EFFICACY OF DIFFERENT MICROBIAL ANTAGONISTS IN CONTROLING FUSARIUM WILT OF TOMATO

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JUNE, 2012

EFFICACY OF DIFFERENT MICROBIAL ANTAGONISTS IN CONTROLING FUSARIUM WILT OF TOMATO

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Registration No. 10-04237

A Thesis
Submitted to the faculty of Agriculture
Sher-e-Bangla Agriculture University, Dhaka
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE
IN
PLANT PATHOLOGY

SEMESTER: JULY-DECEMBER, 2010

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CERTIFICATE

This is to certify that the thesis entitled**,** *"EFFICACY OF DIFFERENT MICROBIAL ANTAGONISTS IN CONTROLING FUSARIUM WILT OF TOMATO"* submitted to the Department of Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka in partial fulfillment of the requirements for the degree of *MASTER OF SCIENCE* **in** *PLANT PATHOLOGY* embodies the results of a piece of bona fide research work carried out by **Md. A. Matin Sarker** bearing Registration No**.** **10-04237** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

I further certify that such help or source of information, as has been availed of during the course of this investigation has been duly acknowledged.

Dated: 20-09- 2012
Place: Dhaka, Bangladesh

.....
Nazneen Sultana
Supervisor

Dedicated
to
My Beloved Parents
and
Elder Brother

ACKNOWLEDGEMENT

All the praises and gratitude are due to the omniscient, omnipresent and omnipotent Almighty Allah, who has kindly enabled the author to complete this research work and complete this thesis successfully for increasing knowledge and wisdom.

The author sincerely desires to express his deepest sense of gratitude, respect, profound appreciation and indebtedness to his research Supervisor, ***Nazneen Sultana****, Associate Professor and Chairman, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for her kind and scholastic guidance, untiring effort, valuable suggestions, inspiration, co-operation and constructive criticisms throughout the entire period of research work and the preparation of the manuscript of this thesis.*

The author expresses heartfelt gratitude and indebtedness to his Co-supervisor, ***Dr. Md. Rafiqul Islam****, Professor, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for his co-operation, criticisms on the manuscript and helpful suggestions for the successful completion of the research work.*

Special thanks and indebtedness to all the respective teachers of the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for their valuable teaching, sympathetic co-operation and inspiration throughout the period of the study.

Thw research work is a project funded by UGC. So, the author is specially thankful to UGC for funding the project.

The author thankfully remembers the students of the Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for their cooperation in the entire period of study. The author also expends his thanks to all the staff of the

Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for their help and cooperation during the research work.

The author also likes to give thanks to all of his friends for their support and inspiration throughout his study period in Sher-e-Bangla Agricultural University, Dhaka.

The author also expresses his thanks specially to Mamun-Or-Rashid, Monir vai, Shimul, Jabbar, Gaziul Haque and Dhriti apu for their cordial support, cooperation and inspiration in preparing this thesis.

Finally, the author found no words to thank his parents and his elder brother for their unquantifiable love and continuous support, their sacrifice, never ending affection, immense strength and untiring efforts for bringing his dream to proper shape. They were constant source of inspiration, zeal and enthusiasm in the critical moment of his studies.

Dated: June, 2012 *The Author*

Place: SAU, Dhaka.

EFFICACY OF DIFFERENT MICROBIAL ANTAGONISTS IN CONTROLING FUSARIUM WILT OF TOMATO

ABSTRACT

The experiment was conducted in the Disease Diagnostic Laboratory and Net house of Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka during the period of July 2011-June 2012 to search suitable antagonistic microorganism against *Fusarium oxysporum* f. sp. *lycopersici* causing Fusarium wilt of tomato.Twenty probable antagonistic bacterial isolates were isolated from rhizosphere soil and screening was done against *F. oxysporum* f. sp. *lycopersici* to detect the promising antagonistic microorganism. One antagonistic fungal isolate *Trichoderma harzianum* also collected from laboratory of Bangladesh Agricultural Research Institute (BARI), Gazipur and assayed as antagonist against *F. oxysporum* f. sp. *lycopersici*. Out of twenty bacterial isolates, only four isolates (Iso-1, Iso-2, Iso-3 and Iso-4) were found effective in dual culture method. Out of all test organisms, *Trichoderma harzianum* produced the highest percent inhibition zone (75.75 %) and the lowest percent 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%). In pot culture, the efficacy of microbial antagonist in controlling Fusarium wilt of tomato was done by root dipping and soil drenching method. The highest disease incidence was observed in control (40% in 20 DAT and 60% in 30 DAT) and the lowest was observed in Iso-1 and *T. harzianum* (0% in 20 DAT, 10% in 60 DAT). Disease severity was also the lowest (PDI 0.00% in both 10 DAT and 20 DAT and 3.33% in 60 DAT) in Iso-1. Thus Iso-1 can be used as a bio-control agent against *F. oxysporum* f. sp. *lycopersici* causing wilt of tomato.

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Chapter I

Introduction

INTRODUCTION

Tomato *(Lycopersicon esculentum*) is one of the most popular and important commercial vegetable crops grown throughout the world. It is rich in vitamins A, B and C. Bangladesh produces 137 thousand metric tons of tomato per year from 47.96 thousand acres of land with an average yield of 2855 kg/acre (BBS, 2008). Many diseases and disorders can affect tomatoes during the growing season. *Fusarium oxysporum* f. sp. *lycopersici* (FOL) is a highly destructive pathogen of both greenhouse and field grown tomatoes in warm vegetable production areas. The disease caused by this fungus is characterized by wilted plants, yellowed leaves and minimal or absent crop yield. There may be a 60 to 70% yield loss (Kirankumar *et al.*, 2008).

Fusarium wilt is one of the most serious diseases in 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). It becomes one of the most prevalent and damaging disease wherever tomatoes are grown intensively because the pathogen can persist indefinitely in infested soils (Agrios, 1997). *Fusarium* root rot causes severe high damage in tomato plants especially those grown in old soils (El-Fahham, 1993). The excessive misuse of a wide range of fungicides has led to it being harmful to the environment and increases the resistant pathogen populations (Ozgonen *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.

For the control of *Fusarium oxysporum*, sufficient work has not done regarding biological control in Bangladesh. Though some effective fungicides are available in market, a little achievement has been attained with these chemicals (Ahmed, 1985 & Ahmed, 1986). But works on biological control are scantly.
As this pathogen is soil borne, biological control could be the suitable measure against it. It is attractive in an environmental and economic sense on account of 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).

For the exploitation to the potential of biocontrol agents against target pathogens systematic research on survey, isolation, identification and multiplication are needed.

Objectives:

Considering the above facts and points, the present research program has been designed with the following objectives:

a) To search suitable antagonistic microorganism against *Fusarium oxysporum* f. sp. *lycopersici.*

b) To test the effectiveness of antagonistic microorganisms in controlling Fusarium wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici.*

Chapter II

Review of literature

REVIEW OF LITERATURE

Literature Related to Fungal Antagonism

Kirankumar *et al.* (2008) reported that *Fusarium oxysporum* f. sp. *lycopersici* (FOL) is a highly destructive pathogen of both greenhouse and field grown tomatoes in warm vegetable production areas. The disease caused by this fungus is characterized by wilted plants, yellowed leaves and minimal or absent crop yield. There may be a 60 to 70% yield loss.

Morsy (2005) treated tomato plants by *Bacillus subtilis* only and/or *Trichoderma harzianum* and showed that it has high bio-control activity against damping off and root rot disease and gave high yield of tomato.

Benitez *et al.* (2004) has proved *Trichoderma* spp. to be useful in the control of phyto-pathogens affecting different crops.

Weller *et al.* (2002) reported that the natural control of several phyto-pathogens is based on the presence of suppressive soils where several bio-control microorganisms belonging to *Trichoderma*, *Pseudomonas* and *Bacillus* genera are detected.

Sivan and Chet (1989) observed *T. harzianum* to reduce the chlamydospore germination rate of both *F. oxysporum* f. sp. *vasinfectum* and *F. oxysporum* f. sp. *melonis.*

Mukhopodhyay (1989) demonstrated increased growth of several crop plants in the presence of biological agents. These responses may be caused by a direct effect to the plant (as bio-fertilizer) or by control of some undiagnosed plant pathogens. Increase plant height, fresh and dry weight of wheat (1.5 times),

cucumber (75.2 times), and radish (1.9 times) was achieved when plant growth promoting fungi (PGPF) *Trichoderma*, *Fusarium*, *Penicillium* and *Mucor* were used as barley grain inoculums (Hyakumachi, 1994).

Elad *et al*. (1986), Papavizas and Lewis (1985), Liu and Baker (1980), and Wells *et al*. (1972) reported that among the soil-borne fungi *Trichoderma* spp. are the most promising biological agent to control plant disease .

Literature Related to Bacterial Antagonism

Matar *et al.* (2009) studied the antagonistic and inhibitory activity of *Bacillus subtilis* isolate G-GANA7 (Gene Bank accession No. EF583053) obtained from Abo-Homos in Egypt against six fungal isolates belonging to four different genera *Rhizoctonia solani, Helminthosporium* sp., *Alternaria* sp. and *Fusarium oxysporum.*

Selvakumar *et al.* (2008) reported that *Exiguobacterium acetylicum* strain 1P (MTCC 8707) is a rhozospheric, Gram positive, rod shaped, yellow pigmented bacterium isolated from an apple orchard rhizospheric soil and in separate in-vitro assays. It was found that volatile compound produced by the bacterium was potent in inhibiting the hyphal development of *Rhizoctonia solani, Sclerotium rolfsii, Pythium* and *Fusarium oxysporum* by 45.55%, 41.38%, 28.92 % and 39.74%, respectively.

Filippi *et al.* (2008) reported thirty two bacteria isolated from soil samples antagonistic to a number of phytopathogenic fungi. One bacterial strain, designed as M51, appeared to be particularly active towards *F. oxysporum*, *in-vitro* and *in-vivo* and it was inhibitory *in-vitro* to three other *Fusarium* spp. used.

Chung *et al.* (2008) reported new methods to control Fusarium wilt of tomato and Phytophthora blight of pepper by *Bacillus subtilis* isolate ME488. They concluded that *B. subtilis* ME488 has potential for biological control of pathogens of tomato possibly due to the production of antibiotics.

Adesina *et al.* (2007) studied the antagonistic potential of soil bacteria towards *R. solani* AG3 and *F. oxysporum* (Fo3). For this purpose they screened 1,788 isolates from the soils and *in-vitro* 327 isolates were found as antagonists.

Omar *et al.* (2006) studied on two bacterial isolates, *Bacillus megaterium* (C96) and *Burkholderia cepacia* (C91), demonstrated to be antagonistic against *F. oxysporium* f.sp. *lycopersici*, the causal organism of Fusarium wilt of tomato and reported that these isolates reduced disease incidence by 75% and 88%, respectively.

Bevivino *et al.* (2005) reported that *B. cepacia* complex (Bcc) bacteria are naturally present in the rhizosphere of several crop plants and have been found to antagonize a wide range of important plant pathogens.

Mao *et al.* (1998) studied on the bio-control of soil borne disease of tomato caused by *R. solani* and *Pythium ultimum* alone or in combination with *S. rolfsii* and *F. oxysporum* f. sp. *lycopersici* in the green house and field and reported that combined *Gliocladium virens*-3 (Gl-3) + *Burkholderia cepacia*-F(Bc-F) application resulted in greater fresh weight and lower DSI for pepper, and greater fruit yield for tomato than those obtained with either Gl-3 or Bc-F alone.

Reddy (1997) reported that *B. cepacia* can also antagonize and repress many fungal plant Pathogens such as *Fusarium, Pythium, Rhizoctonia, Cylindrocarpum, Botrytis* and *Alternaria.*

Hebbar *et al.* (1997) studied that *Burkholderia cepacia* (syn. *Pseudomonas cepacia*) strain PHQM100 applied as a seed coating was tested growth chamber experiments for its ability to suppress pre-emargence damping-off, and post emergence damping-off in corn induced by *Pythium* and *Fusarium* spp.

Tariq *et al.* (2010) carried out to study the potential of plant rhizosphere associated bacteria for the biocontrol of potato black scurf of disease caused by *Rhizoctonia solani* Khun AG-3. Among twenty eight bacterial strains, nine were found to be antagonistic in vitro, reduced the fungal growth and caused the lyses of sclerotia of *R. solani* in dual culture assay as well as in extracellular metabolic efficacy test.

Goud and Muralikrishnan (2009) tested the antagonistic activity of *Pseudomonas fluorescens* against three phytopathogenic fungi *(Pythium ultimum, Macrophomina phaseolina, Pyricularia oryzae, Fusarium oxysporum).* By using Cross Streak assay, Pour plate, Antifungal assay and Agar well diffusion method and result revealed that it has been producing some inhibitory compound that suppresses the fungal growth.

Kazempour and Anvary (2008) evaluated the antagonistic activity of *Pseudomonas fluorescens* against *Fusarium oxysporum in-vitro* by using dual culture test also by demonstration of antibiotic activity and production of diffusible antibiotic.

Jha and Anjaiah (2007) studied on Metabolites of rhizobacteria antagonistic towards fungal plant pathogens and in this study double layer technique was used for isolation of antagonistic bacteria from rhizosphere against plant pathogenic fungi. Four potential rhizobacteria was selected in dual culture plate method based on their antifungal activity against *F. oxysporum*.

AboElnaga (2006) evaluated the antagonistic activity of *Bacillus subtilis invitro* against *F. oxysporum* by using dual culture test and pot assays.
Brion and Genevieve (1999) tested the antagonistic activity of *Pseudomonas fluorescens* by using Cross streak assay and pour plate method and reported that *P. fluorescens* inhibit the growth of *F. oxysporum*.

Symptoms of Fusarium wilt

Altinok (2005) reported that Fusarium wilting in eggplant includes leaf chlorosis and slight vein clearing on the outer leaflets followed by yellowing and dropping of leaves, discoloration of the stem and death of the above-ground plant parts of some aubergine plants.

Thurston (1998) showed that Fusarium wilting in tomato plant causes wilting plus yellowing of leaves. The leaves turn lifeless immature or brownish-red in tone plus bend upon the stems. They do not tumble off plus sojourn trustworthy to the plant.

Agrios (1997) reported that wilting progresses from lower to upper leaves, followed by collapse of the plant. The symptoms of fungal wilt of tomato plant by *F. oxysporum* f. sp. *melongenae* as the yellowing of lower leaves that appears first, usually affecting the leaflets unilaterally. The affected leaves die and the symptoms continue to appear on successive younger leaves. The plant as a whole is stunted and commonly it eventually goes into permanent wilt of the leaves, which die as they cling of the upright woody stems.

Agrios (1997) showed that Fusarium wilt symptoms begin in tomato and potato as slight vein clearing on outer leaflets and drooping of leaf petioles. Later the

lower leaves wilt, turn yellow and die and the entire plant may be killed, often before the plant reaches maturity. In many cases a single shoot wilts before the rest of the plant shows symptoms or one side of the plant is affected first. If the main stem is cut, dark, chocolate-brown streaks may be seen running lengthwise through the stem. This discoloration often extends upward for some distance and is especially evident at the point where the petiole joins the stem. Potato tubers may show browning of the vascular ring as well as browning at the stem end and decay where stolons are attached. In pepper, lower leaves do not begin to wilt until roots and the base of the stem have already started to decay. Wilting of the entire plant soon follows. Dark brown, sunken, and eventually girdling cankers may be seen at the base of the pepper plant.

Miller *et al.* (1996) conducted an experiment and found that Fusarium wilt starts out looking like vein clearing on the younger leaves and drooping of the older lower leaves, followed by stunting of the plant, yellowing of the lower leaves, defoliation, marginal necrosis and death of the plant. On older plants, symptoms are more distinct between the blossoming and fruit maturation stages.

Critchley (1995) showed that affected tomato plants show yellowing of leaves that progressively wilt and die from bottom to upwards. Woody stem and root tissue of diseased plants turn brown.

Smith *et al*. (1988) reported that browning of the vascular tissue is strong evidence of fusarium wilt. Further, on older plants, symptoms generally become more apparent during the period between blossoming and fruit maturation.

Beckman *et al.* (1981) found stunting of the entire plant and the infected leaves do not abscise but remain attached to the stem.

Identification of Bacteria

Wang *et al.* (2010) reported that *Burkholderia* sp. H-6 inhibited mycelium growth *in-vitro* of 6 plant pathogenic fungi, especially of *Phytophthora capsici, Fusarium oxysporum* and *Sclerotium libertiana.* In this study, they identified *Burkholderia* sp. H-6 based on morphological, physiological and biochemical methods as well as on 16SrDNA analysis. In physiological and biochemical studies they conducted gram stain nature, Production of catalase, the V-p test and nitrate deoxidizing test and also the HR test. The strain H-6 exhibited the following traits: gram negative reaction, catalase positive reaction, the V-P test and nitrate deoxidizing test were also negative, but the HR test positive.

Wang *et al.* (2009) studied on the antagonistic activity of *Bacillus subtilis* and they identified as *Bacillus subtilis* according to its morphological, physiological and biochemical characteristics and 16SrDNA sequence analysis.

Parvathi *et al.* (2009) reported that *Bacillus pumilus* contain emetic toxin gene cesB. In this study for identification of *Bacillus* spp. isolates (isolated from coastal environment of Cochin) were subjected to a series of biochemical tests which included nitrate reduction, anaerobic growth, gas production from glucose, Voges-Proskauer (VP), growth at different NaCl concentrations, temperature and pH ranges, degradation of starch, casein, urea, tween 20, gelatin, chitin, acid production from arabinose, mannitol, xylose, glucose, lactose, citrate utilization and production of DNAse.

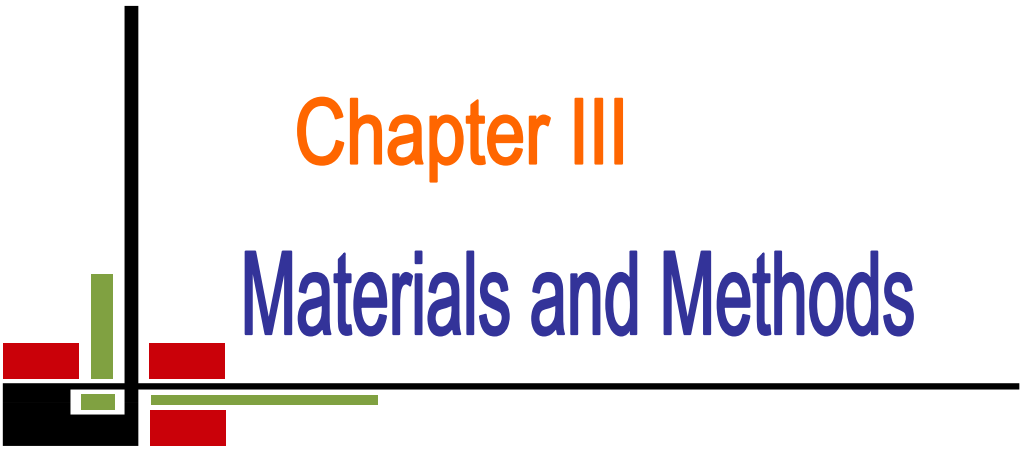
Goud and Muralikrishnan (2009) reported that *Pseudomonas fluorescens* is one of the most suitable bio-control agent in suppressing the phyto-pathogenic fungi and replace chemical fungicides. In this study, they conducted physiological and biochemical tests such as colony character, motility, Gram's stain nature, production of fluorescent pigment, gelatin liquefaction, Starch

hydrolysis and utilization of nitrate to authenticated the isolated organism as *P. fluorescens*.

Kazempour and Anvary (2008) worked on isolation of *Fusarium oxysporum* antagonistic bacteria and cloning of its phenazine carboxylic acid genes and in this study antagonistic isolates of bacteria were identified by biochemical, physiological, and biological tests and PCR.

Quan *et al.*(2006) reported that *Burkholderia cepacia* strain CF-66 has strong antifungal activity against *Fusarium oxysporum*. In this, they conducted different physiological and biochemical tests as well as on 16SrDNA analysis for characterization of *Fusarium oxysporum* strain CF-66. In physiological and biochemical studies, they conducted gram stain test, Indole production, Urease production, starch hydrolysis, nitrate deoxidizing test for identification of strain CF-66.

Abo-Elnaga (2006) reported that the usage of *Bacillus subtilis* as seed treatment reduced the percentage of wilting incidence of tomato under greenhouse conditions and in this study he identified the isolated bacteria using the following tests- shape of cells, motility, Gram's staining, aerobiosis, starch hydrolysis, gelatine liquefaction, nitrate reduction, acetyl methyl carbinol production (VP test) fermentation, reactions with mannitol, glucose, sucrose, arabinose, xylose and lactose and pigment production.



Chapter III

Materials and Methods

MATERIALS AND METHODS

3.1 Experimental Site:

The experiment was carried out in the Disease Diagnostic Laboratory & Net house of Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka.

3.2 Time of Experiment:

This study was conducted during the period of July 2011-June 2012.

3.3 Collection of Seeds:

Seeds of tomato variety BARI Tomato-2 were collected from Bangladesh Agricultural Research Institute (BARI), Gazipur.

3.4 Determination of Prevalence of seed borne fungi of BARI Tomato-2 on blotter method:

The collected seed samples of tomato were analyzed for the presence of major seed borne fungal pathogens by blotter method following the International Rules for Seed Testing (ISTA, 1996).

3.5 Isolation and purification of *Fusarium oxysporum* f. sp. *lycopersici*:

3.5.1 Preparation of Potato Dextrose Agar (PDA)

PDA was prepared as described by Islam (2009) and poured in 500 ml glass bottles and sterilized in an autoclave at 121^0C under 15 PSI pressure for 15 minutes. The media were acidified with 30 drops of 50% lactic acid per 250 ml medium to avoid the contamination of bacteria.

3.5.2 Isolation of *Fusarium oxysporum* f.sp. *lycopersici*

The procedure of this method is outlined below:

(i) Petri pates 1/3 full with sterile PDA (Potato Dextrose Agar) medium was prepared where lactic acid was added for isolation of fungi (@1drop of 50% acid / plate).

(ii) The seeds were surface sterilized by dipping for a few minutes in 10% Clorox solution.

(iii) Seeds were kept in the petriplate (the lid was lifted slowly just high enough to make the operation easy). Twenty five seeds were kept in each petri-plate. Each plate was labelled, placed in paper bags and incubated at a temperature 20- 25^0 C for a few days to allow the pathogen to grow out of the seed surface on the medium.

(iv) When the growth was developed around the seed, a bit of the advancing margin of the colony or mycelial growth was transferred to fresh agar plates for purification and sub-culture and then to agar slants for stock cultures (Ashrafuzzaman, 1976).

3.6 Collection of Antagonistic Fungi

The antagonistic *Trichoderma harzianum* was collected from Bangladesh Agricultural Research Institute (BARI), Gazipur.

3.7 Isolation and purification of probable antagonistic bacteria from soil by using soil dilution plate technique

3.7.1 Collection of soil (rhizosphere) sample

The soil samples were collected from the Plant Pathology Field, Sher-e-Bangla Agricultural University, Dhaka. Five soil samples were randomly collected from the rhizosphere of tomato field at different growth stages. Soil collection was made with the help of an augur of one inch diameter at the depth of six

inches. At the time of collection, the surface of the top soil was scrapped to remove dry soil and other superficial plant debris. Each composite sample was kept in cellophane bag with proper labeling. After collection, the bags were kept under shade.

3.7.2 Preparation of Nutrient Agar (NA)

Nutrient agar media was prepared according to the method followed by Hossain (2006). Twenty eight gram Nutrient agar mixed well in 1000 ml distilled water. It was then autoclaved at 121^0 C under 15 PSI pressure for 15 minutes.

Dilution plate technique was carried out as described by Islam (2009) for isolation of soil bacteria.

3.7.5 Preparation of working sample

For every dilution of soil samples, working sample was prepared from the composite sample that was made after the soil sample collection at the different growth stages of Tomato plant.

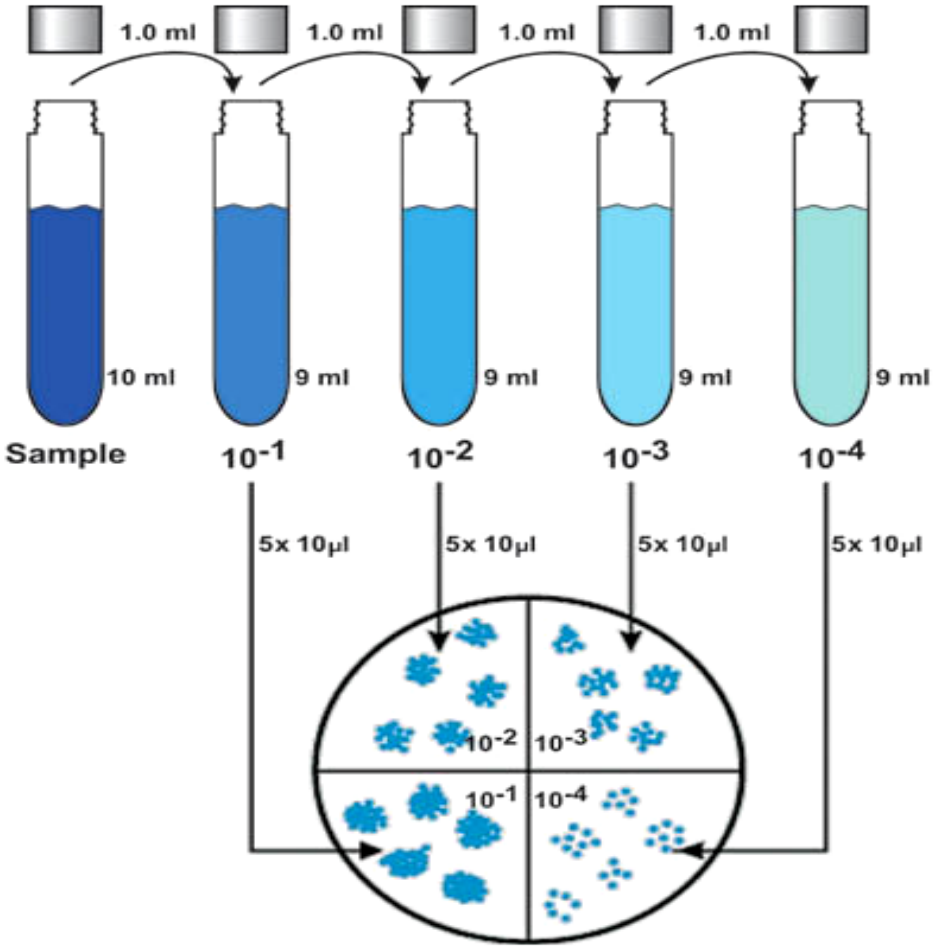
3.7.6 Preparation of soil suspension

One gram of soil was placed in the test tube containing 9 ml of sterile water and stirred thoroughly for few minutes in order to obtain a uniform 1:10 dilute soil suspension. This was used as stock solution resulting 10^{-1} dilution. One ml of 1:10 stock suspension was transferred with the help of sterile pipette into the second test tube containing 9 ml sterile water and shaken thoroughly resulting 10^{-2} solution. This final dilution was made up to 10^{-4}.

3.7.7 Isolation and purification of bacteria

Twenty ml warm melted NA medium (approx. 45^0C) was poured in each sterile petri-plate. 0.1 ml of diluted soil suspension was placed at the centre of NA medium plate and spreaded with an L-shaped glass rod. The inoculated NA plates were incubated for 3-7 days at room temperature (25^0C). After

incubation of the inoculated NA plates, bacteria appeared with various types of colony colors. Then the bacterial colonies were selected and isolated depending on their colony colors and streaked on NA medium separately. Again the streaked NA media plates were incubated at room temperature for 2 days. Further transfers were done for purification.



Flow chart1. Dilution plate technique for isolation of Bacteria.

3.8 *In-vitro* antagonism of isolated bacteria against *F. oxysporum* f. sp. *lycopersici*:

In-vitro antagonism of bacteria was done to check their antagonistic activity. Bacteria were screened out for their antagonistic activity against isolated fungus by following dual culture method.

Dual culture method

In this study, dual culture method was carried out as described by Azadeh *et al.* (2010). A plug of mycelium of isolated fungus (5 mm diameter) was plated at the centre of the petri-dish containing 25 ml PDA, then the bacterium was streaked 3 cm away from the agar plug at both sides towards the edge of the plate by a loop loaded with 48 hr old bacterial culture grown at room temperature on NA media. All the tested plates were incubated at 28^0C for 7 days and antagonistic effect was determined by measuring the longest and shortest free growth zone between the bacteria and fungi. Control trial was done without streaking of bacteria in the plate.

Measurement of % Inhibition

Inhibition percentage of the *F. oxysporum* f. sp. *lycopersici* was calculated based on the growth of the pathogen on NA plates in absence of antagonistic following the formula as suggested by Sundar *et al.* (1995).

$$\% \text{ Growth inhibition} = \frac{X-Y}{X} \times 100$$

Where,

X = Mycelial growth of the *F. oxysporum* f. sp. *lycopersici* in absence of antagonist bacteria i.e. control

Y = Mycelial growth of the *F. oxysporum* f. sp. *lycopersici* in presence of antagonist bacteria

3.9 *In-vitro* antagonism of *Trichoderma harzianum* against *F. oxysporum* f. sp. *lycopersici*:

An *in-vitro* study was conducted to find out the antagonistic effect of *Trichoderma harzianum* against *F. oxysporum* f. sp. *lycopersici* on PDA by dual culture technique (Sundar *et al.*, 1995). Discs of mycelium (5 mm diameter) of each of the selected fungal cultures were cut from the edge of an actively growing fungal colony with a cork borer (5mm diameter). One 5 mm blocks of *Trichoderma harzianum* culture (7 days old) was transferred to the plate containing PDA one side and another block of same size of *F. oxysporum* f. sp. *lycopersici* (9 days old) was placed at the other side of the plate. The plates only with the discs of *F. oxysporum* f. sp. *lycopersici* in the center were used as control plate. The plates were then incubated at room temperature (25±2°C) until the mycelium of *F. oxysporum* f. sp. *lycopersici* in control plate covered the whole plate. After then the diameter of the colonies were measured.

Measurement of % Inhibition

Inhibition percentage of the *F. oxysporum* f. sp. *lycopersici* was calculated based on the growth of the pathogen on PDA plates in absence of antagonistic fungal cultures following the formula as suggested by Sundar *et al.* (1995).

$$\% \text{ Growth inhibition} = \frac{X-Y}{X} \times 100$$

Where,

X = Mycelial growth of the *F. oxysporum* f. sp. *lycopersici* in absence of antagonist (*Trichoderma harzianum*) i.e. control

Y = Mycelial growth of the *F. oxysporum* f. sp. *lycopersici* in presence of antagonist (*Trichoderma harzianum*)

Assessment of percentage mycelial growth inhibition of *F. oxysporum* f. sp. *lycopersici* by *Trichoderma harzianum* was done by the following key:

Inhibition (%)	Grade
≥ 90	Very strong
75-90	Strong
50-74.9	Moderate
30-49.9	Weak
15-29.9	Very weak
0-14.9	No inhibition

3.10 Characterization of Bacteria:

3.10.1 Morphology of the bacteria

3.10.1.1 Study of Colony color

For morphological characters, colony color, shape and surface textures were carefully studied and recorded as all bacterial isolate developed after 24hrs of incubation in NA medium.

3.10.1.2 Gram's Reaction

Gram's Staining

It was done on a clean slide, dried a thinly spreaded bacterial film in air without heat.Then lightly flamed the underside of the slide twice to fix the bacteria to the slide. Then the smear was flooded with Crystal violet solution for 1 minute. It was washed with tap water for a few seconds and excess water removed by air. Then the smear was flooded with Iodine solution (Lugol's Iodine) for 1 minute and then washed with tap water for few seconds and excess water removed by air.

After that the smear was decolorized with 95% Ethanol for 30 seconds and again washed with tap water and dried by air. Then the smear was counterstained with 0.5% Safranine for 10 seconds and washed briefly in tap

water and excess water was removed by air. Finally it was examined under microscope at 40x.

KOH solubility Test:

On glass slide a loop-full of bacteria from a well grown colony was mixed with a drop of 3% aqueous KOH. Mixing was continued for less than 10 seconds. A toothpick was used for picking bacteria from a colony as well as for mixing it. 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).

3.10.2. Different Biochemical Tests

Different chemical tests were done for each antagonistic bacterial isolate such as Starch hydrolysis, Citrate utilization test, Catalase test, Oxidase test, Pecteolytic test and Gelatin liquefaction test.

Starch hydrolysis

A nutrient agar plate containing 2% soluble starch was inoculated with the bacterium isolate to be tested. Then incubated at optimum temperature for at least 48 hours. After inoculation, the plate was flooded with Lugol's iodine and observed.

Catalase test

One colony of the organism from the agar plate was taken on a slide onto which one drop of 3% H_2O_2 (Hydrogen Peroxide) was added and observed.

Oxidase Test

A portion of the test organism was picked up from the agar plate with a sterile wooden toothpick onto the wet oxidase disk (containing tetramethyl-p-phenylene-diamine dihydrochloride. Formation of a dark purple color developed within 5-10 seconds indicted a positive test for oxidase.

Pecteolytic test

Potato tubers were disinfected with 99% ethanol, cut up into slices of about 7-8 mm thick, and then placed on moistened sterile filter paper in sterile Petri dishes. 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. Examination was done for 5 days after inoculation. Two slices were inoculated for each isolate.

Citrate Utilization Test:

A portion of the test organism was picked up from the agar plate with a sterile inoculating loop and streaked into Simmon's citrate agar slants. Following incubation at 30^{o}C for 24 hours changing of the green bromothymol blue indicator positive results.

Gelatin liquefaction test:

One loop-full bacterial culture was inoculated with a sterile straight wire stabbed into the media and incubated at 30^{o}C for 24 hours. Gelatin liquefied microorganisms is detected by the formation liquid culture in the presence of 4^{o}C refrigerator.

Tobacco Hypersensitivity test:

Hypersensitivity test of the isolated bacteria from the soil were done in tobacco plant as described by Wick (2006). Aqueous suspensions of the isolated bacteria were prepared separately from a 24hr culture plates. The suspensions (10^{8}cfu/ml) were injected into the intercellular space of the lower leaves with a 25+ gaugle needle and syringe separately in separate tobacco leaves.

3.11 Efficacy of seed treatment with microbial antagonists in prevalence of *Fusarium oxysporum* f. sp. *lycopersici*

Treatments of the experiment:

The treatments were-

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*

T_6= Control (Untreated)

Seed treatment with bacterial antagonist

The seeds were first treated with microbial antagonists in a specific way. First of all, 24hr aged bacterial culture was made on NA media. Then bacterial cultures were scraped out and 10^8 cfu/ml suspensions were made in sterile distilled water in a beaker for each treatment. Then, 400 seeds for each beaker were dipped in those beakers for four hours. After that, 400 seeds surface sterilized with Chlorax @ 3% & washed in distilled water served as control.

Seed treatment with *T. harzianum*

T. harzianum was collected from laboratory of Bangladesh Agricultural Research Institute (BARI) & multiplied in PDA medium in 9 cm Petridis. 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 spores suspension of bio-agent (*T. harzianum*) for 1hr.

Prevalence of *F. oxysporum*

Prevalence of *F. oxysporum* in treated and untreated seeds was determined by blotter method following International rules for seed testing (ISTA, 1996). Four hundred seeds were tested. Seeds were surface sterilized by 3% chlorax (seeds were dipped into 3% chlorax for 30 seconds, then wash 3 times with distilled water). Ten 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. Germination of the seeds was also recorded.

3.12. Efficacy of root dipping in suspension of microbial antagonists in prevalence of Fusarium wilt of tomato

Treatments of the experiment:

The treatments were-

T_1= Root dipping with Bacterial Isolate 1 (Iso-1)

T_2= Root dipping with Bacterial Isolate 2 (Iso-2)

T_3= Root dipping with Bacterial Isolate 3 (Iso-3)

T_4= Root dipping with Bacterial Isolate 4 (Iso-4)

T_5= Root dipping with *Trichoderma harzianum*

T_6= Control (Untreated)

Effect of pre-plant treatment of roots of tomato seedlings on Fusarium wilt was evaluated under net-house condition under the following way-

Preparation of soil

The soil was prepared by sterilizing with 40% formaldehyde solution. At first, soil was mixed with formaldehyde and kept covered with polythene sheet for three days. After then, the sheet was kept out and the soil was pulverized. Then it was kept for three days. Thus, the soil was prepared.

Raising of seedlings

Then, after preparing the soil, the seeds were sown at a constant rate. It was then kept for 15 days for rising. Time to time observation and watering was done daily and whenever necessary.

Seedling Treatment

15 Days old seedlings were then lifted from soil. Suspensions of antagonists were made as per the ways of step 3.11. Then, 10 seedlings for each treatment were dipped in a separate beaker for 4 hrs.

Preparation of Pots

The plastic pots were bought from the market which has the capacity to fill with 2 Kg of soil. Before preparation, soil was sterilized by 40% formaldehyde & used it as base soil. Then soil: Cow dung @19:1 was mixed and the pots were filled with that soil.

Transplanting of seedling

The seedlings were then transplanted in pots. It was then nourished for 5 days to put up with transplant injury.

Inoculation of *Fusarium oxysporum*

Conidial suspension of *Fusarium oxysporum* was made and then soil drenching was done by this suspension (1 ml conidial suspension containing 1.25×10^7 conidia/ml). Ten plants were inoculated for each treatment. After inoculation, the pots were kept in a net house.

Computation of disease severity and disease incidence

Pot experiment for tomato plant was set following Randomized Complete Block Design (RCBD) with six treatments and three replications. Ten plants were inoculated for each treatment. The Records on expression of symptom on leaves were taken after inoculation at an interval of 10 days up to 30 days. Infection was expressed in percentage. To calculate the percent disease incidence, number of plants wilted per total number of plants was counted.

Percent disease incidence was calculated by the following formula as described by Mansoor *et al.* (2007):

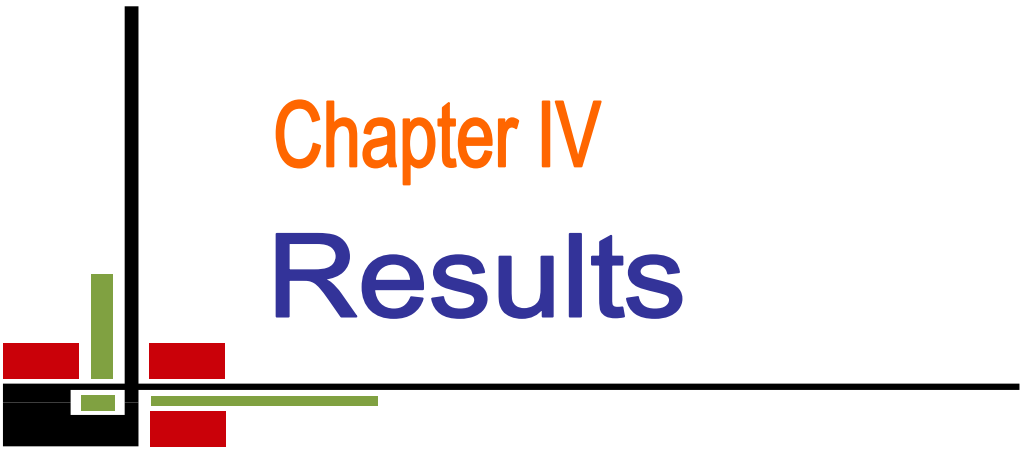
$$\% \text{ Disease incidence} = \frac{\text{Number of plants wilted}}{\text{Total number of plants}} \times 100$$

Wilt severity was recorded based on a 0-3 scale, where 0= no wilt; 1= 1-50% leaves wilted; 2= more than 50% leaves wilted; 3= entire plant wilted or dead (Furuya *et al.* 1997). The percent disease index (PDI) was computed using a formula (Sing, 1984):

$$\text{PDI} = \frac{\sum (\text{Disease class} \times \text{Plant number in that class})}{\text{No. of Plants assessed} \times \text{highest score of the scale}} \times 100$$

Statistical analyses

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).



Chapter IV

Results

RESULTS

4.1 Determination of Prevalence of seed borne fungi of BARI Tomato-2 on blotter method:

To determine the prevalence of seed borne *Fusarium oxysporum* f. sp. *lycopersici* on BARI Tomato-2, blotter test (Fig. 1) was done. It was found that, the seeds were affected by *Fusarium oxysporum* f. sp. *lycopersici* (Fig. 2) in an excessive rate (11.25%). The other fungi were *Aspergillus flavus* (12%) (Fig. 3) and *Curvularia* sp. (5.25%) (Fig. 4), respectively.

4.2 Isolation of and purification of *Fusarium oxysporum* f. sp. *lycopersici* from tomato seeds:

Fusarium oxysporum f. sp. *lycopersici* was isolated and purified from infected seeds in PDA plate. The pure culture is shown in Fig. 2.

4.3 Pathogenicity test of *Fusarium oxysporum* :

The isolated *Fusarium oxysporum* was tested for its pathogenic capability against tomato plant. The result indicated that, the tested fungal isolate was able to produce infection on tomato plants. *Fusarium oxysporum* was found strongly virulent because it caused 93.33 % infection on tomato plants (14 plants diseased out of 15).

4.4 Isolation and purification of probable antagonistic bacteria from soil by using soil dilution plate technique:

Twenty bacterial isolates were isolated from soil samples using dilution plate technique and purified on NA media. Of them, only four have the antagonistic

capability. The bacterial isolates were named as Iso-1 (Fig. 5), Iso-2 (Fig. 6), Iso-3 (Fig. 7) and Iso-4 (Fig. 8), respectively.

Fig. 1 Seed Health Test on Blotter paper

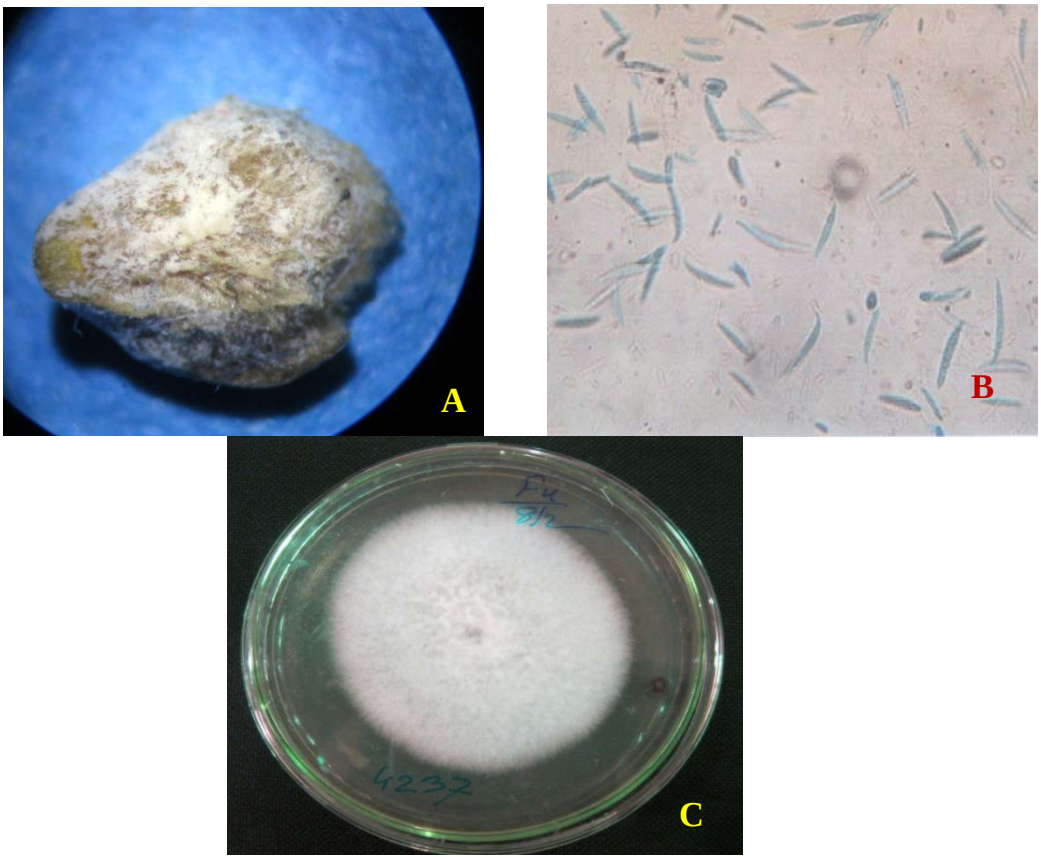


Fig. 2 Growth of *Fusarium oxysporum* f. sp. *lycopersici*

A. On incubated tomato seed under stereo microscope at 50x
B. Conidia of *Fusarium oxysporum* f. sp. *lycopersici* under compound microscope at 400x
C. Pure culture of *Fusarium oxysporum* f. sp. *lycopersici* on PDA

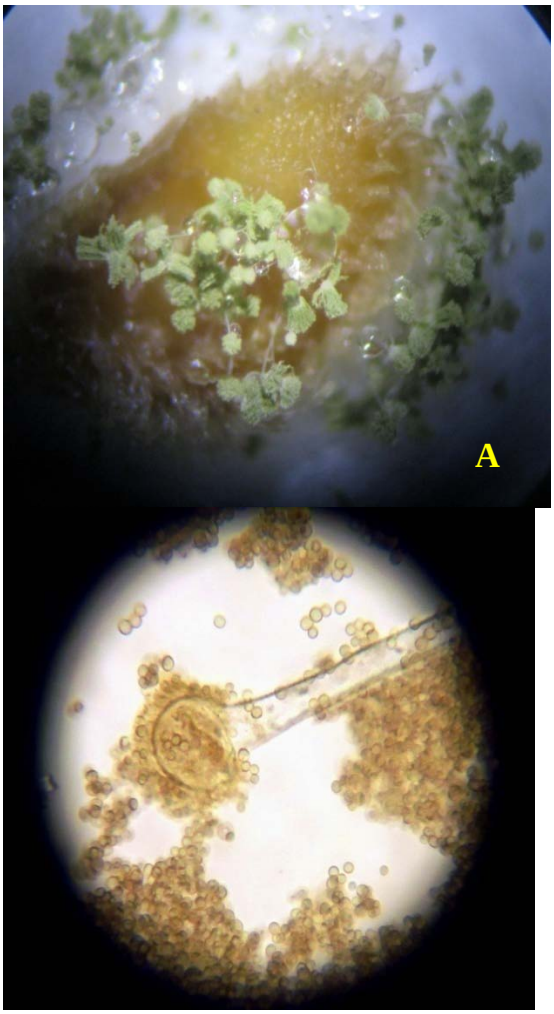


Fig. 3 Growth of *Aspergillus* sp.

A. On surface of incubated tomato seed under stereo microscope at 50x
B. Conidia of *Aspergillus* sp. under compound microscope at 200x

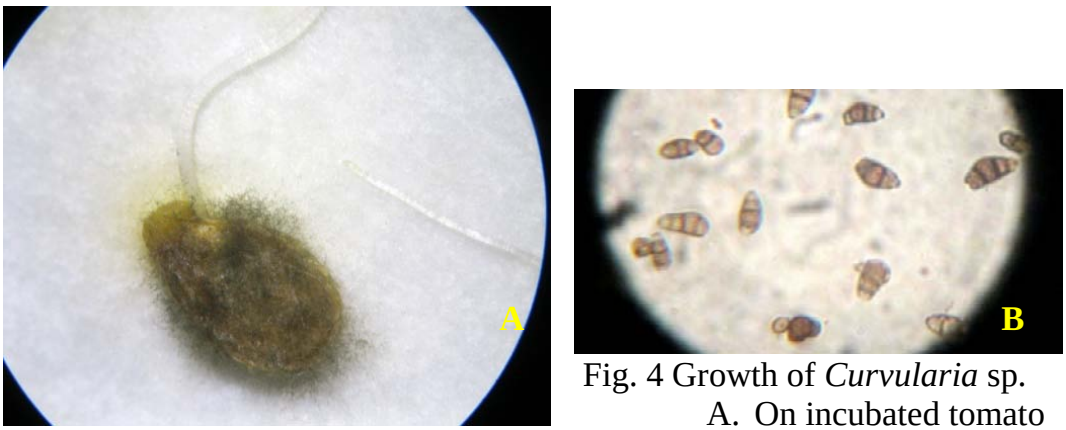


Fig. 4 Growth of *Curvularia* sp.

A. On incubated tomato seed under stereo microscope at 50x

B. Conidia of *Curvularia* sp. under compound microscope at 400x

Fig. 5 Pure culture of Iso-1

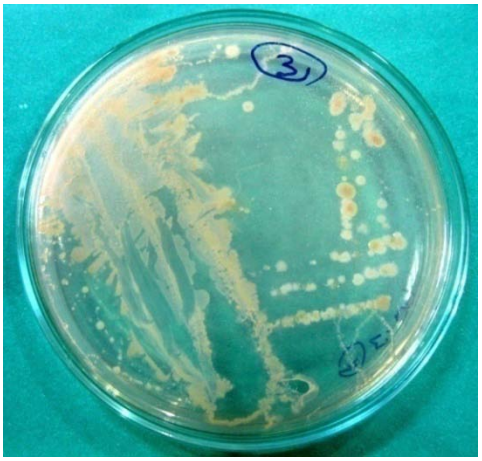


Fig. 6 Pure culture of Iso-2

Fig. 7 Pure culture of Iso-3

Fig. 8 Pure culture of Iso-4

Plate 1. Pure culture of different bacterial antagonists on NA medium

4.5 *In-vitro* antagonism of isolated bacteria against *F. oxysporum* f. sp. *lycopersici*:

The isolated bacteria were allowed to dual culture with *F. oxysporum* f. sp. *lycopersici* to assess their antagonistic capability. It was found that, four bacterial isolates out of twenty have the ability to inhibit the growth of *F. oxysporum* f. sp. *lycopersici* in dual culture. They were designated as Iso-1, Iso-2, Iso-3 and Iso-4. Thus, those isolates showed the antagonistic properties. It was estimated that, 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%) (Table-1 and Fig. 9-12).

4.6 *In-vitro* antagonism of *Trichoderma harzianum* against *F. oxysporum* f. sp. *lycopersici*:

An *in-vitro* study was conducted to find out the antagonistic effect of *Trichoderma harzianum* (Fig. 14) against *F. oxysporum* f. sp. *lycopersici* on PDA by dual culture technique (Fig. 15). The highest (75.75%) percent of inhibition *F. oxysporum* f. sp. *lycopersici* was observed in case of *Trichoderma harzianum* among all the microbial antagonists studied (Table-1). According to the key of Sundar *et al*. (1995), it can be said that, *Trichoderma harzianum* is a strong antagonist against *F. oxysporum* f. sp. *lycopersici.*

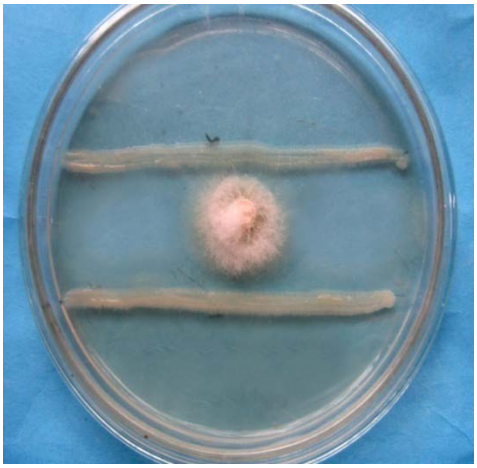


Fig. 9 *F. oxysporum* with Iso-1

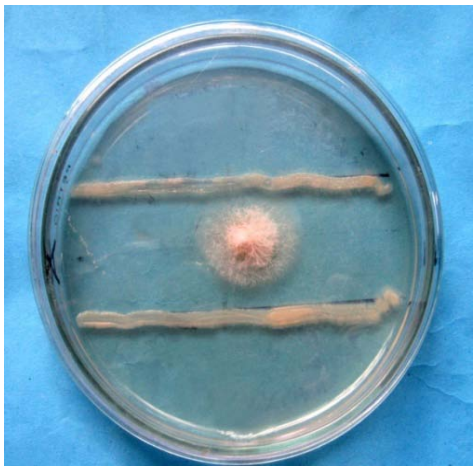


Fig. 10 *F. oxysporum* with Iso-2

Fig. 11 *F. oxysporum* with Iso-3

Fig. 12 *F. oxysporum* with Iso-4

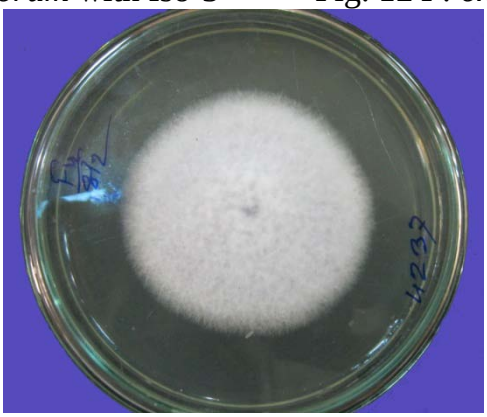


Fig. 13 *Fusarium oxysporum* f. sp. *lycopersici* on NA medium (Control)

Plate 2. Dual culture of *Fusarium oxysporum* f. sp. *lycopersici* with four isolates

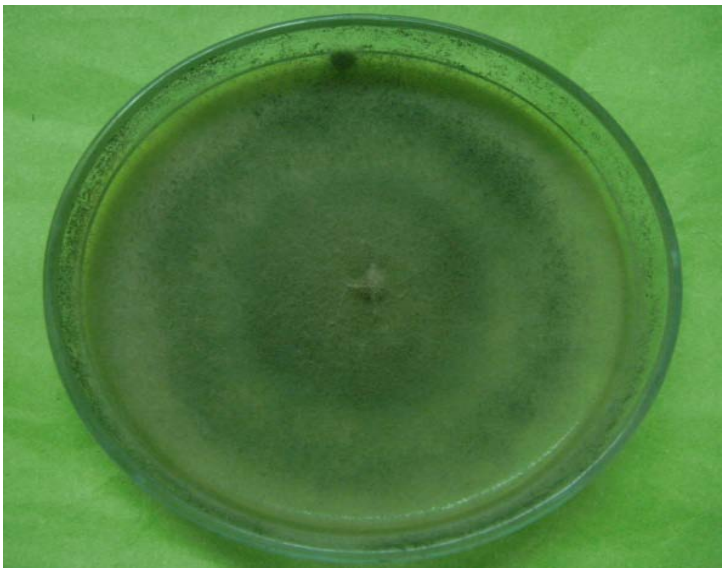


Fig. 14 Pure culture of *Trichoderma harzianum*

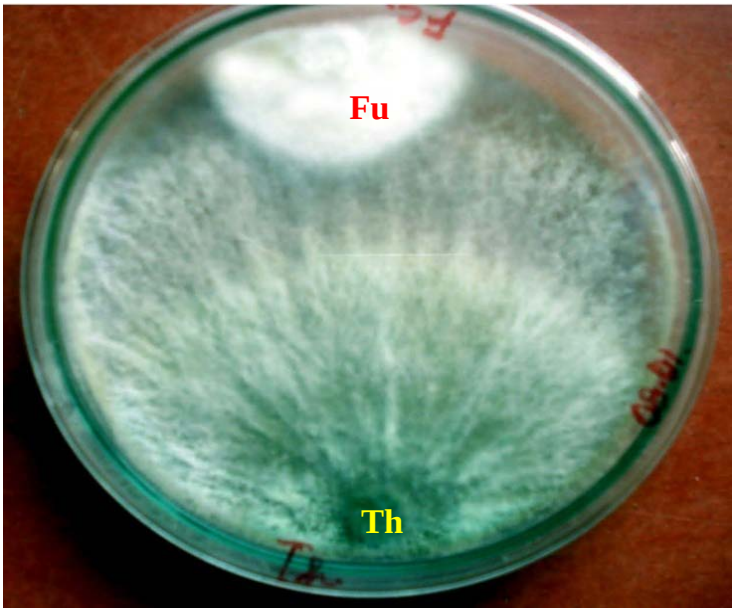


Fig. 15 Dual culture of *Fusarium oxysporum* f. sp. *lycopersici* with *Trichoderma harzianum* (Fu = *Fusarium oxysporum* and Th = *Trichoderma harzianum*)

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 sp.	75.75 a
$LSD_{(0.05)}$	2.944
CV%	3.68

4.7 Characterization of Bacteria

Morphological characters

In morphological test, Iso-1 showed creamy white and other three showed slight yellow colored colony on NA medium after 24 hrs of incubation. All of them had domed shaped, raised colony.

Biochemical characters

Gram's staining

In Gram's staining, all antagonistic bacteria yielded negative reaction and under microscope, all the bacteria produced pink color, straight and curved rod with no particular arrangement (Fig. 16).

KOH solubility Test

In KOH solubility test, all the bacterial isolates showed strands of viscid materials in repeated strokes as described by Suslow *et al.* (1982). These results showed positive in KOH solubility test that supports the result of Gram's staining test (Fig. 17) i.e. all the bacterial isolates were gram negative.

Starch hydrolysis

In starch hydrolysis test, all the bacterial isolate except Iso-4 made clear zone after giving Lugol's Iodine in agar plate containing 2% soluble starch (Table-3). This result proved that, except Iso-4, other three isolates showed positive result (Fig. 18).

Catalase test

In catalase test, all the four isolates showed the positive result in reaction with H_2O_2 (Table-3). That is, all the isolates formed bubble while mixing with 3% H_2O_2 (Fig. 19).

Oxidase Test

Among all the four isolates, Iso-2 formed dark purple color immediately after picking of bacteria on to the oxidase disk. This result proved that, Iso-2 is strongly positive in oxidase test (Table-3). While Iso-3 and Iso-4 produced dark purple color after 20 seconds. Thus these two were slowly positive in oxidase test. Finally, Iso-1 showed negative result i.e. did not form any color in oxidase disk (Fig. 20).

Pecteolytic test

In pecteolytic test, all the bacterial isolate showed negative result (Fig. 21). All bacterial isolates were unable to produce rot in potato slice.

Citrate Utilization Test:

All the three isolates except Iso-1 showed the positive result i.e. changed Simmon's Citrate Agar slants color from green to bromothymol blue (Table-3). Only Iso-1 could not do this (Fig. 22).

Gelatin liquefaction test

All the four isolates showed positive result in Gelatin liquefaction test. All the isolates formed liquid culture in the presence of $4^{o}C$ refrigerator. Thus, there was no variation among all the isolates in case of Gelatin liquefaction test (Table-3).

Tobacco hypersensitivity test

The bacterial isolates obtained from soil were not positive in hypersensitivity reaction on tobacco plants. The result indicated that, all the four isolates were saprophytic i.e. non-pathogenic (Table-3).

Kreig (1923) and Schaad (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*.

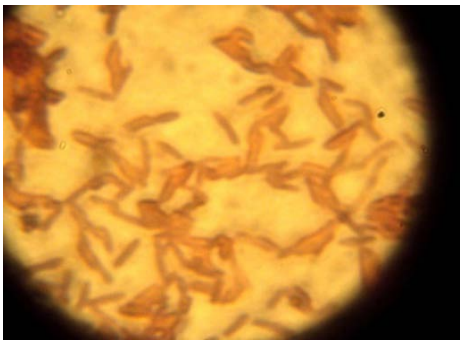


Fig. 16 Gram's staining reaction

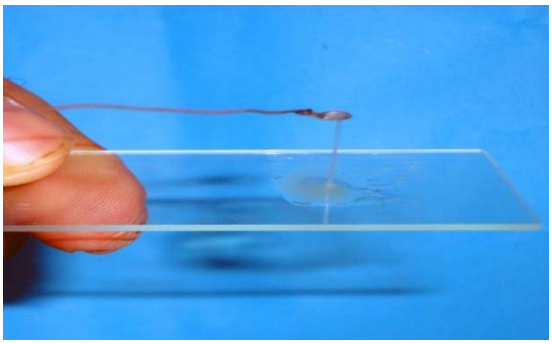


Fig. 17 KOH solubility test

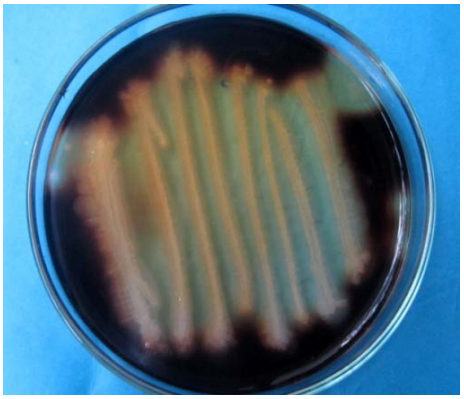


Fig. 18 Starch Hydrolysis

Fig. 19 Catalase test

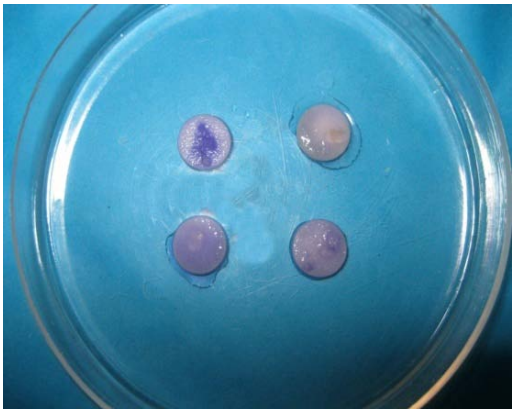


Fig. 20 Oxidase test

Plate 3. Biochemical test results of different bacterial isolates

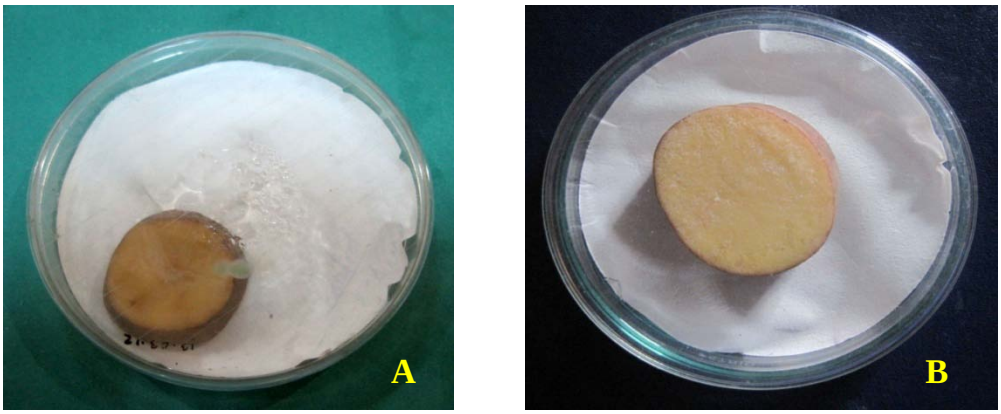


Fig. 21 Pecteolytic test
A. Bacteria inoculated
B. Control

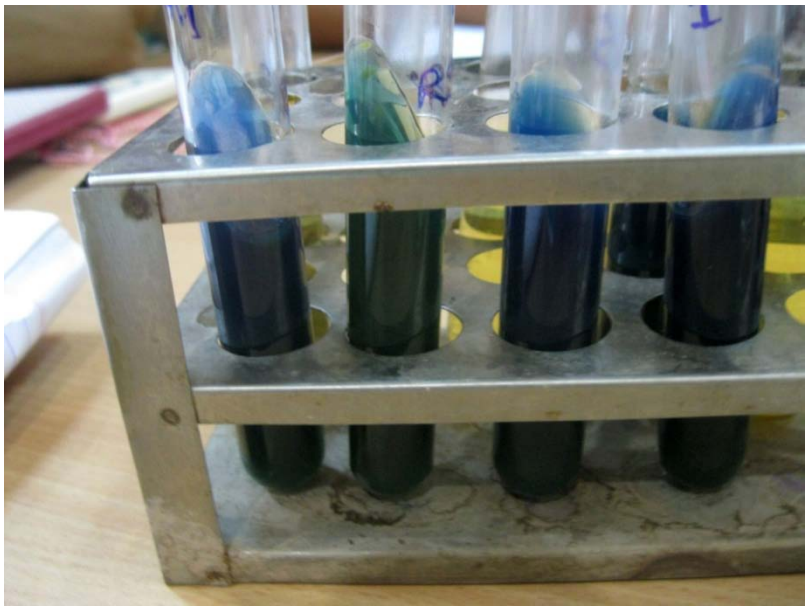


Fig. 22 Citrate Utilization test

Plate 4. Biochemical test results of different bacterial isolates

Table 2. Biochemical characteristics of used antagonistic bacteria

Characters	Name 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

4.8 Efficacy of seed treatment with microbial antagonists in prevalence of *Fusarium oxysporum* f. sp. *lycopersici*

All the tested antagonistic microorganisms significantly reduced the incidence of *F. oxysporum* in tomato seeds (Fig. 23). About 11.25% incidence of *Fusarium oxysporum* was recorded in non treated seeds where as 0.58% incidence was found both in Iso-1of antagonistic bacteria and *Trichoderma* sp. treated seeds (Table 4).

4.9 Efficacy of root dipping in suspension of microbial antagonists on prevalence of Fusarium wilt of tomato

To evaluate the antagonistic potential of isolated antagonistic bacteria and collected antagonistic fungi against Fusarium wilt of tomato, a pot experiment (Fig. 24) was conducted under net house condition. Data were recorded three times at 10, 20 & 30 DAT. The result revealed that, Iso-1 could significantly suppress disease incidence and severity than those of other microbial antagonists. No disease incidence was recorded in tomato seedlings treated with bacterial isolate Iso-1 and *Trichoderma harzianum* upto 20 days after transplanting. Only 10% plant was infected after 30 DAT, while disease incidence was 60% in case of control. In case of severity, the efficacy of Iso-1 was superior to all other bacterial antagonists; PDI was calculated 3.33% after 30 days of transplanting (Table-5).

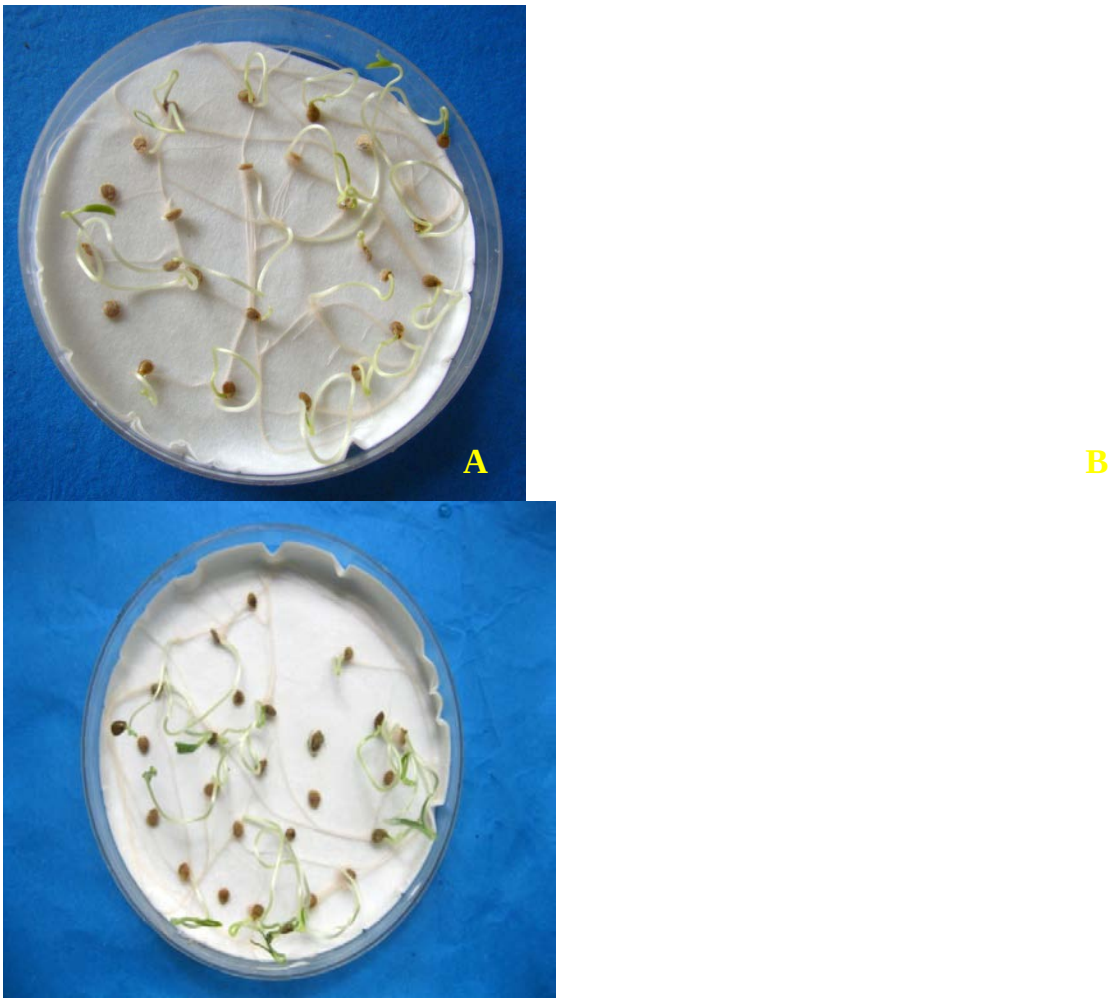


Fig. 23 Efficacy of seed treatment with selected microbial antagonists in prevalence of *Fusarium oxysporum* f. sp. *lycopersici*

A. Tomato seeds treated with antagonistic bacteria and

B. Untreated control

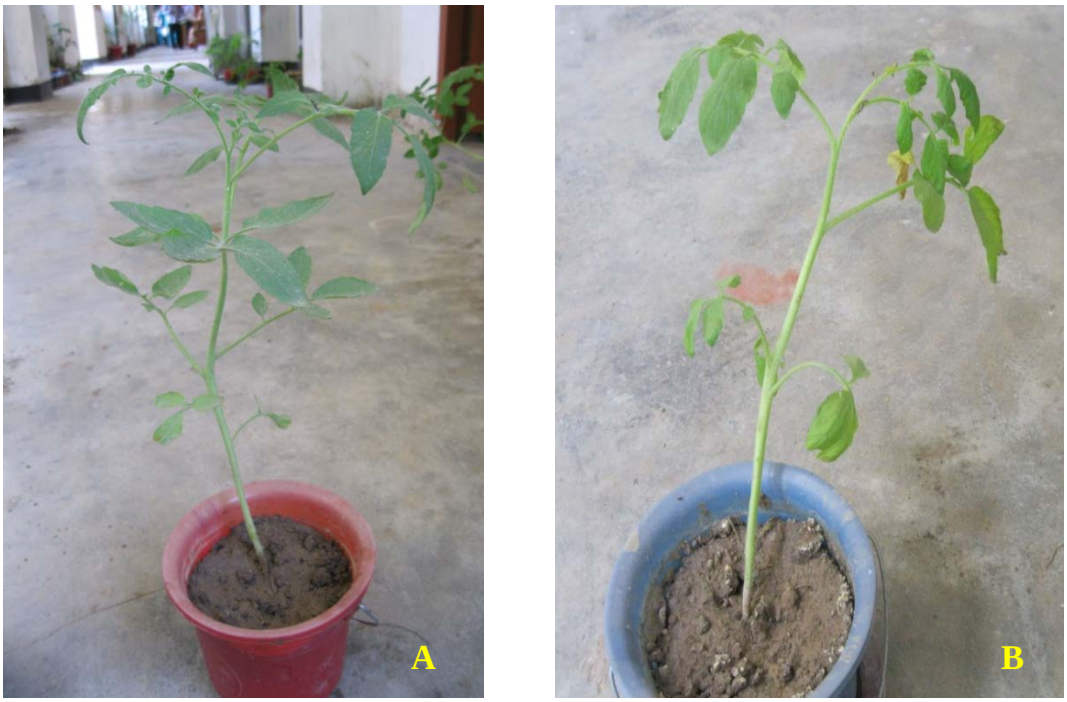


Fig. 24 Effect of root dipping in suspension of microbial antagonists on prevalence of Fusarium wilt of tomato

A. Healthy plant (treated)

B. Wilted plant (untreated)

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

Name of Antagonists	% Germination	Prevalence of seed borne fungi		
		Aspergillus	*Fusarium*	*Curvularia*
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 are not significantly different (p=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*

T_6= Control (Untreated)

Table 4. Efficacy of root dipping in suspensions of selected microbial antagonists on prevalence of *Fusarium oxysporum* f. sp. *lycopersici*

Treatments	% Disease Incidence			PDI		
	10 DAT	20DAT	30DAT	10 DAT	20DAT	30DAT
T_1	0	0.00 d	10.00 d	0	0.00 d	3.33 d
T_2	0	10.00 c	20.00 c	0	3.33 c	13.32 c
T_3	0	10.00 c	30.00 b	0	3.33 c	19.98 b
T_4	0	20.00 b	20.00 c	0	6.66 b	13.32 c
T_5	0	0.00 d	10.00 d	0	0.00 d	3.33 d
T_6 (Control)	0	40.00 a	60.00 a	0	13.32 a	40.96 a
$LSD_{(0.05)}$	0	7.427	7.427	0	1.939	1.037
CV %	0	9.62	9.33	0	9.32	4.06

Means within the same column with a common letter are not significantly different (p=0.05) by DMRT

T_1= Root dipping in bacterial suspension (Iso-1)

T_2= Root dipping in bacterial suspension (Iso-2)

T_3= Root dipping in bacterial suspension (Iso-3)

T_4= Root dipping in bacterial suspension (Iso-4)

T_5= Root dipping in suspension of *Trichoderma harzianum*

T_6= Control (Untreated)

Chapter V

Discussion

DISCUSSION

This study was conducted to isolate suitable microbial antagonist from the rhizospheric soil of tomato field and to determine promising antagonist against *Fusarium oxysporum* f. sp. *lycopersici*.

In this study, twenty bacterial isolates were isolated from the soil sample using dilution plate technique among them, four showed antagonistic activity against *Fusarium oxysporum* f. sp. *lycopersici*. Screening of soil bacteria was done by dual culture technique. Among the bacterial antagonists, the tested four isolates were able to inhibit the growth of *Fusarium oxysporum* f. sp. *lycopersici*. Thus it was assumed that, the four isolates have more or less ability to control *Fusarium oxysporum* f. sp. *lycopersici.*

Further, to identify the bacteria isolates those showed antagonistic activity against *Fusarium oxysporum* f. sp. *lycopersici*, morphological and biochemical tests were done. In Gram's staining test all four isolates gave gram negative reaction that was found rod shaped structure under compound microscope without any particular arrangement and also gave positive reaction in KOH solubility test. Both these test confirmed that all the four isolates were Gram negative bacteria. In starch hydrolysis, all the bacterial isolates showed positive reaction except Iso-4. In catalase test, all the four isolates showed the positive result in reaction with 3% H_2O_2. Among all the four isolates, Iso-2 formed dark purple color immediately after picking of bacteria on to the oxidase disk, while Iso-3 and Iso-4 produce dark purple color after 20 seconds. Iso-1 did not form any color in oxidase disk. In pecteolytic test, all the bacterial isolates showed negative result. All the three isolates except Iso-1 showed the positive result i.e. changed Simmon's Citrate Agar slants color from green to bromothymol blue in citrate utilization test. All the four isolates showed positive result in Gelatin liquefaction test. In this study, hypersensitivity test of the isolated bacteria was also done. It was observed that, all bacterial isolates shown negative hypersensitivity reaction on tobacco leaves. Umesha *et al.* (2008) reported that hypersensitive response is an important, definitive test to identify plant pathogenic bacteria. Plant pathogenic bacteria will show positive

hypersensitive response. Similar reports were given by Hossain (2006) and Wick (2006). Kreig (1923) and Schaad (2001) reported that all the above characteristics are similar with the characteristics of different species of *Pseudomonas*. Biochemical characteristics of the present antagonisti bacterial isolates suggested that, they may be some species of *Pseudomonas*.

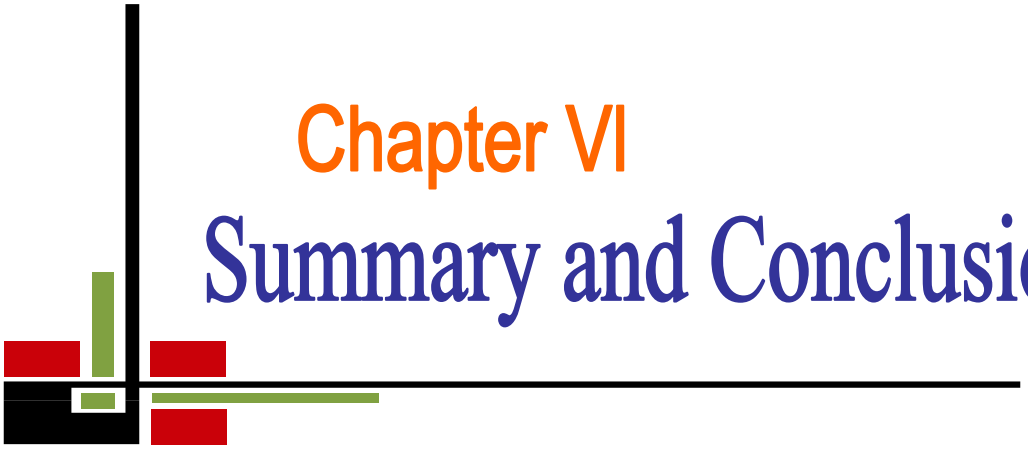
The tested *Trichoderma harzianum* caused 75.75% growth inhibition of *F. oxysporum* f. sp. *lycopersici* and its growth inhibition against *F. oxysporum* f. sp. *lycopersici* was very strong which is supported by Sundar *et al.* (1995) and Deshmukh and Raut (1992) who reported *Trichoderma harzianum* grew over the colonies of *Fusarium oxysporum*. Chabbi and Matrod **(**2002) achieved 77% growth inhibition of *Fusarium oxysporum* with *Trichoderma harzianum.* Xu *et al*. (1993) also observed the hyphal growth inhibition of *Fusarium oxysporum* by two isolates of *T. harzainum* T82 and NF9.

The capability of causing wilt disease by *F. oxysporum* f. sp. *lycopersici* isolate was confirmed by artificial inoculation to tomato plant. The result is supported by the findings of Begum (2007) who observed that *F. oxysporum* f. sp. *lycopersici* was able to produce wilting symptoms in tomato plants. The finding is also supported by the finding of Altinok (2005) who reported wilting in tomato plants in Turkey by *F. oxysporum* f. sp. *lycopersici.* Soil level inoculation method i.e. soil drenching method was followed to inoculate the tomato plant varieties by *F. oxysporum* f. sp. *lycopersici*. Soil level inoculation method was successfully used for the production of collar rot of sunflower caused by *Sclerotium rolfsii* (Babar, 1999). Soil drenching method showed highly susceptible reaction for the inoculation of wilt causing organism of tomato plants. The reliability of soil drenching method is also supported by Sitaramaiah and Sinha (1984) and Rahman (1992) who used the same method for inoculation of tomato.

Seed treatment was performed to evaluate the efficacy of microbial antagonists against prevalence of *Fusarium oxysporum* f. sp. *lycopersici* in seeds. The result revealed that, Iso-1 could significantly suppress prevalence of *F. oxysporum* f. sp. *lycopersici* over all the tested antagonistic microorganisms. About 11.25% infection was caused by *F. oxysporum* f. sp. *lycopersici* in non treated seeds where 0.58% infection was found both in Iso-1 and *Trichoderma harzianum* treated seeds.

It was observed that, the four bacterial isolates have the ability to inhibit the growth of *Fusarium oxysporum*. To evaluate the antagonistic potential of isolated antagonistic bacteria and collected *Trichoderma harzianum*, a pot experiment was conducted by root dipping of tomato seedlings in suspensions of microbial antagonists and *F. oxysporum* f. sp. *lycopersici* was inoculated by soil drenching. Data were recorded three times at 10, 20 & 30 DAT. The result revealed that, Iso-1 could significantly suppress disease incidence and severity more than that of other microbial antagonists. *Trichoderma harzianum* also showed better performance regarding disease of Fusarium wilt. No disease incidence was recorded in case of Iso-1 and *Trichoderma harzianum* upto 20 days after transplanting. After 30 days after transplanting, % disease incidence and PDI were recorded 10% and 3.33%, respectively. The present findings agreed with the findings of Weller *et al.* (2002).

This result clearly indicates that the isolate Iso-1 have the strong antagonistic effect and could be used as an eco-friendly management against *F. oxysporum* f. sp. *lycopersici*.



Chapter VI

Summary and Conclusion

SUMMARY AND CONCLUSION

The experiment was conducted at Plant Pathology Laboratory and net house of SAU, Dhaka during July 2011 to June 2012 to control *Fusarium oxysporum* f. sp. *lycopersici in-vitro* and Fusarium wilt of tomato seedlings in pot experiment. For this purpose, twenty bacterial isolates were isolated from rhizosphere soil. Among the bacterial isolates, four were found to inhibit the growth of *F. oxysporum* f. sp. *lycopersici.* They were isolates Iso-1, Iso-2, Iso-3 and Iso-4. One fungal bio-control agent *Trichoderma harzianum* was also used in this experiment.

Fusarium oxysporum was isolated and purified from tomato seeds. Pathogenicity test showed that, *F. oxysporum* f. sp. *lycopersici* have the strong ability to cause disease in tomato plant.

In-vitro bioassay showed that among all the microbial antagonists, *Trichoderma harzianum* was able to strongly inhibit the growth of *F. oxysporum* f.sp. *lycopersici.* Among the bacterial isolates, Iso-1 has the most capability to inhibit the growth of *F. oxysporum* f.sp. *lycopersici.*

In morphological test, Iso-1 produced creamy white colored colony in NA medium & others are slight yellow. All showed gram negative reaction in Gram's staining test. They also showed positive reaction in KOH solubility test. In biochemical test, all the bacterial isolate except Iso-4 made clear zone in starch hydrolysis test. In catalase test, all the four isolates showed the positive result in reaction with 3% H_2O_2. Among all the four isolates, Iso-2 formed dark purple color immediately after picking of bacteria on to the oxidase disk. In pecteolytic test, all the bacterial isolates showed negative result. All of the three isolates except Iso-1 showed the positive result i.e. changed its color from green to bromothymol blue in citrate utilization test. All of the four isolates showed positive result in Gelatin liquefaction test. All of the bacterial isolates shown negative reaction on tobacco leaves. These above result revealed that, they may be different species of *Pseudomonas*.

Efficacy of four bacterial antagonistic isolates and *T. harzianum* isolates was determined against *F. oxysporum* and Fusarium wilt of tomato seedlings in seed treatment and pot experiment, respectively. Among all the microbial antagonists, *Trichoderma harzianum* was able to strongly inhibit the growth of *F. oxysporum* f. sp. *lycopersici.* Among the bacterial isolates, Iso-1 has the most capability to inhibit the growth of *F. oxysporum* f. sp. *lycopersici.* In pot experiment, no disease incidence was recorded upto 20 days after seedling transplanting. Disease incidence and PDI were recorded 10% and 3.33%, respectively after 30 days of seedling transplanting when the tomato seedlings were treated with Iso-1 and *T. harzianum*. This result clearly indicates that, in case of fungus, *Trichoderma harzianum* and in case of bacteria, Iso-1 has the antagonistic effect and those can be used as bio-control agent.

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APPENDIX-A

ANALYSIS OF VARIANCE

1. Analysis of variance of the data on anti-fungal activity of Antagonists studied against *Fusarium oxysporum* in Dual culture method

Sources of Variation	Degrees of Freedom	Mean Square Value
		% Inhibition
Replication	2	3.582
Antagonists	4	1210.579
Error	8	2.172

2. Analysis of variance of the data on effect of different microbial antagonists on percent germination and prevalence of seed borne fungi in BARI tomato-2

Sources of Variation	Degrees of Freedom	% Germination	Mean Square Value		
			Prevalence of seed borne fungi		
			Aspergillus	*Fusarium*	*Curvularia*
Replication	2	1.601	0.608	0.045	0.406
Treatments	5	35.262	53.162	8.881	2.756
Error	10	0.994	0.433	0.241	0.031

3. Analysis of variance of the data on efficacy of root dipping with microbial antagonists in prevalence of *Fusarium oxysporum* f. sp. *lycopersici*

Sources of Variation	Degrees of Freedom	Mean Square Value					
		% Disease Incidence			PDI		
		10 DAT	20DAT	30DAT	10 DAT	20DAT	30DAT
Replication	2	0	16.667	66.667	0	0.248	3.838
Treatments	5	0	680.00	1050.0	0	63.207	676.067
Error	10	0	16.667	16.667	0	1.136	0.325

APPENDIX-B

PREPARATION OF MEDIA AND REAGENTS

Preparation of Gram's staining reagents:

i) Gram's Crystal violet (Hucker's modification):

Solution A: Crystal violet (90% dye content)	2.0 g
Ethyl alcohol	20.0 ml
Solution B: Ammonium oxalate	0.8 g
Distilled water	80.0 ml

Solution A and B in equal volume to prepare crystal violate solution.

ii) Gram's Iodine (Gram's modification of Lugol's solution):

Iodine	1.0 g
Potassium iodide (KI)	2.0 g
Distilled water	300.0 g

Add iodine after KI is dissolved in water to prepare Gram's Iodine solution.

iii) Gram's alcohol (decolorizing agent)

Ethyl alcohol (95%)	98 ml
Acetone	2 ml

iv) Safranin (counter stain)

Safranin (2.5% solution in 95% ethanol)	10 ml
Distilled water	100 ml

Preparation of KOH solubility reagent:

3% aqueous solution of KOH was prepared from the KOH granules

Preparation of Starch hydrolysis media and reagent:

i) Culture medium

Nutrient broth (Difco)	8.0 g
Soluble potato starch	10.0 g
Bacto agar (Difco)	15.0 g
Distilled water	1000 ml

ii) Reagent (Lugol's iodine)

Iodine	5.0 g
Potassium iodide	10.0 g
Distilled water	100 ml

Preparation of Catalase reagent:

3% aqueous solution of H_2O_2 was prepared from the H_2O_2 absolute solution

Preparation of Oxidase reagent:

1% aqueous solution of N,N,N,'N-tetramethyl-p-phenylene dihydrochloride

Preparation of Simmon's citrate agar medium:

$MgSO_4.7H_2O$	0.2 g
$NH_4H_2PO_4$	1.0 g
K_2HPO_4	2.0 g
Sodium citrate	2.0 g
NaCl	5.0 g
Bromothymol blue	80.0 mg
Agar	15.0 g
Distilled water	1000 ml

Preparation of Gelatin medium:

Gelatin	120g
Distilled water	1000 ml