Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

J E Z S Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(3): 767-770

Received: 02-01-2020
Accepted: 31-01-2020

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In-vitro evaluation of potential bio agents on hatching and mortality of root knot nematode, *Meloidogyne incognita*

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Abstract
The present study was conducted to evaluate the efficacy of indigenous bacterial [*Bacillus amyloliquefaciens* (Ba-14.5), *Bacillus subtilis* (Bs-13.9 and Bs-13.19)] and fungal bioagents [*Trichoderma asperellum* (Tr- 9 and Tr-15)] on root-knot nematode, *Meloidogyne incognita* under *in vitro* conditions. Cell free culture filtrate of these isolates significantly inhibited the egg hatching and caused juvenile (J2) mortality of root knot nematode at 25, 50, 75 and 100% culture filtrate concentrations. In this study, bacterial bioagents were more virulent to second stage juveniles (J2) compared to fungal bioagents. At 24h exposure period, cent percent mortality was recorded in the culture filtrate prepared from *B. subtilis* (Bs-13.9) and *B. amyloliquefaciens* (Ba-14.5) and 74.66 % mortality of J2 was recorded from *B. subtilis* (Bs-13.19). Whereas, in case of fungal bioagents 88.66% and 85% mortality of J2 was recorded at 72 hours after exposure (HAE) period in *T. asperellum* (Tr-9) and *T. asperellum* (Tr-15) respectively. All bioagents significantly suppressed the egg hatching and was maximum in *B. amyloliquefaciens*, Ba-14.5 (86.00%) followed by *B. subtilis*, Bs-13.9 (82.33%) and *B. subtilis*, Bs-13.19 (80.33%). Similarly, the maximum suppression by fungal bioagents in egg hatching of nematodes was observed in *T. asperellum*, Tr-9 (84.66%) followed by *T. asperellum*, Tr-15 (78.66%). The culture filtrate studies revealed that all the five biocontrol agents were effective in suppressing the egg hatching of *M. incognita* and percentage mortality of second stage juveniles (J2) increased with increase in time of exposure to cell free culture filtrate as well with increase in concentration. Further studies to be conducted in pot and field conditions to evaluate the efficacy of these bioagents against root knot nematode, *M. incognita.*

Keywords: *Meloidogyne incognita*, biocontrol agents, culture filtrate, bacteria, fungi, *B. amyloliquefaciens, B. subtilis, T. asperellum*

1. Introduction
Chinese potato, *Plectranthus rotundifolius* belongs to the mint family Lamiaceae and is native to tropical Africa. It is an important minor tuber crop and cultivated mainly for its edible tubers which have special flavour and taste and are mainly used as vegetable (Sathyaseelan and Sudharmaidevi, 2012) [23]. It is cultivated in India, Sri Lanka, Malaysia, Indonesia and parts of Africa. In India, it is widely grown in the states of Tamilnadu, Kerala and Karnataka (Doraipandian, 1973 [6]; Hrishi and Mohankumar, 1976) [10]. The tubers are not only rich in starch, but also in minerals like calcium, iron and vitamins including thiamine, riboflavin, niacin and ascorbic acid (Anju *et al*., 2017) [2]. It is widely used in the treatment of stomach disorders, vomiting, diarrhoea, mouth and throat infection, wounds, burns, abdominal pain, insect bites and other sensory disorders. It can also improve immunity and body's defence mechanism. Several biotic and abiotic stresses resulted in the yield reduction of chinese potato. Among biotic stresses, root knot nematode (*Meloidogyne incognita*) is a serious pest on chinese potato and the infected plants exhibit serious swellings or galls in the roots resulting in suppressed roots, stunted growth and wilting. The damage caused by root knot nematode has resulted 36% yield loss in the country (Senthamarai *et al*., 2006) [24]. Sometimes, severe infestation leads to malformation of tubers, unfit for consumption as well as for storage.
Carbofuran, a solely available nematicide for the management of root knot nematode but their use is strictly prohibited in Kerala. Moreover, the indiscriminate and extensive use of pesticides has posed a serious problem of pollution in the ecosystem and development of resistance in the nematodes. Biopesticides based on living microorganisms, plant extracts and other natural compounds represent non chemical alternatives and eco-friendly in nature

(Kumar *et al.*, 2018) [16]. Among biopesticides, soil dwelling microorganisms like bacteria and fungi have been successfully used as bioagents against plant parasitic nematodes and appears to be a promising alternative strategy in the management of root knot nematode (Hallmann *et al*., 2001 [8]; Meyer *et al*., 2004 and Hussain *et al*., 2017) [11]. Several studies reported that the potential use of bacterial bioagents like *Bacillus* and *Pseudomonas* against the root knot nematode by means of metabolic by-products, enzymes and toxins (Martinez *et al*., 2014) [18]. The effects of these toxins include the suppression of nematode reproduction, egg hatching and juvenile survival, as well as direct killing of nematodes (Zuckerman & Jasson, 1984 [28]; Siddiqui and Shaukat, 2003 [25]; Meyer, 2004) [18]. Fungal bioagents like *T. viride* proved to be effective against root knot nematode, *Meloidogyn*e species and they are able to enhance plant growth and reduce nematode damage (Meyer *et al*., 2004) [19]. Similarly, *Paecilomyces lilacinus* is strongly parasitic to all stages of development, especially the eggs proved its efficacy as effective bio-control agent on *Meloidogyne* spp. on various crops (Jatala, 1986 [12]; Rao *et al*., 1999 [22]; Khan *et al*., 2009 [15]; Brand *et al*., 2010 [5]). In the present study, the efficacy of indigenous bacterial and fungal bioagents were tested against root knot nematode under *in vitro* conditions.

2. Materials and Methods

2.1 Collection of egg masses

The *M. incognita* infected roots were collected from chinese potato growing fields in ICAR-CTCRI, Thiruvananthapuram. The roots were washed gently under tap water and uniform sized egg masses were collected using sterile forceps. These egg masses were then surface sterilized with 0.1% sodium hypochlorite for 10 seconds followed by five washings with sterile water.

2.2 Extraction of second stage juveniles (J2)

The surface sterilized egg masses were placed over a double layered tissue paper supported by a coarse aluminium wire gauge kept on a petri dish containing fresh water, just enough to keep the tissue paper wet. Freshly hatched second stage juveniles were harvested from the Petri dishes and concentrated for the present study.

2.3 Preparation of culture filtrates of fungal and bacterial bio agents:

The potato dextrose broth (PDB) for fungal agents and nutrient broth (NB) for bacterial agents were prepared, inoculated with respective bio agents in 100 ml conical flasks followed by incubation at 30 °C in a shaker for 48 hrs. The cultures were centrifuged at 6000 rpm for 20-30 minutes. The supernatant was kept as a stock solution of cent percent concentration. Next grade of 75, 50, and 25 percent concentration were made by dilution with distilled water.

2.4 Effect of different bio agents on juvenile mortality

One ml of nematode suspension containing 100 J2 was placed in 3 ml of culture filtrates of each fungal and bacterial strain in small Petri plates. The numbers of dead juveniles were were recorded at 24, 48 and 72h of exposure. Juveniles inoculated in sterilized distilled water were served as control. Percentage mortality of second stage juveniles was calculated according to formula (Abbott, 1925) [1].

Nematode Mortality (%) = [(m – n)/(100-n)] X 100 Where; m and n indicate the mortality (%) in treatments and control, respectively.

2.5 Effect of bioagents on egg-hatching

Three milliliters of each concentration of fungal and bacterial culture filtrates was transferred to sterile petri-dishes of 5 cm diameter. Egg masses of root knot nematode were collected and surface sterilized using 0.1% sodium hypochlorite for 30 s and rinsed with sterile distilled water for atleast three times. Each petri plate was placed with three egg masses containing culture filtrate of each concentration and incubated at room temperature. Three egg masses of root knot nematode placed in distilled water served as control. The number of juveniles (J2) hatched in all the four concentrations were counted for 5 days using a stereoscopic microscope. The percentage suppression in hatching of juveniles (J2) was calculated by 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.

2.6 Data analysis

Data of the present study was subjected to statistical analysis of variance (ANOVA) and means compared according to Duncan's multiple range test (Duncan 1955) [7] by using SPSS 21 software.

3. Results and Discussion

Percentage J2 mortality of four different concentrations *viz*., 25, 50, 75 and 100 percent culture filtrates of isolated bacterial and fungal bioagents were given in Table 1 and Table 2. The results presented in both the tables clearly indicate that mortality of second stage juveniles of *M. incognita* was not observed in distilled water (control) and very less mortality of J2s observed in nutrient agar and nutrient broth. However, all the concentrations of fungal and bacterial bioagents showed a significant effect on J2 mortality. Juveniles mortality was found to be directly proportional to the concentration and duration of exposure. The results presented in Table 1 revealed that toxic metabolites of bacterial bioagents showed dramatic effect on mortality of second stage juveniles at all different concentrations. At 24h exposure period, cent percent mortality was observed in the 100% culture filtrate prepared from *B. subtilis* (Bs-13.9) and *B. amyloliquefaciens* (Ba-14.5) followed by 74.66 % J2 mortality in *B. subtilis* (Bs-13.19). Compared to all bacterial bioagents, *B. amyloliquefaciens* (Ba-14.5) showed more potential compared to other two isolates of *B. subtilis*. It showed 100% mortality after 48 HAE of second stage juveniles (J2) even at 75% culture filtrate concentration compared to other two isolates of *B. subtilis*. Deformation of nematode body and formation of globular structures occurred inside the killed nematode. Similar observations were also observed in several bacterial strains like *B. subtilis*, *B. cereus*, *P. aeruginosa*, *P. polymyxa* which significantly killed the second stage juveniles (J2) of root knot nematode (Batool *et al.*, 2013 [4]; Sohrabi *et al*., 2018 [26]; Soliman *et al*., 2019) [27].

Results in Table 2 revealed that two tested fungal bioagents were effective in killing of second stage juveniles (J2) of root knot nematode. 71% mortality of second stage juveniles was observed by 100% culture filtrate of *T. asperellum* (Tr-9) followed by *T. asperellum* (Tr-15) showing 61% mortality of J2s after 24 HAE (Table 2). 88. 66% and 85.00% mortality of J2 was observed with 100% culture filtrate of *T. asperellum*

(Tr-9) and *T. asperellum* (Tr-15) respectively after 72 HAE. Our results were also in agreement with Hooper, 1993 [9] and Rajinikanth *et al*., 2016 [21] where they reported that mortality of root knot nematode increased with increase in exposure time as well as the concentration of culture filtrate. Mortality of second stage juveniles by these bioagents might be due to release of lytic enzymes like chitinases, lipases and acetic acid in the filtrates that cause breakdown of nematode cuticle proteins (Annapurna *et al*., 2018) [3].

Four different concentrations *viz*., 25, 50, 75 and 100 per cent of isolated bacterial and fungal bioagents inhibited hatching of juveniles from egg masses of root knot nematode (Table 3). Results of studies showed that the maximum suppression in egg hatching was observed in *B. amyloliquefasciens*, Ba-14.5 (86.00%) followed by *B. subtilis*, Bs-13.9 (82.33%) and *B. subtilis*, Bs-13.19 (80.33%). Similarly, the maximum suppression by fungal bioagents in egg hatching of nematodes was observed in *T. asperellum*, Tr-9 (84.66%) followed by *T. asperellum*, Tr-15 (78.66%). The culture filtrate studies revealed that all the tested biocontrol agents were effective in suppressing the egg hatching of *M. incognita*.

Deformation of juveniles was observed in most of the eggs in the present study. These observations suggested that the inhibitory effect of the isolated bioagents on hatching of the nematode larvae might be due to the nematotoxic metabolites like chitinase and other lytic enzymes like proteases and lipases that cause break down of egg shell and facilitate egg penetration for successful establishment (Li *et al*., 2005 [17]; Kalele *et al*., 2010) [13].

Our findings are in conformity with observations of Niknam and Dhawan, 2002 [20]; Khan and Gowswami, 1999 [14] who reported inhibition of egg hatching and larval mortality by fungal and bacterial antagonists**.** Hence, these bioagents can be exploited further in development of formulations and evaluation under field conditions.

Table 1: Effect of bacterial bioagents on mortality of second stage juveniles of root-knot nematode, *Meloidogyne incognita*

Time	**24h**				**48h**				**72h**			
Concentration	**25**	**50**	**75**	**100**	**25**	**50**	**75**	**100**	**25**	**50**	**75**	**100**
B. subtilis (Bs-13.9)	54.33^{c}	64.00^{c}	73.00^{d}	100.00^{d}	57.00^{d}	69.00^{d}	75.33^{d}	100.00^{d}	64.00^{d}	74.00^{d}	80.00^{c}	100.00^{d}
B. subtilis (Bs-13.19)	40.33^{b}	47.00^{b}	60.00^{c}	74.66^{c}	54.00^{c}	65.33^{c}	69.00^{c}	87.33^{c}	60.00^{c}	71.00^{c}	80.66^{c}	92.00^{c}
B. amyloliquefaciens (Ba-14.5)	62.66^{c}	83.00^{d}	95.33^{e}	100.00^{d}	70.33^{e}	84.00^{e}	100.00^{e}	100.00^{d}	75.00^{e}	90.00^{e}	100.00^{d}	100.00^{d}
Nutrient Broth	8.66^{a}	8.66^{a}	8.66^{b}	8.66^{b}	11.00^{b}	11.00^{b}	11.00^{b}	11.00^{b}	12.00^{b}	12.00^{b}	12.00^{b}	12.00^{b}
Distilled water	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}

Table 2: Effect of fungal bioagents on mortality of second stage juveniles of root-knot nematode, *Meloidogyne incognita*

Time	**24h**				**48h**				**72h**			
Concentration	25	50	75	100	25	50	75	100	25	50	75	100
T. asperellum (Tr-9)	12.00^{c}	31.66^{d}	47.00^{c}	71.00^{d}	12.33^{b}	53.66^{d}	60.33^{d}	75.33^{c}	31.00^{c}	68.66^{c}	76.00^{c}	88.66^{a}
T. asperellum (Tr-15)	9.66^{b}	28.00^{c}	47.00^{c}	61.00^{c}	11.00^{b}	51.00^{c}	57.00^{c}	73.00^{c}	28.00^{c}	65.00^{c}	73.00^{c}	85.00^{c}
Nutrient Agar	10.33^{bc}	10.33^{b}	10.33^{b}	10.33^{b}	10.66^{b}	10.66^{b}	10.66^{b}	10.66^{b}	13.66^{b}	13.66^{b}	13.66^{b}	13.66^{b}
Distilled water	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}

*Data are means of four replicates. Means, in each column, followed by the same letter are not significantly different ($P \leqslant 0.05$)

Table 3: Effect of fungal and bacterial bio agents on hatching inhibition of second stage juveniles of root-knot nematode, *Meloidogyne incognita*

Time (After 5 days)	**Hatching inhibition (%)**			
Fungal and bacterial bioagents	25	50	75	100
T. asperellum (Tr-9)	58.33^{d}	71.00^{c}	74.00^{c}	84.66^{cd}
T. asperellum (Tr-15)	56.00^{cd}	67.66^{bc}	71.66^{bc}	78.66^{b}
B. subtilis (Bs-13.9)	53.33^{bc}	67.33^{bc}	72.33^{c}	82.33^{bc}
B. subtilis (Bs-13.19)	51.33^{b}	65.00^{b}	3.00^{a}	80.33^{bc}
B. amyloliquefaciens (Ba-14.5)	55.66^{cd}	68.00^{bc}	72.66^{c}	86.00^{d}
Potato Broth	3.00^{a}	3.00^{a}	3.00^{a}	3.00^{a}
Nutrient Broth	3.00^{a}	3.00^{a}	3.00^{a}	3.00^{a}

*Data are means of four replicates. Means, in each column, followed by the same letter are not significantly different (P ⩽ 0.05)

4. Conclusion

From the results it can be concluded that all the tested fungal and bacterial bioagents were able to control root knot nematode by suppression of egg hatching and mortality of second stage juveniles under invitro conditions. Further studies are required to be conducted to validate its effectiveness under pot and field conditions and also further investigations are needed for the isolation and characterization of nematicidal compounds produced by these bioagents.

Acknowledgements

The authors thank to Central Tuber Crops Research Institute for supporting and conducting the research work.

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