



Isolation of antagonistic *Trichoderma* spp. against selected phytopathogenic fungi from the field soils in Kelantan

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ABSTRACT

Aims: *Trichoderma* is a soil borne mycoparasitic fungus which comprises a number of fungal strains that act as biocontrol agent. In this present study have investigated the diversity of *Trichoderma* in different cultivated soils in state of Kelantan. The effectiveness of biocontrol agent of isolated *Trichoderma* species was evaluated against the phytopathogen of *Pyricularia oryzae*, *Fusarium oxysporum*, and *Ganoderma boninense*.

Methodology and results: The identification experiment was carried out on the basis of morphological characteristics as colony appearance, shapes and size of conidia, the branching patterns of conidiophores and phialides. Then, the effectiveness of biocontrol agent of *Trichoderma* species against the pathogens was tested in plate assay experiment. A total of 11 isolates were obtained from three different cultivated soils from Jeli (J), Machang (M) and Pasirmas (P). Morphological studies data identified as two groups of *Trichoderma* species as *Trichoderma harzianum*, and *T. koningii*. All the isolates showed the antagonistic activity against the pathogens while *T. harzianum* strain THMPA1 showed highest antagonistic activity of 80.00% against *P. oryzae* and in *T. koningii* strain TKMPA3 group showed highest antagonistic activity of 69% against *P. oryzae*.

Conclusion, significance and impact of study: Among the 11 isolates of *Trichoderma*, the species of *T. harzianum* strain THMPA1 was the best as biocontrol agent against *P. oryzae*. Thus, diversity of *Trichoderma* species study is important to find promising species isolation of *Trichoderma* species which will be influenced in future to sustainable crop production and maintain green environment.

Keywords: Biocontrol, *Trichoderma*, *Ganoderma boninense*, *Fusarium oxysporum*, *Pyricularia oryzae*

INTRODUCTION

Biological diversity (biodiversity) encompasses the variety of life forms occurring in nature. According to Hawksworth (2001), fungi are a major component of biodiversity, essential for the survival of other organisms and are important in global ecological processes. Fungi are important in the decomposition of plant debris because of their ability to derive carbon and energy requirements from the breakdown of dead and decaying plant cell walls, cellulose and lignin (Lynd *et al.*, 2002). The saprophytic fungal species of *Trichoderma* can frequently found in agricultural soils for involving carbon fixation in soil during the process of decomposer. The fungus is fast growing as well as competitive nature compare to other fungi and produced hydrolytic enzyme to hydrolyse substrates from soil; with these characteristics make *Trichoderma* as good decomposer. Thus, the diversity of *Trichoderma* is an important factor in soil as well as for agriculture. Besides,

the role as decomposer the genus of *Trichoderma* also act as biocontrol agent against many phytopathogens (Srivastava *et al.*, 2015). Besides, the beneficial effects of fungus are many fungi also affect the agriculture production and economy. Like, *Pyricularia oryzae*, which caused leaf blast disease in rice (Suryadi *et al.*, 2013). The disease result in yield loss and subsequently reduce the seed quality (Hai *et al.*, 2007). While, the fungus *Fusarium oxysporum* is a phytopathogenic for several plants including banana plant which is usually call Panama disease. Panama disease is the most highly destructive disease affecting commercial and subsistence of banana production throughout the banana producing areas of the world (Ploetz and Pegg, 2000; Monila *et al.*, 2009). *Ganoderma boninense* cause basal stem rot (BSR) disease in oil palm (*Elaeis guineensis* Jacq) (Fee, 2011). *Ganoderma boninense* is a threat for palm oil industry (Naher *et al.*, 2015). Above mentioned all the pathogens controlled by chemical treatment. Due to

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environment concern the chemical approach is not eco-friendly. Using biocontrol approach is an eco-friendly option which control the pathogen without the negative effect on the plant and environment. Thus, this study was determined to identify natural resource of species diversity of *Trichoderma* from local cultivated soil and evaluated the biocontrol effectiveness against *Pyricularia oryzae*, *Fusarium oxysporum* and *Ganoderma boninense*.

MATERIALS AND METHODS

Sample collection

The soil samples were collected from different cultivated soils at Jeli, Machang, and Pasirmas, Kelantan state in Malaysia. The soil samples were randomly taken at the depth of 10 cm from four cardinal points of each cultivated fields of chili, pineapple, and rice by using a cock borer. From this soil core, approximately 200 g of the soil samples were taken out and kept in the polyethylene bags, sealed in boxes and labeled with the information of the collection sites. Then, the labeled samples were stored set at 4 °C.

Isolation of fungi by soil dilution technique

A 10 g of soil was weighed from 200 g of soil sample for each site. The isolation of fungi was done by mixing 10 g of soil sample with 100 mL of distilled water in a conical flask. After mix the soil with water, the conical flasks were covered with aluminium foil before agitated in electrical shakers (Jeio Tech, Korea) at 100 rpm for 10 min. Serial dilution technique was used to isolate the fungi from soil. Soil serial dilution was carried out using 1 mL of soil suspension and 9 mL of distilled water conducted up to different serial dilutions of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} in final volume of 10 mL and from there 1 mL of diluted soil was poured into the petri dish which previously contained Dichloran Rose Bengal Chloramphenicol (DRBC) media. DRBC media was prepared as 5.00 g of Peptone, 10.00 g of Dextrose, 1.00 g of potassium phosphate, 0.50 g magnesium sulfate, 0.05 g of Rose bengal, 15.00 g of agar of the constituent were added into 500 mL of distilled water in the conical flask. The experiment was conducted in three replicates. The dilution plate was observed daily for the 7 days to examine the diversity of the fungus. After the 7 days, the yellowish green with the same growth appearance of the colonies were selected as *Trichoderma* sp. for culture on PDA plate. Then, slide culture technique (Johnson, 1946) was used for the observation and identification of *Trichoderma* sp. A thin section of PDA agar containing the fungus was fixed on microscopic slide which was cover with the coverslip and the slide was observed under the light microscope for identification of *Trichoderma* species.

Morphological identification

Trichoderma species identification were performed based on morphological anatomy according to Gams and Bissett

(2002) and using interactive key for strain identification at <http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm>. Identification of macro-morphological characteristics were analysed by: colony colour, mycelium density, growth rate and pattern. While the micro-morphological characteristics analysed as: the size, shape and color of the conidia, the branching pattern of the conidiospore, the presence of chlamydospores and phialides.

Pure culture of the pathogen

Three pathogens of *Ganoderma boninense* (Pat.), *Fusarium oxysporum* (f. sp. cubense), and *Pyricularia oryzae* (Cavara) were collected from the slant stock culture. Each fungus of a thick mycelium agar disc was taken from stock culture by using sterile loop and then placed into a Petri dish contained PDA. The culture was stored at the room temperature ± 28 °C for full colony growth.

Antagonistic activity of isolated *Trichoderma* sp. against pathogen

A 6 mm diameter agar disc of pathogen was taken from the edge of actively growing pure culture and placed 1 cm inside from the edge of PDA containing petri dish as size of 8.8 cm. The samples were allowed to grow for 3 days by which time the colony will be reaching to 2 cm. Then a 6 mm diameter disc was taken from pure culture of isolated *Trichoderma* sp. and then placed on the opposite site of petri dish containing each pathogen separately, while control plate was contained only pathogen. The experiment was conducted for 5-7 days. Finally, the antagonistic activity of the *Trichoderma* sp. was be measured by using the formula of Percentage Inhibition of Radial Growth (PIRG).

$$\text{PIRG} = \frac{R1 - R2}{R1} \times 100$$

Where PIRG = Percentage Inhibition of Radial Growth
R1 = the radial growth of the *G. boninense*, *F. oxysporum* and *P. oryza* in the absence of the isolated *Trichoderma* spp. (control plate)
R2 = the radial growth of the *G. boninense*, *F. oxysporum* and *P. oryza* in the presence of the isolated *Trichoderma* spp.

RESULTS

Isolation of *Trichoderma* colony

A total of 100 fungal isolates were successfully isolated from soil samples that were collected from cultivated soils in Jeli, Machang and Pasirmas in the state of Kelantan. About 20 of colonies as *Trichoderma* out of 200 microbial colonies were selected based on morphological appearance on RBA media. After that, isolates were

screened based on the similarity of the colony appearance and 11 colonies were selected as *Trichoderma* species for morphological studies.

Morphological analysis

Specific identification by morphology

Differences in macro- and micromorphological

characteristics of the *Trichoderma* species was summarized in Table 1. This includes the distinct differences on morphological characteristics of *Trichoderma* species which are the shape of phialospores, shape of phialides and branching of conidiospores. Based on the observation of seven colonies, this study found two different types of *Trichoderma* species which were *T. harzianum* and *T. koningii*.

Table 1: The macro- and micromorphological characteristics of *T. harzianum* and *T. koningii*.

Species	Isolate Code	Phialides	Conidio-phores	Phialospores	Chlamydo-spores	Colony Color on PDA
<i>T. harzianum</i>	THMPA1	Cylindrical; enlarge in the middle	Paired branches along the main axis	Subglobose to short obovoid	Globose; granulate	Whitish green to dull green
	THMPA2					
	THPPA4					
	THJVT1					
	THJVT2					
<i>T. koningii</i>	TKMPA3	Narrower at the base and tapering toward apex	Branched and erects	Oblong to narrowly ellipsoidal	-	Whitish green to dark green
TKMPB4						
TKPPA1						
TKPPB2						
	TKPPB3					

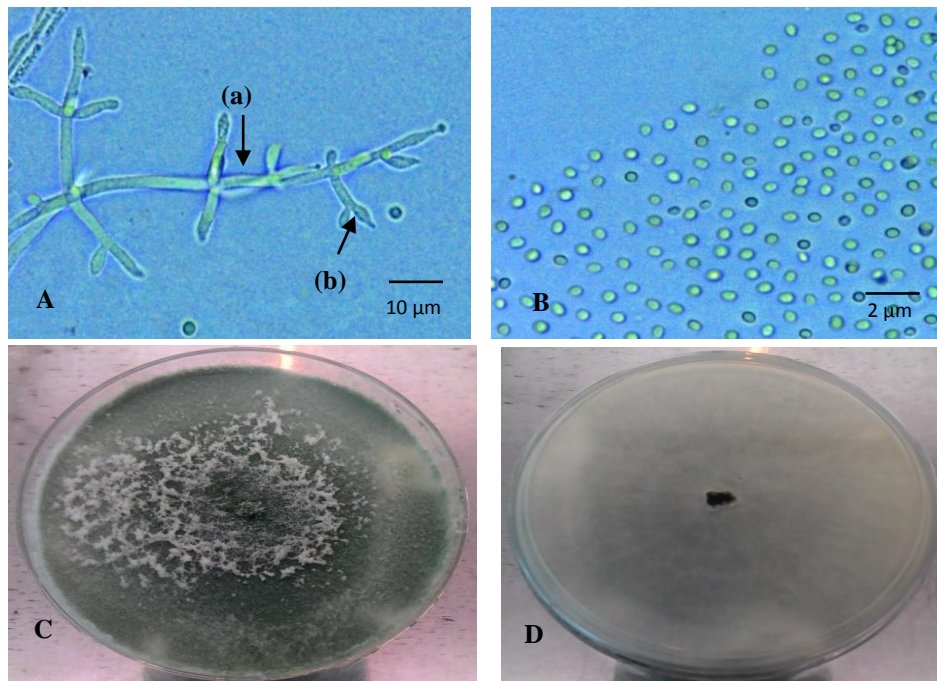


Figure 1: Morphological characteristics of *T. koningii*. A, the branches of conidiophores (arrow a), Phialides (arrow b); B, phialospores; C, colony features from front view on PDA; D, reverse side of the culture plate.

Morphological characteristic of Trichoderma koningii

Five out of 11 colonies were identified as *Trichoderma koningii*. The morphological characteristics are shown in

Figure 1. The conidiophores of *T. koningii* was observed as branched and erects [Figure 1A (a)] typically consisting of a strongly developed central axis with a very uniform branching pattern. The phialides were mainly arranged in

divergent whorls of two to three which arise at or near the tip of the main axis. The shape of phialides was somewhat swollen and flask-shaped, narrow at the base and tapering toward apex [Figure 1A (b)]. The phialospores of *T. koningii* were oblong to narrowly ellipsoidal shape with the size of 2 µm as shown in Figure 1B. The colony appearance shown that the colours changed slowly from whitish green to greenish green, and

finally to dark green (Figure 1C) and the reverse side or bottom of the culture showed no pigmentation on the PDA (Figure 1D). Besides, the colony of *T. koningii* formed moderately well concentric rings with compact to rather loose tuft texture. The growth rate of the *T. koningii* colonies on PDA at room temperature were considerably high ranging between 1.6 – 2.6 cm/day and the mycelia may covered the whole plate within five days.

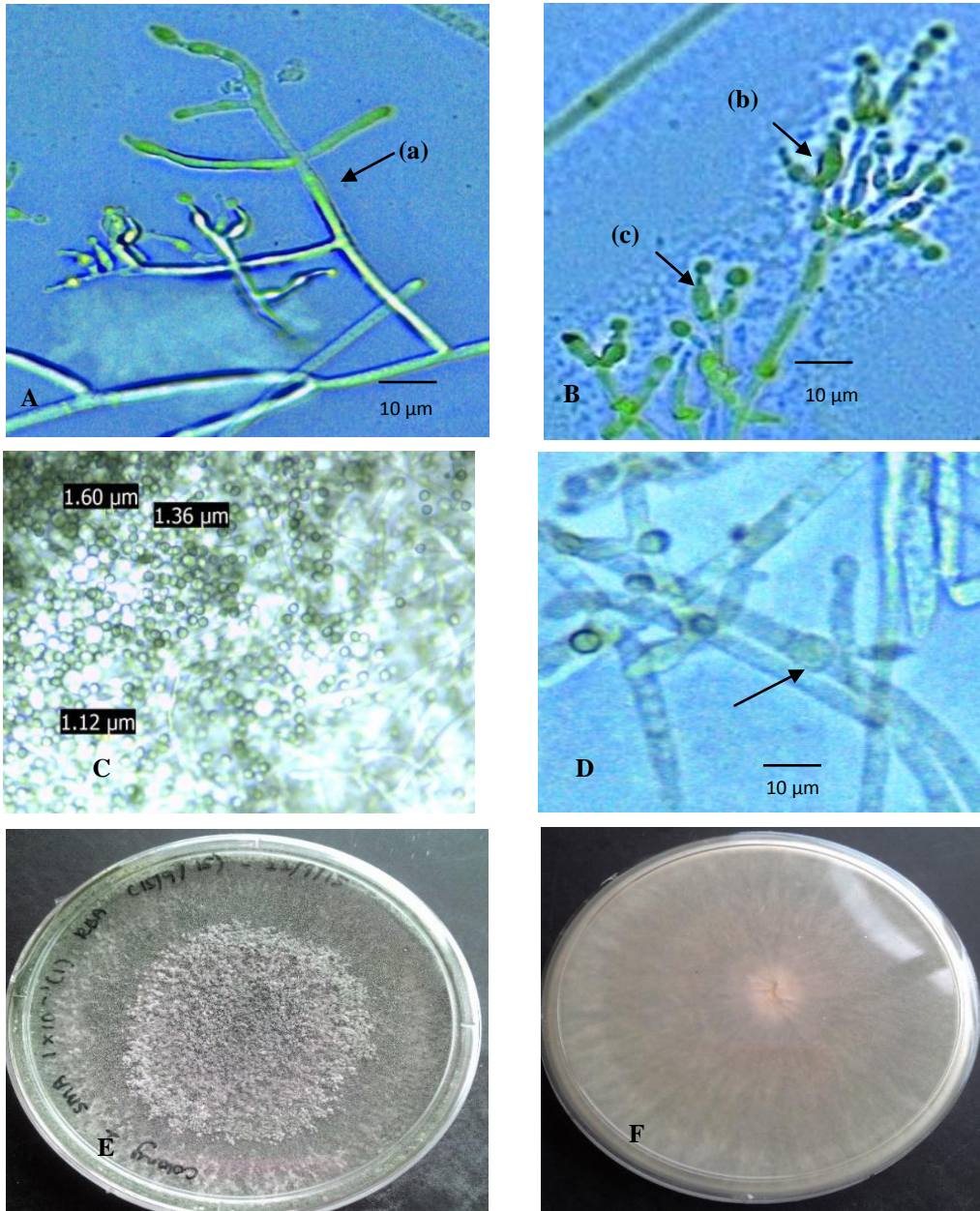


Figure 2: Morphological characteristics of *T. harzianum* (isolate THMPA2). A, the branches of conidiophores (arrow a); B, phialides (arrow b, c); C, phialospores; D, chlamydospores; E, colony features from front view on PDA; F, reverse side of the culture plate.

Morphological characteristic of Trichoderma harzianum

Six colonies were identified as *T. harzianum*. All the morphological characteristics are shown in Figure 2. The formation of conidiophores in *T. harzianum* was in the form of paired branches along the main axis as in Figure 2A. Their phialides generally bends towards the apex with the form of cylindrical shape which enlarge in the middle (Figure 2B). The phialospores shapes of the *T. harzianum* were subglobose to short obovoid and the spore size which is from 1.0 µm to 1.6 µm as shown in Figure 2C. Moreover, *T. harzianum* produced granulated chlamydospores with a globose shape (Figure 2D). The colonies appearance of *T. harzianum* were whitish green to dull green in color and (Figure 2E) with no pigmentation produced through the PDA (Figure 2F). In addition, the colonies form 1–2 concentric rings of conidial zone on the surface of the colony and it was composed of rather loose or compact tufts. Colonies of *T. harzianum* grew rapidly on PDA at room temperature with growth rate ranged between 2.1–3.2 cm/day and cover the whole plate within four days.

Antagonistic activity of isolated *Trichoderma* spp.

The antagonistic effect of all *Trichoderma* isolates were tested against pathogenic fungi, of *P. oryzae*, *G. boninense* and *F. oxysporum* on PDA at room temperature for seven days. The percentage inhibition of radial growth (PIRG) of all three pathogens by *Trichoderma* spp. are presented in Table 2. The study found highest antagonistic activity in *T. harzianum* MPA1 with the inhibition percentage of 86.04% against *P. oryzae*. The second highest antagonistic activity of 71.09% observed in *T. harzianum* MPA1 against the same pathogen of *P. oryzae*, while in species of

Table 2: Antagonistic activity of selected *Trichoderma* isolates against three causal pathogens of *Pyricularia oryzae*, *Fusarium oxysporum* and *Ganoderma boninense*.

Species Name	PIRG in %	Pathogens
<i>T. harzianum</i> MPA1	86.04	<i>P. oryzae</i>
<i>T. harzianum</i> MPA2	71.09	<i>P. oryzae</i>
<i>T. koningii</i> MPA3	69.57	<i>P. oryzae</i>
<i>T. koningii</i> MPB3	69.57	<i>P. oryzae</i>
<i>T.harzianum</i> JV1	53.74	<i>F. oxysporum</i>
<i>T. harzianum</i> JV2	54.7	<i>F. oxysporum</i>
<i>T. harzianum</i> JV3	57.26	<i>F. oxysporum</i>
<i>T. harzianum</i> PA4	60.86	<i>G. boninense</i>
<i>T. koningii</i> PB4	59.00	<i>G. boninense</i>
<i>T. koningii</i> PB2	59.33	<i>G. boninense</i>
<i>T. koningii</i> PA1	45.00	<i>G. boninense</i>

Trichoderma koningii, the highest antagonistic activity of 69.57% was found in both of *T. koningii* MPA3 and *T. koningii* MPA4. All of these isolates were isolated from Pasirmas, Kelantan. This study noted that all the highest antagonistic activities were found in those isolated from Pasirmas cultivated soil among the other two places of

Jeli and Machang. The second highest antagonistic activity observed in those isolated from Jeli cultivated soil and then lowest was from Machang cultivated soil. The reason is not clear why the isolated strains showed some different activities intensity although all three locations were the same state of Kelantan area. According to Tronsmo and Dennis (1978) reported that temperature affect the growth of *Trichoderma* species and also their metabolic activity especially the production of volatile antibiotics and enzymes which involving in the process of mycoparasitism activity. This current study only focused on antagonistic activity from the selected isolated *Trichoderma* species, no data have been recorded from Jeli, Pasirmas and Machang in terms of difference between the temperatures of this three locations.

DISCUSSION

Trichoderma is soil borne fungus, in this study all isolated fungus obtained from soil in three different locations. *Trichoderma* is a facultative saprophytic fungus whereby the saprophytic fungus is typically a soil fungus (Srivastava *et al.*, 2015). Soil dilution plate technique was used to isolate of *Trichoderma* colony, which is good technique for soil fungus. For the isolation and identification of fungus, it is important to use soil dilution technique with proper media. This study used DRBC media to isolate *Trichoderma* colony from soil. DRBC media contained Rose Bengal dye that functions as antibiotic to inhibit bacterial growth when isolate fungal colony from soils (Himedia, 2011). About 200 colonies were grown in culture plates. Based on the morphological features such as colour appearance, colony growth it was suspected that 20 colonies as *Trichoderma*. Slide culture was used as for anatomical study for identification of the fungus. Slide culture method is suitable technique for microbial identification. Since 1969, morphological characteristics have been used to characterize and distinguish *Trichoderma* species (Rifai, 1969; Gams and Bissett, 2002). There were two species as *T. harzianum* and *T. koningii* were positively identified in the group of *Trichoderma* species (Table 1). The hyphal branching patterns of these two species (Figure 1 and 2) were different which showed the difference between the species. The branching formation of conidiophore difference is one of distinguish characteristics for species identification (Samuels *et al.*, 2002).

Trichoderma species not only decomposer in soil, they are also a good biocontrol agent against many plant pathogen (Harman *et al.*, 2006). John and the colleagues had found that *Trichoderma* sp. controlled the pathogens of *Fusarium oxysporum* and *Pythium* sp. (John *et al.*, 2010). Thus, in our study, the antagonistic activity of some selected isolates of *Trichoderma* species of *T. harzianum* and *T. koningii* against the pathogenic species of *P. oryzae*, *F. oxysporum*, and *G. boninense* was evaluated and we had found that the activity of *T. harzianum* strain THMPA1 against *P. oryzae* is the highest which is about 86.04%. Gouramanis (1997) found that *T. harzianum* (GPO-80) inhibited the mycelial growth

of *P. oryzae* about 71% and conidial germination about 88%. In another study, the endophytic fungi of *Phaialemonium curvatum* and *Phaeosphaeriopsis musae* were found to be inhibiting the growth of *P. oryzae* about 66.6% and 63.3%, respectively (Suada *et al.*, 2012). While, the isolated of *T. harzianum* strains JV1, 2, 3 showed the antagonistic activities against *F. oxysporum* and *T. koningii* strains TK PB4, PB2 showed the antagonistic activities against *G. boninense*. Though the antagonistic activities were in the range of 50%, according to Soyong (1988) suggested that that more than 50% of PIRG is considered as a good biocontrol agent. In conclusion, *T. harzianum* MPA1 was found to be the best against *P. oryzae*.

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