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Evaluating the efficacy of *Trichoderma* spp and *Bacillus substilis* as biocontrol agents against *Magnaporthe grisea* in rice

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Abstract

Rice blast causes yield losses to rice farmers worldwide. Although this problem is currently being addressed through the use of resistant rice varieties, fungicide and rotation farming, these methods alone do not form a durable, long lasting solution in mitigating disease. Here we obtained *Trichoderma* isolates from soil and tested their efficacy as biocontrol agents against *Magnaporthe grisea*. Twenty-two *Trichoderma* isolates were identified and used in dual culture assays to determine antagonistic ability before isolates showing promise were administered to rice plants grown in greenhouse conditions where the efficacy of these isolates was tested individually or as dual inoculums with *Bacillus substilis* UKM1. The results showed that the dual inoculation of biocontrol agents caused significant ($p \leq 0.05$) inhibition of *M. grisea* as compared to a single agent. In addition, we observed the inhibitory effect of *Trichoderma* T2 under SEM where the hypha of the biocontrol agent coiled and possibly enzymatically degraded the pathogen. *Trichoderma* T2 in combination with *B. substilis* UKM1 gave the highest reduction in pre-post damping off, disease infection and disease severity caused by *M. grisea* in greenhouse conditions. From the greenhouse experimentations, the biocontrol agents were more efficient in inhibiting rice blast when applied prior to transplantation as compared to when applications were made at seed setting stage.

Keywords: *Magnaporthe grisea*, biocontrol, dual plate culture, hyphal coiling, *Trichoderma spp, Bacillus substilis* UKM1.
Abbreviations: PDA_potatoe dextrose agar, SEM_scanning electron microscope, IRRI_International Rice Research Institute.

Introduction

Magnaporthe grisea (anamorf *Pyricularia grisea* Sacc. synonym *Pyricularia oryzae* Cav.) causes rice blast disease in rice cultivation areas worldwide (BPS, 2010; Chin, 1975; Kato, 2001). The disease causes yield losses from between 1-100% in Japan (Kato, 2001), 70% in China, 21-37% in Bali Indonesia (Suprapta and Khalimi, 2012), and 30-50% in South America and Southeast Asia (Baker et al., 1997; Scardaci et al., 1997). Disease severity has increased recently due to the use of intensive agronomic practices that favor disease development. Blast disease severity is triggered by excess of N fertilization (Faria et al., 1982; Correa-Victoria et al., 2004) as well as rainfall and high humidity. Cultural practice, cultivating resistant varieties and the use of synthethic fungicides are the three strategies used to control rice blast (BPTP, 2009; Ghazanfar et al., 2009; IRRI, 2010). Although the use of resistant cultivars is known to be the most effective control strategy, *Pyricularia oryzae* develops new races rapidly which results in the breakdown of rice resistance. Thus the use of resistant cultivar is limited to a certain place and time (BPTP, 2009). Based on this reason, the use of resistant cultivar should be combined with other control strategies that are environmentaly friendly and effective. The control of this disease through fungicide application however has adverse effects on the environment and negatively affects the soil microbiota. These chemicals either induce the generation of mutant varieties or reduce or suppress other microbial populations. Studies have shown that various types of microorganisms are a potential substitute for inorganic chemical compounds (fertilizer and pesticides) that can be applied in the field in a wide scale. A number of microbes have been reported to be effective as biological control agents of plant diseases i.e. Bacillus, Bdellovibrio, Dactylella, Gliocladium, Penicillium, Pseudomonas, and Trichoderma (Fravel, 1988). Soil microorganisms associated with the rhizospheres of plants have been known to contribute in many processes in the soil which in turn may influence the plants growth and progression (Tilak et al., 2005; Shimoi et al., 2010). Unfortunately, most microorganisms used as biocontrol 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 biocontrol agents is largely due to the utilization of single bio-control agents to suppress single plant-pathogen under any environmental condition. Single biocontrol agents are not likely to be active in all soil environments in which they are applied or against all pathogens that attack the host plant (Altomare et al., 1999; Hafedh et al., 2005; Hafedh et al., 2006; Harman and Kubicek 1998; Kumar 1995; Lewis and Papavizas 1987; Tamimi and Hadwan 1985; Ziedan 1998; Ziedan and Elewa

2000). Trichoderma has a wide spectrum of biotypes that ranges from effective soil colonizers to non-plant symbionts (Cardona and Rodriguez 2006; Meraj-ul et al., 2012) that live in the rhizosphere and are able to successfully colonize the plant epidermis. They have been used as biocontrol agents against plant pathogens (Abeysingne 2007; Andrei et al., 2012; Harman et al., 2004; Sundaramoorthy et al., 2012) and are believed to antagonize plant pathogens through competition for substrate, antibiosis, and parasitism (Belen Suarez et al., 2005).

Several investigators have pointed out that *Trichoderma* and *B. subtilis* species are appealing candidates for control of blast disease in rice. A lot of studies have described using *B. subtilis* individually or in combination with other microorganisms like *Trichoderma* spp. or chemical fungicide to control sheath blight and rice blast in rice plants (Abeysingne 2007; Andrei et al., 2012; Lixuan et al., 2008; Yang et al., 2009). *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 biocontrol agents, continuous screening of new isolates is needed for effective formulation of biocontrol agents against specific pathogens. However obtaining the best concoction that consists of different antagonistic organisms that work under different field condition is required before any bioagent can be developed commercially (Baha 2002; Harman 2000; Ou 1985). Here we present the isolation and characterization of the antagonistic properties of *Trichoderma* isolates derived from soil. These isolates were then scored for pre-post damping off, disease incidence and severity individually or in dual inoculation with *B. substilis* UKM1 in greenhouse experiments using rice and our local Malaysian field isolate of *M. grisea*. The effect of these isolates in controlled and field like conditions was compared and data derived was statistically analyzed. In addition, the reaction between *Trichoderma* T2 and *M. grisea* in dual culture assays was observed under SEM to shed some light on the mode of antagonism between these cultures.

Results

In vitro study of biocontrol agents and their effect on *M. grisea*

The twenty-two isolates had differences in efficiency of suppressing radial growth of *M. grisea* four days post-inoculation. Table 1 shows the antagonistic activity of all twenty-two isolates. The best antagonistic results were obtained with *Trichoderma* isolates T2, T3, T4, T6, T7, T9, T11, T18 and T22. These isolates scored highest (+++) in suppression of the pathogen. This shows that the above *Trichoderma* isolates prevented the growth of *M. grisea* by causing 100% coverage/over growth of the 9 cm plates as seen in Fig. 1. *Trichoderma* isolates that scored (+++) covered the whole plate within 4 days. Some of these isolates (T1, T5, T8, T13, T14, T16, T20 and T21) caused moderate inhibition (++) while others (T10, T12 and T19) showed low levels of inhibition (+). Isolate T15 showed no influence towards the suppression of radial growth of the pathogen. Fig. 2A represents the interaction between *M. grisea* and *B. subtilis* UKM1, a bacterial biocontrol agent that was isolated from a previous studies. The bacterial culture within the wells inhibited the progression of the fungus. Fig. 2B shows the entire 9 cm petri dish is covered completely by *M.grisea* 7 days post subculturing. The inhibition zone observed in Fig. 2A was calculated roughly to be 16 % in comparison with control plates. Inhibition zones were calculated 7 days post incubation according to the formula by Mojica-Marin et al. (2008). No growth of fungus was observed beyond the inoculated wells 14 days post experiment.

Biological control agents and their effect on M. grisea in greenhouse study

Although isolates with the highest antagonistic activity have been identified via the dual culture method, all 22 *Trichoderma* isolates were used in the greenhouse experiment as it has been reported previously that the dual culture data may not be replicable in greenhouse or field experiments. The experiments were designed to test: (1) The effect of *Trichoderma* isolates on the growth rate of rice plants (2) The inhibitory effect of *Trichoderma* isolates and or *Bacillus subtilis* UKM1 on pathogen when used individually or in dual inoculations, and (3) The ability of the best isolates to function in field like conditions.

The first set of experiments tested the efficiency of the *Trichoderma* isolates in enhancing growth and seedling germination in rice. The experiment conducted under greenhouse conditions found that *Trichoderma* isolates showed different levels of growth enhancement and increased Pre and Post seedlings emergence in the MR219 rice variety ($p \leq 0.05$) (Fig. 3 A-D). The data presented indicates that the isolates induced seedling progression (Fig. 3A). There have been previous reports that showed *Trichoderma* isolates were able to induce growth of plants in various environments and conditions (Alfredo and Aleli, 2011; Bell et al., 1982; Harman, 2000; Schuster and Schmoll, 2010). Certain isolates (T2, T5, T7, T11 and T21) showed enhanced growth 60 days post transplanting by approximately 4.3 to 8.7 % (Fig. 3A) when. treated with *Trichoderma* spp. only. Therefore *Trichoderma* isolates do have a positive impact in improving growth. This observation will require further validation through growth and yield related experiments in both greenhouse and field conditions. In the presence of *M. grisea*, these isolates were efficient in reducing progression of disease (Fig. 3B and D).

The greenhouse experiment showed that *Trichoderma* isolates T2, T3, T7, T8, T11 and T21 were effective in inducing significant pre emergence damping off in seedlings in the presence of *M. grisea* ($p \leq 0.05$) (28.6 to 50.3 %) (Fig. 3B). The post emergence damping off showed that *Trichoderma* isolates T5 and T21, resulted in significant (12.3 %; Fig. 3D**).** Although isolates T8 and T21 exhibited moderate degree of antagonistic activity in dual culture experiments (Table 1), these isolates provided better antagonistic values against *M. grisea*, which indicates a possibility of effective mycoparasitism in soil against pathogen (Fig. 4B and D) (Alfredo and Aleli, 2011; Bell et al., 1982; Harman, 2000; Schuster and Schmoll, 2010). Certain isolates (T2, T7, T9, T11 and T21) showed identical results in the dual culture technique and the greenhouse experimets. *Trichoderma* isolate T2 significantly reduced the disease incidence parameter ($p \leq 0.05$) (33.3 %) compared to treatment with pathogen only (100 %) (Fig. 4B). Majority of the isolates showed significant reduction (18% to 57.7%) in disease severity in comparison to control (88.7 %) (Fig. 4D). Inoculation of *Trichoderma* isolates and *B. substilis* UKM1 showed that isolates T2, T3, T4, T5, T7, T8, T9 and T11 reduced pre emergence damping off (4.3 % for all of them) while control untreated with *Trichoderma* isolates only

scored 29.3 % (Fig. 5A). Post emergence of disease in seedlings was recorded as 4.3 to 12 % for the above isolates

Table 1. Antagonistic activity between *Trichoderma* spp. and *Magnaporthe grisea* under laboratory condition.

Treatments	**Degree of antagonism after 4 days
T2+ *Magnaporthe grisea*	+++
T3+ *Magnaporthe grisea*	+++
T4+ *Magnaporthe grisea*	+++
T6+ *Magnaporthe grisea*	+++
T7+ *Magnaporthe grisea*	+++
T9+ *Magnaporthe grisea*	+++
T11+ *Magnaporthe grisea*	+++
T18+ *Magnaporthe grisea*	+++
T22+ *Magnaporthe grisea*	+++
T8+ *Magnaporthe grisea*	++
T20+ *Magnaporthe grisea*	++
T14+ *Magnaporthe grisea*	++
T17+ *Magnaporthe grisea*	++
T5+ *Magnaporthe grisea*	++
T13+ *Magnaporthe grisea*	++
T21+ *Magnaporthe grisea*	++
T16+ *Magnaporthe grisea*	++
T1+ *Magnaporthe grisea*	++
T12+ *Magnaporthe grisea*	+
T19+ *Magnaporthe grisea*	+
T10+ *Magnaporthe grisea*	+
T15+ *Magnaporthe grisea*	-

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

**According to scale by Alfredo and Aleli (2011) that involve four degrees:

(+++) The antagonistic fungus was able to grow over the pathogen and pathogen growth completely inhibited. (++) The pathogen growth completely inhibited, but antagonist was not able to grow over the pathogen. (+) Mutual inhibition initially, but antagonist was overgrown by pathogen. Pathogen growth not inhibited, antagonist was overgrown by pathogen.

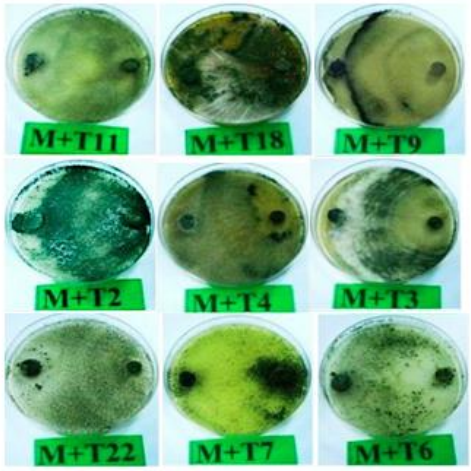

Fig 1. Effect of different treatments on growth of pathogen.

as compared to control untreated with *Trichoderma* only (Fig. 5C and D). In observing the disease infected plants, isolates T2 and T7 showed the most significant reduction in disease incidence i.e. 12 % for both in control (Fig. 6A) and 33 and 33.3 % respectively for both when *M.grisea* is present (Fig. 6B). Fig. 6 shows that the percentage of disease severity was also significantly reduced ($p \leq 0.05$) for isolates T2 and T7 where the values for both were 4.3 % in control (Fig. 6C) and 8.7 % (Fig. 6D) in pathogen infested soil.

From the experiments conducted and presented above it was observed that the inhibition of *M.grisea* was higher in dual inoculation. Therefore we selected the top six *Trichoderma* spp (T2, T7, T8, T9, T11 and T21) that worked effectively with *B. substilis* UKM1 to inhibit *M. grisea* in control conditions for use in (non-autoclaved field soil). Figure 7 shows that the antagonistic effects of the six *Trichoderma* isolates were higher when non autoclaved soil was employed where isolates T2 and T11 had the most significant differences ($p \leq 0.05$) in pre-post emergence parameters (4.3 % for all readings) (Fig. 7 A and B).

The disease incidence and severity data showed high reduction in *M. grisea* infections (Fig. 7C and D). *Trichoderma* T2 was the most effective antagonist in reducing disease incidence and severity parameters (22 and 4.5 % respectively) (Fig. 7 C and D) compared to infections in natural treatment (data not shown). Figure 8 shows MR219

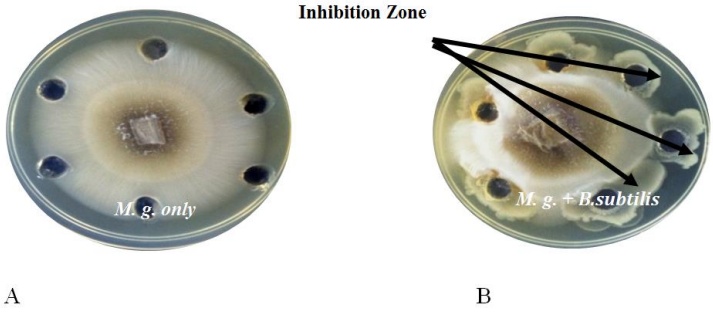

Fig 2. Antagonistic interaction between *Bacillus subtilis* UKM1 and *M. grisea*. (A) *M. grisea* only as control, the mycelium covers the 9 cm plate over a 4-day period. (B) *B. subtilis* UKM1 and *M. grisea*, the arrows refer to the inhibition zones by *B. subtilis* UKM1. The inhibition zone was scored 7 days from culture.

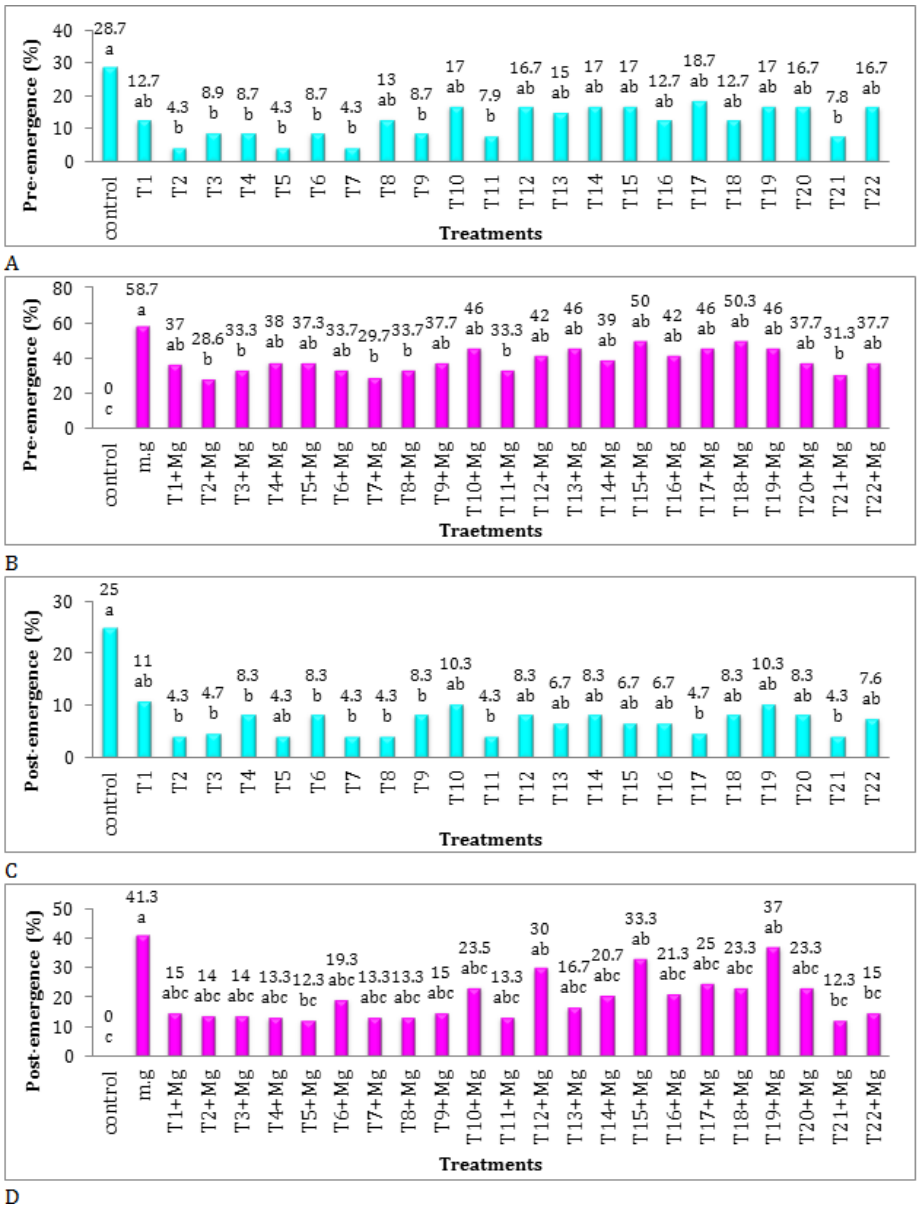

Fig 3. Comparison between pre (A & B) and post emergence (C & D) damping off in rice seedlings treated with 22 *Trichoderma* isolates, and *Trichoderma* spp. in combination with *Magnaporthe grisea* under greenhouse condition. Numbers in each column that have same alphabet do not differ significantly from each other at $p \leq 0.05$ according to Duncan's multiple range tests. Pre and post-emergence test were conducted in triplicate, according to Ziedan (1998).

rice plants treated with the top six *Trichoderma* spp in combination with *B. substilis* UKM1. The plants exhibit good growth with rich green foliage (Fig. 8A and B). Figure 8C shows the MR219 plantlet artificially inoculated with *M. grisea* in the presence of the dual inoculum of biocontrol agents. The plants survived to seed setting stage with minimal disease symptoms. This can be seen clearly by comparing with the untreated plants which showed infected sheaths, stunted growth and no or lack of seed setting at the same age (Fig. 8D and E).

Observation of reaction between Trichoderma T2 and M. grisea under SEM

A previous study conducted between *T. harzianum* and *R. solani* reported that mycoparasitic mechanism in early stages could not be observed because the interaction starts soon after penetration of hyphal cell walls by *Trichoderma* spp. Therefore time factor is crucial in observing the process under a microscope. The mechanism of mycoparasitism is not very clear between *Trichoderma* T2 and hyphae of *M. grisea* (Elad et al., 1983). Figure 9B shows the interaction between the mycelium of *Trichoderma* isolate T2 and *M. grisea* using the scanning electron microscope (SEM). The electron-micrographs show the hyphal coiling of *M. grisea* by *Trichoderma* T2. Hyphal coiling has been reported by several researchers as the mode of action of *Trichoderma* on pathogenic fungi (Van Eck, 1978; Mondal and Hyakumachi, 1998). Chitinolytic assays conducted on isolate T2 showed high endochitinase activity and this enzyme is reported as a CWDE that can break down cell walls of organisms (Kalaivani et al., 2014). We believe the pathogen may have been degraded by this enzyme as we were not able to reisolate *M. grisea* from the dual culture plates (Fig. 9).

Discussion

In recent years the use of bioformulations in crop protection has gained great interest as a safe and effective solution to the chemical alternative (Suryadi et al., 2013). This study has looked into the efficiency of single and dual bioinoculums in controlling rice blast disease at the greenhouse level.

Antagonistic effect of Trichoderma isolate T1 to T22 in dual culture plates

Through the dual culture technique we observed that all twenty two isolates had varying levels of inhibition on pathogen growth. The rate of inhibition was calculated as percentage of overgrowth of *Trichoderma* in petri dish. *Trichoderma* isolates T2, T7, T8, T9, T11 and T21 showed the highest degree of inhibition (+++). As observed in the dual culture assays, the antagonist out grew the pathogen in the petri dish within four days (Fig. 1). Fuji et al. (1978) and Vinale et al. (2008) reported that *Trichoderma* spp. produced secondary metabolites such as antibiotics (6-pentyl-alpha-pyrone (6pp), isocyanide derivatives), acids (heptelidic and koningic acid), peptaibols and cell wall degrading enzymes (CDWE) that are implicated in inhibits of radial growth of many phytopathogenic fungi (Verma et al., 2007)**.**

Antagonistic effect of Bacillus substilis UKM1 against M. grisea

Bacillus is a genus of Gram positive bacteria that produces endospores and is a potential biological control agent due to its resistance to heat and drought conditions (Wayne et al., 2000). *B. substilis* UKM1 while presenting some inhibitory effect (16%) on the growth of the phytopathogen (Fig. 2), it was not as efficient as the *Trichoderma* isolates (T2, T7,T8, T9, T11 and T21 – produced 80-100% inhibition) in reducing radial growth on its own. Therefore *B. substilis* UKM1 is not an effective biocontrol agent against *M. grisea*. These values are low compared to other *B. substilis* biocontrol isolates such as *B. subtilis* NSRS 89-24 which resulted in approximately 60 % inhibition of *P. grisea* in dual culture test (Harman, 2000; Leelasuphakul et al., 2006). It was also reported that *B. subtilis* produced > 50% inhibition of radial growth of pathogens where this inhibition was experimentally attributed to the release of several antifungal metabolites such as subtilin, bacitracin, bacillin and bacillomycin (Moubarak and Abdel-Monaim 2011; Soleimani et al., 2005; Zaghloul et al., 2007).

Hyphal interaction between Trichoderma T2 and M. grisea

Trichoderma T2 was grown on a dual culture plates and harvested for SEM analysis when both cultures were in contact. Macroscopic observation of the fungal growth in dual cultures revealed that the pathogens growth was inhibited soon after contact with the antagonist. As observed by the SEM analysis, *Trichoderma* was found growing and coiling around the hyphal structure of *M. grisea*. Based on microscopic, dual culture assays and biochemical analysis (chitinolytic activity) (Kalaivani et al., 2014) we believe that the mechanism by which *Trichoderma* antagonizes the pathogen is by mycoparasitism which involves the production of enzymes and secondary metabolites. Specific compounds such as chitinolytic enzymes are able to degrade the pathogens cell walls and inhibit the production and release of active compounds by the pathogens. This was further substanciated by the inabiliy to reisolate the pathogen from the dual culture plates 4-days post incubation.

Greenhouse experiments for single and dual biocontrol applications

From the greenhouse analysis conducted using the 22 *Trichoderma* isolates, six isolates showed improved pre and post emergengence in rice when used alone and in combination with the pathogen (Fig 3). This therefore indicates that *Trichoderma* is a good candidate at increasing growth though this would require further study growth, yield and developmental studies. Similarly when scored for disease incidence and disease severity the same isolates showed the most promise in reducing loss of plants due to disease (Fig. 4). Figures 5 and 6 showed the effect of dual inoculation on pre-post emergence, disease incidence and disease severity. The reduction in all four parameters scored in dual incoculation indicates that it is a more efficent way to control *M. grisea*. The antagonistic effect of the *22 Trichoderma* isolates against *M. grisea*, individually or in combination with *B. subtilis* UKM1 were more evident in uncontrolled soil conditions. The reason for this could be that *Trichoderma* is a saprophytic fungus, and the non-autoclaved soil contained a rich source of several microorganisms from which it may obtain food and other chemical exudates to increase its growth, sustainance and effectiveness. Therefore the results show that the six *Trichoderma* isolates can be used under field condition successfully when a suitable formulation has been obtained for field use against pathogens. *Trichoderma* and *Bacillus* effect plant pathogens

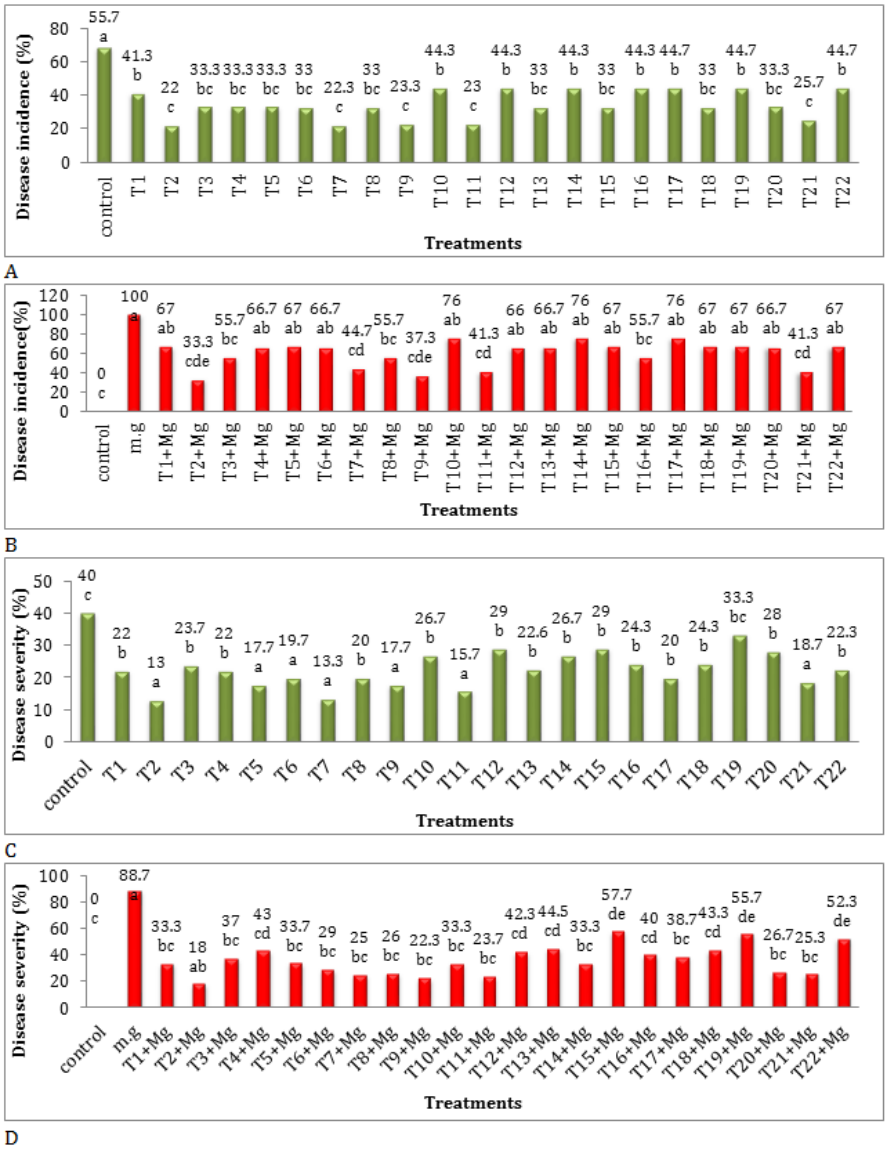

Fig 4. Comparison of disease infected (A & B) and disease severity (C & D) in plants treated with *Trichoderma* only and plants treated with *Trichoderma* and *Magnaporthe grisea*. Numbers in each column that have same letter do not differ significantly from each other at p ≤0.05 according to Duncan's multiple range test. Experiment was conducted in triplicate. Percentage of disease infected was scored after 60 days from sowing according to Woltz and Arthur (1973).

by attacking and linking the causal agents through sugar linkages and release of extracellular enzymes such as protease, cell wall degrading enzymes (CDWEs) and lipase. They also produce and secrete siderophores and hydrogen cyanide that are toxic to microorganisms (Wang et al., 2009). The combination of biological active compounds produced by both organisms collectively may be the contributing factor to the higher level of affectiveness of dual inoculum compared to single.

Materials and Methods

Plant material

MR219, an *Oryza sativa* L. indica variety, that is widely planted in paddy fields in Malaysia, was used in this study. The rice seeds were placed on filter paper in petri dishes and incubated at 40 ^{0}C for 48 hours to break their dormancy. The filter paper was then moistened and the seeds were left for 3 days at 28 ^{0}C to germinate. The germinated seeds were transferred to pots containing sterilized soil and allowed to grow for two weeks.

Plant inoculation

The *M. grisea* isolate was cultured on Potato Dextrose Agar (PDA) and incubated at 28 ^{0}C for 5 days. The plantlets were then inoculated with 5 mm fungal plugs of *M. grisea* culture. The plugs were placed at the branch of the stem and wrapped with cotton moistened with distilled water and 0.5% gelatin. Humidity and moisture was maintained through the use of aluminum foil. The disease symptoms appeared 72 hours post inoculation (hpi), at which point the aluminum foil was removed and the leaves were harvested (Park et al., 2008; Plodpai et al., 2013).

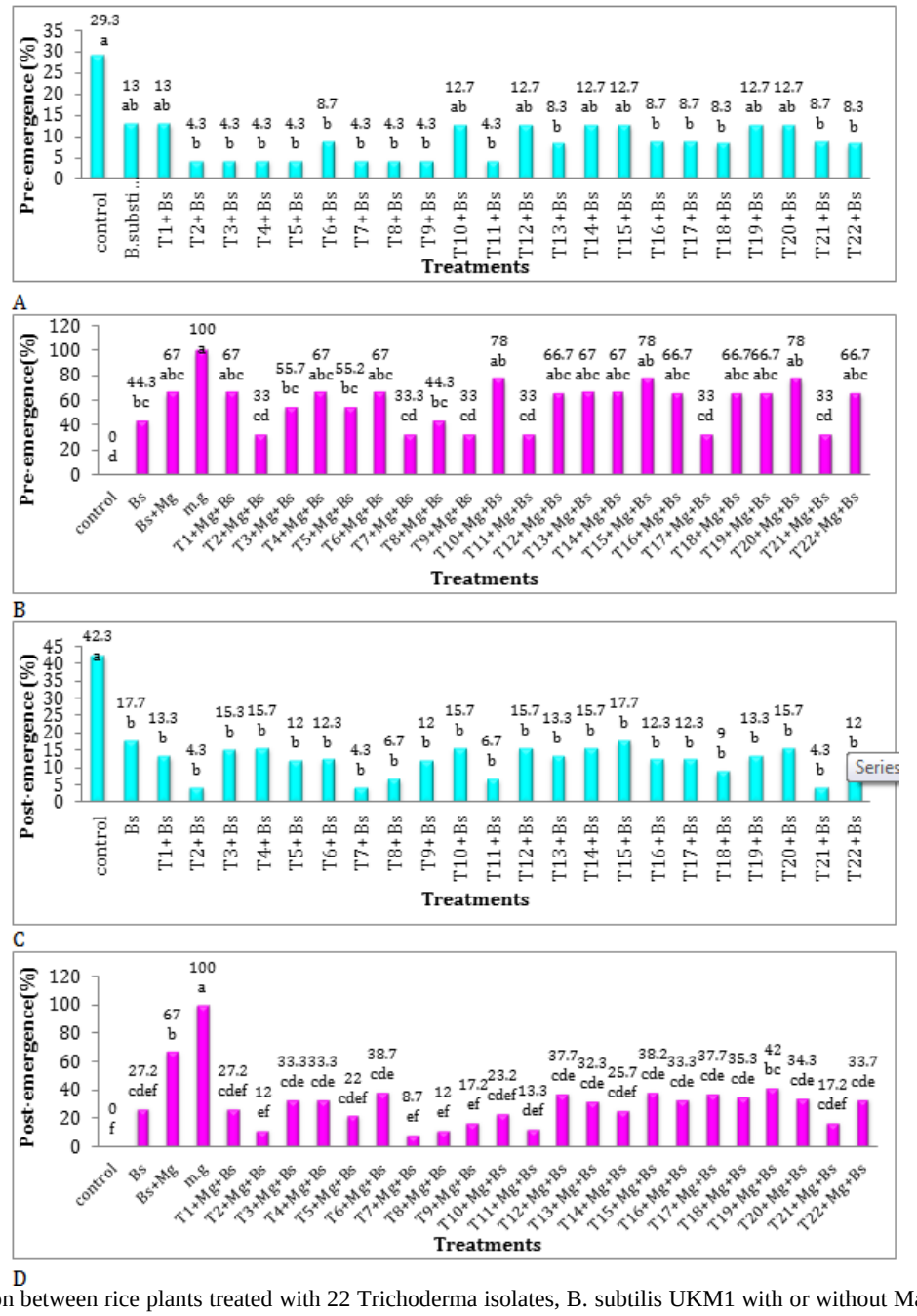

Fig 5. Comparison between rice plants treated with 22 Trichoderma isolates, B. subtilis UKM1 with or without Magnaporthe grisea under greenhouse condition. Pre (A & B) and post emergence (C & D) damping off in seedlings. Numbers in each column that have same letters do not differ significantly from each other at p ≤0.05 according to Duncan's multiple range tests. Pre and post-emergence experiments were conducted in triplicate for each isolate according to Ziedan (1998).

Isolation of Trichoderma spp.

A serial dilution was conducted on soil sample obtained from the National Forest Reserve, Malaysia (Hamdia and Kalaivani, 2013). Fungal cultures obtained were maintained on PDA. Pure cultures of fungal isolates was examined macro and microscopically to determine isolates that were *Trichoderma* spp. Macro and microscopic examination of cultures showed that there were 22 different isolates of *Trichoderma* obtained from the soil sample and these were designated isolate Trichoderma T1 to T22.

Antagonistic activity between Trichoderma isolates and M. grisea

Antagonistics studies were conducted using dual culture technique. Each PDA plate was divided equally into two portions where in one portion a 5 mm *M. grisea* fungal plug was placed while the second half was inoculated with 5mm fungal plugs of any one of the T1 to T22 isolates (Hamdia and Kalaivani, 2013). The plates were incubated at 28^0C for 4 days and the antagonistic activity was scored according to the scale developed by Alfredo and Aleli, (2011).

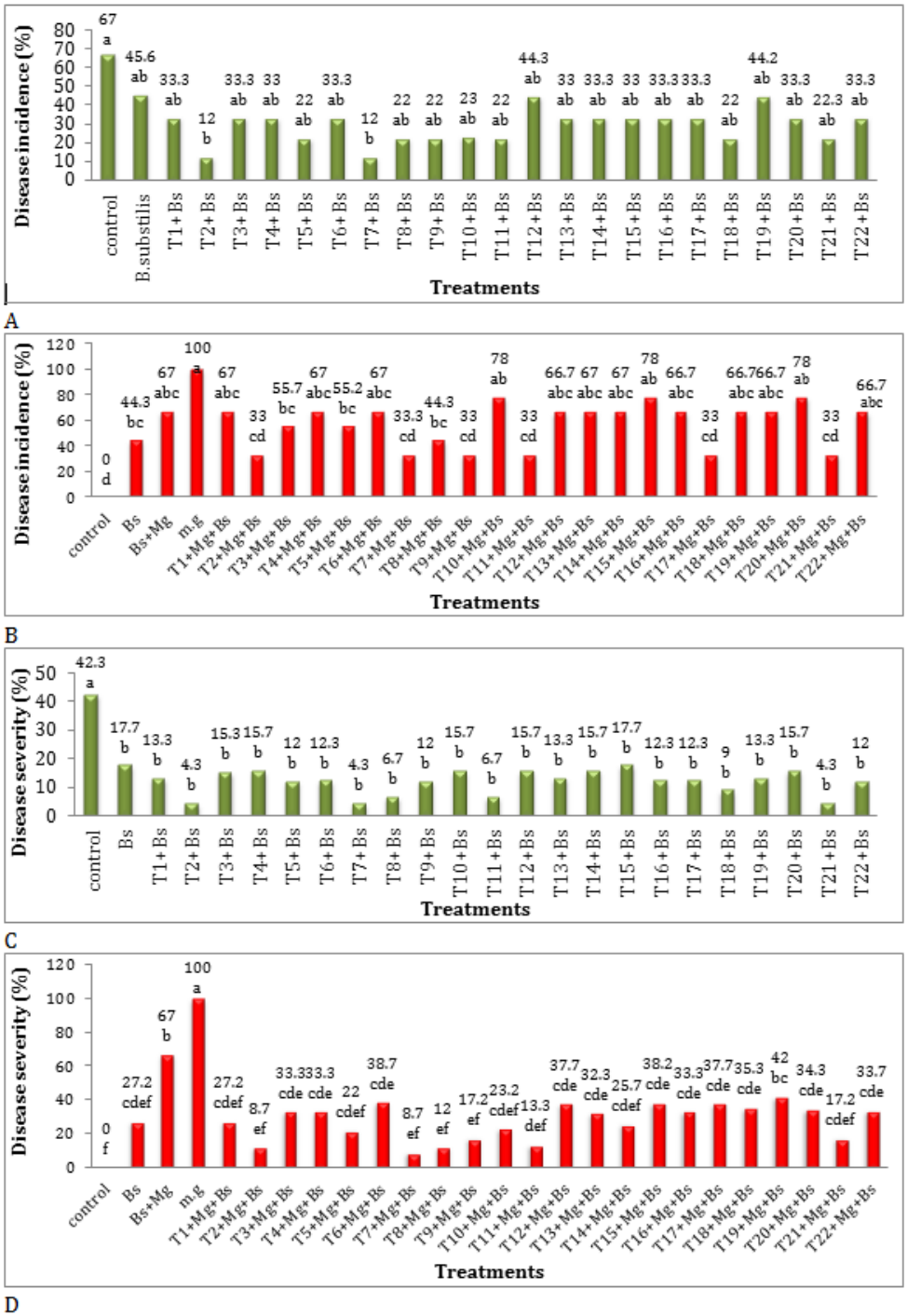

Fig 6. Comparison between rice plants treated with 22 *Trichoderma* isolates and *B. subtilis* UKM1 in combination with or without *Magnaporthe grisea* under greenhouse condition. (A & B) Disease infected and(C & D) disease severity. Numbers in each column that have same letters do not differ significantly from each other at p ≤0.05 according to Duncan's multiple range tests. Three replicates for each isolate. Percentage of disease infected was scored after 60 days from sowing according to Woltz and Arthur (1973).

Preparation of Trichoderma isolate for observation under scanning electron microscope (SEM)

Fresh cultures from interaction area between *Trichoderma* T2 and *M. grisea* was used for observation under the scanning electron microscope. Small specimens were cut by hand with a sharp razor and sliced to 1cm^2 sections. The samples were put into separate vials and fixed with 4% glutaraldehyde for 12-24 hours at 4 ^{0}C. The samples were washed three times with phosphate buffer (PBS) and then dehydrated in an alcohol series of 50%, 70%, 80%, 85%, 90%, 95%, and three changes of 100% alcohol followed by three changes of 100% acetone for 30 min each. The specimen was than stuck to a stub coated with gold or colloidal silver via a sputter coater. The coated specimens were viewed under the scanning electron microscope (SEM - XL 30, Philips) operated at 10 to 15 Kv at various magnifications to obtain the best images.

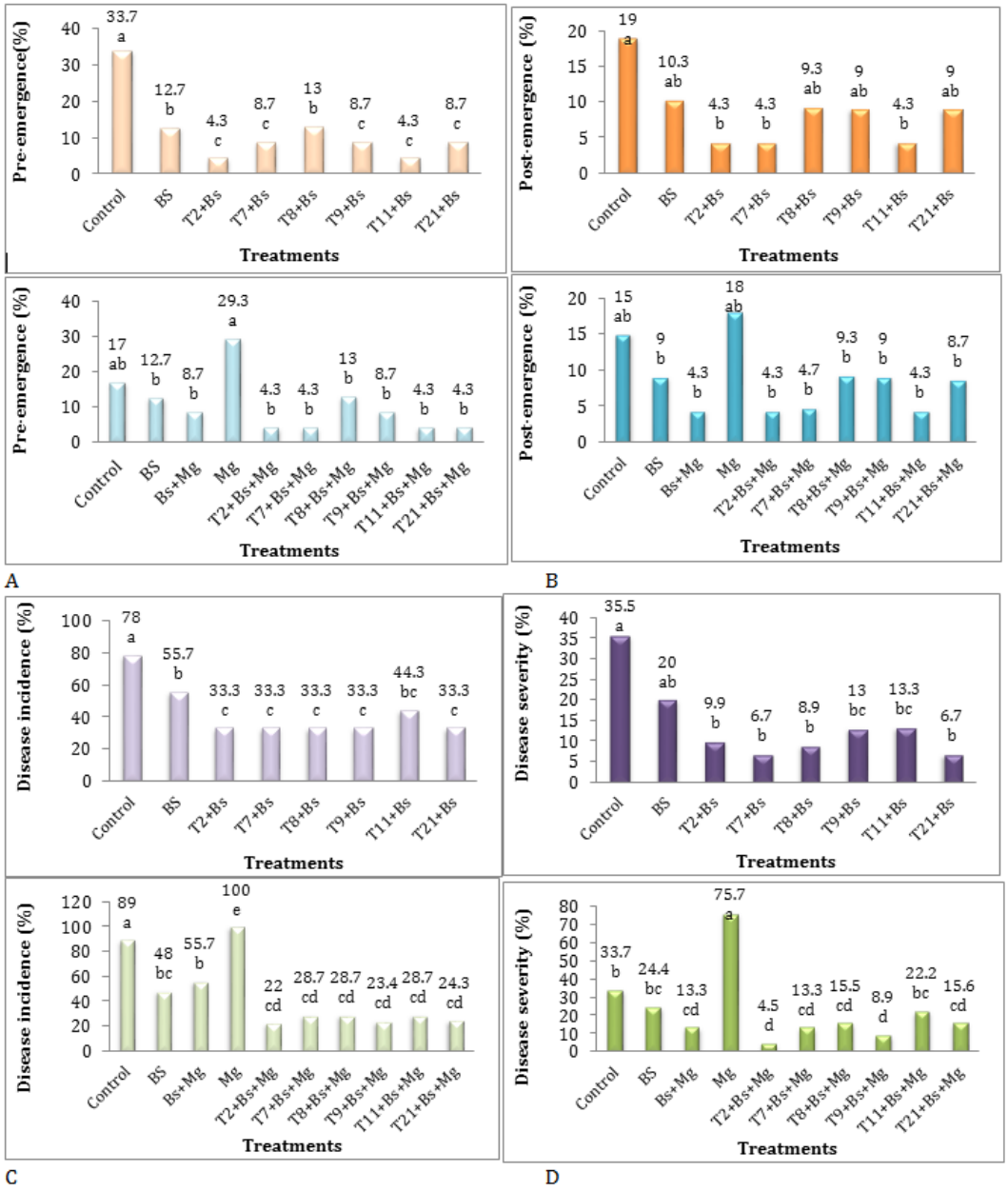

Fig 7. Comparison between rice plants treated with six *Trichoderma* isolates with or without *Magnaporthe grisea* under greenhouse condition (A) pre and (B) post emergence damping off, (C) disease infected and (D) disease severity. Numbers in each column that have same letter do not differ significantly from each other at p ≤0.05 according to Duncan's multiple range test. 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).

Magnifications of 500X to 10,000X were used for spores and mycelium morphology studies.

Greenhouse experiments

As for the greenhouse experiment, the following experimental design was establihed. Experiments was conducted as follows: Control without microbes, *M. grisea* only, *B. subtilis* UKM1 only, 22 *Trichoderma* isolates only (tested individually), *B. subtilis* UKM1 + 22 *Trichoderma* isolates (tested individually), *M.grisea* + 22 *Trichoderma* isolates (tested individually), *M. grisea* + *B. subtilis* UKM1 + 22 *Trichoderma* isolates (tested individually). The concentration of *Trichoderma* isolates and *M. grisea* was determined via Haemocytometer (Duncan, 1995; Goswani and Kistler, 2004). The *M. grisea* inoculum was set at $1x10^7$ spore/mL while Trichoderma spp inoculum was set at $1x10^8$ spore /mL. *B. subtilis* UKM1 inoculum size was set at $2x10^8$ cell /mL (Harman and Kubicek, 1998). The soil was moistened and mixed thoroughly every other day for 1 week. Seedlings were then sown into the infested pots (8 seedlings/pot). Seedlings were visually examined for any

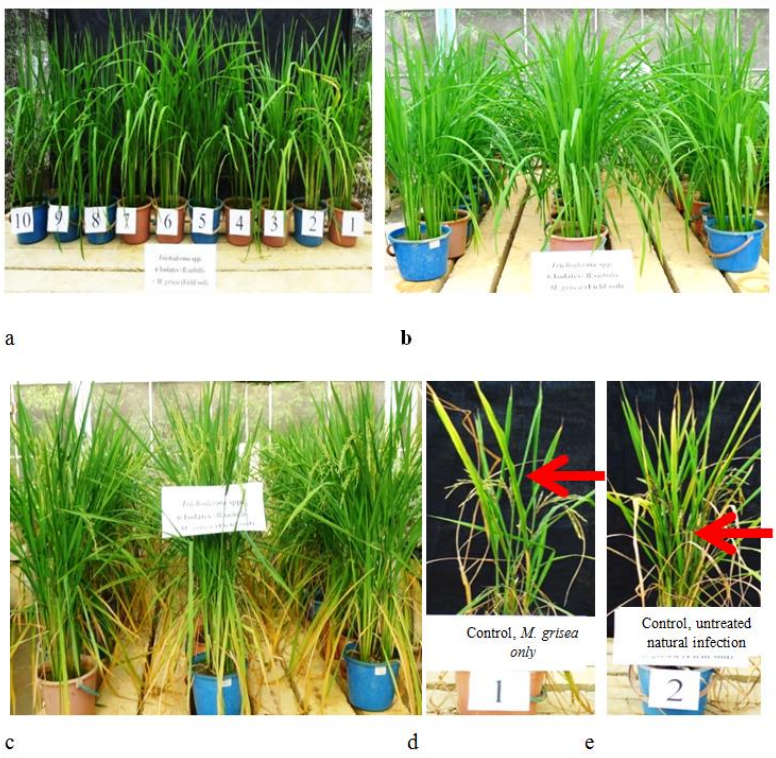

Fig 8. Effect of dual inoculation against *M. grisea*. (a) Plants inoculated individually with six *Trichoderma* spp. + *B. subtilis* UKM1 + *M. grisea*. (1) *M. grisea* only, and showing initial symptoms (2) Control natural infection (3) *Bacillus subtilis* UKM1 only (4) *B. substilis* UKM1 and *Trichoderma* T2 (5) Isolate T2 (6) Isolate T7 (7) Isolate T8 (8) Isolate T9 (9) Isolate T11, and (10) Isolate T21. (A) 6 *Trichoderma* isolates + *M. grisea*. (B) 6 *Trichoderma* isolates + *B. substilis* UKM1 inoculation pre *M. grisea* inoculation (C) 6 *Trichoderma* isolates + *B. subtilis* UKM1 + *M. grisea* at seed setting stage in the end of growth (D) Control, *M. grisea* only. (E) Control, untreated natural infection (field soil). The arrows in (D & E) refer to diseased tissue.

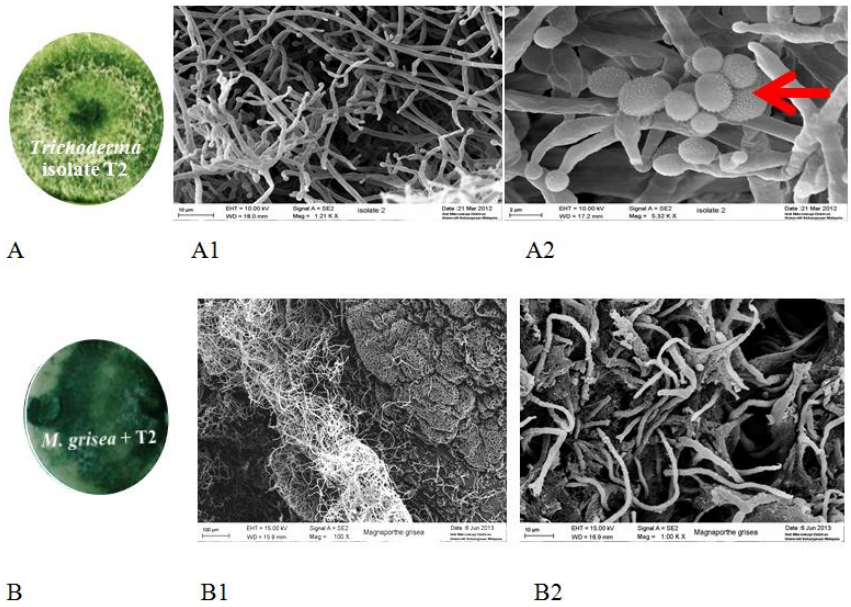

Fig 9. (A) *Trichoderma* isolate T2 on PDA plate (A1) Branches, hyphae, chlamydospores and conidia in more than one point (polyplastic). (A2) The arrow refers to the ornamented walls (verricose) of spores that accumulate in balls, and sporophore that carry many flask-shaped monophialidic. (B) Dual culture plate showing interaction between *Trichoderma* T2 and *M. grisea*. T2 covers the growth of *M. grisea* > 90 %, right side of the plate has *Trichoderma*, and left for *M. grisea*. (B1 & B2) SEM of tip region of *Trichoderma* isolate T2 and *M. grisea*, branches of T2 completely associated and coiling around hyphae of *M. grisea*.

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:

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

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

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

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

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

Disease severity was assessed 60 days from planting in greenhouse 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 leaves, 4=whole plant wilted, 5= plant is dead. The percentage data obtained from observations and calculated using the formulae above were statistically evaluated using a randomized complete block design via the Analysis of Variance (Three Way ANOVA) .Three replications were used for each parameter calculated. The ANOVA analysis was conducted via the Statistical Analysis System (SAS) versi 8.0.

Conclusion

This study shows that the *Trichoderma* isolates obtained from our screening were effective in inhibiting *M. grisea*. Isolate T2 was the most effective and produced higher levels of inhibition of disease incidence and severity when used as dual inoculum with *B. substilis* UKM1. Overall the best results were with T2 and *B. substilis* UKM1 in uncontrolled environment.

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