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Efficacy of Some Rhizosphere Microorganisms in Controlling Fusarium Wilt of Tomato (*Lycopersicon esculentum*)

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Abstract

An experiment was conducted at the Disease Diagnostic Laboratory and Net House of Plant Pathology Department, Sher-e-Bangla Agricultural University, Dhaka during July 2011 to June 2012 to investigate the efficacy of some antagonistic rhizosphere microorganisms against *Fusarium oxysporum* f. sp. *lycopersici* causing Fusarium wilt of tomato. Probable 20 antagonistic bacterial isolates and one antagonistic fungal isolate (*Trichoderma harzianum*) from rhizosphere soil were screened out against *F. oxysporum* f. sp. *lycopersici*. Out of 20 bacterial isolates, only four (Iso-1, Iso-2, Iso-3 and Iso-4) were found effective in dual culture method. Out of all antagonists, *Trichoderma harzianum* produced the highest inhibition zone (75.75 %), while the lowest inhibition zone (28.39 %) was produced by Iso-2. In blotter method, the highest prevalence of *Fusarium oxysporum* was observed in control (11.25%), while the lowest was in Iso-1 (0.58%) treated seeds.

Keywords: Rhizosphere microorganisms, control, fusarium wilt, tomato

1. Introduction

Tomato (*Lycopersicon esculentum*) is one of the most popular and important commercial vegetable grown throughout the world. Bangladesh produced 324 thousand metric tons of tomato from 104 thousand hectares of land (BBS, 2011). Many diseases and disorders can affect tomatoes during the growing season. Fusarium wilt is one of the serious diseases of tomato throughout the world, especially in Bangladesh. This disease is caused by *Fusarium oxysporum* f. sp. *lycopersic*i leading to serious economic losses (Snyder and Hansen, 1940).

There may be 60 to 70% yield loss due to this disease (Kirankumar *et al.*, 2008). The excessive misuse of a wide range of fungicides has resulted this to be harmful to the environment and increased the resistant pathogen populations (Ogzonen *et al.*, 2001). *F. oxysporum* f. sp. *lycopersici* becomes resistant to those chemical fungicides. For this reason, alternative methods to control the disease have to study with emphasis on biological control using fungi or bacteria to reduce fungicide application and decrease cost of production. As this pathogen is soil borne, biological control could be a suitable measure against it. It is attractive in an environmental and economic sense offering durable, safe and cost effective means to control the diseases as an alternative to chemicals (Merriman and Russell, 1990). Moreover biological control is less disruptive to ecosystem than that of chemical pesticides (Cook and Baker, 1983).

Despite economic importance, sufficient information has not been generated so far about the management of this worldwide prevalent disease. Therefore, the study was conducted to search for suitable antagonistic rhizosphere microorganisms against it.

2. Materials and Methods

For studying the antagonistic ability of *Trichoderma herzianum* and some bacterial antagonists against *Fusarium oxysporum* f. sp. *lycopersici* the antagonistic fungus as well as the bacterial isolates was isolated from rhizosphere soil. Bacteria were isolated by dilution plate method of Goszczynska and Serfontein (1998) and colony was purified on NA plates. Similarly, *T.herzianum* was isolated and purified on PDA plates following a standard method (Ashrafuzzaman, 1976). For the study *F. oxysporum* f. sp. *lycopersici* was isolated from seeds of tomato variety BARI tomato-2, collected from Bangladesh Agricultural Research Institute, Gazipur. Prevalence of other fungi on the collected seeds was also studied by blotter method (ISTA, 1996). The antagonistic ability of the bacterial isolates was studied *In-vitro* following a dual culture method described by Azadeh *et al*. (2010). A 5 mm plug of the fungus *F. oxysporum* f. sp. *lycopersici* at the centre of a petri-dish containing 25 ml PDA, then the test bacterial isolate was streaked 3 cm away from the fungal plug both sides towards the edge of the plate by a loop loaded with 48 hr old bacterial culture. A control was maintained without streaking of bacteria in the plate. The test plates were then incubated at 28^0C for 7 days. Similarly, for testing the antagonism of *T. herzianum* the dual culture technique described by Sundar *et al.* (1995). Discs of 5mm diameter of both the test fungi were cut with a sharp cork borer from the growing edge of the culture plate. A 5mm block (7 days old) of *T. herzianum* was placed to PDA plate on one side and another block of *F. oxysporum* f. sp. *lycopersici* was placed at the other side. A plate only with the disc of *F. oxysporum* f. sp. *lycopersici* at the centre was used as control. The test plates were then incubated at room temperature (25 ± 2^0C) until the mycelium of *F. oxysporum* f. sp. *lycopersici* covers the whole plate. The inhibition percentage of *F. oxysporum* f. sp. *lycopersici* was calculated using the formula suggested by Sundar *et al.* (1995).

2.1. Characterization of Bacteria

Bacterial isolates were characterized through morphological and biochemical studies. Morphological characters like colony color, shape and surface textures were carefully studied and recorded using 24 hrs of cultured bacterial isolates on NA medium.

2.1.1. Gram's staining reaction

Part of a young colony was heat fixed on a glass slide. It was flooded with Crystal violet solution for 1 minute and washed with running tap water, excess water was removed by air drying. Then it was flooded with Iodine solution (Lugol’s Iodine) for 1 minute, washed with tap water and excess water was removed by air drying. After that it was decolorized with 95% Ethanol for 30 seconds, again washed with tap water and air dried. Then it was counterstained with 0.5% Safranine for 10 seconds and washed briefly under tap water, excess water was removed by air drying. Finally it was examined under microscope at 100X magnification.

2.1.2. Biochemical characters

For potassium hydroxide test, a loop-full of bacteria from a well grown colony was mixed with a drop of 3% aqueous KOH on a glass slide. A sterilized needle was raised a few centimeters from the glass slide and repeated strokes to have strands of viscid materials as described by Suslow *et al.* (1982). For Starch hydrolysis test, a nutrient agar plate containing 0.2% soluble starch was inoculated with the bacterium isolate to be tested, incubated for at least 48 hours, flooded with Lugol’s iodine solution and observed. For catalase test, bacterial isolates were taken on a slide on which

one drop of 3% H_2O_2 (Hydrogen Peroxide) was added and observed. For oxidase test, a portion of the test organism was picked up on the wet oxidase disk containing tetramethyl-p-phenylene-diamine dihydrochloride and color changed was obsrerved. For pecteolytic test, potato tubers were disinfected with 99% ethanol, cut into slices of about 7-8 mm thick and placed on moistened sterile filter paper in sterile petri-dish. Bacterial cell suspension was pipetted into a depression cut in the potato slices. One potato slice pipetted with sterile water was treated as control. Development of rot on the slices was examined 24–48 h after incubation at 25°C. For citrate utilization test, a portion of the test organism was streaked into Simmon's citrate agar slants. Following incubation at 30°C for 24 hours color change was observed. For gelatin liquefaction test, one loop-full bacterial culture was stub inoculated into the media and incubated at 30°C for 24 hours. Gelatin liquefied microorganisms is detected by the formation of liquid culture in the presence of 4°C refrigerator. Hypersensitivity test of the isolated bacteria was done in tobacco plant as described by Wick (2006), where aqueous suspensions of the isolated bacteria at 10^8cfu/ml were injected into the intercellular space of the lower leaves.

2.2. Efficacy of seed treatment with microbial antagonists

For seed treatment with bacterial antagonist, bacterial cultures were scraped out and 10^8 cfu/ml suspensions were made in sterile distilled water in a beaker for each treatment. 400 seeds for each beaker were dipped in those beakers for four hours. For seed treatment with *T. harzianum,* spore suspension was made by scraping the 10-15 days old culture substrate with the help of blender @ one petriplate in 250 ml water to adjust the concentration 10^7 conidia/ml solution. Collected tomato seeds were dipped in the spore suspension of *T. harzianum* for 1hr. Four hundred seeds were surface sterilized with Chlorax @ 3% and washed in distilled water served as control.

The study comprised of six treatments viz. T_1= Seed treatment with Bacterial Isolate 1 (Iso-1), T_2= Seed treatment with Bacterial Isolate 2 (Iso-2), T_3= Seed treatment with Bacterial Isolate 3 (Iso-3), T_4= Seed treatment with Bacterial Isolate 4 (Iso-4), T_5= Seed treatment with *Trichoderma harzianum* and T_6= Control (Untreated).

Prevalence of *F. oxysporum* in treated and untreated seeds was determined by blotter method (ISTA, 1996). Twenty five seeds were placed on three layers of moist blotting paper (Whatman No. 1) in each glass petri-dish. The petri-dishes were incubated at 25±1ºC under 12/12 hrs light and darkness cycle for 7 days. Each seed was observed in order to record the presence of fungus 7 days after incubation based on growth habit. The results were presented as percent incidence and germination of the seeds were also recorded.

Data collected during experimental period were tabulated and analyzed following statistical package MSTAT-C. Treatment means were compared with Duncan's Multiple Range Test (DMRT) (Gomez and Gomez, 1984).

3. Results and Discussion

In blotter test it was found that the seeds were infected by *Fusarium oxysporum* f. sp. *lycopersici* (11.25%), *Aspergillus flavus* (12%) and *Curvularia* sp. (5.25%), respectively. White, fluffy mycelial colony of *Fusarium oxysporum* f. sp. *lycopersici* was isolated and purified from infected tomato seeds in PDA plate (Fig. 1). Similar types of colony were found by Begum (2007) and Altinok (2005).

Altogether 20 bacterial isolates were collected from soil samples using dilution plate technique and purified on NA medium. Of them, only four (Iso-1, Iso-2, Iso-3 and Iso-4) were recorded as antagonistic and were found to inhibit the growth of *F. oxysporum* f. sp. *lycopersici* in dual culture. The highest (34.83%) growth of *F. oxysporum* f. sp. *lycopersici* was inhibited by Iso-1 which was followed by Iso-4 (31.41%), Iso-3 (29.96%) and Iso-2 (28.39%) as shown in Table 1 and Fig. 2.

Among all the microbial antagonists studied the highest (75.75%) inhibition of *F. oxysporum* f. sp. *lycopersici* was observed in case of *Trichoderma harzianum* (Table 1 and Fig. 2). Sundar *et al.* (1995) and Deshmukh and Raut (1992) reported that *Trichoderma harzianum* grew over the colonies of *Fusarium oxysporum*. Chabbi and Matrod (2002) achieved 77% growth inhibition of *Fusarium oxysporum* with *Trichoderma harzianum.*

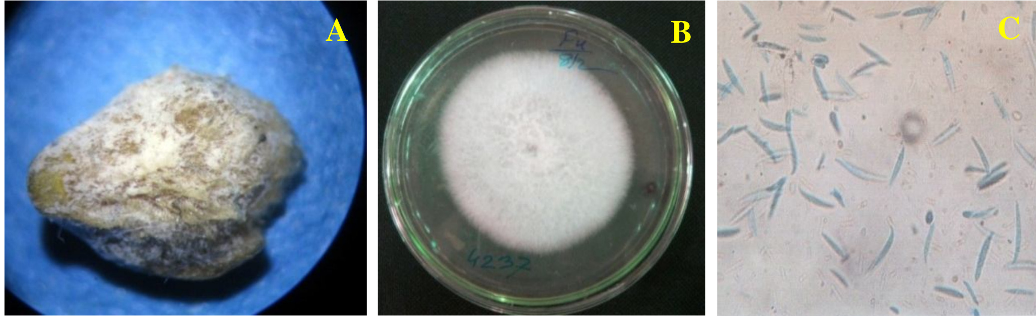

Fig. 1. A. *Fusarium oxysporum* f. sp. *lycopersici* on tomato seed under stereo microscope (50x)
B. Pure culture of *Fusarium oxysporum* f. sp. *lycopersici* on PDA
C. Conidia of *Fusarium oxysporum* f. sp. *lycopersici* under compound microscope (40x)

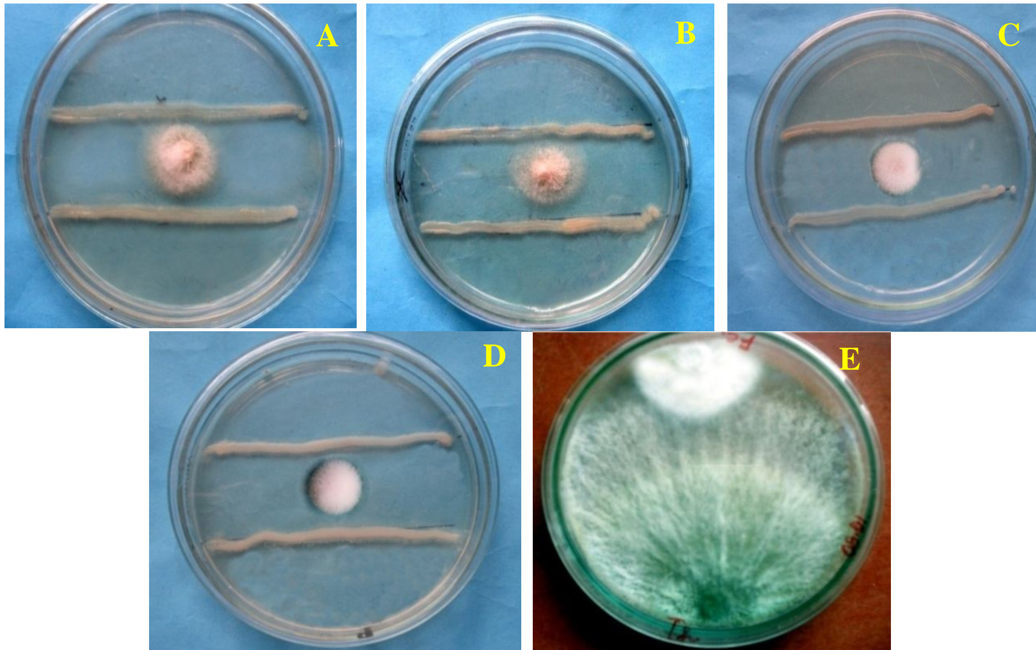

Fig. 2. Dual culture of *Fusarium oxysporum* f. sp. *lycopersici* with different rhizosphere microorganisms; A. Bacterial Iso-1, B. Bacterial Iso-2, C. Bacterial Iso-3, D. Bacterial Iso-4 and E. *Trichoderma harzianum*

Table 1. Anti-fungal activity of antagonists studied against *Fusarium oxysporum* f. sp. *lycopersici* in dual culture method

Name of Antagonists	% Inhibition
Iso-1	34.83 b
Iso-2	28.39 d
Iso-3	29.96 cd
Iso-4	31.41 c
Trichoderma harzianum	75.75 a
$LSD_{(0.05)}$	2.944
CV%	3.68

Data having common letter (s) do not differ significantly ($p \leq 0.05$) by DMRT.

3.1. Characterization of Bacteria

Bacterial isolate Iso-1 showed creamy white, but other three showed slight yellow colored colony on NA medium after 24 hrs of incubation. All of them had dome shaped, raised colony.

In Gram's staining, all antagonistic bacteria showed negative reaction and all the bacteria produced pink color, straight and curved rod with no particular arrangement (Table 2). All the bacterial isolates showed strands of viscid materials in repeated strokes in KOH solubility test. All the bacterial isolates except Iso-4 made clear zone after giving Lugol's iodine in agar plate containing 0.2% soluble starch. All the isolates formed bubble while mixing with 3% H_2O_2. Iso-2 formed dark purple color immediately while Iso-3 and Iso-4 produced after 20 seconds of picking the bacteria on to the oxidase disk and Iso-1 give negative result (Fig. 3 A). All bacterial isolates were unable to produce rot in potato slice. All the three isolates except Iso-1 changed Simmon's citrate agar slants color from green to bromothymol blue (Fig. 3 A). All the isolates formed liquid culture in 4°C refrigeration. The bacterial isolates obtained from soil were not positive in hypersensitivity reaction on tobacco plants. Kreig (1923) and Schaad *et al.* (2001) reported that all the above characteristics are similar with the characteristics of different species of *Pseudomonas*. Thus, the isolates may be different species of *Pseudomonas*. Similar reports were given by Hossain (2006) and Wick (2006).

Fig. 3. A. Oxidase test and B. Citrate utilization test for different bacterial isolates

Table 2. Biochemical characteristics of used antagonistic bacteria

Tests	Reaction of the antagonistic bacteria			
	Iso-1	Iso-2	Iso-3	Iso-4
Gram's staining reaction	-	-	-	-
KOH solubility Test	+	+	+	+
Starch Hydolysis	+	+	+	-
Catalase Test	+	+	+	+
Oxidase Test	-	++	+	+
Pecteolytic Test	-	-	-	-
Citrate Utilization Test	-	+	+	+
Gelatin liquefaction test	+	+	+	+
Tobacco Hypersensitivity Test	-	-	-	-

++ = Strongly positive, + = Positive, - =Negative

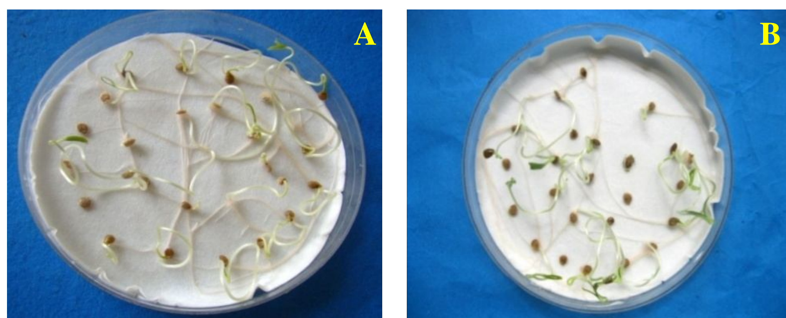

Fig. 4. Efficacy of seed treatment with selected microbial antagonists on incidence of *Fusarium oxysporum* f. sp. *lycopersici*; A. Tomato seeds treated with antagonistic bacteria and B. Untreated control

Table 3. Effect of different microbial antagonists on germination and incidence of seed borne fungi of BARI tomato-2 seed

Name of antagonists	Germination (%)	Incidence of seed borne fungi (%)		
		Aspergillus spp.	*Fusarium* spp.	*Curvularia* spp.
T_1	91.67 a	1.250 c	0.583 c	2.250 b
T_2	86.00 b	4.250 b	3.500 bc	2.250 b
T_3	85.53 b	4.167 b	3.500 bc	0.333 c
T_4	86.67 b	11.00 a	3.917 b	2.333 b
T_5	85.00 b	1.750 c	0.583 c	2.333 b
T_6 (Control)	81.00 c	12.00 a	11.25 a	5.250 a
$LSD_{(0.05)}$	1.814	1.197	0.8931	0.3203
CV %	1.16	9.01	9.49	8.32

Means within the same column with a common letter (s) are not significantly different ($p \leq 0.05$) by DMRT.
[T_1= Seed treatment with Bacterial Isolate 1 (Iso-1), T_2= Seed treatment with Bacterial Isolate 2 (Iso-2), T_3= Seed treatment with Bacterial Isolate 3 (Iso-3), T_4= Seed treatment with Bacterial Isolate 4 (Iso-4), T_5= Seed treatment with *Trichoderma harzianum* and T_6= Control (Untreated).]

4. Conclusions

The present study revealed that among all the microbial antagonists, *Trichoderma harzianum* was highly effective and among the bacterial isolates, Iso-1 was highly effective against *F. oxysporum* f. sp. *lycopersici.* Different biochemical tests revealed that the bacterial isolates may be different species of *Pseudomonas*. Therefore, *Trichoderma harzianum* and bacterial isolate, Iso-1 could be used as biocontrol agents against *F. oxysporum* f. sp. *lycopersici.*

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