Research Journal of Applied Sciences 8 (1): 72-81, 2013
ISSN: 1815-932X
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Evaluating the Efficacy of *Trichoderma* Isolates and *Bacillus subtilis* as Biological Control Agents Against *Rhizoctonia solani*

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Abstract: The aim of this study is to determine the effectiveness of the Trichoderma isolates as a biological control agent against *Rhizoctonia solani* Kuhn. *In vitro* tests of antagonistic activity and ability of Trichoderma isolates against *R. solani* via the dual culture technique showed that isolates 6, 7, 8, 20, 13, 22 and 17 had high antagonistic activities with reduced radial growth of pathogen. The Trichoderma isolates were used in control experiment under greenhouse conditions where inoculation with Trichoderma isolates 2, 4, 7 and 11 were effective in inducing significant increase in pre and post emergence seedlings (4.33, 4.33, 4.33 and 8.33%) and also contributed towards an increase in seed germination (4.67, 4.67, 4.33 and 4.33%). The suppression results of Trichoderma isolates 2, 7 and 9 on *R. solani* growth showed disease incidence of approximately 33.33% while disease severity results for the same isolates were 20, 15.67 and 20%, respectively. These readings recorded a significant difference ($p = 0.05$) than those obtained when the soil was artificially inoculated with *R. solani* only where the observed values for disease incidence and severity were 100 and 77.67%, respectively. The combination treatment between Trichoderma isolates and *Bacillus subtilis* showed all Trichordema isolates were able to reduce pre and post-emergence of disease in seedlings in control experiment ($p = 0.05$). In the combination treatment; isolates 2 and 7 had the highest reduction in disease incidence (both 11%) and severity (both 4.33%) after 60 days post transplanting; the concoction appeared to enhance the growth of plants. Trichoderma isolates 2, 7, 8, 9, 11 and 21 in combination with *B. subtilis* had excellent suppression of pre (8.67, 8.33, 13, 8.67, 8.67 and 8.67%) and post (9, 8.67, 9.33, 14, 9 and 14%) emergence of disease in *R. solani* inoculated soil. This shows an overall reduction of disease incidence of between 22-33% and severity of 15.33-22% when the Trichoderma isolates were used in combination with *B. subtilis* to suppress *R. solani* infestations.

Key words: *Rhizoctonia solani*, rice, biological control, Trichoderma isolates, *Bacillus subtilis*

INTRODUCTION

Rice is subject to diseases such as rice blast, rice sheath blight, bacterial blight and rice viral diseases (Manandhar and Yami, 2008; Mostapha, 2004). The degree of devastation caused by each of these diseases is different in different rice growing countries. Two major problems worldwide for rice farmers are the rice blast which has been reported to cause 100% yield losses in countries such as Malaysia and sheath blight that has resulted in losses up to 50% of yield in countries such as Thailand, Indonesia, Malaysia and China (Mostapha, 2004; Lai Van *et al*., 2001; Hiroshi *et al*., 1999).

Rhizoctonia solani Kuhn, the causative agent for fungal sheath blight causes large devastations in the rice growing regions. Currently it is considered the most serious disease in some rice planting countries. In addition to causing great devastation in yield, the pathogen survives for several months in the soil as sclerotia (Coley-Smith *et al*., 1974) or in the seed of infected plants and thus is able to infect rice plants as mycelia or re-emerge in newly grown plants (Alfredo and Aleli Cornelia, 2011; Shalini and Kotasthane, 2007; Tan *et al*., 2007; Wiwattanapatapee *et al*., 2007; Qin and Zhang, 2005; Pfahler and Petersen, 2004). Researchers have been involved in understanding the process of infection and spread of this disease, the generation of disease resistant rice varieties, the isolation of disease resistance and regulatory genes, the generation of transgenic lines and the use of pesticides in reducing the incidence of disease (Park *et al*., 2008). The most commonly used method of control is the application of fungicides. This however has detrimental effects on the environment, ecology, health of handlers, economics of production as well as the generation of new more virulent strains.

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Therefore, alternatives such as the application of bio-control agents and the engineering of environment have been studied for a more green approach at controlling diseases caused by various plant pathogens. The development of stable cost-effective and easily applied bio-control formulations is critical for the advancement of biological control in all areas of pest management. Unfortunately, most microorganisms used as bio-control agents have relatively narrow spectrum of activity compared to synthetic pesticides and often exhibit inconsistent performance under particular agro-ecosystem. The inconsistent performance of the bio-control agents is due among other reasons to the utilisation of single bio-control agents to suppress single plant-pathogen under any environmental conditions. Single bio-control agent is not likely to be active in all soil environments in which they are applied or against all pathogens that attack the host plant (Hafedh *et al*., 2006, 2005; Ziedan and Elewa, 2000; Altomare *et al*., 1999; Harman and Kubicek, 1998; Ziedan, 1998; Kumar, 1995; Lewis and Papavizas, 1987; Tamimi and Hadwan, 1985).

Several investigators have pointed out that Trichoderma and *B. subtilis* species are appealing candidates for biological control. The first observation of the bio-control ability of *Trichoderma* sp. against *R. solani* was reported by Weindling (1932) and a lot of studies have described using *B. subtilis* individually or in combination with other microorganisms or chemical fungicide to control sheath blight in rice plants, (Yang *et al*., 2009; Kondoh *et al*., 2001). Trichoderma and Bacillus strains are able to grow in a wide range of pH and are available in all soil types. They are capable of secreting hydrolytic enzymes and causing mycoparasitism of fungal pathogens of plants. In order to identify successful bio-control agents, researchers need to continuously screen for effective formulations of bio-control agents against specific pathogens.

The aim of this study is to reduce or replace the use of synthetic fungicides and inorganic fertilizer through the preparation of multi-bioagent inoculations that consists of compatible strains with antagonistic activity and the ability to promote growth (Siameto *et al*., 2010; Cummings *et al*., 2009; Kumar *et al*., 2009; Chittihunsa *et al*., 2007; Wiwattanapatapee *et al*., 2007). In this study, fungi from soil were isolated and analysed as candidates for bio-control activities. These isolates were used individually and in combination with *B. subtilis* UKM1 to determine the best combination of multi-bioagents for use against rice sheath blight pathogen *Rhizoctonia solani.*

MATERIALS AND METHODS

All the screening of microbial isolates and greenhouse studies were conducted at Universiti Kebangsaan Malaysia. Soil samples obtained from Taman Negara Merapoh, in the State of Pahang in Malaysia was used as source to screen for microorganisms that exhibit antagonistic activities. Researchers limited the screen of all microorganisms derived from soil to those that were identified as Trichoderma sp. mainly as these species have shown good antagonistic activity in previous research reports and work well independently and in combination with *B. subtilis* as bio-control agents.

Laboratory experiments: The 1 g of soil obtained from the National Forest Reserve was dissolved in 100 mL of distilled water, homogenised and vortexed for 15 min and 1 mL of the suspended soil solution was added to 9 mL of sterilised distilled water in a tube (10^{-1}). The content of this tube was vortexed and 1 mL was subsequently transferred aseptically to another 9 mL sterilised distilled water in a tube (10^{-2}) and this serial dilution was continued to dilution 10^{-6}. About 1 mL of dilutions obtained from dilutions of 10^{-4}-10^{-6} was applied aseptically on Potato Dextrose Agar (PDA) plates via spread plate technique. These plates were then incubated for 5 days at (28±2°C) and observed for fungal growth for 4-5 days. Individual colonies were isolated and sub-cultured on Rose Bengal Agar plates to obtain pure culture of the fungi. These fungi were then subjected to macroscopic and microscopic evaluation to identify the isolates that were *Trichoderma* sp. The results of the microscopic analysis identified twenty two Trichoderma isolates that were designated T1 to T22. The pure cultures from Trichoderma sp. were kept on Potato Dextrose Agar (PDA) slants in 4°C until further use.

Antagonistic between Trichoderma isolates and *Rhizoctonia solani*: Antagonistics studies were conducted using dual culture technique. The PDA plates were incubated for 4 days at 28°C. Each plate was divided into two equal portions and a 5 mm disk (1 week old fungal culture) of each isolate was placed on one side of the plate and the other had a 5 mm disk *R. solani.*

Antagonistic activity between *Bacillus subtilis* and *Rhizoctonia solani*: *Bacillus subtilis* UKM1 was cultured in sterilised flasks contain 100 mL Nutrient Broth (NB). A single colony of bacteria was transferred from Nutrient Agar (NA) plate to the broth in aseptic manner. These cultures were grown in a 37°C incubator shaker with

agitation at 150 rpm for 36 h. The 9 mL petri dishes were cultured with 5 mm disc of *R. solani*. The fungi were allowed to grow until the diameter reached 3 cm from the centre. Holes were made in the potato dextrose agar surrounding the *R. solani* culture via a sterilised cork-borer. The 10 μL of *B. subtilis* suspension was added into each hole and 10 μL of distilled water was added as control (Chittihunsa *et al*., 2007). The 3 days post-incubation, antagonistic activity against the pathogen was evaluated on solid medium by measuring the inhibition percentage using the following equation (Mojica-Marin *et al*., 2008).

$$\text{Inhibition}(\%) = \frac{\text{Fungal growth (cm)}}{\text{Control growth}} \times 100$$

Greenhouse experiments: The greenhouse experiments were conducted using all the 22 Trichoderma isolates that were tested with *R. solani* in the laboratory. Though the laboratory experiments provided us with the isolates that showed excellent antagonistic ability, researchers repeated the experiments with all isolates as there may be differences in reactivity shown on agar and in the soil.

The inhibitory ability of various isolates on rice plant was studied in pots. Pots (15 cm diameter) were filled with 2 kg clay loam soil (Clay: 30%, Sand; 47.15%, Silt: 20.85%) that was sterilised at a 121°C/1.5 kg/cm^2 for 1.5 h for all experiments. Trichoderma isolates and *R. solani* were prepared by sub-culturing on PDA. *B. subtilis* was cultured on Nutrient Agar (NA). The soil was then inoculated with *R. solani* and left for a period of 1 week. The same process was repeated for *Trichoderma* sp. and *R. solani* co-inoculations, Trichoderma, Bacillus and *R. solani* co-inoculation and Bacillus and Trichoderma co-inoculations. The following experiments were conducted: Control without microbes, *R. solani* only, *B. subtilis* only, 22 Trichoderma isolates only (tested separately), *B. subtilis* + Trichoderma 22 isolates (tested separately), *R. solani* + Trichoderma 22 isolates (tested separately) and *R. solani* + *B. subtilis* + Trichoderma 22 isolates (tested separately). The concentration of Trichoderma isolates in whole experiments determined via Haemacytometer (Kiraly *et al*. (1974) were 1×10^8 spore/mL and *B. subtilis* was 2×10^8 as estimated according to Hafedh (2001) and Abdou *et al*. (1979). The *R. solani* inoculum size was determined as the average number of sclerotia in 4 fields/dish. Pots were moistened and mixed thoroughly every other day for the duration of 1 week.

Rice seeds were placed on filter paper containing petri dishes and incubated at 40°C for 48 h to break their dormancy. The filter paper was then moistened and the seed were left for 3 days at 28°C to induce germination.

The seeds were sown in the infested pots as stated above. These seeds were then sown in the infested pots at the rate of 8 seeds/pot. Once the post-emergence observations have been made on these seedlings, the number of plants per pot was reduced to three. The experiments were conducted in triplicate. Pre-emergence was measured after 1 week from planting and post-emergence was recorded 2 weeks after transplanting (Ziedan, 1998). These pots were observed over a period of time (9 weeks) to assess disease symptoms caused by *R. solani*. The data for pre-emergence and post-emergence dampind off is calculated using the following equations:

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

$$\text{Post-emergence damping off}\ (\%) = \frac{\text{No. of killed plants}}{\text{Total number of emerged plants}} \times 100$$

Percentage of disease incidence was scored after 60 days from sowing according to the following formula in greenhouse:

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

Disease severity was assessed after 60 days from planting in greenhouse via the 0-5 scale developed by Woltz and Engelhard (1973). Where a score of 0 = healthy plants, 1 = yellowing characteristic, 2 = wilting of one third leaves, 3 = wilting of two third leaves, 4 = whole plant wilted and 5 = plant is dead:

$$\text{Disease severity}\ (\%) = \frac{\text{Total (Number of plants in class } 0\times0) + \ldots + (\text{Number of plants in class } 5\times5)}{\text{Total plants}\times3} \times 100$$

The data obtained from the observation were statistically evaluated using a randomised complete design, via a Duncan's multiple rating test (Peterson, 1985).

RESULTS AND DISCUSSION

***In vitro* study:** The results of antagonistic activity of different isolates of Trichoderma against *R. solani* was analysed via the dual culture technique. The antagonistic activity was scored according to the Alfredo and Aleli Cornelia (2011) scale. The twenty two isolates varied in their ability to reduce radial growth of *R. solani* 4 days post-inoculation. Antagonistic activity of isolates 2, 6, 7, 8, 20, 13, 17 and 22 in Table 1 had the highest antagonistic activity (+++ degree). This test showed Trichordema inhibited the growth of *R. solani* by causing 80-100% coverage/over growth of the 9 cm petri dishes (Fig. 1). *R. solani* could not be re-isolated from these plates.

Certain Trichoderma isolates as mentioned above were able to colonise *R. solani* within 96 h and these results were better in comparison with previous study (Goes *et al*., 2002). Isolates 3, 4, 5, 9, 11, 18 and 21 showed moderate antagonistic activity towards *R. solani* (++ degree). Though isolates 1, 2, 12, 14, 15, 16 and 19 showed some inhibitory activity; they recorded the lowest level of inhibition and was scored as + degree. Isolate 10 was determined as the weakest antagonistic isolate against *R. solani*.

Figure 2 shows the results obtained when we used *R. solani* together with *B. substilis*. Here we see clear zones surrounding the *R. solani* plug in the middle of the plate (Fig. 2b). *R. solani* is a fast growing fungus and can grow rapidly and cover the entire 9 cm petri dish in 4 days (Fig. 2a). However, as we can see from Fig. 2b, the growth of *R. solani* was completely inhibited and sharply affected by bacteria *B. subtilis*, inhibition zone was roughly 68% in comparison with control treatment. This result was

Table 1: Antagonistic test between Trichoderma isolates and *Rhizoctonia solani* under laboratory conditions

Treatments	Degree of antagonism after 5 days**
T6+*Rhizoctonia solani*	+++*
T7+*Rhizoctonia solanii*	+++
T8+*Rhizoctonia solani*	+++
T20+*Rhizoctonia solani*	+++
T13+*Rhizoctonia solani*	+++
T22+*Rhizoctonia solani*	+++
T17+*Rhizoctonia solani*	+++
T3+*Rhizoctonia solani*	++
T4+*Rhizoctonia solani*	++
T11+*Rhizoctonia solani*	++
T18+*Rhizoctonia solani*	++
T5+*Rhizoctonia solani*	++
T9+*Rhizoctonia solani*	++
T21+*Rhizoctonia solani*	++
T16*Rhizoctonia solani*	+
T2+*Rhizoctonia solani*	+
T12+*Rhizoctonia solani*	+
T14+*Rhizoctonia solani*	+
T15+*Rhizoctonia solani*	+
T19+*Rhizoctonia solani*	+
T1+*Rhizoctonia solani*	+
T10+*Rhizoctonia solani*	-

*Mean of three plates (9 cm diameter) were used as replicates for each treatment; **According to Alfredo and Aleli scale (2011) that involve four degrees; +++: The antagonistic fungus was able to grow over the pathogen and pathogen growth completely inhabited; ++: The pathogen growth completely inhabited but antagonist was not able to grow over the pathogen; +: Mutual inhibition initially but antagonist was overgrown by pathogen. Pathogen grow not inhibited, antagonist was overgrown by pathogen

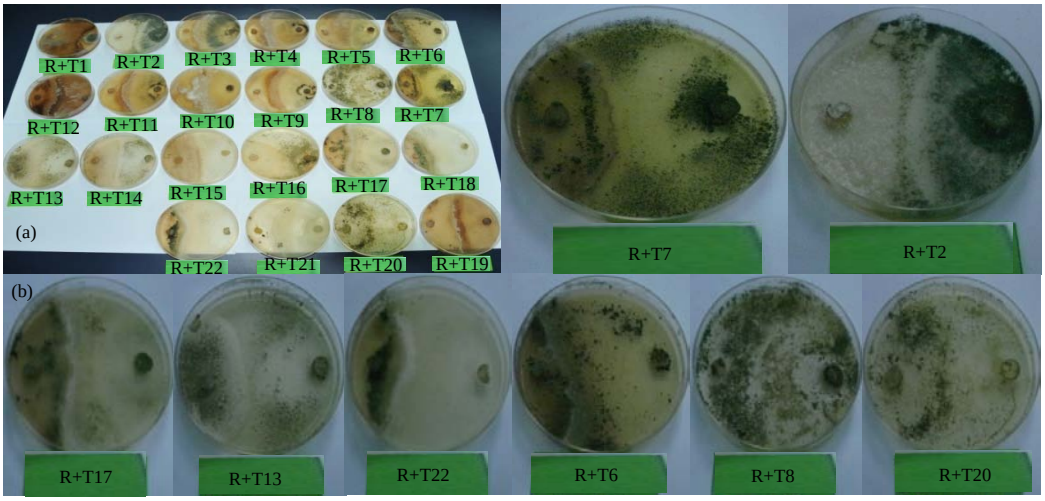

Fig. 1: a) Antagonistic interaction between 22 isolates of Trichoderma and *Rhizoctonia solani*; b) Trichoderma isolates with high Antagonistic activity against *R. solani* were on the left side of the plate and Trichoderma on the right

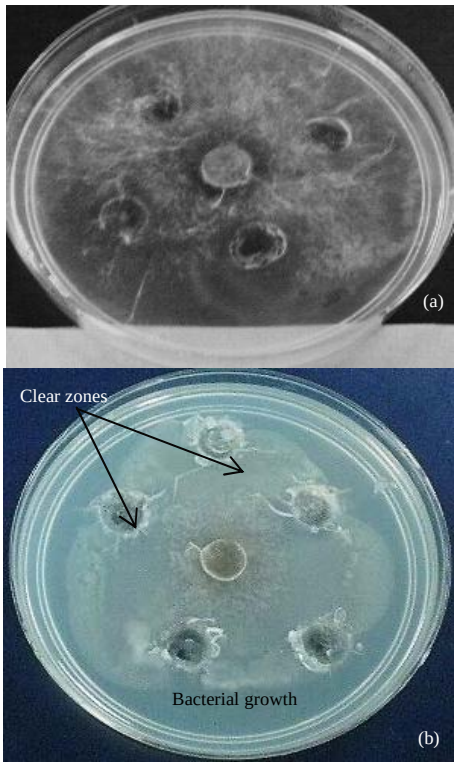

Fig. 2: Antagonistic interaction between *Bacillus subtilis* and *Rhizoctonia solani*; a) *Rhizoctonia solani* only as control; b) *Bacillus subtilis* and *Rhizoctonia solani*

Table 2: The efficiency of Trichoderma isolates under greenhouse conditions

Treatments	Pre- emergence**	Post- emergence***	Disease incidence**	Disease severity***
Control	28.67*^{a}	25.00^{a}	55.67****^{a}	40.00^{a}
T1	12.67****^{a}	11.00^{ab}	44.33*^{a}	22.00^{a}
T2	4.33^{b}	4.67^{b}	22.00^{a}	18.00^{a}
T3	8.67^{ab}	4.33^{b}	33.33^{a}	15.67^{a}
T4	4.33^{b}	4.67^{b}	33.00^{a}	17.67^{a}
T5	12.67^{ab}	8.33^{ab}	33.33^{a}	22.00^{a}
T6	8.67^{ab}	4.33^{b}	33.00^{a}	17.67^{a}
T7	4.33^{b}	4.33^{b}	22.33^{a}	13.33^{a}
T8	13.00^{ab}	4.33^{b}	33.00^{a}	20.00^{a}
T9	8.67^{ab}	4.33^{b}	22.00^{a}	15.67^{a}
T10	17.00^{ab}	10.33^{ab}	44.33^{a}	26.67^{a}
T11	8.33^{b}	4.33^{b}	33.00^{a}	15.67^{a}
T12	16.67^{ab}	14.00^{ab}	44.33^{a}	29.00^{a}
T13	13.00^{ab}	9.33^{ab}	44.67^{a}	22.33^{a}
T14	17.00^{ab}	10.33^{ab}	33.00^{a}	26.67^{a}
T15	17.00^{ab}	14.33^{ab}	44.33^{a}	29.00^{a}
T16	12.67^{ab}	10.33^{ab}	33.00^{a}	24.33^{a}
T17	16.67^{ab}	10.00^{ab}	44.33^{a}	20.00^{a}
T18	12.67^{ab}	9.00^{ab}	33.33^{a}	24.33^{a}
T19	17.00^{ab}	10.33^{ab}	55.33^{a}	33.33^{a}
T20	16.67^{ab}	8.33^{ab}	44.33^{a}	31.00^{a}
T21	8.67^{ab}	4.33^{b}	33.00^{a}	20.00^{a}
T22	16.67^{ab}	11.33^{ab}	44.67^{a}	22.33^{a}

****Numbers in each column that have same letter do not differ significantly from each other at p = 0.05 according to Duncan's multiple range test. *Three replicates for each isolate. **Pre and ***Post-emergence according to Ziedan (1998)

similar to earlier research findings that showed *B. subtilis* greatly inhibited to the radial growth of *R. solani* (Yang *et al*., 2009).

Greenhouse study

Trichoderma isolates only: The greenhouse experiments were conducted in pots. In the experiments conducted under greenhouse conditions, researchers found that Trichoderma isolates showed a different level of growth enhancement and increased seeds germination in pre and post emergence of seedlings ($p \leq 0.05$). Antagonistic test exhibited some isolates induced seedling progression. There have been previous reports that showed that Trichoderma isolates were able to induce growth under different conditions (Bell *et al*., 1982; Harman, 2000; Schuster and Schmoll, 2010; Alfredo and Aleli Cornelia, 2011). When treated with *Trichoderma* sp. there were certain isolates (2, 4, 7 and 11) that enhanced growth 60 days post transplanting by approximately 4.33-8.33% (Table 2). The only isolates that showed identical results in the dual plate technique and the greenhouse were isolates T2 and T7.

Trichoderma isolates and *R. solani*

Dual plate results: The data from Table 1 shows that isolates 10, 12, 15 and 19 have lower antagonistic activity levels in both the dual assay and greenhouse studies. Trichoderma isolates had good mycoparasitism and induction of resistance against the pathogen. Verma *et al*. (2007) reported that the induction of resistance in the plants was a result of enzymatic activity of different proteins such as chitinase, glucanase and protease. These proteins were able to digest the fungal cell wall and lyse them completely. On the other hand, there were certain isolates that showed good antagonistic activity on plates but did not show similar activity when tested in the greenhouse. This is in agreement with the research finding of other groups that had good antagonistic activity on plate but did not produce the same level of inhibition in soil (Grosch *et al*., 2007; Jash and Pan, 2007; Cundom *et al*., 2003).

In addition earlier studies have indicated that inoculation Dense ID has a high impact on disease incidence and severity in plants. Tan *et al*. (2007) found that a higher Dense ID increased the disease progression. This therefore indicates that for us to make valid comparisons of the efficiency of the isolates against those that have been studied previously, researchers would have to use the same density of microbe as inoculum. In the studies; the inoculum density has been in accordance to previous reports.

Table 3: Antagonistic activity of Trichoderma isolates and seedling blight (*Rhizoctonia solani*) greenhouse conditions

Treatments	Pre-emergence**	Post-emergence***	Disease incidence**	Disease severity***
Control	00.00*^{cd}	00.00^{b}	0.00^{c}	0.00^{d}
Rhizoctonia solani	58.33^{a}	33.33^{a}	100.00*^{a}	77.67^{a}
T1+*Rhizoctonia solani*	41.67****^{abc}	15.00^{ab}	67.00****^{ab}	35.67 ^{abcd}
T2+*Rhizoctonia solani*	25.33^{c}	11.00^{ab}	33.33^{bc}	20.00^{bcd}
T3+*Rhizoctonia solani*	37.50^{abc}	13.33^{ab}	55.67^{ab}	33.33^{abcd}
T4+*Rhizoctonia solani*	33.33^{bc}	13.33^{ab}	44.33^{bc}	28.67^{bcd}
T5+*Rhizoctonia solani*	25.17^{c}	13.33^{ab}	55.33^{ab}	24.33^{bcd}
T6+*Rhizoctonia solani*	37.50^{abc}	12.33^{ab}	55.67^{ab}	29.00^{bcd}
T7+*Rhizoctonia solani*	25.17^{c}	11.00^{ab}	33.33^{bc}	15.67^{cd}
T8+*Rhizoctonia solani*	25.33^{c}	13.33^{ab}	44.33^{bc}	24.33^{bcd}
T9+*Rhizoctonia solani*	25.33^{c}	12.33^{ab}	33.33^{bc}	20.00^{bcd}
T10+*Rhizoctonia solani*	41.67^{abc}	21.67^{ab}	78.00^{ab}	42.33^{abcd}
T11+*Rhizoctonia solani*	25.17^{c}	11.00^{ab}	44.33^{bc}	26.67^{bcd}
T12+*Rhizoctonia solani*	45.83^{abc}	16.67^{ab}	77.67^{ab}	44.33^{abcd}
T13+*Rhizoctonia solani*	50.00^{ab}	13.33^{ab}	66.67^{ab}	26.67^{bcd}
T14+*Rhizoctonia solani*	33.33^{bc}	13.33^{ab}	66.67^{ab}	37.67^{abcd}
T15+*Rhizoctonia solani*	41.67^{abc}	27.67^{ab}	78.00^{ab}	64.67^{ab}
T16+*Rhizoctonia solani*	33.50^{bc}	14.00^{ab}	67.00^{ab}	40.33^{abcd}
T17+*Rhizoctonia solani*	33.33^{bc}	17.67^{ab}	78.00^{ab}	44.33^{abcd}
T18+*Rhizoctonia solani*	37.50^{abc}	15.00^{ab}	55.67^{ab}	24.33^{bcd}
T19+*Rhizoctonia solani*	37.50^{abc}	17.67^{ab}	78.00^{ab}	51.33^{abc}
T20+*Rhizoctonia solani*	29.33^{bc}	18.00^{ab}	77.67^{ab}	53.33^{abc}
T21+*Rhizoctonia solani*	37.50^{abc}	12.33^{ab}	44.33^{bc}	22.33^{bcd}
T22+*Rhizoctonia solani*	33.67^{bc}	17.67^{ab}	55.67^{ab}	29.00^{bcd}

****Numbers in each column that have same letter do not differ significantly from each other at p = 0.05 according to Duncan's multiple range test; *Three replicates for each isolate in each replicate 3 plants. **Pre and ***Post-emergence according to Ziedan (1998)

Greenhouse study: In the study conducted to determine the Trichoderma isolates that were able to suppress *R. solani* growth, isolates 2, 5, 7, 8, 9 and 11 were the best isolates to reduce pathogen growth in pre and post- emergence of disease in seedlings by approximately 25.33, 25.17, 25.17, 25.33, 25.33, 25.17 and 11, 13.33, 11, 13.33, 12.33, 11%, respectively compared with *R. solani* treatment only which yielded pre and post-emergence of 58.33 and 33.33% (Table 3). The results of disease incidence and severity revealed that isolates 2, 7 and 9 dramatically reduced disease incidence to 33.33% and severity to 15.67-20%, respectively 60 days post transplanting as compared with artificial infection treatment (incidence-100% and severity-77.67% (Table 3) (Lahlali and Hijri, 2010).

Trichoderma isolates and *B. subtilis*

Dual plate results: Results of the dual inoculation by Trichoderma isolates and *B. subtilis* on *R. solani* showed that isolates 2, 7, 8, 9, 11 and 21 had excellent suppression of pathogen. They were the highest in reducing pre (8.67, 8.33, 13, 8.67, 8.67 and 8.67%) and post (9, 8.67, 9.33, 14, 9 and 14%) emergence of disease in seedlings, respectively as compared with when the plants were exposed to the pathogen alone (54.33% (pre) and 38.67% (post)) (Table 4). The percentage of infected plants and disease severity exhibited that isolates 2, 4, 7, 8, 9, 11 and 21 had significantly (p = 0.05) suppressed *R. solani* and reduced disease incidence (22, 44, 22, 33, 22.33 and 33%) and severity (15.33, 26.67, 17.67, 19.67, 20, 22 and 22%), respectively. The disease incidence and severity with *R. solani* only was 100 and 77.67%, respectively 60 days of post transplanting (Table 4).

Greenhouse study: The combination treatment between Trichoderma isolates and *B. subtilis* showed isolates 2, 3, 4, 5, 7. 8, 9 and 11 had a high significant difference (p = 0.05) in pre and post-emergence of disease in seedlings (4.33, 4.33, 4.33, 4.33, 4.33, 4.33, 4.33 and 4.33%) and (4.67, 4.67, 4.67, 4.33, 4.67, 4.33, 4.33 and 4.33%) compared with non-treated control (29.33%) and (28.67%) (Table 5). The beneficial effect of these isolates was shown in the lower degree of disease incidence and severity demonstrated. The study indicates that isolates 2 and 7 have the highest reduction in disease incidence and severity (both 11%) and (both 4.33%), respectively after 60 days of transplanting. In addition, these isolates seemed to enhance the plants growth in all treatments (Table 5). The results from Table 5 show that there was no clear significant difference between theses isolates for disease severity. It has been reported that *B. subtilis* has high ability to supply the plant with some protection through the production of glucanase enzyme. This enzyme works compatibly with Trichoderma isolates and is known to inhibit a wide range of plant pathogens and one such organism is *Rhizoctonia solani*.

Results obtained from the *in vitro* experiments of antagonistic activity of different isolates of Trichoderma against *R. solani* in this study showed that the Trichoderma isolates alone and in combination with

Table 4: Antagonistic activity of Trichoderma isolates and *Bacillus subtilis* under greenhouse conditions

Treatments	Pre-emergence**	Post-emergence***	Disease incidence**	Disease severity***
Control	29.33****a	28.67^{a}	67.00^{a}	42.33^{a}
Bacillus subtilis alone	13.00*ab	14.00^{ab}	44.33****ab	17.67^{b}
T1+*Bacillus subtilis*	13.00^{ab}	9.67^{b}	33.00*ab	13.33^{b}
T2+*Bacillus subtilis*	4.33^{b}	4.67^{b}	11.00^{b}	4.33^{b}
T3+*Bacillus subtilis*	4.33^{b}	4.67^{b}	33.33^{ab}	15.33^{b}
T4+*Bacillus subtilis*	4.33^{b}	4.67^{b}	33.00^{ab}	15.67^{b}
T5+*Bacillus subtilis*	4.33^{b}	4.33^{b}	22.00^{ab}	11.00^{b}
T6+*Bacillus subtilis*	8.67^{b}	4.67^{b}	33.33^{ab}	11.33^{b}
T7+*Bacillus subtilis*	4.33^{b}	4.67^{b}	11.00^{b}	4.33^{b}
T8+*Bacillus subtilis*	4.33^{b}	4.33^{b}	22.00^{ab}	6.67^{b}
T9+*Bacillus subtilis*	4.33^{b}	4.33^{b}	22.00^{ab}	11.00^{b}
T10+*Bacillus subtilis*	12.67^{ab}	5.67^{b}	33.00^{ab}	15.67^{b}
T11+*Bacillus subtilis*	4.33^{b}	4.33^{b}	22.00^{ab}	6.67^{b}
T12+*Bacillus subtilis*	12.67^{ab}	14.00^{ab}	44.33^{ab}	15.67^{b}
T13+*Bacillus subtilis*	8.33^{b}	11.00^{b}	33.00^{ab}	13.33^{b}
T14+*Bacillus subtilis*	12.67^{ab}	8.33^{b}	33.33^{ab}	15.67^{b}
T15+*Bacillus subtilis*	12.67^{ab}	10.33^{b}	33.00^{ab}	17.67^{b}
T16+*Bacillus subtilis*	8.67^{b}	9.33^{b}	33.33^{ab}	11.33^{b}
T17+*Bacillus subtilis*	8.67^{b}	9.00^{b}	33.33^{ab}	11.33^{b}
T18+*Bacillus subtilis*	8.33^{b}	5.67^{b}	22.00^{ab}	9.00^{b}
T19+*Bacillus subtilis*	12.67^{ab}	9.67^{b}	44.67^{ab}	13.33^{b}
T20+*Bacillus subtilis*	12.67^{ab}	4.67^{b}	33.33^{ab}	15.67^{b}
T21+*Bacillus subtilis*	8.67^{b}	4.67^{b}	22.33^{ab}	4.33^{b}
T22+*Bacillus subtilis*	8.33^{b}	10.00^{b}	33.33^{ab}	11.00^{b}

Table 5: Antagonistic activity of Trichoderma isolates and *Bacillus subtilis* in reducing seedling blight under greenhouse conditions

Treatments	Pre-emergence**	Post-emergence***	Disease incidence**	Disease severity***
Control	0.00^{f}	0.00^{c}	0.00^{c}	0.00^{c}
Bacillus subtilis with *Rhizoctonia solani*	45.83*ab	20.00^{abc}	67.00****ab	48.67^{ab}
Rhizoctonia solani only	54.33^{a}	38.67^{a}	100.00*a	77.67^{a}
T1+*Bacillus subtilis*+*Rhizoctonia solani*	17.00****def	13.00^{abc}	55.33^{ab}	24.33^{bc}
T2+*Bacillus subtilis*+*Rhizoctonia solani*	8.67^{ef}	9.00^{bc}	22.00^{bc}	15.33^{bc}
T3+*Bacillus subtilis*+*Rhizoctonia solani*	12.67^{def}	12.33^{abc}	55.67^{ab}	28.67^{bc}
T4+*Bacillus subtilis*+*Rhizoctonia solani*	8.67^{ef}	14.00^{abc}	44.33^{bc}	26.67^{bc}
T5+*Bacillus subtilis*+*Rhizoctonia solani*	17.00^{def}	11.00^{bc}	55.67^{ab}	26.67^{bc}
T6+*Bacillus subtilis*+*Rhizoctonia solani*	13.00^{def}	9.67^{bc}	55.67^{ab}	24.33^{bc}
T7+*Bacillus subtilis*+*Rhizoctonia solani*	8.33^{ef}	8.67^{bc}	22.00^{bc}	17.67^{bc}
T8+*Bacillus subtilis*+*Rhizoctonia solani*	13.00^{def}	9.33^{bc}	33.00^{bc}	19.67 ^{bc}
T9+*Bacillus subtilis*+*Rhizoctonia solani*	8.67^{ef}	14.00^{abc}	22.33^{bc}	20.00^{bc}
T10+*Bacillus subtilis*+*Rhizoctonia solani*	29.33^{bcde}	18.00^{abc}	67.00^{ab}	22.00^{bc}
T11+*Bacillus subtilis*+*Rhizoctonia solani*	8.67^{ef}	9.00^{bc}	33.00^{bc}	22.00^{bc}
T12+*Bacillus subtilis*+*Rhizoctonia solani*	25.00^{bcde}	16.67^{abc}	66.67^{ab}	46.33^{ab}
T13+*Bacillus subtilis*+*Rhizoctonia solani*	29.33^{bcde}	12.33^{bc}	55.67^{ab}	35.33^{bc}
T14+*Bacillus subtilis*+*Rhizoctonia solani*	29.33^{bcde}	11.33^{bc}	66.67^{ab}	37.67^{bc}
T15+*Bacillus subtilis*+*Rhizoctonia solani*	33.33^{bcd}	19.67^{abc}	67.00^{ab}	49.00^{ab}
T16+*Bacillus subtilis*+*Rhizoctonia solani*	16.67^{def}	12.67^{abc}	55.33^{ab}	40.00^{abc}
T17+*Bacillus subtilis*+*Rhizoctonia solani*	16.67^{def}	14.00^{abc}	66.67^{ab}	42.00^{ab}
T18+*Bacillus subtilis*+*Rhizoctonia solani*	12.67^{def}	12.67^{abc}	55.67^{ab}	22.33^{bc}
T19+*Bacillus subtilis*+*Rhizoctonia solani*	41.67^{ab}	27.67^{ab}	55.33^{ab}	37.67^{bc}
T20+*Bacillus subtilis*+*Rhizoctonia solani*	17.00^{def}	20.00^{abc}	55.67^{ab}	40.00^{abc}
T21+*Bacillus subtilis*+*Rhizoctonia solani*	8.67^{ef}	14.00^{abc}	33.00^{bc}	22.00^{bc}
T22+*Bacillus subtilis*+*Rhizoctonia solani*	21.00^{cdef}	14.00^{abc}	55.67^{ab}	26.67^{bc}

****Numbers in each column that have same letter do not differ significantly from each other at p = 0.05 according to Duncan's multiple range test. *Three replicates for each isolate. **Pre and ***Post-emergence according to Ziedan (1998)

B. subtilis could be used effectively to control *R. solani* (Siameto *et al*., 2010; Kumar *et al*., 2009). These microorganisms achieved control of the *R. solani* by growing quickly and arresting the growth of the pathogen. In this study, researchers observed microscopically that there was hyphal interaction between the mycoparasite Trichoderma isolates and coiling of the mycelia of the Trichoderma species and lysing of the *R. solani* mycelia. As mentioned above there are many means by which these organisms may overcome the pathogen and it's efficiency as biocontrol agent reported by a large group of researchers (Tamimi and Hadwan, 1985; Lewis and Papavizas, 1987; Kumar, 1995; Gupta *et al*., 1995; Harman and Kubicek, 1998; Altomare *et al*., 1999; Hafedh *et al*., 2005, 2006; Prasad and Kumar, 2011).

The greenhouse study showed that the combination of Trichoderma isolates and *B. subtilis* provided the

highest reduction in disease incidence and severity. This researchers believe may result in a systemic resistance within the plant that may be a result of some molecule or chemical exudate that elicits a defense mechanism in the host plant. Pre-emergence damping off caused by *R. solani* occurred at a greater incidence than post-emergence damping off and most plants that were inoculated by Trichoderma had tolerance to infection by *R. solani*. This result is similar to previous studies that recorded the effective use of *T. harzianum* with *B. subtilis* to control *R. solani* in different plants in greenhouse conditions (Cummings *et al*., 2009; Yang *et al*., 2009; Brasier and Webber, 1987). The poor performance of some isolates from this study can be due to the poor growth of the antagonist in the greenhouse conditions. Factors such as seed type, soil type and rhizophere activity in soil can determine the effectiveness of a biocontrol agent. *R. solani* population has been reported to increase in sandy loam soils and reduced in clay loam soils (Khan and Sinha, 2005) and the population of microbes surrounding a root system varies with plants, soil type and the environmental conditions at that locale (Verma *et al*., 2007; Hafedh, 2001; Harman and Kubicek, 1998).

CONCLUSION

In this experiment, researchers have managed to isolate and determine the most efficient Trichoderma isolate against the plant pathogen *R. solani* In addition we have determined the best Trichoderma and *B. subtilis* combination that provides the highest reduction in disease incidence and severity. The way forward from here would be to test these combinations in field conditions to determine the efficiency of these concoctions. Researchers hope that we may be able to determine the most efficient concentration of these isolates to use in the field to act as a pathogen suppressor as well as a soil conditioner.

ACKNOWLEDGEMENTS

Researchers would like to thank the Ministry of Agriculture Malaysia for the Science Fund awarded (UKM 05-01-02 SF1013) to conduct this research. In addition the Ministry of Higher Education has provided some assistance to this research through the awarded grant LRGS/TD/2011/UPM-UKM/KM/01.

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