

***Trichoderma* and *Bacillus* as Biocontrol Agents against *Fusarium* in Rice**

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This study was conducted to determine the effectiveness of *Trichoderma* and *Bacillus* isolates used individually or in combination in reducing the disease incidence of *Fusarium* spF2 in rice. The antagonistic activity of twenty-two *Trichoderma* isolates was evaluated individually or in combination with *Bacillus subtilis* UKM1 against *Fusarium* sp F2 via the dual culture technique. The dual culture technique showed highest reduction in radial growth of pathogen by isolates T2, T3, T4, T5, T7, T8, T9, T11 and T21. This was then followed by greenhouse experiments that evaluated four parameters: Pre and Post emergence of seedlings, disease incidence and disease severity on treated and non treated MR219 rice varieties that were inoculated with *Fusarium* sp F2. The results of this experiment showed that rice plants treated with biocontrol agents showed significant reduction of pathogen ($p \leq 0.05$). The greenhouse experiments further selected isolates T2, T4 and T21 as the best candidates based on disease severity scores. However when co-inoculated with *B. subtilis* UKM1 isolates T2, T7, T8, T9 and T18 resulted in higher reduction of disease incidence (22, 11, 11, 22 and 22 %) and severity (8.67, 6.7, 4.3, 11 and 9 %) 60 days post transplantation.

Key words: *Fusarium* sp, biocontrol, *Trichoderma* sp., *Bacillus subtilis*, rice, co-inoculated.

Control of pathogens, especially soil borne pathogens is difficult due to their ability to adapt to ecological niches, impact a huge range of host, and its ability to produce resistant spores that increase their survivability under environmental pressures (Thabet *et al.*, 2008). As such research over the past decade has been focused towards the use of biological control agents in controlling disease amongst agriculturally significant crops, especially diseases caused by soil borne pathogens (Zarandi *et al.*, 2009).

Several investigators have implied that *Trichoderma* and *B. subtilis* species are appealing candidates for control of blast disease and sheath

blight disease of rice. Studies have described the use of *B. subtilis* individually or in combination with other microorganisms like *Trichoderma* spp. or chemical fungicide in controlling diseases in rice (Yang *et al.* 2009). *Bacillus* and *Trichoderma* have been utilized as commercial biocontrol agents. However obtaining the best concoction that consists of different antagonistic organisms that works under different field condition is required before any bioagent can be developed commercially (Baha, 2002; Harman, 2000). In addition to being able to grow in a wide range of pH and different soil types, *Trichoderma* and *Bacillus* strains are known to secrete hydrolytic enzymes and antimicrobial products that assist with the process of mycoparasitism. In order to identify successful biocontrol agents, continuous screening for new antagonistic isolates is essential for the development of new and more effective biocontrol agents against specific or a broader

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spectrum of pathogens.

Fusarium sp is considered one of the most important groups of Ascomycetous fungi that is commonly isolated from soil and plant debris (Kraft *et al.*, 1981). It is distributed worldwide and is the causal agent that results in severe economic losses due to yield and quality reduction in infected crops (Desjardins, 2006; Leslie and Summerell, 2006; Nelson *et al.*, 1983). This genus of phytopathogen causes vascular wilts or root rots in many economically important vegetables, field crops and trees (Leslie and Summerell, 2006). As such an efficient control system is required to reduce losses incurred by this pathogen group.

Field infections of *Fusarium sp* have been controlled thus far by the use of pesticides. Although this method is currently the mostly widely used to control *Fusarium* infections, it is not a long-term solution to the prevalence of diseases in crops due to expense, concerns on risks of exposure, residual effects, toxicity to non-target organisms and other health and environmental hazards. Therefore, recent efforts have been focused on developing eco-friendly, safe, long lasting and effective methods of control against many phytopathogens to facilitate management of plant diseases (Georgopoulos, 1987; Poornima, 2011).

Here we aim to test the efficiency of 22 *Trichoderma* isolates individually and in combination with *B. subtilis* UKM1 to determine the best biocontrol concoction that may work to inhibit the growth and proliferation of *Fusarium sp* F2. Experiments were designed via the dual culture assays and greenhouse experimentations were conducted using either un-autoclaved and autoclaved soil.

MATERIALS AND METHODS

Isolation of *Trichoderma* spp.

A serial dilution was conducted on soil samples obtained from the National Forest Reserve, Malaysia (Hamdia and Kalaivani, 2013). Fungal cultures obtained were maintained on potato dextrose agar (PDA). Pure cultures were analyzed macro and microscopically and these examinations showed that there were twenty two different isolates of *Trichoderma* derived from the soil samples and these were designated isolates T1 to

T22.

Pathogenicity test of the *Fusarium sp* isolates Soil Inoculation

Rice plants were pre-screened with three *Fusarium sp* (F1, F2 and F3) isolates to determine the isolate with the highest disease severity score for use in the following experiments. Soil used in this study was clay loam (Clay: 30%, Sand; 47.15%, Silt: 22.85%), sterilized at a 121°C /1.5 Kg/cm². The soil was then infested with the pathogenic isolates individually, watered and kept moistened and mixed thoroughly every other day for the duration of one week. All isolates were maintained on PDA plates. The concentration of *Fusarium sp* used in this experiment was determined via Haemocytometer (Kiraly *et al.*, 1974). Soil inoculum of 1×10⁷ spore/mL was prepared for all *Fusarium spp*.

Seed dormancy was broken and the seeds were left for 3 days at 28°C to induce germination. These seedlings were then sown into infested pots and were visually examined for signs of infection seven days post transplantation. The Pre and Post-emergence damping off data was collected two weeks post transplantation. The data for pre-emergence and post-emergence damping off was calculated using the following formula:

$$\% \text{ Pre-emergence damping off} = \frac{\text{No. of seedlings with no signs of emergence}}{\text{No. of seeds sown}} \times 100$$

$$\% \text{ Post-emergence damping off} = \frac{\text{No. of plants killed}}{\text{Total No. of plants with disease emergence}} \times 100$$

Disease incidence and severity were determined 60 days from planting according to the following formula:

$$\% \text{ Disease incidence} = \frac{\text{No. infected plants}}{\text{Total No of Plants in Experiment}} \times 100$$

$$\% \text{ Disease severity} = \frac{\text{Total (number of plants in class \{(0 \times 0) + ... + (number of plants in class (5 \times 5))\}}}{\text{Total plants} \times 3} \times 100$$

Disease severity was assessed via the 0 – 5 scale developed by Woltz and Arthur (1973) where a score of 0 = healthy plants, 1 = yellowing characteristic, 2 = wilting of one third leaves, 3=wilting of two third of leaves, 4=whole plant wilted, 5= plant is dead. The data obtained was statistically evaluated using a randomized complete design, via a Duncan's Multiple Rating

Test (Duncan *et al.*, 1995).

Antagonistic Activity between *Trichoderma* isolates and *Fusarium* sp F2

Antagonistics studies were conducted using dual culture technique. Each PDA plate was divided equally into two portions where a 5 mm *Fusarium* sp F2 (the most virulent isolate based on disease severity score from experiment 2.2) fungal plug was placed in one half while the other was inoculated with a 5mm fungal plugs of isolate T1 to T22 (individual isolate tested at a time) (Hamdia and Kalaivani, 2013). The plates were incubated for 4 days at 28°C, the antagonistic activity was scored according to the scale developed by Alfredo and Cornelia (2011).

The antagonistic activity of the 22 isolates of *Trichoderma* was tested under greenhouse conditions in autoclaved soil (control conditions). The following experiments were conducted: Control without microbes, *Fusarium* sp F2 only, *B. subtilis* UKM1 only, 22 *Trichoderma* isolates only (tested separately), *B. subtilis* UKM1+ 22 *Trichoderma* isolates (tested separately), *Fusarium* sp F2+ 22 *Trichoderma* isolates (tested separately), *Fusarium* sp F2+ *B. subtilis* UKM1+ 22 *Trichoderma* isolates (tested separately). The concentration of *Trichoderma* isolates used was 1×10^8 spore /mL while the inoculum size of *B. subtilis* UKM1 was set at 2×10^8 cell /mL (Abdou *et al.*, 1979). *Fusarium* sp F2 inoculum size was 1×10^7 spore/mL. Germinated seeds were transferred to infested pots and pre and post emergence observations were made. Six *Trichoderma* isolates that showed the best results from the antagonistic test were used in the greenhouse experiments in natural conditions (unautoclaved soil). The data obtained from the observation were statistically evaluated using a randomized complete design, via a Duncan's Multiple Rating Test (Duncan, 1995).

RESULTS

In vitro Studies via Dual Culture Technique

The twenty-two (22) *Trichoderma* isolates varied in their ability to reduce radial growth of *Fusarium* sp F2 four days post-inoculation. The best antagonistic potential was displayed by *Trichoderma* isolates T2, T11, T7, T8, T9, T21, T5,

T3, and T4 (Table 1). All these isolates scored 3+ (top range score) in the degree of inhibition by causing 80 to 100% coverage/over growth in the 9cm petri dishes (Fig. 1). We believe *Trichoderma* isolates that scored 3+ parasitized *Fusarium* sp F2 as the pathogen could not be re-isolated from these plates. Scanning electron micrographs of the dual culture plates showed that the cultures

Greenhouse Experiments in Natural Conditions

Six *Trichoderma* isolates with the best antagonistic results from dual culture technique as well as in greenhouse controlled (sterilized soil – data not shown) was selected for use in greenhouse experiment conducted in natural condition (unsterilized soil). The combination treatment between the six *Trichoderma* isolates and *Fusarium* sp F2 showed high levels of reduction in pre and post emergence damping off in seedlings (Fig. 2A & B). As for the disease incidence and severity parameters (Figure 2C & D), data obtained from this study indicates that *Trichoderma* isolates T2, and T8 resulted in significant ($p \leq 0.05$) reduction of *Fusarium* sp F2 infection (33, 33% and 6.7, 8.9% respectively). The study showed all *Trichoderma* isolates had high

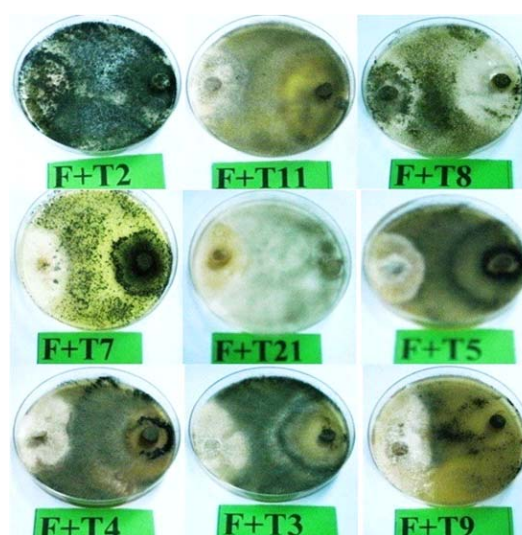


Fig. 1. Antagonistic activity between *Trichoderma* isolates and *Fusarium* sp F2 via dual culture technique. The best *Trichoderma* isolates that had highest antagonistic activities core3+ against *Fusarium* sp F2 are shown here. The right side of the plate was inoculated with *Trichoderma* spp. while the left *Fusarium* sp F2. Isolates presented in order of efficacy against *Fusarium* sp F2.

potential to suppress the pathogen, but they varied in their antagonistic activity.

The effect of combined treatment with *Trichoderma* and *B. subtilis* UKM1 showed significant difference ($p \leq 0.05$) in post emergence

damping off in seedlings where *Trichoderma* isolates T2 and T21 gave the highest suppression (4.3 and 4.7% respectively) compared with soil infested with *Fusarium sp* F2 only (18%) (Fig. 3A&B). A decrease was observed in disease

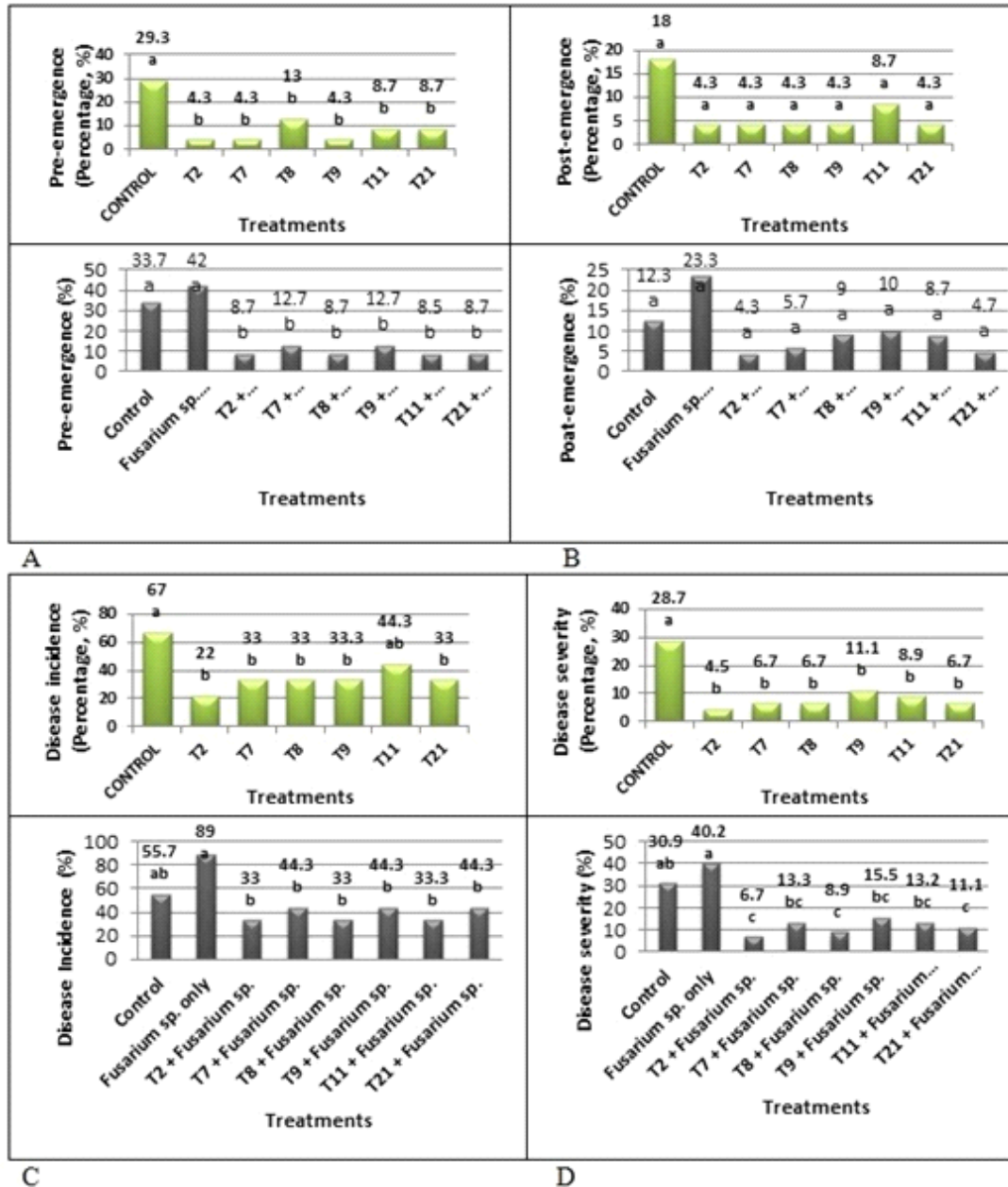


Fig. 2. Comparison between rice plants treated with six *Trichoderma* isolates with or without *Fusarium sp* F2 under green house condition (A) pre and (B) post emergence damping off, (C) disease infected and (D) diseaseseverity. Numbers in each column that have same letters do not differ significantly from each other at $p < 0.05$ according to Duncan's multiple range test. Pre and post emergence damping off was determined according to Ziedan (1998). Percentage of disease infected and severity were scored after 60 days from sowing according to Woltz and Arthur (1973).

incidence and severity, in *Fusarium sp* F2 infested soil treated with *B. subtilis* UKM1 and *Trichoderma* isolates T2 (22% and 6.7% respectively) (Figure3C

& D), and most of the plants that were inoculated with this concoction had a tolerance to infection by *Fusarium sp* F2. The greenhouse experiments

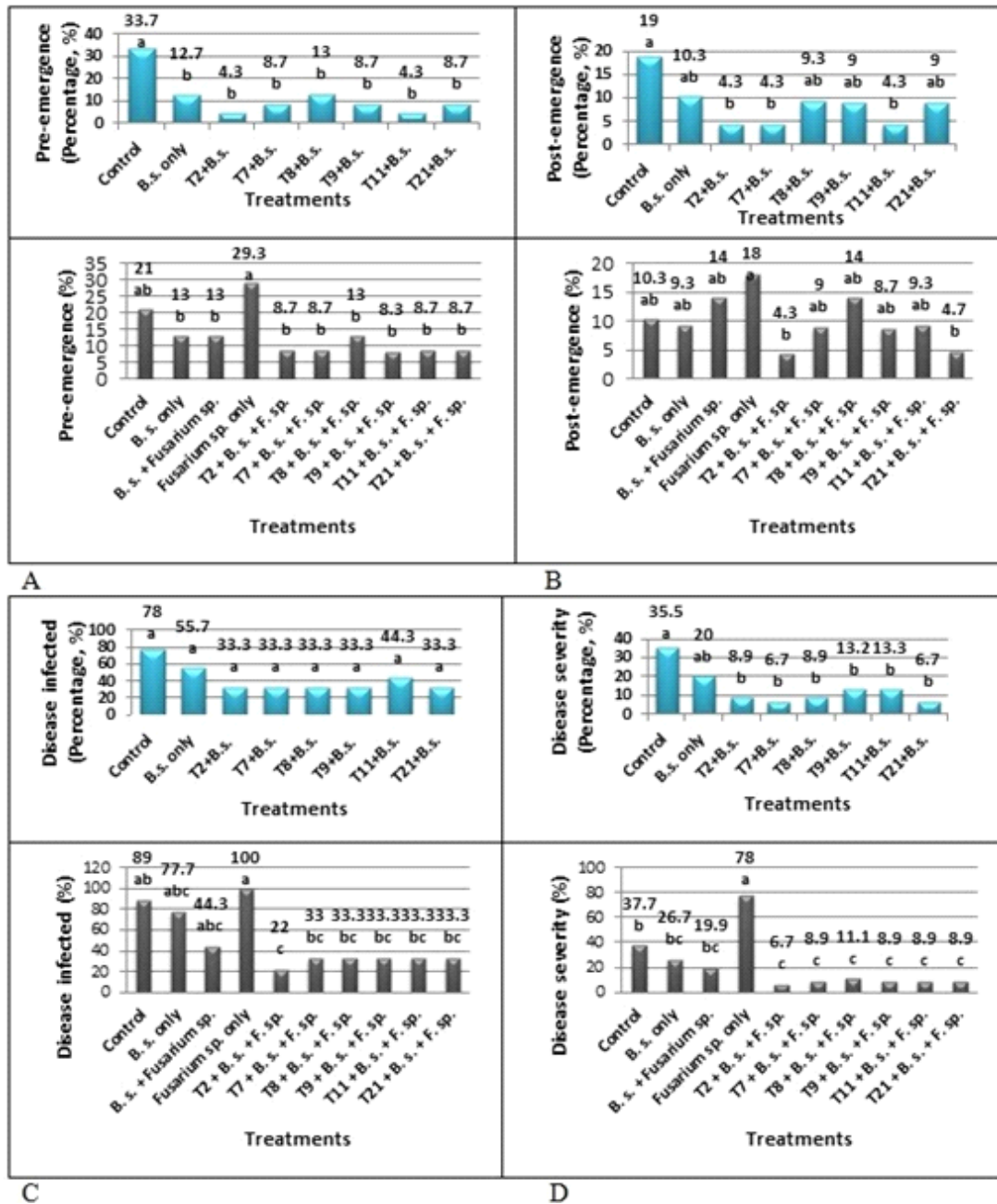


Fig. 3. Comparison between rice plants treated with six *Trichoderma* isolates, and *B. subtilis* UKM1 in combination with or without *Fusarium sp* F2 under green house condition (A) pre and (B) post emergence damping off. (C) Disease infected and (D) disease severity. Numbers in each column that have same letters do not differ significantly from each other at $p < 0.05$ according to Duncan's multiple range test. Pre and post emergence damping off was determined according to Ziedan (1998). Percentage of disease infected and severity were scored after 60 days from sowing according to Woltz and Arthur (1973).

showed that a combination between *Trichoderma* isolates and *B. subtilis* UKM1 provided the best reduction in disease incidence and severity in both sterilized and non-sterilized soil infections. The antagonistic ability of the top six *Trichoderma* isolates in combination with *B. subtilis* UKM1 against *Fusarium* sp F2, in non-autoclaved soil

(Field soil) was more effective in suppressing *Fusarium* sp F2 in comparison with soil inoculated with *Trichoderma* isolates alone. Figure 4 shows that the treatment with biocontrol agents managed to mitigate the disease symptoms caused by *Fusarium* sp on rice. Figure 4A and 4C show rice plants that have been treated by the six

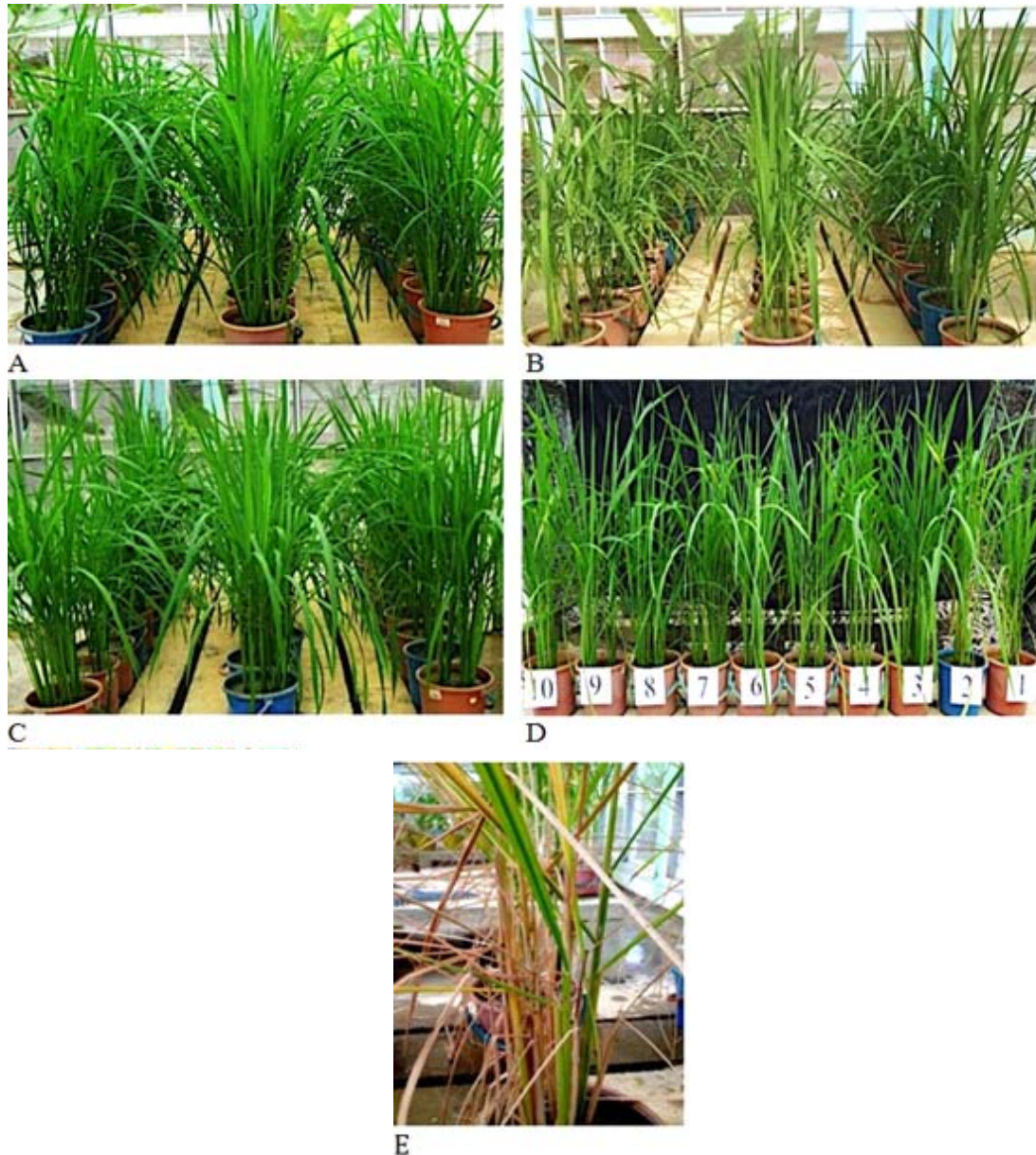


Fig. 4. A. Rice plants inoculated with the top six *Trichoderma* isolates only; B. Rice plants inoculated with combination of *Trichoderma* isolate and *Fusarium* sp F2; C. Rice plants co-inoculated with *Trichoderma* isolate and *B. subtilis* UKM1; D. Rice plants inoculated with *Trichoderma* sp + *B. subtilis* UKM1 + *Fusarium* sp F2 and, E. Rice plant inoculated with *Fusarium* sp F2 only.

Trichoderma isolates and the dual inoculation of *Trichoderma* and *B. subtilis* UKM1 respectively. The plants did not show a significant difference in growth and development in the absence of a pathogen. They were a luscious green with higher levels of panickles compared to the control untreated and those infected with the pathogen. This therefor indicates that the organisms may be functioning as growth regulators in addition to their ability to suppress pathogens. However when *Fusarium* sp F2 was added to the soil, the co-

inoculated samples (Figure 4D) showed healthier plants compared to those treated with *Trichoderma* isolates only (Figure 4B).

DISCUSSION

This study was initiated to isolate and identify antagonists towards a Malaysian isolate of *Fusarium* sp.. Here we report the analysis on the ability of our soil isolates to act as biological control agents against *Fusarium* sp. The inoculation with *Trichoderma* isolates only or in combination with *B. subtilis* UKM1 showed higher numbers of germinated seeds as compared with control non-treated plants. There have been previous reports that showed certain *Trichoderma* isolates were able to induce growth under different conditions (Harman *et al.*, 2004; Alfredo and Cornelia, 2011; Bell *et al.*, 1982; Hamdia and Kalaivani, 2013). Ashraf *et al.* (2005) and Palet *et al.* (2013) stated that *Trichoderma* spp. enhanced the plant growth directly or indirectly by producing several enzymes against plant pathogens and accelerated the degradation of organic crops and materials as nutrient source to enhance growth of plants.

In our study we found that the antagonistic activity of *Trichoderma* isolates were higher in field soil (non-autoclave soil) as compared to autoclaved soil. *Trichoderma* is a saprophytic fungus, and the non-autoclaved soil probably contains a rich source of several microorganisms that encourages microbe-microbe interactions beneficial to the plant root system resulting in better nutrient absorption and inhibition of certain organisms (Abeysingne, 2007; Ashraf *et al.*, 2005; Belen *et al.*, 2005; Cardona and Rodriguez, 2006; Hamdia and Kalaivani, 2013). The interaction between *Trichoderma* and other soil organisms include various mechanisms: such as antibiosis and mycoparasitism (Harman *et al.*, 2004; Vinale *et al.*, 2008; Yedidia *et al.*, 2003). *Trichoderma* spp. can obtain nutrients directly from living tissues of pathogen cells by hyphal coiling, or by connection with host through small pore formation (Elad *et al.*, 1993; Harman *et al.*, 2004). Our electron micrographs showed that the *Trichoderma* isolates were coiling around the hyphae of the pathogen and probably digesting or constricting the pathogen to death. This is supported by our inability to isolate the pathogen from the plate.

Table 1. Antagonistic activity between *Trichoderma* spp. and *Fusarium* spF2 in dual culture assays. The antagonistic results are presented in order of efficacy

Treatment	*Degree of antagonism after 4 day**
T2+F.solaniF2	3+
T11+F.solaniF2	3+
T8+F.solaniF2	3+
T7+F.solaniF2	3+
T21+F.solaniF2	3+
T5+F.solaniF2	3+
T4+F.solaniF2	3+
T3+F.solaniF2	3+
T9+F.solaniF2	3+
T6+F.solaniF2	2+
T20+F.solaniF2	2+
T18+F.solaniF2	2+
T14+F.solaniF2	2+
T19+F.solaniF2	2+
T17+F.solaniF2	2+
T22+F.solaniF2	2+
T16+F.solaniF2	2+
T12+F.solaniF2	2+
T13+ F.solaniF2	2+
T1+F.solaniF2	1+
T10+F.solaniF2	-
T15+F.solaniF2	-

*Mean of three plates (9cm diameter) were used as replicates for each treatment

**According to scale by Alfredo and Aleli (3) that involve four degrees:

3+ The antagonistic fungus was able to grow over the pathogen and pathogen growth completely inhibited.

2+ The pathogen growth completely inhibited, but antagonist was not able to grow over the pathogen.

1+ Mutual inhibition initially, but antagonist was overgrown by pathogen.

-Pathogen growth not inhibited, antagonist was overgrown by pathogen

Treatment with *B. subtilis* UKM1 resulted in post emergence of 14% (Figure 3B), disease incidence of 44.3% (Figure 3C) and disease severity of 19.9% (Figure 3D). However, when used in combination with the six top *Trichoderma* isolates (T2, T7, T8, T9, T11, T21), *B. subtilis* UKM1 resulted in significant reduction of pre and post damping off, disease incidence and disease severity values (Figure 3). Lippi and Monaco (1994) also reported similar findings with *B. subtilis*, where they report that the release of several antifungal metabolites such as subtilin, bacitracin, bacillin and bacillomycin have an inhibitory effect on fungal pathogens (Harman, 2000; Papavizas, 1985). Ebtsam *et al.* (2009) and Gia *et al.* (2006) reported that *B. subtilis* and *Trichoderma* sp were efficient at controlling diseases caused by *Fusarium* sp on tomato. In addition the co-inoculation recorded higher growth parameters, and production than single inoculation (Borrero *et al.*, 2004). As in other research findings, our research concurs with the efficiency of co-inoculation as a strategic approach to control plant diseases (Gong *et al.*, 2006; Yang *et al.*, 2009). However these isolates function better as growth regulators than they do as biocontrol agents based on the data and physical observations of treated plants in this study.

Based on our microscopic analysis, dual culture assays and biochemical analysis (chitinolytic activity) we believe that the mechanism by which our *Trichoderma* isolates antagonizes the pathogen is by mycoparasitism (Fahmi *et al.*, 2012; Kalaivani *et al.*, 2014; Leslie and Summerell *et al.*, 2006) that involves the production of cell wall degrading enzymes (CWDE) such as chitinases and glucanases. In addition researchers have reported that antibiotics (6-pentyl- α -pyrone (6pp), isocyanide derivatives), acids (heptelidic and koningic acid), and peptaibols are secondary metabolites produced by *Trichoderma* spp. to inhibit radial growth of many phytopathogenic fungi (Elad *et al.*, 1982; Elad *et al.*, 1983; Elad *et al.*, 1993; Vinale *et al.*, 2008). The antagonistic activity as seen on the co-inoculation technique in this study shows the possibility of a combination inoculum as a more efficient inhibitor of *Fusarium* sp F2.

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