The Journal of Plant Protection Sciences, **2**(2) : 35-44, 2010

Bio-management of root knot nematode and root rot disease by antagonistic fungi and rhizobacteria

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A B S T R A C T

Effects of four fungi and six rhizobacteria isolates from the rhizosphere of vegetable crops was studied for suppression of root knot nematode, *Meloidogyne incognita* and wilt fungus, *Fusarium oxysporum* f. sp. *lycopersici* on tomato in laboratory and pot culture experiment. All the isolates were isolated by serial dilution and cultured in liquid protein supplemented broth medium and culture filtrates (CF) were prepared. In *in-vitro* test single eggmasses of uniform size were kept in 5ml CF in 5cm diameter Petri plates. Antifungal activity was tested by dual culture of wilt fungus with antagonist fungus and bacteria isolates in PDA media plates. In *in-vivo* test soil was amended with antagonist and wilt fungal and bacterial isolates 2 x 10^6 CFU/ml and 2 x 10^8 CFU/ml respectively as talc formulation @ 0.2% (w/w). Twenty one days old tomato seedlings were transplanted after dip treatment in antagonist fungal and bacterial CF in earthen pots filled with amended soil. Seven days after transplanting, the seedlings were inoculated with second stage juveniles of *M. incognita* @ $2J_2$/g soil**.** The exposure of *M. incognita* eggs and juveniles to the culture filtrates showed high inhibition in egg hatching and caused high mortality of juveniles than the control ($P< 0.05$). The microorganisms which caused high nematicidal activity also showed antifungal activity in dual plate culture test *in vitro*. All the antagonists showed reduction in root knot and root rot disease and promote plants growth as the length and weight of root and shoot in pot trials on tomato plants.

Keywords: Antagonistic fungi, root knot nematode, wilt fungus, bio-management

Introduction

Soil borne disease caused by bacteria, fungi and nematodes create a major economic loss in agricultural crops. The infection in roots by soil borne root infecting fungi such as *Fusarium oxysporum, Rhizoctonia solani* and *Pythium ultimum* and root knot nematode, *Meloidogyne incognita* causes root rot and root knot disease complex which reduces the yield and substantial mortality of the plants (Ghaffar 1995). The economic loss caused by nematodes alone is estimated at US $ 100 billion worldwide. Also annual estimated crop losses due to nematodes in India, has been worked out to be about Rs. 242.1 billion (Jain *et al.* 2007).

Synthetic pesticides have been successfully used to control soil borne plant pathogens. Although in many cases these pesticides appear to be the most economical and efficacious means of controlling plant pathogens, toxicological, environmental and sociological concerns have led to drastic reductions in the availability of these chemicals. These restrictions have forced farmers to look for an integrated system that makes use of other means of disease control to prevent the infection of fungi as well as nematodes.

The present study was initiated to determine the biological control of soilborne plant pathogens by antagonistic microorganisms (fungi and bacteria) as a potential nonchemical means of plant disease control. One of those organisms, *Trichoderma* spp., which is an active mycoparasites, has been considered a biocontrol agent of foliar disease (Elad *et al.* 1993), soilborne disease (Papavizas 1985) and root knot nematode diseases (Kerry 1990). The antagonistic species of some other genera of fungi as *Aspergillus* spp., and entomopathogenic fungi *Beauveria bassiana*

also interact with other fungi, including plant pathogenic fungi and root knot nematodes. The antagonistic nature of fungal species from the genus *Trichoderma* spp. and *Aspergillus* spp. are common, found in almost all types of soil. Some reports of nematode antagonism were also reported from Entomopathogenic fungi *Beauveria bassiana* and bacteria *Bacillus subtilis*. The antagonism of these microorganisms involves several mechanisms, such as competition for nutrients, antibiosis, and production of fungal cell wall and nematode cuticle degrading enzymes as the main mechanism. Several reports showed suppression of root knot disease by *Trichoderma* spp. (Rao *et al.* 1996; Sharon *et al*. 2001; Spiegal & Chet 1998), *Aspergillus* spp. (Siddiqui *et al*. 2001, 2004) and few reports by *Beauveria bassiana* (Ekanayake & Jayasundara 1994; Liu *et al*. 2008) successfully.

The rhizobacteria *Bacillus subtilis* produces spores which are resistant to unfavorable conditions (Doi 1989) and also more adaptable than other bacteria to formulation, storage and field application. The potential of *Bacillus* against phytopathogenic fungi (Berger *et al*. 1996; Ryder *et al*. 1999; Knox *et al*. 2000; Wulff *et al*. 2002; Yu *et al.* 2002; Collins & Jacobsen, 2003) and root knot nematodes has been reported previously (Li *et al*. 2005). The metabolites of *Bacillus subtilis* are also able to induce systemic resistance and plant growth on varieties of plant species (Cook & Baker 1983). In view of the dual importance (Plant growth promotion and disease control), present study was conducted to examine the effects of Plant Growth Promoting Rhizobacteria (PGPR) in the control of root infecting fungi and the root knot nematode on tomato plants.

Materials and Methods

Culture of root knot nematode

The egg masses were removed from the monoxenic culture of *M. incognita* and thoroughly washed 3-4 times in sterile water. The content was poured onto moistened double layer tissue paper on (5 cm dia.) Petri plates filled with sterile water and incubated at 28 ± 2^0C in BOD for 2 days for emergence of juveniles (IJ_2). Hatched juveniles collected in a beaker were used for *in vitro* and *in vivo* experiment.

Soil fungi and bacteria culture

Soil fungi and bacteria isolates were cultured by serial dilution of collected root adhered soil samples from vegetable cultivated fields of some districts of Meerut region (U.P.). The fungi were isolated by pouring 0.5ml of serially diluted solution (10^4 dilutions to 10^7) of each step on Potato Dextrose Agar media and incubated at 25 ± 2^oC. After two repeated subculturing selected isolates of fungi were mounted in Lactophenol- Cotton Blue stain (Thomas Baker, India). Prepared mounts were examined under Phase Contrast Microscope and identified with the help of identification keys (Barron 1977) on the basis of colonial morphology, shape of spore or conidia (oval or round), type of sporangiophore and type of mycelium (septate or non septate).

Isolation of rhizobacteria was done by pouring 0.5ml of serially diluted solution (10^4 dilutions to 10^7) of each step on Medium 523 Agar plates (selective media for *Bacillus* spp.). All the plates were incubated at 28 ± 2^0C in BOD for 5 days and after repeated subculturing of single colonies on Nutrient Agar media plates, the purified isolates were selected for gram staining and examined under Phase Contrast Microscope for identification.

In-vitro Experiments

Nematicidal activity

Total eleven isolates of fungi and sixteen strains of *Bacillus subtilis* rhizobacteria were isolated from the rhizosphere of surveyed vegetable crops. Five to ten days old colonies of bacteria and fungi were inoculated in three different protein supplemented broth media *viz.* chitin and collagen (Himedia). Culture media composition (per liter) was used as given by Galper *et al.* (1990). The composition of media was as Protein Supplement (2g); $CaCl_2.2H_2O$ (0.1g); K_2HPO_4 (0.8g); $MgSO_4.7H_2O$ (0.3g); Yeast Extract (0.1g). To obtained cell free Culture Filtrate (CF), the inoculated flasks were incubated in BOD separately at 25±2°C and 14 days for fungi and at 28±2°C and 10 days for bacteria and thereafter centrifuged (at 2800 x g for 20 minute). The Content was filtered through Whatman filter paper No. 1 (Siddiqui *et al.* 2001). To determine the effect of CF on egg hatching, single eggmasses of uniform size were collected from monoxenic culture of *M. incognita* infected tomato roots and sterilized in 0.1% mercuric chloride solution and kept in 5 ml CF in Petri plates (5 cm diameter). The eggmasses kept in growth medium without the test fungi and bacterium served as control. For counting of juveniles small squares were made on outside of the bottom of Petri plates by permanent marker. All the plates of CF were replicated four times and incubated at 28±2°C in a BOD incubator for 5 days. After incubation the plates were examined under binocular stereoscopic microscope. All the hatched juveniles were counted and percent inhibition in hatching over control was estimated.

Mortality of juveniles was estimated by taking fifty freshly cultured J_2 of *Meloidogyne incognita*, in 5 ml CF of isolated fungi, bacteria and in growth medium (as control) in Petri plates and incubated at 28±2°C in BOD for 5 days. After incubation the plates were examined under binocular stereoscopic microscope and mortality was confirmed by probing the needle, if they did not move, considered dead (Cayrol *et al.* 1989).

Antifungal activity

The antifungal activity was tested against wilt fungus *Fusarium oxysporum* f. sp. *lycopersici* by employing dual culture plate method on PDA media (Li *et al*. 2005). In order to determine the antagonistic effects of fungi, fresh culture agar discs (5 mm diameter) of fungi were streaked on one side of the Petri plates and test fungus on another side of the plate. Antagonism of rhizobacteria isolates was determined by placing the 9 mm culture agar disc (upside down) of *Fusarium oxysporum* f sp. *lycopersici* in the centre and four sterile filter paper discs were placed in equal distance to the test fungus, with 10 µl rhizobacteria suspension grown in nutrient broth poured on each paper disc (Akköprü & Demir 2005). All the plates replicated four times and incubated at 28±2°C for a week. After incubation period all type of reaction or zone of inhibition were measured by growth measuring scales (Himedia). The isolates which produced clear zone of inhibition or lysis of fungal mycelium considered effective (Siddiqui *et al.* 2001). The antagonistic inhibition in growth zone of *Fusarium oxysporum* were measured and calculated by applying the following formula (Bora & Özaktan 1998):

$$\text{Growth inhibition (\%)} = \frac{\text{Diameter of Control Fungus - Diameter with Antagonist}}{\text{Diameter of Control Fungus}} \times 100$$

In-vivo Pot experiment

Sandy loam soil collected from the fields of Chaudhary Charan Singh University, Meerut, (U.P.) was filled in earthen pots (15 cm diameter) at 2 kg per pot and after sterilization amended with fungal and bacterial formulations at 0.2% (w/w). Fungi were cultured on sorghum seeds (2 x 10^6 CFU/ml) soaked in 5% sucrose solution and bacterial suspension (2 x 10^8 CFU/ml) was mixed with talc powder (40ml suspension in 200g talc powder). Twenty one day-old seedlings prepared in seedling trays sterilized by 1% mercuric chloride solution and after rinse in sterile water dipped in fungal CF and bacterial CF with 1% Carboxy Methyl Cellulose (as adhesive) for five minutes. Seven days after transplanting, the seedlings were inoculated with second stage juveniles of *M. incognita* $2J_2$/g soil by making the holes of about 3-4 cm depth around the stem and covered with top soil. Each treatment was replicated four times and irrigated as needed.

The data collected on *in-vitro* and *in-vivo* study were statistically analyzed by analysis of variance (ANOVA) and significant differences among treatments were tested by LSD test at probability levels of 5% ($LSD_{0.05}$) using SPSS statistical software.

Results

Out of sixteen isolates of *Bacillus subtilis*, only six and out of eleven fungi only four (*Trichoderma harzianum, Beauveria bassiana, Aspergillus fumigatus* and *Aspergillus niger*) were found antagonist to *M. incognita.* The suppression in egg hatching of *M. incognita* by *B. subtilis* was recorded highest in CF of Bs_1 (87.37%) and followed by CF of Bs_2, Bs_3, Bs_7, Bs_5 and Bs_8 and juvenile mortality was also recorded highest in CF of Bs_1(90.35%) and followed by Bs_2, Bs_7, Bs_5, Bs_3 and Bs_8 (Table- 1; fig. 1). It was observed that the *B. subtilis* strains from tomato, brinjal, okra, cucumber, bottle guard, bitter guard, pumpkin and chilli performed better nematicidal activities against *M. incognita*.

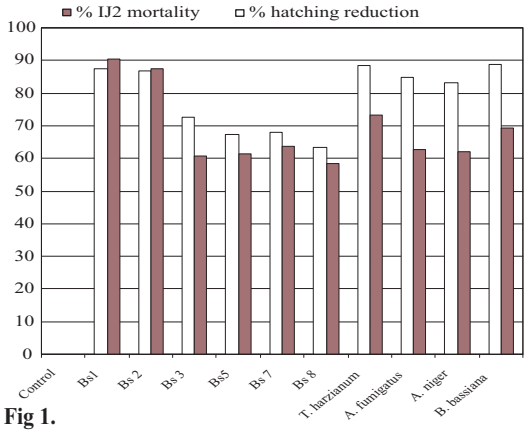

Fig 1.
Comparative efficacy of culture filtrates of different antagonistic fungi and strains of *B. subtilis* on *M. incognita* eggs and juveniles

The maximum percent reduction in egg hatching was observed in the CF of *B. bassiana* (88.88%), followed by *T. harzianum* (88.51%) (Table- 2; fig. 1), *A. fumigatus* (84.68%), *A. niger* (83.22%). The mortality of J_2 of *M. incognita* was recorded ($P< 0.05$) in CF of *T. harzianum* (73.32%), *B. bassiana* (69.32%), *A. fumigatus* (62.66%) and *A. niger* (62.0%).

The inhibition of growth zone of *F. oxysporum* f sp.*lycopersici* was found highest with *A. fumigatus* (59.19%) and followed by *T. harzianum* (55.75%). All the six antagonistic strains of *B. subtilis* (Bs1, Bs2, Bs3, Bs5, Bs7 and Bs8) showed 100% antifungal activity against the phytopathogenic fungi, *F. oxysporum.* In the culture media all bacterial strains grown well and no growth of wilt fungus was observed at five days of incubation period in BOD (Table-1 & 2; Fig. 2).

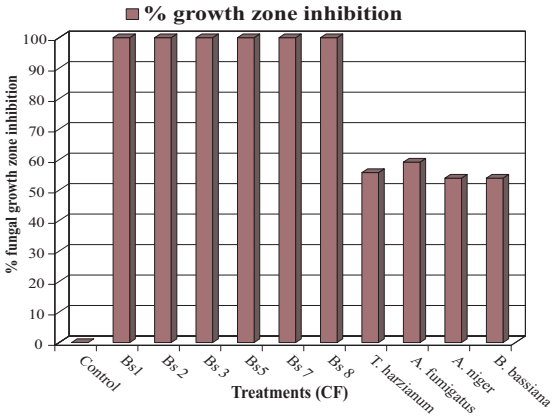

Fig 2. Comparative efficacy of antagonistic fungi and bacteria strains against *F. oxysporum* f.sp. *lycopersici* as % growth zone inhibition.

In pot experiments, study, two most effective strains of *B. subtilis* (Bs_1 and Bs_2) and three fungi (*T. harzianum, A. fumigatus* and *B. bassiana*) were tested against *F. oxysporum* f sp. *lycopersici* and *M. incognita* on tomato plants. All the antagonists showed significant suppression of *F. oxysporum* and *M. incognita* as compared to untreated inoculated control tomato plants ($P< 0.05$). *A. fumigatus* showed better plant growth as total plant length, total fresh and dry weight with least difference than *T. harzianum, B. bassiana and B. subtilis*

Table 1.
In-vitro nematicidal and antifungal activity of bacterial strains

S. No.	Treatments (CF)	*Meloidogyne incognita*				*Fusarium oxysporum* f sp. *lycopersici*
		Hatching of juvenile (IJ_2)	Reduction in hatching over control (%)	IJ_2 mortality	IJ_2 mortality over control (%)	Inhibition of growth zone* (%)
1.	Control (media)	192.66	0.0	0.0	0.0	0.0
2.	Bs1	24.33	87.37	40.75	90.35	100. 00
3.	Bs 2	25.66	86.68	39.25	87.4	100. 00
4.	Bs 3	53.0	72.49	27.25	60.73	100. 00
5.	Bs 4	109.33	43.26	17.25	34.66	-
6.	Bs5	63.0	67.29	27.75	61.46	100. 00
7.	Bs 6	98.33	48.97	18.75	37.32	-
8.	Bs 7	61.66	67.99	26.25	63.68	100. 00
9.	Bs 8	70.33	63.49	28.75	58.51	100. 00
10.	Bs 9	102.0	47.06	21.25	47.4	-
11.	Bs 10	97.33	49.48	17.0	37.77	-
12.	Bs 11	138.0	28.37	9.75	21.46	-
13.	Bs 12	128.0	33.56	10.0	22.22	-
14.	Bs 13	108.0	43.94	14.0	31.11	-
15.	Bs 14	127.33	33.9	40.75	25.17	-
16.	Bs 15	121.0	37.19	39.25	33.33	-
17.	Bs 16	138.66	28.02	27.25	30.35	-
	LSD (P<0.05)	8.57	-	3.59	-	-

$B_{S\ (1\text{-}16)}$ - Strains of *Bacillus subtilis* * Done only of effective antagonists bacterial strains

strains. The reduction in gall formation was also found high (77.67%/ RKI-1) in *A. fumigatus* treated plants and significantly followed by both strain of *B. subtilis* (76.48% and 76.04%; RKI-1), *T. harzianum* (73.95 %; RKI-2) and *B. bassiana* (73.66%; RKI-2) (Table–3; Fig. 3&4).

Discussion

In the present study six isolates of *B. subtilis* and four fungi *T. harzianum, A. fumigatus A. niger* and one entomopathogenic fungus, *B. bassiana* were found effective against the wilt fungus *F. oxysporum* and root knot nematode

Table 2.
In-vitro nematicidal and antifungal activity of fungal strains

S. No.	Treatments (CF)	*Meloidogyne incognita*				*Fusarium oxysporum* f sp. *lycopersici*
		Hatching of juvenile (IJ_2)	Reduction in hatching over control (%)	IJ_2 mortality	IJ_2 mortality over control (%)	*inhibition of growth zone (%)
1.	Control (media)	182.50	0.00	0.00	0.00	0.00
2.	*T. harzianum*	21.00	88.51	36.50	73.32	55.75
3.	*T. glaucum*	62.66	65.70	21.50	42.66	-
4.	*T. lignorum*	61.33	66.43	27.00	54.00	-
5.	*A. fumigatus*	28.00	84.68	31.00	62.66	59.19
6.	*A. niger*	30.66	83.22	31.00	62.00	53.93
7.	*A. flavus*	42.66	76.65	28.00	56.00	-
8.	*B. bassiana*	20.33	88.88	34.75	69.32	53.93
9.	*Fusarium oxysporum*	55.33	69.71	20.50	40.66	-
10.	*Rhizoctonia solani*	57.66	68.44	19.50	39.32	-
11.	*Pythium ultimum*	73.66	59.68	22.25	44.66	-
12.	*Penicillium expansum*	78.33	57.12	15.75	31.32	-
	LSD (P<0.05)	8.88	-	4.22	-	-

* Inhibition of growth zone was done only of effective antagonists fungal stains

Table 3.
Effect of different treatments on disease complex of *Meloidogyne incognita* and *Fusarium oxysporum* f.sp. *lycopersici* on tomato plants

Treatment	Plant length (cm)			Plant fresh weight (g)			Plant dry weight (g)			No. of gall / root system	No. of eggmass /root system	RKI
	Shoot	Root	Total	Shoot	Root	Total	Shoot	Root	Total			
T1	31.20	16.30	47.5	19.65	9.41	29.06	13.20	3.38	16.58	0.0	0.0	0.0
T2	19.61	11.74	31.35	13.63	6.62	20.25	6.35	1.95	8.30	168.0	151.5	**4**
T3	30.35	15.84	46.19	19.08	9.06	28.14	12.85	3.02	15.87	43.75 (73.95)	29.25	**2**
T4	30.21	17.30	47.51	19.42	9.35	28.77	13.20	3.25	16.45	37.50 (77.67)	26.50	**1**
T5	29.63	16.62	46.25	18.91	8.89	27.8	12.02	2.89	14.91	44. 25 (73.66)	30.25	**2**
T6	29.24	21.04	50.28	19.32	9.20	28.52	13.11	3.05	16.16	39.5 (76.48)	27.5	**1**
T7	28.72	20.81	49.53	18.96	8.56	27.52	12.0	2.85	14.85	40.25 (76.04)	30.25	**1**
LSD (P<0.05)	2.90	3.76	-	3.18	1.84	-	1.90	0.53	-	8.64	7.12	0.61

N = nematode; *Fo = Fussarium* oxysporum; *Af = Aspergillus fumigatus; Th = Trichoderma harzianum; Bb = Beauveria bassiana;* Bs = *Bacillus subtilis,* T1 = Healthy Control, T2 = Infseted Control (N+ Fo), T3 = N+ Fo+ *Th,* T4 = N+ Fo+ *Af,* T5 = N+ Fo+ *Bb,* T6 = N+ Fo+ Bs_1, T7 = N+ Fo+ Bs_2, Values in parentheses are percent reduction in gall formation

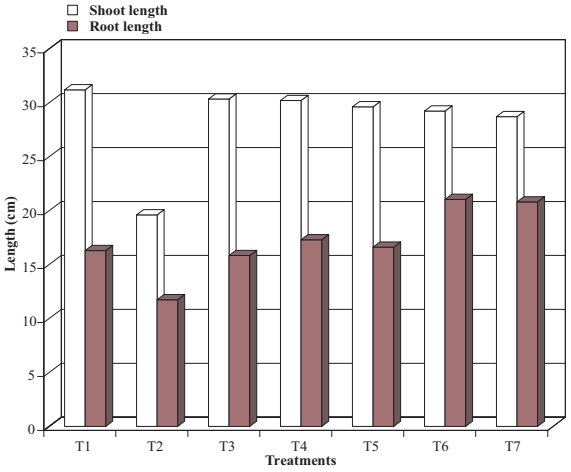

Fig 3. Effect of different treatments on shoot and root length of tomato plants infested by *Meloidogyne incognita* and *Fusarium oxysporum* f.sp. *lycopersici*

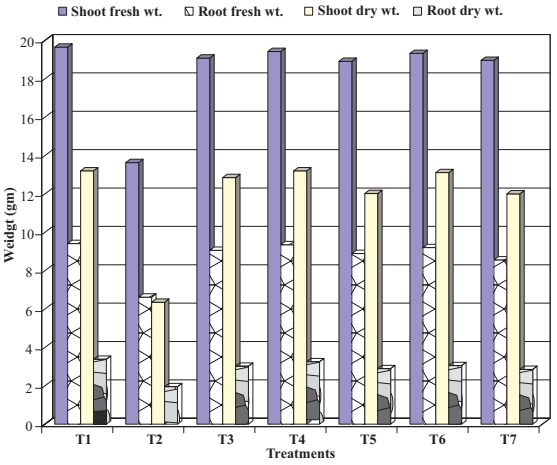

Fig 4. Effect of different treatments on fresh and dry weight of shoot and root of tomato plants infested by *Meloidogyne incognita* and *Fusarium oxysporum* f.sp. *lycopersici*

M. incognita in-vitro study as well as pot experiment study. *T. harzianum* strains are known to produce chitinase which play important role in the antifungal and nematicidal activity because chitin is the main component of the cell wall of the fungal pathogens and also of the nematode cuticles. Chitinase from *T. harzianum* have been characterized previously by De la Cruz *et al.* (1992) and Haran *et al.* (1995). The fungus *B. bassiana* is more prevalent in agriculture soil and exhibit many attributes that determine its virulence toward the host by producing degradative enzymes. The best known enzyme is subtilisin like serine protease which play important role in cuticle penetration of soil pathogens (St. Leger 1995). Liu *et al.* (2008) reported that the culture filtrates of *B. bassiana* strongly inhibited egg hatch and toxic to J_2 of *M. hapla.* Sun *et al.* (2006) observed that *B. bassiana* colonize *Meloidogyne* spp. eggs and females with 100% parasitism rate. The group of *Aspergillus* spp. also reported to secrete some mycotoxins with or without aflatoxins which are toxic to soil pathogens and it was known that *A. fumigatus* and *A. terreus* produces metabolites without aflatoxins. Therefore *A. fumigatus* may be a promising antagonist to pathogenic fungi and nematodes. Khan *et al.* (1984) studied the effect of culture filtrates of eight species of *Aspergillus* on the hatching and mortality of *M. incognita*. Culture filtrates of *A. niger, Pythium debarianum, Fusarium oxysporum, F. solani* and *F. moniliforme* found efficacious *in-vitro* on mortality of root knot nematode *M. incognita* (Radwan 2007). Culture filtrates of *Bacillus subtilis* and *Aspergillus fumigatus* have been studies and significant percent reduction on hatching and mortality of *M. incognita* have been observed (Satyandra & Chaubey 2007). Reddi *et al.* (2008) isolated nematophagus fungi from the rhizosphere of vegetable crops and eggmasses of *Meloidogyne* spp. and found that *A. fumigatus* and *A. niger* were dominated in rhizosphere of healthy tomato plants. In present study, it was noted that the strains of *B. subtilis* had suppressive effects on root knot nematode egg hatching, mortality and on growth of *F. oxysporum in-vitro* study as well as *in-vivo*

study. The total growth of tomato plants as length and weight were observed highest in *B. subtilis* treated plants and followed by fungal microorganisms as compared to untreated inoculated control tomato plants with no significant difference ($P< 0.05$). The rhizobacteria dominate the pathogens by various mechanisms such as competition for key nutrients, antibiosis and induced resistance and then positively affect plant growth with their promoting (PGPR) properties (Kloepper 2003). The field application of *B. subtilis* against *M. incognita* promoted growth of tomato plants by the solubilization of non-utilizable forms of phosphates present in the soil into forms which are readily utilized by plants (Khan & Tarannum 1999). The phosphate solubilizers also released some metabolites in soil, which suppressed fungal multiplication and survival. The metabolites may have been absorbed by the roots and became systemic in the plant, thereby inhibiting fungal pathogenesis inside the root tissue that not only led to decrease in the wilt severity but also led to a growth-promoting effect (Khan & Khan 2001; 2002).

Acknowledgement

Authors are thankful to Head of Department of Zoology, CCS University, Meerut for providing the laboratory facility. Department of Science & Technology (DST) is thankfully acknowledged for financial assistance by sanctioning the research project (SR/SO/AS-32/2003) to AKC and fellowship to SK.

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