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Application of chitosan and *Trichoderma* against soil-borne pathogens and their effect on yield of tomato (*Solanum lycopersicum* L.)

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Abstract

Plant diseases need to be controlled to uphold the quality and load of yields produced by grower's across the world. A variety of approaches may be used to halt, alleviate or control plant disease. Uses of different biological control agents regulate the growth of plants in a miraculous form. The present study was conducted to reduce soil-borne diseases caused by *Fusarium oxysporum* f. sp. *lycopersici*, *Sclerotium rolfsii*, *Rhizoctonia solani* and to increase the yield of tomato field under natural epiphytotic conditions through the application of *Trichoderma harzianum* and chitosan based-treatments at different methods. Isolate *T. harzianum* pb27 displayed 88.56%, 90.58%, 89.28% inhibition rate and chitosan 800 ppm showed 78.61%, 81.11%, 77.22% inhibition rate on PDA plate against the highly virulent isolate of *F. oxysporum* f.sp. *lycopersici* (FOL), *S. rolfsii*, and *R. solani,* respectively. *Trichoderma*-fortified compost along with foliar spray of chitosan and seedling roots dipped both in chitosan solution and *Trichoderma* spore suspensions appeared to be best in controlling the post-emergence seedling mortality (81.83%) caused by soil-borne pathogens. Similarly the same treatment was also found the most promising for the management of different soil-borne diseases of tomato including *Fusarium* wilt caused by *F. oxysporum* f. sp. *lycopersici*, collar rot/southern blight caused *by S. rolfsii*, dry root rot or wet root rot caused by *R. solani*. In addition, all treatments significantly increased yield and yield contributing components of tomato. Therefore, integration of two or three components is advised as one of the most worthy methods in order to effectively control the disease and improve crop performance.

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Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most popular and nutritious vegetables across the world. It ranks second next to potato in acreage and production among the vegetables crops grown in Bangladesh (Anonymous, 2009). Although the total cultivated area of tomato in our country have increased gradually over last few years but the productivity is still very low 9.66 t/ha compared to the average of the world yield 33.68 t/ha (FAO, 2012). Diseases are the major limiting factor in tomato production in Bangladesh. Soil-borne pathogens predominantly *Sclerotium rolfsii*, *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *lycopersici*, and *Pythium* spp. are very threats in tomato production both at seedling stage and mature stage in the field (Jones *et al*., 1991). In this regards, Bangladesh must need to improve disease control strategies with minimizing the production cost for chemical pesticides and fertilizers.

Although application of fungicides is primarily considered as the most effective method in controlling different soil-borne fungi but it can be involved in many problems due to health risk concerns and environmental pollution. Thus, there is a growing need to develop alternative approaches for the management of soil-borne diseases. An acceptable approach that is being actively investigated involves the use of bio-agents such as *Trichoderma* and bio-active substances like chitosan in controlling soil-borne fungi (Mandal and Ray, 2011, Akram and Anjum, 2011, Bautista-Banos *et al.,* 2006). The fungus *Trichoderma*, a natural soil-inhabiting genus, has been used successfully to control diseases of tomato (Nawar, 2005). The beauty of *Trichoderma* is that it can be used to combat almost every pathogenic fungus that people want to control. Moreover, several species of *Trichoderma* is now established to enhance pant growth as well as to increase yield significantly (Stewart and Robert, 2015; Kotasthane *et al.,* 2015). Besides, the biodegradable nature of natural compounds derived from animal and plants have also been interested for the plant pathologist to mitigate damages caused by pathogens. Among them, chitosan- a polysaccharide extracted from the shells of crustaceans, such as shrimp, crab and other sea crustaceans has become a useful appreciated compound due to its fungicidal effects and elicitation of defense mechanisms in plant tissues (Terry and Joyce, 2004). Chitosan and its derivatives display antibiotic activity against microorganisms including bacteria and fungi as well as it can also increase growth and yield (Russell, 2013; Anusuya and Sathiyabama, 2016).

Soil-borne diseases are complicated to control in field-grown tomatoes because the pathogen rapidly colonizes soil and persists for long periods. However, an integration of the different management strategies may help to reduce the impact of soil-borne fungal diseases. Management of many fungal pathogens in different pathosystems through the application of *Trichoderma* or chitosan individually is well documented (Abd-El-Kareem *et al*., 2006, Nawar, 2005). Though research on the application of bio control agents began in the mid- 1990's, but results from documented field trials on the application of *Trichoderma* or chitosan in controlling vegetable diseases are scanty. Therefore, the application of *T. harzianum* individually or in combination with chitosan was introduced in this study as an integrated management approach. Considering the above mentioned facts, the present study was carried out with the specific objectives such as (i) to optimize the dose of chitosan for controlling the major field diseases of tomato, (ii) to evaluate the efficacy of natural chitosan and *Trichoderma*, individually or in combination, against the soil-borne pathogens of tomato under lab and field conditions, and (iii) to study the effect of chitosan and *Trichoderma* on yield promoting components and yield of tomato.

Materials and methods

Microorganisms and plant materials

A total of 20 isolates of *Trichoderma harzianum* used in this study are listed in Table 2. The isolates were collected from soils of different crop fields at Pabna district in Bangladesh. Four isolates of each of the test pathogens *F. oxysporum* f. sp. *lycopersici,*

Rhizoctonia solani and *Sclerotium rolfsii* were isolated from the infected region of tomato crops grown in Plant Pathology Field Laboratory of the Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur, Bangladesh. Seed samples of Tomato variety "Manik" (BARI Tomato-1) was used as the test plant.

Isolation and identification of T. harzianum isolates

Soils samples were collected from rhizosphere region particularly carrot, radish, tomato, potato, brinjal, Chilli, soybean and pea field in Pabna district of Bangladesh. Soils were collected from at least three place of a particular crop field and mixed together to make a composite sample. *Trichoderma* spp. was isolated from individual samples following the soil dilution plate technique (Mian, 1995). Ten gm of soils from each sample was mixed with 90 ml of sterile water in a sterile conical flask and the content was stirred with a magnetic stirrer for about 20 minutes. A series of soil dilution prepared in flask were agitated on a vortex for two minutes for thoroughly mixing. The process was repeated until the 5 folds serial dilutions were made. Then 5 ml of each dilution was incorporated on a plate with PDA modified by 100 ppm streptomycin sulfate. The soil suspension on PDA plate was then spread evenly using a turntable. The plates were incubated for 5-7 days at room temperature (25±2 ° C). The fungus was purified on PDA following hyphal tip culture technique (Tuite, 1969). A total of 20 fungal isolates were identified as *T. harzianum* on the basis of growth, colony and morphological characters using the standard key (Barnett and Hunter, 1998). The other isolated fungi were discarded. The isolates of the pure culture of *T. harzianum* were preserved by using PDA slants at 10°C in refrigerator as stock culture for future use.

Isolation, Identification and inocula preparation of F. xysporum f. sp. *lycopersici, R. solani and S. rolfsii*

Each of the isolates of test pathogens such as *F. oxysporum* f. sp. *lycopersici*, *R. solani* and *S. rolfsii* were isolated from the infected tomato plants following the tissue planting method (Mian, 1995). Briefly, the diseased specimens were washed in distilled water to remove sand and soil particles. After that, the specimens were cut into small pieces (5mm) along with healthy and dead tissue and surface sterilized with 0.1% NaOCl for 2 minutes and rinsed in sterilized water for three times. Four pieces of them were blotted dry to remove excess water and plated on water agar. The characteristic colonies of the isolates were transferred to freshly acidified PDA plates and aseptically maintaining equal distance. After 5 days incubation, these isolates were purified by selecting a single hyphal tip (less than 1.5 mm) of each isolate (Tuite, 1969). The fungal isolates were identified following the standard key (Barnett and Hunter, 1998).

Inocula of target pathogens were prepared on autoclaved moist wheat grain following the procedures of Chang and Hsu (2008) with some modifications. Wheat grains (500g) were soaked in water for 24 hours and excess water was drained out. About 100 g of water soaked grains were taken into a 500-ml Erlenmeyer flask separately for each of the test pathogen, sealed by cotton plug and were sterilized in an autoclave. Sterilized wheat grains were inoculated with 10 mycelial disks (5 mm in diameter) obtained from the edge of a 3-day-old PDA culture plate of *F. oxysporum* f. sp. *lycopersici*, *R. solani* and *S. rolfsii* followed by incubating them at room temperatures (25 - 28°C) shaken by hand at 2-3 days interval for proper colonization. The completely colonized wheat bran was air dried for 2 days and stored at 4 °C for until use.

Pathogenicity Test

Inocula of each isolate of *F. oxysporum* f.sp. *lycopersici*, *R. solani* and *S. rolfsii* were thoroughly mixed with sterilized soil at the rate of 20 g /kg soil in separate earthen pot. Earthen pots were filled with sterilized soil at the rate of 1.5 kg/pot. An un-inoculated pot was also included as control where only sterilized soil was used. Eight pieces of tomato seeds were sown in each pot. Disease development was observed regularly and recorded at 10 to 25 days after sowing to evaluate the effect of pathogens in causing pre-emergence and post-emergence seedling mortality.

The causal agent of pre-emergence seedling mortality was confirmed after re-isolation of the pathogen from un-germinated seeds and the infected roots of tomato, respectively.

In vitro screening of T. harzianum isolates for their antagonistic effect on the radial growth of F. oxysporum f. sp. *lycopersici, R. solani and S. rolfsii*

To select the most effective antagonist, a total of 20 isolates of *T. harzianum* were tested for their antagonistic effect against *F. oxysporum* f. sp. *lycopersici*, *R. solani* and *S. rolfsii* on potato dextrose agar (PDA) medium by Dual Plate Culture technique (Dhingra and Sinclair, 1995). Briefly, one young mycelial disc (5 mm in diameter) of individual isolate of *T. harzianum* and of test pathogen were placed simultaneously on the edge of the each PDA plate at opposite direction. The plates only with the discs of test fungal pathogen in the centre were used as controlled plate. The growth of the colonies of each tested pathogens were measured after the complete growth of the control plates. The antagonistic potential of different *Trichoderma* isolates were assessed by calculating the inhibition of the radial growth of test pathogens using the formula (Kotasthane *et al*., 2015): % Inhibition of growth = $\frac{X-Y}{X} \times 100$, Where, X= Mycelial growth of the pathogen in absence of *T. harzianum* (control), and Y= Mycelial growth of the pathogen in presence of *T. harzianum*. The fungal isolates which inhibited above 75% absolute inhibition of the test pathogens were grouped as very strong, 60-75% inhibition as strong, 50-60% inhibition as moderate, 30-50% inhibition as weak, 15-30% inhibition as very weak and 0-15% inhibition of the pathogens were grouped as no inhibition. Isolate of *T. harzianum* having very strong suppressive effect on mycelial growth of *F. oxysporum* f.sp. *lycopersici*, *R. solani* and *S. rolfsii* were selected for further study and preserved for further use in the PDA slant at 10°C.

Preparation of T. harzianum- fortified compost with poultry refuges

Based on the result in *invitro* screning, the highly effective antagonist *T. harzianum* Pb27 was used against the selected soil-borne virulent pathogenic isolates of *F. oxysporum* f. sp. *lycopersici*, *R. solani* and *S. rolfsii* in the field experiment. Before setting the experiment, the isolate of *T. harzianum*-fortified compost composition was prepared using poultry refuges separately in a 1.0 m x 1.0m x 1.5m composting pit and covered with polythene sheet. The compost pit was prepared with 40 kg poultry refuges and after 45 days of decomposition, 2.5 kg of wheat grain colonized *T. harzianum* Pb27 inocula prepared previously. Compost pits were allowed for 90 days for decomposition and degradation following the procedure of the preparation of standard quality compost.

In vitro evaluation of chitosan for their inhibitory effect on the radial growth of F. oxysporum f. sp. *lycopersici, R. solani* and *S. rolfsii*

Chitosan used in this study was water-soluble solution and collected from Bangladesh Atomic Energy Commission (BAEC). It was extracted from the shell of quick growing sea shrimp irradiated with γ-ray as described by Rashid *et al.,* 2012. After several preliminary evaluations with the lower chitosan concentration such as 100, 200, 400 and 800 ppm were evaluated on PDA plate against the test pathogens. Chitosan was added to conical flasks containing sterilized PDA before solidification and rotated gently then poured into sterilized Petri plates (9 cm diameter). Plates were individually challenged at the centre with equal agar plug (5 mm in diameter) taken from *F. oxysporum* f.sp. *lycopersici, R. solani* and *S. rolfsii* 7-day-old cultures and incubated at 25 ± 2°C. Mean colony diameter was measured when the control plates, where no chitosan was used, reached full growth. Based on the laboratory results, the most antagonist chitosan 800 ppm was selected for field experiment.

Individual and integrated effect of T. harzianum pb27, chitosan and Trichoderma- fortified compost in controlling different soil-borne pathogens and improving yield of tomato in the field

A field experiment under natural epiphytotic condition was conducted to determine the individual and integrated effect of the most effective

T. harzianum isolate, an organic amendment and a natural aminopolysaccharide on soil-borne diseases of tomato in the Plant Disease Diagnostic Clinic Research field under Plant Pathology Department of BSMRAU during 2014-2015. To do this, we selected *T. harzianum* pb-27, chitosan and *Trichoderma*-fortified compost from our preliminary screening studies and evaluated their effect on soil-borne diseases and yield improvement of tomato in a non-sterile field soil. Prepared *T. harzianum* fortified compost @ 6 ton per hectare (As suggested by Tamil Nadu Organic Agriculture Portal) was mixed in the field one week before tomato seedling transplantation depending on the laid out of the experimental treatments. Three weeks old tomato seedling's roots grown on seed bed were dipped individually in Chitosan 800 ppm solution and *Trichoderma* pb27 spore suspension (5 x 10^6 per ml) for 30 minutes and the chitosan suspension was sprayed on experimental tomato plants at their vegetative and flowering growth stages. The following combinations of treatments were allotted randomly to each block.

T_1=Control without *Trichoderma* or chitosan
T_2= Tomato seedlings spray with chitosan solution
T_3=Seedling roots dipped in chitosan solution
T_4= Seedling roots dipped in *Trichoderma* spore suspensions
T_5= *Trichoderma*-fortified compost applied in the field
T_6= *Trichoderma*-fortified compost + Seedling roots dipped in *Trichoderma* spore suspensions
T_7= *Trichoderma*-fortified compost + Chitosan spray
T_8= *Trichoderma*-fortified compost + Seedling roots dipped in Chitosan solution
T_9=*Trichoderma*-fortified compost + Seedling roots dipped in *Trichoderma* spore suspensions + Chitosan spray
T_{10}=*Trichoderma* fortified compost + Seedling roots dipped in *Trichoderma* spore suspensions + seedling roots dipped in Chitosan solution
T_{11}= *Trichoderma* fortified compost + Seedling roots dipped in *Trichoderma* spore suspensions + seedling roots dipped in Chitosan solution + Chitosan spray

For raising tomato seedlings, the soil was well prepared through mixing fertilizers and cow dung. The seeds of tomato "Manik" (BARI Tomato-1) were sown in the seed bed and fifteen days aged apparently healthy tomato seedlings were planted in the field in each plot having the size of 2.5 m X 4.0 m where row to row and plant to plant distance was 50 cm. Weeding, irrigation and other intercultural operations were done as and when necessary until the maturity of plants.

Data Collection

Tomato plants were observed regularly immediately after transplantation to record the incidence of post emergence seedling mortality/damping off, different diseases caused by *R. solani*, *S. rolfsii* and *F. oxysporum* f.sp. *lycopersici* until two weeks of the field growth. Infected Tomato plants were identified based on characteristic symptoms of the diseases. The causal agents of the recorded diseases were identified on isolation of the pathogen from the infected leaves, plants and roots. The disease incidence was recorded continuously at 3 days interval from transplanting to final harvest. Observations were made by selecting five plants randomly from each plot. Diseases of the crop were calculated by using the following formula and expressed as percentage:

$$\% \text{ Disease incidence} = \frac{\text{Number of infected plants}}{\text{Total number of plant observed}} X 100$$

$$\text{Per cent disease index (PDI)} = \frac{\text{Summation of all rating}}{\text{Number of plant observed} \times \text{Maximum rating in the scale}} \times 100$$

Disease severity was rated as 0-4 scale in which 0= no symptoms, 1=1-25%, 2=26-50%, 3=51-75% and 4=76-100% of plant organ covered with lesions (Randall, 1980).

Yield and yield components including number of fruits, individual fruit weight were also recorded randomly taken five plants from each replication of all the treatments attain after certain maturity.

Harvesting was done at four different times as tomato fruits were not mature and ripen at the same time.

Experimental design and data analysis

For the *in vitro* assay, the plates were arranged in Completely Randomized Design (CRD) and the field experiment was laid out in a Randomized Complete Block Design (RCBD) with 4 replications. Data recorded on disease components, yield promoting factors and yield were analyzed statistically using the MSTAT-C computer programme after proper transformation whenever necessary. The means were compared following Duncan's Multiple Range Test (DMRT).

Results

Pathogenicity test for the selection of isolates of the test pathogens

The pathogenicity tests of the randomly selected four isolates of each pathogen *F. oxysporum* f. sp. *lycopersici*, *R. solani* and *S. rolfsii* in the pot culture was done to select the most virulent isolate of each pathogen for the screening of *Trichoderma*.All the isolates of the tested pathogens were virulent but variable in causing total seedling mortality of tomato ranged from 62.96 – 93.19 % (Table 1). The isolates TR-1, TF-1 and TS-1 were found as the highly virulent isolates of *R. solani*, *F. oxysporum* f. sp. *lycopersici* and *S. rolfsii*, respectively. The virulence of the isolates SR-1, SF-2 and SS-2 were significantly lower against total mortality of tomato seedlings in comparison to the highly virulent isolates of the tested pathogens.

Table 1. Pathogenicity test of *Fusarium oxysporum* f. sp. *lycopersici*, *Rhizoctonia solani* and *Sclerotium rolfsii* isolates against Tomato variety 'Manik'.

Fungi with isolates	% Mortality*		
	Pre-emergence	Post-emergence	Total mortality
F. oxysporum f.sp. *lycopersici* (SF-1)	51.85	18.52	70.37 c
F. oxysporum f.sp. *lycopersici* (SF-2)	51.85	11.11	62.96 d
F. oxysporum f.sp. *lycopersici* (TF-1)	66.67	18.52	85.19 a
F. oxysporum f.sp. *lycopersici* (TF-2)	62.96	14.81	77.77 b
R. solani (SR-1)	52.55	14.81	67.36 d
R. solani (SR-2)	59.26	11.41	70.67 c
R. solani (TR-1)	78.54	11.11	89.65 a
R. solani (TR-2)	66.64	7.30	73.94 b
S. rolfsii (SS-1)	55.15	14.82	69.97 c
S. rolfsii (SS-2)	51.85	16.52	68.37 c
S. rolfsii (TS-1)	78.37	14.82	93.19 a
S. rolfsii (TS-2)	66.67	11.11	77.78 b
Control	0.00	0.00	0.00

*Means within a column having a common letters do not differ significantly (P=0.05) by DMRT.

In vitro evaluation of T. harzianum isolates for their antagonistic effect on the radial growth of F. oxysporum f. sp. *lycopersici, R. solani and S. rolfsii*

To observe the antagonistic effect of *T. harzianum* a total of selected 20 isolates were tested against the virulent isolates of *F. oxysporum* f. sp. *lycopersici*, *R. solani* and *S. rolfsii* on Potato Dextrose Agar (PDA) by dual culture plate technique. All the 20 isolates of *T. harzianum* showed more than 50% inhibition of radial growth of the tested pathogens as compared with the control (Table 2 and Fig. 1.).

Among the tested isolates, *T. harzianum* Pb27 showed the highest inhibition of the radial growth in case of all the tested pathogens. The lowest 52.99% followed by 57.40% growth inhibition against *R. solani*, 62.10% followed by 62.54% radial growth inhibition against *F. oxysporum* f. sp. *lycopersici*, and 60.73% followed by 61.40% inhibition of radial growth was observed against *S. rolfsii*. Based on the screening the highly antagonist *T. harzianum* Pb27 was selected to prepare the *Trichoderma* spore suspension and *Trichoderma*-fortified compost.

Table 2. Screening of *Trichoderma harzianum* isolates against *Fusarium oxysporum* f. sp. *lycopersici*, *Rhizoctonia solani* and *Sclerotium rolfsii* by dual culture technique.

Isolates of antagonist	% Inhibition		
	F. oxysporum f. sp. *lycopersici*	*R. solani*	*S. rolfsii*
T. harzianum pb21	62.10	73.7	63.32
T. harzianum pb22	85.23	62.55	67.38
T. harzianum pb23	72.22	75.55	66.67
T. harzianum pb24	68.94	73.57	56.66
T. harzianum pb25	66.98	62.57	79.52
T. harzianum pb26	78.98	64.56	77.52
T. harzianum pb27	88.56	90.58	89.28
T. harzianum pb28	63.57	52.99	65.55
T. harzianum pb29	62.54	69.66	76.12
T. harzianum pb30	75.22	80.00	79.33
T. harzianum pb31	76.43	66.65	65.58
T. harzianum pb 32	68.98	74.55	73.57
T. harzianum pb33	74.00	68.88	78.48
T. harzianum pb34	72.59	67.03	65.92
T. harzianum pb35	67.81	82.33	61.47
T. harzianum pb36	76.66	57.40	60.73
T. harzianum pb37	67.57	70.00	62.24
T. harzianum pb38	81.29	73.14	72.99
T. harzianum Pb39	74.67	67.33	71.50
T. harzianum Pb40	68.89	73.76	78.83
Control	9.00 cm	9.00 cm	9.00 cm

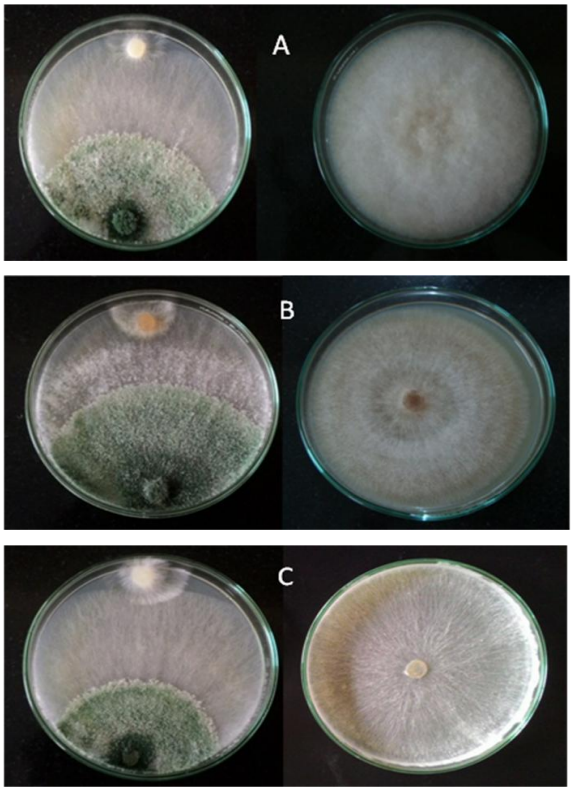

Fig. 1. Radial growth inhibition of different test pathogens by *T. harzianum* isolate pb27 on PDA plate following dual culture technique A) *Trichoderma* and *Fusarium oxysporum* f. sp. *lycopersici* B) *Trichoderma* and *Rhizoctonia solani* C) *Trichoderma* and *Sclerotium rolfsii*.

In vitro evaluation of chitosan for their inhibitory effect on the radial growth of F. oxysporum f. sp. *lycopersici, R. solani and S. rolfsii*

The inhibitory effect of chitosan against the virulent isolates of *F. oxysporum* f. sp. *lycopersici*, *R. solani* and *S. rolfsii* was tested *in vitro* by using four concentrations i.e. 100, 200, 400 and 800 ppm (Fig. 2.). Result presented in Table 3 indicates that all tested concentrations have significantly reduced the mycelial growth of tested pathogen. The highest reduction was obtained with chitosan used at 800 ppm where the mycelial growth was reduced by up to 78.61%, 81.11% and 77.22% of *F. oxysporum* f. sp. *lycopersici*, *R. solani* and *S. rolfsii*, respectively. Therefore chitosan 800 ppm was selected for the field experiment.

Individual and Integration effect of T. harzianum and chitosan in controlling different soil-borne diseases and improving yield of tomato in the field

To control various soil-borne diseases of tomato developed under natural epiphytotic condition, *Trichoderma* spore suspension as root dipped and chitosan 800 ppm both as spray and root dipped were used either individually or in combination with *Trichoderma*-fortified compost.

Table 3. Effect of Chitosan against *Fusarium oxysporum* f. sp. *lycopersici*, *Rhizoctonia solani* and *Sclerotium rolfsii* on PDA plate.

Treatments	*F. oxysporum* f. sp. *lycopersici*		*R. solani*		*S. rolfsii*	
	Radial growth(cm)	% inhibition	Radial growth(cm)	% inhibition	Radial growth(cm)	% inhibition
Control	9.00 e	-	9.00 e	-	9.00 e	-
Chitosan 100 ppm	4.85 d	46.22	7.05 d	21.67	7.85 d	12.77
Chitosan 200 ppm	3.75 c	58.33	5.25 c	41.67	6.70 c	25.56
Chitosan 400 ppm	2.675 b	70.28	2.45 b	72.78	4.05 b	55.00
Chitosan 800 ppm	1.925 a	78.61	1.70 a	81.11	2.05 a	77.22

*Means within a column having a common letters do not differ significantly (P=0.05) by DMRT.

In all the treatments, post-emergence seedling mortality was significantly lower in comparison to the untreated control (T_1) where no *Trichoderma* or chitosan was applied in any form (Table 4). Significantly the lowest seedling mortality was observed when all the components were integrated in the treatment T_{11} where *Trichoderma* and/or chitosan suspension were used in different forms along with the individual application of *Trichoderma*-fortified poultry refuge compost. However, statistically no difference was found between T_{10} and T_{11} for seedling mortality. Other treatments (T_4, T_3 and T_2) showed similar effect on seedling mortality of tomato but superior to the control and inferior to all other treatments (Table 4).

Moreover, a total of three diseases mainly Fusarium wilt caused by *F. oxysporum* f. sp. *lycopersici*, Rhizoctonia dry root rot or wet root rot caused by *R. solani*, and collar rot or southern blight caused by *S. rolfsii*, were recorded during the production of the crop in the field. The disease incidence of fusarium wilt was also observed significantly the highest reduction in the same (T_{11}) followed by T_{10} where except foliar spray with chitosan was not applied in comparison to the control treatment T_1 (Table 5). However, both the treatments (T_{10} & T_{11}) were found significantly identical and appeared superior in observing the disease incidence of Rhizoctonia root rot, and collar rot or southern blight of tomato as compared to control (T_1) treatment. Interestingly, disease severity expressed was highest in fusarium wilt among the soil-borne diseases in case of all treatments. Disease severity was significantly lower but identical in the treatments T_8, T_9, T_{10}, and T_{11} for fusarium wilt and T_7, T_8, T_9, T_{10}, T_{11} for both Rhizoctonia root rot, and collar rot or southern as compared to control (T_1).

Similarly, the effect of *Trichoderma* and chitosan on the yield component and total yield of tomato were varied depending on the treatments combinations (Table 6). The highest numbers of fruits and yield were observed in T_{11}, where integrated treatments with *T. harzianum* pb27 spore suspension for root dipping, chitosan suspension for spray and root dipping along with *Trichoderma*-fortified poultry refuge compost were incorporated; the lowest was in control plots (T_1), where untreated tomato seedling were sown in field soil. Significantly the highest yield (50.99%) was increased in the treatment T_{11} followed by 47.08% % increased yield in the treatment T_{10}. Other treatments (T_7, T_8, and T_9) showed a statistically similar effect on tomato yield but inferior to the treatment T_{11} and T_{10}. However, all the supplements added treatments increased yield significantly in comparison the untreated control treatment.

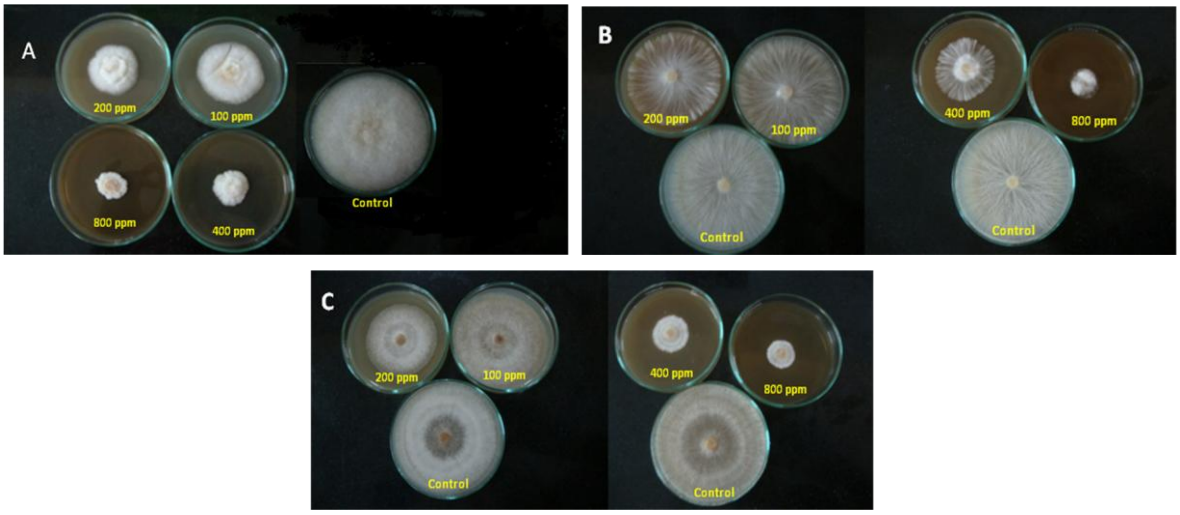

Fig. 2. Growth inhibition of different pathogens by Chitosanin on PDA plate A) Chitosan and *Fusarium oxysporum f.* sp. *lycopersici* B) Chitosan and *Sclerotium rolfsii* C) *Chitosan* and *Rhizoctonia solani*.

Table 4. Effect of *Trichoderma* and chitosan in controlling seedling mortality of tomato in open field.

Treatments	Post-emergence mortality (%)*	Reduction of total mortality (%)
T_1=Control without *Trichoderma* or chitosan	18.33 a	------
T_2= Tomato seedlings spray with chitosan solution	14.00 b	23.62
T_3=Seedling roots dipped in chitosan solution	13.33 b	27.27
T_4= Seedling roots dipped in *Trichoderma* spore suspensions (5 x 10^6 spores ml^{-1})	13.33 b	27.27
T_5= *Trichoderma* fortified compost applied in the field	10.33 c	35.89
T_6= *Trichoderma* fortified compost + Seedling roots dipped in *Trichoderma* spore suspensions	10 .00 c	45.44
T_7= Trichoderma fortified compost + Chitosan spray	6.00 d	79.54
T_8= *Trichoderma* fortified compost + Seedling roots dipped in Chitosan solution	6.67 d	63.61
T_9=*Trichoderma* fortified compost + Seedling roots dipped in *Trichoderma* spore suspensions + Chitosan spray	5.75 d	63.44
T_{10}=*Trichoderma* fortified compost + Seedling roots dipped in *Trichoderma* spore suspensions + seedling roots dipped in Chitosan solution	3.75 e	68.63
T_{11}= *Trichoderma* fortified compost + Seedling roots dipped in *Trichoderma* spore suspensions + seedling roots dipped in chitosan solution + Chitosan spray	3.33 e	81.83

*Means in a column followed by the same letters does not differ significantly (p= 0.05) according to Duncan's multiple range test.

Table 5. Effect of *Trichoderma* and chitosan on disease incidence of soil-borne disease of tomato in the field condition.

Treatments	*Fusarium* wilt*		*Rhizoctonia* root rot*		collar rot/ southern blight*	
	% Disease incidence	PDI	% Disease incidence	PDI	% Disease incidence	PDI
T_1	35.0 a	34.0 a	25.4 a	27.0 a	27.6 a	24.3 a
T_2	30.0 b	27.5 b	21.0 b	22.5 ab	25.3 ab	20.0 ab
T_3	30.0 b	26.3 b	19.3 b	20.0 abc	24.0 bc	19.3 ab
T_4	24.7 c	23.0 bc	15.8 c	18.8 bc	21.9 c	19.0 abc
T_5	20.3 d	20.3cd	12.1 d	17.6 bcd	18.5 d	17.0 abc
T_6	18.0 e	17.0 de	10.2 de	15.5 bcde	16.3 de	15.5 bcd
T_7	13.4 f	14.4 def	8.5 ef	12.5 cdef	14.0 ef	12.3 cde
T_8	10.0 gh	11.3 efg	7.6 f	8.8 ef	10.9 gh	8.8 de
T_9	11.0 fg	12.5 efg	8.4 ef	10.0 def	12.9 fg	11.0 cde
T_{10}	9.2 h	8.8 fg	6.3 fg	7.5 ef	8.8 hi	7.8 de
T_{11}	5.0 i	8.0 g	5.0 g	6.3 f	7.5 i	6.3 e

*Means within same column followed by common letter(s) are not significantly different (P=0.05) by DMRT. Mean disease severity of four replicates, rated 0-4, in which 0= no symptoms, 1=1-25%, 2= 26-50%, 3= 51-75%, and 4=76-100% of the organ covered with lesions.

Table 6. Effect of *Trichoderma*-fortified compost and *chitosan* on the yield and yield component of tomato in the field.

Treatments	Total no. of fruit*	Individual fruit wt* (g)	Yield* (ton /h)	% increased yield
T_1	79.00g	41.56 d	8.74 f	-
T_2	83.77f	43.72 cd	9.83 ef	11.03
T_3	88.67e	44.99 bcd	10.61 de	17.55
T_4	95.75 d	43.80 cd	11.20 de	21.87
T_5	97.00 d	44.53 cd	11.52 d	24.08
T_6	115.0 b	46.15 bcd	114.00 c	27.10
T_7	115.7 b	47.72 abcd	14.72 c	40.57
T_8	118.8 b	50.15 abc	15.86 bc	44.86
T_9	119.7 b	50.52 abc	16.13 bc	45.76
T_{10}	120.0 b	51.71 ab	16.53 ab	47.08
T_{11}	126.8 a	52.71 a	17.86 a	50.99

*Means within same column followed by common letter(s) are not significantly different (P=0.05) by DMRT.

Discussion

Seedling mortality caused by soil-borne pathogens such as *F. oxysporum* f. sp. *lycopersici*, *R. solani* and *S. rolfsii* is primarily known as one of the serious threats in tomato cultivation. These pathogens are particularly exigent because they often stay alive in soil for many years and can significantly decrease the yield and quality of vegetable crops.

The experiment was conducted to reduce the post-emergence seedling mortality and diseases of tomato at different growth stages caused by several soil-borne fungal pathogens and improve yield of tomato in the field under natural epiphytotic conditions through the application of *T. harzianum* and chitosan based-treatments at different combinations.

In the pot culture, we observed the disease mortality caused by *F. oxysporum* f. sp. *lycopersici*, *S. rolfsii*, *R. solani*. As warmth-dependent pathogens, they are most frequently occurred in Bangladesh soil. In recent years, seedling mortality of different vegetable crops including tomato caused by these pathogens was also reported by several investigators (Begum and Bhuiyan, 2007; Akhter *et al.*, 2015). In the current study, ample intra-isolate variation in pathogenicity was recorded in case of all the tested pathogens. Previously, researchers highlighted the value of combining several morphological and pathological attributes to distinguish between strains (Parmeter, 1970) but later it was reported that these characters were more distinctive among the isolates when they were genetically diverse (Demers *et al.*, 2015, Xie *et al.*, 2014; Zhou *et al.*, 2009). Therefore, seedling mortality disease in tomato fields in Bangladesh is occurred due to the mixture of pathomorphologically incongruent groups of the tested pathogens. These results are decisive for controlling seedling mortality disease in Bangladesh.

In vitro assay clearly revealed that antagonists *T. harzianum* showed radial mycelium growth inhibition of highly virulent isolate *F. oxysporum* f.sp. *lycopersici*, *R. solani* and *S. rolfsii*, although different isolates displayed varying levels of antagonism against the pathogens. Moreover, the lab studies also showed that chitosan was also able to inhibit the growth of these pathogens. Many researchers also reported the significant effect of *T. harzianum* against *R. solani* and *S. rolfsii* infecting many other crops in Bangladesh (Uddin *et al.,* 2011; Akhter *et al.,* 2015; Faruk and Rahman, 2015). The species of *Trichoderma* are early colonizers of substrates and reduce the activity of other fungi simply by substrate occupation and exhaustion (Martin and Loper, 1999) and this would be in accord with the present observations. The variation among the different isolates of *T. harzianum* may be occurred due to their genetic makeup for the antagonistic activity (Shanmugam *et al.,* 2008, Kumar *et al.*, 2011), virulence factor such as metabolites (Shentu *et al.*, 2014), degree of virulence, trichodene (Malmierca *et al.*, 2015) etc. A variety of extracellular lytic enzymes such as chitinase and β-(1,3)-glucanase produced by *T. harzianum* seems to cooperate in parasitism (Kumar *et al.,* 2012) and there may be a link between the ability to inhibit the pathogens and the production of these enzymes. Our results revealed that the radial mycelial growth of the pathogens was restricted within the contact area or interface zone, indicating the interference of the mycelium of plant pathogenic fungi by *T. harzianum*. The fungicidal activity of chitosan has been well documented both in *invitro* and *in situ* studies. It is believed that the polycationic nature of this compound is the key to its antifungal properties and that the length of the polymer chain enhances its antifungal activity (Hirano and Nagao, 1989). In this respect Abd-El-Kareem *et al.,* 2006 showed that chitosan @ 6 g/L completely inhibited the linear growth of all tomato root rot fungi. Moreover, there is strong evidence that mycelial growth can be inhibited or retarded when the growth media of fungi are amended with chitosan (Oliveira *et al.*, 2012; Bautista-Banos *et al.*, 2006). Based on the *in vitro* screening result, chitosan 800 ppm was used both as spray and root dipped either individually and in combination with *Trichoderma*-fortified compost for the field experiment.

Research has demonstrated that biological control of soil-borne diseases of tomato has been successful in some instances under greenhouse. However, it is so difficult to control in field-grown tomatoes using the single methods because these pathogens rapidly colonizes and persists for long periods in the soil. In this concern, we applied an integrated management strategy that combined the use of *Trichoderma*-fortified compost along with chitosan foliar spray and transplants root dipped in both *Trichoderma* and chitosan suspension was found to be significantly more superior in reducing seedling mortality incidence, other soil-borne diseases such as wilt and root rot and improving yield in tomato when compared to dual and individual application of them. Different mechanisms have been implied as being responsible for the action of individual bio-agents and chitosan.

Trichoderma is considered as potential microbial antagonists as they are effective a range of economically important phytopathogenic fungi. The bio-control activity of *T. harzianum* includes mycoparasitism, antibiosis, lysis, competitive, metabolites secretions, enhancement of plant growth and modulation of induced resistance (Schirmböck *et al*., 1994; Howell, 2003). Several studies revealed that *T. harzianum* strains produced some metabolites that inhibited growth of fungi and pre-treatment of soil with *Trichoderma* significantly reduced the severity of tomato root rot diseases (Alamri *et al*., 2012; Altinok and Erdogan, 2015) which are accordance in our results. Besides, chitosan obtains different properties, *i.e.* its inhibitory effect against pathogenic fungi (Bautista-Banos *et al*., 2006) and its ability to be potent elicitors of plant defense reactions (Amini, 2009). Many workers used chitosan in reducing diseases of various crops (Abd-El-Karem *et al*., 2006; Ramírez-Arrebato *et al*., 2016). The reduction in tomato root rot incidence obtained in our study might be attributed due to indirect effect of chitin treatments and its elicitor defense response in plants. Additionally, tomato plants treated with chitosan might be increased enzymes activities (Abd-El-Karem *et al*., 2006) which can promote the growth of the plant. Chitosan may release volatiles for chitin decomposition such as ammonia that can suppress some soil-borne fungi (Hora and Baker, 1972). In addition, the natural disease suppressive effects of composts are due to increase in microbial biomass and it aids in their introduction and establishment into the soil for sustained bio-control activities of soil microbiota (Hoitink and Boehm, 1999). The increased of tomato yield obtained in the present study might be happened due to the decline in disease incidence and promotion of plant growth as influenced by fertilizer-like effect of chitosan and *Trichoderma* (El-Tantawy, 2009; Rahman, 2013). These results are also in accordance with many other findings highlighting that *T. harzianum* (Stewart and Robert, 2015) and chitosan based-treatments enhance growth of tomato (Howell, 2003). It could be suggested that combined treatments between *Trichoderma* and chitosan in different forms might be used commercially as easily, safely and applicable cheap method for controlling tomato root diseases under field conditions.

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