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Evaluation of potential bio-control agents on root-knot nematode *Meloidogyne incognita* and wilt causing fungus *Fusarium oxysporum* f.sp. *conglutinans in vitro*

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Indigenous strains of *Trichoderma viride* (ITCC No. 6889), *Pseudomonas fluorescens* (ITCC No. B0034) and *Purpureocillium lilacinum* (ITCC No.6887) were isolated from undisturbed forest eco-system of Southern India. These three bio-mediators were evaluated for their antagonism towards root knot nematode, *Meloidogyne incognita* and *Fusarium oxysporum* f.sp. *conglutinans in vitro*. Cell free culture filtrate of these strains significantly inhibited the egg hatching and caused juvenile (J_2) mortality of *M. incognita* at 25, 50, 75 and 100% concentrations. Maximum inhibition in egg hatching and juvenile mortality were recorded in *P. lilacinum* as 94.21 and 91.28%, respectively after 120 h. It was followed by *T. viride* and *P. fluorescens* which recorded 92.72 and 91.46% and 89.12 and 90.14% inhibition in egg hatching and juvenile mortality, respectively after 120 h. Antagonism of *T. viride* on *F. oxysporum* was recorded maximum on the 5th day as 45.82%. Similarly, the antagonism on the 5th day for both the bio-agents of *P. lilacinum* and *P. fluorescens* were recorded as 45.26 and 44.19%, respectively.

Key words: Biocontrol agents, culture filtrate, *Fusarium oxysporum*, *Meloidogyne incognita*.

INTRODUCTION

Root-Knot nematodes are causing a notable damage to a wide range of vegetable crops causing significant yield loss in tropical and sub-tropical agriculture (Sikora and Fernandez, 2005). The symptoms of nematode disease are manifested by the formation of root galls accompanied by stunted growth, chlorosis and loss of viability of the plant (Babu et al., 1999). *Fusarium* wilt is soil borne fungal pathogen which can sustain many years in the soil without a host (Ignjatov et al., 2012). *F. oxysporum* has a worldwide distribution and causes severe root rot or vascular wilt in ample range of plant families (Enya et al., 2008; Lievens et al., 2008; Michielse and Rep, 2009). This fungal pathogen infects the seed and early stages of seedling growth, causing seed decay and damping-off (Punja et al., 2004).

Trichoderma viride is an effective bio-control agent

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against numerous soil borne plant pathogens and can easily colonize plant rhizosphere and helps in the plant growth promotion (Verma et al., 2007; Savazzini et al., 2009; John et al., 2010). *T. viride* proved to be effective against root knot nematode, *Meloidogyne* species and reduced its damage on several crops (Meyer et al., 2001; Abd et al., 2007). Various reports on nematophagous fungus *Purpureocillium lilacinum* prove its efficacy as effective bio-control agent on *Meloidogyne* spp. on various crops (Jatala, 1986; Rao and Reddy, 1994; Rao et al., 1999; Khan and Williams, 1998; Mohd et al., 2009; Brand et al., 2010). It has more rate of occurrence in tropical and subtropical areas (Morgan-Jones et al., 1984; Chen et al., 1996).

Pseudomonas fluorescens is a Plant Growth Promoting Rhizobacteria (PGPR) effective against soil borne pathogens including root-knot nematodes (Perveen et al., 1998; Siddiqui et al., 1999; Rao et al., 2002; Rao, 2007; Otsu et al., 2004). The concept of PGPR has been documented with the isolation of many bacterial strains which exhibit the desirable characteristics of root colonization, disease suppression, plant growth stimulation and biocontrol (Molla et al., 2001; Beneduzi et al., 2008).

Process of DNA extraction from fungal cultures eliminates many unknown interfering substances which allow to identify species specific organisms using ribosomal DNA by PCR (Don et al., 2000; Bryan et al., 1995). Currently, many methods are available for the isolation of fungal genomic DNA (Plaza et al., 2004; Melo et al., 2006). *Pseudomonas* species were isolated from soil eco-system that was naturally suppressive to many plant diseases like *Fusarium* wilt and black rot of tobacco (Thomashow et al., 1990; Raaijmakers and Weller, 1998; De Boer et al., 1999; William et al., 1991).

Culture filtrates of bio-control agents are reported to be antagonistic to a wide range of plant parasitic nematodes in *in vitro* (Reibinger, 1995; Hallaman and Sikora, 1994; Meyer et al., 2004; Vu, 2005; Abd et al., 2007). In the current investigation, efforts were made to isolate the bio-agents and evaluate the effect of three bio-agents, namely, *T. viride*, *P. fluorescens* and *P. lilacinum* against *M. incognita* and *Fusarium oxysporum* f.sp. *conglutinans* in *in vitro*.

MATERIALS AND METHODS

Isolation of bio-agents, collection of *M. incognita* egg mass and *F. oxysporum*

Cultures of *T. viride* (ITCC No.6889), *P. fluorescens* (ITCC No. B0034) and *P. lilacinum* (ITCC No. 6887) were maintained on nutrient agar (NA) (Himedia chemicals, India) for bacterial cultures and Potato dextrose agar (PDA) (HIMEDIA chemicals, India) for fungi by cryopreservation method (Sudheer, 2010). The isolates were sub-cultured and used for further study. Root knot nematode culture was obtained from infected cauliflower plants grown in farmers field (Doddaballapur, Bengaluru rural district, Karnataka, India). Identification of *M. incognita* was confirmed by perineal cuticular pattern (PCP) under stereo microscope and used for further studies. *F. oxysporum* f. sp. *conglutinans* was isolated from fusarium infected cauliflower plants. Preparation of inoculum was made by culturing the *F. oxysporum* isolate in PDA amended with streptomycin sulphate for 7 days at 25 ± 2°C.

DNA isolation from *T. viride*, *P. lilacinum* and *P. fluorescens* and PCR

Genomic DNA was isolated from cultures of *T. viride* and *P. lilacinus* as per the protocol of Raeder and Broda (1985). Bacterial genomic DNA of *P. fluorescens* was isolated using Nucleospin tissue extraction kit (Macherey-Nagal, Germany). Polymerization Chain Reaction (PCR) of 25 µl PCR mixture was prepared for molecular identification of *T. viride* and *P. lilacinum* with the following protocol. Final PCR volume contained 19.7 µl of PCR grade water, 0.5 µl of each forward and reverse primers (0.2 pmol; Bio-serve Pvt. Ltd, India), 0.5 µl of dNTPs (10 mM; Fermentas Inc, Canada), 2.5 µl (10X) of Taq buffer (Bangalore Genei Pvt.Ltd, India), and 0.3 µl of Taq DNA polymerase (Bangalore Genei Pvt.Ltd, India) and 1 µl of template DNA (25 ng/µl). PCR amplifications were carried out in Eppendorf master cycler gradient (vapo.protect, Germany). Amplification reactions were performed in master cycler with heated lid. The primer pairs ITS F (TCCGTAGGTGAACCTGCGG) and ITSR (TCCTCCGCTTA-TTGATATGC) were used for the amplification of region including the ITS 1, 5.8 S and ITS 2 (Hurtado et al., 2008). The initial denaturation for 5 min at 94°C was followed by 35 cycles of 45 s at 94°C, 35 s at 43°C, 40 s at 72°C and a final extension of 10 min at 72°C.

16s rDNA amplification of *P. fluorescens* was carried out using universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) (Weisburg et al., 1991) in thermo cycler (Eppendorf vapo.protect, Germany) using the aforementioned components which followed for final PCR mixture of 25 µl except 16s primers and DNA template (25 ng/µl). The PCR cycle of initial denaturation was done at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 45 s, primer annealing at 51°C for 35 s and elongation at 72°C for 40 s. Final extended elongation was done for 10 min at 72°C. The obtained PCR products were gel electrophoresis on 1.5% agarose gel (Figures 1 and 2).

The PCR products were sequenced at Bio-serve, Hyderabad, Telangana state, India. All the sequenced PCR products were confirmed using NCBI mega blast for its species identity of ITS region and 16s region. The molecular identified strains were submitted at NCBI.

Effect of culture filtrate of *P. fluorescens* on egg hatching of *M. incognita*

A single colony from pure culture of *P. fluorescens* taken from 24 h old culture plates was inoculated into 50 ml of sterilized King's B broth (HIMEDIA chemicals, India) in 100 ml Erlenmeyer flasks. These flasks were incubated in a shaker incubator at 150 rpm speed and 37°C for 24 h. The bacterial growth after 24 h was tested for their luminosity under transilluminator at 250 to 260 nm. *P. fluorescens* culture filtrate was obtained by centrifugation (Eppendorf refrigerated centrifuge 5415) at 10,000 rpm for 15 min at 4°C. The supernatant culture filtrate was collected and passed through syringe filter of 0.22 µm (Millipore PVDF Durapore 13 mm diameter). Consequently, collected culture filtrate was tested for the absence of any viable cell and used to study the effect on egg hatching and juvenile mortality of *M. incognita*.

P. fluorescens culture filtrate was made into four concentrations of 100, 75, 50 and 25% by adding sterile distilled water. Three millilters of each concentrations of culture filtrate was transferred to sterile Petri-dishes of 5 cm diameter. *M. incognita* egg masses

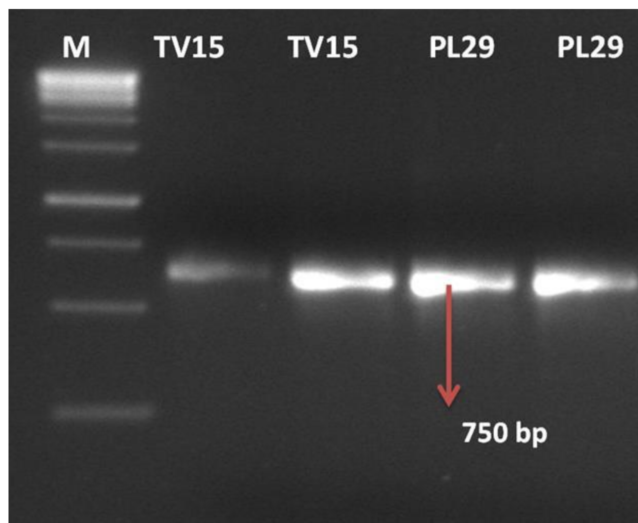

Figure 1. PCR amplified ITS region of *T. viride* and *P. lilacinum*.

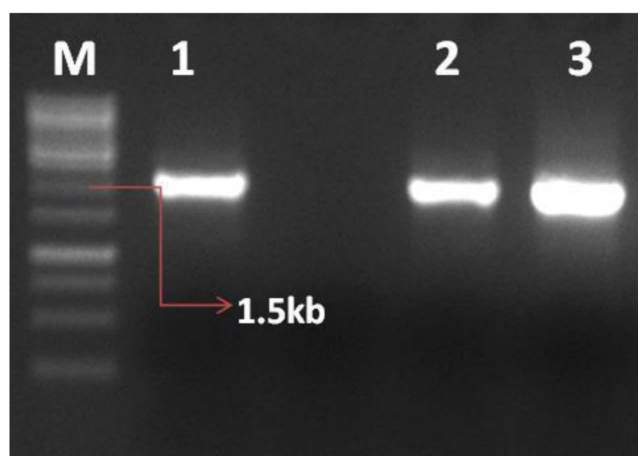

Figure 2. PCR amplified 16s region of *P. fluorescens* (1 to 3).

were collected and surface sterilized using 0.1% sodium hypochlorite for 30 s and rinse the treated egg masses with sterile distilled water. Each petri dish was placed with five egg masses containing culture filtrate of each concentration and incubated at room temperature. *M. incognita* egg masses placed in King' B and distilled water served as control. The number of juveniles (J_2) hatched in all the four concentrations were recorded at 24, 48, 72, 96 and 120 h of exposure.

The percentage suppression in hatching of juveniles (J_2) was calculated using the following formula:

Percentage of hatching suppression = [1- (Ht/Hc)] ×100

Where, Ht is the number of juveniles hatched in treatment and Hc is the number of juveniles hatched in control.

Effect of culture filtrate of *P. fluorescens* on mortality of *M. incognita* juveniles

Culture filtrate was made into four concentrations of 100, 75, 50 and 25% by adding sterile distilled water. Three millilters culture filtrate of each concentration was transferred to sterile Petri-dishes of 5 cm diameter. Freshly hatched 100 *M. incognita* juveniles (J_2) were placed in each Petri dish and incubated at room temperature (25 to 30°C). Petri dishes containing sterile water and autoclaved King's B broth placed with juveniles (J_2) served as control and treatment.. Total number of dead nematodes were counted after 24, 48, 72, 96 and 120 h of exposure and percentage mortality of juveniles was calculated.

Effect of culture filtrate of *T. viride* and *P. lilacinum* on egg hatching of *M. incognita*

Freshly sub-cultured *T. viride* and *P. lilacinum* of 5 mm disc were inoculated in 100 ml sterilized potato dextrose broth – PDB (HIMEDIA chemicals, India) in 250 ml Erlenmeyer flask. The flasks were incubated at 27 ± 1°C for 8 days. From each culture, 50 ml of broth containing 2.8 × 10^6 spores (CFU/ml) was centrifuged at 13000 rpm at 4°C for 20 min (Eppendorf refrigerated centrifuge 5415). The obtained pellet was discarded and supernatant was collected which passed through 0.45 µm syringe filter (Millipore PVDF Durapore 13 mm diameter). The culture filtrate thus obtained was tested for the absence of any fungal spores by plating it on PDA. *T. viride* and *P. lilacinum* culture filtrate was made into four concentrations of 100, 75, 50 and 25% by adding sterile distilled water. They were tested for hatching and J_2 mortality as per the aforementioned procedure for *P. fluorescens*.

Effect of *T. viride* and *P. lilacinum* antagonists against *F. oxysporum* f.sp. *conglutinans* in *in vitro*

Five day old cultures of *T. viride* (TV-15), *P. lilacinum* (PL-29) and *F. oxysporum* discs (5 mm diameter) were grown on PDA and punched in the periphery in 90 mm petri plates. Each culture was inoculated separately on PDA plates as control. Cultures of *T. viride* with *F. oxysporum* and *P. lilacinum* with *F. oxysporum* were inoculated separately on PDA plates at 20 mm distance from periphery of the petriplate as per dual culture method. Each treatment was replicated thrice and incubated at 27±2°C for antagonistic study. Observations were recorded on growth of each fungal culture for 5 days. The percentage of inhibition of *F. oxysporum* was calculated (Vincent, 1927).

PI =C-T/C×100

Where, PI is the percentage inhibition over control, C is the control of *F. oxysporum* without *T. viride* (mm), and T is the growth of *F. oxysporum* with *T. viride* (mm).

Effect *of P. fluorescens* antagonists against *F. oxysporum* f.sp. *conglutinans* in *in vitro*

Both the cultures of 1 day old *P. fluorescens* and 5 days old *F. oxysporum* were inoculated on Nutrient Agar (NA) and PDA plates (90 mm), respectively. As per dual culture method, *P. fluorescens* was inoculated by spread plate method on PDA plate. *F. oxysporum* disc (5 mm) was inoculated in the centre of PDA plate. Half streak of *P. fluorescens* and 5 mm *F. oxysporum* disc inoculated 20 mm distance from periphery of the plate in the same PDA plates were also maintained for antagonistic study. The same procedure for *T. viride* and *P. lilacinum* antagonistic study was followed.

Table 1. Effects of the cell-free culture filtrate of *T. viride* on egg hatching and mortality of *M. incognita* in *in vitro*.

Concentration (h)	% Suppression in hatching (T)					% J_2 Mortality (T)				
	24 h	48 h	72 h	96 h	120 h	24 h	48 h	72 h	96 h	120 h
25%	51.25 (45.71)	52.41 (46.37)	53.28 (46.88)	54.72 (47.71)	55.18 (47.97)	56 (48.45)	65.13 (53.81)	67.41 (55.20)	68.23 (55.71)	69.72 (56.62)
50%	62.21 (52.09)	63.54 (52.87)	64.11 (53.20)	64.98 (53.74)	65.72 (54.16)	68.51 (55.95)	70.28 (56.96)	71.37 (57.66)	73.25 (58.86)	75.12 (60.09)
75%	69.18 (56.29)	70.35 (57.00)	71.83 (57.94)	72.64 (58.48)	73.84 (59.24)	76.23 (60.84)	78.52 (62.52)	79.25 (62.97)	81 (64.23)	82.51 (65.34)
100%	81.24 (64.34)	82.16 (66.37)	83.08 (65.98)	84.31 (66.84)	92.72 (74.83)	83.15 (65.80)	85.48 (67.64)	86.41 (68.58)	88.27 (70.12)	91.46 (73.05)
Media alone (PDB)	11.02 (19.29)	12.27 (20.30)	13.48 (21.51)	14.13 (22.00)	15.09 (22.80)	10.46 (18.79)	11.35 (19.63)	12.51 (20.66)	13.18 (21.26)	14.64 (22.42)
Control (Distilled water)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)

	CD (0.01)	SED	CD (0.01)	SED
C	2.24	0.85	2.19	0.83
T	2.05	0.78	2.00	0.76
CxT	5.02	1.91	4.91	1.87

Figures in parentheses are arc sine transformed values.

RESULTS

Molecular identification of bio-agents using gene specific primers

Ribosomal DNA internal transcribed spacers region was amplified using fungal genus specific ITS-1 and ITS-4 primers (Figure 1). Bacterial strain was amplified using 16s primers (Figure 2). Molecular identification of these PCR amplified strains were confirmed using NCBI mega blast. *T. viride* and *P. lilacinum* matching to National Centre for Biotechnology Information (NCBI) were 94 and 96%, respectively and *P. fluorescens* was 97%. These strains were submitted at NCBI with the following accession numbers *T. viride* - KP271026, *P. lilacinum*-KP271028 and *P. fluorescens* - KP27102.

Effect of culture filtrates of three bio-agents on *M. incognita*

Results of studies on culture filtrates showed that the efficacy of the isolates of *T. viride*, *P. lilacinum* and *P. fluorescens* on hatching of *M. incognita* eggs and J_2 mortality increased with the increase in concentration of the culture filtrates. These investigations clearly indicated that as the duration of exposure to culture filtrate of the bacterium and fungal cultures increased, suppression in hatching and mortality of juveniles (J_2) also increased. The maximum suppression in egg hatching was recorded in *P. lilacinum* as 94.21% and juvenile mortality as 91.28% after 120 h at 100% concentration (Table 1). It was followed by *T. viride* which recorded the inhibition in egg hatching as 92.72%, and juvenile mortality as 89.12% after 120 h (Table 2). In *P. fluorescens*, suppression in egg hatching and juvenile mortality was recorded as 91.46 and 90.14%, respectively after 120 h (Table 3).

Effect of bio-agents antagonism on *F. oxysporum*

T. viride showed antagonistic activity on *F. oxysporum* on the 5th day which was recorded as 45.82%. It was followed by *P. lilacinum* and *P. fluorescens*, for which the antagonism was recorded on the 5th day as 45.26 and 44.19% (Figure 3).

DISCUSSION

During the experimental investigations, native strains of *T. viride*, *P. lilacinum* and *P. fluorescens* were isolated from different regions of South India. The identity of these isolates were confirmed through molecular techniques and evaluated for their antinemic and antifungal activity *in vitro*. The culture filtrate studies revealed that all the bio-control agents were effective in suppressing the egg hatching of *M. incognita* and causing J_2 mortality which increased with increase in time of exposure of eggs to cell free culture filtrate as well with increase in concentration.

Deformation of juveniles was observed in most of the eggs in the present study. The results

Table 2. Effects of cell-free culture filtrate of *P. lilacinum* on egg hatching and mortality of *M. incognita* in *in vitro*.

Concentration (h)	Suppression in hatching (%)					J2 Mortality (%)				
	24 h	48 h	72 h	96 h	120 h	24 h	48 h	72 h	96 h	120 h
25%	53.17 (46.81)	54.38 (47.51)	55.09 (47.92)	57.34 (49.26)	58.71 (50.04)	58.24 (49.74)	69.31 (56.37)	71.33 (57.63)	73.48 (59.00)	75.08 (60.05)
50%	65.08 (53.78)	66.41 (54.58)	67.26 (55.10)	68.43 (43.83)	69.12 (56.25)	72.24 (58.21)	74.04 (59.38)	74.81 (59.89)	76.15 (60.83)	77.34 (61.57)
75%	71.27 (57.59)	72.41 (58.32)	73.81 (59.23)	74.08 (59.40)	75.38 (60.27)	78.13 (62.28)	79.14 (63.12)	80.24 (65.01)	82.40 (65.23)	84.52 (66.86)
100%	83.07 (65.78)	85.24 (67.97)	86.18 (68.25)	87.92 (69.95)	94.21 (76.16)	82.04 (64.93)	84.25 (66.64)	85.13 (67.33)	89.14 (70.83)	91.28 (72.92)
Media alone (PDB)	10.42 (18.78)	11.07 (19.38)	12.46 (20.60)	13.51 (21.49)	13.05 (21.11)	11.14 (19.19)	12.21 (20.42)	13.06 (21.15)	13.68 (21.69)	14.04 (21.99)
Control (Sterile water)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)

	CD (0.01)	SED	CD (0.01)	SED
C	1.99	0.76	3.45	1.31
T	1.82	0.69	3.15	1.20
C×T	4.46	1.70	7.72	2.95

Figures in parentheses are arc sine transformed values.

Table 3. Effects of the cell-free culture filtrate of *P. fluorescens* on egg hatching and mortality of *M. incognita* in *in vitro*.

Concentration (h)	% of Hatching suppression					% J2 Mortality				
	24 h	48 h	72 h	96 h	120 h	24 h	48 h	72 h	96 h	120 h
25%	52.08 (46.18)	53.49 (46.99)	53.94 (47.26)	55.04 (47.89)	55.78 (48.31)	52.64 (46.51)	63.05 (52.64)	64.22 (53.26)	65.18 (53.84)	66.52 (54.67)
50%	61.04 (51.37)	62.13 (52.03)	63.46 (52.81)	63.91 (53.08)	64.76 (53.59)	64.04 (53.15)	65.17 (53.85)	66.54 (54.66)	67.25 (55.10)	68.84 (56.19)
75%	65.24 (53.90)	66.37 (54.65)	67.19 (55.10)	68.46 (55.86)	69.81 (56.84)	72.64 (58.49)	73.42 (58.97)	74.49 (59.67)	76.21 (60.68)	78.55 (62.51)
100%	79.28 (63.05)	81.44 (64.51)	82.06 (64.98)	83.05 (65.76)	89.12 (70.83)	80.28 (63.68)	81.27 (64.37)	83.22 (65.84)	84.76 (67.05)	90.14 (71.76)
Media alone (NB)	10.18 (18.37)	11.64 (19.91)	12.08 (20.28)	13.34 (21.39)	14.24 (22.12)	11.52 (19.80)	12.41 (20.58)	13.08 (21.08)	14.64 (22.44)	15.16 (22.89)
Control (Sterile water)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)

	CD (0.01)	SED	CD (0.01)	SED
C	1.95	0.74	2.09	0.80
T	1.78	0.68	1.91	0.73
C×T	4.37	1.67	4.69	1.79

Figures in parentheses are arc sine transformed values.

indicated the production of nematicidal compounds in the culture filtrates. These nematicidal compounds produced by *T. viride*, *P. fluorescens* and *P. lilacinum* seemed to play an important role in causing nematode mortality. Many soil borne nematode trapping fungi, endoparasitic fungi, parasites of nematode eggs and cysts were reported to produce toxic metabolites against nematodes (Li et al., 2007). In earlier reports,

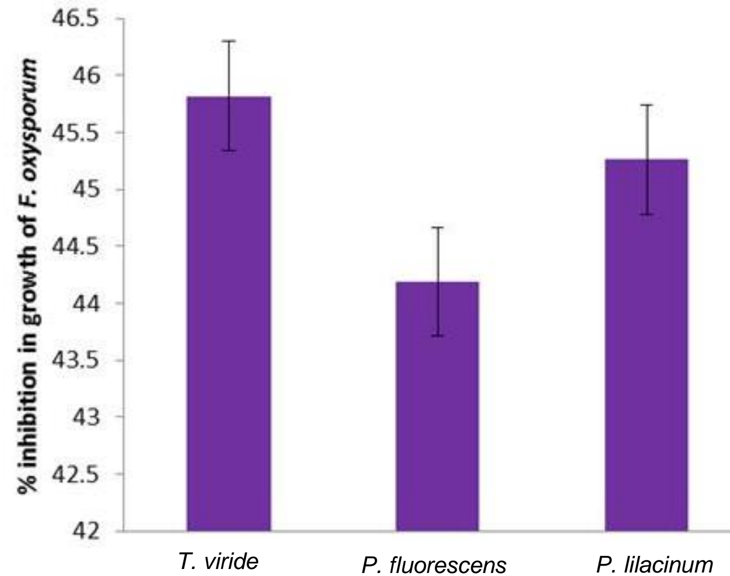

Figure 3. Effect of *T. viride*, *P. fluorescens* and *P. lilacinum* antagonism on growth inhibition of *F. oxysporum* in *in vitro.*

isolates of *P. lilacinum*, an egg parasite of root knot and cyst nematode, showed potential nematoxic activity (Shamim et al., 2012).

These observations also fall in line with experimental evidence as indicated by Regina et al. (1998) and Hanna et al. (1999) who reported that mortality of *M. incognita* increased with increase in exposure time as well as the concentration of culture filtrate. Bin et al. (2005) showed that culture filtrates of rhizobacterium are heat stable and resistant to extreme pH values, which suggested that the antibiotic rather than protein might be responsible for the nematicidal activity.

Antagonism was observed in 3 bio-control agents, namely, *T. viride, P. lilacinum* and *P. fluorescens* against *F. oxysporum in vitro*. *Trichoderma* species are widely used as biocontrol agents to reduce the disease incidence caused by plant pathogenic fungi and many soil borne pathogens (Papavizas, 1985; Sivan and Chet, 1986). Pau et al. (2012) investigated the effect of antagonism on *P. lilacinum* in *in vitro*. The culture filtrates and their antagonism proved the biocontrol efficiency of these microbes. Results on antagonism clearly indicated the effect of bio-agents in *in vitro* on *F. oxysporum*. More antagonism was recorded with *T. viride* followed by *P. lilacinum* and *P. fluorescens*. Hence, it can be exploited further in development of formulations and evaluation under field conditions.

Conclusion

The findings on the effect of culture filtrates of three bio-agents, namely, *T. viride*, *P. fluorescens* and *P. lilacinum* showed on root-knot nematode *M. incognita* in *in vitro.* The experiments also proved the antagonism of these bio-control agents on suppression of *F. oxysporum* f.sp. *conglutinans*. For the resultant data, the effect on *M. incognita* and on *F. oxysporum* was an apparent indication to control the nematode induced disease complex in *in vivo* in cauliflower (Rajinikanth et al., 2013) by application of eco-friendly bio-control agents of bio-nematicide (*P. lilacinum*), bio-fungicide (*T. viride*) and bio-bactericide (*P. fluorescens*).

Conflict of Interests

The authors have not declared any conflict of interests.

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