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Potential of *Trichoderma* spp. as Biological Control Agents Against Bakanae Pathogen (*Fusarium fujikuroi*) in Rice

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ABSTRACT

Bakanae disease is a major rice disease caused by *Fusarium fujikuroi*. Infected plants show slender and hyper elongated internodes due to the over-production of gibberellic acid. Application of *Trichoderma* spp. as biocontrol agent is gaining attention due to high capability in hyperparasitize the soil borne pathogen. The studies aimed to screen and evaluate the bio-efficacy of *Trichoderma* spp. with antagonistic activities against *F. fujikuroi* and plant growth-promoting properties. All the 65 *Trichoderma* isolates were isolated from healthy rice rhizosphere soil. Thirty eight out of 65 *Trichoderma* isolates exhibited more than 45 Percentage of Inhibition Radial Growth (PIRG) against *F. fujikuroi* in dual culture plate testing. All selected *Trichoderma* isolates were further *in vitro* screened for antagonistic testing: volatile compounds production and hydrogen cyanide production and plant growth-promotion properties: IAA production and phosphate solubilization. Twelve *Trichoderma* isolates were selected for further evaluation on antagonistic activity against *F. fujikuroi*, germination rate, plumule and radical lengths and vigor index. Finally, seven of the most potential *Trichoderma* isolates were selected for greenhouse evaluation. The bakanae disease incidence and disease severity in rice plant treated with respective selected *Trichoderma* isolates were significant reduced as compared with untreated plant. However, there was no significant increase in plant height between *Trichoderma* inoculated and uninoculated plants. Moreover, rice plant treated with *Trichoderma* T61 showed significantly increase in total plant dry biomass as compared to untreated plants. The selected *Trichoderma* isolates have potential to be developed as biological control agent against *F. fujikuroi* and also as an alternative for bakanae management.

Key words: *Trichoderma* spp., bakanae disease, *Fusarium fijikuroi*, biocontrol, plant growth promotion

INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food crop in many Asian countries. Rice production is required to increase by 25%, in order to meet the estimated population of around 8 billion in year 2025. However, worldwide rice production is threatening by diseases, included bakanae disease caused by *F. fujikuroi*. The over production of fusaric and gibberellic acids by *Fusarium fujikuroi* are responsible for the symptoms of bakanae disease (Kandhari, 2010). Affected rice seedlings are thin, pale yellowish green, elongated abnormally and some will die before producing grains. Some other

symptoms are discoloration at the lower nodes and the production of adventitious roots in one or more nodes above the water level. Infected plants in some cases are severally stunted instead of elongated (Kandhari, 2010) and affected the productivity. In Malaysia, yield losses about 30-50% have been reported in direct-seeded rice field and under heavy infestation; lodging of infected plants may occur and caused total yield loss (Watanabe *et al*., 2000).

Recently, the application of biological control agent in plant disease management is gaining huge momentum in crop production systems. Various studies have been conducted to focus on application of plant growth-promoting microorganism to improve plant health and productivity in various crops. *Trichoderma* spp. were reported have the ability to reduce several plant diseases by inhibiting plant pathogens mainly found in the soil roots, through antagonistic and mycoparasitic potential (Viterbo *et al*., 2010). For instance, studies conducted by Ha (2010) in Vietnam, indicated that *Trichoderma* spp. had the ability to suppress growth of fungal pathogens and enhance plant growth. However, no research has been conducted to evaluate the bio-efficacy of *Trichoderma* spp. in plant growth promotion and bakanae disease management especially in rice variety MR 219. The objectives of the study were to screen and to evaluate the bio-efficacy of *Trichoderma* spp. with antagonistic activities against *F. fujikuroi* and plant growth-promoting properties in rice variety MR 219. The application of *Trichoderma* spp. is an alternative in bakanae management toward sustainability in rice production systems.

MATERIALS AND METHODS

Isolation of *Trichoderma* spp.: *Trichoderma* spp. were isolated from the rhizosphere soils of healthy rice plants collected from Kedah, Kelantan and Terengganu, Malaysia. *Trichoderma* Medium E agar (TME) was used to isolate the *Trichoderma* spp. with serial dilution technique. The soil solution was shaking for 30 min. The serial dilution of 10^{-1}-10^{-4} was prepared by sequentially transferring 1 mL of the solution samples into respective test tubes containing 9 mL of sterile distilled water. A 0.1 mL of each solution was then pipette onto TME agar and evenly spread using sterilized bent glass rod. The Petri dishes ware incubated at 28±2°C for seven days (Naidu *et al*., 2010). After incubation, colony forming unit was enumerated as CFU mL^{-1}. All colonies were further transferred to Potato Dextrose Agar (PDA) for maintaining. Distinct morphological characteristics of *Trichoderma* spp., such as shape, size and texture were observed and identified based on morphological and cultural characteristics (Soesanto *et al*., 2011).

Antagonistic activities: The pure cultures of *Fusarium fujikuroi* was obtained from Plant Protection Department of University Putra Malaysia (UPM), Serdang, Selangor. The antagonistic activities of *Trichoderma* spp. against *F. fujikuroi* were evaluated using dual culture plate testing, volatile compounds production and Hydrogen cyanide (HCN) production.

Dual culture plate testings: Five mm diameter of mycelial disc from the margin of the seven days-old culture of *F. fujikuroi* was transferred onto PDA plates and allowed to grow for two days before introducing the respective *Trichoderma* isolates. The *F. fujikuroi* disc was placed approximately 4 cm apart from the respective *Trichoderma* spp. The experimental unit was arranged in completely randomized design with five replications. In control, only *F. fujikuroi* was placed on the PDA plate. Inoculated plates were incubated at 28±2°C for seven days. The inhibition zone was measured when the mycelium of *F. fujikuroi* in control plates had reached the edge of the

plates. The suppression effect of all *Trichoderma* spp. isolates were evaluated by using Percentage Inhibition in Radial Growth (PIRG) of *F. fujikuroi* based on the following formula (Gaigole *et al*., 2011).

PIRG value:

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

R1 = Radial growth of *F. fujikuroi* in the absence of the antagonist (control)
R2 = Radial growth of *F. fujikuroi* in the presence of the antagonist (treatment)

Volatile compounds production: Five millimeter mycelial disc of *F. fujikuroi* and *Trichoderma* spp. obtained from the margin of the seven days-old culture of *F. fujikuroi* and *Trichoderma* spp. were transferred onto PDA plate. Both discs of *F. fujikuroi* and the *Trichoderma* spp. were placed on the opposite (placed inversely) at equal distance from the centre of plates. A PDA plate without *Trichoderma* spp. isolate paired with *F. fujikuroi* served as control. The pairs of each plate (without the lids) were sealed together with parafilm and incubated at 28±2°C for seven days. The radius of *F. fujikuroi* disc was recorded and the percentage inhibition of radial growth was determined after seven days of incubation by using the same formula as described in dual culture plate testing.

The most potential *Trichoderma* spp. was evaluated based on *in vitro* antagonistic and volatile substances (Dubey *et al*., 2007). Five replications were conducted for each treatment.

Hydrogen cyanide (HCN) production: For HCN production, *Trichoderma* spp. was grown on Tryptic Soy Agar (TSA) supplemented with 4.4 g L^{-1} of glycine for two days. White filter paper discs were cut in the same size and soaked in picric acid solution (0.5% picric acid in 2% (w/v) sodium carbonate in 1 L of water). The sheets of filter papers were placed on the upper lid of each plate. The plates were sealed with Parafilm and incubated for seven days at 28±2°C. After incubation, HCN production was observed by the colour changes of the filter paper from yellow to light brown or reddish brown which indicated the production of HCN (Meera and Balabaskar, 2012). The coloured filter paper was then eluted by placing the filter paper in a clean test tube containing 10 mL distilled water and the optimum density (absorbance) was measured at 625 nm by using spectrophotometer (Manwar *et al*., 2011). Five replications were maintained for each isolate.

Indole acetic acid (IAA) production: Five discs of each *Trichoderma* spp. were transferred into respective universal bottles containing 10 mL of Potato Dextrose Broth (PDB) and incubated on the incubator shaker for 24 h. After 24 h of incubation, 1 mL of fungal inoculum was transferred into 250 mL conical flask containing 100 mL of sterile PDB with 5 mL of 0.2% (w/v) L-tryptophan and incubated at 28±2 °C for 72 h. Conical flask without *Trichoderma* spp. served as controls or blanks. A 1.5 mL of aliquot was sampled and centrifuged at 3,000 rpm for 30 min, 1 mL of the supernatant was then added with two drops of orthophosphoric acid and 4 mL of salkowskis reagent (50 mL, 35% perchloric acid; 1 mL 0.5 M ferric chloride, $FeCl_3$). To determine the amount of IAA produced from the isolates, the colour density (absorbance) was measured at 535 nm using spectrophotometer (Noori and Saud, 2012). The IAA produced was compared to the standard graft and expressed as μg mL^{-1}.

Phosphate solubilizing activity: All *Trichoderma* spp. isolates were screened for inorganic phosphate solubilization. The respective fungal isolates were cultured in PDB for seven days, 100 μL of the mycelium broth were spotted onto National Botanical Research Institute's Phosphate (NBRIP) medium containing inorganic phosphate, respectively by using a micro pipette. The inoculated plates were incubated at 28±2°C for seven days. After incubation, the colonies with clear halo zones (solubilizing zone) around colony indicated positive solubilization of mineral phosphate. Phosphate solubilization activities were screened by measuring the clearing zone surrounding the fungal colony as phosphate solubilization index (Noori and Saud, 2012).

$$\text{Phosphate solubilization index} = \frac{A}{B} \times 100\%$$

A = Total diameter (*Trichoderma* colony+halo zone)
B = Diameter of *Trichoderma* colony

Selection of potential *Trichoderma* spp.: The selection of potential *Trichoderma* spp. was conducted based on mean separation by LSD using Statistical Product and Service Solutions (SPSS) programme version 20.0. The potential antagonistic and plant growth-promotion *Trichoderma* spp. were further confirmed by seed germination testing and rice seedling vigor index before evaluated under greenhouse conditions. The antagonistic activity of the selected *Trichoderma* isolates was conducted as the method described previously.

Rice seed germination testing: Rice seeds variety MR219 were surface sterilized for 5 min with 1% sodium hypochlorite solution followed by three times of rising with sterile distilled water. *Trichoderma* spp. inoculums (1×10^8 CFU mL^{-1}) were prepared by using haemocytometer. Sterilized rice seeds were immersed in respective *Trichoderma* suspension for 1 h and 45 min. Rice seeds immersed in distilled water served as control. Ten *Trichoderma* inoculated rice seeds were placed in a petri dish layered with moisten filter paper and incubate at 28±2°C under dark condition. Germinated seeds were recorded at the 3rd day after incubation. Germination rate was calculated as the formula below:

$$\text{Seed germination rate} = \frac{\text{No. of germinated seed}}{\text{Total No. of seeds}} \times 100\%$$

The experiment was conducted twice with five replicates per treatments. The germination rates were analyzed by Friedman test, Least Significant Different (LSD) (Bandyopadhyay *et al*., 2003).

Rice seedlings vigor index: The rice seedlings from the germination testing were further assessed at the 5th day after incubation for plumule and radical lengths. The germination rate, plumule and radical lengths were used to calculate for vigor index using the following formula (Farooq *et al*., 2005):

$$\text{Vigor index (\%)} = (\text{Plumule length} + \text{Radical length}) \times \text{Germination rate}$$

Second selection of the potential *Trichoderma* spp.: The selection of potential *Trichoderma* spp. was conducted as the method mentioned above. The seven most potential *Trichoderma* isolates (T40, T43, T45, T46, T50, T59 and T61) were selected for bio-efficacy evaluation under greenhouse conditions.

Bio-efficacy evaluation of the selected *Trichoderma* spp.: The bio-efficacy of the selected *Trichoderma* isolates was conducted under greenhouse conditions using rice variety MR 219. The sterilized rice seeds were soaked in the suspension of *F. fujikuroi* (1×10^8 CFU mL^{-1}) for 24 h before sowing. Rice seeds soaked in distilled water served as control. Ten *F. fujikuroi* treated rice seeds were sown in poly bags containing 4 kg of top soil. The pots were maintained under greenhouse conditions. The treatment of selected *Trichoderma* isolates was presented in the Table 1.

At 7 days after sowing, the rice seedlings were inoculated with respective *Trichoderma* inoculums (1×10^8 CFU mL^{-1}) through soil drenching at 10 mL per pot. The experimental units were arranged in a Randomized Complete Block Design (RCBD) with five replications per treatment. The day and night temperatures in greenhouse at University Malaysia Terengganu ranged 30.3-35.1 and 23.3-30.6°C, respectively. Rice seedlings were fertilized every two weeks with 3 g/poly bag of N:P:K = 21:21:21 and irrigated daily with tap water (Nur Ain Izzati *et al*., 2008).

The bakanae disease severity, incidence and growth parameters of rice seedlings in the glasshouse were evaluated at vegetative stage: 35 days after sowing (MacLean *et al*., 2002). The disease incidence was calculated by using the formula as described by Teng and James (2001):

$$\text{Disease incidence (\%)} = \frac{\text{Total No. of infected plants per pot}}{\text{Total No. of plants per pot}} \times 100\%$$

The disease symptoms were evaluated based on to the disease scales from 0-4 (Table 2). The Disease Severity Index (DSI) was calculated following the calculation described by Ooi (2002) using the following equation:

$$\text{DSI} = \sum \frac{\text{No. of plants in specific scale} \times \text{disease scale}}{\text{Total No. of plants}}$$

$$= \sum \frac{(n\times0)+(n\times1)+(n\times2)+(n\times3)+(n\times4)}{\text{Total No. of plants}\,(n)}$$

Data analysis: All treatments were arranged in Randomized Complete Block Design (RCBD), consisting five replications for each treatment. Post Hoc test- Least Significant Different (LSD) was

Table 1: Treatment of the selected *Trichoderma* isolates

Representative code	Treatments
Control 1	Rice plant without any inoculation
Control 2	Rice plant inoculated with *F. fujikuroi*
Treatment 1	Rice plant inoculated with *F. fujikuroi*+T40
Treatment 2	Rice plant inoculated with *F. fujikuroi*+T43
Treatment 3	Rice plant inoculated with *F. fujikuroi*+T45
Treatment 4	Rice plant inoculated with *F. fujikuroi*+T46
Treatment 5	Rice plant inoculated with *F. fujikuroi*+T50
Treatment 6	Rice plant inoculated with *F. fujikuroi*+T59
Treatment 7	Rice plant inoculated with *F. fujikuroi*+T61

Table 2: Bakanae disease symptoms scoring scale

Disease scale	Disease symptoms
0	Healthy and uninfected plants (no external symptoms)
1	Normal growth but leaves beginning to show yellowish-green
2	Abnormal growth, elongated, thin and yellowish-green leaves, seedlings also shorter or taller than normal
3	Abnormal growth, elongated, chlorotic, thin and brownish leaves, seedlings also shorter or taller than normal
4	Seedlings with fungal mass on the surface of infected plants or die

applied when one way ANOVA revealed significant differences ($p \leq 0.05$). All statistical analyses were performed with Statistical Product and Service Solutions (SPSS) programme version 20.0.

RESULTS

A total of 65 *Trichoderma* spp. were obtained from the healthy rice rhizosphere soil obtained from Kedah, Kelantan and Terengganu, Malaysia. All isolates were confirmed as *Trichoderma* spp. based on morphological and cultural characteristics. Dual culture plate testing was used as a benchmark to evaluate the antagonistic capability of *Trichoderma* isolates against *F. fujikuroi*. Thirty eight out of 65 *Trichoderma* isolates exhibited excellent antagonistic activity against *F. fujikuroi* with PIRG value more than 45% (Table 3). *Trichoderma* isolate T40 was identified as the strongest antagonist against *F. fujikuroi* with significantly high of PIRG value (77.33%). Five *Trichoderma* isolates (T8, T9, T37, T40 and T41) exhibited PIRG value for more than 55% (Table 3 and Fig. 1).

For volatile compounds production testing, all *Trichoderma* isolates tested shown inhibition effect against *F. fujikuroi*. Isolate T59 exhibited the highest inhibition effect against *F. fujikuroi*

Table 3: Antagonistic activities and plant growth-promotion properties of *Trichoderma* isolates

No. of isolates	*Trichoderma* code	Dual culture plate testing (PIRG %)	Volatile compound production testing (PIRG %)	HCN production testing (nm)	IAA production testing (μg mL^{-1})	Phosphate solubilization activity testing (index)
1	T2	50.67^{bcd}	30.55 ^{bcdefg}	0.01^{jk}	18.31^{g}	0
2	T3	52.00^{bcd}	22.22^{efghi}	0.04^{fghi}	0^{r}	0
3	T4	48.00^{bcd}	15.28 hi	0.04^{fghi}	18.19^{h}	0
4	T5	53.33^{bcd}	22.22^{efghi}	0.05^{efg}	0^{r}	0
5	T6	48.00^{bcd}	26.38^{bcdefghi}	0.04^{fghi}	0^{r}	0
6	T7	52.00^{bcd}	29.16 ^{bcdefg}	0.01^{jk}	14.94^{j}	0
7	T8	56^{bcd}	22.22^{efghi}	0.01^{jk}	15.44^{i}	0
8	T9	58.67^{bc}	19.45^{efghi}	0.02^{ijk}	0^{r}	0
9	T12	50.67^{bcd}	16.66 ^{ghi}	0^{k}	30^{d}	0
10	T13	48^{bcd}	19.44^{efghi}	0.04^{fghi}	0^{r}	0
11	T16	50.67^{bcd}	13.89^{i}	0.03^{ghij}	0^{r}	0
12	T19	53.33^{bcd}	27.78^{bcdefgh}	0.03^{fghi}	6.88^{m}	0
13	T28	52^{bcd}	25^{bcdefghi}	0 k	0 r	0
14	T29	48^{bcd}	22.22^{efghi}	0.03^{hij}	27^{e}	0
15	T37	57.33^{bcd}	33.33^{abcd}	0.04^{efgh}	0^{r}	0
16	T38	45.33^{d}	26.39^{bcdefghi}	0.05^{def}	3.75^{o}	0
17	T39	54.67^{bcd}	30.55^{bcdefg}	0.05^{efgh}	0^{r}	0
18	T40	77.33^{a}	26.38^{bcdefghi}	0.02^{hij}	0^{r}	0
19	T41	60.00^{b}	27.77^{bcdefgh}	0.03^{ghij}	35.63 ^{c}	0
20	T42	46.67^{cd}	18.05^{fghi}	0.02^{hij}	0^{r}	0
21	T43	52^{bcd}	23.61^{cdefghi}	0.05^{efg}	49.38 ^{b}	0
22	T44	52^{bcd}	25^{cdefghi}	0.07^{cde}	6.25 ^{n}	0
23	T45	45.33^{d}	16.67^{ghi}	0.08^{bc}	7.5 ^{l}	0
24	T46	45.33^{d}	31.94^{abcde}	0.12^{a}	0^{r}	0
25	T47	45.33^{d}	20.83^{efghi}	0.07^{bcd}	10.63 ^{k}	0
26	T48	50.67^{bcd}	30.55^{bcdef}	0.08^{bcd}	0^{r}	0
27	T49	48^{cd}	26.38^{bcdefghi}	0.12^{a}	20.63 ^{f}	0
28	T50	46.67^{cd}	29.16^{bcdefg}	0.09^{b}	0^{r}	0
29	T51	52^{bcd}	29.16^{bcdefg}	0.04^{efgh}	3.13^{p}	0
30	T52	45.33^{d}	29.16^{bcdefg}	0.09^{b}	2.5^{q}	0
31	T54	45.33^{d}	31.94^{abcde}	0.05^{efg}	0^{r}	0
32	T56	49.33^{bcd}	23.61^{cdefghi}	0.05^{efg}	0^{r}	0
33	T59	48^{bcd}	44.44^{a}	0.03^{hijkl}	0^{r}	0
34	T61	48^{bcd}	38.89 ^{ab}	0.03^{jkl}	93.75^{a}	0
35	T62	45.33^{d}	36.11^{abcd}	0.05^{efg}	0^{r}	0
36	T63	45.33^{d}	25^{cdefghi}	0.03^{ghij}	0^{r}	0
37	T64	45.33^{d}	20.83 ^{efghi}	0.04^{fghi}	0^{r}	0
38	T65	45.33^{d}	37.50^{abc}	0.05^{def}	0^{r}	0

Means within column with same letters are not significantly different by Least Significant Different (LSD) test using SPSS at $p \leq 0.05$, Each value represents the mean of five replications of two independent experiments

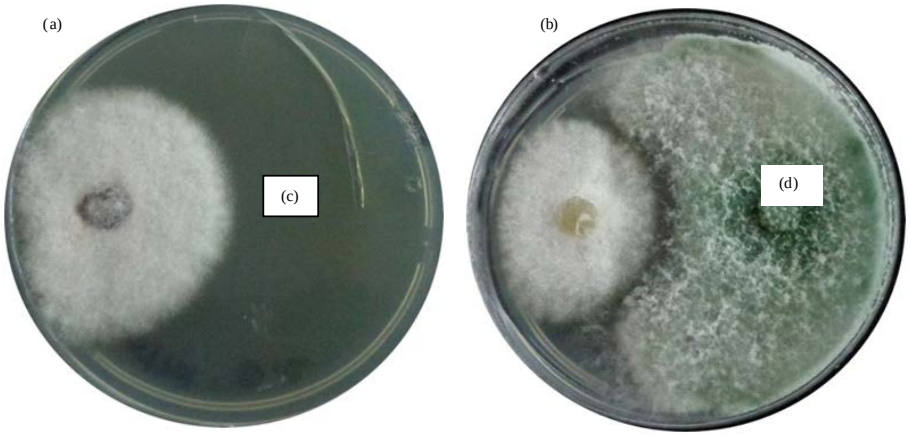

Fig. 1(a-b): *Fusarium fujikuroi* colony in control plate (a): Suppression effect of *Trichoderma* spp. against *F. fujikuroi* in dual culture plate testing, (b) Inhibition effect against *F. fujikuroi* after seven days of incubation at 28±2°C, (c): Colony of *Fusarium fujikuroi* and (d): Colony of *Trichoderma* isolate

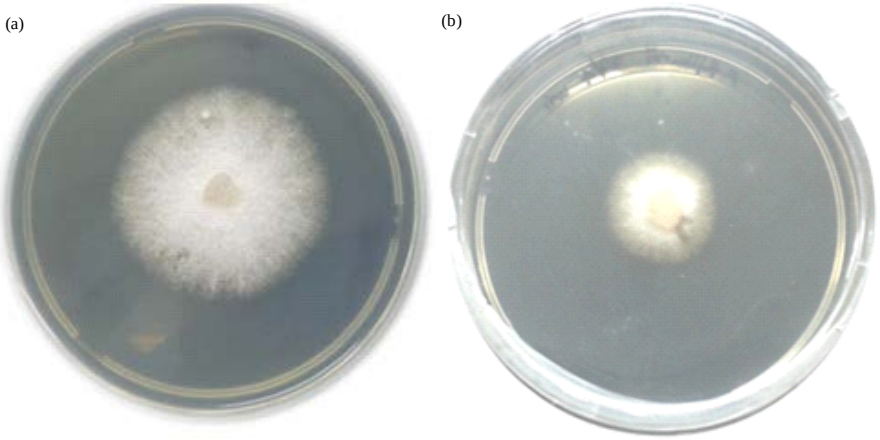

Fig. 2(a-b): (a) *Fusarium fujikuroi* colony in control plate and (b) Suppression effect on *F. fujikuroi* colony through the production of volatile compounds by *Trichoderma* isolate after seven days of incubation at 28±2°C

with PIRG value 44.44% followed by isolate T61 (38.89%), T65 (37.50%), T62 (36.11%) and T37 (33.33%) (Table 3 and Fig. 2). Only 35 *Trichoderma* isolates shown positive HCN production with the formation of orange colour on the picric acid treated filter paper. The density of the colour indicated the presence of cyanide activity. *Trichoderma* isolates: T46 and T49 have shown the significantly high colour density (Table 3).

The plant growth-promotion activities of *Trichoderma* isolates were assessed based on Indole Acetic Acid (IAA) production and capability in phosphate solubilization. Seventeen out of 38 *Trichoderma* isolates were able to produce IAA ranged 2.5-93.75 μg mL^{-1} (Table 3). *Trichoderma* T61 produced significantly high IAA, 93.75 μg mL^{-1}. However, none of the *Trichoderma* isolates screened capable in phosphate solubilizing on NBRIP agar (Table 3).

From the *in vitro* screenings conducted, twelve most potential *Trichoderma* isolates: T9, T40, T41, T43, T45, T46, T49, T50, T52, T59, T61 and T65 were selected based on the

Table 4: Antagonistic and plant growth-promotion testing of the selected potential *Trichoderma* isolates

Isolates	Dual culture plate testing (PIRG %)	Germination rate (%)	Plumule length (cm)	Radical length (cm)	Vigor index (%)
Control	-	96.67^{a}	2.38^{ab}	4.58^{ab}	67.88^{ab}
T9	81.98^{a}	100.00^{a}	2.24^{ab}	4.84^{ab}	70.83^{ab}
T40	84.99^{ab}	100.00^{a}	2.73^{a}	5.41^{a}	81.37^{a}
T41	81.98^{b}	100.00^{a}	2.35^{ab}	4.17^{ab}	65.17^{abc}
T43	84.99^{ab}	100.00^{a}	2.92^{a}	5.05^{ab}	79.70^{a}
T45	89.49^{a}	100.00^{a}	2.30^{ab}	4.44^{ab}	67.43^{abc}
T46	87.99^{a}	96.67^{a}	2.03^{ab}	3.77^{ab}	57.62^{abc}
T49	84.99^{ab}	100.00^{a}	2.31^{ab}	4.21^{ab}	65.13^{abc}
T50	87.99^{a}	100.00^{a}	1.65^{b}	2.48^{b}	41.27^{c}
T52	85.99^{ab}	100.00^{a}	1.83^{b}	2.98^{b}	48.13^{c}
T59	91.98^{a}	100.00^{a}	2.34^{ab}	5.15^{a}	74.87^{ab}
T61	86.99^{a}	100.00^{a}	1.93^{ab}	3.07^{ab}	50.00^{bc}
T65	77.98^{b}	96.67^{a}	2.35^{ab}	3.74^{ab}	60.28^{abc}

Means within column with same letters are not significantly different by Least Significant Different (LSD) test using SPSS at $p \leq 0.05$. Each value represents the mean of five replications of two independent experiments

Table 5: Bio-efficacy of the selected *Trichoderma* isolates under greenhouse conditions

Treatments	Inoculated isolates	Disease incidence (%)	Disease Severity Index (DSI)	Plant height (cm)	Dry biomass (g)
Control	-	0.00^{d}	0.10^{c}	18.24^{c}	0.53^{ab}
Control	*F. fujikuroi*	90.00^{a}	2.53^{a}	18.69^{abc}	0.47^{b}
Treatment 1	*F. fujikuroi*+T40	39.63^{b}	1.15^{b}	22.14^{a}	0.56^{ab}
Treatment 2	*F. fujikuroi*+T43	22.50^{bc}	0.78^{b}	21.04^{ab}	0.48^{b}
Treatment 3	*F. fujikuroi*+T45	21.94^{bc}	0.80^{b}	21.51^{abc}	0.51^{b}
Treatment 4	*F. fujikuroi*+T46	25.00^{bc}	0.58^{bc}	20.08^{abc}	0.52^{b}
Treatment 5	*F. fujikuroi*+T50	24.76^{bc}	0.76^{b}	21.33^{abc}	0.54^{ab}
Treatment 6	*F. fujikuroi*+T59	20.74^{c}	0.84^{b}	19.31^{abc}	0.58^{ab}
Treatment 7	*F. fujikuroi*+T61	30.00^{bc}	1.03^{b}	20.20^{abc}	0.67^{a}

Means within column with same letters are not significantly different by Least Significant Different (LSD) test using SPSS at $p \leq 0.05$. Each value represents the mean of five replications

mean separation conducted. The selected isolates were further tested for germination rate, vigor seedling index, plumule and radical lengths.

The germination rate on rice seeds inoculated with *Trichoderma* isolates and control were no significant different. However, *Trichoderma* isolates: T9, T40, T41, T43, T45, T49, T50, T52, T59 and T61 improved germination rate to 100% as compared to control, T46 and T65 at 96.67%, respectively (Table 4). For plumule and radical lengths, no significant increments were observed in *Trichoderma* inoculated seedlings compared to control. However, the rice seeds inoculated with T50, T52 and T61 stunted the growth of rice seedling with vigor index of 41.27, 48.13 and 50.00%, respectively (Table 4). Rice seeds inoculated with T43 shown highest plumule length (2.92 cm) and which contributed to the highest vigor index (79.70%) (Table 4).

Seven most potential *Trichoderma* isolates were selected for greenhouse evaluation. *Trichoderma* T45, T46, T50, T59 and T61 were selected based on prominent antagonist activity against *F. fujikuroi* and T40, T43 and T59 on growth performances (Table 4). Rice plant inoculated with respective *Trichoderma* isolates shown significantly low bakanae disease incidence and disease severity index (Table 5). Plant heights in all *F. fujikuroi* inoculated seedlings were not significantly different. Generally, rice plant inoculated with both *F. fujikuroi* and *Trichoderma* isolates were slightly higher than those in control (Fig. 2). In addition, plant dry biomass for *Trichoderma* T61 inoculated seedlings found significantly higher than rice seedlings inoculated with only *F. fujikuroi*, *F. fujikuroi* with T43, T45 and T46, respectively (Table 5).

DISCUSSION

All 65 isolates obtained from rhizosphere soil were confirmed as *Trichoderma* spp.: *T. harzianum* and *T. virens*. The *Trichoderma* spp. were reported abundant in the healthy rice

rhizosphere soil (Harman *et al*., 2004). The variable in suppression capability of *Trichoderma* isolates obtained against soil-borne fungal plant pathogen (*F. fujikuroi*) was also reported by Consolo *et al*. (2012). In dual culture plate testing, the mycelia of *Trichoderma* spp. extended outgrowths of *F. fujikuroi* representing a chemo-attractive mechanism of interaction between two isolates (Sharma, 2011). The biocontrol potential of *Trichoderma* spp. against phytopathogenic *Fusarium* was highly suggested by Calistru *et al*. (1997). This is associated with the strong saprotrophic and mycoparasitic behavior of *Trichoderma* spp. in colonizing the root epidermis and even a few cell layers below the epidermis (Harman *et al*., 2004). For instance, *Trichoderma* spp. were commonly use for effectively control of fungal pathogen in various fruit crops (Svetlana *et al*., 2010).

Besides, several mechanisms were also reported responsible for the suppression of pathogenic fungal including competition, antibiotic and metabolite production (Compant *et al*., 2005) and this was in line with our findings where the growth inhibition of *F. fujikuroi* was associated with the production of the volatile compounds and HCN. However, the PIRG values from the volatile compounds production were much more lower than those in dual culture plate testing for all the *Trichoderma* isolates tested. Our results were supported by Kucuk and Kivanc (2003), where the volatile metabolites inhibition effect of *Trichoderma* isolates found lower than non-volatile metabolites. In addition, the production of HCN by *Trichoderma* isolates was reported as an important antifungal feature for soil borne fungi pathogen management. This was due to the microbial produced cyanide can acts as a general metabolic inhibitor to avoid predation or competition without harming the host plant (Noori and Saud, 2012.). Moreover, hydrogen cyanide was also reported effectively in blocking the cytochrome oxidase pathway and which is highly toxic to microorganisms at picomolar concentrations (Manwar *et al*., 2011).

Plant growth-promoting properties such as auxins production and phosphate solubilizing are important in regulating plant and root growth in various crops. IAA produced by *Trichoderma* spp. were found important for root growth and to increase seedling quality (Gravel *et al*., 2007). Potential IAA-producing isolate (T61) was identified in the current study. This isolate is highly potential to be developed as plant growth promoter as it produced higher IAA concentration (93.75 μg mL^{-1}) as compared to those reported by Kotasthane *et al*. (2015) with only 30.08 μg mL^{-1} and Salas-Marina *et al*. (2011) with 27 μg mL^{-1}. However, no P-solubilization activity was observed in NBRIP plate assay for all the *Trichoderma* isolates tested. This was in contrast with El-Katatny (2004), who reported that *Trichoderma* isolates are relatively good in P-solubilization.

The present study evaluated the growth promoting ability of *Trichoderma* isolates in rice seeds based on seed vigor index and seedlings dry biomass. It was observed that some *Trichoderma* isolates work as plant growth promoter and some even seen to be detrimental. The same observation was reported in association to the plant genetic background, the interaction with the *Trichoderma* isolate (Tucci *et al*., 2011) and the species tested. Therefore, the potential *Trichoderma* isolate as plant growth promoter in the current work cannot be generalized to all other planting materials. However, *Trichoderma* spp. are multifunctional plant symbionts for enhancing germination rate and increasing seedling lengths of pepper, bean, radish, tomato, pepper and cucumber (Avis *et al*., 2008; Oyarbide *et al*., 2001; Yossen *et al*., 2003). Moreover, the present investigation shows that the use of *Trichoderma* T61 exhibited the highest dry biomass of rice seedlings was explained in relation to the high IAA production. Gravel *et al*. (2007) demonstrated

Trichoderma induce plant Indole Acetic Acid (IAA) production and helps to improve root growth and seedling quality. The application of *T. harzianum* was reported to increase fresh shoot weight, root weight and/or root length in peas (Naseby *et al*., 2000), increase root area by 95%, root length by 75%, dry weight by 80%, shoot length by 45% and leaf area by 80% in cucumber when compared to the control (Yedidia *et al*., 2001). The mechanisms involved in the stimulation of plant growth by *Trichoderma* include interactions with plant roots similar to mycorrhizae, in which *Trichoderma* penetrates and colonizes root tissues without eliciting specific defense responses against the colonizing strain (Yedidia *et al*., 2001). However, in our study not all *Trichoderma* isolates enhance plant growth and is might be due to the suppression effect of the metabolite produced.

The effectiveness of *Trichoderma* spp. in controlling plant pathogens have been proven in many studies and through direct benefits of these fungi on plant growth and production (Avis *et al*., 2008). This was in agreement with our findings, where rice plants inoculated with *Trichoderma* spp. significantly reduce bakanae disease incidence and disease severity under greenhouse conditions. Various mechanisms were suggested to involve, such as induced systemic resistance in plants, antibiosis, competition for nutrients and/or space and mycoparasitism using lytic enzymes (Tseng *et al*., 2008). Lytic enzymes of *Trichoderma* such as cellulases, chitinases, glucanase and proteases are partially induced before direct contact with the host (Foreman *et al*., 2003). Besides, *Trichoderma* interaction with plant roots creates a sensitized state in the plant allowing it to respond more efficiently to subsequent pathogenic attack (Shoresh and Harman, 2008). Sharma (2011) demonstrated *Trichoderma* spp. as promising antagonist against *F. fujikuroi* by parasitized and lysed the mycelium of *F. fujikuroi*. *Trichoderma* spp. have evolved multiple mechanisms that result in improvements in plant resistance to disease and plant growth and productivity (Harman *et al*., 2004). Therefore, the application of *Trichoderma* spp. in crop disease management is gaining more attention and the development of *Trichoderma* T40, T43, T45, T46, T50, T59 and T61 as biocontrol agents is potential to be an alternative in bakanae management.

CONCLUSION

The development of biological control approach of bakanae disease using *Trichoderma* spp. is potential to reduce the agrochemical usage. In the present study, the potential isolates (T40, T43, T45, T46, T50, T59 and T61) increased plant resistance against *F. fujikuroi* in rice variety MR219. However, further studies on plant defense mechanisms and disease suppression were suggested especially under natural field conditions.

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