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Trichoderma SPECIES DIVERSITY IN RHIZOSPHERE SOILS AND POTENTIAL ANTAGONISM WITH *Fusarium oxysporum*

DIVERSIDADE DE ESPÉCIES DE Trichoderma EM SOLOS DE RIZOSFERA E POTENCIAL ANTAGONISMO COM Fusarium oxysporum

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ABSTRACT: In this study six different *Trichoderma* species were isolated form rhizosphere soils of paddy, banana, oil palm, rubber, vegetables and grass land soils. The species are *T. harziaum, T. viride, T. koningii, T. asperrelum,* and *T. parareesei*. The morphological study as pigmentation, colony growth and anatomical studies such as, conidiation appearances, size of conidia, conidiophores branching pattern, shapes of phialides, absent or present of chlamydiophores were carried out to identify the species of *Trichoderma*. The *Trchoderma harzianum* species were abundant in soil while *T. viren* was the second highest in the soil. All species showed the antagonistic activity against *Fusarium oxysporum*. While, *T. parareesei* showed the highest antagonistic 91.10 % activity against *F. oxysporum*, reported as best antagonism agent for phytopathogen.

KEYWORDS: Antagonistic activity. Diversity. *Fusarium oxysporum*. *Trichoderma.*

INTRODUCTION

Fungi play the important roles in ecosystem and economic for development in nature. Decomposer for soil or disease causing in plant or animal fungi have numerous effect in both sectors. One of the most diverse groups of organisms on Earth is kingdom fungi because they are integral ecosystem agents that cycle soil carbon cycling and plant nutrition. Most of the fungi are entirely multicellular, heterotrophic and known as decomposers of nutrients (VERMA, 2012). *Trichoderma* is the genus of fungus that are economical important fungus as for decomposer in soil, celluloses or hemicellualases enzyme production for industrial, antibiotic production or act as biocontrol agent (SEKHAR et al., 2017). *Trichoderma* is soil borne fungus thus ubiquitous in all agricultural soil. As soil received the plant and animal residues, the soil microorganisms start to decompose the material. *Trichoderma* basically role as decomposer for cellulose, hemicellulose and chitin, however, the fungus also paly symbionts with plant and parasitic to other fungi (HARMAN, 2004). During the symbiotic situation, *Trichoderma* act as enhancer in plant in different sectors. Plant received advantages from *Trichoderma*, a) act as security that means protect plant form pathogenic fungi by direct encounter as *Trichoderma* grow towards the pathogenic fungus then coil around the it and kill the fungus or hydrolyse the fungus hypha (HARMAN et al., 2004), b) in direct protection *Trichoderma* induced systemic resistance in plant, (HARMAN et al., 2004), and c) *Trichoderma* stay in root niche, the typical hyphal extension of *Trichoderma* tiger or stimulates the roots growth and bunches (HARMAN et al., 2004, NAHER et al., 2011). With this agreement found in oil palm plant which roots bunches were expand when oil palm treated with *Trichoderma harziaum* mulch compare to control plant (NAHER et al., 2012, 2014). LEE et al. (2016) reported that *Trichoderma* species produced volatile organic compounds in conjunction to enhance plant growth. In their study they found that Tomato biomass increased >99%, plant length and lateral roots also increased in corporation with *Trichoderma viride* treatment (LEE et al., 2016). Other than that, the presence of *Trichoderma* species in soil act as pesticides to kill the larvae and nematode in the soil. *Trichoderma* species penetrate the nematode body by forming haustoria like structure and colonize internally causing the death of nematodes (ZAIDI; SINGH, 2013). The role as fungicide the fungus control many phytopathogenic fungi of *Rhizoctonia solani* in potato plant, *Ganoderma boninense* in oil palm seedlings, *Fusarium oxysporum* in banana plant (NAHER et al., 2014; RAHMAN et al., 2014; ZHANG et al., 2014;). The role of *Trichoderma* as biofungicides and biofertilizer depends on the effectiveness of *Trichoderma* species. Thus, the isolation of *Trichoderma* species diversity is very importance. Hence, this research was conducted to identify the *Trichoderma* diversity in various rhizosphere soil to

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find the effective biocontrol agent against *Fusarium oxysporum*, a phytopathogen in several plant diseases.

MATERIAL AND METHODS

Soil sample collection

Rhizosphere soil samples were taken from different cultivated area as paddy, rubber, oil palm, banana plantation. The soil samples were collected at depth of 10 cm away from the surface and at the rhizosphere area. About 150 gram of soil for each site was taken by using soil auger, and then placed the soil into plastic zipper bag and kept in fridge at 4˚C until used.

Media preparation

In this study two different medium as DRBA (Dichoran Rose Bengal Chloramphenicol) and PDA (Potato Dextrose Agar) media were used. DRBA media is good for soil fungal isolation (NAHER et al., 2016). While DPA was used for further culture process. A 19 gram of PDA powder and a 31.6 gram of DRBC were weighed and diluted in 500 ml distilled water in conical flask in separately. Both conical flasks were stirred with hot plate for few minutes to dissolve the powder completely. Then, both medium was autoclaved at 121˚C, 15 psi for 15 minutes.

Soil suspension and Soil serial dilution preparation

Soil suspension prepared as 10 g of soil samples were dissolve in 100 ml of sterilized distilled water and then mixed the composition using rotary shaker at 100 rpm for 10 minutes. The soil suspension was used to prepare soil serial dilution to isolates the *Trichoderma* colony from soils. Serial dilutions were prepared as follows 1 ml of soil suspension was added in 9 ml of sterilized distilled water to make first dilution of 10^1 from there subsequent dilutions prepared labelled as 10^2, 10^3, 10^4, and 10^5.

Soil culture

Soil culture was performed to isolate the Trichoderma colony from soil. Under the sterile condition, 1 ml of each dilution (10^1, 10^2, 10^3, 10^4, and 10^5) were pipetted into petri dish in separately followed with about 9 ml DRBC agar solution was added into the plates in three replicates. The media was swirled gently to mix the solution and let it harden. After the media solidify, the plates was sealed with parafilm.

Isolation of Trichoderma colony

The diverse fungal growth on DRBA agar plate was observed daily and calculated the colony formed. The visible fungal colonies formed were identified based on macromorphology characteristics as for primary selection of *Trichoderma* colony and then isolated into PDA media to obtain a pure culture.

Slide culture observation

A slide culture technique was done to identify the species of *Trichoderma* under light microscope. Under sterile condition, a sterile cotton swab and filter paper were placed in the petri dish and a microscopic slide was placed on cotton swab. Three to five drops of distilled water was dropped on the filter paper to keep the environment in the petri dish moisture for fungal growth. About 6 mm x 6 mm size of PDA agar was cut and placed at the centre on the microscopic slide. Four side of agar square were inoculated with spores of mycelia of fungus and cover with microscopic cover slit. The petri dish was covered and incubated at room temperature for two days to let the mycelia to grow.

Identification of *Trichoderma* species

Trichoderma species identification was carried out based on macro and micro-morphological characteristics. For macro-morphological characteristics colony growth, pigmentation. The micromorphology characteristic examination of fungus such as conidia, conidiophores, philiades, pigmentation and spore structure was identified under light microscope for identification key of *Trichoderma* species (GAMS; BISSETT, 2002). Identification key for *Trichoderma* species was used to compare and differentiate between species.

Fusarium oxysporum culture

The stock culture of *Fusarium oxysporum* was collected from tissue culture laboratory (UMK Jeli campus) which was isolated from banana plant in another study. The mycelial tips was transferred into a new PDA plate to obtain the pure culture of *F. oxysporum*.

Antagonistic activity of *Trichoderma* isolates against *F. oxysporum*

An agar disc with diameter of 6 mm of newly grown pure culture *Fusarium oxysporum* was taken and placed from 1 cm of the petri dish wall. The plates was leaved for three days to growth of *Fusarium oxysporum*. Then *Trichoderma* species was introduced into the same plate but opposite site

of the *Fusarium oxysporum*. Whereas, same technique was used for control plate but without received Trichoderma. The experiment was conducted in three replicates. All plates were left for 6 to 7 days to allow the colony grow. The linear growth and Percentage Inhibition of Radial Growth (PIRG) of *Fusarium oxysporum* was measured and calculated using formula by Kamala and Indera (2014) and descriptive assessment of antagonistic activity scaled by SOYTONG (1988).

$$\text{PIRG (\%)} = \frac{R1-R2}{R1} \text{ X } 100$$

Where:

R1 = the radial growth of *Fusarium oxysporum* in control plate without *Trichoderma* species.

R2 = the radial growth of *Fusarium oxysporum* with the present of *Trichoderma* species

Data collection

The percentage of inhibition was considered as the antagonistic activity of *Trichoderma* species against *Fusarium oxysporum*. Data of PIRG was recorded during the 10 days of incubation period. The antifungal properties of *Trichoderma* strains considered as good bio control agent if the PIRG shows more than 50% (RAHMAN et al., 2011).

RESULTS

***Trichoderma* isolation from soil**

A total of 355 diverse colonies were grown from the soil suspension culture. Based on the macro morphological characteristic of *Trichoderma* species, the pigmentation of colony was the primary selection for isolation of *Trichoderma* colony from soils. *Trichoderma* species had been found from all rhizosphere soil. The visible colonies on petri dish were identified based on its yellowish and greenish colour of mycelia on DRBC agar and 16 colonies of *Trichoderma* have been identified from the collected samples (Figure 1 and Table 1) in which three colonies were found from paddy field, one colony from rubber, two from scallion, two from oil palm plantation, two from eggplant, two mugbean, two colonies from tomato cultivated soil, one from pineapple cultivated soil, and one colony banana plantation shown in Table 1 and Figure 1. The colonies were subculture on PDA media for pure culture preparation and identification of *Trichoderm* species.

Table 1. Colony, dilution, origin and isolation code of *Trichoderma* from soil culture

Trichoderma colony	Dilution	Origin of *Trichoderma*	Code
Colony 1	$1x10^{-1}$	Rhizosphere of Paddy	P1
Colony 2	1×10^{-2}	Rhizosphere of Paddy	P2
Colony 3	$1x10^{-2}$	Rhizosphere of Paddy	P3
Colony 4	$1x10^{-2}$	Rhizosphere of Long Bean	LB4
Colony 5	$1x10^{-2}$	Rhizosphere of Long Bean	LB5
Colony 6	$1x10^{-1}$	Rhizosphere of Eggplant	E6
Colony 7	$1x10^{-1}$	Rhizosphere of Eggplant	E7
Colony 8	1×10^{-1}	Rhizosphere Scallion	S8
Colony 9	1×10^{-1}	Rhizosphere Scallion	S9
Colony 10	1×10^{-1}	Rhizosphere of Oil palm	OP10
Colony 11	1×10^{-1}	Rhizosphere of Oil palm	OP11
Colony 12	1×10^{-4}	Rhizosphere of Pineapple	P12
Colony 13	$1x10^{-3}$	Rhizosphere of Rubber	R13
Colony 14	$1x10^{-1}$	Rhizosphere of Banana	B14
Colony 15	$1x10^{-3}$	Rhizosphere of Tomato	T15
Colony 16	$1x10^{-3}$	Rhizosphere of Tomato	T16

Figure 1. Isolation of *Trichoderma* colonies from soil grown on DRBC media. The rhizosphere soil were; A& B: paddy C&D: Long bean; E: Eggplant; F&G: Scallion; H&I: Oil palm; J: Pinneaple; K&L: Rubber and M: tomato.

Pure culture of *Trichoderma* colonies on PDA media

The selected colonies on DRBC agar plates were sub-culture on PDA plate for identification of isolates of *Trichoderma* species. Based on the physical observation on PDA plate, *Trichoderma* species had an irregular form, flat elevation and undulate margin which makes them different from other fungi colony (Figure 1). Other than that, *Trichoderma* has greenish or yellowish- green colony. The formation of the colonies was faster where they took less than five days to fully colonize the media plate. The greenish or yellowish-green colour usually on three to four days after growth. The reverse color appears when the *Trichoderma* species are fully matured and become whitish, pale green or tan and sometimes can be yellow (GAMS; BISSETT, 2002). Hence, these physical observation characterise as macro-morphological feature of *Trichoderma* species. Meanwhile, the slide cultures were carried out on microscopic characterization for identification of *Trichoderma* species. The characteristics of isolated *Trichoderma* species have been summarized in Table 2.

Table2. Micro- and macromorphological characteristics of *Trichoderma* isolates

Characters	Isolates					
	P1&P2, S8&9,LB4,5, B14, T15,&16	P3	E6&7	OP10, R13	OP11	P12
Colony growth	Faster growth (0.7cm/d)	Moderate growth (0.5cm/d)	Moderate growth (0.6cm/d)	Moderate growth (0.6cm/d)	Rapid growth (0.9cm/d)	Moderate growth (0.6cm/d)
Colony colour	Dark green to yellowish green with dense white mycelia pustulates	Dark green with few white aerial mycellia	Yellowish-white to dull green	Yellowish-white to dull green	Blue green to yellowish green	Dark green with dense white mycelia pustulates
Conidiophore	Broad, verticillate, frequent branching. Short branches phialides	A central stripes with symmetrically paired branches. The second branch were observed but not for third branches	Narrow branching. The branches arise at pairs with the terminal conidiophores are longer than other.	The base of conidiophores are sterile and have no branches but fertile at apices	Variables, Branched and erects	Narrow and flexuous
Phialides	3 to 4 phialides in whorls. Ampuliform and lageniform forming at dense area	Paired primary branches formed in nearly 90° to main axis, phialides may be solitary or held in whorls of two to three. Langeniform in effuse area and ampuliform at dense area.	2 to 3phialides in with closely lageniform to subulate divergent phialides.	3 to 6 phialides in with closely appressed which only form at apices of branches. Most phialides were ampuliform and langeniform.	Pair phialides. Lageniform; more or less ampulliform	Solitary, verticillate, more / less lageniform
Conidia	Subglobose green, smooth conidia	Globose, obovoid dark green, smooth conidia	Subglobose to ellipsoid	Subglobose, ellipsoid dark green	Slight cylindrical and abundant	Subglobose
Chlamydosphore	Present	Present	Not observed	Present	Present	Present
Species	*T.harzianum*	*T.asperellum*	*T.pararesei*	*T.virens*	*T.koningii*	*T.viride*
New coded	THP1, THP2, THS8, THS9, THLB4, THLB5, THB14, THT15 & THT16	TAP3	TPE6 &TPE7	TVOP10, TVR13	THOP11	TVP12

Trichoderma harzianum

The colonies of *Trichoderma harzianum* were initially formed white mycelia which then changed into yellowish green or dark green on the progression of its maturation and spreading. As for growth, the fungus showed faster growth 0.7 cm per day (Table 2). Due to the faster colonization of mycelia, formation of white aerial mycelia and green colour colony, the characteristics made the species was easier to be identified. Conidiophores of *Trichoderma harzianum* were formed in paired along the main branches and axis (Figure 3; A-C). The conidiophores branching patterns was broad, verticillate, and frequent branching with a degree of 90˚ (Figure 3C) One branching verticillate usually had three to four of phialides (Figure 3 B). Phialides were characteristically elongate and lageniform in shapes (Figure 3; A-B). At the end of phialides or subterminal cell of conidiophores, conidia were formed with a shape of globose (Figure 3D and 3F) to subglobose (Figure 3E). The chlamydospores were observed to have globose (figure 3H) to subglobose shapes (Figure 3G and 3I). The full growth with conidiation shown in figure 3;J-L. From the observation, the isolates of PDY 1,2; S 8,9; LB4,5; T15,16 and B14 were identified to belong *Trichoderma harzianum* species and all the isolate later coded as THP1, THP2, THS8, THS9, THLB4, THT15, THT16 and THB14, respectively (Table 2).

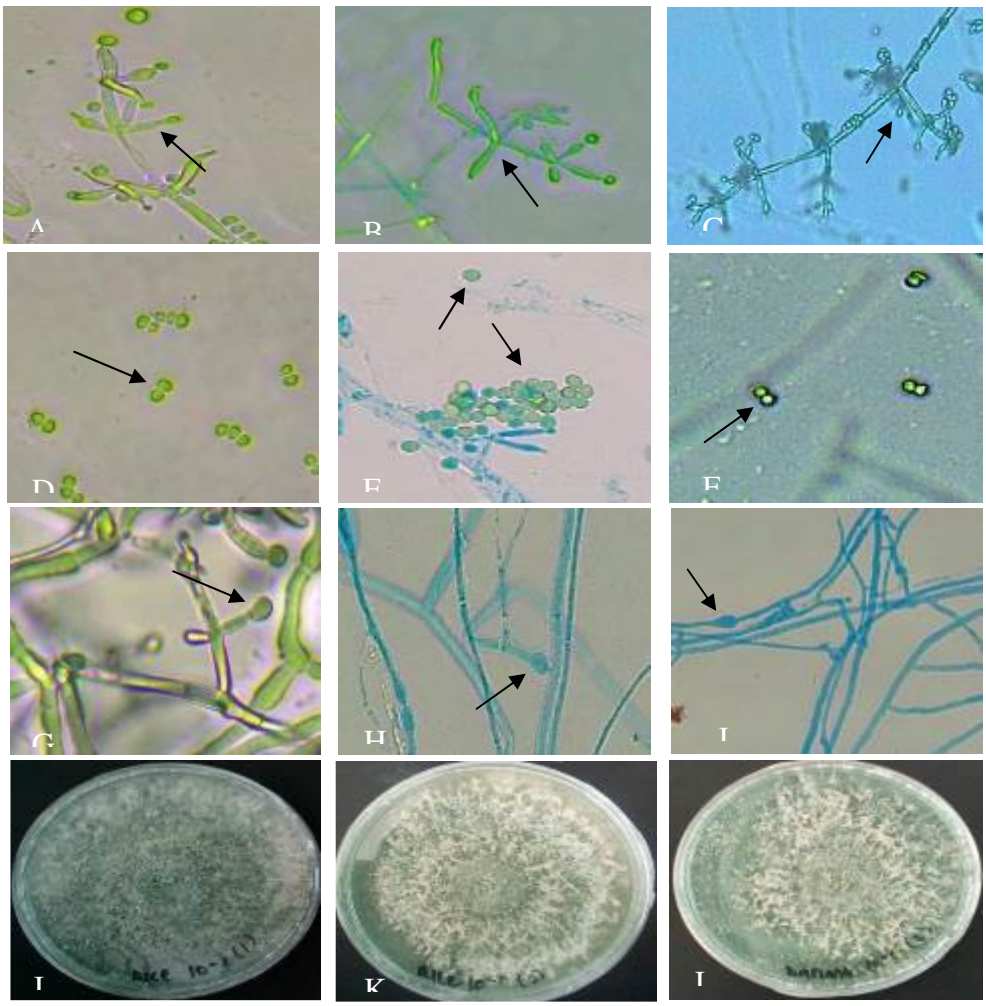

Figure 3. Microscopic morphological characteristic of *T.harzianum* in isolated colonies P1,2;S 8,9;LB4,5;T 15,16 and B14 (THP1, THP2, THS8, THS9, THLB4, THT15, THT16 and THB14). A-C: phialides held in whorl of three to four with the shapes of langeniform (arrow), D-F: globose,subglobose and subcylindrical conidia (arrow), G-I: globose to subglobose chlamydospores (arrow), J-L: conidiation of *Trichoderma harzianum.* (Under microscope Leica: 40X)

Trichoderma asperellum

The colony of P3 was shown the feature of *T. asperellum*. As the fungal growth was moderate 0.5cm/day (Table 2). The colony of *T.asperellum* forms less white pustules mycelium compare to *T. harzianum* (Figure 4i). The conidiation was

predominantly effused and powdery. Most of the conidiophores of *T. asperellum* were formed symmetrically paired along the main branches and axis (Figure 4c). The conidiophores branching patterns was broad, verticillate, and almost 90˚ angle (Figure 4d (2)). One branching verticillate usually had two to four of phialides (Figure 4;a-d). The second branch were observed but not for the third branches (Figure 4e). Phialides were characteristically elongate divergent lageniform (Figure 4e) and can be ampuliform in dense area (Figure 4e) in shapes. The terminal phialides were usually more elongated than another side of phialides (Figure 4 b and d (1)). The special shapes of terminal phialides were used to differentiate between *T. harzianum* with *T. asperellum* in this study. At the end of phialides or subterminal cell of conidiophores, conidia were formed with a shape of globose (Figure 4 f (2)) to obovoid (Figure 4f (1)). Meanwhile, the conidia colour showed dark green under a light microscope. The formation of chlamydospores was observed to have subglobose (Figure 4 g) and globose (Figure 4 h). They mostly found in the middle of hyphae (Figure 4 g-h). Based on the characteristic stated above, the isolates of P was identified to belong in *Trichoderma asperellum* and later coded as TAP3 (Table2)

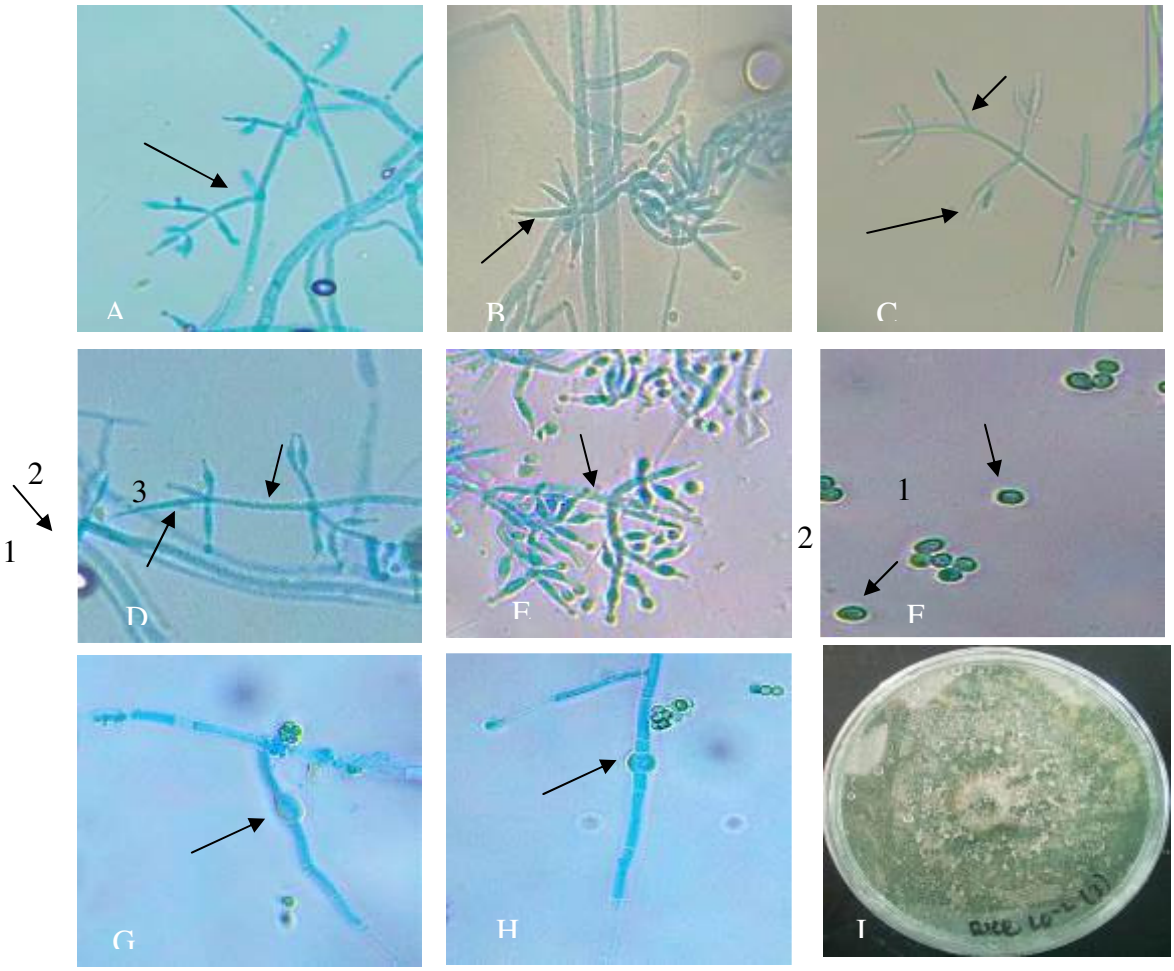

Figure 4. Morphological characteristic of *Trichoderma asperellum* in isolated colony P3 (TAP3). A: unpaired branch (arrows), B-C: Two to four phialides held in paired and unpaired with subulate shapes of phialides (arrow), D1and D2: The terminal phialides was longer than other phialides (arrow), D 3: Conidiophores broad branching pattern with 90˚(arrow), F (1-2): Obovoid to globose conidia with dark green (arrow), G-H: Subglobose to globose chlamydospores (arrow), I: Conidiation of *Trichoderma asperellum* on PDA plate. (Under microscope Leica: 40X).

Trichoderma virens

The isolates OP10 and R13 feature were *T. virens* similarity. The anatomical studies on conidiophores formed were many branches (Figure 5B) with aggregate phialides at the end of conidiophores (figure 5A). The branches were mostly unpaired and smoothly bending (Figure 5C). The base of conidiophores was sterile and have no

branches while fertile at the apex towards phialides (figure 5B). The phialides were not spreading but closely packed together forming like a cluster of slender phialides. There were about 3 to 6 phialides in closely appressed (Figure 5D) while the philiadosphores forming abundantly at the apices. The phialides were lageniform to subulate in shape (figure 5; C-D). The conidia were globose to ovoid in shape and the conidia colour was bluish green (Figure 5; E-F). The formation of chlamydospores was rare and difficult to observe. The chlamydospores were identified to form subglobose in shape (Figure 5; G-H) and can be found in the middle of branches (Figure 5G) or at the end of hyphae (Figure 5H). Based on the characteristic stated, isolates of OP10 and R13 identified to belong in *Trichoderma virens* which coded as TVOP10 and TVR13 (Table 2).

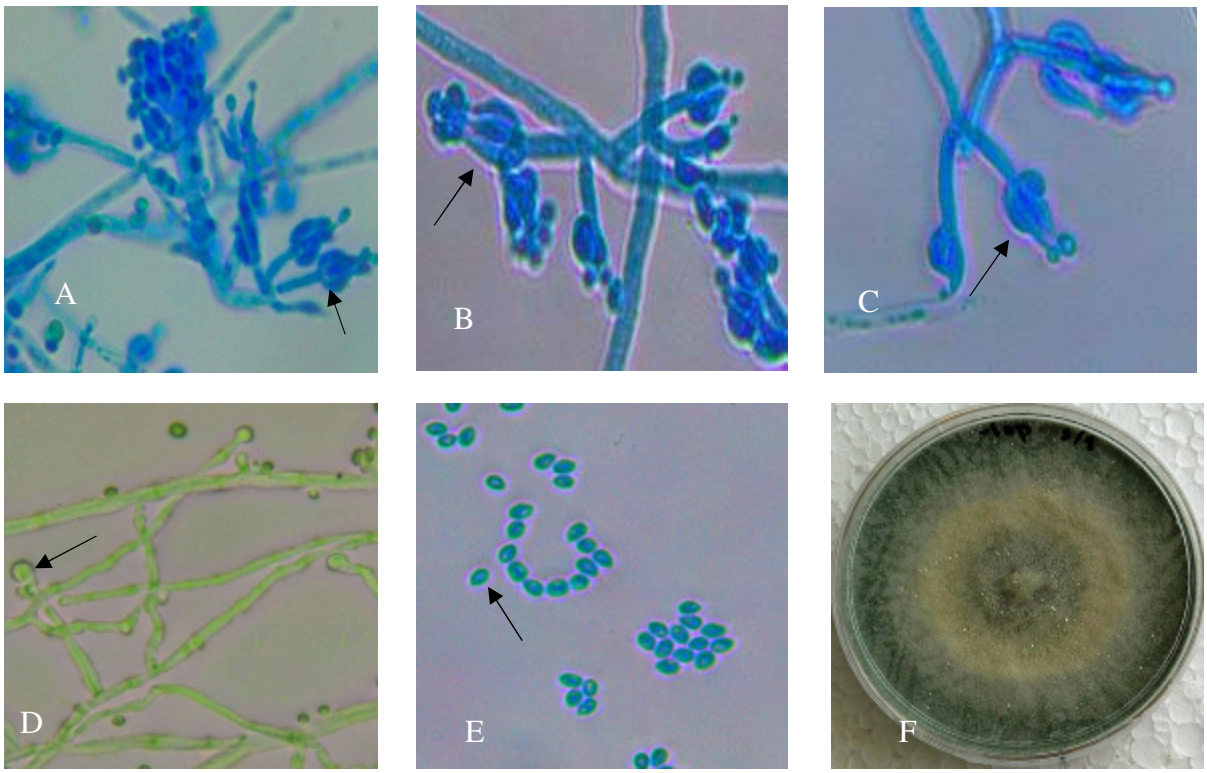

Figure 5. Morphological characteristics of *T. virens* isolated colony 3(TVOP10, TVR13). A-C: The Phialides and the branches of conidiophores (arrows); D: Chlamydospores (arrows); E: Phialospores (arrow). F: Conidiation of *Trichoderma virens* on PDA plate. (Under microscope Leica: 40X).

Trichoderma koningii

The isolated colony OP11 macro-mophological and micro-morphological feature showed as *T. koningii*. The micro-morphological studies on conidiophores were paired primary branches which were usually formed in nearly 90° to the main axis and phialides tend to be cylindrical to sharply constrict at the tips (Figure 6; A-C). Most of the chlamydospores in other isolates were formed on the hyphal tips. However, in *T. koningii* the chlamydospores were found within the hyphae (Figure 6D). The phialospore showed as subcylindrical to narrow ellipsoid in *T. koningi* (6E). Based on these characteristics the colony OP11 belongs to Trichoderma koningii which coded as TKOP11 (Table2).

Trichoderma viride

The isolated colony OP11 on macro-morphological and micro-morphological feature showed as *T. viride*. The features on conidiophores showed as long, straight, solitary and fertile apices (Figure 7; A to C). In this study all *Trichoderma* isolates were found to produce chlamydospores after 7 days. In colony OP11 the chlamydospores observed as unicellular and appeared globose to subglobose (Figure 7D). Most of the chlamydospores were formed on the hyphal tips. The phialospores of were subglobose to obovoid or ellipsoid (Figure 7E). Based on there morphological characteristics the colony OP11 identified as *Trichoderma viride* and coded as TVOP11 (Table 2).

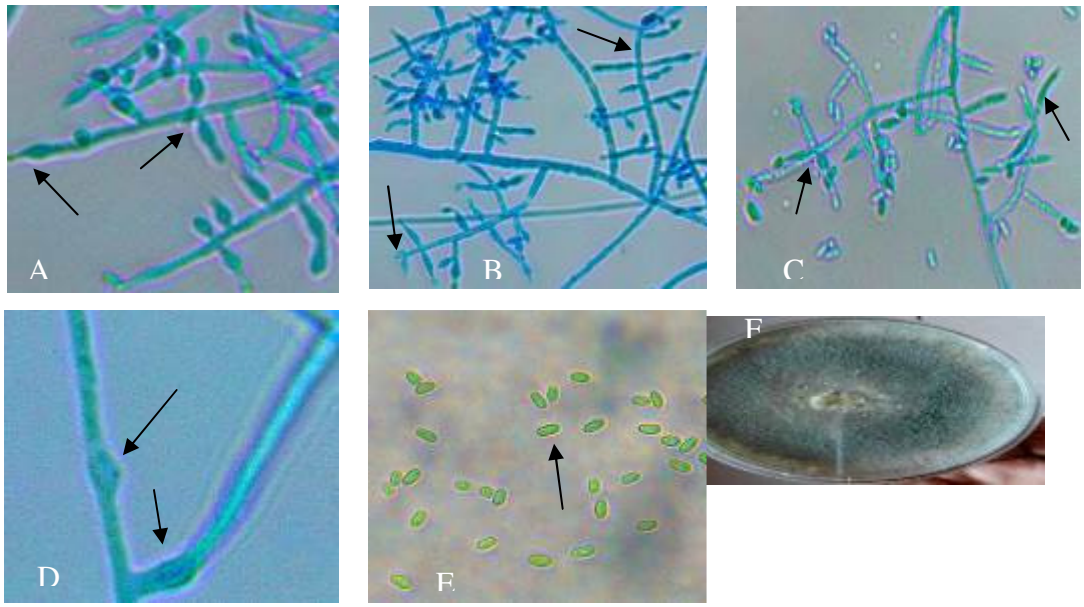

Figure 6. Morphological characteristics of *T. koningii* in isolated colony OP11 (TKOP11). A-C: The Phialides and the branches of conidiophores (arrows); D: Chlamydospores (arrows); E: Phialospores (arrow). F: Conidiation of *Trichoderma koningii* on PDA plate. (Under microscope Leica: 40X).

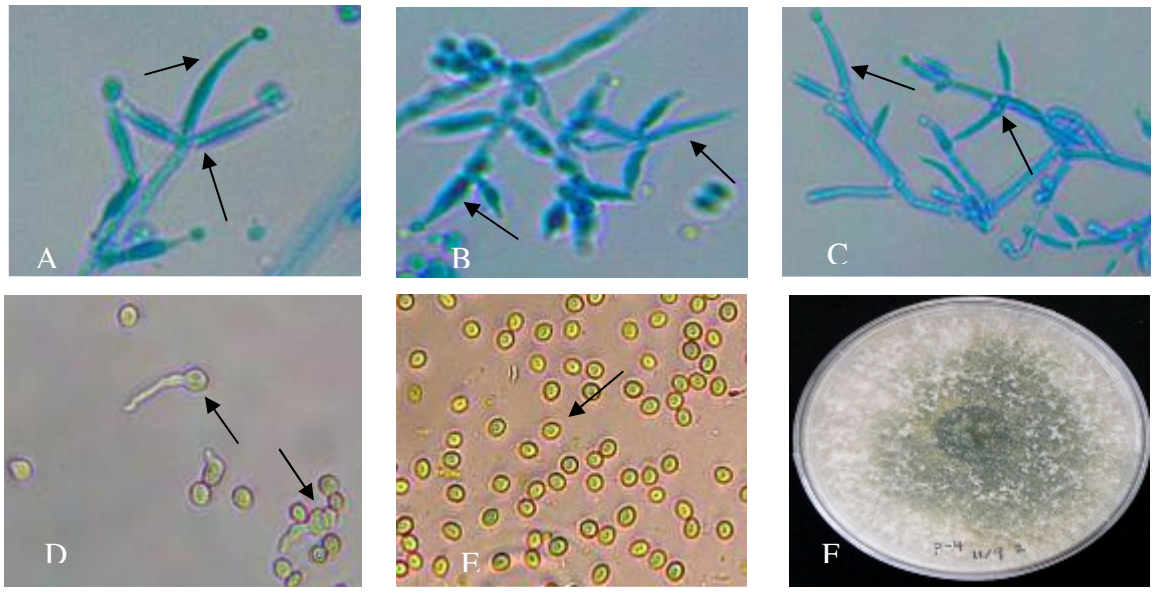

Figure 7. Morphological characteristics of T. viride isolated colony P12 (TVP12). A-C: The Phialides and the branches of conidiophores (arrows); D: Chlamydospores (arrows); E: Phialospores (arrow). F: Conidiation of *Trichoderma viride* on PDA plate. (Under microscope Leica: 40X).

Trichoderma parareesei

The isolated colonies EG6 and EG7 on macro-morphological and micro-morphological feature showed as *T. parareesei*. The conidia of colonies E6 or E7 appeared yellowish green in colour, uniformly ellipsoid, and smooth structure when observed (Figure 8A). The conidia, conidiophores, and phialides of *Trichoderma parareesei* are modestly similar to *Trichoderma reesei* because they came from the same ancestry group. Nevertheless, the conidiophores structure of *Trichoderma parareesei* was longer and branching compare to *Trichoderma reesei* (Figure 8B), (ANTANASOVA *et al*., 2010). The conidiophore revealed narrow branching and branches that arise paired with conidiophores which are longer and the bases are sterile however fertile at the branches (Figure 8C). The conidiation of EG6 or EG7 are

found predominantly effused and in an abundant form after fourth day of inoculation, thus they possessed rapid growth on the PDA media plates (Figure 8E). Based on these characteristics the colonies E6 and E7 refer as *T. parareesei* coded as TPE6 and TPE7 (Table 2).

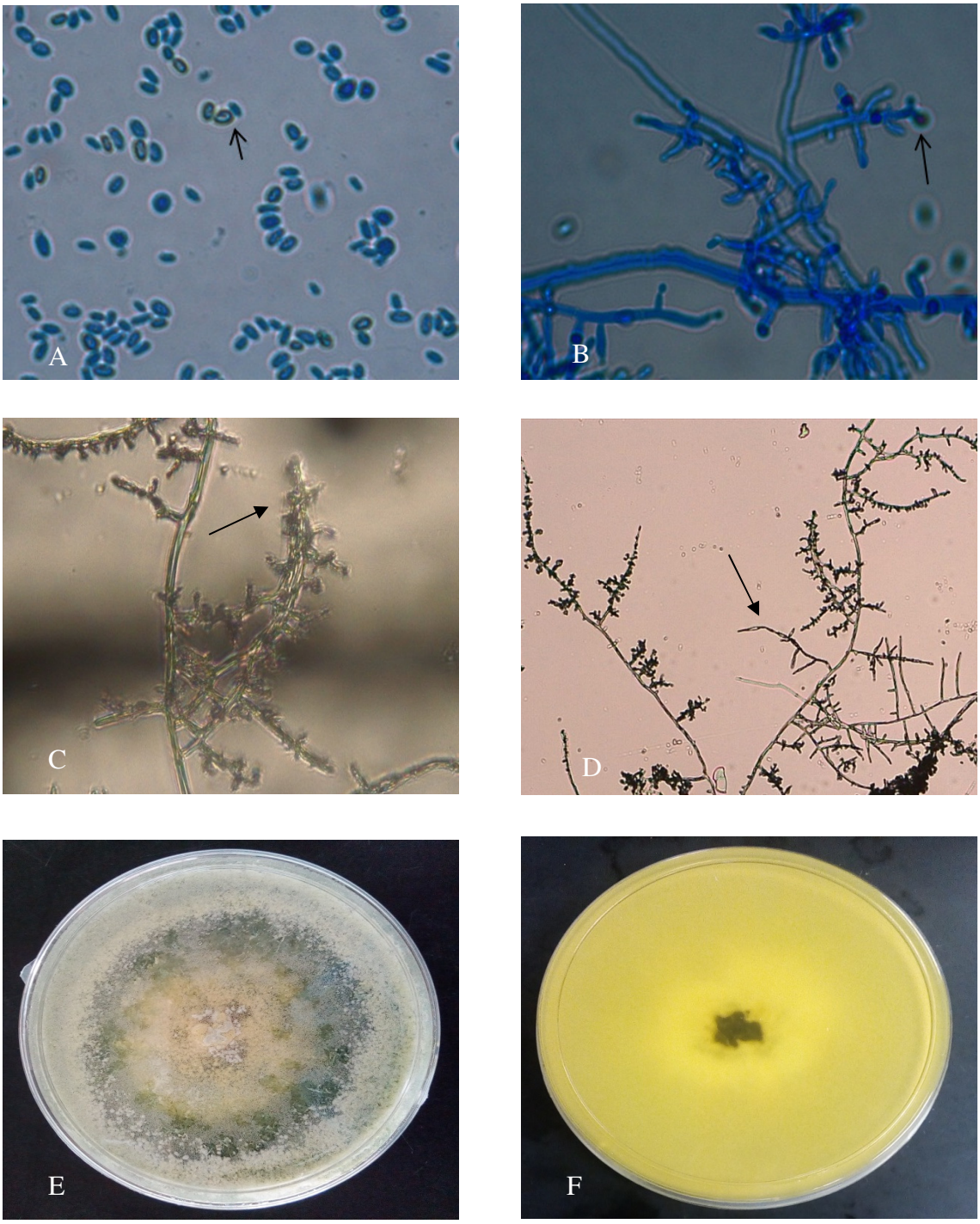

Figure 8. Morphological characteristics of *T. parareesei* colonies E6/E7 (TPE6 and TPE7). A: Conidia (arrow); B: Phialides (arrow); C: Conidiophores (arrow); D: Colony feature from front view on PDA; E: Bottom of PDA plate. Magnification: (Under microscope Leica: 40X).

Antagonistic activities of *Trichoderma* isolates against *F. oxysporum*

The antagonistic activities of *Trichoderma* isolates were tested against *F. oxysporum* on PDA at 25°C for 10 days. In all the dual culture plates tested, the contact zone was a curve, with concavity oriented towards the pathogenic fungi. The curvature of the contact area between the colony of antagonistic fungi and the pathogenic fungi is depend on the growth rate of colonies in the same plate. The curvature will be not occurred if the two colonies have same growth rate. In this study among

the 16 isolates, the isolate colony E7, identified as *Trichoderma parareese*i strain TPE7 showed the strong highest antagonistic activity 91% against the *F.oxysporum* (Table3). The second highest antagonistic activity was 89% in same species (colony 6) *T. parareesei* strain TPE6 (Table3). The third highest antagonistic 76% found in colony LB4, identified as *T. harzianum* strain THLB4 (Table3). The rest of colonies found the antagonistic activities between the ranges 68%-74% except colony OP10 (*T. virens* strain TVOP10) shown in Table 3.

Table 3. Antagonistic activities of *Trichoderma* isolates against *Fusarium oxysporum*

Isolate code	*Trichoderma* species	Percentage of Antagonistic activity of *Trichoderma* against *F. oxysporum*
THPY1	T. harzainum	70.18
THPY2	T. harzainum	70.18
THS8	T. harzainum	68.18
THS9	T. harzainum	71.28
THLB4	T. harzainum	76.09
THLB5	T. harzainum	73.91
THT15	T. harzainum	74.34
THT16	T. harzainum	74.43
THB14	T. harzainum	71.57
TAPY3	T. asperellum	74.16
TVOP10	T. virens	58.7
TVR13	T. virens	73.17
TKOP11	T.koningii	71.40
TVP12	T. viride	70.65
TPE6	T. parareesei	89.13
TPE7		91.0

DISCUSSION

Biofungicide and biofertilizer for using the management of plant diseases are increasing in agriculture. The reason the biofungicide or biofertilizer control the disease in sustainable way and without any negative impact for the environment. In nature there are many microbial agents that cause disease in plant while some microbes are beneficial for plant. The genus of *Trichoderma* is one of beneficial fungus for plant that can found in soil. The species diversity is important for managing of phytopathogenic fungi. In this study different cultivated soils such as paddy, eggplant, tomato, oil palm, banana, rubber, were collected to isolate of *Trichoderma* species. The collected soils were culture on DRBC media to isolate *Trichoderma* colony from soil. Around 16 colonies showed the similarity feature of *Trichoderma* species (Table 1). Morphological identification of macro and micro studies identified six different species namely *T. harzianum*, *T. virens*, *T. viride*, *T. asperellum*, *T. parareesei*, and *T. koningii*. The colonies of PDY1,2; S8,9; LB4,5;T15,16 and B14 were dark green colour with dense white aerial mycelia formed on top of the plates. The conidiation was fertile pustules formation which a dense form of mycelia between 1 to 5 mm diameters formed giving a compact conidiophores (PLESSIS, 2015). GAMS; BISETT (2002) had stated that the *Trichoderma harzianum* formed a granular or powdery conidiation with a tuft or pustulates white fringes on plates Figure 2 (J-L). Thus, these colonies identified as *T. harzianum* with strains as THP1, THP2, THS8, THS9, THLB4, LTLB5, THT15, THT16 and THB14. While the colony PDY3, refer as *T. asperellum* strain TAPD3 due to its colour formation. *Trichoderma asperellum* has been classified under clade *Trichoderma paschybasium*, it does not mean to have same characteristics with *Trichoderma asperelloides* which also from the same clade (PLESIS, 2015). Wu et al. (2017) had stated the morphology mycelium of *Trichoderma asperellum* was coarse and dark green conidia stated to form at the centre of the colony while, the *Trichoderma asperelloides* was showing smooth and dark green conidia under light microscope (WU et al., 2017). However, the colonies OP10 and R-13 identified as *T. virens*, refer as strain TVOP10 or R13 was relatively different from other *Trichoderma* isolates because the dull green colour only formed after four days of inoculation and the conidiation was loosely packed tuft (Figure 5F). The colony OP11 refer as *T.*

koningii strain TKOP11, the colony at the commencement blue green to dull green sporulation (Figure 5F1 and 5F2) which similar with the finding of Shekhar et. al. (2017). The colony of OP12 the colour pigmentation was dark green which similar with *T. viride*. The colony E6,E7 refer as *T.parareesei*, dull pale green showed the similar findings of Shekhar et. al. (2017). Putty (2010) in his analysis state that *Thrichoderma* were producing various types of enzymes such as cellulases and chitinases which can give influences to the colour changes of mycelia during its growth (PUTTY, 2010). However, the green colour possesses by *Trichoderma* species can help researchers to identify and differentiate the *Trichoderma* species with other soil fungus easily while problem to differentiate between the *Trichoderma* species. Thus, anatomical or micromorphological studies on conidia, conidiophores, phialides, phialospore, chalamydosphores are very important to identification of *Trichoderma* species.

The conidia of *Trichoderma harzianum* can be differentiated from other *Trichoderma* isolates easily because the size were smaller and the colour were light green compare to *Trichoderma asperellum* and *Trichoderma virens* (SAVITHA; SRIRAM, 2015). In other situation, *Trichoderma asperellum* was similar with *Trichoderma viride* due to their warted structure but the conidia of *Trichoderma asperellum* was having less warted and more ovoidal than *Trichoderma viride* (SAMUEL et al, 1999).

In addition, the pattern of conidiophores and shapes of phialides were also the important key for identification. This is because some of species of *Trichoderma* formed different pattern and shapes of conidiophores and phialides. In this study, the colonies of PDY1,2;S 8,9; LB4,5; T15 and T16 were frequent branching conidiophores with more solitary phialides formed in whorls of 3 to 4 and mostly were ampulliform to lageniform at the dense area with 3 to 4 phialides (Figure 3; A-C) which refer as *Trichoderma harzianum* (GAMS; BISETT, 2002; PLESIS, 2015). Conidiophores of another isolates B14 (coded THB14) in species of *Trichoderma harzianum* was a verticillate pyramidal structure with nearly to 90˚ angle of paired branched (Figure 3C). HUI (2013) reported that *Trichoderma harzianum* species possess solitary phialides which sometimes can be paired and unpaired in nearly 90˚ to the main axis. The colony of PDY3 conidiophores were narrowly branching and the branches arise at pairs and unpaired along the main branches with the terminal conidiophores were more elongate than other (Figure 3; B-D). There were 2 to 3 phialides in pairs and shapes were divergent lageniform phialides which identified as *T. asperellum*. Apparently, the conidiophores in colony of OP10 and R13 were sterile at the base and have no branches but fertile at the end of conidiophores (Figure 5: A-B). The phialides were aggregate and closely appressed in 3 to 6 phialides (Figure 4: A) as similar pattern with *T. virens*. In colony OP11 the conidiophores formed from the main axis and branching occurred at nodes which diverged from the stipe at approximately right angles. Second degree branching were also observed bearing lageniform phialides (Figure 6: A-C). While, the conidia looked oblong and smooth-walled, these characteristics refer as *T. koningii* (Figure 6) similar with findings of PLESSIS (2015). The colony of P12 (coded TVP12) conidiophore narrow and flexuous with primary branches arising at regular intervals, mostly paired or in whorls of three (Figure 7; A-C). The phialides solitary, or 2-4 verticillate, more or less lageniform, often curved (Figure 7; A-C) and the conidia was dark green, smooth, subglobose to obovoid as shown in Figure 7E. The different characteristics of this colony was it produced sweet coconut smell. Plessis (2015) reported that *Trichoderma* species produce distinctive coconut-like odours that belong to *T. viride* clade. In this research, this isolate P12 has a distinctive aromatic odour resembling coconut at fresh plate culture. GAMS; BISSETT (2002) also mentioned that the production of sweet coconut smell could be a characteristic of a species identification. Hence, the colony P12 identified as *T. viride* strainTVP12 (Figure 7). The colonies E6 and E7 (coded TPE6 and TPE7) appeared yellowish green in colour, uniformly ellipsoid, and smooth structure. Taxonomy characteristics found that *Trichoderma parareesei* occasionally secreted yellow pigment in the agar media due to production cellulase and variably superposed bright yellow-green to dull green pustules which are similar to *Trichoderma reesei* (ATANASOVA et al., 2010). Thus, these colonies can be identified at *T. parareesei*. The conidia, conidiophores, and phialides of *Trichoderma parareesei* are modestly similar to *Trichoderma reesei* because they came from the same ancestry group. Nevertheless, the conidiophores structure of *Trichoderma parareesei* was longer and branching than *Trichoderma reesei* (Figure 8B), (ANTANASOVA et al., 2010).

Trichoderma is one of most primitive fungus that long been using as biocontrol agent, however, researcher are still isolating *Trichoderma* species for most and advance agent/strain for biocontrol (NAGLOT et al., 2015). To biocontrol

study the plate assay technique is most suitable to identify promising agent at preliminary level. In this study among the 16 isolates, *T. parareesei* both strains were highest as 91% in strain TPE7 and 89% in strain TPE6 (Table 3) showed antagonistic activity against *F. oxysporum* compare to *T. harzianum*, *T. vir*ens, *T. koningii*, and *T. viride*. Very few studies have been conducted to test biocontrol activity of *T. parareesi*, nevertheless, the study of Atanasova et al., (2010) reported that *T. parareesi* inhibited the growth of *Lepidium sativum*, the pathogen for garden cress. The beneficial effect of *T.parareesei* was observed against *Pythium irregulare*, *Rhizoctonia solani*, and *Botrytis cinerea* in plate assay as well as the interaction between *T.parareesei* and tomato plants (RUBIO et. al., 2014). Besides the control of these pathogen the fungus gave mutualistic relation in tomato plant to enhance lateral root development and enhanced Jasmonic acid defence gene expression in tomato plant (RUBIO et. al., 2014). The second highest antagonistic activity 76.09% was *T. harizaum* group in strain THLB4, while the others were in range 70-74% except T. virens OPTV which showed 58.7% antagonistic activity in Table 3. The biocontrol activity might be differ based on the situation of pathogen and biocontrol agent. In this study *T.parareesei* showed the best promising species against *Fusarium oxysporum*.

CONCLUSIONS

Trichoderma are attractive fungal species in agriculture prospectus. The most talent point of this fungus is biocontrol potential, side by side mutualism in plant relationship especially enhanced plant development and plant defence immune. Thus, isolating of these fungus still on going to find which may reveal most promising biocontrol agent against versatile pyhtopathogen and might be potentially in industrial application.

Among the six different species the *T.parareesei* was exhibited most promising species against the phytopathogen of *Fusarium oxysporum*.

RESUMO: neste estudo foram isoladas seis espécies de trichoderma isoladas de solos rizosféricos de arrozais, bananeiras, dendezeiros, seringueiras, hortaliças e pastagens. As espécies são t. harzianum, t. viride, t. koningii, t. asperellum, and t. parareesei. O estudo morfológico como pigmentação, crescimento de colônias e estudos anatômicos como aparências de conidiação, tamanho de conídios, padrão de ramificação dos conidióforos, formas de phialides, ausência ou presença de clamidósporos foram realizados para identificar as espécies de trichoderma. As espécies de trichoderma harzianum foram abundantes no solo enquanto as de t. viren foram a segunda mais frequente no solo. Todas as espécies apresentaram atividade antagônica contra o fusarium oxysporum. Enquanto t. parareesei apresentou a maior atividade antagônica de 91,10% contra f. oxysporum, relatado como melhor agente antagonista para fitopatógeno.

PALAVRAS-CHAVE: Atividade antagônica. Diversidade. Fusarium oxysporum. Trichoderma.

REFERENCES

ATANASOVA, L.; JAKLITSCH, W.M.; KOMON.,ZELAZOWSKA,. M.; KUBICEK, C.P.; DRUZHININA, I .S. 2010. Clonal species *Trichoderma parareesei* sp. nov. likely resembles the ancestor of the cellulose producer *Hypocrea jecorina/T. reesei*. **Appl. Environ. Microbiol.,** v. 76, p. 7259–7267. 2010.

GAMS, W.; BISSETT, J. Morphology and identification of *Trichoderma*. In Christian, P. K., & Gary, E. H. *Trichoderma and Gliocladium Volume 1, Basic biology, taxonomy and genetics.* **Taylor and Francis London**, p. 3-31, 2002.

HARMAN, G. E.; LORITO, M.; LYNCH, J. M. Uses of Trichoderma spp. to alleviate or remediate soil and water pollution. In (Lakskin, A. I., W. J. Bennett and G.M Gadd (eds.) **Advances in Applied Microbiol**., v. 56, p. 313-327. 2004. https://doi.org/10.1016/S0065-2164(04)56010-0

HUI, T. S. Morphological Characterization and sequence analysis of 5.8s-its region of *trichoderma* species. Bachelor Science. Thesis. **University Tunku Abdul Rahman. Kuala Lumpur**. 2013.

NAGLOT, A.; GOSWAMI, S.; RAHMAN, I.; SHRIMALI, D.D.; KAMLESH, D.; YADAV, K.; VIKAS K. GUPTA, A., J., R., H. K. GOGOI, H. K.; VEER, V. Antagonistic potential of native *Trichoderma viride* strain against potent tea fungal pathogens in North East India. **The plant Pathol. J**., v. 31, p. 278-289. 2015. https://doi.org/10.5423/PPJ.OA.01.2015.0004

NAHER, L.; HO, C. L.; TAN, S. G.; YUSUF, U. K.; ABDULLAH, F. Cloning of transcripts encoding chitinases from *Elaeis guineensis* Jacq. and their expression profiles in response to fungal infections. **Physiolog. Molecul. Plant Pathol**., v. 76, p. 96–103.2011.

NAHER, L.; ABDULLAH, F.; HO, C. L.; YUSUF, U. K.; TAN, S. G. Biocontrol agent *Trichoderma harzianum* strain FA1132 as an enhancer for oil palm growth. **Pertanika J.Tropical Agricul. Sci**., v. 35, p. 173–182. 2012.

NAHER, L.; YUSUF, U. K.; TAN, S. G.; SIQQIQUEE, S.; ISLAM, R. M. In vitro and In vivo Biocontrol Performance of *Trichoderma harzianum* Rifai on *Ganoderma boninense* Pat. Related to Pathogenicity on Oil Palm (*Elaeis guineensis* Jacq.). **J. Pure Appl. pure Microbiol.,** v. 8, p. 973-978. 2014.

PLESSIS, I. L. D. The diversity of *Trichoderma* spp. in South Africa. Degree of Master of Science in the Faculty of Natural Sciences. Thesis. **Stellenbosch University**. 2015.

PUTTY, M. Trichoderma. Retrieved on 2017 from The Environment Reporter EM Lab websites: https://www.emlab.com/s/sampling/env-report-04-2010.html. 2010.

RAHMAN, M. M.; ALI, A. M.; ISLAM, M. M.; NAHER, L.; AHMAD, I. Evolution of disease development and potential biocontrol activity of *Trichoderma* sp. against *Rhizoctonia solani* on potato. **Bioscience J**., v. 30, p. 1008-1117. 2014.

RAHMAN, A.; BEGUM, M. F.; RAHMAN, M.; BARI, M. A.; ILIAS, G. N. M.; ALAM, M. F. Isolation and identification of *Trichoderma* species from different habitats and their use for bioconversion of solid waste. **Turkey J. Biolog**., v. 35, p. 183-194.2011.

RUBIO, B. M.; QUIJADA, M. N.; REREZ, E.; DOMINAGUEZ, S.; MONTE, E.; HERMOSA, R. Identifying Beneficial Qualities of *Trichoderma parareesei* for Plants. **Appl. Environ. Microbiol**., v. 6, p. 1864-1873. 2014. https://doi.org/10.1128/AEM.03375-13

SAVITHA, M. J.; SRIRAM, S. Morphological and molecular identification of *Trichoderma* isolates with biocontrol potential against *Phytophthora* blight in red pepper. **Pest Managem. Horticul. Ecosyst**., v. 21, p. 194-202.2015.

SEKHAR, C. Y.; AHMMED, K. S.; PRASAD, T. N. V. K. V.; DEVI, R. S. J. Identification of Trichoderma species based on morphological characters isolated from rhizosphere of groundnut (arachis hypogaea l). **Int. J. Sci. Environ**., v. 6, p. 056-2063.2017.

SOYTONG, K. Identification of species of *Chaetomium* in Philippines and screening for their biocontrol properties against seed Born Fungi of rice. Ph.D. Thesis. *Department Plant Pathology*, ULPB, College, Laguna, **Philippines**. 1988.

VERMA, H. K. A Textbook of Fungi. Random Publication. **New Delhi:India**. p. 711. 2012.

ZHANG, N.; ZHANG, J. X.; RAZA, W.; YANG, X. M.; ZERUAN, Y.; SHEN, R.; HUANG, Q. W. Suppression of Fusarium wilt of banana with application of bio-organic fertilizers. **Pedosphere**, v. 24, p. 613-624. 2014. https://doi.org/10.1016/S1002-0160(14)60047-3