall degrading enzymes (CWDEs) such as chitinases and glucanases. When pathogenic fungi are present, CWDEs lyses the cell wall of pathogens and release cell wall oligomers from pathogens. The degradation products from pathogens further induce the expression of mycoparasitic gene expression (Vinale et al., 2007). After that, *Trichoderma* species grow towards pathogens. When *Trichoderma* species come into contact with pathogenic fungi, they attach and coil around the pathogens, and a specialized pressing organ known as appressoria will be formed to infect pathogens. Holes can be produced at the site of appressoria, and *Trichoderma* hyphae enter into the lumen of target fungi. As a result, the pathogenic fungi can be killed (Harman et al., 2004).

Furthermore, *Trichoderma* species can be the active colonizers of their habitats because they can produce a wide variety of secondary metabolites, including antibiotics and other natural compounds (Vinale et al., 2007).

According to Ghisalberti and Sivasithamparam (1991), secondary metabolites produced by *Trichoderma* can be classified into three categories: (i) volatile antibiotics such as 6-pentyl-α-pyrone (6PP), (ii) water soluble compounds such as heptelidic acid and (iii) peptaibols which are classified under a class of linear oligopeptides, and shown to inhibit β-glucan synthase in pathogenic fungi (Ben ífez et al., 2004). As a result of the inhibition, pathogens are prevented from reconstructing their cell walls which are degraded by β-glucanase produced by *Trichoderma*. This also allows the β-glucanase to act more effectively (Vinale et al., 2007).

Thangavelu et al. (2004) who tested the potential of *Trichoderma* species in controlling the Fusarium wilt of banana reported that *T. harzianum* isolate Th-10 was most effective in inhibiting the mycelial growth of *Fusarium in vitro*. Soil application of *T. harzianum* Th-10 in dried formulation was shown to be effective in suppressing the disease. The efficacy was comparable to that of the fungicide carbendazim.

2.4.2 *Trichoderma*-plant Interactions

Trichoderma species are usually found colonizing plant root ecosystems, establishing symbiotic relationship with plants. However, colonization of the root tissues are only limited at the root cortex due to the deposition of callose which restrict the penetration of hyphae. The callose barriers made *Trichoderma* become harmless to the plants (Vinale et al., 2007). However, elicitors produced by *Trichoderma* species during penetration stimulate the

activation of plant defence system, causing an increase in the production of defence-related plant enzymes, such as chitinase, glucanase, and enzymes associated with the biosynthesis of phytoalexins. This has been shown in the plants treated with *Trichoderma* (Ben ífez et al., 2004; Vinale et al., 2007). Some of the induced resistances in plants are localized, while most of them are systemic, where the control of plant disease happens at a site distant from *Trichoderma* (Harman et al., 2004).

Furthermore, presence of *Trichoderma* species at the root ecosystems had shown to enhance plant root development (Harman et al., 2004; Ben ífez et al., 2004; Vinale et al., 2007). This in turn increase drought tolerance of the plants, and may improve the resistance of plants towards compacted soils. Besides that, *Trichoderma* species are capable in controlling deleterious microbes that reduce root development. *Trichoderma* species are resistant to the cyanide produced by these deleterious microbes, and even able to remove the microbes from the root zone through mycoparasitic effects. Therefore, the *Trichoderma*-plant interactions are always associated with improvements in plant yield and biomass. For example, maize treated with *Trichoderma* strain T-22 had shown to increase about 5% in average yield (Harman et al., 2004).

2.5 Internal Transcribed Spacer (ITS)

There are several molecular methods to characterize fungi species, including isozymes analysis, restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD) and DNA sequencing

(Lieckfeldt et al., 2002). Sequence analysis of the ITS region is one the famous method among these molecular characterization methods.

In eukaryotic cells, rRNA cistrons made up of 18S, 5.8S and 28S rRNA genes (Figure 2.3) are transcribed by RNA polymerase I. Then, RNA splicing of the cistrons will remove the two internal transcribed spacers flanking the 5.8S gene. The two spacers, together with the 5.8S gene, are normally referred to as the ITS region (Schoch et al., 2012). The rRNA genes are universally conserved, while the ITS region and intergenic spacer (IGS) are highly variable (Lieckfeldt et al., 2002). The ITS region and IGS region are the fastest evolving regions and they may vary among species within a genus. Thus, the sequences of these regions can be used for identification of closely related species (White et al., 1990). From previous studies, different *Colleotrichum* species, including *C. gloeosporioides, C. musae* and *C. truncatum* were successfully identified based on the ITS1 and ITS2 regions amplified. In addition, phylogenetic relationships between the *Colleotrichum* isolates were also studied (Photita et al., 2005).

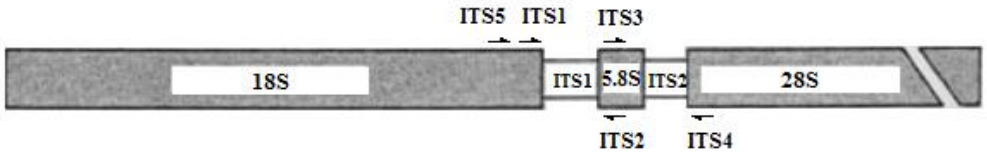

Figure 2.3: schematic representation of rDNA showing the 18S, 5.8S and 28S genes with ITS1 and ITS2 regions flanking 5.8S gene. Priming sites for ITS primers are indicated with arrows (Adapted and modified from White et al., 1990).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample Collection

A total of 15 samples were collected from different collection sites in Kampar and Penang. Twelve of them were rhizosphere soil, two were compost and one was humus. The soil samples were taken from a depth of 10 to 15 cm around the rhizosphere area of fruit plants, including mango, ciku, durian, yam, pineapple, amra fruit, dragon fruit, pomelo, nona, lime and banana trees. The soil samples were collected in plastic bags, sealed in boxes and labeled with information of collection sites and origin of samples. Then, the samples were transported to the laboratory and processed within 24 hours.

3.2 Isolation of *Trichoderma* spp.

Serial dilution technique was used to dilute the samples collected. The samples were homogenized and 10 g of the samples were weighed and used to carry out serial dilution. Thousand times (10^{-3}) and ten thousand times (10^{-4}) dilution of each sample was prepared. Then, 100 µL of each diluted sample was pipetted onto Malt Extract Agar (MEA, Conda Pronadisa) plates amended with 0.12 g/L neomycin (Merck) and 0.09 g/L streptomycin (Bio Basic Inc.) and spread evenly using a sterile hockey stick. The inoculated MEA plates were then incubated at 28℃ for 4 to 7 days. The plates were observed daily.

Visible fungal colonies were transferred to new Potato Dextrose Agar (PDA, Merck) plates and incubated at 28 ℃ for 5 days.

Preliminary screening for *Trichoderma* species was carried out by observing both macroscopic and microscopic features of the fungal colonies. For macroscopic screening, the growth rate and colours of colonies were examined. For microscopic screening, slides were prepared. Mycelia from each isolate were taken from PDA plate and spread onto a clean slide mounted with a drop of water, covered with cover slip and then observed under a light microscope (Leica) using 400X magnification. The branching patterns of conidiophores, and the shapes and sizes of conidia were examined. The macroscopic and microscopic features were compared to the characteristics described by Samuels et al. (2002a).

3.3 Single Spore Isolation

Spore suspension was prepared by inoculating small piece of mycelia with conidia into a Bijoux bottle containing sterile distilled water and shook vigorously. After that, one loopfull of spore suspension was streaked on Water Agar (WA, Merck) in a zig-zag manner. The inoculated WA plates were incubated at 28 ℃ for 24 – 48 h. After that, well isolated colonies germinated from single conidia were subcultured to new PDA plates. The inoculated plates were incubated at 28 ℃ for 3 – 5 days and used as inoculums for further studies and storage of fungal cultures.

3.4 Stock Culture

Half-strength PDA slants prepared in universal bottles were used for the storage of fungal cultures. Small pieces of mycelia from each pure culture were picked up using an inoculating needle and inoculated on the surface of agar slants. The inoculated slants were incubated at 28 ℃ for 5 days. After 5 days, the inoculated slants were stored at 4 ℃ until use.

3.5 Morphological Characterization

An interactive key provided by Samuels and his coworkers at http://nt.ars-grin.gov/taxadescriptions/keys/FrameKey.cfm?gen=Trichoderma was used for morphological identification of *Trichoderma* isolates. The morphological characteristics that were submitted for comparison include the colony appearance and pigmentation, the presence or absence of sweet coconut odour, growth rate at 35 ℃, the presence or absence of pustules on Corn Meal Dextrose Agar (CMD, Conda Pronadisa), the sizes of conidia, the branching patterns of conidiophores, and the presence or absence of chlamydospores.

3.5.1 Colony Characters

Colony appearance and pigmentation of *Trichoderma* isolates were studied on PDA plates. By using a cork borer, 3 mm mycelial plugs were cut and transferred to the center of the agar plates. The inoculated agar plates were then incubated at 28 ℃ for 7 days. The cultures were observed daily and

colonies that produced sweet coconut odour were noted. The experiment was conducted with duplicates for each culture.

3.5.2 Growth Rate

The growth rate of each *Trichoderma* isolate was studied on CMD and PDA plates. Mycelial plugs (3 mm) from the margin of growing fungal colonies were cut and placed at the center of the 9 cm vented agar plates. The culture plates were then incubated at 28 ℃ and 35 ℃, respectively. A duplicate plate for each isolate was prepared. For culture plates incubated at 28 ℃, the diameter of each colony was measured every 24 hour-interval until the agar plates were fully colonized. For culture plates incubated at 35 ℃, the diameter of each colony was measured every 24 hour-interval for 4 days. The growth trial was repeated once independently and the average diameter of the colonies was taken from the two independent growth trials. Besides that, the ability of isolates to produce pustules on CMD were noted.

3.5.3 Microscopic Features

Trichoderma isolates were inoculated on PDA and incubated at 28 ℃ prior to microscopic identification. Culture plates that incubated for 3 – 5 days were used to observe branching patterns of conidiophores, whereas culture plates that incubated for 7 – 10 days were used to observe the chlamydospores. A slide of each isolate was prepared by placing a small piece of mycelia onto a drop of distilled water on a slide and the mycelia was gently dispersed using

an inoculating needle. The slide was then observed under a light microscope (Leica) with 400X magnification. Observations focused on the sizes, shapes and colours of conidia, the branching patterns of conidiophores, and the appearance of chlamydospores. The sizes of conidia were measured using an ocular micrometer. Twenty five measurements were taken and the average sizes of conidia were calculated.

3.6 Molecular Analysis

3.6.1 DNA Extraction

For DNA extraction, fungal mycelia were inoculated onto PDA plates overlaid with two pieces of sterile dialysis membranes. A duplicate plate was prepared for each *Trichoderma* isolate. The inoculated plates were then incubated at 28℃ for 2 days. After 2 days, genomic DNA for each *Trichoderma* isolate was extracted using DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions.

First, the mycelia cultured on the dialysis membrane were harvested and ground into fine powder using a pestle and mortar with sufficient amount of liquid nitrogen added. After that, about 23 mg to 25 mg of ground mycelial powder was then weighed in a 1.5 mL microcentrifuge tube. Next, 400 µL of Buffer AP1 (lysis buffer) and 4 µL of RNase A were added into the microcentrifuge tubes, vortexed and incubated at 65℃ for 10 minutes. During the incubation period, the tubes were inverted 2 to 3 times. Then, 130 µL of Buffer AP2 (precipitation buffer) was added into the tubes, vortexed and

incubated for 5 minutes on ice. The lysate was centrifuged for 5 minutes at 14,000 rpm. The supernatant was then transferred into a QIAshredder spin column and centrifuged for 2 minutes at 14,000 rpm.

After that, the flow-through was transferred into a new 1.5 mL microcentrifuge tube. Then, 1.5 volume of Buffer AP3/E (binding buffer) was added to the tubes and the mixture was mixed by pipetting. 650 µL of the mixture was transferred into a DNeasy Mini spin column and centrifuged for 1 minute at 8000 rpm. The flow-through was discarded and this centrifugation step was repeated once with the mixture. After that, the spin column with DNA bound was placed into a new 2 mL collection tube, and 500 µL of Buffer AW (wash buffer) was added, followed by centrifugation at 8000 rpm for 1 minute. This step was repeated once by adding 500 µL Buffer AW and centrifuged for 2 min at 14,000 rpm after the flow-through was discarded. The spin column was transferred to a new 1.5 mL microcentrifuge tube.

Lastly, 100 µL of Buffer AE (elution buffer) was added, incubated at room temperature for 5 minutes and centrifuged for 1 minute at 8000 rpm. The above mentioned step was repeated once to obtain 200 µL of genomic DNA. The genomic DNA was then stored in -20 ℃ until use. Gel electrophoresis was carried out to visualize the genomic DNA.

3.6.2 PCR Amplification of 5.8S-ITS Region

ITS1 and ITS2 regions together with 5.8S gene in rDNA were amplified using primer pair ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') according to Hermosa et al. (2000) with modifications. PCR amplification was conducted in 25 µl reaction mixtures containing 1X PCR buffer (*DreamTaq*™ Green Buffer), 2 mM $MgCl_2$, 0.08 µM of each primer, 160 µM of each deoxynucleotide triphosphate, 1.25 U of *Taq* DNA polymerase (*DreamTaq*™ DNA Polymerase) and 4 – 10 ng of genomic DNA using a thermocycler (TPersonal, Biometra). The primer pair was obtained from 1st BASE while other PCR reagents were obtained from Fermentas. The 5.8S-ITS region was amplified with an initial denaturation for 5 minutes at 95℃, followed by 35 cycles of denaturation at 94℃ for 1 minute, annealing of primers at 60℃ for 30 seconds, and extension at 72℃ for 30 seconds, and the amplification was completed with one cycle of final extension at 72℃ for 5 minutes.

3.6.3 Visualization of PCR Products

To visualize the PCR products, 4 µL of aliquots were electrophoresed in 1% agarose gels using 1X TBE running buffer at 80 V (700 mA) for 45 minutes. The approximate sizes of the amplified regions were estimated by referring to a 100 bp DNA ladder (GeneRuler, 100 bp Plus DNA Ladder, Fermentas). The gels were stained in ethidium bromide for 10 minutes and visualized by using an UV transilluminator. Photographs were taken by using Ingenius Syngene Bio Imaging from Syngene.

3.6.4 Purification of PCR Products

PCR products of each *Trichoderma* isolate were purified using QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instruction. Buffer PB (binding buffer) was added to PCR products in a 5:1 portion and mixed by repeated pipetting. To bind DNA, the mixture was transferred to QIAquick column placed in a 2 mL collection tube and centrifuged for 1 minute at 13,000 rpm. The flow-through was discarded and the QIAquick column was placed back into the same collection tube. Then, 750 µL of Buffer PE (wash buffer) was added to the QIAquick column and centrifuged for 1 minute at 13,000 rpm. The flow-through was again discarded and the QIAquick column was placed back in to the collection tube. The QIAquick column was centrifuged once more to remove the remaining wash buffer. Then, the QIAquick column was placed in a sterile 1.5 mL microcentrifuge tube and 50 µL of Buffer EB (elution buffer) was added to the center of the QIAquick membrane. The tube was centrifuged for 1 minute at 13,000 rpm to elute the purified PCR products.

The purified PCR products were electrophoresed in a 1% agarose gel in 1X TBE buffer. The concentrations of the PCR products were determined by using NanoPhotometer P300 (Implen). Lastly, the purified PCR products of each isolate were sequenced using forward primer ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and reverse primer ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3').

3.6.5 Sequence Analysis of 5.8S-ITS Region

The nucleotide sequences of 5.8S-ITS region were aligned using Molecular Evolutionary Genetics Analysis (MEGA) version 5.10. The forward and reverse sequences were checked and edited manually when needed. Then, a consensus sequence was generated from each alignment made. The sequences were then compared with the sequences deposited in GenBank database using Basic Local Alignment Search Tool (BLAST), where a nucleotide blast program was chosen. Besides, the 5.8S-ITS sequences were compared to a specific database for *Trichoderma* using *Trich*OKEY 2 program, which available online from the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (ISTH, www.isth.info) (Druzhinina et al., 2005).

3.7 Antagonistic Test against *Fusarium* spp.

The antagonistic effects of each *Trichoderma* isolate against *Fusarium oxysporum* and *Fusarium solani* were tested according to the steps described by Zhang and Wang (2012) with modifications. Both *F. oxysporum* and *F. solani* tested were isolated from dry rotted potato and shown to have strong pathogenicity activity.

Dual culture technique was used to conduct the antagonistic test. The *Trichoderma* isolates and *Fusarium* species to be tested were cultured separately on PDA for 7 days. After 7 days, 5 mm mycelial plugs (taken from the edge of fungal colonies) of each species to be tested were transferred to PDA plates using cork borer. The mycelial plugs of *Trichoderma* spp. and

Fusarium spp. were placed 2.5 cm apart from each other on a PDA surface. PDA plates inoculated with *Fusarium* spp. were included as negative controls. The antagonistic tests were conducted in duplicate. All culture plates were incubated at 28℃ and observations were made daily for 5 days. The percentage of inhibition (I%) on the mycelial growth of *F. oxysporum* and *F. solani* were calculated using this formula:

$$I\% = \frac{r1 - r2}{r1}$$

where r1 is the radius of *Fusarium* away from *Trichoderma* isolate, while r2 is the radius of *Fusarium* towards *Trichoderma* isolate (Abadi 1990).

CHAPTER 4

RESULTS

4.1 Isolation of *Trichoderma* spp.

A total of 11 fungal isolates were successfully isolated from different samples. Five isolates, namely DUR2, DUR5, DUR6, DUR9 and POM1 were from Penang while six isolates, namely BNN2, YAM1, MG3, PPY1, PPY12 and PTT were from Kampar, Perak. Preliminary screening was carried out which showed that these fungal isolates were *Trichoderma* species. The types of samples and the isolate codes were shown in Table 4.1.

Table 4.1: Isolate codes, origins of *Trichoderma* species and the types of sample used.

Isolate Code	Origin of *Trichoderma* spp.	Types of Sample
BNN2	Kampar, Perak	Rhizosphere soil
DUR2	Penang	Rhizosphere soil
DUR5	Penang	Rhizosphere soil
DUR6	Penang	Rhizosphere soil
DUR9	Penang	Rhizosphere soil
YAM1	Kampar, Perak	Rhizosphere soil
MG3	Kampar, Perak	Rhizosphere soil
POM1	Penang	Rhizosphere soil
PPY1	Kampar, Perak	Humus
PPY12	Kampar, Perak	Humus
PTT	Kampar, Perak	Compost

4.2 Morphological Characterization

Morphological identification of the potential *Trichoderma* isolates was performed using an online interactive key (Samuels et al., 2002a) based on the colony appearance and pigmentation, the presence or absence of sweet coconut smell, growth rate at 35℃, the presence or absence of pustules on CMD, the sizes of conidia, the branching patterns of conidiophores, and the presence or absence of chlamydospores.

4.2.1 Colony Characters

Colony characters of *Trichoderma* isolates were studied using 7 days old PDA cultures that were incubated at 28℃ and 35℃, respectively. At 28℃, all *Trichoderma* isolates grew well and formed conidia within 4 days. The conidial production in BNN2 was diffused, dispersed and at the same time formed concentric rings. The colour of mature conidia in BNN2 was light green. For YAM1, the mature conidia appeared to be grayish green, and no concentric ring was observed. The conidia of YAM1 tend to concentrate at the center of the colony. For isolates MG3, POM1, PPY1, and PPY12, dark green conidia tend to form in pustules, and arranged in concentric rings. More concentric rings were observed in these colonies compared to that in colonies of BNN2. In the colonies of DUR2, DUR5, DUR6 and DUR9, however, no concentric rings were observed. Their conidial productions were restricted to the center of the colonies, diffused, and appeared to be yellowish green (Figure 4.1).

The colonies of all *Trichoderma* isolates grown at 35℃ have different appearance compared to their duplicates grown at 28℃. At 35℃, all *Trichoderma* isolates were able to grow well except YAM1. The colonies that able to grow at 35℃ were found to form more concentric rings compared to those grown at 28℃. In addition, the concentric rings were thinner when grown at 35℃. Besides that, colonies grown at 35℃ appeared flatter, more compact and less cottony (Figure 4.1).

Colonies of PTT were found to produce diffusible yellow pigments. These pigments caused the PDA to turn yellowish. The production of diffusible yellow pigments was not found in other *Trichoderma* isolates.

Furthermore, Samuels et al. (2002a) stated that the odour produced by the colonies can be the characteristic of a species. In this study, only YAM1 was found to produce a smell resembling coconut, thus distinguishing this isolate from the others.

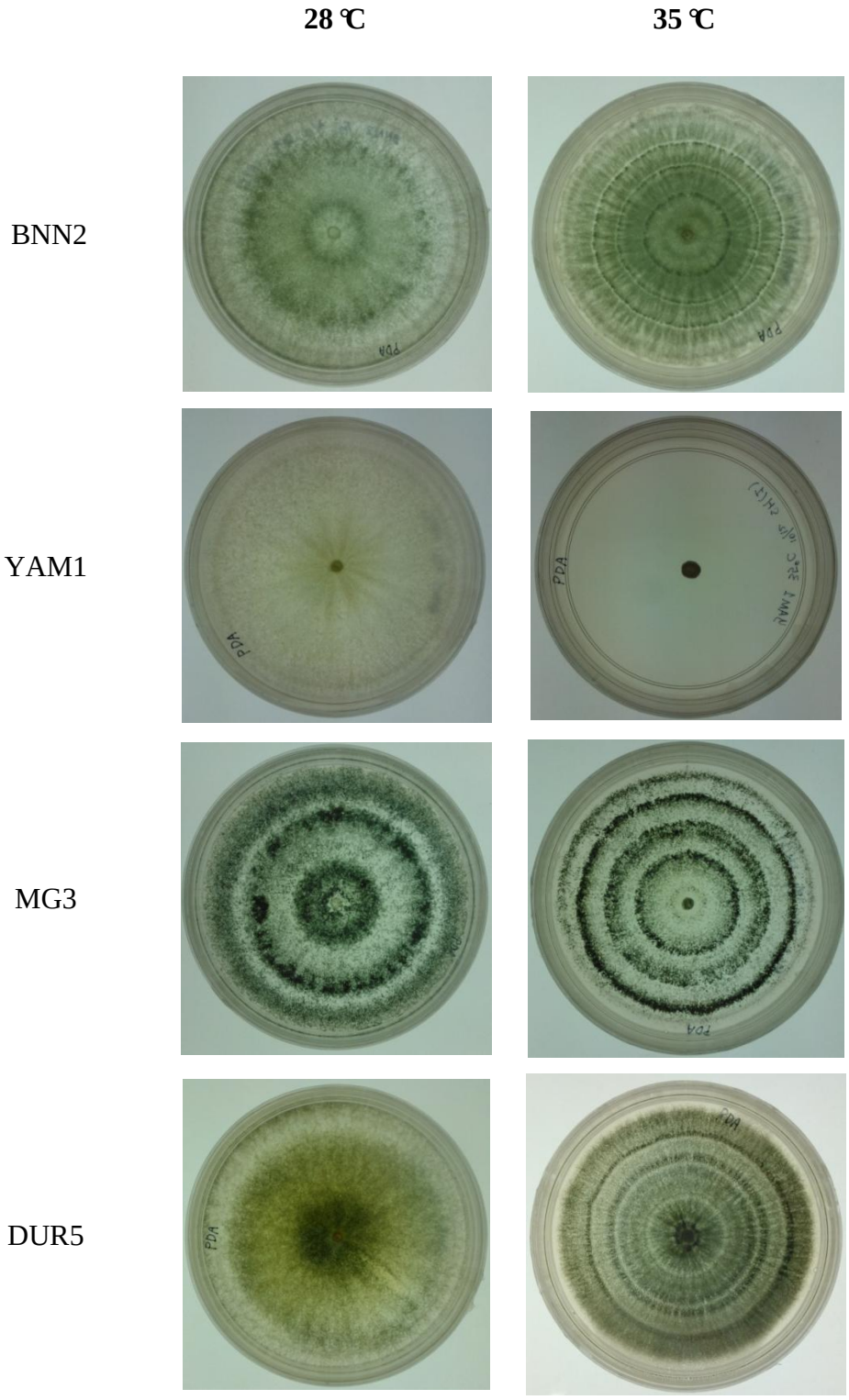

Figure 4.1: Representative plates of *Trichoderma* isolates cultured at 28 ℃ and 35 ℃.

4.2.2 Growth Rate

The averaged colony diameters of each *Trichoderma* isolates were calculated and tabulated in Table 4.2 to Table 4.5. The graphical presentations of the growth rates of *Trichoderma* isolates cultured on PDA at 28 ℃ and 35 ℃ were shown in Figures 4.2 and 4.3, respectively. Other than that, the growth rates of *Trichoderma* isolates grown on CMD at 28 ℃ and 35 ℃ were presented in Figures 4.4 and 4.5, respectively.

When *Trichoderma* isolates were cultured on PDA at 28 ℃, nearly all isolates had the same growth rate except isolates PTT and PPY1. PTT grew faster than any other isolates at 28 ℃ on PDA. The fast growth rate of PTT was more obvious after 48 h (2 days). However, PPY1 grew slightly slower than any other isolates at 28 ℃ on PDA and fully colonized the 9 cm vented PDA plate on day 4. All isolates except PPY1 fully colonized the PDA plates on day 3.

Greater differentiation in growth rate was observed when *Trichoderma* isolates were cultured on PDA at 35 ℃. In this case, YAM1 can be clearly distinguished from other isolates since it showed the slowest growth rate among all. YAM1 had very slow growth in the first 3 days, and its growth ceased after day 3. Besides that, PTT can be differentiated from all other isolates because it grew relatively fast compared to other isolates. Overall, all isolates showed slower growth rate at 35 ℃ than at 28 ℃.

When *Trichoderma* isolates were cultured on CMD at 28 ℃, these isolates established faster growth compared to their growth on PDA at 28 ℃. Six

isolates, namely BNN2, DUR2, DUR5, DUR6, DUR9 and PTT were found to fully colonize the CMD plate in day 2. The other isolates (MG3, POM1, PPY1, PPY12 and YAM1) also grew rapidly and CMD plates were fully colonized in day 3. In day 2, YAM1 could be distinguished from other isolates as it had slightly slower growth than other isolates. However, other isolates can hardly be differentiated from one another at 28 ℃ on CMD based on growth rate. The ability of *Trichoderma* isolates to produce pustules on CMD at 28 ℃ was also determined. All isolates were able to produce green pustules on CMD except isolate BNN2.

On CMD at 35 ℃, the growth rate of all *Trichoderma* isolates were slower compared to their growth rate on CMD at 28 ℃. Nonetheless, their growth rate on CMD at 35 ℃ was somewhat faster than their growth rate on PDA at 35 ℃. In day 3, PTT was able to fully colonize the CMD plate at 35 ℃. On day 4, another four isolates also managed to fully colonize the CMD plate. There were some similarities observed based on the growth rate of *Trichoderma* isolates grown on PDA and CMD at 35 ℃ (Figure 4.3 and Figure 4.5). Firstly, PTT had the fastest growth rate in both conditions. Secondly, YAM1 had the slowest growth rate, and its growth stopped after day 3. The stunted growth of YAM1 could distinguish it from other isolates. Thirdly, all isolates except PTT and YAM1 had moderate growth rate and difficult to differentiate from one another.

Table 4.2: Averaged colony diameters of *Trichoderma* isolates cultured on PDA at 28 ℃ from day 1 to day 4.

Isolate Code	Colony diameter (cm)			
	Day 1	**Day 2**	**Day 3**	**Day 4**
BNN2	2.850	7.100	FC*	FC
DUR2	2.825	7.025	FC	FC
DUR5	2.350	5.875	FC	FC
DUR6	2.750	6.950	FC	FC
DUR9	2.650	7.000	FC	FC
MG3	2.850	6.300	FC	FC
POM1	3.100	7.025	FC	FC
PPY1	3.000	6.650	8.525	FC
PPY12	2.925	6.875	FC	FC
YAM1	2.725	6.150	FC	FC
PTT	3.600	8.500	FC	FC

***** FC = fully colonized (9 cm).

Table 4.3: Averaged colony diameters of *Trichoderma* isolates cultured on PDA at 35 ℃ from day 1 to day 4.

Isolate Code	Colony diameter (cm)			
	Day 1	**Day 2**	**Day 3**	**Day 4**
BNN2	1.350	2.850	4.650	6.150
DUR2	1.300	2.900	5.500	7.600
DUR5	1.500	4.200	6.300	8.300
DUR6	1.950	4.600	6.750	8.500
DUR9	1.800	4.450	6.750	8.550
MG3	1.600	3.500	5.300	7.000
POM1	2.100	4.350	5.450	5.950
PPY1	1.550	4.150	5.850	7.400
PPY12	1.850	4.100	5.550	6.600
YAM1	0.600	0.900	1.025	1.025
PTT	2.550	6.200	FC*	FC

***** FC = fully colonized (9 cm).

Table 4.4: Averaged colony diameters of *Trichoderma* isolates cultured on CMD at 28 ℃ from day 1 to day 3.

Isolate Code	Colony diameter (cm)		
	Day 1	**Day 2**	**Day 3**
BNN2	4.350	FC*	FC
DUR2	3.675	FC	FC
DUR5	3.425	FC	FC
DUR6	3.650	FC	FC
DUR9	3.700	FC	FC
MG3	3.250	7.350	FC
POM1	3.300	7.325	FC
PPY1	3.300	7.425	FC
PPY12	3.150	7.350	FC
YAM1	2.850	6.475	FC
PTT	3.775	FC	FC

***** FC = fully colonized (9 cm).

Table 4.5: Averaged colony diameters of *Trichoderma* isolates cultured on CMD at 35 ℃ from day 1 to day 4.

Isolate Code	Colony diameter (cm)			
	Day 1	**Day 2**	**Day 3**	**Day 4**
BNN2	1.95	4.15	6.45	8.10
DUR2	1.60	3.70	5.50	6.80
DUR5	2.00	4.85	7.20	FC
DUR6	2.25	5.45	7.95	FC
DUR9	2.25	5.15	7.25	FC
MG3	1.65	3.70	5.50	6.90
POM1	2.35	4.35	5.30	6.45
PPY1	2.15	4.45	6.45	7.65
PPY12	2.25	5.35	8.00	FC
YAM1	0.85	1.65	2.10	2.15
PTT	2.60	6.60	FC*	FC

***** FC = fully colonized (9 cm).

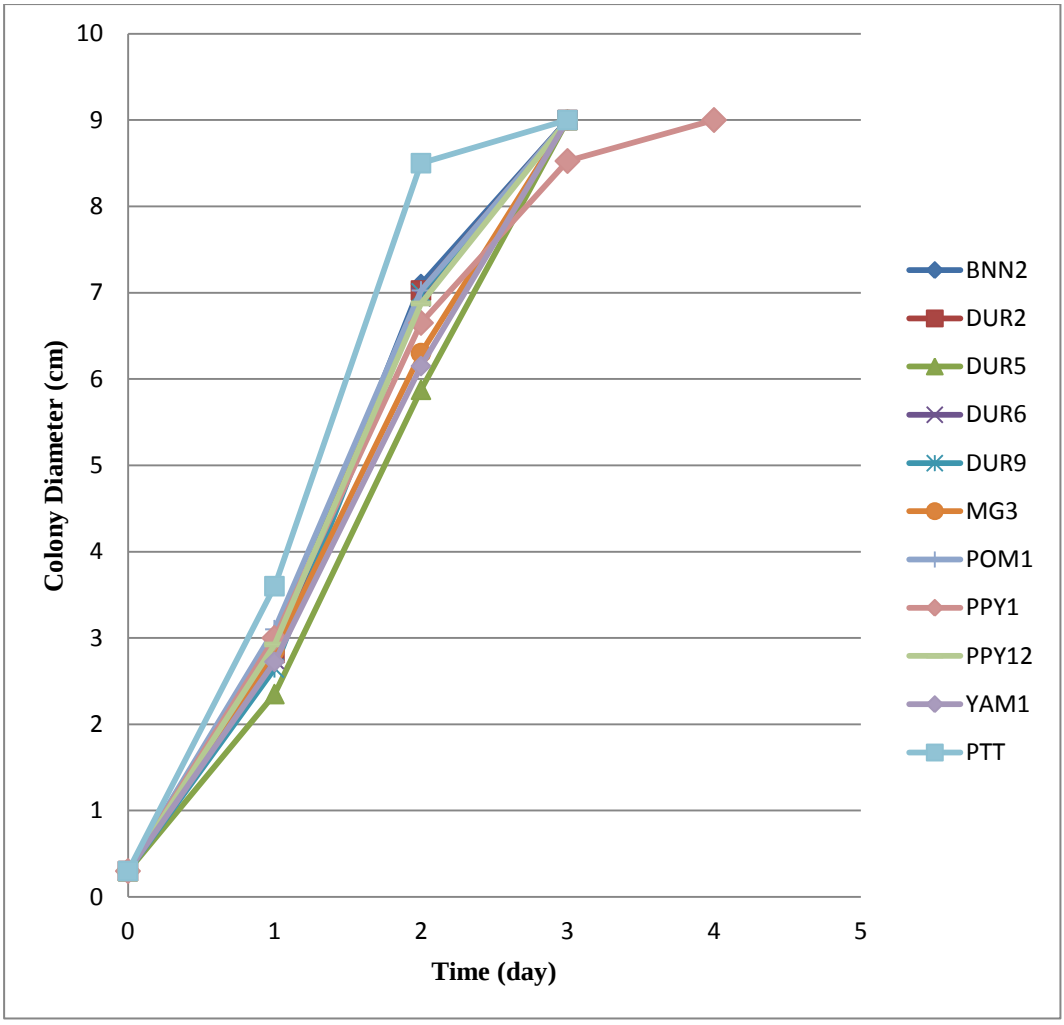

Figure 4.2: Colony diameter of *Trichoderma* isolates grown on PDA at 28 ℃ from day 1 to day 4.

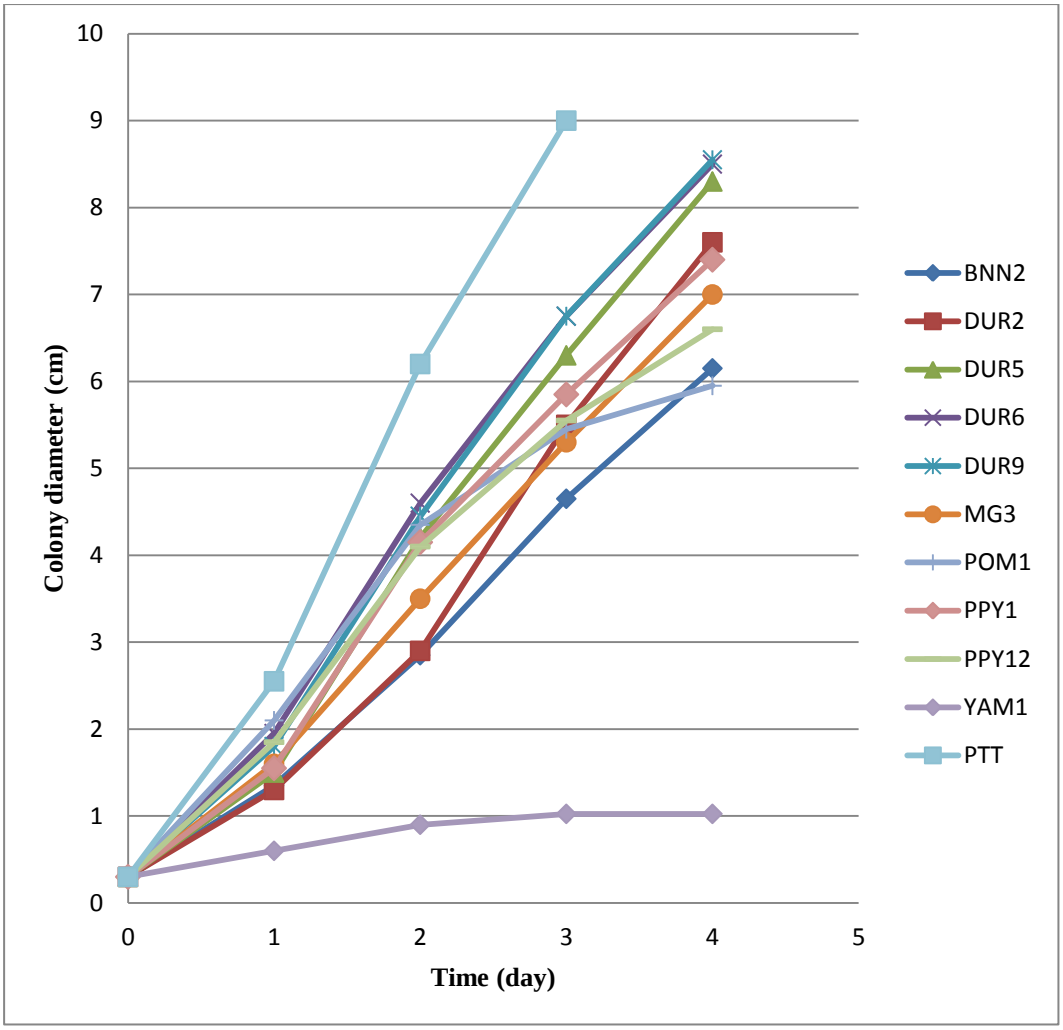

Figure 4.3: Colony diameter of *Trichoderma* isolates grown on PDA at 35 ℃ from day 1 to day 4.

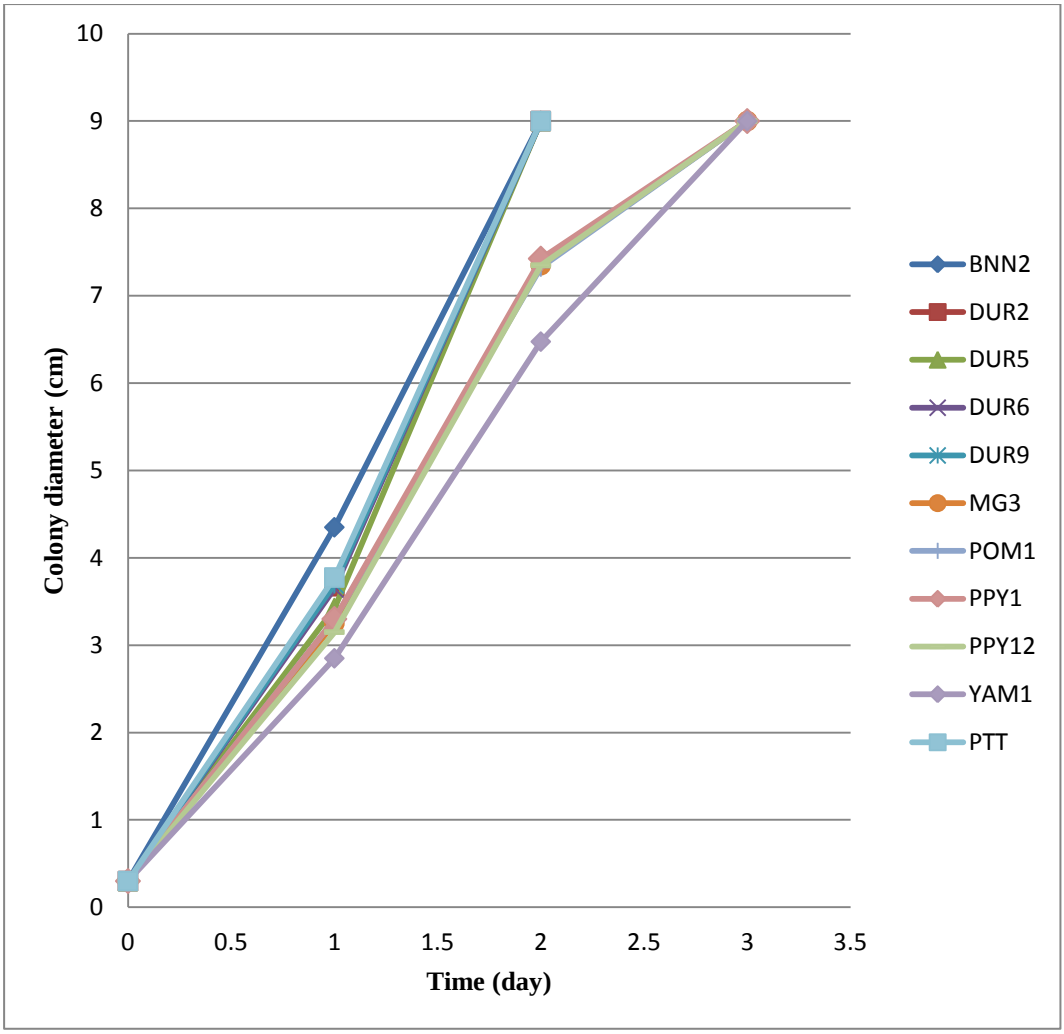

Figure 4.4: Colony diameter of *Trichoderma* isolates grown on CMD at 28 ℃ from day 1 to day 3.

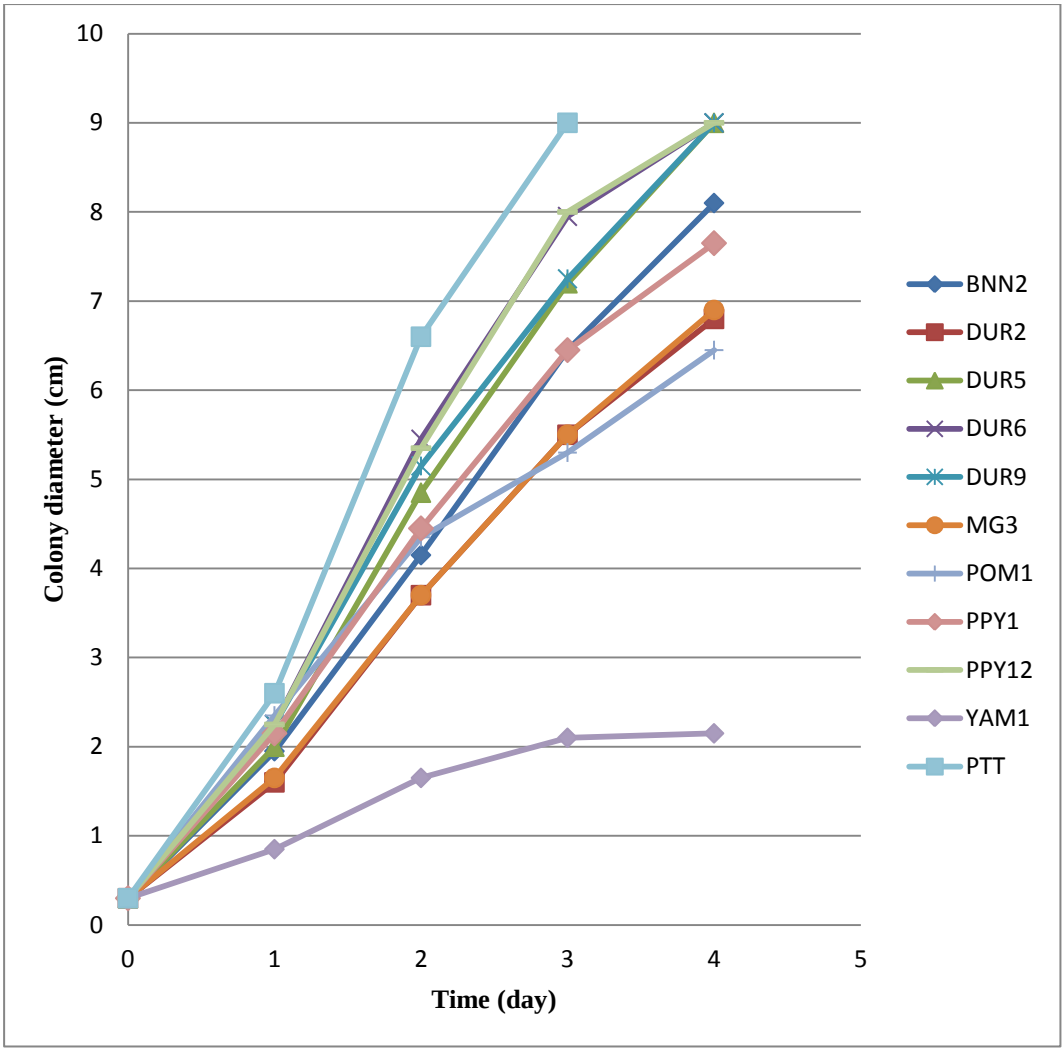

Figure 4.5: Colony diameter of *Trichoderma* isolates grown on CMD at 35 ℃ from day 1 to day 4.

4.2.3 Microscopic Features

The microscopic features of *Trichoderma* isolates were observed under light microscope with 400X magnification. The colours, shapes and sizes of conidia were presented in Table 4.6. The conidiophores and chlamydospores of *Trichoderma* isolates were shown in Figures 4.7 and 4.8, respectively.

Under light microscope, the colours of conidia of all *Trichoderma* were found to be green. The different intensities of greens colours (light green, yellowish green, dark green and grayish green) of mature conidia observed on PDA plate can hardly be observed under light microscope. Both BNN2 and YAM1 have subglobose to ellipsoidal conidia. However, the conidia of BNN2 are wider than those of YAM1. On the other hand, isolates MG3, POM1, PPY1 and PPY12 have both globose and subglobose conidia, but more subglobose conidia were observed compared to globose conidia. The conidial sizes of these isolates were almost similar, except the conidia size of PPY1, which tends to be slightly smaller than conidia of other isolates. Different from other isolates, only globose conidia were observed in DUR2, DUR5, DUR6, DUR9 and PTT. The conidia diameters of these isolates ranged from 2.6 to 2.9 μm.

Conidiophores of PTT, DUR2, DUR5, DUR6 and DUR9 were shown in Figure 4.6A-E. These isolates have conidiophores with paired primary branches (Figure 4.6A and Figure 4.6E). Their phialides are flask-shaped and normally held in whorls of two to three phialides. Conidiophores of PPY1, POM1, MG3 and PPY12 were presented in Figure 4.6F-I, showing their paired primary branches which were usually formed in nearly 90 ° to the main

axis. Their phialides may be solitary (Figure 4.6F) or held in whorls of two to three (Figure 4.6F-I). Those phialides that held in whorls normally are flask-shaped, while solitary phialides tend to be cylindrical and sharply constricted at the tips. In conidiophores of YAM1, long, straight, solitary and fertile apices were observed (Figure 4.6J). Besides that, YAM1 also has unpaired primary branches and flask-shaped phialides held in whorls of two to three (Figure 4.6K). For BNN2, the primary branches of conidiophores tend to branch towards the tips, and usually not form in pairs (Figure 4.6L). The flask-shaped phialides are closely appressed, usually arised in whorls of two to three. Solitary phialides was not observed.

All *Trichoderma* isolates were found to produce chlamydospores after 7 days. All chlamydospores observed were unicellular and appeared globose to subglobose (Figure 4.7). Most of the chlamydospores were formed on the hyphal tips. However, chlamydospores of isolate MG3 were found on both hyphal tips and within the hyphae (Figure 4.7H, pointing with arrow).

The results of morphological identification using an online interactive key were presented in Table 4.6. From the results obtained, BNN2 was identified as *T. virens* while YAM1 was identified as *T. strigosum*. For DUR2, DUR5, DUR6, DUR9 and PTT, they were identified as *T. harzianum*. Other than that, MG3, POM1, PPY1 and PPY12 were identified as *T. asperellum*.

Table 4.6: The identities of *Trichoderma* isolates identified using an online interactive key and the colours, shapes and averaged sizes of conidia of *Trichoderma* isolates.

Isolate Code	*Trichoderma* spp.	Conidial Colour	Conidial Shape	Conidial Size[a] (µm)	
				Length	Width
BNN2	*T. virens*	Green	Subglobose to ellipsoidal	4.50	3.25
YAM1	*T. strigosum*	Green	Subglobose to ellipsoidal	4.45	2.85
MG3	*T. asperellum*	Green	Globose to subglobose	4.45	3.40
POM1	*T. asperellum*	Green	Globose to subglobose	4.45	3.45
PPY1	*T. asperellum*	Green	Globose to subglobose	4.00	3.05
PPY12	*T. asperellum*	Green	Globose to subglobose	4.50	3.30
DUR2	*T. harzianum*	Green	Globose	2.90[b]	
DUR5	*T. harzianum*	Green	Globose	2.85[b]	
DUR6	*T. harzianum*	Green	Globose	2.80[b]	
DUR9	*T. harzianum*	Green	Globose	2.70[b]	
PTT	*T. harzianum*	Green	Globose	2.60[b]	

[a] The conidial sizes are taken from the average of 25 measurements.
[b] The length and width of the conidia are the same, thus these values are the diameters of globose conidia.

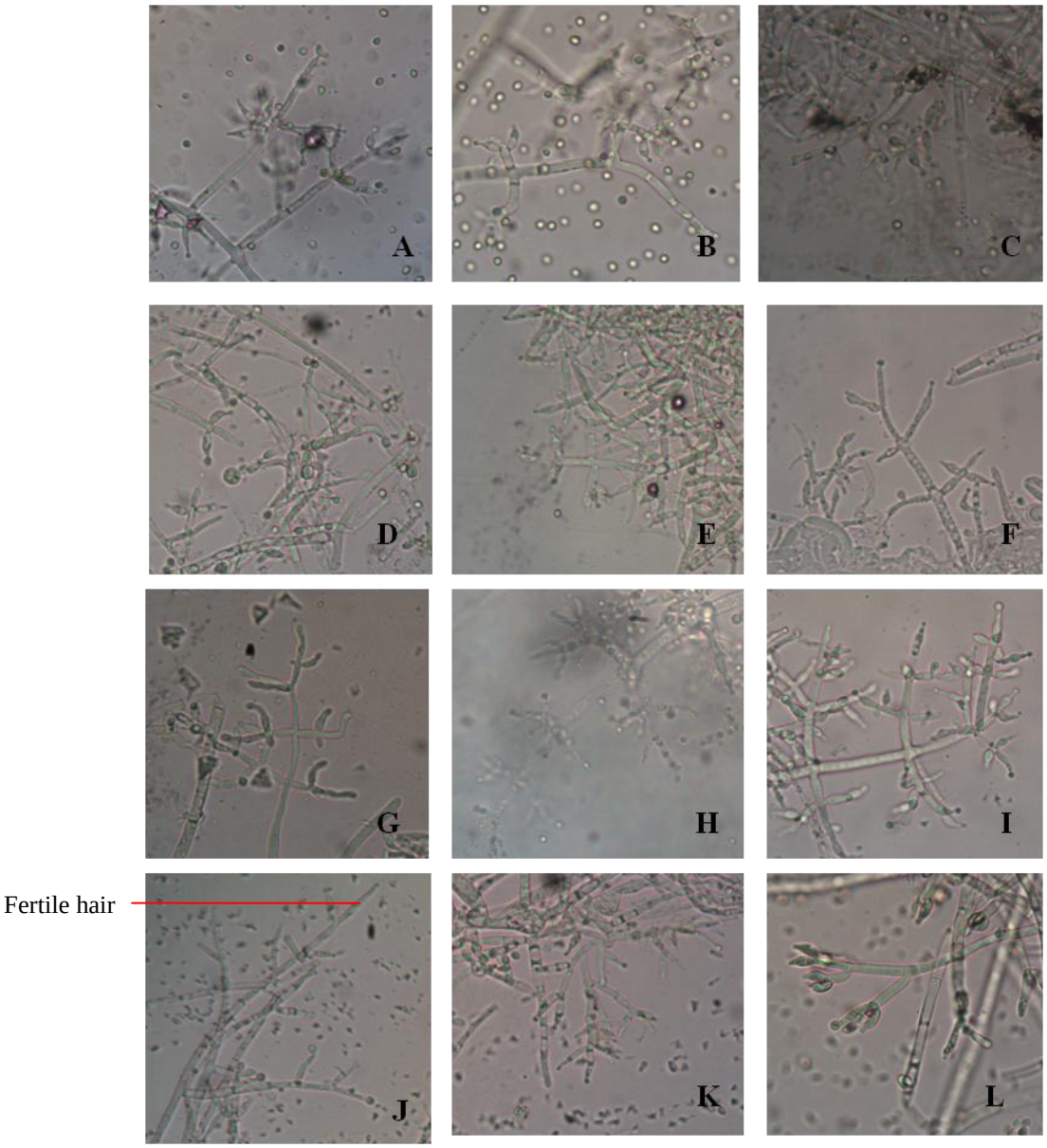

Figure 4.6: Conidiophores of *Trichoderma* isolates (400X magnification). A–E (PTT, DUR9, DUR2, DUR5, DUR6): Paired primary branches, phialides held in whorls of two to three. F–I (PPY1, POM1, MG3, PPY12): Paired primary branches formed in nearly 90 °to main axis, phialides may be solitary or held in whorls of two to three. J (YAM1): Fertile hair with long, straight, solitary and fertile apices. K (YAM1): Unpaired primary branches branching towards tips, phialides held in whorls of two to three. L (BNN2): Unpaired primary branches branching towards tips, with closely appressed phialides arised in whorls of two to three.

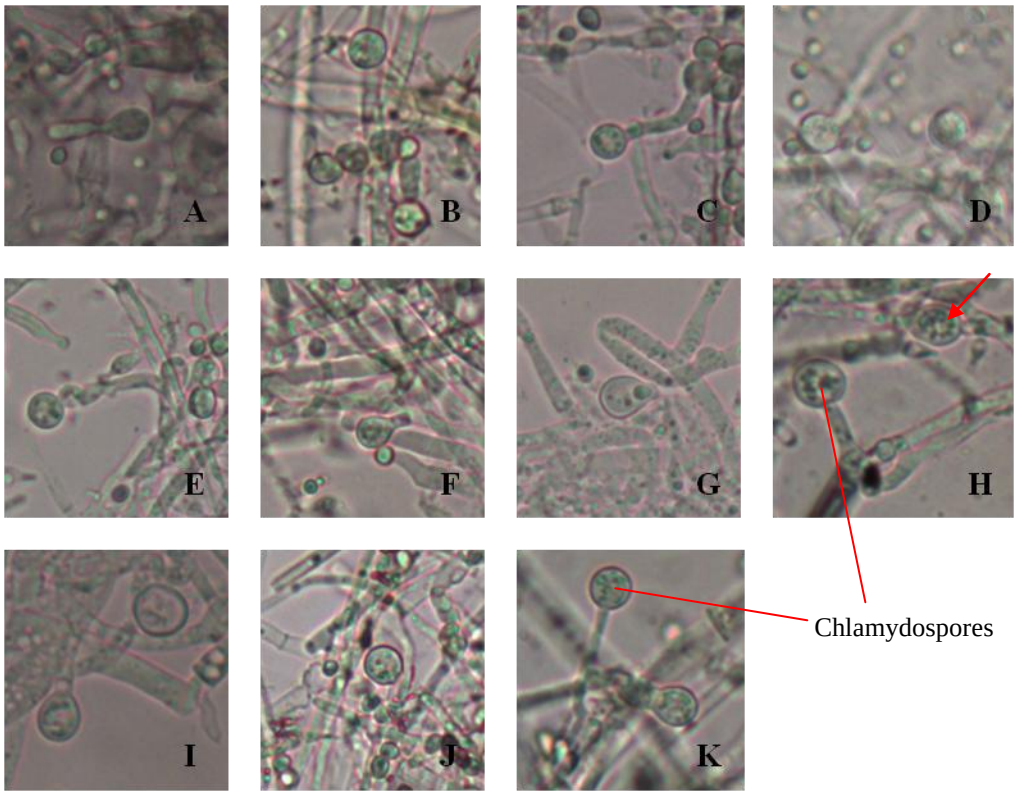

Figure 4.7: Chlamydospores of *Trichoderma* isolates (400X magnification), A: DUR2; B: PTT; C: DUR6; D: DUR9; E: DUR5; F: POM1; G: PPY1; H: MG3; I: PPY12; J: YAM1; K: BNN2. Chlamydospores within the hyphae (arrow).

4.3 Molecular Analysis

4.3.1 DNA Extraction

Genomic DNA of all *Trichoderma* isolates was successfully extracted. The purities of all genomic DNA (A_{260}/A_{280}) ranged from 1.65 to 1.77.

4.3.2 PCR Amplification of 5.8S-ITS Region

An approximately 600 bp of 5.8S-ITS DNA fragment was successfully amplified from all *Trichoderma* isolates (Figure 4.8). The concentrations of the purified PCR products ranged from 14.5 ng/µL to 23.5 ng/µL.

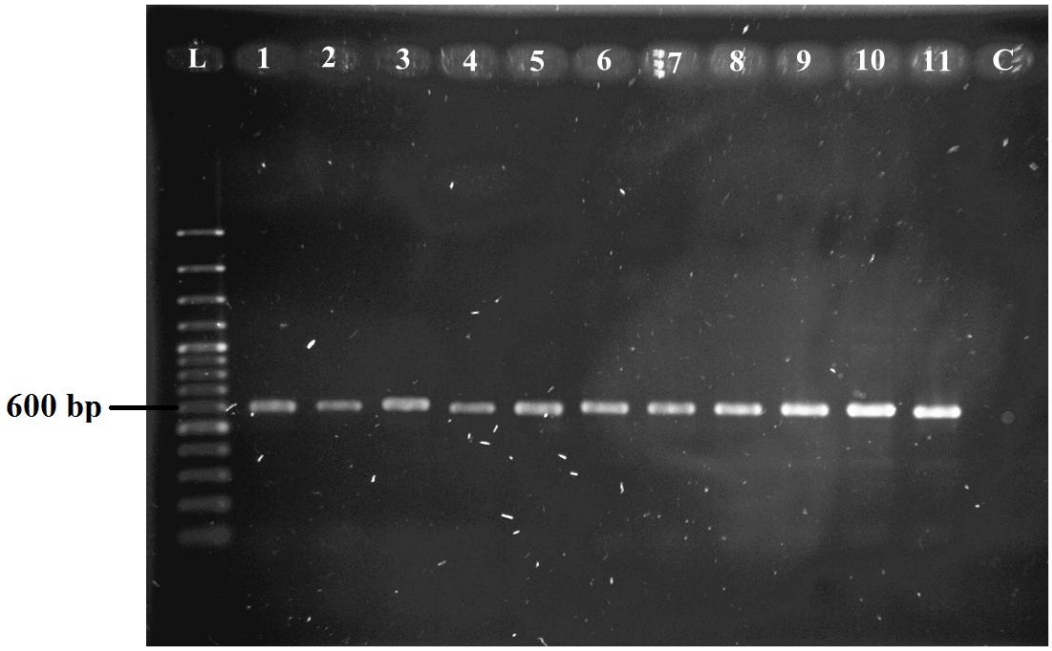

Figure 4.8: 600 bp 5.8S-ITS region amplified for all *Trichoderma* isolates using primer pair ITS1 and ITS4. L: 100 bp DNA ladder; Lane 1: POM1; Lane 2: YAM1; Lane 3: PTT; Lane 4: PPY1; Lane 5: PPY12; Lane 6: DUR2; Lane 7: DUR5; Lane 8: DUR6; Lane 9: DUR9; Lane 10: BNN2; Lane 11: MG3; C: control.

4.3.3 Sequence Analysis of 5.8S-ITS Region

PCR products amplified from all *Trichoderma* isolates were sequenced. They could be aligned and a consensus sequence was generated from each alignment made (Appendix A). Then, BLAST and *Trich*OKEY 2 program were used to determine the species identity of *Trichoderma* isolates. The BLAST and *Trich*OKEY search results were presented in Table 4.7.

According to the BLAST results, five isolates (DUR2, DUR5, DUR6, DUR9 and PTT) were identified as *T. harzianum*, four isolates (MG3, POM1, PPY1 and PPY12) were identified as *T. asperellum*, one isolate (BNN2) was identified as *T. virens* while another isolate (YAM1) was identified as *T. strigosum*.

*Trich*OKEY search was also used to assess the reliability of BLAST results. Based on the *Trich*OKEY results obtained, 10 out of 11 isolates (except YAM1) were in agreement with the results obtained from GenBank database. However, YAM1 was only identified until genus level.

Table 4.7: BLAST and *Trich*OKEY search results for all *Trichoderma* isolates.

Isolate Code	BLAST Results			*Trich*OKEY Results
	Species Identified	Accession no.	Percentage of Homology (%)	
DUR2	*T. harzianum*	KC330218.1	99	*T. harzianum*
DUR5	*T. harzianum*	KC330218.1	99	*T. harzianum*
DUR6	*T. harzianum*	KC330218.1	99	*T. harzianum*
DUR9	*T. harzianum*	KC330218.1	99	*T. harzianum*
PTT	*T. harzianum*	KC139308.1	100	*T. harzianum*
MG3	*T. asperellum*	KC243781.1	100	*T. asperellum*
POM1	*T. asperellum*	JX677933.1	100	*T. asperellum*
PPY1	*T. asperellum*	GU198313.1	100	*T. asperellum*
PPY12	*T. asperellum*	KC243781.1	100	*T. asperellum*
BNN2	*T. virens*	HQ608079.1	100	*T. virens*
YAM1	*T. strigosum*	EU718081.1	100	An unidentified species of *Trichoderma*

4.4 Antagonistic Test against *Fusarium* spp.

Antagonistic effects of all *Trichoderma* isolates were tested against *F. oxysporum* and *F. solani* on PDA at 28℃ for 5 days. In all the dual culture plates tested, the contact zone was a curve, with concavity oriented towards the pathogenic fungi. In the negative control plates, only *Fusarium* species were inoculated (Figure 4.9 and Figure 4.10). The averaged inhibition percentage (I%) of mycelial growth for *F. oxysporum* and *F. solani* were presented in Table 4.8.

Among all *Trichoderma* isolates, DUR9 (*T. harzianum*) exhibited the lowest inhibition to the mycelial growth of *F. oxysporum* with an inhibition percentage of 35.71% whereas PPY1 (*T. asperellum*) showed the highest inhibition percentage (66.67%) against the growth of *F. oxysporum.* In the antagonistic test against *F. solani*, DUR2 (*T. harzianum*) exhibited the lowest antagonistic capacity with inhibition percentage of 25% while PPY1 (*T. asperellum*) exhibited the highest percentage of inhibition, 65.52%. Overall, all *Trichoderma* isolates showed the ability to inhibit the mycelial growth of *F. oxysporum* and *F. solani* with at least 25% of inhibition.

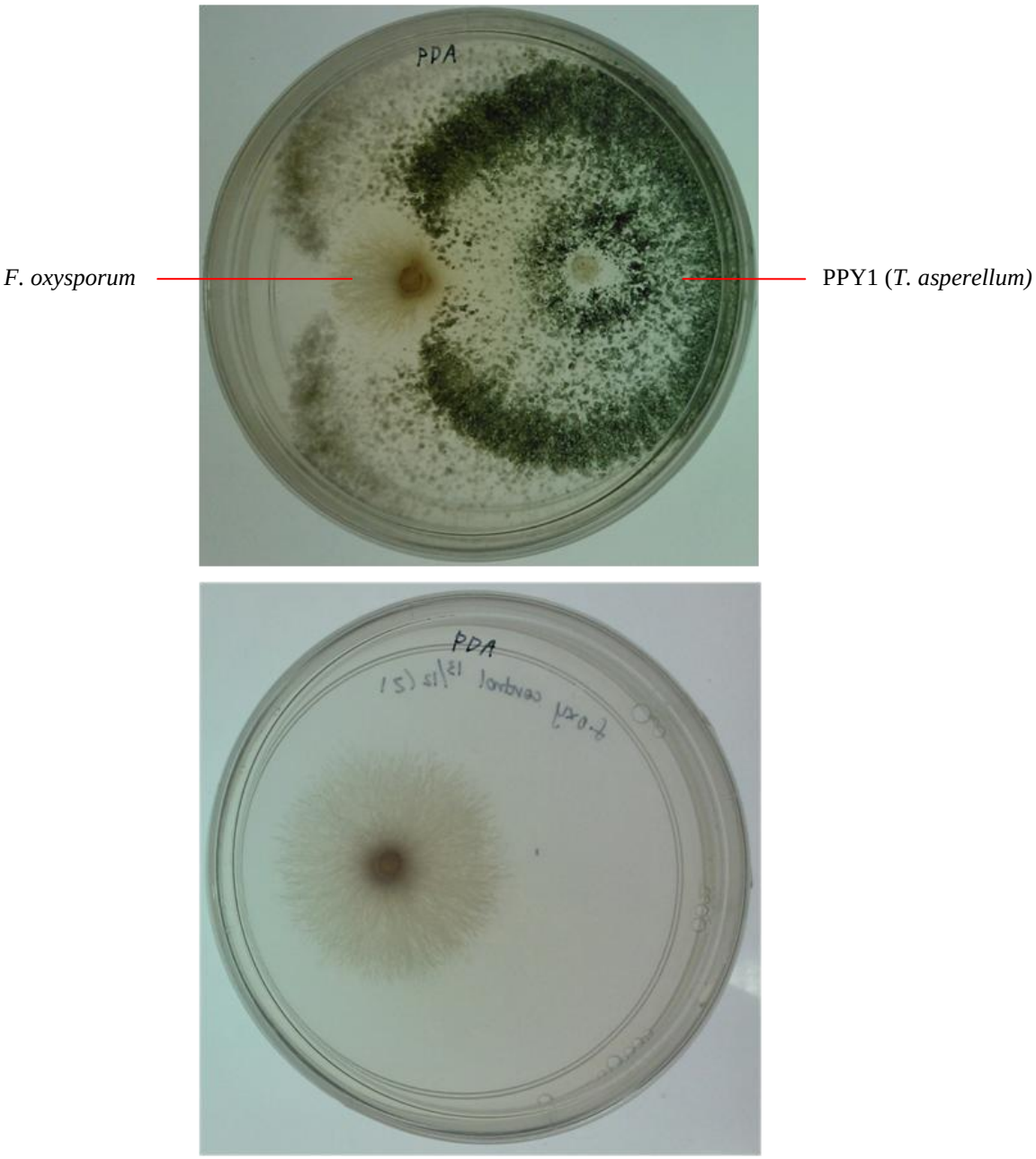

Figure 4.9: Antagonistic test of *Trichoderma* isolates against *F. oxysporum.* Top: Dual culture of *F. oxysporum* and PPY1; Bottom: Negative control.

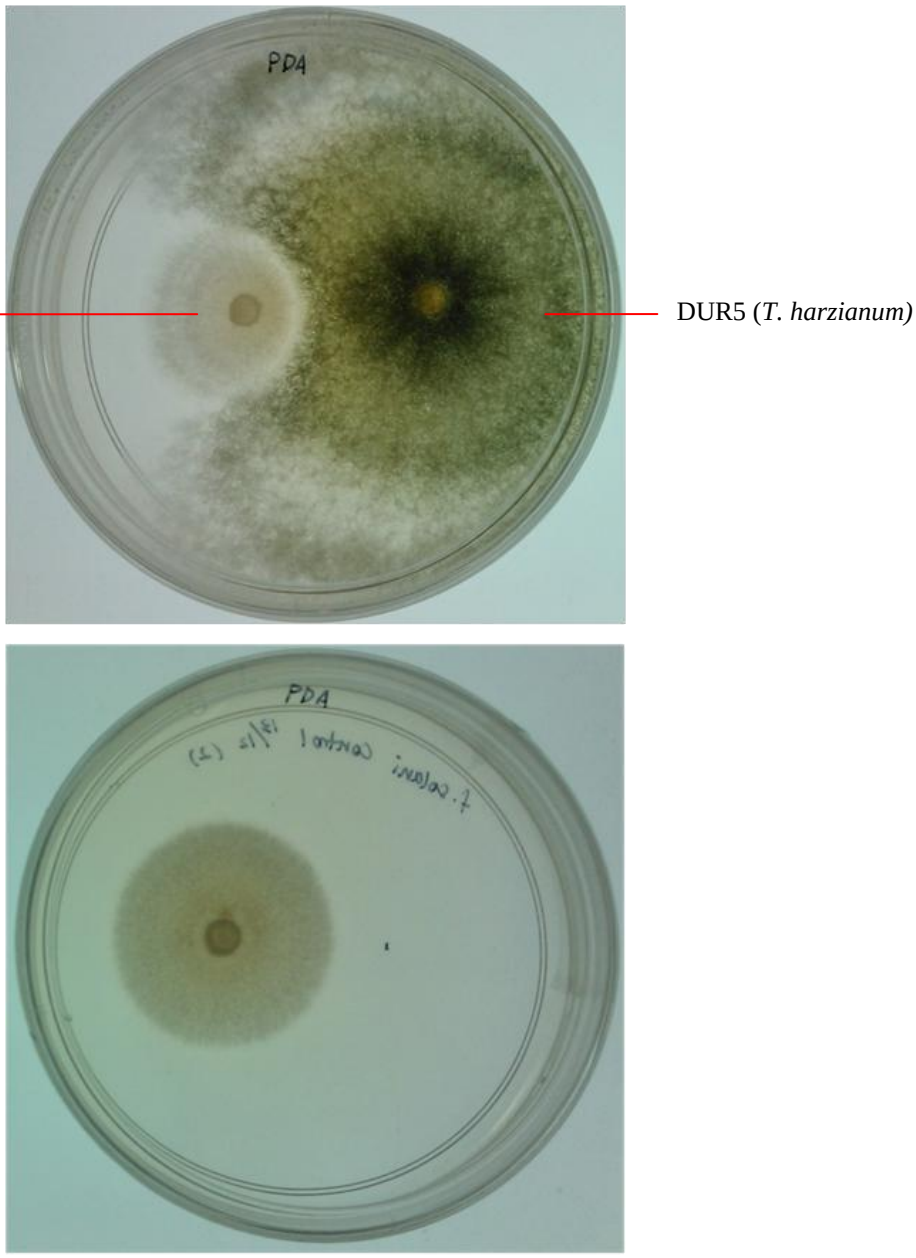

Figure 4.10: Antagonistic test of *Trichoderma* isolates against *F. solani.* Top: Dual culture of *F. solani* and DUR5; Bottom: Negative control.

Table 4.8: Average inhibition percentage of mycelial growth for *F. oxysporum* and *F. solani* by different *Trichoderma* isolates.

***Trichoderma* isolate**	**Average percentage of inhibition against *F. oxysporum* (%)**	**Average percentage of inhibition against *F. solani* (%)**
DUR2 (*T. harzianum*)	54.29	25.00
DUR5 (*T. harzianum*)	40.00	52.94
DUR6 (*T. harzianum*)	52.63	38.46
DUR9 (*T. harzianum*)	35.71	30.43
PTT (*T. harzianum*)	39.29	58.62
MG3 (*T. asperellum*)	52.94	50.00
POM1 (*T. asperellum*)	40.00	44.44
PPY1 (*T. asperellum*)	66.67	65.52
PPY12 (*T. asperellum*)	43.33	48.00
BNN2 (*T. virens*)	61.11	51.85
YAM1 (*T. strigosum*)	60.00	58.06

CHAPTER 5

DISCUSSION

5.1 Species Identification

Morphological characterization was conventionally used in the identification of *Trichoderma* species, and it remains as a potential method to identify *Trichoderma* species (Anees et al., 2010; Gams and Bissett 2002; Samuels et al., 2002a). Therefore, macroscopic and microscopic features of *Trichoderma* isolates were studied in this project.

The growth rate of *Trichoderma* isolates on PDA and CMD cultured at 28 ℃ and 35 ℃ were studied. Their growth rate at 28 ℃ on both PDA and CMD did not provide much information in distinguishing the isolates because they had almost similar growth rate at this temperature. However, Samuels (2004) mentioned that the ability of *Trichoderma* isolate to grow at 35 ℃ is useful in identification of some *Trichoderma* species. In this study, YAM1 isolate can be distinguished from other isolates due to its restricted growth at 35 ℃. Besides that, PTT had the fastest growth rate compared to other isolates at 35 ℃. Thus, PTT can be differentiated from other isolates based on its fast growth rate.

Besides macroscopic characteristics and growth rate, microscopic features of *Trichoderma* isolates are also important morphological keys in the identification of *Trichoderma* species. The microscopic features that are

frequently studied include the shapes and sizes of conidia, the branching patterns of conidiophores, the shapes and sizes of phialides, and the production of chlamydospores (Anees et al., 2010; Gams and Bissett 2002; Samuels et al., 2002a).

In this study, the descriptions of the shapes of conidia were not really useful in identifying most of the isolates due to the confusion caused by the use of different terms in different literatures in describing the shapes of the conidia. In addition, no systematic rule was established in defining the shapes of the conidia. The description of the shapes of conidia may be subjective, and thus it may be imprecise to be used in the identification of *Trichoderma* species. However, the measurements of conidial size were relatively useful in species identification especially in the identification of *T. harzianum* species. The relatively small conidia size of *T. harzianum* (2.6-2.9 μm) was the key to distinguished *T. harzianum* isolates from other *Trichoderma* species. However, the species identity of other isolates cannot be directly identified based on their conidial sizes because more than one *Trichoderma* species were found to have similar conidial sizes as these isolates. Nevertheless, conidial sizes of these isolates were useful in narrowing down the possible number of *Trichoderma* species in the progress of species identification.

Furthermore, the branching patterns of conidiophores also served as a distinguishing characteristic for *Trichoderma* species. Some of the *Trichoderma* species possess paired primary branches in their conidiophores while others might have primary branches formed in nearly 90 ° to the main

axis of conidiophores, or branching towards the tips (Samuels et al., 2002b). In this study, the appearance of conidiophores of *T. virens* isolate (BNN2) was different from other isolates. Its primary branches were not form in pairs and tend to branch towards the tips. Moreover, the phialides of this isolate were closely appressed (Figure 4.6L), which was not observed in any other isolates. Hence, based on the arrangement of conidiophores and phialides, *T. virens* isolate can be differentiated from other isolates. Other than that, the fertile hair (Figure 4.6J) observed in *T. strigosum* isolate (YAM1) was not found in other isolate. Based on this characteristic, *T. strigosum* isolate can be differentiated from other *Trichoderma* isolates. Gams and Bissett (2002) also mentioned that the production of sweet coconut smell could be a characteristic of a species. In this study, *T. strigosum* isolate (YAM1) were found to produce an odour resembling sweet coconut smell, which was contributed by the volatile compounds (6-pentyl-α-pyrone) produced by the colonies (Klein and Eveleigh 2002).

The production of chlamydospores by all *Trichoderma* isolates was also observed. All *Trichoderma* isolates were found to produce chlamydospores within 10 days. The appearance of chlamydospores did not provide much information for the identification of *Trichoderma* isolates as all the chlamydospores observed were uniform in appearance (Figure 4.7). The chlamydospores of all *Trichoderma* isolates were unicellular, globose to subglobose, and usually formed on the hyphal tips. However, evaluation on the presence or absence of chlamydospores within 10 days can be a morphological key for the identification of a *Trichoderma* species (Samuels et

al., 2002a). In this study, the presence of chlamydospores in *T. virens* isolate (BNN2) is important in differentiating it from *T. crassum*, a *Trichoderma* species that has the same conidiophore features as *T. virens*. According to Samuels et al. (2002a), *T. crassum* did not produce chlamydospores within 10 days.

By combining the morphological characteristics observed, species identity of *Trichoderma* isolates could be determined by using an online interactive key for strain identification provided by Samuels and his coworkers at http://nt.ars-grin.gov/taxadescriptions/keys/FrameKey.cfm?gen=Trichoderma. Isolate BNN2 was identified as *T. virens*, YAM1 was identified as *T. strigosum*, while DUR2, DUR5, DUR6, DUR9 and PTT were identified as *T. harzianum*. For isolates MG3, POM1, PPY1 and PPY12, the presence of pustules on CMD was an important characteristic that differentiated them from *T. pseudokoningii* and *T. saturnisporum*, allowing them to be identified as *T. asperellum*.

However, information from morphological study alone is insufficient to precisely identify a *Trichoderma* species because *Trichoderma* species have relatively few morphological characters and limited variation that may cause overlapping and misidentification of the isolates (Anees et al., 2010). Besides that, morphological characteristics are influenced by culture conditions (Diguta et al., 2011). Therefore, there is a necessity to use molecular technique to compensate for the limitations of morphological characterization.

In this study, DNA sequencing of the 5.8S-ITS region was carried out. The ITS region is one of the most reliable loci for the identification of a strain at the species level (Kullnig-Gradinger et al., 2002). By comparing the sequences of the 5.8S-ITS region to the sequences deposited in GenBank, all of the *Trichoderma* isolates can be identified to species level with homology percentage of at least 99% (Table 4.7). However, Druzhinina and Kubicek (2004) mentioned that GenBank database contain many sequences of *Trichoderma* isolates which may have been incorrectly identified and occurred under a false name. Hence, *Trich*OKEY search tool, a program that specifically compare ITS1 and ITS2 sequences to a specific database for *Trichoderma* generated from only vouchered sequences were used to assess the reliability of BLAST results. *Trich*OKEY was recently used by many literatures and resulted in successful identification of *Trichoderma* isolates (Anees et al., 2010; Migheli et al., 2009). From the *Trich*OKEY results obtained (Table 4.7), all isolates except YAM1 were identified, and the results were in agreement with the BLAST results. Isolate YAM1, however, was identified as an unknown *Trichoderma* species. Thus, morphological data of YAM1 in this case were especially important to check the species identity of YAM1. By comparing to the morphological characteristics described (Samuels et al., 2002a; Gams and Bissett 2002), YAM1 was identified as *T. strigosum*. The restricted growth of YAM1 at 35 ℃, its conidial size and its production of sweet coconut smell were the important characteristics for the successful identification of YAM1 isolate.

Morphological and molecular approaches were shown to play important roles in the identification of *Trichoderma* isolates. Each approach has its own limitations and strengths. By combining morphological and molecular approaches, all *Trichoderma* isolates were successfully identified. The results obtained from the morphological interactive key, BLAST and *Trich*OKEY search tools were found to be in agreement. Among the eleven isolates, five *T. harzianum* (DUR2, DUR5, DUR6, DUR9 and PTT), four *T. asperellum* (MG3, POM1, PPY1 and PPY12), one *T. virens* (BNN2) and one *T. strigosum* (YAM1) were identified.

From the colony appearances observed, the *Trichoderma* isolates obtained can be classified into four groups (Figure 4.1). Morphological features of each *Trichoderma* isolate within the same group were very similar. Therefore, isolates within each group most probably belong to the same species. Results obtained from the morphological interactive key, BLAST search tool, and *Trich*OKEY were in agreement and isolates within the same group were identified as same species.

5.2 Antagonistic Test against *Fusarium* spp.

The antagonistic capacities of all *Trichoderma* isolates against *F. oxysporum* and *F. solani* were tested using dual culture method. In all the dual culture plates, the contact zone appeared as a curve, with concavity oriented towards *Fusarium*. The curvature of the contact area between the colony of antagonistic fungi and the colony of pathogenic fungi in the same PDA plate

depend on the growth rate of the colonies. If one colony has a faster growth rate than the other, a curve in the contact zone will most probably be observed. However, if the two colonies have the same growth rate, a straight line would be observed when mycelia from both fungi come into contact (Petrescu et al., 2012). Moreover, all *Trichoderma* isolates exhibited inhibition to the mycelial growth of both *F. oxysporum* and *F. solani* prior to mycelial contact. This could be due to the production of diffusible components, such as lytic enzymes or water-soluble metabolites, by *Trichoderma* isolates (Grondona et al., 1997; Anees et al., 2010). These components such as chitinases and glucanases were always secreted by *Trichoderma* in low level, so that they can act against the pathogenic fungi before mycelial contact, thus increasing the antagonism of *Trichoderma*. The degraded cell wall components from *Fusarium* were then induced the expression of gene related to mycoparasiticism, allowing *Trichoderma* to be more antagonistic (Vinale et al., 2007).

Furthermore, the antagonistic effects of all *Trichoderma* isolates were determined through the average inhibition percentage of mycelial growth of pathogenic fungi. This approach was frequently used and shown to be a useful way in assessing the antagonistic potential of the antagonistic fungi (Abadi 1990; Grondona et al., 1997; Petrescu et al., 2012).

From the antagonistic test against *F. oxysporum*, all isolates were found to have inhibition on the mycelial growth of *F. oxysporum*, with the lowest inhibition percentage of 35.71% by DUR9 (*T. harzianum*), and highest

inhibition percentage of 66.67% by PPY1 (*T. asperellum*). In the antagonistic test against *F. solani*, all isolates were also shown to inhibit the growth of *F. solani*. DUR2 (*T. harzianum*) established the lowest inhibition percentage, 25% whereas PPY1 (*T. asperellum*) showed the highest inhibition percentage, 65.52%. Overall, all *Trichoderma* isolates have the ability to inhibit the mycelial growth of *F. oxysporum* and *F. solani*. Nevertheless, PPY1 isolate, which was identified as *T. asperellum,* was the most efficient isolate in inhibiting the mycelial growth of the pathogenic fungi tested. This was in agreement with Ommati and Zaker (2012) who evaluated the antagonistic capacities of *T. atroviride, T. asperellum, T. longibrachiatum, T. harzianum* and *T. brevicompactum* against *F. oxysporum.* They found that *T. asperellum* had the highest inhibition on the growth of *F. oxysporum*. Besides that, they showed that *T. asperellum* could reduce potato wilt disease incidence by 70.5% under green house conditions. Furthermore, *T. asperellum* was also reported as an efficient biological control agent in controlling Fusarium wilt in tomato caused by pathogenic strain of *F. oxysporum* (Cotxarrera et al., 2002; Segarra et al., 2010).

In this study, it was also found that the antagonism of different isolates that belong to the same species varied. Isolates PPY1 and POM1 which were both identified as *T. asperellum,* showed big difference in their inhibition percentage (26.67%). Similarly, this phenomenon was also observed among isolates DUR2, DUR5, DUR6, DUR9 and PTT which were identified as *T. harzianum.* This situation was also reported by Anees et al. (2010). This phenomenon could be due to the different biological control mechanisms

established by the isolates (Anees et al., 2010). Another possible explanation for this situation is that different isolates may have different efficiency in expressing the genes responsible for antagonistic activities. The isolates that can express these genes more rapidly and efficiently are usually better antagonists (Scherm et al., 2009).

5.3 Future Studies

By analyzing the 5.8S-ITS sequences, most of the *Trichoderma* species can be identified. Nonetheless, some closely related species, especially *Trichoderma* species from section *Trichoderma* may not be distinguished, because some of them may share the sequences of their ITS regions. (Samuels 2006) Therefore, a multigene approach, where two or more unlinked loci are used in species identification, is recommended (Anees et al., 2010). Usually, a combination of the 5.8S-ITS region and *tef1* gene is used (Migheli et al., 2009; Anees et al., 2010). According to Samuels (2006), identification of closely related species is more reliable by using *tef1* gene compared to the 5.8S-ITS region. The *tef1* gene, which encodes for the translational elongation factor 1-α, contains the fourth and fifth introns that are highly variable among closely related species (Druzhinina and Kubicek 2004). Besides, the *tef1* gene is also more variable compared to the 5.8S-ITS region. Therefore, *tef1* gene of *Trichoderma* isolates can be sequenced in future to further confirm the identities of *Trichoderma* isolates, especially for isolate YAM1.

Other than that, more morphological characteristics of these *Trichoderma* isolates can be studied. These morphological characteristics include the length and width of phialides, the sizes of chlamydospores, the presence or absence of fertile hair, and the growth rate on synthetic nutrient agar (SNA) at 35℃ (Samuels et al., 2002b; Anees et al., 2010). Further characterization based on these morphological characteristics could help to enhance the credibility and reliability of the morphological identification results.

Lastly, more studies need to be conducted before the antagonistic *Trichoderma* isolates can be used as biological control agents in agricultural field. Field trials or *in vivo* tests can be carried out to check the antagonism of *Trichoderma* isolates, especially PPY1, in the presence of host plants. In a study carried out by Thangavelu et al. (2004), the most antagonistic isolate determined *in vitro*, the *T. harzianum* Th-10, was used to conduct *in vivo* tests. Soil application of this isolate in dried formulation was shown to effectively suppressed Fusarium wilt disease in bananas.

CHAPTER 6

CONCLUSION

A total of 11 *Trichoderma* isolates were isolated from rhizosphere soils, humus and compost. They were successfully characterized and identified by a combination of morphological and molecular approaches. The morphological characters that were useful in the species identification include growth rate at 35℃, the size of conidia, the branching patterns of conidiophores, the arrangement of phialides, the production of sweet coconut smell, the presence or absence of chlamydospores, and the ability of *Trichoderma* isolates to form pustules on CMD. In molecular approach, sequence analysis of 5.8S-ITS region were carried out. The sequences of 5.8S-ITS region were compared to sequences deposited in GenBank and *Trich*OKEY database by using BLAST and *Trich*OKEY 2 program, respectively. The results from both approaches were found to be in agreement. Among the 11 isolates, five *T. harzianum*, four *T. asperellum*, one *T. virens* and one *T. strigosum* were identified.

From the results of the antagonistic test, all *Trichoderma* isolates were shown to have the ability to inhibit the mycelial growth of *F. oxysporum* and *F. solani* with at least 25%. Among these *Trichoderma* isolates, PPY1, which was identified as *T. asperellum*, had the biggest potential to be used as a biological control agent because it had the highest antagonistic effects on the mycelial growth of *F. oxysporum* and *F. solani* with inhibition percentage of 66.67% and 65.52%, respectively. More studies such as *in* vivo tests have to

be conducted using these *Trichoderma* isolates, especially PPY1, before they can be used as a biological control agent in agricultural field.

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APPENDIX A

Consensus sequences of *Trichoderma* isolates

BNN2:

CGAGTTTACA ACTCCCAAAC CCAATGTGAA CGTTACCAAA CTGTTGCCTC GGCGGGATCT
CTGCCCCGGG TGCGTCGCAG CCCCGGACCA AGGCGCCCGC CGGAGGACCA ACCAAAACTC
TTATTGTATA CCCCCTCGCG GGTTTTTTAC TATCTGAGCC ATCTCGGCGC CCCTCGTGGG
CGTTTCGAAA ATGAATCAAA ACTTTCAACA ACGGATCTCT TGGTTCTGGC ATCGATGAAG
AACGCAGCGA AATGCGATAA GTAATGTGAA TTGCAGAATT CAGTGAATCA TCGAATCTTT
GAACGCACAT TGCGCCCGCC AGTATTCTGG CGGGCATGCC TGTCCGAGCG TCATTTCAAC
CCTCGAACCC CTCCGGGGGG TCGGCGTTGG GGATCGGCCC TTTACGGGGC CGGCCCCGAA
ATACAGTGGC GGTCTCGCCG CAGCCTCTCC TGCGCAGTAG TTTGCACACT CGCATCGGGA
GCGCGGCGCG TCCACAGCCG TTAAACACCC CAAACTTCTG AAATGTTGAC CTCGGATCAG
GTAGGAATAC CC

DUR2:

AACTCCCAAA CCCAATGTGA ACGTTACCAA ACTGTTGCCT CGGCGGGATC TCTGCCCCGG
GTGCGTCGCA GCCCCGGACC AAGGCGCCCG CCGGAGGACC AACCAAAACT CTTTTTGTAT
ACCCCCTCGC GGGTTTTTTA TAATCTGAGC CTTCTCGGCG CCTCTCGTAG GCGTTTCGAA
AATGAATCAA AACTTTCAAC AACGGATCTC TTGGTTCTGG CATCGATGAA GAACGCAGCG
AAATGCGATA AGTAATGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT TGAACGCACA
TTGCGCCCGC CAGTATTCTG GCGGGCATGC CTGTCCGAGC GTCATTTCAA CCCTCGAACC
CCTCCGGGGG GTCGGCGTTG GGGATCGGCC CTCCCTTAGC GGGTGGCCGT CTCCTAAATA
CAGTGGCGGT CTCGCCGCAG CCTCTCCTGC GCAGTAGTTT GCACACTCGC ATCGGGAGCG
CGGCGCGTCC ACAGCCGTTA AACACCCAAC TTCTGAAATG TTGACCTCGG ATCAGGTAGG
AATACCC

DUR5:

TACAACTCCC AAACCCAATG TGAACGTTAC CAAACTGTTG CCTCGGCGGG ATCTCTGCCC
CGGGTGCGTC GCAGCCCCGG ACCAAGGCGC CCGCCGGAGG ACCAACCAAA ACTCTTTTTG
TATACCCCCT CGCGGGTTTT TTATAATCTG AGCCTTCTCG GCGCCTCTCG TAGGCGTTTC
GAAAATGAAT CAAAACTTTC AACAACGGAT CTCTTGGTTC TGGCATCGAT GAAGAACGCA
GCGAAATGCG ATAAGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT CTTTGAACGC
ACATTGCGCC CGCCAGTATT CTGGCGGGCA TGCCTGTCCG AGCGTCATTT CAACCCTCGA
ACCCCTCCGG GGGGTCGGCG TTGGGGATCG GCCCTCCCTT AGCGGGTGGC CGTCTCCTAA
ATACAGTGGC GGTCTCGCCG CAGCCTCTCC TGCGCAGTAG TTTGCACACT CGCATCGGGA
GCGCGGCGCG TCCACAGCCG TTAAACACCC AACTTCTGAA ATGTTGACCT CGGATCAGGT
AGGAATACCC GCT

DUR6:

TTTACAACTC CCAAACCCAA TGTGAACGTT ACCAAACTGT TGCCTCGGCG GGATCTCTGC
CCCGGGTGCG TCGCAGCCCC GGACCAAGGC GCCCGCCGGA GGACCAACCA AAACTCTTTT
TGTATACCCC CTCGCGGGTT TTTTATAATC TGAGCCTTCT CGGCGCCTCT CGTAGGCGTT
TCGAAAATGA ATCAAAACTT TCAACAACGG ATCTCTTGGT TCTGGCATCG ATGAAGAACG
CAGCGAAATG CGATAAGTAA TGTGAATTGC AGAATTCAGT GAATCATCGA ATCTTTGAAC
GCACATTGCG CCCGCCAGTA TTCTGGCGGG CATGCCTGTC CGAGCGTCAT TTCAACCCTC
GAACCCCTCC GGGGGGTCGG CGTTGGGGAT CGGCCCTCCC TTAGCGGGTG GCCGTCTCCT
AAATACAGTG GCGGTCTCGC CGCAGCCTCT CCTGCGCAGT AGTTTGCACA CTCGCATCGG
GAGCGCGGCG CGTCCACAGC CGTTAAACAC CCAACTTCTG AAATGTTGAC CTCGGATCAG
GTAGGAATAC CC

DUR9:

CGAGTTTACA ACTCCCAAAC CCAATGTGAA CGTTACCAAA CTGTTGCCTC GGCGGGATCT
CTGCCCCGGG TGCGTCGCAG CCCCGGACCA AGGCGCCCGC CGGAGGACCA ACCAAAACTC
TTTTTGTATA CCCCCTCGCG GGTTTTTTAT AATCTGAGCC TTCTCGGCGC CTCTCGTAGG
CGTTTCGAAA ATGAATCAAA ACTTTCAACA ACGGATCTCT TGGTTCTGGC ATCGATGAAG
AACGCAGCGA AATGCGATAA GTAATGTGAA TTGCAGAATT CAGTGAATCA TCGAATCTTT
GAACGCACAT TGCGCCCGCC AGTATTCTGG CGGGCATGCC TGTCCGAGCG TCATTTCAAC
CCTCGAACCC CTCCGGGGGG TCGGCGTTGG GGATCGGCCC TCCCTTAGCG GGTGGCCGTC
TCCTAAATAC AGTGGCGGTC TCGCCGCAGC CTCTCCTGCG CAGTAGTTTG CACACTCGCA
TCGGGAGCGC GGCGCGTCCA CAGCCGTTAA ACACCCAACT TCTGAAATGT TGACCTCGGA
TCAGGTAGGA ATACCC

MG3:

CGAGTTTACA ACTCCCAAAC CCAATGTGAA CGTTACCAAA CTGTTGCCTC GGCGGGGTCA
CGCCCCGGGT GCGTCGCAGC CCCGGAACCA GGCGCCCGCC GGAGGAACCA ACCAAACTCT
TTCTGTAGTC CCCTCGCGGA CGTATTTCTT TACAGCTCTG AGCAAAAATT CAAAATGAAT
CAAAACTTTC AACAACGGAT CTCTTGGTTC TGGCATCGAT GAAGAACGCA GCGAAATGCG
ATAAGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT CTTTGAACGC ACATTGCGCC
CGCCAGTATT CTGGCGGGCA TGCCTGTCCG AGCGTCATTT CAACCCTCGA ACCCCTCCGG
GGGATCGGCG TTGGGGATCG GGACCCCTCA CACGGGTGCC GGCCCCTAAA TACAGTGGCG
GTCTCGCCGC AGCCTCTCCT GCGCAGTAGT TTGCACAACT CGCACCGGGA GCGCGGCGCG
TCCACGTCCG TAAAACACCC AACTTTCTGA AATGTTGACC TCGGATCAGG TAGGAATACC
C

POM1:

CGAGTTTACA ACTCCCAAAC CCAATGTGAA CGTTACCAAA CTGTTGCCTC GGCGGGGTCA
CGCCCCGGGT GCGTCGCAGC CCCGGAACCA GGCGCCCGCC GGAGGAACCA ACCAAACTCT
TTCTGTAGTC CCCTCGCGGA CGTATTTCTT ACAGCTCTGA GCAAAAATTC AAAATGAATC
AAAACTTTCA ACAACGGATC TCTTGGTTCT GGCATCGATG AAGAACGCAG CGAAATGCGA
TAAGTAATGT GAATTGCAGA ATTCAGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC
GCCAGTATTC TGGCGGGCAT GCCTGTCCGA GCGTCATTTC AACCCTCGAA CCCCTCCGGG
GGATCGGCGT TGGGGATCGG GACCCCTCAC ACGGGTGCCG GCCCCGAAAT ACAGTGGCGG
TCTCGCCGCA GCCTCTCCTG CGCAGTAGTT TGCACAACTC GCACCGGGAG CGCGGCGCGT
CCACGTCCGT AAAACACCCA ACTTTCTGAA ATGTTGACCT CGGATCAGGT AGGAATACCC

PPY1:

CGAGTTTACA ACTCCCAAAC CCAATGTGAA CGTTACCAAA CTGTTGCCTC GGCGGGGTCA
CGCCCCGGGT GCGTCGCAGC CCCGGAACCA GGCGCCCGCC GGAGGAACCA ACCAAACTCT
TTCTGTAGTC CCCTCGCGGA CGTATTTCTT TACAGCTCTG AGCAAAAATT CAAAATGAAT
CAAAACTTTC AACAACGGAT CTCTTGGTTC TGGCATCGAT GAAGAACGCA GCGAAATGCG
ATAAGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT CTTTGAACGC ACATTGCGCC
CGCCAGTATT CTGGCGGGCA TGCCTGTCCG AGCGTCATTT CAACCCTCGA ACCCCTCCGG
GGGATCGGCG TTGGGGATCG GGACCCCTCA CACGGGCGCC GGCCCCTAAA TACAGTGGCG
GTCTCGCCGC AGCCTCTCCT GCGCAGTAGT TTGCACAACT CGCACCGGGA GCGCGGCGCG
TCCACGTCCG TAAAACACCC AACTTTCTGA AATGTTGACC TCGGATCAGG TAGGAATACC
C

PPY12:

CGAGTTTACA ACTCCCAAAC CCAATGTGAA CGTTACCAAA CTGTTGCCTC GGCGGGGTCA
CGCCCCGGGT GCGTCGCAGC CCCGGAACCA GGCGCCCGCC GGAGGAACCA ACCAAACTCT
TTCTGTAGTC CCCTCGCGGA CGTATTTCTT TACAGCTCTG AGCAAAAATT CAAAATGAAT
CAAAACTTTC AACAACGGAT CTCTTGGTTC TGGCATCGAT GAAGAACGCA GCGAAATGCG
ATAAGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT CTTTGAACGC ACATTGCGCC
CGCCAGTATT CTGGCGGGCA TGCCTGTCCG AGCGTCATTT CAACCCTCGA ACCCCTCCGG
GGGATCGGCG TTGGGGATCG GGACCCCTCA CACGGGTGCC GGCCCCTAAA TACAGTGGCG
GTCTCGCCGC AGCCTCTCCT GCGCAGTAGT TTGCACAACT CGCACCGGGA GCGCGGCGCG
TCCACGTCCG TAAAACACCC AACTTTCTGA AATGTTGACC TCGGATCAGG TAGGAATACC
CG

PTT:

CGAGTTTACA ACTCCCAAAC CCAATGTGAA CGTTACCAAA CTGTTGCCTC GGCGGGATCT
CTGCCCCGGG TGCGTCGCAG CCCCGGACCA AGGCGCCCGC CGGAGGACCA ACCAAAACTC
TTATTGTATA CCCCCTCGCG GGTTTTTTTT ATAATCTGAG CCTTCTCGGC GCCTCTCGTA
GGCGTTTCGA AAATGAATCA AAACTTTCAA CAACGGATCT CTTGGTTCTG GCATCGATGA
AGAACGCAGC GAAATGCGAT AAGTAATGTG AATTGCAGAA TTCAGTGAAT CATCGAATCT
TTGAACGCAC ATTGCGCCCG CCAGTATTCT GGCGGGCATG CCTGTCCGAG CGTCATTTCA
ACCCTCGAAC CCCTCCGGGG GGTCGGCGTT GGGGATCGGC CCTGCCTTGG CGGTGGCCGT
CTCCGAAATA CAGTGGCGGT CTCGCCGCAG CCTCTCCTGC GCAGTAGTTT GCACACTCGC
ATCGGGAGCG CGGCGCGTCC ACAGCCGTTA AACACCCAAC TTCTGAAATG TTGACCTCGG
ATCAGGTAGG AATACCC

YAM1:

CGAGTTTACA ACTCCCAAAC CCAATGTGAA CGTTACCAAA CTGTTGCCTC GGCGGGGTCA
CGCCCCGGGT GCGTCGCAGC CCCGGAACCA GGCGCCCGCC GGAGGAACCA ACCAAACTCT
TTACTGTAGT CCCCTCGCGG ACGTTTTTAC TTACAGCTCT GAGCAAAAAT TCAAAATGAA
TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC AGCGAAATGC
GATAAGTAAT GTGAATTGCA GAATTCAGTG AATCATCGAA TCTTTGAACG CACATTGCGC
CCGCCAGTAT TCTGGCGGGC ATGCCTGTCC GAGCGTCATT TCAACCCTCG AACCCCTCCG
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CGCGTCCACG TCCGTAAAAC ACCCAACTTT CTGAAATGTT GACCTCGGAT CAGGTAGGAA
TACCC