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Evaluation of different bio-agents on larval mortality and egg hatching of *Meloidogyne graminicola* causing root knot disease in rice

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Abstract
An experiment was conducted to test the efficacy of metabolites (Culture filtrate) of bio-agents at two concentrations *viz.* 25%, 50% against larval motility and egg hatching of *Meloidogyne graminicola* casing root knot disease in rice under *In vitro* condition. The bioagents *viz*., *Bacillus subtilis*, *Pseudomonas fluorescens*, *Paecilomyces lilacinus*, and two isolates of *Trichoderma (Trichoderma* isolates S13 and *Trichoderma* isolates S7) tested in this study. All the tested bioagents significantly increased the larval mortality and reduced the egg hatching of *Meloidogyne graminicola*. Among the tested bioagents maximum (90.00%) larval mortality and minimum (48.33%) egg hatching was recorded at 50% concentration. While, at 25% concentration maximum (75.00%) larval mortality and minimum (55.00%) egg hatching was recorded in culture filtrate of *Trichoderma* isolate S13 after 72 hr of inoculation. Minimum 78.33% larval mortality and Maximum 58.33% egg hatching was recorded in culture filtrate of *Paecilomyces lilacinus* whereas, in case of untreated control 1.67% larval mortality and 88.33% egg hatching was recorded after 72 hour of J_2 inoculation.

Keywords: Bio-agents, larval mortality, egg hatching, culture filtrate etc.

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. The rice root-knot nematode, *Meloidogyne graminicola*, is one of the constraints to rice production in Asia and causes significant yield losses in upland and rainfed lowland rice production Soriano *et al*. (2000) [11]. It is likely that *M. graminicola* will further contribute to rice yield decline, as the trend towards intensification of production will support increased nematode population densities. To prevent further rice yield losses due to the nematodes and improve productivity, a sound nematode management scheme is essential.

The utilization of fungal and bacterial bio-agents in the management of nematode parasites is gaining importance. Among the various biocontrol agents, *Paecilomyces lilacinus*, *Trichoderma viride*, *Pseudomonas fluorescens* and *Pasteuria penetrans* have been found to be promising against root-knot nematodes Sharma and Kumar (2005) [9]. 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 bioagents are found increase in attention and use of such bioagents offer an effective, safe, persistent and natural durable protection against *Meloidogyne graminicola* Anita and Samiyappan (2012) [2]. However, many natural enemies attack *Meloidogyne* spp. in the soil and such enemies can be used as bioagents for the effective management of *Meloidogyne* spp Karssen *et al.* (2006) [13]. Among them, fungi and bacteria are unique natural enemies for managing the nematodes in soil. Such bioagents showed their antagonistic activity like predation, parasitism and antibiosis etc., towards *Meloidogyne graminicola*. However, these fungi and bacteria have ability to release the antibiotics, metabolites, protease enzymes *etc*. Sharon *et al.* (2001) [15] in the environment and that caused nematode viability. However, the efficacy of bioagents to reduce the nematode viability varied from species to species. So, one of the means of increasing the potentiality of bioagents is to use the native biocontrol agents Singh *et al.* (2013) [14].

The potential benefits must be examined so that effective native biocontrol agents can be utilized.

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

Materials and Method

Infected rice root samples were collected from Crop Research Centre (CRC), S.V.P. University of Agriculture & Technology, Meerut, (U.P.). The experiment was conducted in a completely randomized design with three replications each treatment. Metabolites of these tested bioagents were tested at two concentrations (25 and 50%) under *in-vitro* condition on percent egg hatching and percent larval mortality. Data were recorded at 24, 48 and 72 hours after inoculation. While, percent egg hatching inhibition was recorded at 72 hour after inoculation.

Collection of juveniles (J_2)

The infected rice roots having galls developed by *Meloidogyne graminicola* were collected. The roots of uprooted rice plants were washed under running tap water. The galls were separated from the root. After that, the galls were transferred to the watch glass and were teased with the help of needle. The crushed root galls with water suspension were observed under sterobinocular microscope for the confirmation that the galls contain juvenile stage of *M. graminicola*. The J_2 stage of *M. graminicola* were separated by spreading the suspension on to a double layer tissue paper placed over wire gauze and then submerged into water in petri plates.

Collection of eggs

The infected rice roots having galls developed by *Meloidogyne graminicola* were collected. The roots of uprooted rice plants were washed under running tap water. The galls were separated from the root. After that, the galls were dissected with a sterilized dissecting needle and egg were hand picked up from the galled root with help of dropper. The picked eggs were kept in sterilized cavity block containing sterilized water.

Preparation of culture filtrates of bio agents

For obtaining the culture filtrate of bio-agents, *Trichoderma* isolate S13, *Trichoderma* isolate S7 and *Paecilomyces lilacinus* were cultured on potato dextrose broth medium and *Pseudomonas fluorescens* on Kings 'B' broth medium while, *Bacillus subtilis* cultured on nutrient broth medium. PDA disk of 0.5 mm size of 7 days old culture of *Trichoderma* isolates-13, and 14 days old culture of *Paecilomyces lilacinus* mycelia whereas a loopful, 3 day old culture of *Pseudomonas fluorescens* and *Bacillus subtilis* were added to flasks containing 200 ml of Potato dextrose broth (PDB), King 'B' broth and nutrient broth medium (NBM), respectively. The inoculated flasks were incubated at 26±2 °C temperature and B.O.D Shaker Incubator. After one week of incubation, culture of *Trichoderma* isolates S13, *Trichoderma* isolates S7, *Paecilomyces lilacinus*, *Bacillus subtilis* and *Pseudomonas fluorescens* was filtered through Whatman No.1 filter paper. The suspension was centrifuged at 5000 rpm for 20 min at 20°C to remove all small part and spores of the fungus. The supernatants of tubes were taken in sterilized culture tubes. Sterilized distilled water was used to maintain the control.

Effect on Juvenile (J_2) Mortality

To study the effect of culture filtrate of the bioagents on larval mortality, 10 ml culture filtrate of the test bioagents in two concentrations (25 and 50%) was separately poured into 5 cm size petri dishes. Three replications were maintained for each treatment. Control petri plates were maintained by adding 10 ml of sterilized distilled water only. All these petri plates were added by l ml suspensions containing 20 freshly hatched J_2 of *M. graminicola* with the help of a flat tipped picking dropper. petri plates were incubated at room temperature. Observations on larval mortality were recorded at 24, 48 and 72 hours after inoculation under stereomicroscope. Percentage of larval mortality was calculated by following formula given by Ahmad *et al.* (2004) [1].

$$\% \text{ larval mortality} = \frac{\text{Total number of larvae killed}}{\text{Total number of larvae inoculated}} X100$$

Effect on Egg Hatching

To study the effect of culture filtrate of different bioagents on nematode egg hatching culture filtrate of the test bioagents in two concentrations (25 and 50%,) was separately poured into 5 cm petri dishes .Three replications were maintained for each treatment. Control petri plates were maintained by adding 10 ml of sterilized distilled water. All these petri plates were added by l ml suspensions containing 20 freshly eggs of *M. graminicola* with the help of a flat tipped picking dropper. These petri plates were incubated at room temperature. Observations on egg hatching were recorded at 24, 48 and 72 hours after inoculation. Whereas egg hatching inhibition was observed 72 hours after inoculation at both 25% and 50% concentration. Percentage of egg hatched and inhibition was calculated by following formula given by N. G. Ravichandra (2010) [8].

$$\% \text{ Egg Hatching} = \frac{\text{No. of hatched juveniles}}{\text{Total number of eggs inoculated}} \times 100$$

$$\text{Hatch inhibition of eggs (\%)} = \frac{\text{Total no. of eggs- hatched No. of eggs}}{\text{Total number of eggs inoculated}} \times 100$$

Results and Discussion

Effect of culture filtrates of bio-agents on juvenile (J_2) mortality of *M. graminicola*

The results revealed (**Table:1**) that culture filtrate of all the tested bioagents *viz. Bacillus subtilis, Pseudomonas fluorescens, Paecilomyces lilacinus,* and two isolates of *Trichoderma (Trichoderma* isolates S13 and *Trichoderma* isolates S7) found effective against larval mortality of *M. graminicola* at 25% and 50% concentration over control. At 25% concentration, maximum 26% larval mortality was recorded in culture filtrate of *Trichoderma* isolates S13 followed by 20% in *Trichoderma* isolates S7, 11.67% in *Pseudomonas fluorescens* and *Bacillus subtilis* whereas, minimum 10% larval mortality was recorded in *Paecilomyces lilacinus* at 24 hours after inoculation. At 48 hours after inoculation maximum 51.67% larval mortality was recorded in culture filtrate of *Trichoderma* isolate S13 followed by 50% in *Trichoderma* isolate S7, 48.33% in both *Pseudomonas fluorescens* and *Bacillus subtilis.* While minimum 45.00% larval mortality was recorded in *Paecilomyces lilacinus.* At 72 hour after inoculation, maximum 75.00% larval mortality was recorded in culture filtrate of *Trichoderma* isolate S13

followed by 73.33%, 70.00% and 68.33% in *Trichoderma* isolate S7, *Bacillus subtilis* and *Pseudomonas fluorescens* respectively. Minimum 65.00% larval mortality was recorded in culture filtrate of *Paecilomyces lilacinus*. Whereas 1.67% larval mortality was recorded in case of control after 72 hour of inoculation.

At 50% concentration, maximum 43.33% larval mortality was recorded in culture filtrate of *Trichoderma* isolates S13 followed by 41.67% in *Trichoderma* isolates S7, 33.33% in both *Pseudomonas fluorescens* and *Bacillus subtilis* whereas, minimum 23.33% larval mortality was recorded in *Paecilomyces lilacinus* at 24 hours after inoculation. At 48 hours after inoculation maximum 70.00% mortality was recorded in culture filtrate of *Trichoderma* isolate S13 followed by 66.67% in *Trichoderma* isolate S7, 65.00% in both *Pseudomonas fluorescens* and *Bacillus subtilis*, while minimum 63.33% in *Paecilomyces lilacinus.* At 72 hour of inoculation, maximum 90.00% larval mortality was recorded in culture filtrate of *Trichoderma* isolate S13 followed by 88.33%, 85.00% and 81.67% in *Trichoderma* isolate S7, *Bacillus subtilis* and *Pseudomonas fluorescens* respectively. Minimum 78.33% larval mortality was recorded in culture filtrate of *Paecilomyces lilacinus.* Whereas 1.67% larval mortality was recorded in case of control at 72 hour.

Table 1: Effect of culture filtrate of different bioagents on larval (J_2) mortality of *Meloidogyne graminicola*

Treatment		25% Concentration						50% Concentration					
		24hrs.		48hrs.		72hrs.		24hrs.		48hrs.		72hrs.	
		No. of dead J_2	% mortality	No. of dead J_2	% mortality	No. of dead J_2	% mortality	No. of dead J_2	% mortality	No. of dead J_2	% mortality	No. of dead J_2	% mortality
T_1	*Bacillus subtilis*	2.33	11.67	9.67	48.33	14.00	70.00	6.67	33.33	13.00	65.00	17.00	85.00
T_2	*Pseudomonas fluorescens*	2.33	11.67	9.67	48.33	13.67	68.33	6.67	33.33	13.00	65.00	16.33	81.67
T_3	*Paeciliomyces lilacinus*	2.00	10.00	9.00	45.00	13.00	65.00	4.67	23.33	12.67	63.33	15.67	78.33
T_4	*Trichoderma isolates* S13	5.33	26.67	10.33	51.67	15.00	75.00	8.67	43.33	14.00	70.00	18.00	90.00
T_5	*Trichoderma isolates* S7	4.00	20.00	10.00	50.00	14.67	73.33	8.33	41.67	13.33	66.67	17.67	88.33
T6	Control	0.00	0.00	0.00	0.00	0.33	1.67	0.00	0.00	0.00	0.00	0.33	1.67
C.D. at 5%		2.260		1.573		2.907		2.315		1.598		2.796	

Table 2: Effect of culture filtrate of different bio-agents on egg hatching of *Meloidogyne graminicola*

Treatment	25% Concentration							50% Concentration						
	24hrs.		48hrs.		72hrs			24hrs.		48hrs.		72hrs.		
	No. of egg hatched	% egg hatching	No. of egg hatched	% egg hatching	No. of egg hatched.	% egg hatching	% Inhibition over control	No. of egg hatched	% egg hatching	No. of egg hatched	% egg hatching	No. of egg hatched	% egg hatching	% Inhibition over control
Bacillus subtilis	2.67	13.33	8.33	41.67	12.67	63.33	36.67	1.33	6.67	6.67	33.33	10.33	51.67	48.33
Pseudomonas fluorescens	2.33	11.67	8.67	43.33	13.00	65.00	35.00	1.67	8.33	7.00	35.00	10.67	53.33	46.67
Paeciliomyces lilacinus	3.00	15.00	9.00	45.00	13.67	68.33	31.67	2.00	10.00	7.67	38.33	11.67	58.33	41.67
Trichoderma isolates S13	2.00	10.00	7.67	38.33	11.00	55.00	45.00	1.00	5.00	5.67	28.33	9.67	48.33	51.67
Trichoderma isolates S7	2.00	10.00	8.00	40.00	11.33	56.67	43.33	1.33	6.67	6.33	31.67	10.00	50.00	50.00
Control	3.67	18.33	13.33	66.67	17.67	88.33		3.67	18.33	13.33	66.67	17.67	88.33	
C.D. at 5%	N.S.		1.861		1.163			2.439		1.719		1.280		

Effect of culture filtrates of bioagents on egg hatching of *M. graminicola*

The Results revealed (**Table:2**) that culture filtrate of all the tested bioagents *viz. Bacillus subtilis, Pseudomonas fluorescens, Paecilomyces lilacinus,* and two isolates of Trichoderma (isolates S13 and S7) were found effective against egg hatching of *M. graminicola* at 25% and 50% concentration over control. At 25% concentration, minimum 10% egg hatching was recorded in culture filtrate of *Trichoderma* isolates S13 and *Trichoderma* isolates S7 followed by 11.67% in *Pseudomonas fluorescens*,13.33% in *Bacillus subtilis* whereas, maximum 15% egg hatching in *Paecilomyces lilacinus* at 24 hours after inoculation. While, 18.33% egg hatching was recorded in case of control after 24 hours of eggs inoculation. After 48 hours of inoculation minimum 38.33% egg hatching was recorded in culture filtrate of *Trichoderma* isolate S13 followed by 40.00% in *Trichoderma* isolate S7, 41.67% in *Bacillus subtilis* and 43.33% *Pseudomonas fluorescens* while, maximum 45.00% egg hatching was recorded on case of *Paecilomyces lilacinus* at 48 hours after inoculation . Whereas, 66.67% egg hatching was recorded in case of control at 48 hour after eggs inoculation. At 72 hour after inoculation, minimum 55.00% egg hatching was recorded in case of *Trichoderma* isolate S13 followed by 56.67%, 63.33% and 65.00% in *Trichoderma* isolate S7, *Bacillus subtilis* and *Pseudomonas fluorescens* respectively. Maximum 68.00% egg hatching was recorded in culture filtrate of *Paecilomyces lilacinus.* While, 88.33% egg hatching was recorded in case of control at 72 hour after eggs inoculation.

Percent eggs hatching inhibition at 72 hour after inoculation, maximum 45.00% egg hatching inhibition was recorded in case of *Trichoderma* isolate S13 followed by 43.33%, 36.67% and 35.00% in *Trichoderma* isolate S7, *Bacillus subtilis* and *Pseudomonas fluorescens* respectively. Minimum 31.67% egg hatching inhibition was recorded in culture filtrate of

Paecilomyces lilacinus at 72 hour after eggs inoculation.

At 50% concentration, minimum 5.00% egg hatching was recorded in culture filtrate of *Trichoderma* isolates S13 followed by 6.67% in *Trichoderma* isolates S7 and *Bacillus subtilis*, 8.33% in *Pseudomonas fluorescens*, whereas, maximum 10% egg hatching in *Paecilomyces lilacinus* at 24 hours after inoculation. While, 18.33% egg hatching was recorded in case of control at 24 hours of egg inoculation. After 48 hours of inoculation minimum 28.33% egg hatching was recorded in culture filtrate of *Trichoderma* isolate S13 followed by 31.67% in *Trichoderma* isolate S7, 33.33% in *Bacillus subtilis* and 35.00% in *Pseudomonas fluorescens* while, maximum 38.33% egg hatching in *Paecilomyces lilacinus* at 48 hour after inoculation. Whereas, 66.67% egg hatching was recorded in case of control after 48 hour of egg inoculation. At 72 hour after inoculation, minimum 48.33% egg hatching was recorded in culture filtrate of *Trichoderma* isolate S13 followed by 50.00%, 51.67% and 53.33% in *Trichoderma* isolate S7, *Bacillus subtilis* and *Pseudomonas fluorescens* respectively. Maximum 58.33% egg hatching was recorded in culture filtrate of *Paecilomyces lilacinus.* While, 88.33% egg hatching was recorded in case of control at 72 hour of egg inoculation.

Percent egg hatching inhibition at 72 hour after inoculation, maximum 51.67% egg hatching inhibition was recorded in case of *Trichoderma* isolate S13 followed by 50.00%, 48.33% and 46.67% in *Trichoderma* isolate S7, *Bacillus subtilis* and *Pseudomonas fluorescens* respectively. Minimum 41.67% egg hatching inhibition was recorded in culture filtrate of *Paecilomyces lilacinus* at 72 hour after eggs inoculation. Similar results were observed by Prasad and Ravichandra (2018) [6] evaluated of isolated indigenous bioagents on inhibition of egg hatching and larval mortality of *M. incognita*. Indigenous bioagents *viz*., *Trichoderma harzianum*, *T. viride*, *Pachonia lecani* with 2×10^6 cfu/g, *Pseudomonas fluorescens* and *Bacillus subtilis* with 1×10^8 cfu/g were tested at 25, 50, 75 and 100 percent concentrations . Among indigenous bioagents tested, maximum inhibition of egg hatching was recorded in *T. harzianum* (23.33%) amounting to 64.82 percent and larval mortality of 69.00% after 72 hours of incubation.

Annapurna *et al*. (2018) [3] reported that the fungal bioagents *viz*., *Trichoderma viride*, *T. harzianum*, *Pochonia chlamydosporia* and *Purpureocillium lilacinum* were screened for their efficacy against *Meloidogyne incognita* under *in-vitro.* In respect of egg hatch inhibition and juvenile mortality, the culture filtrates of these fungal bioagents 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 bioagents, *T. harzianum* was showed highest egg hatch inhibition and juvenile mortality of *M. incognita*. Dose-response models were used in the larval mortality test to determine the concentration of culture filtrate required to kill 50 percent of the juveniles. Singh *et al.* (2019) [3] observed that efficacy of metabolites (culture filtrate) of *Trichoderma* isolate -13, *Paecilomyces lilacinus*, *Bacillus subtilis* and *Pseudomonas fluorescens* was tested against second stage larvae of *Meloidogyne graminicola* at 100%, 75%, 50% and 25%concentrations under laboratory conditions in petri plates. Maximum larval mortality (30%, 45% and 80%) was recorded in case of *Trichoderma* isolate followed by *Paceliomyces lilacinus* (21.65%,36.65% and 60%) and *Pseudomonas fluorescens* (18.35%, 25% and 51%) after 24, 48 and 72 hours after inoculation at 100% concentration respectively. Similar trend in mortality was recorded almost at all concentrations. In case of control (sterilized plain water) 3.35, 8.35 and 13.35% larval mortality was recorded after 24, 48 and 72 hours respectively.

Devi and Bora (2019) [4] studied the effect of culture filtrate of different bacterial isolates on egg hatching and juvenile mortality of root-knot nematodes (*Meloidogyne incognita* race 2) under *in vitro*. Culture filtrate of all the isolates of bacteria significantly induced mortality and inhibition of egg hatching of *M. incognita* juveniles. The highest percentage of inhibition of egg hatching was recorded for *Bacillus thuringiensis* followed by *Bacillus sp*. and *Pseudomonas fluorescens* whereas the highest percentage of mortality of juvenile was recorded for *Bacillus thuringiensis* followed by *Pseudomonas fluorescens*.

Conclusion

Based on the results of the present investigation, it can be concluded that *Trichoderma* isolates (S13 and S7) tested in this study produce good amount of secondary metabolites that is having lethal effect on larval mortality and egg hatching of *Meloidogyne graminicola*. It indicated that the presence of these isolates of *Trichoderma* in the soil may be helpful as a bio agent in the management of this rice root knot nematode.

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