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Compatibility of Fungal and Bacterial Bio-Agents and their Antagonistic Effect against *Fusarium oxysporum* f. Sp. *Lycopersici*

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ABSTRACT

Fusarium oxysporum f. sp. *lycopersici* causing tomato wilt is a common soil borne fungus. Bio-control agents could be used as an eco-friendly approach to effectively control the disease and may be advised to the farmers for profitable organic farming. The fungal (*Trichoderma harzianum*) and bacterial (*Bacillus subtilis* and *Pseudomonas fluorescens*) biological control agents were tested for their compatibility *in vitro* to determine whether they can be used in combination. Absence of inhibition zone indicated that the biocontrol agents were compatible with each other. *Trichoderma harzianum*, *Bacillus subtilis* and *Pseudomonas fluorescens* were tested *in-vitro* for their antagonistic activity against *Fusarium oxysporum* f.sp. *lycopersici.* The antagonistic potentiality of *Trichoderma harzianum* was determined by 25.4% percent inhibition of the growth of the fungal pathogen (*F. oxysporum lycopersici*) in presence of bio-control agent (*T.harzianum*) and the antagonistic activity of bacterial bio-control agents revealed *maximum* Zone of Inhibition (ZOI) with *Bacillus subtilis (*29.9 mm) followed by *Pseudomonas fluorescens* (25.6 mm).

Introduction

Tomato (*Solanum lycopersicum* L.*)* is one of the most popular and widely grown vegetable crops in the world. In 2014, world production of tomatoes was 170.8 million tonnes, with China accounting for 31% of the total, followed by India. The worldwide, tomato productivity is 33.9 MT/ha. In India, tomato occupies an area of 0.88 M ha having the production of 18.26 MT. However, the productivity is only 21.2 MT/ha (Anonymous, 2014). Fusarium wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* causes serious economic loss (Agrios, 2005). The estimated economic losses range from 10 to 80 percent yield loss in tomato producing area of the country (Keshwan and Chaudhary, 1977). The disease is systemic in nature and the pathogen may infect plants at any growth stage. The pathogen is soil as well as seed-borne in nature and causes vascular wilts by infecting plants through the roots and growing internally through the cortex to the stele

(Bowers and Locke, 2000) thereby causing xylem browning or blackening. The pathogen can survive in the soil up to 6 years even in the absence of a host plant.

It is important to investigate the potential of biological control agents in agriculture as these are highly effective, inexpensive with excellent shelf life and serve as a suitable alternative to chemical applications with sustainable disease management without pesticides residues in food stuffs, development of resistance in plant pathogens and appearance of new strains of pathogens. The natural control of several phytopathogens is based on the presence of suppressive soils where several biocontrol microorganisms belonging to *Trichoderma*, *Pseudomonas* and *Bacillus* genera are detected (Weller *et al.,* 2002 and Huang *et al.,* 2005).Numerous bacteria and fungi, including *Trichoderma* isolates, or combinations of microorganisms, collected from field tomato plants have proved to be effective in controlling Fusarium wilt in tomato (Larkin and Fravel, 1998; Srivastava *et al.,* 2010).

Prospects of biological control of soil-borne plant pathogens using the genus *Trichoderma*, as one of the promising bio-control agent, has been described (Morsy *et al.,* 2009; Sabalpara *et al.,* 2009). Successful control of Fusarium wilt in many crops by application of different species of *Trichoderma* has been reported (Bell *et al.,* 1982; Ramezani, 2009). They can also compete with other microorganisms; for example, they compete for key exudates from seeds that stimulate the germination of propagules of plant- pathogenic fungi in soil and, more generally, compete with soil microorganisms for nutrients and/ or space (Chet, 1987).

B. subtilis also produces a variety of biologically active compounds with a broad spectrum of activities toward phytopathogens and that are able to induce host systemic resistance (Bais *et al*., 2004; Stein, 2005; Butcher *et al.*, 2007; Nagorska *et al*., 2007; Ongena *et al.*, 2007; Ongena and Jacques, 2008). Various strains of *B. subtilis* have also been shown to be capable of forming multicellular structures or biofilms (Branda *et al*., 2001; Hamon and Lazazzera, 2001; Bais *et al*., 2004). Due to these beneficial traits, *B. subtilis* is potentially useful as a biological control agent.

Among biocontrol agents, root-associated fluorescent *Pseudomonas* spp. has also received special attention because of its excellent root colonizing ability, potential to produce a wide variety of anti-microbial metabolites, and its induction of systemic resistance (Erdogan and Benlioglu, 2010). Several studies have shown their efficacy as an inoculum (Kloepper *et al.,* 1980; Thomashow and Weller, 1995; Lugtenberg and Dekkers *et al*., 1999; Whipps, 2001; Weller *et al*., 2002; Achouak *et al*., 2004; Hariprasad and Niranjana, 2009; Validov *et al*., 2009). Liquid formulation of *P. fluorescens* Pf1 exhibited higher induction of defense enzymes and reduced the incidence of tomato Fusarium wilt disease (Manikandan and Raguchander, 2014). Fluorescent *Pseudomonas* bacteria have been shown to act against pathogenic agents by synthesizing antibiotic compounds (e.g., Phenazins, Pyrrolnitrine and 2,4- Diacetyl fluoro glucinol) (Keel *et al*., 1992), hydrogen cyanide (Maurhofer *et al*., 1995), lytic enzymes capable of altering the fungal cell wall (Chitinase and Glucanase) and other secondary metabolites (O'Sullivan and O'Gara, 1992). In addition to the antibiotic properties and the trophic competition recognized in these rhizobacteria, there is evidence that fluorescent *Pseudomonas* strains can trigger Induced Systemic Resistance (ISR) in plants, thus assuring a protection against a broad spectrum of phytopathogen agents (Van

Loon *et al*., 1998). With this background information, the present investigation was undertaken to evaluate the compatibility of fungal and bacterial bio-agents and their antagonistic effect against *Fusarium oxysporum* f. sp. *lycopersici.*

Materials and Methods

An *In vitro* experiment was conducted in the Bio-control Laboratory of Plant Pathology Discipline, Chandra Shekhar Azad University of Agriculture And Technology, Kanpur, U.P. to assess the compatibility among *P. fluorescens, Bacillus subtilis* and *Trichoderma harzianum* in order to determine whether they can be used in combination. Thereafter *In vitro* experiments to assess antagonistic effect of each of these biocontrol agents against *F. oxysporum* f. sp. *lycopersici* were also conducted.

Survey, collection, isolation, purification and identification of fungal pathogen

The tomato plants showing typical wilt symptoms were collected from farmer's fields of Kalayanpur, Mandhana and Chaubeypur blocks of Kanpur district. The diseased plants were uprooted, packed in polybags and brought to Bio-control lab for isolation.

Roots of infected tomato plants were cut into small pieces and surface sterilized with 1% Sodium hypoclorite solution for 1 minute. Isolations were made on Petri plates poured with PDA by placing the sterilized root pieces under aseptic conditions using laminar air flow cabinet. These inoculated Petri plates were incubated at 25 ±1ºC in a BOD (Biological Oxygen Demand) incubator. As soon as the growth of pathogens occurred, with the help of the sterilized needle a hyphal bit from the periphery of the growing fungal colony was transferred onto a potato dextrose agar slant, in the laminar air flow cabinet to avoid any chance of contamination. Thus, pure cultures of the fungi growing on these root pieces were prepared. For each fungal colony separate slant was used.

For identification of different fungi/ pathogens the colonies of different fungi growing on potato dextrose agar medium were examined under Light microscope (Olympus). Based on colony colour and growth and type of mycelium, sclerotia and the spores produced, tentatively the colonies of different pathogens were separated. Later on the slides of the pathogens having dark colour colonies were prepared in lactophenol only and of those having cottony white colonies apparently, looking as those of *Fusarium* were prepared with lactophenol- cotton blue stain. The *Fusarium* cultures were separated on PDA medium based on their colony colour, pattern, spore morphology and the conidiophores etc. as described by Booth (1971).

Isolation, purification and identification of *Trichoderma sp*.

Soil samples from 5-6 cm depth were collected from farmer's fields of Kalayanpur, Mandhana and Chaubeypur blocks of Kanpur district in polythene bags. Five soil samples were collected from each location.

For isolation of *Trichoderma* strains, Serial dilution technique (Johnson and Curl, 1972) on Trichoderma selective medium (Elad *et al*., 1981) was followed.

Ten gram soil sample from well pulverized, air dried soil was added into 90mL sterile water in a flask to make 1:10 dilution (10^{-1}). The mixture was vigorously shaken on a magnetic shaker for 20-30 minutes to obtain uniform suspension. One ml of suspension from flask was transferred into a test tube containing 9mL sterile water under aseptic

condition to make 1:100 (10^{-2}) dilution. Further dilution 10^{-3} was made by pipetting 1mL suspension into additional water as prepared above. One mL each liquids of 10^{-3} dilution were transferred into 10 sterile Petri plates, which was previously poured by 15mL sterile PDA medium and spread uniformly. The Petri plates were incubated at 25 ± 1^{0}C for 7 days in an incubator. As soon as the mycelial growth were visible in the PDA culture medium, the hyphal tips from the advancing mycelium were cut and transferred into the culture slants containing PDA medium for further purification and identification of culture. The pure culture of *Trichoderma* sp. was obtained by adopting single spore technique. Trichoderma isolate was identified by light microscope for morphological characters such as the branching pattern of conidiophore, the conidiophore apex elongation, phialides shape, size, structure, and conidial shape**,** using the available literature (Bisset, 1991).

Isolation of antagonistic bacteria (*Pseudomonas fluorescens* and *Bacillus subtilis)*

Serial dilution technique (Johnson and Curl, 1972) was adapted for isolation of *Bacillus* and *Pseudomonas* sp. from rhizospheric soil samples collected from tomato eco-system. One gram of air-dried soil samples were weighed and suspended in 9mL sterilized distilled water and stirred well.

Isolation of *Pseudomonas* sp.

For isolation of *Pseudomonas* sp. one ml of the soil suspension at 10^5, 10^6, 10^7 dilution was spread on Petri plates poured with Pseudomonas specific medium known as King's B medium (Hi media) (King *et al.*, 1954).

The plates were incubated at 30°C for 48 h. *Pseudomonas* colonies were picked from the medium and sub-cultured onto Nutrient Agar slant.

Isolation of *Bacillus* sp.

For isolation of *Bacillus* sp., Nutrient Agar medium (Sigma Al-drich) was used. The plates were incubated at 30°C for 48 h. *Bacillus* colonies were picked from the medium and sub-cultured onto Nutrient Agar slant.

For identification of bacterial bio-agents, two techniques were adopted *viz.* visual observation on Petri dishes and micro-morphological studies. Observation of colony morphology was done such as the shape, size, texture, colony surface markings, elevation, margin type, consistency, colour, translucency or opaqueness and presence of pigments, precipitates or crystals in the medium. For micro-morphological studies, Gram staining method (Gram, 1884) was used. First of all a bacterial smear was prepared on greeze free clean slide, dried in air and then fixed by heat. Staining was done with ammonium oxalate crystal violet for 1 min and then washed in gently in tap water. It is then decolorized with gentle agitation in 95% ethyl alcohol for 30 s, till the blue colour ceased to come out .Further, it is counter stained with Safranine solution for 10s, washed in tap water, dried and examined under the oil immersion objective of the microscope. Appearance of red color revealed the Gram negative nature of the bacterium (*Pseudomonas* sp.) and that of violet color revealed Gram positive nature of the bacterium (*Bacillus* sp.). Cell shape, arrangements, flagellation etc. were also seen the under light microscope.

For identification at species level purified cultures of fungal and bacterial bio-agents were send to ITCC, New Delhi. Based on the

identification report the fungal and bacterial bio-agents were identified as *Trichoderma harzianum, Pseudomonas fluorescens* and *Bacillus subtilis* were used for further studies.

Compatibility among fungal and bacterial bioagents

In vitro compatibility test between *P. fluorescens* and *T. harzianum* or *Bacillus subtilis* and *T. harzianum* using Dual culture plate method described by Siddiqui and Shaukat (2003) was employed.

Accordingly, an overnight culture of *P. fluorescens* and *Bacillus subtilis* grown on Nutrient broth streaked on one side of a Petri-dish containing PDA. The other side of the petri-dish was inoculated with 5mm disc of *T. harzianum* (9 days old). The plates were then incubated at 25±1°C and zone of inhibition (if any) was measured. The test was performed in triplicates.

Compatibility between *Pseudomonas fluorescens and Bacillus subtilis*

The isolates of *Pseudomonas fluorescens* and *Bacillus subtilis* were tested for their compatibility among each other following the method of Fukui *et al*., (1994).

The compatibility was determined for *P. fluorescens* and *B. subtilis* strains using Nutrient Agar medium.

The bacterial strains were streaked horizontally and vertically to each other. The plates were incubated at room temperature (28±2°C) for 72h and observed for the inhibition zone.

Absence of inhibition zone indicated the compatibility with respective bacterial strains and the presence of inhibition zone (if any) indicated the incompatibility.

In vitro screening of fungal bio-agent (*Trichoderma harzianum*) against *Fusarium oxysporum* f.sp. *lycopersici*

To assess *in vitro* effect of *T. harzianum* against *F. oxysporum* f. sp. *lycopersici* a laboratory bioassay using Dual culture technique (Morton and Stroube, 1955) was used. The antagonistic activity of *T. harzianum* against *F. oxysporum* f. sp. *lycopersici* was tested using PDA medium. Five mm disc from 7 days old culture of the pathogen was placed on one end of the Petri dish with the help of sterilized inoculation needle and one day later, 5 mm disc from antagonist culture was inoculated at opposite side, since, *T. harzianum* was fast growing. Petri plates without antagonist served as control. Experiment was replicated thrice. Observations were recorded up to 72 h. and percent growth inhibition was calculated using following formula - Vincent (1927).

Percent growth inhibition =

$$\frac{\text{Growth in control - Growth in treatment}}{\text{Mycelial growth in control}} \text{X100}$$

In vitro screening of bacterial bio-agents (*Pseudomonas fluorescens* and *Bacillus subtilis)* against *Fusarium oxysporum* f. sp. *lycopersici*

Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts. The antagonistic activity of *Pseudomonas fluorescens* and *Bacillus subtilis* against *Fusarium oxysporum* f. sp. *lycopersici* was tested using Well Diffusion Technique (Magaldi, 2004; Valgas, 2007). Twenty mL of PDA media was poured on glass Petri plates and allowed to solidify. The agar plate surface was then inoculated by spreading 1 mL of *Fusarium oxysporum lycopersici* suspension over the entire agar surface. Then, 4 holes with a diameter of 5 mm were punched

aseptically with a sterile cork borer ensuring proper distribution of holes in the periphery and the bacterial bio-agents (suspension having cfu $2x10^8$/mL) were introduced into the wells in different plates. The petri plates without antagonist (*Pseudomonas fluorescens* and *Bacillus subtilis*) served as control. The plates were incubated at 25 ± 1^0C for 5 days and observed for the inhibition zone. Experiment was replicated thrice. The bacterial bio-agent diffuses in the agar medium and inhibits the growth of the pathogen creating a zone of inhibition. The diameter of the zones of inhibition was measured with scale.

Results and Discussion

Compatibility of *T.harzianum* with *Pseudomonas fluorescens* and with *Bacillus subtilis*

The fungal and bacterial antagonist found potential against *Fusarium oxysporum* f. sp. *lycopersici* were tested for their compatibility *in vitro* as described in "Materials & Methods". Absence of inhibition zone around the disk indicated that these two bacterial bio-control agents were compatible with *T. harzianum* (Fig. 1).

Compatibility between *Pseudomonas fluorescens and Bacillus subtilis*

The two bacterial antagonists found potential against *Fusarium oxysporum* f. sp. *lycopersici* were tested for their compatibility *in vitro* as described in "Materials & Methods". Absence of inhibition zone indicated that these two bacterial biocontrol agents were compatible with each other (Fig. 2).

Antagonistic activity of *T. harzianum* against *Fusarium oxysporum* f. sp. *lycopersici* (Dual culture technique)

Trichoderma inhibited the growth of *Fusarium oxysporum* f. sp. *lycopersici* through its ability to grow much faster than the pathogenic fungi thus competing efficiently for space and nutrients. The antagonistic potentiality of *Trichoderma harzianum* was determined by dual culture technique as described in "Materials and Methods". The results are interpreted in terms of percent inhibition of the growth (25.4%) of the fungal pathogen (*F. oxysporum lycopersici*) in presence of bio-control agent (*T.harzianum*) and presented in Table 1 and Figure 3 and 4.

Table.1 Percent inhibition of *F.oxysporum lycopersici* in presence of *T. harzianum*

Treatment	Radial growth of pathogen* (mm)	Percent inhibition in growth
Fol + Th	39.3	**25.4**
Control	52.7	-
SE	**1.5**	
CD @ 5 %	**5.1**	

* Mean of three replications; *Fol – Fusarium oxysporum* f. sp. *lycopersici; Th – Trichoderma harzianum.*

Table.2 Antagonistic activity of bacterial bio-agents against *Fusarium oxysporum* f. sp. *lycopersici* recorded in terms of ZOI (Zone of Inhibition)

Treatment	cfu/ml	ZOI (mm)*
Fol+Pf	$2X10^8$	25.6
Fol+Bs	$2X10^8$	29.9

* Mean of three replications
Fol – Fusarium oxysporum f. sp. *lycopersici*
Pf- Pseudomonas fluorescens
Bs-Bacillus subtilis

Fig.1(A) Compatibility of *T.harzianum* with *B.subtilis* (B) Compatibility of *T.harzianum* with *P.fluorescens*

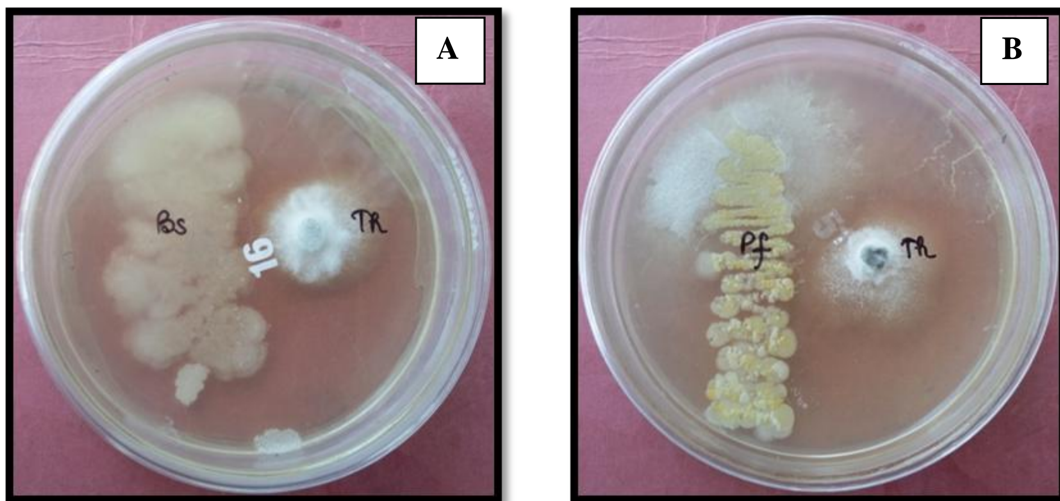


Fig.2 Compatibility of *Pseudomonas fluorescens* with *Bacillus subtilis*

Fig.3(A) Control **(B)** Antagonism of *T. harzianum* against *F. oxysporum f. sp. lycopersici* (Dual Culture)

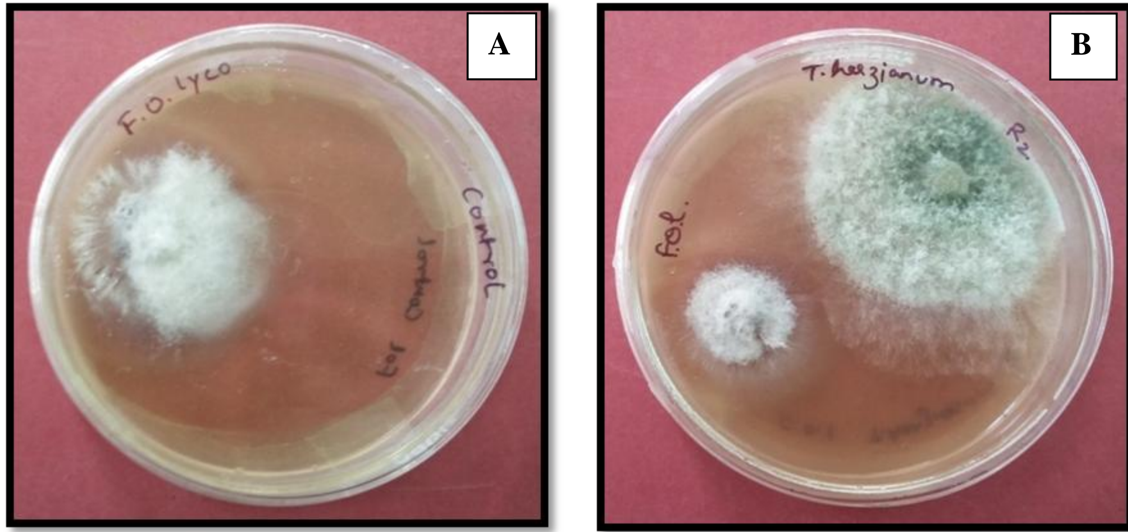


Fig.5 Antagonism of Bacterial bio-agents against *F. oxysporum f.sp. lycopersici* (Well Diffusion Technique) (A) *Bacillus subtilis* (B) *Pseudomonas fluorescens*

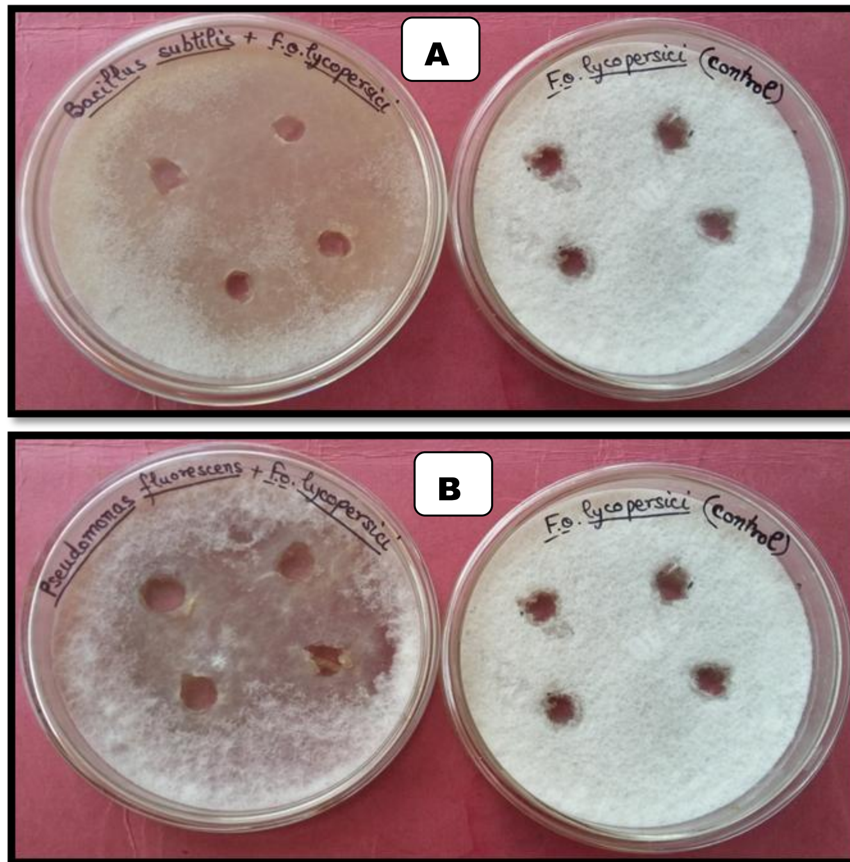
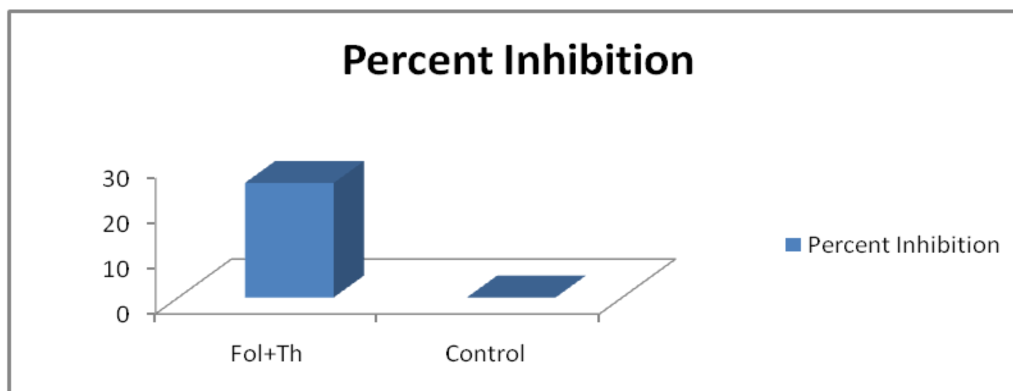


Fig.4 Percent inhibition of *Fusarium oxysporum fsp lycopersici* in presence of *T. harzianum*



Antagonistic activity of bacterial bio-control agents (*Pseudomonas fluorescens* and *bacillus subtilis)* against *Fusarium oxysporum* f. sp. *lycopersici* (Well Diffusion Technique)

The results of antagonistic activity of bacterial bio-control agents (*Bacillus subtilis and Pseudomonas fluorescens*) are presented in table 2 and figure 5. Data presented in table 2 clearly indicated that maximum Zone of Inhibition (ZOI) was recorded with *Bacillus subtilis as* 29.9 mm *followed by Pseudomonas fluorescens as* 25.6 mm.

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