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Evaluation of pathogenicity of indigenous entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) from Mizoram, India, against tobacco cutworm, *Spodoptera litura* (Fabricius, 1775)

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Abstract

Aim: To evaluate pathogenicity of locally isolated entomopathogenic nematodes *Heterorhabditis indica*, *Heterorhabditis baujardi* and *Steinernema sangi* against larvae of *S. litura* (Lepidoptera: Noctuidae).

Methodology: Different concentrations of nematodes (10, 25, 50, 100, 200, 400 and 800 infective juveniles per larva) of *H. indica*, *H. baujardi* and *S. sangi* were used against 3rd, 4th and 5th instar larvae of *S. litura* using Petri dish assay. Pathogenicity test was performed by assessing larval mortality of *S. litura*, penetration of infective juveniles and successive production of nematodes on the larvae of *S. litura*. LC₅₀ and LT₅₀ values were calculated by Probit Regression Analysis using SPSS software 20.0.

Results: All the tested EPN isolates showed high pathogenicity against the pest, with a strong positive correlation between nematode concentrations, inoculation time and larval mortality. Among the isolates, *S. sangi* showed highest pathogenicity against the pest by showing the lowest value of LC₅₀ at 48 hr post-inoculation (9.2, 55.58 and 350.65 infective juveniles for 3rd, 4th and 5th instar larvae, respectively) and LT₅₀ values (24.52, 34.13 and 74.69 hr against 3rd, 4th and 5th instar larvae, respectively) at a dosage of 50 infective juveniles per larva. Besides, the nematodes were produced successfully inside the 5th instar larvae of *S. litura* (57192.86 infective juveniles per larva for *S. sangi* and 72231.13 infective juveniles per larva for *H. baujardi*).

Interpretation: EPN isolates represent good candidates to control *S. litura* and can be considered for integrated pest management (IPM).

Key words: Biological control, Entomopathogenic nematodes, Infective juveniles, Integrated pest management, *Spodoptera litura*

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Introduction

Tobacco cutworm (*Spodoptera litura* F.) is one of the most destructive and serious pests of agricultural crops with widespread distribution throughout tropical and temperate Asia, Australia and Pacific Islands (Feakin, 1973; Kranz et al., 1977). In India, it has a widespread distribution across the land, covering almost all the states (CABI, 2019) and responsible for heavy damage of economically important crops like groundnut (Patil et al., 1996), soybean (Choudhary et al., 2007; Punithavalli et al., 2014; Sharma et al., 2014), cotton (Gedia et al., 2008). The larva is a polyphagous pest (Singh and Jalali, 1997) and reported to infest different varieties of plants, including major agricultural crops such as tobacco, groundnut, taro and castor (Wu et al., 2004; Qing et al., 2006) causing an economic loss of about 26–100 percent on the fields (Dhir et al., 1992; Rao et al., 1993; Isman et al., 2007; Satyagopal et al., 2014).

Excessive use of chemical pesticides became a serious problem as insects have continued to show increased resistance against pesticides (Lohar et al., 1995; Kerns et al., 1998; Whalon et al., 2007). *S. litura*, in particular, has been reported to develop resistance against number of chemical pesticides viz., organophosphate, carbamate, pyrenoids and more towards newly developed chemical insecticides (Armes et al., 1997; Kranthi et al., 2002; Ahmad et al., 2007a, b; 2008; Saleem et al., 2008), resulting in ineffective management that leads to huge loss of agricultural crop production around the world (Ahmad et al., 2007a). Similarly, the harmful effects that chemical pesticides can impose on human health, non-targeted organisms and environment (Gaugler, 1988; Villani and Wright, 1988) need instant action. Therefore, the need for alternative methods of insect pest management has become an important issue and biological control is one of the most ideal alternatives for chemical pest management (Brixey, 1997).

Entomopathogenic nematodes (EPNs) of the genera, *Steinernema* Travassos, 1927 and *Heterorhabditis* Poinar, 1976 are obligate and lethal parasites of insects (Poinar, 1979) that are characterized by their symbiotic association with bacteria, *Xenorhabdus* spp. in steinernematids and *Photorhabdus* spp. in heterorhabditids (Poinar, 1990). EPNs naturally occur in soil and are reported to have widespread geographical and diverse climatic distributions across the globe (Hominick et al., 1996), except Antarctica (Hominick, 2002). The only considered free-living stage of EPNs, infective juveniles (Boemare, 2002), after in contact with the insect host enter through body openings like spiracles, oral cavity, anus and also through cuticle in some cases (Bedding and Molyneux, 1982; Dowds and Peters, 2002). Upon entering the host insect, the infective juveniles release the intestinal harboured symbiotic bacteria and infected host insect dies within 24–48 hr of infection (Poinar, 1979). The infective juveniles may reproduce for about 2 to 3 generations inside the host, and later exit the nutrient depleted cadaver to infect new

insect host in the soil (Kaya, 1990; Grewal and Georgis, 1999). In addition to the primary agents for host insect death, the symbiotic bacteria provide nematodes with nutrition and protection from other micro-organisms that may invade them (Poinar, 1990). With the urgent need for more sustainable eco-friendly strategy of pest management, EPNs have been developed and frequently applied for regulating and controlling of insect pests (Shapiro-Ilan et al., 2002 a; Georgis et al., 2006). The potential of EPNs as biological control agents differ with the process of invasion and evasion of host insects (Simões and Rosa, 1996), host age and nematode strain (Fuxa et al., 1988). For the successful development of EPN as suitable biological control agents, the locally adapted indigenous species of EPNs need to be evaluated where they show higher pathogenic potential against the host insect in comparison to non-native species (Bedding, 1990; Grewal et al., 2002; Lewis et al., 2006). In light of the above, the present study was conducted to study the pathogenicity of three species of EPNs, *Steinernema sangi*, *Heterorhabditis indica* and *H. baujardi* against tobacco cutworm, *S. litura*.

Materials and Methods

Nematode sources: Three species of EPN, *S. sangi* (Phan et al., 2001), *H. indica* (Poinar et al., 1992) and *H. baujardi* (Phan et al., 2003) were isolated from soil by baiting methods (Bedding and Akhurst, 1975). Soil samples were collected from riparian forest in Mizoram, Northeast (NE) India. A total of 400–500 g of soil samples were collected from a depth of 10–15 cm from each collection site (approximately 1 sq. m) and transported to the laboratory. The collected soil samples were baited immediately by using larvae of greater wax moth, *Galleria mellonella*, in a plastic container and kept at room temperature. Larval mortality was checked every 24 hr for 10 consecutive days and dead larvae were kept in modified white trap following the method of Kaya and Stock (1997). Healthy *G. mellonella* were infected again with the extracted infective juveniles, maintained at 28 °C and only the infective juveniles which passed through the re-infection process were collected for further use. The extracted EPNs were stored in the form of infective juveniles with aerated water in an incubator at 12–15 °C.

Collection of *S. litura*: Eggs of *S. litura* were obtained from the Department of Agriculture (Research & Extensions), Government of Mizoram, Aizawl and incubated at 28 ± 2 °C for hatching. The emerged larvae were reared using castor plant leaves and different larval stages (3rd, 4th and 5th) were used for evaluation of their susceptibility against the locally isolated EPNs.

Larval mortality bioassay : Experiment on larval mortality was performed using Petri dish assay following methods of Kaya and Stock (1997). Petri dishes (35 x 10 mm in diameter) were lined with Whatman filter paper No. 1. Different concentrations of nematodes (10, 25, 50, 100, 200, 400 and 800 infective juveniles per larva) were selected for the assay. Each concentration of nematodes in 0.5 ml of deionized water was introduced into the

petri dish and allowed to settle for 30 mins. A single larva of healthy *S. litura* was selected, introduced into the petri dish containing pre-inoculated infective juveniles, sealed with parafilm and kept in an incubator at 28 °C. For each larval stage (3rd, 4th and 5th) of *S. litura*, eight replicas were set for each species of nematode and concentrations of infective juveniles. The experiment was repeated three times. A petri dish lined with filter paper and wetted with deionized water was kept as control for the assay. Mortality of larvae were checked after every 24 hr for five consecutive days. The mortality was confirmed by colour change on the body and smell emanating from the dead cadaver of larvae caused by the symbiotic bacteria.

Penetration of infective juveniles : Dead larvae from the mortality bioassay were dissected after 48 hr of mortality and the total number of infective juveniles that had penetrated the larva were counted under the microscope. This experiment was

performed for different concentrations of nematodes at different larval stages of the insect pest for all the EPN isolates.

Production of infective juveniles : A dosage of 200 nematode infective juveniles per insect larva with 0.5 ml of deionized water was introduced into a petri dish lined with Whatman filter paper No. 1 and allowed to stay for 30 mins. For each species of EPN, 3rd instar larva of *S. litura* was introduced into a petri dish pre-inoculated with nematodes, sealed with a parafilm and incubated at 28 ± 2 °C. The dead larva was rinsed with distilled water and transfer individually in a modified white trap (Kaya and Stock, 1997) for reproduction of nematodes and emergence of infective juveniles. After successive reproduction, the total number of emerged infective juveniles were counted for 20–30 days. A total of eight replicas were set for each species of nematode for all the larval stages of *S. litura* and the experiment was repeated thrice. The same procedure was done for 4th and 5th instar larvae.

Fig. 1: Effect of different concentrations of *S. sangi* and incubation time on *S. litura* mortality (% ± S.E.). Vertical bars represent standard error of means. Bars with same uppercase letter indicates non-significant differences ($P > 0.05$) for different concentrations of *S. sangi* at same inoculation time. Bars with same lowercase letter indicates non-significant differences ($P > 0.05$) for same nematode concentration at different inoculation time. (i) Against 3rd instar larvae, (ii) Against 4th instar larvae and (iii) Against 5th instar larvae.

Fig. 2: Effect of different concentrations of *H. indica* and incubation time on *S. litura* mortality (% $\pm$ S.E.). Vertical bars represent standard error of the means. Bars with same uppercase letter stand for non-significant differences existing for different concentrations of *H. indica* causing *S. litura* mortality at same inoculation time. Bars with same lowercase letter stand for non-significant differences existing among different inoculation time causing *S. litura* mortality for same nematode concentration ($P > 0.05$). (i) Against 3rd instar larvae, (ii) Against 4th instar larvae and (iii) Against 5th instar larvae.

Statistical analysis : Statistical data analysis was performed for all the experimental data and were presented as mean $\pm$ S.E. Student's t-test and One-way analysis of variance (ANOVA) were performed for analyzing significant differences at the level of $P \leq 0.05$. Probit Regression Analysis (using SPSS 20.0 software) was done to calculate the lethal concentration (LC_{50}) and lethal time (LT_{50}) values.

Results and Discussion

The results revealed that the three EPN isolates, *S. sangi*, *H. indica* and *H. baujardi* were highly pathogenic against *S. litura* larvae and the degree of pathogenicity showed significant variations with different concentrations of infective juveniles and incubation time (Fig. 1–3). However, no significant difference was observed among EPN isolates at same concentration of infective juveniles ($P \geq 0.05$). Similar observation was reported by Phan

et al. (2005) where a significant difference was observed between nematode isolates, concentrations of infective juveniles and exposure time to kill insect larvae.

At 50 infective juveniles per larva and 48 hr post-inoculation, *S. sangi* showed the highest pathogenicity with 87.5 %, 68.75 % and 16.67 % larval mortality against 3rd, 4th and 5th larvae; *H. indica* showed 81.25 %, 68.75 % and 12.5 % larval mortality against 3rd, 4th and 5th instar larvae; and *H. baujardi* showed 81.25 %, 62.5 % and 16.67 % larval mortality against 3rd, 4th and 5th larvae, respectively. Statistical analysis revealed significant difference in larval mortality of *S. litura* in the EPN isolates for different larval stages of *S. litura* ($F = 1368.307$; $df = 2, 93$, $P < 0.001$ for *S. sangi*, $F = 1601.249$; $df = 2, 93$, $P < 0.001$ for *H. indica* and $F = 912.064$; $df = 2, 93$, $P < 0.001$ for *H. baujardi*). However, no significant difference in larval mortality of the pest was found among the EPN isolates for same larval stages ($F =$

Fig. 3: Effect of different concentrations of *H. baujardi* and incubation time on *S. litura* mortality (% $\pm$ S.E.). Vertical bars represent standard error of the means. Bars with same uppercase letter stand for non-significant differences existing for different concentrations of *H. baujardi* causing *S. litura* mortality at same inoculation time. Bars with same lower-case letter stand for non-significant differences existing among different incubation time causing *S. litura* mortality for the same nematode concentration ($P > 0.05$). (i) Against 3rd instar larvae, (ii) Against 4th instar larvae and (iii) Against 5th instar larvae.

368.307; $df = 2, 93$, $P > 0.05$ for 3rd instar larvae, $F = 307$; $df = 2, 93$, $P > 0.05$ for 4th instar larvae and $F = 298$; $df = 2, 93$, $P > 0.05$ for 5th instar larvae). The result of laboratory evaluation demonstrated that all the EPN isolates were highly pathogenic against *S. litura* with more pathogenicity towards the 3rd instar larvae.

Larval mortality time (hr): The recorded larval mortality time showed positive correlation with concentration of nematodes and duration of incubation ($r^2 = 0.88$ in *S. sangi*, $r^2 = 0.97$ in *H. indica* and $r^2 = 0.89$ in *H. baujardi*). Mortality of 3rd instar larvae of *S. litura* was first observed at a concentration of 10 infective juveniles during 24 hr post-inoculation (Fig.1–3). Similarly, 50 % larval mortality was caused by both *S. sangi* and *H. indica* and 43.75 % by *H. baujardi* at a concentration of 50 infective juveniles per larva during 24 hr post-inoculation. After 48 hr of incubation, absolute larval mortality of *S. litura* was observed at a concentration of 800 infective juveniles per larva for all the EPN isolates.

After 24 hr of inoculation, larval mortality of 4th instar larvae was initially recorded at a dosage of 10 infective juveniles for all the EPN isolates. With the same incubation time, larval mortality of 5th instar larvae was first observed at 100 infective juveniles per larva which indicates that larval mortality continued to decrease with the advancement of larval stages of the pest. Furthermore, against 5th instar larvae, *S. sangi* caused 100% larval mortality at 100 infective juveniles per larva and, similarly, at 200 infective juveniles per larva by both *H. indica* and *H. baujardi*.

LC_{50} and LT_{50} values were calculated for all the nematode isolates (Table 1, 2). At 50 infective juveniles per larva during 48 hr post-inoculation, the value of LC_{50} on 3rd instar larvae was 9.2, 16.37 and 17.04 infective juveniles per larva for *S. sangi*, *H. indica* and *H. baujardi*, respectively. With the same concentration of infective juveniles, *S. sangi* showed less LT_{50} value (24.52 hr) in comparison to *H. indica* and *H. baujardi* (LT_{50} values of 25.36 and

Fig. 4: Graph showing comparison of mortality (%) of different larval stages of *S. litura* at 50 infective juveniles per larva and 48 hr post-inoculation ($\% \pm$ S.E.). Vertical bars are standard error of means. * and ** represent significant difference of larval mortality (%) at $P \leq 0.05$ and $P \leq 0.01$ level, among different larval stages for same nematode isolate.

Fig. 5: Graph representing percent penetration of IJs for different concentrations of EPN isolates. Correlation analysis showed that total number of infective juveniles penetration into the pest larvae were positively correlated with nematode concentrations for all the EPN isolates ($r^2 = 0.95$ for *S. sangi*, $r^2 = 0.97$ for *H. indica* and $r^2 = 0.89$ for *H. baujardi*). However, no statistically significant variations exist for percentage penetration of infective juveniles for different concentrations of nematodes. (i) *S. sangi*, (ii) *H. indica* and (iii) *H. baujardi*.

Fig. 6: Production of infective juveniles in 5th instar larvae of *S. litura* at a dosage of 200 infective juveniles per larva (Mean ± S.E.). Bars with different uppercase letter stand for significant differences existing between *S. sangi* and *H. indica* at the level of $p \leq 0.01$.

28.06 hr, respectively). *S. sangi* showed highest pathogenicity against 3rd instar larvae of *S. litura* followed by *H. indica* and *H. baujardi*. Gokte-Narkhedkar *et al.* (2019) also reported LC_{50} values of *H. indica* against *S. litura* larvae as 7.37–17.91 IJs at 48 hr of post-inoculation. In terms of LC_{50} and LT_{50} values, *S. sangi* showed the highest pathogenicity against all the larval stages of *S. litura* with lowest LT_{50} and LC_{50} values. In addition, *H. indica* was more pathogenic against 3rd and 4th instar larvae but less pathogenic against 5th instar larvae of *S. litura* as compared to *H. baujardi*. The result showed that *S. sangi* was most pathogenic against *S. litura* as it caused 93.75 % mortality in 3rd and 4th instar larvae and 31.25 % mortality at 400 infective juveniles per larva dose with 24 hr of incubation. The observation is in concordance with Phan *et al.* (2005), who reported that 500 infective juveniles per larva dose of *S. littoralis* caused 92 % larval mortality within 24 hr of incubation with *S. sangi* being the only isolate capable of causing larval mortality against all the evaluated pests.

Host penetration: The total number of infective juveniles that had penetrated the larva were recorded for different concentrations of nematodes for all the EPN isolates at different larval stages of *S. litura* (Fig. 5). Host penetration by the nematodes showed a positive correlation with the applied doses of infective juveniles. It was observed that the percentage penetration of infective juveniles ranged from 10–16 %, 12.65–32 % and 12.51–30 % for *S. sangi*, *H. indica* and *H. baujardi*, respectively, with insignificant difference between them. In contrast, Phan *et al.* (2005) reported that variation occurred in nematode penetration into *S. littoralis* larvae among different EPN isolates.

Production of infective juveniles: The reproductive capacity of three EPN isolates were assessed at a concentration of 200 infective juveniles per larva on different larval stages of *S. litura*. Even though all the nematode isolates were highly pathogenic against *S. litura*, successive progeny production failed to occur on 3rd and 4th instar larvae. Similarly, Gokte-Narkhedkar *et al.* (2019) reported that some species of EPN isolates failed to reproduce successively inside larvae of *S. litura* and concluded that phase change of bacterial symbionts as reason of primary failure. In contrast, Safdar *et al.* (2018) have reported successive reproduction of *H. bacteriophora* inside different larval stages of *S. litura* larvae (2nd, 3rd, 4th and 5th instar larvae). In addition, Han and Ehlers (2001) concluded that lack of important nutrients in host and/or failure of toxins production in phase II bacterial symbiont may not affect development of *H. bacteriophora*. It is therefore suggested that further study on the impact of phase variation of the symbiotic bacteria on the nematode reproduction is necessary.

In the case of 5th instar larvae, successive nematode reproduction was recorded for *S. sangi* and *H. indica* where they produced 57192.86 and 72231.13 infective juveniles per cadaver of larva, respectively (Fig. 6). The reproduction potential of EPNs inside the 5th instar larvae of *S. litura* showed significant variations between *S. sangi* and *H. indica* ($F = 1.125$; $df = 1, 14$; $P < 0.05$). High production of infective juveniles by *H. indica* may be attributed to the hermaphroditic nature of heterorhabditids and smaller size of infective juveniles. The difference in size and behaviour of isolated nematode species may determine the

Table 1: LC₅₀ values of EPN isolates against 3rd, 4th and 5th instar larvae of *S. litura*

Hours of incubation	LC ₅₀ (infective juveniles per larva)								
	<i>S. sangi</i>			<i>H. indica</i>			<i>H. baujardi</i>		
	3 rd instar	4 th instar	5 th instar	3 rd instar	4 th instar	5 th instar	3 rd instar	4 th instar	5 th instar
24	114.07	182.8	995.7	121.19	189.53	1003.3	129.84	193.08	1016.2
48	9.2	55.58	350.65	16.37	60.43	369.28	17.04	61.35	354.69
72	–	–	–	–	–	–	–	–	–
96	–	–	–	–	–	–	–	–	–
120	–	–	–	–	–	–	–	–	–

Table 2: LT₅₀ values of EPN isolates against 3rd, 4th and 5th instar larvae of *S. litura*

Concentrations Infective juveniles per larva	LT ₅₀ (hr)								
	<i>S. sangi</i>			<i>H. indica</i>			<i>H. baujardi</i>		
	3 rd instar	4 th instar	5 th instar	3 rd instar	4 th instar	5 th instar	3 rd instar	4 th instar	5 th instar
10	57.88	59.36	88.77	59.36	59.93	91.89	60.75	59.96	90.15
25	49.08	54.71	83.63	50.01	55.14	87.2	50.57	56.58	85.97
50	24.52	34.13	74.69	25.36	36.81	77.2	28.06	37.43	74.65
100	–	25.36	59.05	–	25.7	63.3	6.63	27.43	61.32
200	–	12.85	42.29	–	14.037	51.53	–	17.7	45.19
400	–	–	36.81	–	–	43.47	–	–	39.93
800	–	–	–	–	–	–	–	–	35.26

successful reproductive ability of nematodes in the host (Loya and Hower, 2003). Likewise, the reproductive potential of six isolates of *Steinernema thermophilum* on *S. littoralis* was 19×10^3 to 47×10^3 infective juveniles per larva (Phan et al., 2005). Safdar et al. (2018) reported that *H. bacteriophora* was capable of successful reproduction 25786, 17500, 12642 and 9652 infective juveniles on 5th, 4th, 3rd and 2nd instar larvae of *S. litura*. Also, successful reproduction of EPN on larvae of *S. litura* was reported by Park et al. (2001) recording higher multiplication rate in heterorhabditidae, with highest multiplication in the 5th – 6th instar (1.3×10^6 infective juveniles per larva). However, Phan et al. (2005) reported that *S. sangi* was capable of successive multiplication inside larvae of *G. mellonella* but failed to do so on larvae of *S. litura*. Similarly, Ali et al. (2008) have documented variations in production of infective juveniles for EPNs on host insects (*Hemicoverpa armigera*, *Galleria melonella* and *Corcyra cephalonica*). Differences in pathogenicity and reproduction potential among EPN isolates may be attributed to species variation, susceptibility of host, symbiotic bacteria and ecological characteristics of EPN. EPN have been successively used against some of the agricultural pests and represent good candidate for component of IPM (Lacey and Georgis, 2012).

The future of human deeply relies on sustainability of agriculture system. Meanwhile, the harmful effects that chemical pesticides can impose on human and non-targeted organisms

indirectly leads to unsustainable agricultural cropping system. To lessen the environmental pressure from the use of conventional insecticides, more approach on safer pest management strategy need to be imposed. Indigenous isolates of EPN are considered more effective than exotic species for pest control in the local environment (Bedding, 1990). So far, two species of EPN, *S. sangi* and *H. baujardi* were reported from Mizoram (Lalramnghaki et al., 2017; Vanlalhlmpuia et al., 2018), and the present assessment of their efficacy against insect pest will contribute to enhancement of biological control method of the region in future.

In conclusion, all the three species of locally isolated EPNs showed high pathogenicity against *S. litura*, capable of causing high rate of mortality in all the selected larval stages which indicated that they are good candidates to be included in the integrated pest management.

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