

Entomopathogenic Nematodes Survey, Persistence in Soil, Reproductive Potential and their Effects on *Meloidogyne incognita*

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ABSTRACT

The increasing cost of nematicides and environmental deterioration are the great challenges to agriculture. Entomopathogenic nematodes (EPNs) could be a choice as biocontrol agent in suppressing the root knot nematode, *Meloidogyne incognita* on tomato and other crops. A total number of 75 soil samples were collected from different locations of Vehari and Faisalabad, Pakistan. In Vehari out of 14 sites, 13 sites were found positive for EPNs, Nematodes were recovered from 19 (54 %) of 35 soil samples obtained from ecologically diverse habitats. From University of Agriculture Faisalabad, EPNs were recovered from 6 (40 %) of 15 soil samples. While, out of 25 samples collected from Ayub Agriculture Research Institute 9(36%) were found positive for EPNs. Both *Steinernema* and *Heterorhabditis* species were found in these locations. The soil samples were also analyzed to indicate the effect of soil texture. Maximum EPNs (36 %) were recovered from sandy loam soil followed by loamy soil. A series of experiments was carried out to unearth EPNs' soil persistence, reproductive potential and their effects on root knot nematodes. All species and strains of EPNs had potential to persist in almost all type of soils. Persistence was observed in sterilized soil sand and clay (1:1). Maximum persistence was found in *S. glaseri* (29.11 %) followed by *S. pakistanese* (28.41 %), *H. indica* (26.93 %), *S. asiaticum* (24.42 %), *S. feltiae* (21.52 %) and *H. bacteriophora* (21.11 %) which showed the least persistence in sterilized soil. All species of both the families *Heterorhabditidae* and *Steinernematidae* did not persist more than 30 % in sterilized soil. Reproductive potential of three EPNs was recorded using the greater wax moth *Galleria mellonella* larvae. *H. indica* showed very high reproductive potential within wax moth followed by *S. asiaticum* and *S. pakistanese*. On the other hand, maximum emerged infective juveniles per day were recovered from *H. indica* followed by *S. pakistanese* and *S. asiaticum*. Suppressive effect of EPNs against root knot nematodes was observed on susceptible tomato cultivars at different concentration. *S. pakistanese* suppress *M. incognita* population more efficiently than *H. indica* at all the levels i.e. 1250, 2500 and 5000 nematodes pot⁻¹. After 35 days egg masses (EM) recovered from *S. pakistanese* treated pots at level 1250 were 232/pot, at level 2500 were 207 and at level 5000 were 135 respectively. Similarly, egg masses recovered from *H. indica* treated plants at level 1250 were 276, at level 2500 were 212 and at level 5000 were 154 EM pot⁻¹ respectively. No EM were recovered from clean plants on contrary 328 EM were recovered from plants with root knot nematodes only.

Keywords: Egg Mass, Root knot Nematode, Entomopathogenic Nematode, Wax Moth

INTRODUCTION

Biological control gained momentum as an important part of integrated pest management system for reducing harmful pesticide use. The entomopathogenic nematodes (EPNs) could be a choice from various biological control agents because they are native to many regions throughout the world with broad host range, have no adverse effects on animals and plants. The application is easy to handle with conventional pesticides equipment. Different species of the genera *Heterorhabditis* and *Steinernema* are effective biological control agents (e.g. Stock, 2005; Abd-Elgawad et al., 2017). They are obligate natural parasites of insects and remain in the soil with no feeding until the infective juveniles (IJS) attack their targets to complete the rest of EPNs life cycle in the insect hosts.

Nematicides limited availability and high cost of nematicidal development have created a need to find alternative methods for plant-parasitic nematode management. Development of alternatives to harmful chemical nematicides is priority for the future nematology (Barker et al., 1994). Researchers reported antagonism between EPNs and plant parasitic nematodes (e.g. Ishibashi and Kondo, 1986; Grewal et al., 1997 and 1999; Jagdale et al., 2009; Abd-Elgawad, 2017).

There is no information on pre and post-infestation applications of entomopathogenic nematodes to suppress *M. incognita* on tomato in Pakistan. The objective of this research was to isolate EPNs and to test EPNs persistence in soil, their reproductive potential as well as to evaluate effect of EPNs on *M. incognita* suppression on tomato using two species of entomopathogenic nematodes.

MATERIALS AND METHODS

Rearing of the greater wax moth (*Galleria mellonella* L.)

Galleria mellonella L. were kept in rearing plastic jars (19x12x12 cm³) or in plastic boxes 1500 ml volume (11 cm diameter and 15 cm height). These Jars were covered with lids containing small holes to avoid suffocation or with muslin cloth. Last instars larvae of *G. mellonella* were separated for culturing six EPN species nematode culture imported from England. Fresh laid eggs were transferred to modified artificial diet which was prepared by mixing oat, wheat, rice and maize porridge (20 g), yeast granules (50 g) in solution of 80 ml warm honey and glycerol (100 g). The eggs hatched within 3-4 days. After 5-6 weeks, larvae reached the last instars and were collected to be used in the experiments. Some larvae were left in the jar to pupate. Two weeks later, the adult females emerged and laid eggs. *Gallerias* reared on this diet were kept at 27 °C in an incubator.

Multiplication of EPNs in the wax moth

Wax moth larvae were used as bait for multiplication of entomopathogenic nematodes. Late-instars larvae of the greater wax moth, *G.mellonella* L. were placed in plastic Petri-dish (9 cm diameter), lined with two pieces of Whatman filter paper. Two ml water containing 300 juveniles were added in each Petri-dish. Petri dishes then sealed with Para-film and placed in incubator at 27 °C in dark for 2 to 3 days until *Galleria* found dead.

Isolation of IJs from dead *Galleria* by White trap method

A modified white trap consisting of a plastic container (9×9×6cm³) filled with distilled water to a depth of 1cm (White, 1927); the bottom of an inverted Petri-dish (5-3.5 cm²

depending on plastic container) was placed in container. A sheet of filter paper (7 cm) was placed on the Petri dish allowing the edge of filter paper to come in contact with water. Afterward, dead larvae were placed on filter paper on the top of the Petri dish and plastic container covered the lid. White traps were further incubated at respective infection temperature until the nematode progeny emerged. Depending on nematode species, IJs started to leave the cadaver 8-20 days after infection. Infective juveniles that moved down through the filter paper into the water were then harvested. Nematode harvested from White trap were collected in beakers, counted and stored in refrigerator at 10 °C in small plastic containers (Woodring and Kaya, 1988).

Preparation of the root-knot nematode

Single egg mass of *Meloidogyne incognita* per each plant was cultured on tomato 'Money Maker' in the greenhouse, then nematode identification was confirmed via perennial patterns of adult females and additional plants were re-infected by *M. incognita* (Taylor and Sasser, 1978). The plants were transferred to big pots (1.5 kg) and maintained for further three months. Eggs of *M. incognita* to be used as inoculum were extracted using a sodium hypochlorite method (Hussey and Barker, 1973).

Soil samples collection and processing

Soil samples were collected to isolate EPNs from 13 different locations in Vehari strict Punjab. Samples were also collected from vegetables cultivated area of University of Agriculture Faisalabad and Ayub Agriculture Research Institute. Total 75 samples were collected from diverse habitats of agricultural areas viz. various vegetables fields, e.g. cotton, sugarcane, maize, cauliflower and okra vegetables preferred where insect attack was more. Soil type was also noted during the study. From each location about 500 g soil sample was collected with a hand shovel from top 10 cm soil profile within the area of about 1x1 m². Samples were brought to laboratory and then stored in refrigerator at 10 °C. For isolation of nematodes from soil samples "Galleria trap" method was used (Mracek, 1980). Soil samples were placed in (9×6 cm²) plastic pots, 100 g in each container. In each pot, five Wax moth *G.mellonella* larvae were placed in close contact with soil through the pressure of the lid. The pots were incubated for 5 to 7 days at 27 °C. Samples were checked daily and the dead larvae collected from the samples; these larvae were cleaned by tissue paper and rinsed properly with tap water after that these dead larvae were transferred to White traps and identified to the generic level using the color of dead larvae (Woodring and Kaya, 1988).

Experiment1: Persistence of EPNs in soil

The quantity 600 g of soil sample consisting of sand and clay (1:1) was mixed thoroughly then autoclaved at 15 psi for 30 min. After that 100 g of soil samples placed in each plastic pot and the soil in pots was moistened. Total six species of EPNs i.e. *Steinernema feltiae*, *S. glaseri*, *S. pakistanese*, *S. asiaticum*, *Heterorhabditis bacteriophora* and *H.indica* were used with three replications each. Three thousand nematodes of each species were added in each pot. After 2 days these soil samples with EPNs were placed on modified white head trays filled with water. Collections of nematode suspensions were made at various time intervals. Completely randomized design was applied for statistical analysis of the data (Gomez and Gomez, 1984).

Experiment 2: Reproductive potential of EPNs in wax moth larvae

Three late-instars larvae of *G.mellonella* were placed in plastic Petri dish (9 cm diameter), lined with two pieces of Whatman filter paper. Two ml water containing

300 IJs were added in each Petri dish. Petri dishes were sealed with para-film and placed in an incubator at 27 °C in dark for 2 to 3 days until galleria were dead. Three species of EPN were used with three replications each. After the death of *Galleria*, they were placed on White trap and waited for recovery of EPN juveniles. Collection of IJs in beakers was made every day or otherwise until there was no or very low recovery. Data were analyzed using Duncan's Multiple Range Test.

Experiment 3: Effect of entomopathogenic nematodes on root knot nematode, *Meloidogyne incognita*

The culture of *S. pakistanese* and *H. indica* was maintained on the wax moth larvae. One month old 'Money Maker' tomato plants were transplanted into soil within 200 ml plastic pots. Both EPN species were applied at the rate of 1250, 2500 and 5000 individual pot⁻¹ at the same time with *M. incognita*. Root knot nematodes were applied at the rate of 1500 second stage juvenile pot⁻¹. Plants without any treatment or receiving only root knot nematodes were kept as control. These plants were randomised on a glass house bench at 27-35 °C and each treatment was replicated five times. Plants were uprooted after 35 days of exposure to root knot nematodes. After washing and taking root-shoot weight, roots were stained in Phloxin B then total number of egg masses was counted. The parameter for the assessment of efficacy of EPN against root knot nematodes were based on number of egg masses/roots, and roots and shoot weight. Data were analyzed using Duncan's Multiple Range Test (Duncan, 1955)

RESULTS AND DISCUSSION

Collection of EPNs

Thirteen locations were found positive for EPNs; no EPN was recovered from one site only. Nematodes were recovered from 19 (54 %) of 35 soil samples obtained from ecologically diverse habitats. These nematodes identified as species of *Steinernema* and *Heterorhabditis*. From University of Agriculture Faisalabad, EPNs were recovered from 6 (40 %) of 15 soil samples. Nematodes isolated from University of Agriculture Faisalabad mostly belong to family *Steinernematidae*. While, out of 25 samples collected from Ayub Agriculture Research Institute 9 (36%) were found positive for EPNs. Both *Steinernema* and *Heterorhabditis* species were found in these locations.

These soil samples were also evaluated for the effect of soil texture on the basis of EPNs distribution. Prevalence of nematodes varied in four soil textures, sandy loam, loam, clay loam and sandy clay loam (Fig.1). Maximum number of EPNs isolates was recovered from sandy loam soils viz. 36 %, followed by 20 % in loamy soils. Whereas no EPNs were recovered from clay loam and sandy clay loam soils. Maximum number of EPNs was recovered from maize followed by sugarcane, okra, watermelon and chilies fields. EPNs were also recovered from the soil taken from the root base of pumpkin, brinjal, cabbage, rice and cotton.

Experiment.1 Persistence of EPNs in soil

Persistence of *S. glaseri*, *S. pakistanese*, *S. asiaticum*, *S. feltiae*, *H. bacteriophora*, *H. indica* declined with the passage of days (Table 1). At the end of experiment maximum persistence was found in *S. glaseri* (29.11 %) followed by *S. pakistanese* (28.41 %), *H. indica* (26.93 %), *S. asiaticum* (24.42 %), *S. feltiae* (21.52 %) and *H.*

bacteriophora which showed least persistence (21.11 %) in sterilized soil. All tested species did not persist more than 8% in sterilized soil. Both *Steinernema* and *Heterorhabditis* persisted in soil but *Steinernema* species persisted more in soil than *Heterorhabditis*.

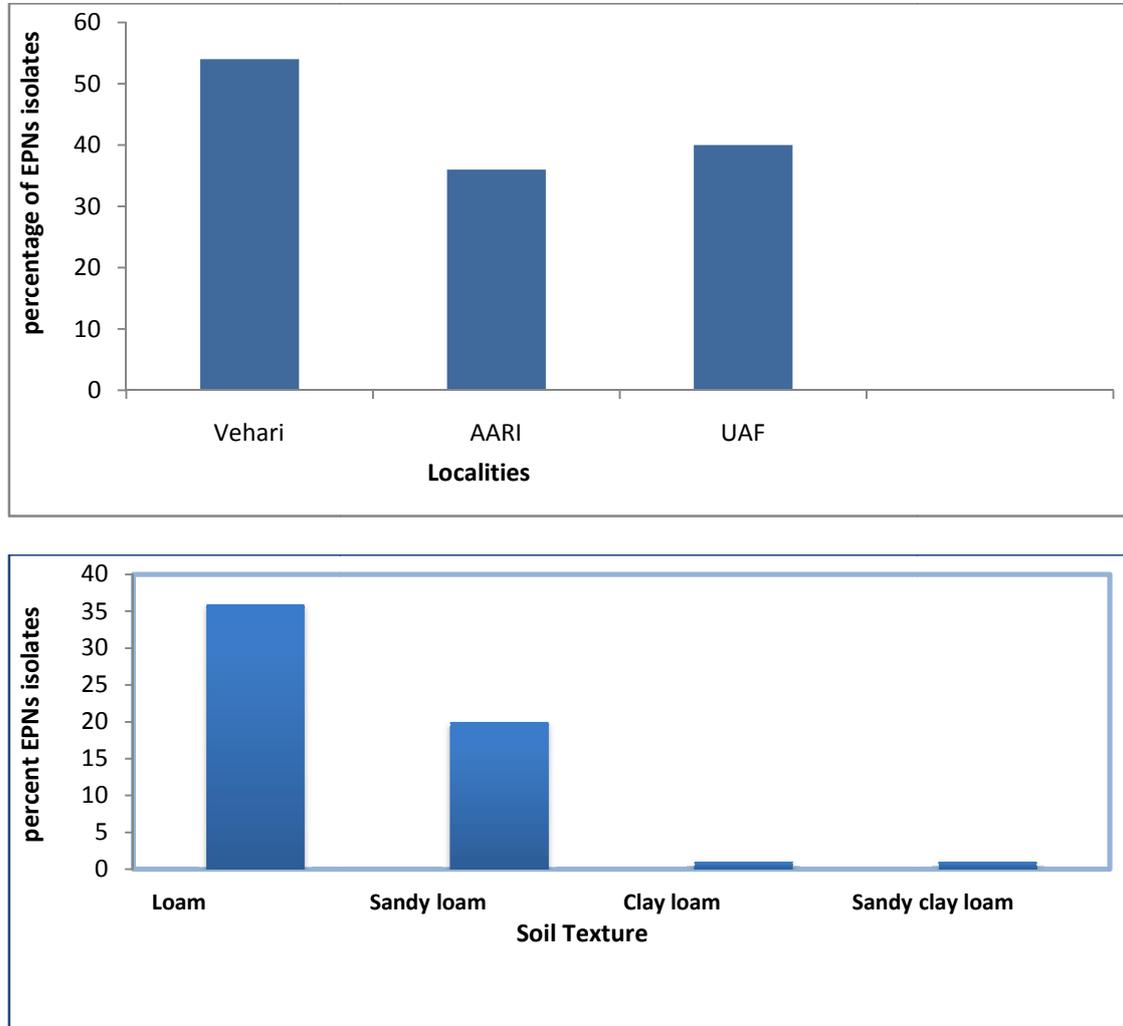


Fig.1.Percentage of recovered entomopathogenic nematode (EPN) isolates relative to localities (upper bar chart) and soil type (lower bar chart). UAF = University of Agriculture Faisalabad; AARI = Ayub Agriculture Research Institute.

On the 6th day of experiment, mean values for different EPNs species ranged from 7.87 to 0.62 %. Maximum persistence mean value (7.87 %) was observed in *S. glaseri* followed by *S.pakistanese* (6.75 %), *S. asiaticum* (5.16 %), *S. feltiae* (3.08 %), *H. bacteriophora* (2.28 %) and minimum mean value was recorded in *H. indica* i.e. 0.62 %.

Experiment 2: Reproductive potential of EPN in the wax moth larvae

Data collected from three different EPN species indicated that emerged IJs among these EPN species were statistically different (Figs.2-4). *H.indica* showed very high reproductive potential within the insect larvae followed by *S. asiaticum* and *S.*

pakistanese. Maximum nematodes per day were recovered from *H. indica*. followed by *S. pakistanese* and *S. asiaticum*. Nematodes reproduce themselves within the cadaver by utilizing their symbiotic bacteria which are used as a food source for nematodes. Presumably, EPNs complete two life cycles within the host.

Table 1. Persistence of six entomopathogenic nematode (EPN) species in soil.

EPN Species	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Means
<i>S. glaseri</i>	14.08 a	10.15 d	8.513 ef	7.957fg	7.930 fg	7.870 e	9.4167
<i>S. pakistanese</i>	10.99 c	12.86 b	7.307 gh	6.940 h	6.717 hi	6.753 g	8.5945
<i>S. asiaticum</i>	9.777 d	8.613 e	5.707 j	5.557 j	4.250 k	5.163 j	6.5112
<i>S. feltiae</i>	6.913 h	6.203 ij	3.870 k	4.143 k	2.477 lm	3.080 l	4.4477
<i>H. bacteriophora</i>	4.003 k	4.253 k	3.097 l	3.077 l	2.517 o	2.283 lm	3.2050
<i>H. indica</i>	2.52 lm	2.390 m	2.157mn	2.273 m	1.620 p	0.6233 no	1.9306
Means	8.04717	7.41150	5.10850	4.99117	4.25183	4.29538	

Each value is a mean of three replicates. Means in a column followed by the same letter are not significantly ($P \leq 0.05$) different according to Duncan's multiple-range test.

Graphical representation of *S. asiaticum* showed the reproductive potential of this species within the insect larvae (Fig.2). Two peaks possibly showed the completion of life cycle within the cadaver. If so, *S. asiaticum* completed two life cycles within the host then came out of the cadaver in millions. *S. pakistanese* showed the completion of only one life cycle within the host, one peak showed the completion of life cycle and then nematodes came out of the cadaver in millions (Fig.3). Graphical presentation showed that *S. pakistanese* reproduced within the cadaver for 12 days while *S. asiaticum* and *H. indica* completed two life cycles within host in more than 20 days. Graphical presentation of *H. indica* showed the completion of two life cycles within host. Nematodes were recovered from the cadaver more than 20 days (Fig.4). After completion of 1st life cycle nematode recovery was more as compared to 2nd life cycle. Maximum nematodes were recovered from the cadaver infected with *H. indica*. Reproductive potential means for all the three species ranged from 96540.2 to 2790 IJs. All these values of reproductive potential means were statistically different from each other.

Experiment 3: Effect of entomopathogenic nematodes on root knot nematode *Meloidogyne incognita*.

Significant suppressive effect *H. indica* and *S. pakistanese* on *M. incognita* was indicated at different EPNs levels on a susceptible tomato cultivar (Table 2). Results showed that *S. pakistanese* suppressed the root knot population more efficiently than *H. indica* at all the tested levels i.e. 1250, 2500 and 5000 nematodes pot⁻¹. After 35days egg masses recovered from *S. pakistanese* at 1250IJs were 232/pot, at level 2500 were 207 and at 5000 IJs were 135.

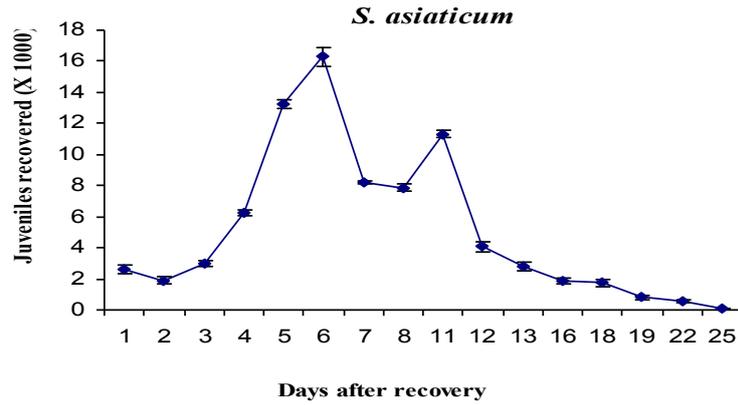


Fig. 2: Reproductive potential of *Steinernema asiaticum* in *Galleria mellonella* larvae.

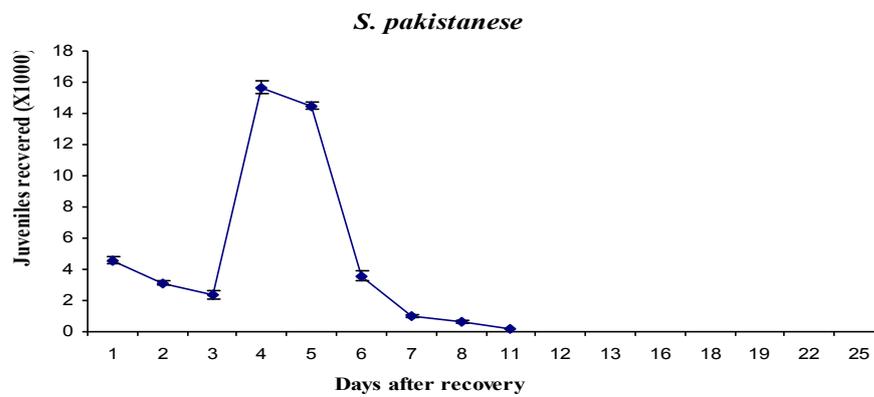


Fig. 3: Reproductive potential of *Steinernema pakistanese* in *Galleria mellonella* larvae.

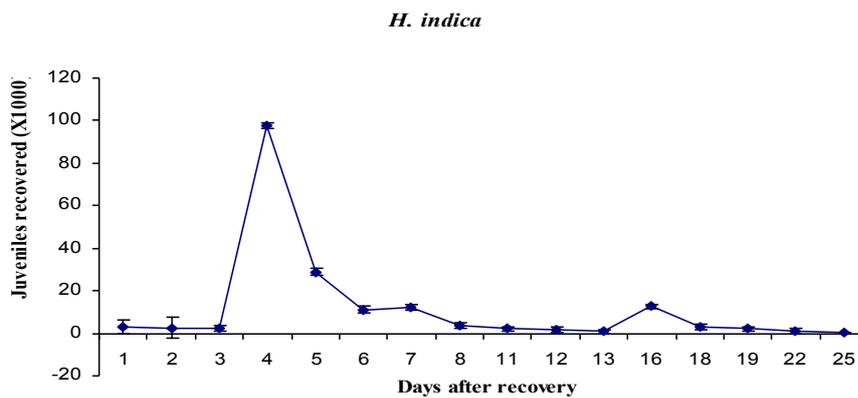


Fig. 4: Reproductive potential of *Heterorhabditis indica* in *Galleria mellonella* larvae.

These results showed that high concentration of EPNs suppressed *M. incognita* population efficiently than low concentration. Similarly egg masses recovered from *H. indica* treated plants at level 1250 were 276, at level 2500 were 212 and at level 5000 were 154 EM/pot. On non-infested (clean) plants, no egg masses were recovered and 328 egg masses were recovered from plants with root knot nematode only.

Table 2. Effect of different densities of entomopathogenic nematode-infective juveniles (IJs) on tomato plant weight and number of root knot nematode, *Meloidogyne incognita*, egg masses (EM) per root system.

Densities of entomopathogenic nematodes									
Treatments	1250IJs			2500IJs			5000IJs		
	Root weight	Shoot weight	EM/ root	Root weight	Shoot weight	EM/ Root	Root weight	Shoot weight	EM/ root
<i>S. pakistanese</i>	1.97a	4.9a	232a	2.7a	5.1a	217a	2.1a	5.3a	135a
<i>H. indica</i>	2.2a	5.4a	276a	2.3a	5.2a	202a	2.3a	5.6a	154a
Control (RKN)	2.4a	4.7a	328b	2.4a	4.7a	328b	2.4a	5.7a	328b
Clean plants	2.3a	5.1a	--	2.3a	5.1a	--	2.3a	5.1a	--

Each value is a mean of five replicates. Means in a column followed by the same letter are not significantly ($P \leq 0.05$) different according to Duncan's multiple-range test.

Our identification of the EPN genera relied on the observation of the colour of the cuticle of dead *Galleria* larva infected by the EPN. That is if the colour of the cuticle of the dead *Galleria* larva is yellowish that indicates the presence of EPN of *Steinernema* species. Whereas the colour of the cuticle of the EPN infected dead *Galleria* larva is brick red means the presence of *Heterorhabditis* species (Woodring and Kaya, 1988). These EPNs may flow freely in sandy loam soil and have easy way to access insect targets. They may also get sufficient amount of oxygen from soil water in loamy soils. Kung et al. (1990) also reported that nematode persistence declined as soil clay contents increased. Choo et al. (1995) mentioned that EPNs preferred loose soils having humus and rich in organic matter. Recovery of most EPNs from sandy loam and loamy soils in the present study was therefore expected.

Our results also confirmed the findings of Barbercheck et al. (1995) who observed that EPNs offspring highest yield was in corn soil due to the presence of plant secondary metabolites. These results also agreed with the results of Ahmad and Hussain (2002) that conducted a survey of entomopathogenic nematodes in Potowar region of Pakistan and reported the presence of entomopathogenic nematodes in various locations of that area. Both *Steinernema* and *Heterorhabditis* species were recovered from the same crops.

Environmental factors that affect nematodes persistence in soil are soil composition, moisture, temperature, and presence of antagonists and competitors. Nematode persistence was prompted in sterilized soil (Kaya, 1990). Generally, *Steinernema* spp. appears to persist longer than *Heterorhabditis* spp. under laboratory and field conditions (Baur and Kaya, 2001). In our study persistence, irrespective of soil type was also generally much lower in the *Heterorhabditis* spp. than in the *Steinernema* spp. Recovery relative to both *Heterorhabditis* spp. rapidly declined during the whole days of experiment. The objective of the present study was to develop a method that could estimate nematode occurrence and persistence in soil in a time efficient way. The effect of soil type on infectivity and persistence varied among the six entomopathogenic nematode species and also followed different trends for

infectivity and persistence. Persistence of *S. glaseri* was highest as compared to all other EPN species; on the other hand *H. bacteriophora* gave very good results. The tested EPN species persisted well in all type of soils but persistence rate was high in loam, sandy loam and clay loam soils. Previous studies showed lesser infectivity of *S. glaseri* (Kung et al., 1990) and *H. bacteriophora* in fine textured soils (Choo and Kaya, 1991). This reduction may be correlated to the decreasing pore space between the smaller soil particles that increasingly hinder nematode movement (Wallace, 1958).

Differences in behavior may be more important factors determining the differences in persistence among nematodes species. Molyneux (1985) observed that in the absence of hosts in sandy soil *S. glaseri* but not *H. bacteriophora* often were not moving, continuous positions. This difference in behavior would add to the difference in persistence between *H. bacteriophora* and *S. glaseri*.

Kung et al. (1990) compared nematode persistence in sand, sandy loam, clay loam, and clay and found that *S. glaseri* and *S. carpocapsae* persistence declined from coarser to finer soils. This observation agreed to assume that EPN interaction for survival is influenced by soil type, humidity, and aeration with nematode survival being shorter in finer-textured soils (higher clay content). Smaller pores and higher water-holding capacity in finer soils result in poorer aeration. In less aerated conditions, aerobically respiring (Burman and Pye, 1980) of EPNs inefficiently utilize food material reserves (Croll and Matthews, 1977) and survive for shorter periods. But in our study all species of *Heterorhabditis* and *Steinernema* persisted well in mixed soil except *H. bacteriophora*.

In conclusion, our data and those of other studies showed that soil parameters can have different effects on different nematode species, amongst other things depending on behavioral and physiological adaptations of those nematode species. It also became clear that we have to be careful with generalizations across soil types based on a limited number of studies. Soils occur in an almost unimaginable variety. Many more factors can affect nematode performance than just soil particle size composition, pH, and organic matter. For example, different soil nutrients could have positive or negative impacts on nematode performance depending on nematode species, nutrient concentration and exposure length (Jaworska et al., 1999).

The rate of 1250, 2500 and 5000 IJspot⁻¹ of *H. indica* and *S. pakistanese*, suppressed *M. incognita* on tomato plants in growth greenhouse experiment. These findings for *Steinernema* species agreed with those of Lewis et al. (2001) and showed that the same rate of *H. indica* and *S. pakistanese* also suppressed *M. incognita* on tomato in both growth chamber and greenhouse trials. Both the symbiotic bacteria like *Xenorhabdus* spp. for *Steinernema* and *Photorhabdus* spp. For *Heterorhabditis*, may be a factor for *M. incognita* suppression, because these symbiotic bacteria produce metabolites that are toxic to nematodes (Hu et al., 1999). Grewal et al. (1999) reported that repellency, and toxicity reduced eggs hatch of *M. incognita* exposed to *Xenorhabdus* spp. cell free extract. They suggested allelo-chemicals produced by *Xenorhabdus* spp. as the cause of antagonism against the root knot nematodes.

CONCLUSION

Considering the high cost of chemical nematicides, EPN might be a viable control measure. for some species of plant parasitic nematodes in some crops like tomatoes

and peanut. Many operators such as the timing of application may be key factors in optimizing their efficacy. Further experiments that account for these factors are needed. Consequently, it is suggested that entomopathogenic nematodes are very efficient bio-control agents in suppressing *M. incognita* on tomatoes.

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