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BIOMANAGEMENT OF NEMATODE-FUNGUS DISEASE COMPLEX IN TUBEROSE USING PLANT GROWTH PROMOTING RHIZOBACTERIA

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ABSTRACT
Pot culture experiments were carried out to study the interactive effect of root knot nematode, *Meloidogyne incognita* and the fungus, *Fusarium oxysporum* in tuberose var. Prajwal. The study revealed significant reduction of plant growth parameters and increased wilt severity in the sequential inoculation of nematodes followed by fungus over the individual inoculation of the fungus. To control the disease complex in tuberose, about four established antagonistic PGPR strains *viz*., *Bacillus subtilis* (Bbv 57, Bs1) and *Pseudomonas fluorescens* (Pfbv 22, Pf 1) were obtained from the culture collection centre, Department of Plant Pathology, Tamil Nadu Agriculture University, India. Crude antibiotics were extracted from the above strains and tested against *M. incognita* and *F. oxysporum in vitro.* The results revealed that 25 per cent concentration of the antibiotics exhibited enhanced antagonistic activity against both the nematode and fungi. The four strains were prepared in liquid formulation and tested against nematode-fungus disease complex in tuberose var. Prajwal under pot culture and field condition. Among the four strains tested, combined application of Bbv 57 and Pfbv 22 (each at 500 ml/ha) at monthly interval recorded maximum efficacy against the disease complex with the induction of high level of defense enzymes over other strains.

Key words: *Bacillus subtilis*, *Fusarium oxysporum*, *Meloidogyne incognita*, *Pseudomonas fluorescens.*

INTRODUCTION
Tuberose (*Polianthes tuberose* L.) is a perennial bulbous ornamental crop, being cultivated in many parts of India for its fragrant flowers which is being used in perfume and cosmetic industries besides being used in garlands and bouquets. Vegetative propagation of tubers of the plant paves way for the entry of many pathogens. Among them, plant parasitic nematodes and wilt inducing fungus contributes majorly for the drastic reduction in the plant yield. Infestation of root knot nematode, *Meloidogyne incognita* was reported to be wide spread in almost all the tuberose growing regions of South India (Rao *et al*., 2001) and known to cause about 10 % reduction in the flower yield (Khan and Parvatha Reddy, 1992). Spike emergence was observed to be delayed (65.07 and 68.33 days) due to the presence of high population of nematodes (100 and 150/cm^3 soil). The delay was extended further (81 days) when *Fusarium* was present along with the nematode. Presence of root knot nematode accelerates the wilt development which reflects in the reduced growth and yield of the crop. Maximum reduction was observed when both the pathogens were present than the presence of either of the pathogens alone. High cost of chemicals involved the management of disease complex provided with their ill effects on the environment made an urge to search for the alternative and reliable eco friendly management strategy for the management of disease complex. Plant growth promoting rhizobacteria (PGPR) are one among the candidate whose potential has been proved against the nematodes and fungus infesting various crops. Efficacy of the PGPR *viz*., *Pseudomonas fluorescens* and *Bacillus subtilis* has been well explored by earlier workers against the disease complex in various crops. Present investigation has been proposed to study the interactive effect of *M. incognita* and *F. oxysporum* in tuberose and to test the efficacy of PGPR strains against nematode-fungus disease complex in tuberose var. Prajwal under pot culture and field condition.

MATERIALS & METHODS
Source and identification of root knot nematode species associated with tuberose
Inoculum of root knot nematode was obtained from the nematode infected galled roots of tuberose collected from the field at Muthukallur, Coimbatore District, Tamil Nadu, India. Galled roots were washed in water to remove the adhering soil particles and the protruding egg mass in the galls were collected under microscope with needle. The collected egg masses were placed in distilled water for hatching. After 24-48 h, freshly hatched juveniles were collected and inoculated into one month old tomato plants (cv. CO3) planted in 5 kg pots filled with sterilized pot mixture (red soil: sand: Farm Yard manure (2:1:1)) to maintain the pure culture of the nematode. The juveniles were allowed to grow for 25-30 days in tomato to pass one generation and after the stipulated time, eggs and juveniles collected from the pure culture were used for *in vitro* and pot culture studies.Root knot nematode females collected from the galled roots were processed for perineal pattern studies to identify the species associated with the tuberose.
Identification of nematode species through perennial pattern studies
Matured *Meloidogyne* females were teased from the root galls and placed on a glass slide. They were cut at the neck

region and all the body tissues were gently pushed out. The cuticle was placed in 45 per cent lactic acid to facilitate further cleaning. The cuticle was carefully trimmed so that 5-10 times the perennial area (area near to vulva) was retained. This was transferred to a fresh drop of glycerol and examined under microscope.

Source and identification of *Fusarium* species associated with tuberose

Wilt infected tuberose plants were collected from the field at Muthukallur, Coimbatore District, Tamil Nadu, India. For isolation of *Fusarium* species, a small section of infected root (5-6 mm) tissues were cut and placed on Potato Dextrose Agar (PDA) medium with an antibacterial agent (Streptomycin sulphate). The plate was incubated for 2-4 days. Conidial culture was prepared from the specimen to confirm the species.

Preparation of conidial culture

Sporulated hyphae was scrapped from the PDA medium and placed on a cavity slide. Few drops of water was added to the slide and mixed well with the hyphae. The slide was checked under microscope to observe the conidial characters.

Study of nematode-fungus disease complex in tuberose under pot culture condition

Uniform sized, healthy bulbs of tuberose var. Prajwal were planted in 5 kg pots filled with sterilized pot mixture (red soil: sand: FYM: 2:1:1). One month after the establishment of plants in the pots, they were being inoculated with the fungus, *Fusarium oxysporum* (50 ml conidial suspension (1000 micro conidia/ml) per pot) and the nematode, *Meloidogyne incognita* (one J_2/g soil) as per the treatment schedule mentioned in Table 1. Inoculation of nematode and fungus was done by carefully adding the homogenous suspension of the two pathogens at the root zone of the plants. Experiments were laid out in completely randomized design (CRD) with four replications during June to July, 2013 and November to December, 2013 in the glass house of Tamil Nadu Agricultural University, Coimbatore, India maintaining the temperature range of 28 to 32ºC.

Experiment was terminated 30 days after inoculation of the two pathogens and observations were recorded on the plant growth (shoot length and weight; root length and weight) and yield parameters (No. of spikes/plant; no. of flowers/spike and flower length). Observations were also recorded on the root population of nematodes *viz*., number of females per g root, number of egg mass per g root and gall index. Gall indices were graded based on 0 to 5 scale (Taylor and Sasser, 1978). Nematode population in the soil was processed as per the method of Cobb (1918) and modified Baermann funnel technique (Schindler, 1961). Per cent wilt incidence due to fungus was assessed by the formula: (Number of wilt infected plants /Total number of plants taken for observation)*100 (Abdul Baki and Anderson, 1973).

Source and maintenance of biocontrol agents

Biocontrol agents *viz*., *Bacillus subtilis* (Bbv 57 and Bs 1) and *Pseudomonas fluorescens* (Pfbv 22 and Pf 1) were obtained from the culture collection centre, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India. These bacterial cultures were subcultured and maintained on Nutrient agar (*Bacillus* strains) and King's B medium (*Pseudomonas* strains) for further experiments.

Extraction of crude antibiotics from PGPR strains

Bacterial cells were grown in Nutrient broth and incubated at 28°C for 3 days. Supernatant was collected after 72 h of incubation by centrifugation at 8,000 rpm for 30 min. Collected supernatant was adjusted to acidic pH 2.0 by adding with concentrated HCl and the mixture was stirred at 100 rpm in an orbital shaker for 8 h. Antibiotic compounds in the supernatant or culture broth were extracted by adding the equal volume of solvent ethyl acetate and it was shaken vigorously for 1 - 2 h. Culture broth was extracted twice with ethyl acetate solvent for complete extraction. Solvent fraction containing antibiotic compounds were combined and concentrated by evaporation in the rotary flash evaporator maintained at 60°C at 80 rpm. Concentrated crude extract of the extracellular lipopeptide compounds were then dissolved in 1 ml methanol: chloroform mixture (1:1) and they were tested *in vitro* against *M. incognita* and *F. oxysporum.*

In vitro* assay of crude antibiotics against *Meloidogyne incognita

Hatching studies

About two ml of crude antibiotic extract of the four bacterial strains were taken at different concentrations *viz*., 5, 15 and 25 per cent in a 50 mm Petri dish and one egg mass of *M. incognita* were placed in each dish and incubated at 28 $\pm$ 2ºC. Egg mass placed in King's B broth, Nutrient broth without bacteria and distilled water served as three ontrols. Experiment was replicated four times in CRD. Observation on number of hatched juveniles was made on 24, 48 and 72 h after exposure.

Mortality studies

The same procedure of hatching studies was followed for mortality studies wherein instead of egg mass, freshly hatched 100 second stage juveniles were placed in each Petri dish. Each treatment was replicated four times in CRD. Observations were recorded on the mortality of juveniles on 24, 48 and 72 h after exposure and per cent mortality was calculated. Inactive nematodes were transferred separately from each dilution into sterile distilled water and kept overnight to check whether mortality was permanent or temporary.

In vitro* assay of crude antibiotics against *Fusarium oxysporum

Fugal disc (5 mm dia) was placed in the centre of sterilized Petri plate containing PDA medium. About 7 mm diameter well was made by punching the media with a sterile cork borer on the four corner of the plate with equal distance. Crude antibiotic extract of different PGPR strains (25 per cent concentration) were poured individually into the wells at the rate of 50 µl per well and the plates were incubated at 28±2 C for 3 days. Inhibitory activity of fungus growth was expressed as per cent growth inhibition by comparing with control using the following formula:

Growth inhibition (%) = (DC – DT)/DC × 100.

Where, DC: diameter of fungus colony in control; DT: diameter of fungus colony in treatment (Pandey *et al*., 1982).

Preparation of liquid formulation of PGPR strains

Liquid formulation of four PGPR strains were prepared by adding one ml of log phase culture of each bacterial strain (8 x 109 cfu/ml) to Nutrient Broth (NB) (1l) amended with glycerol (10 mM) and maintained at room

temperature (28+2oC). Pot culture and field studies were carried out with these formulations.

Evaluation of liquid formulation of PGPR strains against nematode - fungus disease complex under pot culture condition

Efficacy of liquid formulation of four PGPR strains were tested against nematode - fungal disease complex in tuberose var. Prajwal under pot culture condition in the glass house of Department of Nematology, Tamil Nadu Agricultural University, Coimbatore, India. Efficacy of the formulations were compared with the standard chemicals, carbofuran (1 kg a.i/ ha) and carbendazim (0.1 %). An untreated control was also maintained.

Uniform sized healthy bulbs of tuberose var. Prajwal were sown in 5 kg pots filled with sterilized pot mixture (red soil: sand: FYM: 2:1:1) at one bulb / pot. After establishment of plants in the pots, *M. incognita* (1J_2 / g soil) and *F. oxysporum* (50 ml conidial suspension (1000 microconidia / ml)) were introduced into the pots. The treatments were applied through soil drenching (Table 5) 7 days after the introduction of two pathogens in order to establish them firmly in the soil. Biocontrol agents (*Pseudomonas fluorescens,* Pfbv 22 and Pf1 and *Bacillus subtilis,* Bbv 57 and Bs1) were applied at the rate of 1000 ml/ha and the chemical treatments were given at the rate of Carbofuran at 1 kg ai / ha and Carbendazim at 0.05 per cent. Four replicates were maintained for each treatment and the experiment was maintained in CRD. Experiment was terminated 30 days after application of the treatments and the plants were pulled out to assess the effect of liquid formulation against the growth and yield parameters of the plant and the reduction in nematode and fungus load.

Gall index was assessed by counting the number of galls per root system and rating was given based on their numbers (1=no galls; 2=1-25 % galls; 3=26-50% galls; 4=51-75 % galls; 5=76 -100 % galls per root system).

Fusarium wilt incidence was assessed by the formula:

$$\frac{\text{Number of infected plants}}{\text{Total number of plants}} x\ 100$$

Evaluation of liquid formulation of PGPR strains against nematode-fungus disease complex under field condition

Two field trials were conducted in hot spot areas of Coimbatore District (Muthukallur and Karamadai), Tamil Nadu, India during July to September 2014 and December, 2014 to February 2015 to study the efficacy of liquid formulations of PGPR strains against nematode-fungal disease complex in tuberose var. Prajwal. Preplant nematode population was estimated as 325 $\pm$ 25 juveniles / 250 cc soil while wilt incidence was observed to be 68.5 per cent. Randomized block design was followed for the experiments with individual plot size of 5x4 m^2. The treatments were imposed same as followed in pot culture study and given through drip irrigation (Table 6). Each treatment consisted of four replications with approximately 40 plants per replication. Experiment was terminated three months after application of the treatments and observations on growth and yield parameters were recorded. Observation on gall indices and wilt severity was counted. Microscopic observations were recorded on soil and root population of nematodes.

Assay of defense related enzymes

Roots were collected from tuberose var. Prajwal during the termination of the field experiment to assess the variation in the level of defense enzymes due to the application of bioagents. The defense enzymes *viz*., peroxidase polyphenol oxidase, phenylalanine ammonia lyase and total phenols were estimated as per the methods of Hammerschmidt *et al.* (1982); Mayer *et al.* (1965); Dickerson *et al*. (1984) and Zieslin and Ben - Zaken, (1993).

Statistical analysis

Data were pooled and the critical differences (CD) was calculated at P=0.05 to test for significant differences between treatments (T) (Pansey and Sukhatme, 1978). The data were square root and arcsine transformed before analysis.

RESULTS

Identification of *Meloidogyne* species associated with tuberose

Meloidogyne species collected from tuberose were identified by the cuticular markings present in the perennial area of the matured female. The cuticular pattern in the perennial area were observed with high dorsal arch and flattened top (Fig. 1). Based on the cuticular pattern, the species was identified as *M.incognita.*

Identification of *Fusarium* species associated with tuberose

The species was identified based on the characters of microconidia, macroconidia and chlamydospores. Observation under microscope revealed small, oval shaped, single or bicelled microconidia. Hyaline, multicelled macroconidia with 3 septation which were sickle shaped with knotched base at one end. Also, typical chlamydospores produced by the species confirmed the species as *Fusarium oxysporum* (Fig. 2).

Effect of nematode-fungus interaction on growth and yield of tuberose and population load of the pathogens

Nematode and fungus, when inoculated sequentially or combinedly caused significant reduction in growth parameters of tuberose than the individual inoculation of the pathogen (Table 1). Significant reduction of shoot length (27.73 cm), root length (6.18 cm) and their respective weight (8.33g and 1.38 g) were observed when nematodes were inoculated 15 days prior to fungus. The same treatment reduced the number of spikes (1/plant) with reduced flower yield (5.5/spike) (Table 1). Uninoculated control recorded maximum growth and yield parameters of the plant.

TABLE 1. Interaction of *Meloidogyne incognita* and *Fusarium oxysporum* in tuberose var. Prajwal

S. No.	Treatments	Plant growth parameters							Root population			Soil population	Per cent wilt incidence
		Shoot		Root		No. of spikes /plant	No. of flowers /spike	Flower length (cm)	No. of females/g root	No. of egg mass/ g root	Gall index (0-5 scale)	No. of juveniles/ 250 cc soil	
		length (cm)	weight (g)	length (cm)	weight (g)								
1.	Nematode alone (N)	34.93	13.00	8.98	3.38	1.80	9.75	3.90	36.00	29.50	5.00	402.50	2.75
2.	Fungus alone (F)	33.48	12.63	7.88	2.35	1.50	8.75	3.75	0.00	0.00	0.00	0.00	83.25
3.	Concomitant inoculation of nematode and fungus (N+F)	26.68	10.33	6.30	1.42	1.00	6.00	3.58	29.50	22.25	4.50	311.75	85.50
4.	N 15 days before F inoculation	22.73	8.33	6.18	1.38	1.00	5.50	3.55	35.00	25.75	5.00	392.25	86.00
5.	F 15 days before N inoculation	31.85	11.83	6.45	1.75	1.30	6.50	3.60	20.75	15.50	3.75	269.75	84.75
6	Uninoculated control	39.55	14.75	9.00	3.61	2.30	13.00	4.23	0.00	0.00	0.00	0.00	0.00
	CD (0.05)	2.32	1.37	0.80	0.58	0.63	1.62	0.42	0.26	3.31	0.10	0.38	2.19

*Pooled data of two pot culture experiments conducted during June – July, 2013 and November – December, 2013.
Gall index: 1=No galls; 2=1-25 % galls; 3=26-50% galls; 4=51-75 % galls; 5=76 -100 % galls per root system.

TABLE 2. *In vitro* efficacy of crude antibiotics of PGPR strains on egg hatching of *Meloidogyne incognita*

Treatments	Concentration / (No. of hatched juveniles/egg mass)								
	5 %			15 %			25 %		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Bbv57	36.50	55.25	90.00	24.50	35.30	39.80	6.30	7.30	11.00
Bs 1	42.00	62.00	94.50	28.50	43.30	47.50	9.00	11.00	14.50
Pfbv 22	39.00	60.25	92.25	25.80	39.80	44.50	6.80	10.00	12.30
Pf 1	42.75	62.00	95.00	28.80	43.50	51.00	10.00	11.80	15.30
KB broth	48.75	103.25	124.50	50.50	129.50	138.50	51.50	131.80	146.50
Nutrient broth	50.00	112.75	137.50	52.50	133.80	143.00	53.00	137.30	147.30
Dis water	56.00	162.75	198.00	56.00	162.80	198.00	56.00	162.80	198.00
Mean	45.00	88.32	118.82	38.10	84.00	94.60	27.50	67.40	77.80

Mean of four replications, figures in parentheses are square root transformed values

	CD (p=0.05)
Treatment(T)	(0.10)
Concentration (C)	(0.06)
Hour(H)	(0.06)
TXC	(0.17)
CXH	(0.11)
TXH	(0.1743)
TXCXH	(0.3020)

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TABLE 3. *In vitro* efficacy of crude antibiotics of PGPR strains on per cent mortality of *Meloidogyne incognita* juveniles

Days	Concentration / (No. of dead juveniles/100 juveniles)								
	5 %			15 %			25 %		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Bbv57	5.00	14.25	17.00	20.75	36.25	40.75	46.25	63.50	82.50
Bs 1	8.25	11.50	13.50	16.50	23.00	28.50	35.50	45.00	60.75
Pfbv 22	6.75	12.50	15.00	18.00	25.00	33.50	42.00	54.25	71.25
Pf 1	9.00	11.00	12.00	16.25	22.50	25.00	31.25	40.00	54.50
KB broth	2.50	4.00	5.00	5.00	6.75	7.75	8.00	9.75	11.00
Nutrient broth	2.25	3.25	4.50	4.50	5.75	6.50	6.00	8.00	9.25
Dis water	1.25	1.75	3.75	1.25	1.75	3.75	1.25	1.75	3.75
Mean	5.0	8.32	10.11	11.75	17.29	20.82	24.32	31.75	41.86

Values are the mean of four replications; figures in parentheses are arcsine transformed values

	CD (p=0.05)
Treatment(T)	(0.66)
Concentration (C)	(0.43)
Hour(H)	(0.43)
TXC	(1.14)
CXH	(0.74)
TXH	(1.14)
TXCXH	(1.97)

TABLE 4. *In vitro* efficacy of crude antibiotics of PGPR strains against *Fusarium oxysporum*

S.No	Bacterial antagonists	Mycelial growth (cm)	Percent inhibition over control
1.	Bbv57	5.00	45.53
2.	Bs 1	5.21	43.25
3.	Pfbv 22	5.18	43.57
4.	Pf 1	5.35	41.72
5.	Control	9.18	
	CD (0.05)	0.23	

TABLE 5. Effect of liquid formulation of PGPR strains on nematode fungal disease complex in tuberose var. Prajwal under pot culture condition

S. No	Treatments	Shoot len (cm)	Shoot wt (cm)	Root len (cm)	Root wt (cm)	No. of spikes /plant	No. of flowers/spike	Flower length (cm)	Nematode population: Root pop.			Nematode population: Soil pop/ 250 cc soil	Per cent wilt incidence
									Fem./g root	Egg mass/g root	Gall index		
1.	Soil application (SA) of Bbv 57 at monthly interval	45.43	16.47	9.08	4.39	3.67	14.00	4.68	16.33	13.00	2.00	168.33	26.33
2.	SA of Bbv 57 at bimonthly interval	44.43	15.25	8.50	3.11	3.00	13.00	4.07	18.33	14.67	2.67	176.00	32.00
3.	SA of Pfbv 22 at monthly interval	41.40	14.52	8.24	3.08	3.00	12.67	3.42	20.67	17.33	3.00	180.00	33.67
4.	SA of Pfbv 22 at bimonthly interval	38.73	13.59	8.11	3.04	2.67	11.33	3.39	22.00	18.33	4.00	186.00	36.00
5.	SA of Bbv 57 + Pfbv 22 at monthly interval	46.07	16.50	9.67	4.40	4.33	14.33	4.81	15.67	11.67	1.67	166.00	24.00
6.	SA of Bbv 57 + Pfbv 22 at bimonthly interval	44.52	15.60	8.73	3.43	3.33	13.33	4.20	18.33	14.00	2.33	169.67	30.00
7.	SA of Carbofuran +Carbendazim at monthly interval	38.33	12.96	7.54	3.00	2.33	11.67	3.02	22.33	18.67	3.33	184.33	36.00
8.	SA of Carbofuran +Carbendazim at bimonthly interval	38.00	12.81	6.92	2.80	2.00	11.33	3.00	24.33	20.00	3.67	186.67	38.33
9.	Untreated control	25.60	8.45	6.00	2.31	1.67	8.33	2.90	43.67	38.67	5.00	327.00	93.00
	CD (0.05)	1.34	1.19	1.04	0.59	1.14	2.16	0.65	0.28	0.27	0.36	0.19	4.60

*Pooled data of two pot culture experiments carried out during February – March, 2014 and June – July, 2014.
Gall index: 1=No galls; 2=1-25 % galls; 3=26-50% galls; 4=51-75 % galls; 5=76 -100 % galls per root system.

TABLE 6. Effect of liquid formulation of PGPR strains on nematode fungal disease complex in tuberose var. Prajwal under field condition

S. No	Treatments	Shoot len (cm)	Shoot wt (cm)	Root len (cm)	Root wt (cm)	No. of spikes /plant	No. of flowers/spike	Flower length (cm)	Nematode population: Root pop.			Nematode population: Soil pop/ 250 cc soil	Per cent wilt incidence
									Fem./g root	Egg mass/g root	Gall index		
1.	Soil application (SA) of Bbv 57 at monthly interval	118.67	32.59	12.73	4.92	5.33	25.67	6.10	20.33	15.67	3.00	180.67	35.00
2.	SA of Bbv 57 at bimonthly interval	113.40	31.50	12.27	3.68	4.67	24.33	5.63	23.33	18.00	3.00	187.00	36.67
3.	SA of Pfbv 22 at monthly interval	111.87	27.33	12.33	3.46	4.33	24.33	5.60	24.00	19.67	3.33	193.33	42.00
4.	SA of Pfbv 22 at bimonthly interval	107.67	24.67	11.17	3.37	4.00	21.67	5.07	25.67	21.33	3.67	197.00	43.67
5.	SA of Bbv 57 + Pfbv 22 at monthly interval	123.87	34.33	13.21	5.02	5.67	27.67	6.63	17.67	14.33	2.67	177.67	33.00
6.	SA of Bbv 57 + Pfbv 22 at bimonthly interval	117.20	32.20	12.71	3.86	5.00	24.67	5.67	21.33	17.33	3.00	183.33	36.00
7.	SA of Carbofuran +Carbendazim at monthly interval	99.17	25.00	11.13	3.33	3.67	21.33	4.77	24.67	19.33	3.33	197.00	42.67
8.	SA of Carbofuran +Carbendazim at bimonthly interval	97.60	24.24	10.83	3.20	3.33	21.00	4.53	25.00	22.67	3.67	199.67	44.33
9.	Untreated control	79.60	20.32	8.67	3.00	3.00	13.67	3.57	49.33	40.33	5.00	399.67	95.33
	CD (0.05)	8.95	2.42	0.93	0.66	0.87	3.17	0.74	0.34	0.25	0.19	0.22	3.04

*Pooled data of two field experiments conducted during July – September, 2014 and December, 2014 – February, 2015.
Gall index: 1=No galls; 2=1-25 % galls; 3=26-50% galls; 4=51-75 % galls; 5=76 -100 % galls per root system.

Nematode-fungus disease complex and their biomanagement

TABLE 7. Induction of defense enzymes due to the application of PGPR strains in tuberose infested with nematode-fungus disease complex

S. No	Treatments	PO	PPO	PAL	Total phenols
1.	Soil application (SA) of Bbv 57 at monthly interval	1.13	1.06	11.93	1.83
2.	SA of Bbv 57 at bimonthly interval	1.10	1.00	10.33	1.49
3.	SA of Pfbv 22 at monthly interval	1.05	0.96	9.50	1.33
4.	SA of Pfbv 22 at bimonthly interval	1.01	0.92	8.40	1.28
5.	SA of Bbv 57 + Pfbv 22 at monthly interval	1.52	1.16	13.87	2.18
6.	SA of Bbv 57 + Pfbv 22 at bimonthly interval	1.45	1.12	12.97	2.13
7.	SA of Carbofuran +Carbendazim at monthly interval	0.85	0.75	5.03	0.54
8.	SA of Carbofuran +Carbendazim at bimonthly interval	0.83	0.73	4.93	0.53
9.	Untreated control	0.81	0.71	4.83	0.51
	CD (0.05)	0.05	0.04	0.65	0.13

*Pooled data of two field experiments conducted during July – September, 2014 and December, 2014 – February, 2015.

Highest nematode population (36 female/g root; 402.50 juveniles/250 cc soil) with the gall index of 5.0 was recorded in the plants inoculated with nematodes alone while reduced nematode female population (20.75/g root) and soil population (269.75 juveniles/250 cc soil) with the gall index of 3.75 was recorded in the inoculation of fungus 15 days prior to nematodes. Maximum wilt incidence of 86 per cent was observed in the plants that received nematode inoculation 15 days prior to fungus followed by the concomitant inoculation of the two pathogens (85.5 %) while inoculation of fungus alone recorded the wilt incidence of 83.25 per cent (Table 1).

In vitro* effect of crude antibiotics of PGPR strains against *Meloidogyne incognita

Significant reduction in egg hatching was observed in the antibiotic compounds extracted from all the four PGPR strains. Maximum reduction in egg hatching (11/egg mass) was observed in the antibiotic compound (25 % concentration) of Bbv 57 after 72 h of exposure period which was followed by Pfbv 22 (12.3/egg mass). Maximum egg hatching was observed in untreated control (198/egg mass) at the incubation period of 72 h (Table 2).

Maximum juvenile mortality (82.5 %) was observed in antibiotic compound (25 % concentration) obtained from *Bacillus* strain, Bbv 57 after 72 h exposure period which was followed by Pfbv 22 with 71.25 per cent reduction of juvenile population. Lowest juvenile mortality (3.75 %) was recorded in untreated control (Table 3).

In vitro* assay of crude antibiotics against *Fusarium oxysporum

Crude antibiotics of Bbv 57 (25 % concentration) inhibited the mycelial growth (5.0 cm) of *F. oxysporum* by 45.53 per cent which was significantly higher over other strains. It was followed by Pfbv 22 (5.18 cm) with 43.57 per cent inhibition of the pathogen over control. Maximum mycelial growth of the fungus was recorded in untreated control (9.18 cm) (Table 4).

Effect of liquid formulation of PGPR strains on nematode - fungal disease complex in tuberose under pot culture condition

Maximum growth and yield parameters of the plants and least nematode - fungus load was observed in all the biocontrol agents treated plants. Among them, soil drenching of liquid formulation of Bbv 57 + Pfbv 22 (each at 500 ml/ha) at monthly interval recorded maximum growth and yield parameters with lowest nematode population in the soil (166 juveniles/250 cc soil) and root (15.67 females/g root) with the gall index of 1.67. The same treatment recorded lowest wilt incidence (24 %). Application of liquid formulation of Bbv 57 at monthly interval was adjudged as the next best treatment in reducing the nematode - fungal disease complex and increasing the growth and yield of the plant. Untreated control recorded the lowest growth and yield parameters with increased nematode (43.67 females/g root; 327 juveniles/250 cc soil) population and wilt severity (93%) (Table 5).

Effect of liquid formulation of PGPR strains on nematode - fungal disease complex in tuberose under field condition

Under field condition, soil drenching of liquid formulation of Bbv 57 + Pfbv 22 (each at 500 ml/ha) at monthly interval maximized growth and yield parameters (Table 6). The same treatment reduced the incidence of root knot nematode in soil and root (177.67 juveniles / 250 cc soil and 17.67 females/g root) and recorded the least gall index of 2.67. Untreated control recorded maximum nematode population (49.33 females/g root; 399.67 juveniles / 250 cc soil) with the gall index of 5.0 (Table 6). Similarly, application of liquid formulation of Bbv57+ Pfbv 22 at monthly interval recorded lowest wilt incidence of 33 per cent which was 65.38 per cent increase over control while untreated control plot recorded highest wilt incidence of 95.33 per cent.

Induction of defense enzymes in the plants treated with PGPR strains

Highest peroxidase (PO) activity of 1.52 $min^{-1}g^{-1}$ root was observed in the plants that received the monthly application of Bbv 57 + Pfbv22. The same treatment recorded highest activities of poly phenol oxidase (PPO) (1.16 $min^{-1}g^{-1}$ root), phenyl alanine ammonia lyase (PAL) (13.87 $min^{-1}g^{-1}$ root) and phenol (2.18 mg g^{-1} fresh root) (Table 7). Lowest activities of the above defense enzymes were recorded in untreated control.

DISCUSSION

Interaction studies revealed that presence of nematodes increased the wilt severity and caused death of the plants earlier than the individual inoculation of the two pathogens. Early reports of Harris and Ferris, (1991), Jonathan and Gajendran, (1998) and Sankari Meena *et al.* (2015) recorded increased *Fusarium* wilt incidence due to the presence of root knot nematodes. The data reported here indicated that the infection of root knot nematode altered the host morphology and physiology which

favoured quick entry of the fungus into the plant roots. Presence of both nematode and fungus caused greater reduction in growth of the plants which was proved earlier by Shokoohi *et al.* (2004) in different melon varieties.
Crude antibiotic compounds of *Bacillus* and *Pseudomonas* play a significant role in the reduction of both nematode and pathogen population. Role of *Bacillus* and *Pseudomonas* against nematodes and fungus has already been reported by Karimi *et al.* (2012) and Showkat *et al.* (2012). Present study revealed greater reduction in nematode population (eggs and juveniles) and fungus colonies *in vitro* with the application 25 per cent concentration of the antibiotics. Results of Kavitha *et al*. (2012) revealed significant reduction of nematode population with the application of antibiotics of *Bacillus*. Similarly, Siddiqui *et al*. (2000) obtained significant reduction in the juveniles of *M. javanica* using ethyl acetate and hexane fractions of *Bacillus* at different concentrations. Antibiotic compound of *Pseudomonas* had a significant effect over *M. incognita in vitro* (Sankari Meena *et al*., 2013). Ramyabharathi and Raguchander (2014a) recorded the crude antifungal antibiotic compounds of *B. subtilis,* EPCO16 showed a greater efficacy in the suppression of *Fusarium* wilt of tomato. In pot culture and field studies, application of PGPR strains *viz*., *Bacillus* and *Pseudomonas* recorded a significant reduction in fungus and nematode population with increased growth parameters of the plant.
Increased growth of plants in the present study due to the application of *Bacillus* and *Pseudomonas* were in accorded with the studies of Jonathan *et al*. (2005), Senthilkumar *et al.* (2008) and Sankari Meena *et al.* (2014) where they observed enhanced growth parameters of the plants due to the application of above bioagents. Bacterized plants recorded increased plant growth by the production of phyto hormones, antibiotics, reduction of ethylene level and induced systemic resistance (Holland, 1997). Effect of *Bacillus* and *Pseudomonas* on the severity of disease complex has been well documented earlier by Siddiqui and Haque (2001); Siddiqui *et al.* (2001); Shennawy *et al.* (2012) and Rao *et al.* (2014).
Results of the present study suggest that application of *B. subtilis* and *P. fluorescens* would be a valuable candidate for the management of nematode –fungus disease complex infesting tuberose. Hence, it should be added as one of the components in integrated management programmes for the management of disease complex.

CONCLUSION

Interaction studies on nematode-fungus disease complex in tuberose under pot culture studies revealed that the presence of nematode aggravated the infection of secondary fungal pathogen which favours the quick spread of *Fusarium* wilt disease over the individual inoculation of the pathogen. Biomanagement studies were carried out to mitigate the disease complex under pot culture and field condition using PGPR bacteria, *P. fluorescens* and *B. subtilis.* Intensive control of the two pathogens was achieved by the combined application of two PGPR bacteria*, P. fluorescens* (Pfbv 22) and *B. subtilis* (Bbv 57) at monthly interval. From the present study, it is clear that the PGPR biocontrol agents had a greater efficacy in the reduction of nematode and fungal pathogens.

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