In-vitro evaluation of different fungal bio-agents on egg hatchingof *Meloidogyne incognita*causing root-knot disease in tomato

ABSTRACT

Investigation were carried out under *in vitro* condition to evaluate the antagonistic effect of fungal bio-control agents *i.e.,Trichoderma harzianum*, *T. viride*, *Purpureocilliumlilacinum*, *Metarhizium anisopliae* and *Beauveria bassiana*on egg hatching of root-knot nematode, *Meloidogyne incognita*. Bio-control agents were tested at 10^6 and 10^7 dilutions on egg hatching of *M. incognita* after 24, 48, 72, 96 and 120 hrs exposure period as compared to control. *P. lilacinum* 10^7 dilutions and *T. harzianum* 10^6dilutuions (83.90 % and 83.04 %) were found at par and significantly effective on egg hatching inhibition of *M. incognita*. Among different dilutions, *P. lilacinum* at 10^6 dilutions (85.46 %) gave maximum egg hatching inhibition followed by *P. lilacinum* at 10^7 dilutions (83.90 %), *T. harzianum* at 10^6 dilutions (83.04 %) and *T. viride* at 10^6 dilutions (82.00 %) after 120 hrs. *M. anisopliae* at 10^7 dilutions (69.46 %) was found least effective at different period of exposure. Further studies to be conducted in pot and field conditions to evaluate the efficacy of these bio-agents against root knot nematode, *M. incognita*.

Key words: Fungal bio agents, Root knot nematode, Antagonistic, *In vitro*, Egg hatching.

Introduction

Root knot nematodes (*Meloidogyne* spp.) is one of the most important polyphagous pests in agriculture. Among the top five plant pathogens affecting world's food production, root knot nematode is one of the most devastating pathogen of crops. Overall, plant parasitic nematodes (PPNs) cause 21.3 per cent crop losses amounting to Rs. 102,039.79 million (1.58 billion USD) annually. *Meloidogyne incognita* was economically most important root-knot nematode (RKN) causing yield losses of Rs. 6035.2 million in tomato (1). It is likely that *M. incognita* will further contribute to tomato yield decline, as the trend towards intensification of production will support increased nematode population densities. To prevent further tomato yield losses due to the nematodes and improve productivity, a sound nematode management scheme is essential.

The utilization of different fungal bio-agents in the management of nematode parasites is gaining importance. Among the various bio-control agents, *Trichoderma harzianum*, *T. viride*, *Purpureocilliumlilacinum*, *Metarhizium anisopliae* and *Beauveria bassiana*have been found to be promising against root-knot nematodes (2). The application of chemical nematicides will

become prohibited due to not only the increase of resistance in the target pathogen but also caused the environmental hazard. To reduce such condition, the use of bio-agents are found increase in attention and use of such bio-agents offer an effective, safe, persistent and natural durable protection against *M. incognita*(3). However, many natural enemies attack *Meloidogyne* spp. in the soil and such enemies can be used as bio-agents for the effective management of *Meloidogyne*spp(4). Among them, fungal bio-agents are unique natural enemies for managing the nematodes in soil. Such bio-agents showed their antagonistic activity like predation, parasitism and antibiosis etc., towards *M. incognita*. However, these fungal bio-agents have ability to release the antibiotics, metabolites, protease enzymes etc. (5) in the environment and that caused nematode viability. However, the efficacy of bio-agents to reduce the nematode viability varied from species to species. The potential benefits must be examined so that effective bio-control agents can be utilized.

Keeping this in view, the present investigations were undertaken to study the efficacy of culture filtrate of different fungal bio-agents in the managing of *M. incognita* infecting tomato under *in- vitro* condition.

Materials and Methods

Maintenance of pure culture of *Meloidogyne incognita*:

Egg-masses of *M. incognita* were collected from tomato roots and the population was multiplied on a susceptible tomato variety (SL-21) grown in pots containing sterilized soil. This was done six months prior to the start of the experiment. Other intercultural operations were applied as and when needed.

Source and maintenance of fungal bio-control agents:

Fungal biocontrol agents *i.e., Trichoderma harzianum*and *T. viride* was obtained from Department of Plant Pathology, RCA, Udaipur, *Purpureocilliumlilacinum*was obtained from Department of Nematology, RCA, Udaipur and *Metarhizium anisopilae& Beauveria bassiana*was obtained from Department of Entomology, RCA, Udaipur. Pure culture of these fungal bio-agents were maintained on Potato Dextrose Agar media in laboratory for further studies.

Collection of egg-masses:

Egg masses were collected from the tomato plants maintained as pure culture. Roots were dissected with a sterilized dissecting needle and egg masses were hand picked up from the galled root with help of sterilized forceps. The picked egg masses were kept in sterilized cavity block containing 5ml sterilized water.

Surface sterilization of egg masses:

The collected egg masses were surface sterilized in 0.4 per cent sodium hypochlorite (NaOCl) for two minutes (6). Egg masses were washed thoroughly with sterile distilled water until the traces of NaOCl is removed and placed in cavity block for further use.

Extraction of eggs from egg masses

Surface sterilized egg masses were taken in a petridish and subjected to 0.5 % sodium hypochlorite solution for two minutes, with frequent stirring followed by a 30 seconds settleling to dissolve the gelatinous matrix. The eggs released through gelatinous matrix and further disinfested in 0.4 % NaOCl followed by three washing with sterile water. Eggs were then collected on a 500 mesh sieve and washed thoroughly with sterilized distilled water to remove the traces of NaOCl. A measured quantity of suspension was prepared with eggs in the distilled water in a measuring cylinder. The egg suspension was prepared in such a way that 1 ml of it contained 100 eggs. The counting of eggs in the suspension was made by using Hawkshley counting dish. Five aliquots of 1 ml suspension were counted and their average number was multiplied with total volume of suspension prepared.

Preparation of media

Potato Dextrose Agar (PDA)

The ingredients used for preparation of PDA were Peeled potato - 200 g, Dextrose - 20 g, Agar - 20 g, Water - 1000 ml, pH – (6.0 to 6.5). Peeled potatoes were cut into slices and boiled in 500 ml of distilled water, till they are easily penetrated by a glass rod. Filter through double layer muslin cloth and measured amount of dextrose was added to the extract. In another flask, remaining 500ml distilled water was taken and allowed agar-agar to melt by boiling. The molten agar- agar was strained through double layer muslin cloth and mixed with potato extract solution. The volume was made up to 1000ml by adding distilled water. pH of the media was adjusted to

6.0-6.5. The media was poured into culture tubes and conical flask plugged by non-absorbent cotton and then sterilized in autoclave at 121□ for 20 minutes. (7).

Potato Dextrose Broth (PDB)

The potato dextrose broth was also prepared following the same method as describe above except that no agar-agar was added.

Preparation of culture filtrates of bio-agents

For the preparation of fungal culture filtrates, 100ml potato dextrose broth was prepared in 250ml Erlenmeyer flasks as mentioned above and seeded with tested fungal bio-agents. The inoculated flasks were incubated at 25± 2°C for 15days in BOD incubator. Then fungal culture filtrates were obtained by filtering through Whatman filter paper no.1. The filtrates so obtained were further centrifuged at 2000 rpm to remove the extra spores and mycelia. Then supernatants were collected and used in the *in-vitro* studies.

Process of spore counting:

For estimation of spores, pure culture of isolated bio-control agents diluted to 10^6 and 10^7 was used. Haemocytometer was cleaned with ethyl alcohol and left for few minutes to dry. One ml of spore suspension was placed at the centre of the slide and then covered with cover slip. Before counting, the preparation was allowed to slant for 2 minutes for setting of spores. At the bottom of the haemocytometer, ten small squares were selected at random and the spores were counted inside these squares. The bottom was cleaned again and the same procedure was repeated. Estimation of spores/10 squares of haemocytometer for calculation of spores per ml of suspension.

Treatments and experimental layout:

Experiment was conducted to investigate the antagonistic effect of fungal bio-control agents on hatching of root-knot nematode, *M. incognita* under *in-vitro* conditions. The experiment was laid out in a complete randomized design (CRD) with eleven treatments *i.e.,T.harzianum, T. viride, P. lilacinum, M. anisopliae* and *B. bassiana* at 2×10^6 and

2×10^7spore/ml were tried and untreated control were also maintained for comparison purpose and replicated thrice.

Preliminary preparation of experimentation:

Cavity blocks filled with five ml of sterilized distilled water were kept and then uniform sized single sterilized egg mass of *M. incognita* was transferred into them with fungi spore suspension diluted to 10^6 and 10^7 separately. Two drops of 0.1 per cent streptomycin were added to cavity blocks having fungus for avoiding bacterial contamination. One cavity block with sterilized distilled water was maintained for control. After 24, 48, 72, 96 and 120 hrs of exposure observation on hatching and mortality of larvae were recorded under compound microscope.

Hatching Test:

Five ml of spore suspension of bio-control agents in each sterile cavity block was taken. Surface sterilization of *M. incognita* egg masses were done with 0.4 per cent sodium hypochlorite and rinsing was done three times in sterile water. These surface sterilized *M. incognita* egg-masses were transferred into cavity blocks containing spore suspension. One egg-mass/cavity block. Cavity blocks were incubated for 120 hrs and the numbers of hatched juveniles were recorded out for every 24 hrs interval. The percent inhibition in egg hatching was calculated by using formula:

Per cent inhibition of egg hatching = $(C-T/C) \times 100$

Where, C = Number of hatched juveniles in control.

T = Number of hatched juveniles in each concentration of extract.

Results and Discussion

Table: 1.Antagonistic effect of fungal bio control agents on egg hatching of root-knot nematode, *M. incognita* under*in vitro* conditions.

Spore suspension dilution	No. of hatched juveniles after an exposure period				
	24 hours	48 hours	72 hours	96 hours	120 hours
T_1 – *T. harzianum* 2×10^6 spore/ml	24.00	34.33	48.00	56.00	65.33
T_2 – *T. harzianum* 2×10^7 spore/ml	29.66	41.00	54.66	65.66	75.33
T_3- *T. viride* 2×10^6 spore/ml	26.33	38.33	51.00	59.33	69.33
T_4 – *T. viride* 2×10^7 spore/ml	31.00	42.66	59.00	71.00	80.33
T_5 – *P. lilacinum* 2×10^6 spore/ml	19.33	29.00	40.00	48.33	56.00
T_6 – *P. lilacinum* 2×10^7 spore/ml	20.66	30.66	43.00	51.66	62.00
T_7 – *M. anisopliae* 2×10^6 spore/ml	40.33	51.00	72.00	85.33	105.33
T_8- *M. anisopliae* 2×10^7 spore/ml	44.00	55.66	77.66	93.33	117.66
T_9- *B.bassiana* 2×10^6 spore/ml	35.00	44.66	62.00	75.33	91.33
T_{10}- *B.bassiana* 2×10^7 spore/ml	38.00	47.33	66.00	79.33	96.33
T_{11}– Control	83.00	155.00	226.66	292.00	385.33

One egg mass of per cavity block. Data are average value of three replications.

Table 2. Effect of fungal bio-control agents on hatching inhibition of root-knot nematode, *Meloidogyne incognita* under*in vitro*conditions.

Spore suspension dilution	Per cent inhibition of hatching after an exposure period				
	24 hours	48 hours	72 hours	96 hours	120 hours
T_1 – *Trichoderma harzianum* 2×10^6 spore/ml	71.08 (57.47)	77.85 (62.02)	78.82 (62.71)	80.82 (64.16)	83.04 (65.86)
T_2 – *Trichoderma harzianum* 2×10^7 spore/ml	64.26 (53.30)	73.54 (59.08)	75.88 (60.64)	77.51 (61.75)	80.45 (63.85)
T_3- *Trichoderma viride* 2×10^6 spore/ml	68.27 (55.71)	75.27 (60.17)	77.49 (61.74)	79.68 (63.20)	82.00 (64.89)
T_4 - *Trichoderma viride* 2×10^7 spore/ml	62.65 (52.36)	72.47 (58.35)	73.96 (59.33)	75.68 (60.62)	79.15 (62.83)
T_5 - *Purpureocilliumlilacinum* 2×10^6 spore/ml	76.71 (61.18)	81.29 (64.44)	82.35 (65.24)	83.44 (66.08)	85.46 (67.60)
T_6 - *Purpureocilliumlilacinum* 2×10^7spore/ml	75.10 (60.18)	80.21 (63.80)	81.02 (64.34)	82.30 (65.40)	83.90 (66.70)
T_7 - *Metarhizium anisopliae* 2×10^6 spore/ml	51.40 (45.80)	67.09 (54.99)	68.23 (55.69)	70.77 (57.27)	72.66 (58.48)
T_8- *Metarhizium anisopliae* 2×10^7 spore/ml	46.98 (43.26)	64.09 (53.19)	65.73 (54.18)	68.03 (55.58)	69.46 (56.47)

T_9- *Beauveria bassiana* 2×10^6 spore/ml	57.83 (49.50)	71.18 (57.54)	72.64 (58.46)	74.20 (59.48)	76.29 (60.87)
T_{10}- *Beauveria bassiana* 2×10^7 spore/ml	54.21 (47.41)	69.46 (56.48)	70.88 (57.48)	72.83 (58.63)	75.00 (60.06)
T_{11}– Control	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
SEm ±	**1.65**	**1.76**	**2.10**	**2.13**	**1.78**
CD at 5%	**4.70**	**5.08**	**6.07**	**6.15**	**5.15**

Note: Data are per cent of average value of three replications over control.

The fungus *T. harzianum, T.viride, P.lilacinum, M.anisopilae*and *B.bassiana*first colonizes on the gelatinous matrix secreted by female nematode in which eggs were laid, eventually a mycelial network develops and engulfs the nematode eggs. Both mechanical and enzymatic activity involved in the penetration. Following penetration, the fungus grows and proliferates in the eggs in early embryonic development. After depleting all nutrients from the egg, the mycelium penetrates and ruptures the cuticle of the infected egg and then emerges out to infect other eggs in the vicinity.

This experiment was conducted to know the efficacy of fungal bio control agents *i.e.,T. harzianum, T.viride, P.lilacinum, M.anisopilae*and *B.bassiana*at two different dilution (10^6 and 10^7)and distilled water kept as a control. Inhibitory effects on hatching were observed after 24, 48, 72, 96 and 120 hrs exposure period as compared to control. Inhibitions of hatching were recorded and presented in Table1- 2.Among all dilutions, results of studies showed that the maximum suppression in egg hatching was observed in T5 – *P. lilacinum* @ 2×10^6 spore/ml (85.46 per cent), followed by T6 – *P. lilacinum* @ 2×10^7 spore/ml (83.90 per cent), T1- *T. harzianum* @ 2×10^6 spore/ml (83.04 per cent) and T3 - *T. viride*@ 2×10^6 spore/ml (82.00 per cent) whereas T8- *M. anisopliae* @ 2×10^7 spore/ml (69.46 per cent) was found least effective after 120 hrs. The culture filtrate studies revealed that all the tested fungal bio-control agents were effective in suppressing the egg hatching of *M. incognita*.

In-vitro testing of nematode destroying fungi is an essential method for evaluating their antagonistic activity against *M. incognita* and such antagonistic fungi produced metabolites and/ or protease enzymes that affect viability of nematode (8). Nematode egg shell is proteinaceous and chitinous in nature and act as barriers for egg parasitic fungi. To overcome these barriers, these fungal bio-agents produce lytic enzymes *viz.*, proteases, chitinases and lipases that cause

break down of egg shell and facilitate egg penetration for successful establishment (9, 10, 11, 12, 13). The same mechanism might be possessed by tested bio-agents that may have ability to produce such type of enzymes which caused extensive network of hyphae inside the *M. incognita* eggs. Similar result were also observed that egg parasitism by fungal bio-agents and observed conidia of *T. harzianum* to stick on the gelatinous matrix around the *M. javanica* eggs masses with prolific fungal growth inside the eggs as the germinating hyphae penetrated the egg masses for parasitization (14). The present investigation demonstrated early age of *M. incognita* eggs to be more susceptible *to P. lilacinum*infection than the eggs with ready to hatch (15). Further, they observed extensive network of hyphae of *P. lilacinum* that ramified several eggs as recorded in the present investigation as well.

These findings are in agreement with the results of (16) who reported 92.72 per cent inhibition in hatching of root-knot nematode, *M. incognita* by *T. viride*after 120 hrs. (17) showed *T. harzianum* BI most effective for its capacity to reduce the incidence and pathogenicity of the root-knot nematode *M. javanica* on tomato *In vitro* conditions. Parasitism of *M. javanica* eggs by *T. harzianum* BI ranged from 21.00 per cent in control to 84.00 per cent in antagonistic fungi. *T. harzianum* BI reduced nematode damage to tomato. Another similar reports on inhibition of egg hatching P. *lilacinum, T. viride, P. fluorescens* and *P. penetrans* have been reportred (18, 19, 20, 21, 22). The fungal bio-agents *viz., T.viride,* T. *harzianum, P.chlamydosporia*and *P.lilacinum* were screened for their efficacy against *M. incognita* under *in vitro*(2). In respect of egg hatch inhibition and juvenile mortality, the culture filtrates of these fungal bio-agents was tested and found to be effective in inhibition of egg hatch and mortality of juveniles of *M. incognita* at 25, 50, 75 and 100 percent concentrations. Among the bio-agents, *T. harzianum* was showed highest egg hatch inhibition and juvenile mortality of *M. incognita*which are in conformity with present findings. Hence, these bio-agents can be exploited further in evaluation under field conditions.

Conclusion

Based on the results of the present investigation, it can be concluded that *Purpureocilliumlilacinum, Trichoderma harzianum* and *T. viride* tested in this study produce good amount of secondary metabolites that is having lethal effect on egg hatching of *Meloidogyne incognita*. It indicated that the presence of these isolates of *P. lilacinum, T. harzianum* and *T. viride* in the soil may be helpful as a bio agent in the management of this root knot nematode under field conditions.

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