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The Efficacy of *Trichoderma harzianum* T73s as a Biocontrol Agent of Fusarium Ear Rot Disease of Maize

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Abstract

Fusarium ear rot (FER) disease in maize reduces grain quality and yield to an appreciable extent. Based on virulence assay, *F. proliferatum* B202c was the most pathogenic isolate among other species including *F. verticillioides*. This pathogen was challenged in dual culture assays with 72 isolates of *Trichoderma* sp., which were isolated from soil samples. *T. harzianum* T73s showed highest percentage inhibition of 73.10% was further tested for its efficacy to suppress FER under glasshouse conditions. The application of T73s every week, immediately after planting reduced the severity of FER with DSI 0.5% compared with control, 4.75%. Thus, *T. harzianum* T37s can be used as good biocontrol agent and has potential for further tests in the field and on commercial scale. © 2013 Friends Science Publishers

Keywords: *Fusarium proliferatum*; *F. verticillioides*; *Trichoderma harzianum*; Maize; Biological control; Fusarium ear rot

Introduction

Maize (*Zea mays* L.) is monocotyledonous plant that belongs to the grass family (*Poaceae*). Each of maize plant has both female and male parts thus it can be self-pollinated. The male part is tassel, which emerges from the top of plant, while the ear is female part, grows out from a leaf node (Sheridan, 1988). Maize cultivated all over the world is ranked third in the economically important cereals after rice and wheat. It is susceptible to various fungal pathogens and *Fusarium* sp. is one of the most important pathogens. There are several diseases on maize infected by *Fusarium* sp. such as Fusarium ear rot (FER; *F. verticillioides* and *F. proliferatum*), Gibberella ear rot (*F. graminearum*), Fusarium stalk rot (*F. verticillioides*), Gibberella stalk rot (*F. graminearum*) and Fusarium root rot (*F. graminearum* and *F. verticillioides*) and seedling blight (*F. graminearum* and *F. verticillioides*) (Munkvold and O'Mara, 2002; Rodriguez-Brljevich *et al*., 2010; Reyes-Velázquez *et al*., 2011).

FER is one of the destructive diseases in maize, caused by several species of *Fusarium* such as *F. graminearum, F. proliferatum, F. subglutinans* and *F. verticillioides* (Logrieco *et al*., 2002). The general observable symptoms of FER are production of fungal growth in tan or brown color at the tip of ear (Munkvold, 2003). The distributions of FER pathogens are related to environmental conditions such as temperature, humidity, light intensity and wind (Bottalico, 1998). Most of the pathogens are capable to produce mycotoxins such as beauvericin, fumonisin, fusaproliferin and moniliformin (Logrieco *et al*., 2002). These toxins affected 25% of the world food crops, pose potential threat to humans and animals (Weidenbörner, 2001).

For controlling the disease, farmers usually applied synthetic fungicides to the plants. However, these treatments were ineffective because it led to the appearance of environmental contaminations (Jacobsen and Backman, 1993). It also can increase the development of fungicide-resistant pathogens (Moënne-Loccoz *et al*., 1998). Hence, biological control using microbial agents is an alternative way to control plant pathogens because it is eco-friendly and cost-effective. Nowadays, many microbes have been used as biocontrol agent of plant disease including *Trichoderma* sp.

Fungi in the genus *Trichoderma* are promising biocontrol agents against wide range of plant pathogens and can easily be isolated from soils, decaying woods and other forms of plant organic matters (Howell, 2003). They act as biocontrol agents against plant pathogens directly by mycoparasitism production and indirectly by nutrient and space competition, environmental conditions or plant growth modification and defensive promotion (Benítez *et al*., 2004). There are several commercial products of *Trichoderma* sp., which are Bio-Fungus, Trichodex, Binab-T, Root Pro, RootShield, SoilGard, Supresitiv, Trichoject, TUSAL, Trichoderma 2000 and Trieco (Monte, 2001). However, none of them effectively can be used for controlling the FER pathogens.

The use of *Trichoderma* sp. as a biocontrol agent against *Fusarium* sp. showed a high efficacy when tested under *in vitro* and *in vivo* conditions (Mousseaux *et al*., 1998; Dal Bello *et al*., 2002; Thangavelu *et al*., 2004).

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The previous study conducted by Thangavelu *et al*. (2004) found that *T. harzianum* Th-10 successfully inhibited the mycelial growth of *F. oxysporum*, the pathogen of Fusarium wilt under *in vitro* condition. Moreover, the application dried banana leaf added with jaggery (10% w/v) increased the mass production of Th-10 and effectively controlled Fusarium wilt disease at field trials.

Since FER has given the major problems in plantation worldwide and the used of fungicides caused negative effect to environment and animal, therefore serious attention must be paid to find an alternative way to control this disease. Moreover, to date, the biocontrol agent of FER was not well documented. Therefore, the purpose of this study was to screen the pathogens of FER and antagonistic ability of *Trichoderma* sp. to suppress the growth of FER pathogen under *in vitro* and *in vivo* conditions.

Materials and Methods

Isolation and Identification of *Trichoderma* sp.

Twenty-two soil samples were collected from the rhizospheres of different crops plantation such as cabbage, chive, kenaf, oil palm, paddy, rubber and watermelon from six states in Peninsular Malaysia. *Trichoderma* isolates were isolated using Rose Bengal Agar (RBA) and single-spored following Nur Ain Izzati and Abdullah (2008). The pure cultures were maintained in Potato Dextrose Agar (PDA) slants and incubated at room temperature (27±2^{o}C). The isolates were identified at species level based on their morphological characteristics. Macro- and micromorphological appearance were observed following Diba *et al*. (2007).

Virulence Assays

Pure culture of *F. proliferatum* isolates B84c, P191c and P202c and *F. verticillioides* isolates C116c, C121c and P175c were obtained from culture collection of Mycology Laboratory, Department of Biology, Faculty of Science, Universiti Putra Malaysia (UPM). These isolates were originally isolated from different plant parts of maize around four different states in Peninsular Malaysia (Nur Ain Izzati *et al*., 2011).

To confirm the pathogen of FER disease, two-months-old maize plants (Thailand Supersweet variety) were inoculated with conidial suspension of *Fusarium* at concentration of 2×10^6 conidia/mL in 0.02% Tween 20 following procedure as described by Siti Nordahliawate *et al*. (2008). About 1mL conidial suspension was injected into the maize ears using sterile syringe and distilled water was replaced for control. Controls ears were divided into two as follows: C1) inoculated with sterile distilled water; C2) non-inoculated ear. Inoculated plants were incubated for seven days and the symptoms of the FER disease were recorded based on disease scale from 0 to 5 following the scoring system in Table 1 (Lori *et al*., 2008).

Table 1: Disease rating scale used in disease assessment

Grades	Symptom color*
0	No visible symptoms
1	Moccasin
2	Tan1
3	Tan2
4	Tan3
5	Tan4 and decay

*Color chart based on Glynn (2005)

Disease severity index (DSI) was calculated by using the formula modified from Elmer (2002) as follows:

$$DSI = \frac{\sum (A \times n)}{\sum B}$$

A = disease scale (0, 1, 2, 3, 4, 5).
n = number of maize in specific scale.
B = total number of maize.

To ascertain the pathogenicity of *F. proliferatum* and *F. verticillioides*, all inoculated ears with those isolates that showed FER symptoms were re-isolated, single-spored and re-identified. Treatments and control was completed in four replicates and the experiments were repeated at least twice. The DSI data was statistically analyzed using statistical package for the social sciences (SPSS) version 19.0 based on parametric analyses, which is one-way Analysis of variance (ANOVA) test ($p \leq 0.05$) to compare the virulence level of different isolates of *Fusarium* sp. The most virulence isolate, *F. proliferatum* P202c was used to in the subsequent study.

Screening of *Trichoderma* Isolates against FER Pathogen under *in vitro* Condition

All *Trichoderma* isolates were individually challenged for their antagonistic property against P202c. A mycelial plug of isolate P202c and *Trichoderma* isolates with size 8 mm diameter, respectively were placed 5 cm apart on opposite sides of 9 cm PDA culture plate. For the control plate, only isolate P202c was placed in a similar manner without *Trichoderma* culture. The culture plates were incubated at room temperature (27±2^{o}C) for five days (Siddiqui *et al*., 2008).

The ability of *Trichoderma* isolates to inhibit the growth of P202c was assessed by measuring the radius of pathogen colony in the direction towards the *Trichoderma* colony. The fungal growth was transformed into percentage inhibition of radial growth (PIRG) formula by Siddiqui *et al*. (2008) as below:

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

R1= radial growth of pathogen in control plate.
R2= radial growth of the pathogen in treatment plate.

To compare the percentage inhibition of different isolates of *Trichoderma*, the calculated data of PIRG

were subjected to parametric statistics, which is multivariate test ($p \leq 0.05$) by using SPSS version 19.0.

Efficacy of *T. harzianum* T73s as Biocontrol Agent of FER Disease

The best antagonist isolate of *Trichoderma* in the dual culture assay, isolate *T. harzianum* T73s was further tested under in *in vivo* condition. Maize was planted under glasshouse condition at Ladang 2, Faculty of Agriculture, Universiti Putra Malaysia. Daily temperature was in range from 31-33^{o}C and 50-70% relative humidity during the study period.

Maize plants were divided into three controls and four treatments as shows in Table 2, which different in application time of *T. harzianum* T73s. The preparation of conidial suspension (1×10^{7} conidia/mL) of T73s was done following method by Nur Ain Izzati and Abdullah (2008). The fresh conidial suspension was poured on the soil at 1L/bag according to respective application time of treatment until harvesting period at week 12th. P202c was inoculated to maize ears at week 8 and the symptoms were observed after seven days. Protocol of pathogen inoculation, disease assessment and data analysis were done as same as described in virulence assay. All treatments and controls were performed in 20 replicates.

Results

Species Identification of *Trichoderma*

Seventy-two isolates of *Trichoderma* were obtained from soil samples identified according to the identification key based on macroscopic and microscopic characteristics. Three *Trichoderma* sp. identified were *T. hamatum*, *T. harzianum* and *T. koningii*. The most frequently isolated species was *T. harzianum* with 51 isolates followed by *T. koningii* (20 isolates) and *T. hamatum* (1 isolate) (Table 3). Microscopic observations showed that the conidiophores of *T. harzianum* were formed in paired branches along the main axis (Fig. 1A-B). The phialides were cylindrical and enlarge shaped in the middle (Fig. 1C) and chlamydospores

Table 2: Control and treatment of T73s in different application time

Experiment	Pathogen	Application of T73s
C1	dH_2O	-
C2	-	-
C3	P202c	-
T1	P202c	Treatment given every week, immediately after planting*
T2	P202c	Treatment given every 2 weeks, immediately after planting*
T3	P202c	Treatment given every week, after pathogen inoculation**
T4	P202c	Treatment given every 2 weeks, after pathogen inoculation**

*The treatment was applied starting from week 0 until week 12
**The treatment was applied starting from week 8 until week 12

Table 3: The percentage of inhibition growth (PIRG) and antagonistic activity of *Trichoderma* sp

Trichoderma sp.	Isolates	[A]Mean value of PIRG	[B]Antagonistic activity
T. hamatum	A223s	65.16^{yz}	+++
T. harzianum	A190s, A217s, B95s, B98s, B99s, B112s, B130s, B131s, B134s, B136s, B151s, B155s, B158s, B159s, B161s, B166s, B170s, N317s, T66s, T78s	27.70^{cd} - 49.71^{nopqrs}	+
	A240s, B10s, B16s, B20s, B22s, B24s, B25s, B26s, B29s, B30s, B94s, B101s, B302s, C256s, C259s, C261s, C264s, C267s, T63s, T64s, T69s, T72s, T79s, T83s	50.54^{opqrst} - 59.56^{vwxy}	++
	B8s, B139s, B141s, B142s, B144s, N327s, [C]T73s	61.03^{wxyz} - 73.10^{f}	+++
T. koningi	A205s, A221s, A237s, A238s, B124s, B128s, B129s, B138s, B149s, B154s, B165s, B304s, B305s, T71s	20.43^{b} - 49.21^{mnopqr}	+
	B19s, B108s, B156s, B296s, C269s	51.91^{pqrst} - 59.47^{vwxy}	++
	B28s	61.04^{wxyz}	+++
Control	-	0.00^{a}	-

Means for respective fungus with same letter or symbol are not significantly different amongst themselves when Duncan test were used at 0.05 significance level
[A]Percent inhibition of radial growth
[B]Antagonistic activity: ++++ = very high antagonist activity (> 75 PIRG); +++ = high antagonist activity (61 - 75 PIRG); ++ = moderate antagonist activity (51 - 60 PIRG); + = low antagonist activity (< 50 PIRG); - = no antagonistic activity
[C]Isolate was selected for study of its efficacy as biocontrol agent under glasshouse condition

Table 4: Disease severity index assessed in maize ears which treated with T73s at different timing and frequency

Experiment	Disease severity index (DSI)*
Control 1 (C1): - Negative control, non-inoculated and no treatment	0.00^{a}
Control 2 (C2): - Positive control, artificially inoculated but untreated	4.75^{c}
Treatment 1 (T1): - Artificially inoculated with *F. proliferatum* P202c and treated every week, immediately after planting	0.50^{a}
Treatment 2 (T2): - Artificially inoculated with *F. proliferatum* P202c and treated every 2 weeks, immediately after planting	1.90^{b}
Treatment 3 (T3): - Artificially inoculated and treated every week, after inoculated with *F. proliferatum* P202c	4.50^{c}
Treatment 4 (T4): - Artificially inoculated and treated every 2 weeks, after inoculated with *F. proliferatum* P202c	4.55^{c}

*DSI with same letters is not significantly different at amongst themselves when Tukey HSD test were used at 0.05 significance level

were formed in globose and granulate (Fig. 1D). The phialospores are subglobose to short obovoid (Fig. 1E) with overall range of 2.48-3.25µm × 2.2-2.84 µm. The colony growth rate of *T. harzianum* was 2.12-2.45 cm/day on PDA under room temperature. The colony was whitish-green to dull green in color and reverse side was light green in color (Figs. 1F-G). Typically, *T. harzianum* formed distinct ring-like conidial zones with rather loose tufts of colony.

Evaluation for the Most Virulence *Fusarium* Isolates

Out of six *Fusarium* isolates, the highest mean of DSI was exhibited by *F. proliferatum* P202c (4.75) followed by *F. verticillioides* C116c (4.50). The maize ears inoculated with those isolates showed typical symptom of FER with tan in color of kernels after seven days of inoculation. The formation of symptom was only involved a portion of the inoculated kernels and not the whole ear. No visible symptom was observed on control ears. *F. proliferatum* P202c and *F. verticillioides* C116c that were re-isolated from inoculated ears, which showed FER symptom, were identical to the original isolate. *F. proliferatum* P202c was selected for study of the efficacy of *T. harzianum* as biocontrol agent.

***In vitro* Screening for *Trichoderma* Isolates against Pathogen of FER**

Dual culture assay revealed that all tested *Trichoderma* isolates had potential to inhibit the colony growth of pathogen, ranging from 20.43% to 73.10% (Table 3). Based on Noveriza and Quimio (2004), when the value of PIRG was higher than 60%, the antagonist was regarded as promising biocontrol agent. There are seven isolates of *T. harzianum* and a single isolate of *T. hamatum* and *T. koningii*, which showed the promising antagonist against P202c, and inhibition zones were visibly observed. T73s showed the highest inhibition of P202c with PIRG value of 73.10%. Therefore, the efficacy of this isolate was further tested under glasshouse condition.

Effectiveness of T73s on Suppressing FER under Glasshouse Condition

In glasshouse trials, the DSI was used as an indicator of effectiveness of *T. harzianum* T73s in suppressing FER. Low value of DSI on treated plants indicated the presence of disease suppression. The highest DSI value of 4.75 (Table 4) shown by the ears of positive control (C2), which inoculated with pathogen but untreated. The treatment that applied every week, immediately after planting (T1) had successfully suppressed FER disease due to lowest DSI value of 0.50 and statistically different at $p \leq 0.05$ with C1. In addition, DSI values of T2, T3 and T4 were significantly different at $p \leq 0.05$ with C1; hence, these treatments were not successfully suppressed FER disease.

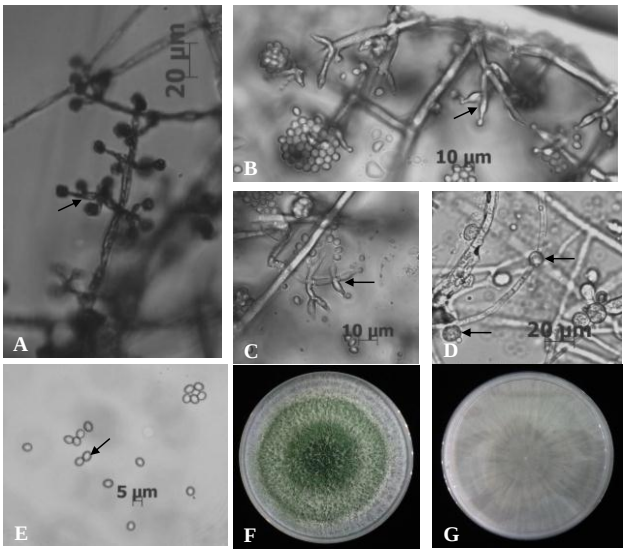

Fig. 1: Morphological characteristics of *T. harzianum* (T73s), A-B: Spore masses and conidiophores; C: Phialides D: Chlamydospores; E: Phialospores; F-G: Colony features on PDA

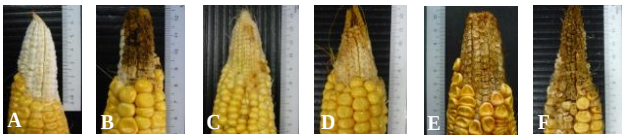

Fig. 2: Disease symptoms on maize ears; disease free on Control 1 (A), symptoms were observed on Control 2 (B), maize which treated with Treatment 1 (C), Treatment 2 (D), Treatment 3 (E) and Treatment 4 (F)

The kernels of infected ears (C2) showed FER symptom with tan in color (Fig. 2). In contrast, no symptoms of FER exhibited by non-inoculated ears of control C1.

Discussion

Trichoderma are free living fungi and usually found in a diverse soil types such as forest, agriculture, prairie, desert soil and salt marsh (Pandya *et al*., 2011). Aggregate species of *Trichoderma* can be distinguished based on macro and microscopic features.

Sampling of *Trichoderma* sp. proved that *T. harzianum* exhibited the highest number of isolates (70.83%) followed by *T. koningii* (27.78%) and *T. hamatum* (1.38%). Similar study by Lim and Teh (1990), which also isolated these species in Malaysian soils, *T. harzianum* from rubber and oil palm, *T. koningii* from rubber and cocoa and *T. hamatum* from cocoa grown on ex-rubber areas. Besides, other species of *Trichoderma* such as *T. longibrachiatum, T. virens* and *T. viride* have also been recovered from the rhizospheres of oil palm and rubber in Malaysia (Siddiquee *et al*., 2009).

Nur Ain Izzati *et al.* (2011) isolated 10 species of

Fusarium from maize that showed typical symptoms of FER in Malaysia. The most prevalent species was identified as *F. proliferatum*, followed by *F. subglutinans* and *F. verticillioides*. This finding supports the results on virulence assay in this study, which found *F. proliferatum* B202c and *F. verticillioides* C116c as the most virulent species on maize as in agreement with Suhaida and Nur Ain Izzati (2010). The association of *F. verticillioides* as a predominant species of FER was recognized for many decades and it seems clear that some previous reports also included *F. proliferatum* as a pathogen (Leslie *et al*., 1990; Logrieco *et al*., 1993; Cotten and Munkvold, 1998; Munkvold, 2003).

Among three species of *Trichoderma* from rhizosphere of different crops, *T. harzianum* exhibited highest number of isolates, which showed a good potential as antagonist. So the isolate of *T. harzianum* was more efficient for manipulating the growth of pathogens in dual culture. Here, all *Trichoderma* isolates gave distinct degree of PIRG values, even among the same species. This is due to the different patterns of induction of every single isolate of *Trichoderma* sp. (Benítez *et al*., 2004). Similar study performed by Sharfuddin and Mohanka (2012) who reported that different isolates of *Trichoderma* were different in their effectiveness in controlling soil borne phytopathogen.

The antagonist effect employed by *Trichoderma* sp. was divided into direct and indirect mechanisms. The interaction between *Trichoderma* sp. and the pathogen as in dual culture studies is by direct mechanism (mycoparasitism) (Benítez *et al*., 2004). Once *Trichoderma* encounter the pathogen, it attaches itself to the pathogen, coil and strangulate their hyphae (Siddiquee *et al*., 2009). Furthermore, *Trichoderma* sp. grows considerably faster compared to pathogens under same conditions on PDA. The rapid growth of *Trichoderma* isolates added an advantage to inhibit the growth of pathogens by competing for space and nutrients even before it deploys the mycotoxins (Kumar *et al*., 2012). Biological activity of *Trichoderma* sp. may be associated with production of lytic enzymes such as chitinases, glucanases and proteases. *Fusarium* cell wall is made up by chitin and their growth inhibition might be due to the inhibition of spore germination and tube elongation by chitinase (Brozóvá, 2004). This study suggests that *Trichoderma* sp. released metabolites, which are toxic and fungistatic to *F. proliferatum.*

The potential of *T. harzianum* T73s as biocontrol agent of FER disease was ultimately tested at the glasshouse with different frequency and time of application. The disease was effectively suppressed when treated with T73s immediately after planting and the treatment was given every week (T1). However, the application of T73s after inoculation of pathogen (T3 and T4) has failed to control this disease. This concludes that early interaction of *T. harzianum* was successfully protecting the maize plants against FER. Bacon *et al*. (2001) who found the ability of *T. harzianum* recovering from maize root to reduce the production of fumonisins by *F. verticillioides* also reported the similar results. The reduction of fumonisin was higher at 85% when *T. harzianum* T73s inoculated simultaneously after inoculation of P202c on maize kernels compared with 7 days after with 72%. A study done by Datnoff *et al*. (1995) also discovered the antagonist effect of *T. harzianum* against *Fusarium oxysporum* at field experiment in 1991 and 1993. Disease severity was reduced to 2.4% and 1.8% when *T. harzianum* was subjected to tomato plants for both years.

Since the antagonist and pathogen were applied to the soil and maize ear, respectively there is no direct contact between these fungi. Hence, we can say that the suppression of FER disease at glasshouse is by indirect mechanism. *Trichoderma* sp. is capable to colonize plant root prior to stimulation of plant growth and plant defense mechanisms. Vinale *et al*. (2008) reported that *Trichoderma* species are capable to colonize plant root and produce compound that changes plant metabolism and stimulate plant defense. After penetrate the plant, they will produce metabolites, which act as elicitors and activate plant defense response. Shoresh *et al*. (2010) reported that these metabolites might be proteins such as peroxidase, xylanase, glucanase, swollenin, cellulose and endochitinase. Later, plants will synthesis phytoalexins that activated defense-gene, known as mycoparasitic gene and induce resistance against pathogen (Benítez *et al*., 2004).

There are several methods for biocontrol agent application such as foliar spray and seed treatment. However, in this study the *Trichoderma* T73s was introduced to the soil, as originally isolated from soil samples. Hence, this isolate is considered has a good ability to compete with other rhizosphere microorganisms for the nutrient. Moreover, to ensure the success in suppressing the disease, it is more preferable to introduce it to the soil, which is its natural habitat. Therefore, it will adapt well at the soil environment and make the suppression of plant disease more efficient.

In conclusion, the most pathogenic species of *Fusarium* to FER disease of maize was *F. proliferatum* followed by *F. verticillioides.* PIRG test revealed that the growth of *F. proliferatum* was successfully inhibited by *T. harzianum* T73s. Moreover, this isolate also showed a good potential as biocontrol agent of FER under glasshouse condition.

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