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Trichoderma Suppresses Pathogenic Fusarium Causing Tomato Wilt in Bangladesh

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Authors' contributions

Author AR has undertaken the molecular study of Trichoderma spp. and wrote the manuscript. Author MK has carried out the molecular characterization of Fusarium. Author MEMF purified the two fungal samples and phenotypically characterized them. Author MAR has edited the manuscript. Author AFMJU grew tomato plants in his field and assisted in collection and isolation of Fusarium. Author ASMN is the expert Pathologist leading the program by providing intellectual guidance and overall supervision.

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ABSTRACT

Aims: Antagonism to *Fusarium* root rot caused by *Fusarium oxysporum* f. sp. *lycopersici* (ACI-FOL7 and ACI-FOL8) by *Trichoderma spp.* was investigated.
Study Design: The study was designed to understand antagonistic properties of locally collected *Trichoderma spp.* against *Fusarium in vitro.*
Place and Duration of Study: The study was done at the laboratories of Advanced Seed Research & Biotech Centre and the study was carried out for 2 years.
Methodology: *Trichoderma spp.* was collected from soil and *Fusarium* was collected from symptomatic tomato plant roots. Pathogenicity assay was done with *Fusarium* and degree of antagonism study was carried out through dual culture *in vitro.* Apart from morphological

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characterization, DNA was extracted from both fungus and confirmation of species was done through fungal specific Internal Transcribed Specer regions.
Results: The Percent Inhibition of Mycelial Growth (PIMG) recorded higher in T_2 (96.7%) followed by T_1 (95.5%) and T_3 (95.3%). The highest PIMG was recorded in ACI-FOL7 and the lowest in ACI-FOL8 (84.4%). Among the *Trichoderma* species (T1, T2 and T3) significant antagonism difference were observed in ACI-FOL8, whereas ACI-FOL7 was highly suppressed by the *Trichoderma* species.
Conclusion: These results reveal that the native *Trichoderma* spp. might have biocontrol potentiality for the suppression of FOL in Bangladesh.

Keywords: Antagonistic effect; bioagents; Fusarium oxysporum; root rot; ITS.

1. INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most important solanacious crop [1] grown in almost all areas of Bangladesh. It has high market value because of its diversified use, nutritional contents and medicinal properties [2], [3]. In Bangladesh, tomato production needs to be increased to meet the demand as the average yield is undesirably low compared to other leading tomato producing countries [4] due to several diseases [5], affecting plant growth and yield. Out of these diseases, wilt of tomato is a serious and increasing threat, causing 60-70% yield loss in Bangladesh [6]. *Fusarium* wilt is typified by a reduction in yield and then eventual death of plants within a short period of time by yellowing on the one side of the leaf, shoot, or branch [7]. A number of factors contribute to tomato wilt worldwide but the most significant is *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) [8,9]. In addition, bacterial such as *Ralstonia Solanacearum* can increase plant stress and accelerate the wilt confirmed in Bangladesh [10], where the fusarial issue remains unclear. The pathogenic factors are difficult to control because no resistant cultivar has been developed. Actually, breeding of disease resistant cultivar has been attempted [11], but it is too time consuming. On the other hand, biological control of *Fusarium* disease was tried by inoculation with non pathogenic isolates of *Fusarium* species in vegetable crops including tomato [12-14]. However, the method is not effective enough to control the disease and has no growth promoting effect.

Fusarium wilt is one of the severe soil-borne fungal diseases, which negatively affects plants growth and yield [15]. It remains as a challenge in terms of disease management [16,17]. Fungicides cannot control this soil borne pathogen; moreover, some other beneficial micro-organisms are killed [3]. In addition, the chemical's toxicity contaminates the whole environment [18] and causes serious risk for the appliers and consumers.

Plant pathogens are successfully controlled by using natural resources in sustainable agriculture [19]. Biological control is now practiced worldwide as a key factor [20]. For example, the *Fusarium* wilt can be reduced by different *Trichoderma* spp., applied in many crops [21], [22,23]. The potentiality of *Trichoderma* species has been reported as one of the most efficient bioconrol agents against various plant diseases [24,25], including tomato wilt [26]. The biocontrol activity of *Trichoderma* spp. comes from their successful parasitic abilities on other fungi [27]. Beside this, enhancement of plant growth of tomato and tobacco has been observed after application of *Trichoderma* in field and greenhouse [28,29]. However, not all the isolates of *Trichoderma* spp. can control the pathogens to the same extent in both *in vitro* and *in vivo* conditions [30,31]. Hence, some specific isolates can effectively control a particular pathogen in an environmentally safe and economically robust way [32].

Therefore, the experiment was carried out to assess the antagonistic ability of native *Trichoderma* spp. in suppressing the populations of FOL in tomato under *in vitro* condition.

2. MATERIALS AND METHODS

2.1 Isolation of *Fusarium* from Sympto-matic Tomato Plant Roots

Infected root regions of tomato plants showing wilt symptoms were collected from the field of Sher-e- Bangla Agricultural University, Dhaka, Bangladesh. The root segments were surface sterilized with 70% (v/v) ethanol for 10 seconds and 10% (v/v) sodium hypochlorite for 10

minutes, and subsequently three washings with sterile distilled water. Then they were placed on Komada's medium [33] in a standard Petri dish and incubated in growth chamber (MMM, Germany) at 25°C for seven to ten days until fungal colonies appeared. Then the colonies were transferred onto Potato Dextrose Agar (PDA) medium (HiMedia Laboratories) at least three times to maintain pure cultures for further studies.

2.2 Pathogenicity Assay

Pathogenicity test of the ten isolates of *Fusarium oxysporum* f. sp. *lycopersici* was carried out using the root dip method described by Author [34]. Tomato seedlings were inoculated with FOL using spore suspension with conidial concentration of $1x10^5$ conidia/ml by root dip method. The diseased plants wilted and dried up while control plants were free from disease. Pathogenicity of each fusarial isolate was scored according to the number of seedlings with root lesion to confirm its identity as root rot pathogen under greenhouse condition.

2.3 Isolation of *Trichoderma* spp. from Rhizospheric Soil

Rhizospheric soil from healthy tomato plants was taken from Sher-e- Bangla Agricultural University, Dhaka, Bangladesh. One gram of soil was added to 1 ml of sterilized distilled water to make a dilution of 10^{-1}. This suspension was then subjected to serial dilution and a dilution of 10^{-5} was attained. One mililitre of each dilution was poured on to *Trichoderma* Specific Medium (TSM) and purified by single spore method. They were identified on the basis of their morphological characters. The purified and identified cultures of *Trichoderma* spp. were maintained on PDA medium and stored at 4°C for further use.

2.4 *In vitro* Screening of *Trichoderma* Antagonists against *Fusarium* by Dual Culture Method

Isolated *Trichoderma* spp. were tested for antagonism against of common plant pathogen *Fusarium* (ACI-FOL7 and ACI-FOL8) by using dual culture techniques as developed by Author [35]. The 5 mm mycelial discs of *Trichoderma* spp. were placed 1 cm away from the edge of the Petri dishes containing autoclaved PDA medium. The mycelial discs (5 mm in diameter) of either ACI-FOL7 or ACI-FOL8 were placed at the opposite side of the Petri dishes. The mycelia discs were also cultured individually on PDA media as control. The plates were incubated at 25°C for a week.

2.5 Degree of Antagonism

The degree of antagonisms between the test pathogens and bio-agents in dual culture was obtained as reported by Author [3]. Percentage of mycelia growth inhibition was calculated according to the formula given below:

$$PI = (dc- dt) \times 100/dc$$

Where, PI= Percent inhibition dc= fungal colony diameter in control sets, dt = fungal colony diameter in treatment sets.

2.6 Recovery of High Molecular Weight of FOL DNA and *Trichoderma* DNA

Fungal mycelium (0.1 g fresh weight) was taken from actively growing colonies and titrated in a 2.0 ml eppendorf tube containing 300 µl puregene cell lysis solution (Gentra systems), and then kept on ice. The mycelium was crushed in the tube with a mini-homogenizer (Pellet Mixture, Samsyo) and 150 µl of 3 M sodium acetate was added. The contents were mixed and incubated at -20°C for 10 minutes. The precipitated slurry was clarified by centrifugation at 14,000 rpm for 5 min. Finally, the clear supernatant was transferred to a clean microcentrifuge tube and the volume of supernatant recorded. An equal volume of isopropanol was added, mixed gently, and the tube incubated for 5 min at approximate room temperature. Precipitated DNA was collected by centrifugation at 14,000 rpm for 5 min. The isopropanol was carefully poured off and the sample was washed in 200 µl of 70% filter-sterilized ethanol for 15 min, and centrifuged at 14,000 rpm for 5 min. The DNA pellet was air-dried prior to resuspension in 70 µl TE (10 mM Tris-HCl and 1 mM EDTA at pH 8) buffer, and stored at 4°C.

2.7 PCR Analysis of *Fusarium* Pathogens and *Trichoderma* Antagonists

Fusarium pathogens (ACI-FOL7 and ACI-FOL8) were confirmed by PCR. PCR (Veriti, Applied Biosystems) targeted the ITS2 regions of nuclear rRNA genes. The 20 µl reaction mixture was

comprised of 4 µl of Taq DNA polymerase 10X reaction buffer, (Promega, Madison, WI), 2.25 µl of 25 mM Mg2+ solution, 1.75 µl of dNTP mix (4 mM each), 1.75 µl of 25 mM ITS3 forward primer (5´ GCATCGATGAAGAACGCAGC 3´) and ITS4 reverse primer (5´ TCCTCCGCTTGATATGC 3´) each, 0.1 µl of Taq DNA polymerase, 1 µl of extracted DNA, and sterile MilliQ water making it up to 20 µl. The PCR program consisted of 35 cycles with a denaturing step of 95°C for 45 s, an annealing step of 50°C for 1 min and a polymerization step of 72°C for 3 min. PCR products were checked by agarose gel electrophoresis and visualized on a UV transilluminator.

The same protocol was followed to identify the ITS1 region in *Trichoderma* spp. using ITS1 (5' TCTGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') primers.

2.8 Data Analysis

The analysis of variance of the studied characters was done by following completely randomized design in Wasp2 software (Web Agri Stat Package 2.0). The mean differences were evaluated finally by Duncan Multiple Range Test (DMRT) at 5% level of significance.

3. RESULTS

In total, ten fusarial isolates (Fig. 1) were isolated from the horticutural fields of Sher-e-Bangla Agricultural University, Bangladesh showing symptoms of *fusarium* wilt (Table 1). Fungal colonies were confirmed based on the examination under light microscope (Fig. 2A). A subset of two isolates (ACI-FOL7 and ACI-FOL8) was selected among them, which showed PCR bands at 350 bp in the ITS2 region (Fig. 2B). Besides, *Trichoderma* isolates (Fig. 3A) were identified according to conidiophores and conidia (Fig 3B) and the ITS1 specific region as shown in Fig. 3C.

The pathogenicity assay yielded natural wilt symptoms. Among the ten tested fungal isolates, ACI-FOL7 and ACI-FOL8 were found highly pathogenic by showing 100% wilt symptoms. Therefore, these two isolates were selected to use for further studies.

Three native isolates (T_1, T_2, and T_3) of *Trichoderma* spp. were screened for their *in vitro* antagonism against *F. oxysporum* f. sp. *lycopersici* by dual culture technique (Table 2). The result indicates that T_2 exhibited the maximum percentage (96.7%) of inhibition over control in case of ACI-FOL7 (Fig. 4A), followed by T_1 (95.5% inhibition rate) and T_3 (95.3% inhibition rate). In addition, T_3 most actively suppressed the growth of ACI-FOL8 by showing 91.33% (Fig. 4B) inhibition compared to T_1 and T_2 obtaining 84.4% and 88.6% of inhibition rates respectively. The lowest colony diameter (2.9 mm) was recorded for growth of ACI-FOL7 in interaction with T_2 (Table 3) whereas, the highest colony diameter was observed in dual culture between ACI-FOL8 and T_3 (14 mm).

4. DISCUSSION

In this experiment, the identity of isolates of *Fusarium oxysporum* f. sp. *lycopersici* was confirmed by obtaining 350 bp band in the ITS2 region. Previous reports revealed similar results showing 350 bp bands were commonly presents in all *F. oxysporum* isolates in PCR-SSCP and RFLP analyses [36-38].

In this study, *Trichoderma* spp. isolates were identified based on the morphological and microscopic observation as well as molecular characterization. Whitish green to green colonies, long and thick conidiophores and conidia were also observed in *Trichoderma* spp. isolation according to [39].

The present evaluation revealed that the tested isolates of *Trichoderma* successfully inhibited the growth of *Fusarium oxysporum* f. sp. *lycopersici* causing tomato wilt. Similar antagonistic effects found by other researchers having success in suppressing colonies of FOL by *T. harzianum*, isolated from rhizosphere soil [40]. Various isolates of *T. harzianum, T. coningi* and *T. viride* suppressed the mycelial growth of some pathogens by covering or overlapping the parasitic colonies in *in vitro* conditions [41-43], indicating the coparasitic behavior [44] of *Trichoderma.* In this experiment, the reason behind the inhibitory effect of these bioagents against the pathogens was not studied. However, the negative effect probably happened due to competition, production of antagonistic substances, and antibiosis [45,46].

The findings of this study showed that the different isolates antagonized the pathogens to various degrees and this assorted effects were demonstrated earlier between and within species of *Trichoderma* against FOL [47] suggesting that the diversity occurred because of the

environment, quality, and quantity of antibiotic or inhibitory substances secreted by the antagonists [48-50]. The degree of antagonism significantly varied between these *Trichoderma* isolates and ACI-FOL8 in the current study, supported by Author [21] who found highly significant interactions between seventy seven tested isolates of *Trichoderma* and pathogens, indicative of the gene involvement of both antagonists and pathogens in regulating antagonism variations.

In this investigation, ninety six percent of growth inhibition was achieved by one of the isolates; whereas highest 87.33% growth inhibition was obtained in a previous study [51]. However, the percentage of inhibition varied between *in vitro* and greenhouse or field trials [29].

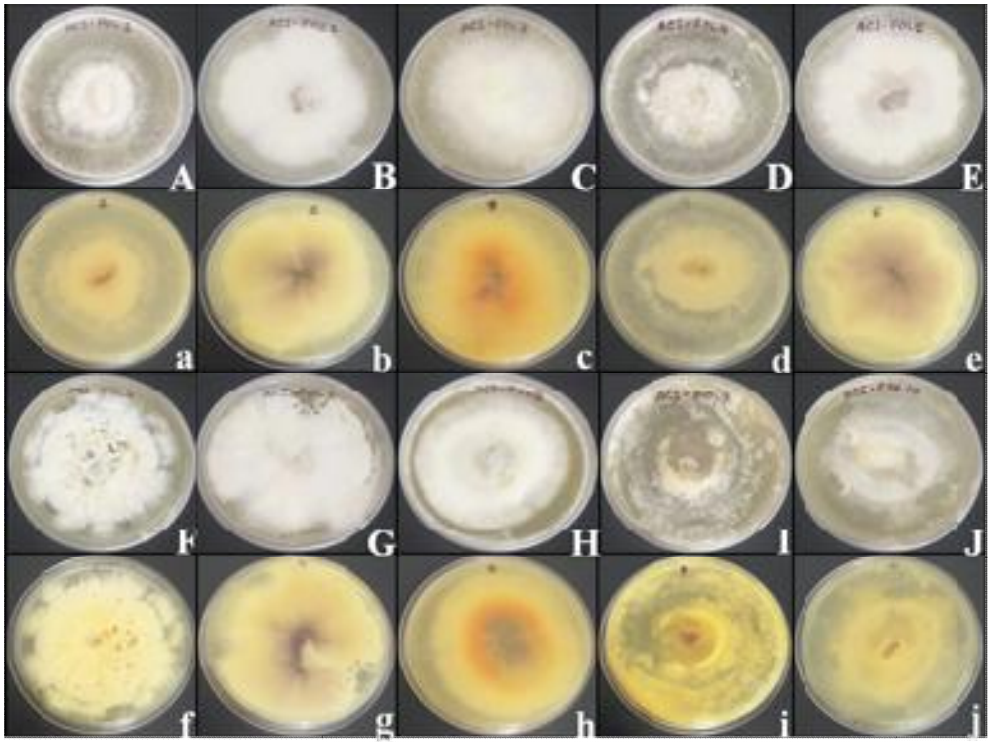

Fig. 1. *Fusarium Oxysporum* f. sp. *lycopersici* (FOL) isolates isolated from horticultural farm, Sher-e-Bangla Agricultural University, Bangladesh. (A-J) show colony colors and growth patterns of FOL isolates while (a-j) show the metabolite colors of FOL isolates

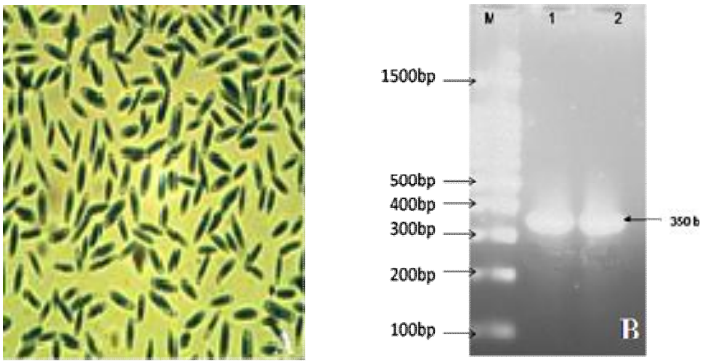

Fig. 2. A. Microscopic view of *Fusarium oxysporum* f. sp. *lycopersici* B. PCR amplification of ITS2 region with specific primers ITS3 and ITS 4. Lanes: 1, ACI-FOL7; 2, ACI-FOL8 and M, 100 bp DNA ladder

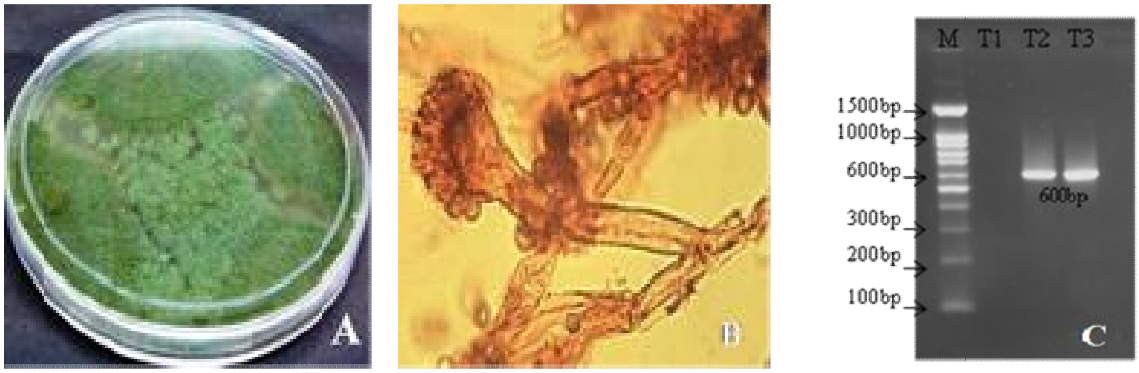

Fig. 3. A. Pure Culture of *Trichoderma* spp.and B. Conidiohpores and conidia of *Trichoderma* spp. under microscope C. Lines T2 and T3 were specifically confirmed through Molecular Characterization of ITS1 region of the rDNA of *Trichoderma*

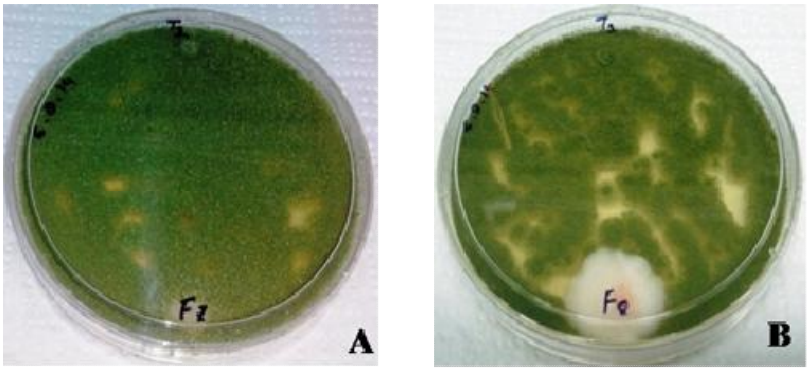

Fig. 4. Antagonistic efficacy of *Trichoderma* spp. against tomato wilts pathogen (FOL) under *in vitro* condition A. ACI-FOL7 and B. ACI-FOL8

Table 1. List of *F. oxysporum* f. sp. *lycopersici* used in this study

SL.	Isolates of FOL	Geographic origin	Colony color	Wilt symptom (%)	Source of isolation
1	ACI-FOL1	Dhaka	Cream Color	50	Tomato root
2	ACI-FOL2	Dhaka	White	50	Tomato root
3	ACI-FOL3	Dhaka	Pale Yellow	60	Tomato root
4	ACI-FOL4	Dhaka	Cream Color	40	Tomato root
5	ACI-FOL5	Dhaka	White	60	Tomato root
6	ACI-FOL6	Dhaka	Light Brown	50	Tomato root
7	ACI-FOL7	Dhaka	Pinkish White	100	Tomato root
8	ACI-FOL8	Dhaka	Light Brown	100	Tomato root
9	ACI-FOL9	Dhaka	Yellowish Brown	50	Tomato root
10	ACI-FOL10	Dhaka	Pale Yellow	40	Tomato root

Table 2. List of *Trichoderma* spp. used in this study

SL.	Isolates of *Trichoderma* spp.	Geographic origin	Colony color	Source of isolation
1	T_1	Dhaka	Dark green	Rhizospheric soil
2	T_2	Dhaka	Dark green	Rhizospheric soil
3	T_3	Dhaka	Dark green	Rhizospheric soil

Table 3. Antagonism by different *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *Lycopersici*

FOL	*Trichoderma* isolates	Colony growth of *Fusarium oxysporum* f. sp. *lycopersici* (mm)*	PIMG
Fusarium oxysporum f.sp. *lycopersici* (ACI-FOL7)	T_1	4.0 a	95.5 a
	T_2	2.9 a	96.7 a
	T_3	4.2 a	95.3 a
	LSD (5%)	1.8	2.0
	Co-variance	49.3	2.1
Fusarium oxysporum f.sp. *lycopersici* (ACI-FOL8)	T_1	14.0 a	84.4 a
	T_2	10.2 b	88.6 b
	T_3	7.8 c	91.3 c
	LSD (5%)	1.5	1.7
	Co-variance	14.5	1.9

**Mean of three replicates was taken. In a column, mean followed by a common letter are not significantly different at the 5% significant level by DMRT*

5. CONCLUSION

It is clear from the study that the isolates of *Trichoderma* have strong antagonistic potential effect as a bio-control agent, which can effectively be used in tomato wilt diseases management to save environmental risk. This will definitely minimize the usage of chemical fungicides. The next phase of this study may involve identifying the various methods of effective *Trichoderma* application.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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