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Full Length Article

Virulence of Entomopathogenic Nematodes against *Meloidogyne incognita* for Invasion, Development and Reproduction at Different Application Times in Brinjal Roots

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Abstract

Keeping in view the staid health and ecological apprehensions coupled with the use of pesticides, entomopathogenic nematodes have the potential to supersede pesticides for the management of various pests. Brinjal is seriously affected by *Meloidogyne incognita.* Research was conducted on two important groups of nematodes, plant parasitic nematodes (RKNs) and insect parasitic nematodes (entomopathogenic nematodes). *Steinernema asiaticum, S. glaseri, Heterohabditis indica* and *H. bacteriophora* were evaluated at different time of application against *M. incognita* for invasion, development and reproduction of *M. incognita*. In a life cycle study, *S. asiaticum* and *H. bacteriophora* were proved more effective in influencing the life cycle of RKNs at all application times than *S. glaseri* and *H. indica.* However all application time of entomopathogenic nematodes (before, with and after) the application of *M. incognita* proved effective as compared to control. *S. asiaticum* and *H. bacteriophora* delayed penetration of nematode juveniles (J2) into roots of brinjal. © 2015 Friends Science Publishers

Keywords: Biological control; Entomopathogenic nematodes; *M. incognita*

Introduction

The economy of Pakistan is based on agricultural. It is the instant major part of economy, contributes more than 21% of GDP and absorbs 45% of employers in the country (Anonymous, 2011).

Eggplant (*Solanum melongena* L.) is an important low-price summer vegetable. Total production of eggplant in Pakistan is 94.3(000) tons. In Punjab (Pakistan), it was cultivated on an area of 4.7(000) ha with an annual production of 59.2 (000) tons during 2009-2010, with the average yield of 12.6 tons/ ha, which is very low as compared to other Asian countries (Anonymous, 2011). Root-knot nematode [*Meloidogyne incognita* (Kofoid and White) Chitwood] is widespread and destructive pathogen of brinjal (Fourie and McDonald, 2000). RKNs attack more than 3000 species of plants, including almost all cultivated plants and reduce world crop production by about 5% losses in individual fields, however may be much higher (Agrios, 2005). Root-knot nematodes *Meloidogyne* (Goeldi spp.) are recognized as important pests of vegetable crops with a host range of about 100 from different cultivated zones of Pakistan (Zaki *et al*., 2000). Among the root-knot nematode, *M. incognita* and *M. javanica* are commonly found in the Punjab (Anwar *et al*., 1991).

Among different nematode management strategies, chemical control has proved generally effective (Barker and Koenning, 1998). But being highly expensive, toxic to plants, livestock, soil micro-flora and fauna (Jairajpuri *et al*., 1990) and development of resistance in pathogen against these chemicals, governments are demanding environmentally safe chemicals with low toxicity and less mobility to avoid ground water contamination and limited effects on non-target organisms. Therefore, the development and implementation of alternative control strategies are needed.

Entomopathogenic nematodes (EPN) belong to families; Steinernematidae and Heterorhabditidae and kill insects (Grewal *et al.,* 1999, 2005). EPNs is also being used as biological control agents against RKN infesting different crops in the field and green house (Shapiro-Ilan *et al*., 2006; Molina *et al*., 2007; Javed *et al*, 2012).

During 1996-2004, studies on distribution, isolation, biology, taxonomy and efficacy of EPNs have been carried out in Pakistan. The Pakistani species i.e. *S. pakistanense* have been classified under bicornatum group, while *S. asiaticum* under carpocapsae group (Nguyen, 2004). The advantages of the use of EPNs are many including low cost, high efficacy, safety for humans and other non-target organisms, preservation of other natural enemies, reduction

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of pesticide residues in food and increased biodiversity in managed ecosystems.

Although there is a very little work has been done on entomopathogenic nematodes in Pakistan. The species *Steinernema asiaticum,* used in these experiments is a new Pakistani species not used against root knot nematode (*M. incognita*) before. Keeping in view the importance of the biological control, it was planned to investigate the biological control of root-knot nematodes through EPNs.

Materials and Methods

Screening of Entomopathogenic Nematodes for the Management of *M. incognita* in Brinjal

The greater wax moth larva *Galleria mellonella* L. (Lepidoptera: Galleridae) was used for nematode baiting and produce progeny of nematode isolates. *G. mellonela* were separated from infected bee hives for nematode culture. The insect culture was reared in 1,500 mL volume glass containers (11 cm diameter and 15 cm height) on an artificial medium (Wiesner, 1993). Diet with galleria was kept at 27ºC in an incubator. After reaching last instars, they were taken out from the diet and used for storage and nematode multiplication. Culture of *S. glaseri*, *H. indica*, *H. bacteriophora* collected from Reading University UK, maintained by Ernesto, and *S. asiaticum* (Local) was maintained on *G. mellonella*. These were reconfirmed on their morphological basis in Plant Nematology laboratory at University of Agriculture, Faisalabad. Larvae were kept at 15°C. EPN were collected from dead *G. mellonella* larvae by modified White trap (White, 1927) and then stored at about 10-15ºC.

Nematodes were always checked for viability before starting experiments. Nematodes were provided oxygen by aerator used for fish aquarium tanks. The number of *M. incognita* used as inoculums in greenhouse experiment always refers to infective *M. incognita.* Only freshly hatched (24-48 h old) were used. For EPNs, only those freshly produced *in vivo* (less than 2 weeks old) were used.

Brinjal was grown into earthen pots containing 240 mL formalin sterilized sandy loam soil (72% sand, 17% silt and 8% clay) (Safdar *et al*., 2012). After four weeks when the plants established their root system, inoculation of plants except control was in the rhizosphere of each plant by making 3-4 holes (Campos and Campos, 2005) and then filled with soil to prevent drying. EPNs were used at the concentration of 2,000 and *M. incognita* was 1000 per plant. Plants inoculated with only *M. incognita* were kept as control. Treatments were replicated three times. EPNs were applied at three different time intervals (at the same time with *M incognita*, 5 days before application of *M incognita* and 5 days after application of *M incognita*) simultaneously in separate 5 mL water suspensions by making separate holes of 1 cm with sharp pointed wood around the base of plants.

Plants were harvested after 35 days to observe the development and reproduction of RKNs factor. After 35 days of inoculation, seedlings of brinjal were soaked along with their pots for 3-4 h. After soaking their roots were gently shaken in a bucket filled with water (washing with a stream of water was avoided to reduce the risk of losing egg masses). After washing shoots of plants were cut off and roots were placed in between two folds of tissue paper to prevent drying. Care was taken in order to limit the loss of small roots and egg masses during the washing procedure. At harvest, to facilitate counting of egg masses, the washed roots were stained with phloxine B (Southey, 1986). Then these were treated 0.1% acid fuchsin to count total number of females (Bridge *et al*., 1982).

After staining, roots were wrapped in tissue paper to prevent drying out during the steps of the procedure of evaluation. Stained egg masses from entire root system were counted under a stereomicroscope (Olympus SZ 61) at 2.5X magnification. The whole root system was rated for galling and egg mass presence on a 0 to 5 scale (Quesenberry *et al*., 1989; Anwar *et al*., 2007), where 0 = no gall or egg masses, 1 = 1-2, 2 = 3-10, 3 = 11-30, 4 = 31-100, and 5 = >100 galls or egg masses per root system. Experiment was repeated three times.

The data of following parameters was recorded on Fresh weight of root, Number of galls/root system, Number of females, Galling index (No. of egg masses) and Nematode reproduction factor [Pf/Pi].

Where Pf = final nematode population at harvest, Pi = initial inoculums (1000). Data were subjected to ANOVA by using SAS statistical software (SAS Institute, Cary, NC, USA, 1988) and significant difference among the treatments was portioned by Least Significant Difference Test (LSD) at probability levels of P = 0.05 (Steel *et al*., 1997).

Effect of EPN on Invasion, Development and Reproduction of RKN (*M. incognita*)

Two species of EPNs, *H. bacteriophora, S. asiaticum* were selected, one from each group based on the results of experiment 1 (Table 1) and these were tested against the invasion and development of root knot nematodes. Brinjal was grown into earthen pot containing 240 mL formalin sterilized soil. After four weeks when the plants established their root system, inoculation was done in different holes with EPNs at the concentration of 2,000 and *M. incognita* 1000 (J2s) per plant in the rhizosphere of each plant by making holes (Campos and Campos, 2005) and then filled with soil to prevent drying. Three replicated pots for each treatment were arranged in completely randomized design and maintained at 25-30^{o}C in green house. Invasion/development of root knot nematodes in brinjal plant was studied by staining the roots with acid fuchsin (Bridge *et al*., 1982). Data was recorded after 7, 17 and 35 days to observe the invasion, development and reproduction of various stages of *M. incognita*. RKN alone treated plants

were as control. Experiment was repeated three times. The data was recorded on fresh root weight and No. of females per root system. Data were subjected to ANOVA by using SAS statistical software (SAS Institute, 1988) and significant difference among the treatments was portioned by Least Significant Difference Test (LSD) at probability levels of P = 0.05 (Steel *et al*., 1997).

Results

Screening of Entomopathogenic Nematodes for the Management of *M. incognita* in Brinjal

A preliminary trial was carried out to study entomopathogenic nematodes effects on life cycle of *M. incognita* and its development in a host plant brinjal. The objective of this study was to evaluate the response of *Steinernema glaseri*, *Heterohabditis indica*, *H. bacteriophora* and *S. asiaticum* by applying at different time intervals (before, at the same time and after the application of root knot nematodes) for the management *M. incognita* on brinjal (P<0.05). It was assessed by using plant root weight and nematode development parameter (number of galls, egg masses, number of females and reproduction factors).

Root weight of brinjal varied significantly among all treatments. All the EPNs species were significantly different in their effect with respect to root weight (Fig. 1a) as compared to control. When compared average means of EPNs species at all times of applications with root knot nematodes, the maximum root weight was observed in *H. indica,* followed by *S. glaseri, H. bacteriophora* and *S. asiaticum* respectively. Minimum root weight was recorded in *H. bacteriophora* (3.83 g) followed by *S. asiaticum* (3.84 g) statistically similar (p<0.05) in all time of application with root knot nematodes. Whereas maximum root weight (5.66) was observed in control where only root knot nematodes were applied.

All the treatments gave significant reduction in root weight as compared to control treatments where only root knot nematodes were applied (p<0.05). It was observed that there was direct relationship of root weight and number of galls. Maximum root weight was observed in the treatment where the number of galls was the maximum.

At all intervals, all the entomopathogenic species varied in their effect as the number of galls is concerned. All the EPNs species used, proved effective in reducing the number of galls as compared to control. *S. asiaticum* found to be the most effective at all application time with root knot nematodes followed by *H. bacteriophora*. Less number of galls was recorded in *S. asiaticum* (152) and it was significantly different from other treatments p<0.05. It was followed by *H. bacteriophora* (170), *H. indica* (179) and *S. glaseri* (192) respectively (Fig. 1b). All the application times of entomopathogenic nematodes proved effective (statistically similar) as for as the no. of galls are concern as compared to control.

Table 1: Effect of different species of entomopathogenic nematodes on invasion and development of RKN in Brinjal (after 7 Days)

Treatments	Root Weight (g)	J2s[1]	dJ2s[2]
S. asiaticum +RKN	1.38[NS]	81.3b	31.3b
S. asiaticum	1.37	0.0a	0.0a
H. bacteriophora +RKN	1.37	123.7c	71.7c
H. bacteriophora	1.38	0.0a	0.0a
RKN alone	1.38	159.7d	166.7d

[1]J2s= 2nd stage Juveniles, [2]dJ2s= developing 2nd stage Juveniles, [3]dJ4s= developing 4th stage Juveniles
Numbers followed by different letters are significantly different from each other at p<0.05
Data is mean of three replications

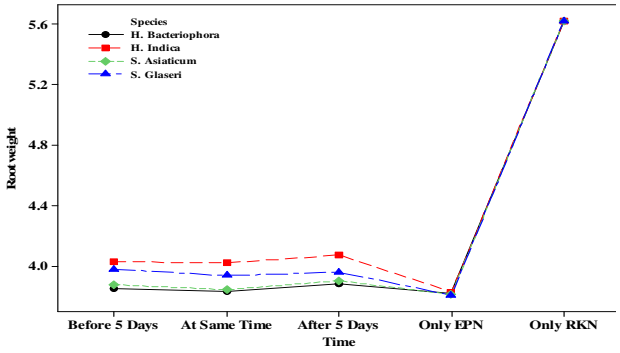

Fig. 1a: Effect of EPN species at different times on root weight of RKN
LDS at P=0.05, Species = 0.0333, time = 0.0372, specie*species = 0.0744

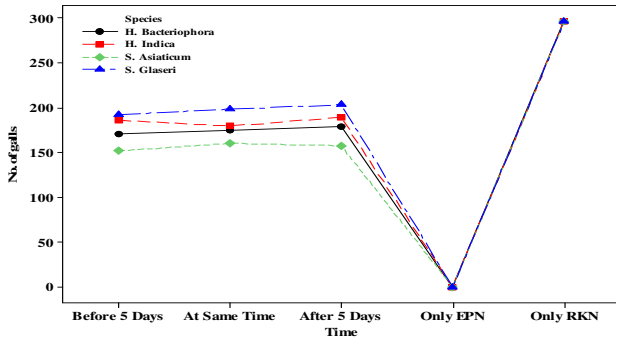

Fig. 1b: Effect of EPN species at different times on number of galls of RKN
LDS at P=0.05, Species = 2.1025, time = 2.3507, specie*species = 4.7014

Fig. 1c shows that the maximum number of females was recorded in control. All the entomopathogenic nematode species varied in their effects on the number of females. Time of application did not affect the number of females (p<0.05). Maximum number of females was observed in *S. glaseri* (228), followed by *H. indica* (221), *H. bacteriophora* (197) and *S. asiaticum* (180) respectively and were significantly (P<0.05) lower as compared to control treatment (322) as given in Fig. 1c (p<0.05).

As for as the number of egg masses are concerned, the

best treatment was *S. asiaticum* (92) followed by *H. bacteriophora* (116), *S. glaseri* (127) and *H. indica* (153) and these were significantly different from each other at $P<0.05$. While the number of egg masses were maximum (323) in the control where only root knot nematodes were applied (Fig. 1d). Time of application did not significantly affect the number of egg masses ($p<0.05$).

Similar trend was observed with reproduction factors. Fig. 1e shows that *S. asiaticum* was the best treatment in reducing the reproduction factors of root knot nematodes with values of 3.026 at all-time intervals $P<0.05$. Highest reproductive potential of root knot nematodes with values of 19.407 were observed with root knot nematodes (control). All the treatments demonstrated upright results as compared to control. Final population of root knot nematodes and rate of reproduction were directly proportional to each other.

Effect of EPNs on Invasion, Development and Reproduction of RKN (*M. incognita*)

Two species, *H. bacteriophora and S. asiaticum* (one from each group) were selected from experiment number 1, on the basis of their effectiveness against *M. incognita* and reevaluated for their efficacy against invasion, development and reproduction of *M. incognita* at 7, 17 and 35 days of harvesting. Both entomopathogenic nematodes (*H. bacteriophora and S. asiaticum*) and root knot nematodes were applied at same time.

At the first harvest after seven days of inoculation of root knot nematodes and EPNs, root weight did not differ significantly ($P<0.05$). The reason behind that most of the nematodes were invading the root system but not developing into galls.

Numbers of J2s of root knot nematodes in roots of brinjal were reduced significantly in *S. asiaticum* and *H. bacteriophora* as compared to control. Minimum number of J2s were recorded in *S. asiaticum* (81.3) followed by *H. bacteriophora* (123.7), while the maximum number of J2s were recorded in control treatment (159.7) where only RKNs were applied. Minimum number of dJ2s were recorded in *S. asiaticum* (31.3) followed by *H. bacteriophora* (71.7) and were significantly ($P<0.05$) lower as compared to control (166.7) as shown in Table 1

At second harvest, after 17 days of application, root weight was recorded minimum in *S. asiaticum* and *H. bacteriophora* (statistically similar) as compared to control where it was maximum (3.67 gm). Root weight was in relation to the number of nematodes invaded into the root. Number of dJ2s in all the treatments was significantly different ($p<0.05$) as compared to control. These were recorded the maximum in *H. bacteriophora* (43), minimum in *S. asiaticum* (17), while it was intermediate in control (31). As far as the number of dJ4s were concerned, they were recorded minimum in *S. asiaticum* (67) followed by *H. bacteriophora* (130) and recorded maximum in control (208). Lesser number of dJ2s and higher number of dJ4s in

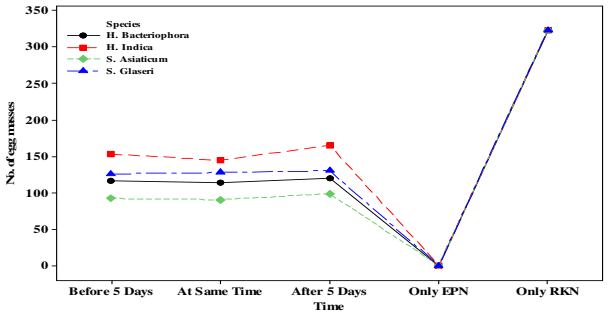

Fig. 1c: Effect of EPN species at different times on number of females of RKN
LDS at P=0.05, Species = 2.2566, time = 2.5230, specie*species = 5.0459

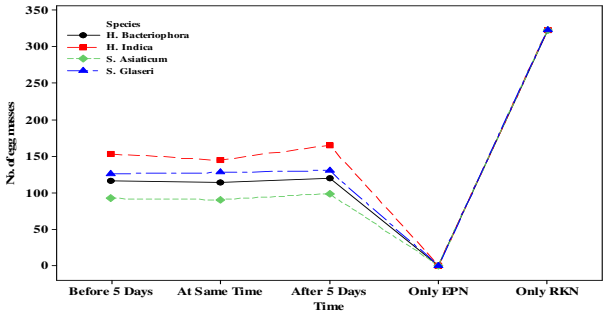

Fig. 1d: Effect of EPN species at different times on number of egg masses of RKN
LDS at P=0.05, Species = 2.4065, time = 2.6906, specie*species = 5.3811

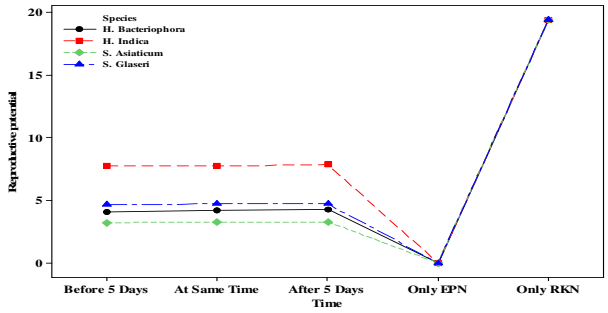

Fig. 1e: Effect of EPN species at different times on reproductive potential of RKN
LDS at P=0.05, Species = 0.1287, time = 0.1439, specie*species = 0.2879

control treatment as compared to *H. bacteriophora* indicate that most of the dJ2s had been passed to the next stage (dJ4s) in control treatment (Table 2). The minimum number of females was recorded in *S. asiaticum* (31) followed by *H. bacteriophora* (34) statistically similar and were significantly ($P<0.05$) lower as compared to control treatment (93) as given in Table 2.

At the final harvest after 35 days, root weight of brinjal plants was recorded by applying *H. bacteriophora* and *S. asiaticum* at same time along with root knot nematodes. Influence of both entomopathogenic nematodes species and root knot nematodes alone were also studied.

Table 3 shows that both the entomopathogenic nematodes species were not significantly differed ($p<0.05$) in their effect with respect to root weight. The minimum root weight was recorded in *S. asiaticum* (3.79 g) fallowed by *H. bacteriaphora* (3.84 g) statistically similar and were found to be most effective species influencing the root weight as compared to root knot nematodes (5.64 g).

J2s and dJ2s found on roots were neglected at this stage. (As the purpose of research was to study only one generation and these were probably of juveniles who were hatched out because there was not any J2s present at second harvest (17 days), which showed that all the J2s had been passed to the next stage).

Numbers of dJ4s were significantly different from each other. These were minimum in *S. asiaticum* followed by *H. bacteriophora,* while maximum number of dJ4s was observed in control ($p<0.05$).

More a less similar trend was observed in case of number of females, these were recorded minimum in *S. asiaticum* (108) followed by *H. bacteriophora* (201). Maximum females were counted in control (329), where only the root knot nematodes had been applied ($p<0.05$) as shown in Table 3.

Discussion

Plant parasitic root knot nematodes are the most notorious parasites having their presence around the globe, but excessively inhabiting in areas having damp conditions. Root knot nematodes (RKN) not only damage the plants by drawing away nutrients of diseased plants but also affect the fruit quality. Normally, at seedling stage because tissues of the plants roots are tender therefore, the invasion of nematodes is more resulting in heavy losses. Nematodes cause injury usually by entering into the infected tissues, this also facilitates other pathogens to enter through these openings and expedite the losses.

Plants have two types of resistance against RKN including pre-infection and post resistance. Pre-infection resistance is because of the presence of root containing toxic or antagonistic chemicals, which create resistance against puncturing of root knot nematodes in roots (Bendezu and Starr, 2003), whereas an early hypersensitive reaction (HR) operates in post-infection resistance which averts establishment of feeding sites leading to resistance to avoid development of nematodes in host tissues (Haynes and Jones, 1976; Bendezu and Starr, 2003). This is mostly considered that giant cells developed which restrict the activity of xylem. Secondly, rate of photosynthetic activity, because of less light interception and carbohydrate synthesis is also disturbed. This forces the plant to produce more roots in response to the attack of nematode to overcome the damage (Trudgill, 1992).

The objective of this study was to manage the root-knot nematode exploiting the potential of entomopathogenic nematodes *H. indica, H. bacteriophora and S. glasseri*.

Table 2: Effect of different species of entomopathogenic nematodes on invasion and development of RKN in Brinjal (after 17 Days)

Treatments	Root Weight (g)	J2s[1]	dJ2s[2]	dJ4s[3]	No. of females
S. asiaticum + RKN	2.67b	0	17b	67.3b	31.3c
S. asiaticum	2.32a	0	0a	0.0a	0.0a
H. bacteriophora +RKN	2.69b	0	43d	130.3c	34.3c
H. bacteriophora	2.32a	0	0a	0.0a	0.0a
RKN alone	3.67d	0	31c	208.3d	93.7d

[1]J2s= 2nd stage Juveniles, [2]dJ2s= developing 2nd stage Juveniles, [3]dJ4s= developing 4th stage Juveniles
Numbers followed by different letters are significantly different from each other at $p<0.05$
Data is mean of three replications

Table 3: Effect of different species of entomopathogenic nematodes on invasion and development of RKN in Brinjal (after 35 Days)

Treatments	Root weight (g)	J2s[1]	dJ2s[2]	dJ4s[3]	No. of females
S. asiaticum+RKN	3.84c	117b	36.3b	6.33b	108.7b
S. asiaticum	3.43b	0.0a	0.0a	0.00a	0.0a
H. bacteriophora+RKN	3.79c	177c	83.0c	7.33c	201.7c
H. bacteriophora	3.43b	0.0a	0.0a	0.00a	0.0a
RKN alone	5.64d	312d	311.0d	10.33d	329.0d

[1]J2s= 2nd stage Juveniles, [2]dJ2s= developing 2nd stage Juveniles, [3]dJ4s= developing 4th stage Juveniles
Numbers followed by different letters are significantly different from each other at $p<0.05$
Data is mean of three replications

Effect of entomopathogenic nematodes on root knot nematodes (*M. incognita*) were studied by designing different experiments under *in vitro* conditions. Impact of application time on root knot nematodes in brinjal was studied. Different species of EPN preciously have been reported to control RKN. Pre and post application of *S. riobravis* and *S. feltiae* @ 25 IJs/cm^2 and before application of *H. bacteriophora* against *M. incognita* suppresses the entry and egg production (Perez and Lewis, 2002). Similar results were obtained under *in vitro* conditions when *H. bacteriophora, H. indica, S. asiaticum* and *S. glaseri* were applied at the rate of 2000IJs/pot. It was revealed that all the species of EPN tested in experiment significantly suppressed *M. incognita* by affecting the invasion of J2, egg production, number of females and the reproductive potential of RKN at all different times of applications. But our findings are not in line with the results of Perez and Lewis (2004) who concluded that only pre-infestation application was effective; in our research it was found that application of EPNs before at the same time and after the application of root knot nematodes was effective likewise. Our revelations confirming the previous studies in which plant parasitic nematodes were effectively controlled by entomopathogenic nematodes (Somasekhar *et al.,* 2000; Perez and Lewis, 2004). However, application time and species of EPN influence the suppressive effect of EPN against root-knot nematodes (Perez and Lewis, 2002).

In second experiment conducted in pots, *S. asiaticum*

and H. bacteriophora were investigated for invasion, development and reproduction of root knot nematodes in brinjal roots. Treatments were terminated at various time intervals over a period of 35 days. Reduction was recorded in root knot nematodes along with reduced number of galls per root system, number of egg masses and number of females in EPN treated roots. From this, it was inferred that it might be due to crowding effect of EPN, nematicidal properties of metabolites and their allopathic effects which was influencing the life cycle and maturity of RKN (Hu *et al.,* 1999; Lewis *et al*., 2001). Different factors, like competition between nematode groups for CO_2, space in rhizosphere and other root exudates assist the entomopathogenic nematodes to create suppressive effects on plant-parasitic nematodes (Robinson, 1995; Tsai and Yeh, 1995).

Furthermore, behavioral response of nematodes, increased population of natural enemies and predators in the production of allelochemicals by the entomopathogenic nematodes and symbiotic bacteria complex are also considered important factors enhancing the potential of EPN against RKN (Lewis *et al*., 2001). Present study will be helpful for the management of root knot nematodes.

Conclusion

Attack of *M. incognita* seriously affected the brinjal shoot and especially root growth. However, application time of entomopathogenic nematodes (before, with and after) were quite effective in reducing the population of *M. incognita* as compared to control.

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