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In vitro bioassay of *Meloidogyne incognita* juveniles against biocontrol agents

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Abstract
Biological control is considered as new efficient method that becomes widely used for controlling plant parasitic nematodes, as aim to decrease the extent of environment degradation and the effect of the excessive toxic nematicides. So, this study was done to investigate the role of four biocontrol agents against *Meloidogyne incognita* juveniles. *In vitro* nematicidal activity of bioagents against *M. incognita* juveniles was evaluated and juvenile mortality was recorded periodically at 24, 48 and 72 hrs. The mortality was found at 24 hrs and from then on, it increased with increase in time. The results showed that maximum mean mortality of juveniles was observed in *Paecilomyces lilacinus* (67.77%) culture followed by *Trichoderma harzianum* (54.89%), *Pseudomonas fluorescens* (50.00%) and the least mortality was observed in *Bacillus subtilis* (48.89%). The results of *in vitro* experiments indicated that, all tested biocontrol agents have a greatly significant effectiveness for juvenile mortality *M. incognita*.

Keywords: Biocontrol agents, *In vitro*, juvenile, *Meloidogyne incognita*, mortality

Introduction
Betelvine (*Piper betle* L.) is a perennial climber belongs to family *Piperaceae*, which is believed to be originated in central and eastern Malaysia. It is valued as both mild stimulant and for its medicinal properties.

Soil borne pathogens are important in the context of betelvine production as they cause heavy yield losses. Various plant parasitic nematodes are known to cause reduction in crop yields, among the plant parasitic nematodes the root knot nematode (*Meloidogyne incognita*) and reniform nematode (*Rotylenchus reniformis*) are the most destructive in all the betelvine growing areas, while *Helicotylenchus microcephalus* was found to be dominant in Maharashtra [10]. So far, 35 genera of plant parasitic nematodes have been reported to be in association with betelvine crop [18]. The yield losses due to the diseases caused by *M. incognita* alone ranged from 16.8 to 50.2 per cent [3].

The roots of nematode infested vines are very much prone to other soil borne plant pathogen infection *viz*., *Sclerotium rolfsii, Colletotrichum capsici, Phytophthora palmivora, Rhizoctonia solani, Fusarium solani* and *Xanthomonas campestris* pv. *betlicola*. The higher density of nematodes especially, *M. incognita* and *R. reniformis* helps in the easy entry of soil-borne plant pathogens, which may further lead to the wilt complex. The extent of loss due to association of wilt causing fungi with root knot nematode *M. incognita* is up to100 per cent [16].

In recent years, several fungal and bacterial bio-agents like *Aspergillus terreus, Aspergillus niger, Pachonia* sp., *Trichoderma harzianum, Trichoderma viride, Paecilomyces lilacinus, Pseudomonas fluorescens, Pasturia penetrans, Bacillus subtilis, Penicillium* sp. are being tested for managing root knot nematodes [6]. Since, fungi, bacteria and nematodes occur together in the rhizosphere, the toxic metabolites naturally produced by microorganisms like fungi and bacteria may be responsible for keeping low levels of nematode populations. Keeping in view of the above mentioned, the study has been taken up to evaluate the nematicidal potentials of four biocontrol agents *viz*., *P.lilacinus, T. harzianum, P. fluorescens* and *B. subtilis* against second stage juveniles (J2) of *M. incognita* under *in vitro* condition which can be further used in root knot nematode management at field conditions.

Material and methods
To assess the nematicidal properties of biocontrol agents, *in vitro* experiment was carried out in the plant pathology laboratory of UAHS, Shivamogga. Nematicidal effect of cultural filtrates of *P. lilacinus, T. harzianum, B. subtilis* and *P. fluorescens* (with 2 x 10^8 cfu/ml) were

studied on juveniles of *Meloidogyne incognita* at room temperature (28 ± 1° C).

Four different bioagents *P. lilacinus, T. harzianum, B. subtilis* and *P. fluorescens* were obtained from department of plant pathology and were grown on the broth media. The flasks were incubated for 21 days at 28 ± 1° C. After incubation the contents of the flasks were filtered through Whatman No.1 filter paper and filtrate was used within 24 hours.

The inoculum of root-knot nematode *M. incognita* was collected from naturally infested betelvine crop in field and single egg mass was used to raise pure culture. *M. incognita* mass culturing was done on roots of tomato plants (*Lycopersicon esculentum*). *M. incognita* egg masses were extracted from severely infected tomato roots using a 1.5% NaClO solution and subsequently washed with distilled water. The surface-sterilized egg masses were incubated in sterile water for 3–5 days at 25 °C. Using Baermann funnel method second-stage juveniles (J2s) were obtained [5]. The freshly hatched J2s were used for nematicidal assay. 50 freshly hatched juveniles were transferred to petri plates containing 10 ml culture filtrate of *P. lilacinus, T. harzianum, B. subtilis* and *P. fluorescens* with sterile water as control. Each treatment was replicated thrice and inoculated plates were incubated for 24, 48 and 72 hrs at 28 ± 1^0 C.

The control was maintained by transferring 50 juveniles to a petri plate containing sterile distilled water without bioagents. After 24, 48 and 72hrs the number of dead and live nematode was counted under sterio binocular microscope and percent mortality in each petri plate was calculated by using the formula given by Ashoub (2010) [4]. The data from the two experiments were subjected to analysis of variance (ANOVA) and standard deviations and critical differences (CD) at P = 0.05 were calculated.

$$\text{Per cent disease incidence} = \frac{\text{Number of dead juveniles}}{\text{Total number of juveniles}} \text{ X100}$$

Results and discussion

Nematicidal activity of biocontrol agents against *M. incognita* juveniles was evaluated and the results of mortality of nematode as function of time are presented in Table 1. The results presented in the table against 24, 48 and 72 hrs are the average values of the three replications, each set of replication containing 50 second stage juveniles in them (J_2).

Juvenile mortality was recorded periodically at 24, 48 and 72 hrs. Initial mortality was observed at 24 hrs of incubation and from then on, it increased with increase in time. Maximum mean mortality of juveniles was observed in *Paecilomyces lilacinus* (67.77%) culture followed by *Trichoderma harzianum* (54.89%) and the least mortality was observed in *Bacillus subtilis* (48.89%). Highest mortality of 64.00 and 67.33 per cent was recorded by *P. lilacinus* after 24 and 48 hrs of incubation which is significantly superior over all other treatments followed by *T. harzianum* (49.33 and 54.67%) respectively. Minimal larval mortality was recorded by *Bacillus subtilis* (46 and 48.67%).

Same trend continued for 72hrs incubation also where, maximum larval mortality of 72.00 per cent recorded in *P. lilacinus* (72%) which is significantly (11.33%) superior over all other treatments and the minimal larval mortality was recorded by *B. subtilis* (52%). Among the four bioagents tested against *M. incognita* J2s, fungal biocontrol agents *P. lilacinus* and *T. harzianum* were found to be most effective in causing larval mortality than bacterial antagonists *B. subtilis* and *P. fluorescens*.

The per centage mortality of juvenile (J2) was found to increase with the increased exposure to time. *In vitro* studies have established presence of toxic substances in fungal culture media, These nematicidal compounds produced by *T. harzianum, P. lilacinus, P. fluorescens* and *B. subtilis* seemed to play an important role in causing nematode mortality, which kill or immobilize the juveniles of root knot nematode. (Alam *et al*., 1973; Sakhuja *et al*., 1978; Mani and Sethi, 1984) [1, 15, 11]. The culture filtrates and their antagonism proved the biocontrol efficiency of these microbes. Pau *et al*. (2012) [14] showed the effect of antagonism of *P. lilacinus* under *in vitro*. To infect J2, *P. lilacinus* needs to overcome the cuticle of nematode. Once the cuticle is penetrated by fungal hyphae, the nematode get paralyzed, invaded and digested [20]. Metabolites in culture filtrate of *P. lilacinus* have been detected and screened such as paecilotoxin [12], acetic acid [8] and leucinostatins [13]. These metabolites may potentially cause nematicidal effect toward J2 of nematodes.

Trichoderma spp. is utilized in the production of a number of antibiotics, such as trichoderin A, trichodermol and harzianolide. Successful parasitism of the nematode by *Trichoderma* requires mechanisms to facilitate penetration of the nematode cuticles or eggshells. *Trichoderma* produces molecules such as 6-pentyl α-pyrone, VOCs and enzymes [17] that can attack the cuticle of nematodes. The involvement of lytic enzymes has long been suggested and demonstrated in *Meloidogyne* parasitism [19]. Also, its hyphae form a physical barrier, which is a difficult step for nematodes locomotion which makes it immobile. The culture filtrates of rhizobacterium are heat stable and resistant to extreme pH values, which suggested that they are antibiotic and their protein might be responsible for the nematicidal activity [7]. These observations are in conformity with the earlier workers, Karmakar (2004) [9] and Anjum and Reddy (2013) [2].

Table 1: Effect of bioagents on juvenile mortality of *M. incognita*

Bioagents	Juvenile mortality (%)			
	24hrs	48hrs	72hrs	Mean
Trichoderma harzianum	49.33 (44.64)*	54.67 (47.70)	60.67 (51.19)	54.89 (47.84)
Paecilomyces lilacinus	64.00 (53.16)	67.33 (55.18)	72.00 (58.09)	67.77 (55.47)
Pseudomonas fluorescens	46.67 (43.11)	48.00 (43.88)	55.33 (48.09)	50.00 (45.02)
Bacillus subtilis	46.00 (42.73)	48.67 (44.26)	52.00 (46.17)	48.89 (44.38)
Control	0.00 (0.00)	4.67 (12.17)	7.33 (15.69)	4.00 (9.29)
	S.Em±		CD at 1%	
Bioagents (B)	0.51		1.99	
Hour (H)	0.39		1.54	
BxH	0.88		3.45	

Conclusion

The per centage mortality of juvenile was found to increase with the increased exposure to time. All the bioagents have shown maximum mortality at 72 hrs of exposure compared to 24 and 48 hrs. This antagonistic effect against *M. incognita* juvenile is due to permeability changes of juvenile cuticle which is characterized by its selective permeability and the visual abnormalities probably were due to the effect of fungal toxic metabolites produced by the bioagents.

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