Pathogenic and reproductive potential of three Himalayan entomopathogenic nematode strains (Nematoda: Heterorhabditidae) and the commercial strain of *Heterorhabditis indica* against *Spodoptera litura*

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Summary. *Spodoptera litura* larvae were exposed to 10, 20, 30 and 40 infective juveniles (IJ) of each species of entomopathogenic nematodes (EPN): *Heterorhabditis bacteriophora* (HRJ), *Heterorhabditis* sp. (HSG), *Heterorhabditis* sp. (HKM)) and a commercial formulation of *H. indica* for four different time periods and they were found to be susceptible to all the EPN tested. Appreciably acceptable performance was attained by *H. bacteriophora* (HRJ), which exhibited 91.7% mortality of *S. litura* larvae in 96 h exposure period against third instar larvae, while *H. indica* produced 83.0% mortality. All the tested EPN species were also found to reproduce within the host and produce first generation infective juveniles. This study concluded that all the three tested indigenous EPN species produced > 90.0% mortality of third instar larvae of *S. litura*. All tested EPN were also found to replicate within the host and maximum production of IJ was recorded in *H. indica* ($26.3 \pm 3.76 \times 10^3$ IJ larva^{-1}) and *H. bacteriophora* (HRJ) ($26.0 \pm 1.73 \times 10^3$ IJ larva^{-1}) at the concentration of 40 IJ larva^{-1}.

Key words: India, pathogenicity, reproduction, tobacco caterpillar.

Spodoptera litura Fabricius, commonly known as tobacco caterpillar, is a polyphagous pest widely distributed throughout South and East Asia and Oceania within climate types ranging from tropical to temperate regions (Shu *et al.*, 2017), native to India and South-East Asia (Waterhouse & Norris, 1987) and causes considerable damage to field crops such as cotton, corn, groundnut, soybean, tobacco and vegetables (Patel *et al.*, 1971; Hill, 1983; Smith *et al.*, 1997). It is also a significant pest of crops such as eggplants, sweet peppers and tomatoes in protected cultivation (glasshouses and vinyl-houses) (Nakasuji & Matsuzaki, 1977; Vashisth *et al.*, 2012). Intensive crop production includes indiscriminate use of pesticides, resulting in serious adverse environmental and human consequences (Ahmad *et al.*, 2008; Gill *et al.*, 2012; Mostafalou & Abdollahi, 2013; Tong *et al.*, 2013; Sang *et al.*, 2016), thus necessitating the deployment of alternative methods for *S. litura* management (Denholm & Rowland, 1992; Kranthi *et al.*, 2002; Nauen & Denholm, 2005). Insecticides of biological origin or biopesticides are now being increasingly sought and considered as promising alternatives to chemical insecticides (Mills, 2014; Orr & Lahiri, 2014). Vegetable growers are also now intending to use alternatives to insecticides, or a combination of tactics, rather than depending solely on insecticides. Being cash crops, the utilisation of biological pest control agents, such as entomopathogenic nematodes (EPN), has stimulated a great interest worldwide in Integrated Pest Management (IPM) of vegetable crops with a view to producing vegetables free from pesticide residues.

The entomopathogenic nematodes belonging to the families Steinernematidae and Heterorhbditidae are used worldwide for the biological management of insect-pests in different agroecosystems (Kaya & Gaugler, 1993; Bedding *et al.*, 1993; Grewal & Georgis, 1998; Kaya *et al.*, 2006; Lacey & Georgis, 2012; Vashisth *et al.*, 2013). EPN possess broad host range, high virulence, host seeking capability, ease of mass production, recycling ability and are also safe to the environment (Gaugler & Kaya, 1990; Vashisth *et*

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al., 2013). Previous studies have shown that the vulnerability of different developmental stages of insect hosts show great variations to distinct species or strains of EPN (Bedding & Molineux, 1982; Geden *et al.*, 1985; Fuxa *et al.*, 1988; Glazer *et al.*, 1991; Smits *et al.*, 1994; Jansson, 1996; Simoes & Rosa, 1996; Khatri-Chhetri *et al.*, 2011; Holajjer *et al.*, 2014). When investigating a feasible management approach for any insect pest, it is always advisable to match the correct indigenous EPN isolate against the target species. The indigenous isolates of EPN are more likely to be adapted to the local climatic conditions and host population. An ideal EPN species or isolate should also have good potential to recycle and propagate in the insect host. Surveys for isolation of EPN were conducted (Vashisth *et al.*, 2015), and pathogenic strains of *Heterorhabditis* spp. were collected from soil samples of sub-temperate to temperate zones of Himachal Pradesh, India. In initial experiments, quick knockdown of larvae of *Corcyra cephalonica* and *Galleria melonella* was observed within 48 h post exposure. Therefore, the present studies were undertaken to study the comparative pathogenicity and reproduction potential of EPN species/ strains from different regions in Himachal Pradesh and commercially available *H. indica* Poinar, Karunakar & David, 1992 for management of *S. litura*. The virulence of these EPN to tobacco caterpillar larvae, and their reproduction in insect hosts was studied under laboratory conditions.

MATERIAL AND METHODS

Nematode sources. Three EPN populations originally isolated from Sangla (3215 m a.s.l.), Kamand (2421 m a.s.l.) and Rajgarh (2119 m a.s.l.) areas of Himachal Pradesh, India, were maintained in culture on last instar larvae of *Galleria melonella* at Nematology laboratory, Department of Entomology, CSK HPKV, Palampur, India. The nematode identification was based on morphometric characteristics of the infective juvenile (IJ), male and hermaphrodite female as described by Uribe-Lorio *et al.* (2005) and its comparison with the data from original descriptions (Adams & Nguyen, 2002; Liu, 1994; Nguyen & Smart, 1996; Poinar, 1990). The indigenous strains of EPN were identified as *Heterorhabditis bacteriophora* Poinar, 1976 (HRJ), *Heterorhabditis* sp. (HSG) and *Heterorhabditis* sp. (HKM).

For experimental study, the commercial strain of *H. indica* was extracted from commercial formulation and reared similar to local nematodes under laboratory conditions on late instar larvae of *G. melonella* at 25°C, as described by Woodring & Kaya (1988). The IJ that emerged from wax moth larvae cadavers were collected using modified White traps (Kaya & Stock, 1997), and stored in darkness at 15°C in deionised water. Before being used for assay, the IJ were allowed to acclimatise for 1 h at room temperature and their viability was checked by observation of movements under a stereomicroscope.

Insect sources. The larvae of *S. litura* were collected from polyhouses on capsicum in April, 2012. These larvae were fed on cabbage leaves in plastic jars (18 × 15 cm) until adult emergence. The emerging adults were transferred to a glass chimney in pairs for oviposition covered with muslin cloth, containing 15% honey solution on cotton swabs and crumpled paper, and placed in small Petri dishes. The female moths laid eggs in clusters frequently on the muslin cloth and the crumpled paper. The eggs were stored in plastic jars along with their oviposition substrates and observed for hatching. Neonate larvae were transferred to cabbage and maintained in plastic jars (7 × 4.5 cm) in groups up to the second instar. During third instar, the larvae were separated, and 10-15 larvae were reared in a single jar of 18 × 15 cm. The full-fed larvae were moved to jars filled with a mixture of soil + sand up to 10-15 cm for pupation. After sexing, the male and female pupae were stored in separate jars until adult emergence. The culture so obtained was used for various studies. The mass culturing was done under controlled conditions at 25 ± 5°C and 75 ± 5% relative humidity under 16:8 hours (L:D) photoperiod.

Table 1. Details of entomopathogenic nematodes tested against *Spodoptera litura*.

Nematode	Location/ Source	Larval instar treated
Heterorhabditis sp. (HSG)	Sangla (Kinnaur)	III – V
Heterorhabditis sp. (HKM)	Kamand (Kullu)	III – V
H. bacteriophora (HRJ)	Rajgarh (Sirmaour)	III – V
H. indica	NBAII, Bengaluru (Karnataka)	III – V

Larval mortality bioassay. The three local EPN obtained during the survey along with commercially available formulation of *H. indica* were tested for their efficacy against *S. litura* (Table 1). Larval mortality bioassays were carried out in Petri dishes (9.5 cm diam.) lined with double layer of Whatman No. 1 filter paper, following the methods of Kaya & Stock (1997). Nematodes in 1.0 ml of deionised water were added to the filter paper in concentrations of 10, 20, 30 and 40 IJ larva^{-1}. After 30 min, a single larva of each instar (third, fourth and fifth) of *S. litura* was placed individually in the

Petri dish. At a given time, all EPN were tested against a each instar. The dishes were sealed with parafilm and maintained in a climate controlled chamber at 27 ± 2°C in the dark. For each nematode species and concentration, there were 10 replicates and the experiment was repeated thrice. Untreated controls were identical to the treatment except that no IJ were added. Larval mortality was checked at every 24 h for up to 96 h of exposure period. The cause of larval death was confirmed by change in body colour of the cadaver to different reddish tones and production of bioluminescence under darkness (Grimont *et al.*, 1984; Stock & Goodrich-Blair, 2012), due to the presence of different species of symbiotic bacteria (*Photorhabdus*) (Stock, 1993), and later was confirmed by dissecting the cadaver.

Reproduction of EPN. Three specimens of third instar larvae of *S. litura* were exposed to 10, 20, 30 and 40 IJ concentrations of each EPN in separate Petri dishes and total number of IJ produced per larva for up to a period of 10 days was counted. In brief, the nematode-infected dead insect larvae were removed from dishes, rinsed in deionised water and transferred individually on to White traps for their emergence from the body (White, 1927). The larvae were collected daily for up to a period of 10 days, until the emergence of IJ stopped and the total number of IJ produced per larva was then determined. There were 10 replicates for each nematode species and concentration and the experiment was repeated thrice. To each concentration, one Petri dish, prepared as described above but without IJ served as control.

Statistical analysis. The insect mortality was corrected using Abbott's (1925) formula:

$$\text{Corrected mortality \%} = \frac{\text{mortality\% in treatment - mortality\% in control}}{\text{100 - \% mortality in control}} \times 100$$

Data were subjected to analysis of variance using Genstat Version 14.0 (VSN International Ltd.; www.vsni.co.uk). Significance of differences between the isolates was tested by the F-test, while the treatment means were compared by least significant differences (LSD) at $P < 0.05$.

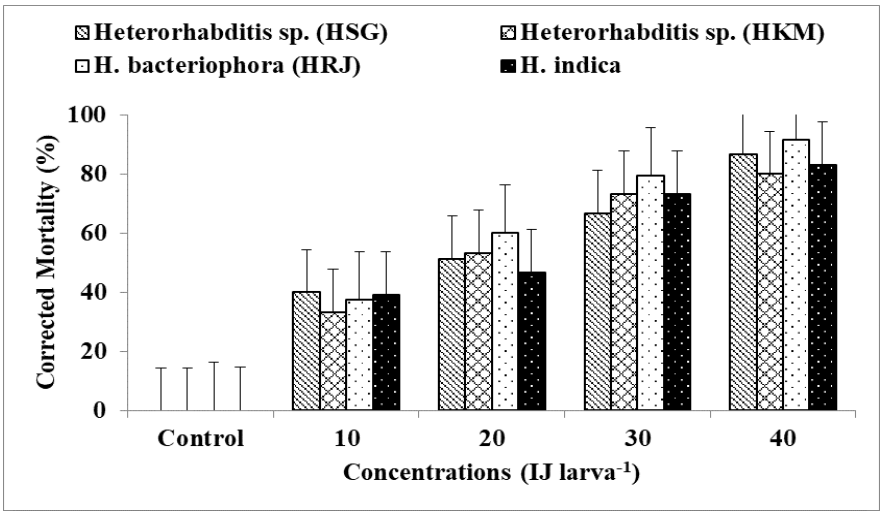
Table 2. Values of correlation coefficient (r) between concentration/time with mortality of *Spodoptera litura* larvae.

Species/Strain	Value of correlation coefficient (r) for III, IV and V instar larvae					
	Concentration of IJ × mortality at 96 h			Time × mortality at 96 h		
	III	IV	V	III	IV	V
Heterorhabditis sp. (HSG)	0.9944	0.9730	0.9759	0.9467	0.9859	0.9699
Heterorhabditis sp. (HKM)	0.9798	0.9479	0.9898	0.9795	0.9693	0.9827
H. bacteriophora (HRJ)	0.9905	0.9795	0.9759	0.9272	0.9607	0.9971
H. indica	0.9769	0.9845	0.9759	0.9944	0.9959	0.9971

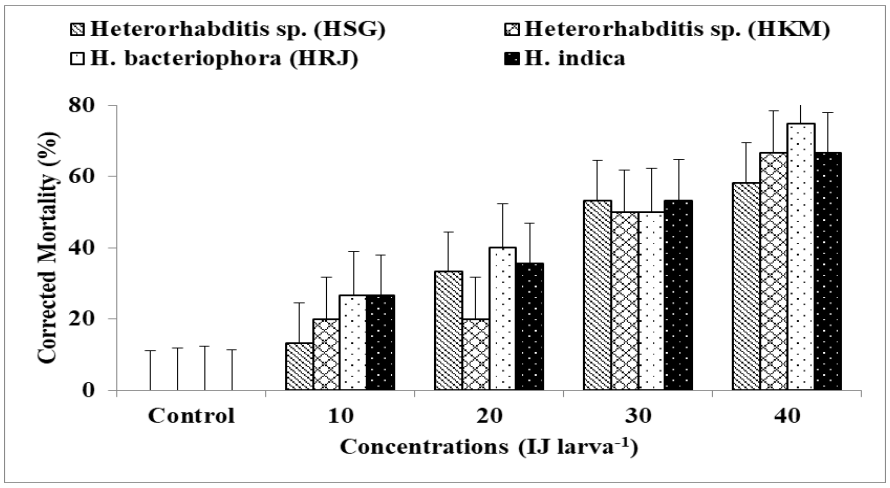
IJ = infective juveniles.

RESULTS

The third instar larvae of *S. litura* were found to be susceptible to all the three tested indigenous EPN and commercially available *H. indica* (Fig. 1A). However, the degree of susceptibility of insect larvae to nematode infection varied according to exposure period in a concentration dependent manner. The data revealed a positive correlation between the tested doses of IJ and/or time with larval mortality, for all the tested local EPN isolates and *H. indica* (Table 2). The data revealed significant differences in mortality rate of *S. litura* for each nematode species: population species (S) ($F = 42.07$, df = 3, $P = < 0.001$), exposure (E) period ($F = 461.21$, df = 3, $P = < 0.001$) and population (P) number ($F = 313.17$, df = 3, $P = <0.001$). There were significant differences in mortality of third instar larvae of *S. litura* across EPN isolates. *Heterorhabditis bacteriophora* (HRJ) caused the maximum larval mortality (91.7%) after 96 h of exposure at concentration of 40 IJ larva^{-1} (Fig. 1) in comparison to the other local and commercial EPN isolates. Larval mortality due to *H. bacteriophora* (HRJ), however, continued to increase further with an increase in exposure time and 100% larval mortality was recorded only after 120 h at 40 IJ larva^{-1}. Among the local EPN, *H. bacteriophora* (HRJ) was the most virulent irrespective of exposure time. The bio-efficacy of different isolates of EPN against fourth instar larvae of *S. litura* differed significantly across the dosages. Maximum mortality was caused by *H. bacteriophora* (HRJ) (75.0%) after 96 h of treatment, followed by *H. bacteriophora* (HRJ) (Fig. 1B). The data clearly revealed significant differences in mortality rate of *S. litura* for each nematode species: population species (S) ($F = 135.44$, df = 3, $P = < 0.001$), exposure (E) period ($F = 255.98$, df = 3, $P = < 0.001$) and population (P) number ($F = 8.32$, df = 3,



A



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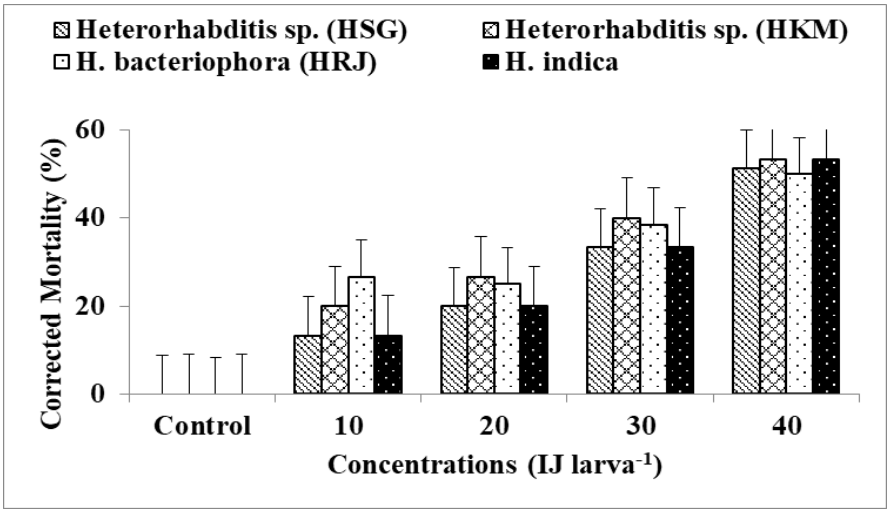

C

Fig. 1. The percentage mortality of *Spodoptera litura* larvae following exposure to different concentrations of infective juveniles (IJ) of nematodes under laboratory conditions. (A) Third instar; (B) Fourth instar; (C) Fifth instar.

P = < 0.001). Against fifth instar larvae of *S. litura* the bio-efficacy of tested EPN isolates differed significantly from each other. Local EPN isolate, *Heterorhabditis* (HKM) isolated from Kamand recorded the maximum larval mortality (53.3%) in fifth instar larvae of *S. litura* after 96 h of inoculation at a concentration of 40 IJ larva^{-1} in comparison to other two local isolates (Fig. 1C). The maximum mortality was observed with local EPN isolate, *Heterorhabditis* (HKM) and commercial formulation of *H. indica* (53.3%). The data revealed significant differences in mortality rate of *S. litura* for each nematode species: population species (S) (F = 2.18, df = 3, P = < 0.001, exposure (E) period (F = 159.40, df = 3, P = < 0.001) and population (P) number (F = 114.64, df = 3, P = < 0.001). In order to study the reproduction of EPN, the tobacco caterpillars were exposed to 10, 20, 30, and 40 IJ larva^{-1} of each nematode species. Following host mortality, the emerging IJ from host cadavers were collected and counted. The data indicated that all the four test EPN were able to penetrate and propagate in the insect larvae and successfully produced IJ (Fig. 2A-D). All nematode species exhibited a linear relationship between the concentrations of IJ applied and total number of IJ produced per infected larva. In this study, *H. indica* and *H. bacteriophora* (HRJ) produced significantly greater number of infective juveniles per insect larva than the other two nematode species (Fig. 2A-D). For *H. bacteriophora* (HRJ) and *H. indica*, maximum production of infective juveniles per larva (26.3 ± 3.76 × 10^3 IJ larva^{-1} and 26.0 ± 1.73 × 10^3 IJ larva^{-1}, respectively) was obtained at a concentration of 40 IJ larva^{-1}. The minimum IJ production was recorded for *Heterorhabditis* sp. (HKM); it increased linearly with an increase in IJ concentration, reaching to its maximum of 11.3 ± 4.78 × 10^3 IJ larva^{-1} at a concentration of 40 IJ larva^{-1}.

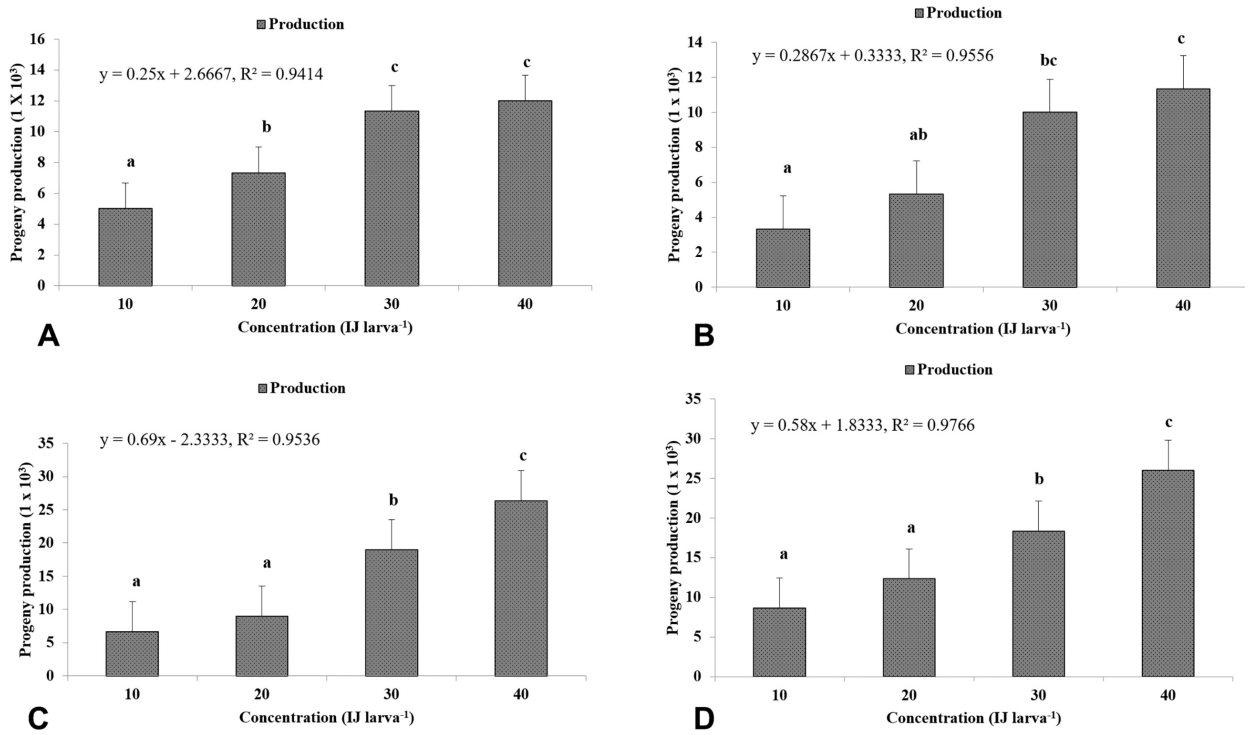

Fig. 2. Production of first generation infective juveniles (IJ) in *Spodoptera litura* larvae at different dosages of IJ. (A) *Heterorhabditis* sp. (HSG); (B) *Heterorhabditis* sp. (HKM); (C) *H. bacteriophora* (HRJ); (D) *H. indica*. **P < 0.01%; Means shown by the same letter are not significantly different (P > 0.05).

DISCUSSION

The aim of this study was to evaluate the comparative pathogenicity and reproductive potential of three indigenous strains of EPN (*H. bacteriophora* (HRJ), *Heterorhaditis* sp. (HSG) and *Heterorhaditis* sp. (HKM)) isolated from the cultivated lands, forest and orchard soils in

Himachal Pradesh, India in relation to the commercial formulation of *H. indica* procured from NBAII, Bengaluru against the third, fourth and fifth instar larvae of *S. litura*, a serious polyphagous pest in northern India.

Each EPN isolate was assessed for its pathogenicity on the basis of dose and time required to cause mortality of *S. litura* larvae and also on the basis of the ability of the EPN to propagate within the body of infected hosts and produce infective juveniles. *Spodoptera litura* larvae were relatively susceptible to indigenous and commercial available EPN, and there was a positive correlation between the concentration of IJ and larval mortality for all tested nematodes.

Among local EPN *H. bacteriophora* (HRJ) showed maximum virulence and our present findings on pathogenicity of EPN against the larvae of *S. litura* are in accordance with previous results on *S. litura* (Rajkumar *et al.*, 2003). Besides the innate qualities of any EPN species, their bio-efficacy in causing pathogenicity is greatly influenced by their dosage (Hominick & Reid, 1990). Many researchers have reported a positive correlation between the nematode concentration and host mortality (*e.g.*, Forschler & Nordin, 1988; Glazer & Navon, 1990; Peters & Ehlers, 1994; Divya *et al.*, 2010). Differences in pathogenicity of EPN species or strains have also been reported for many other insect species (Forschler & Nordin, 1988; Griffin *et al.*, 1989; Divya *et al.*, 2010; Vashisth *et al.*, 2013) and pathogenicity also depends upon many biotic and abiotic factors like viability, behaviour, invasion and penetration ability of the IJ (Gaugler, 1988; Lewis *et al.*, 1992; Glazer *et al.*, 2001), as well as post-penetration behaviour and reproduction (Kaya & Gaugler, 1993). Thus, different EPN species have been found to differ in their virulence against a specific insect host (Forschler & Nordin, 1988; Griffin *et al.*, 1989). In the present work, the emerging IJ were collected from host cadavers and counted after the host mortality. The data showed that all the three local species were able to invade and propagate within the host and produce IJ, similarly to the commercially available EPN species, *H. indica*. Reproduction and recycling of EPN in host insects plays an important role in their persistence in the soil, and also in their overall effectiveness in insect-pest management (Harlan *et al.*, 1971; Georgis & Hague, 1981). Existing knowledge about the reproduction and recycling of EPN is considered important in determining the time and dose of subsequent EPN application, which may be useful in reducing the cost of application. The data in this study suggest that following application, all the tested species of EPN were able to cause host death and propagate within the insect host and produce IJ. In such studies, the major drawback is that the soil environment makes it impossible to observe EPN *in situ* and non-soil systems only help to understand the behavioural studies (Kaya & Gaugler, 1993).

In conclusion, the evidence obtained in this study suggests that all the three local tested indigenous species of EPN are virulent enough to produce significant mortality to the larvae of *S. litura*. Furthermore, all EPN tested can also reproduce in the infected host and produce first generation IJ. Considering these attributes, a cost-effective and efficient mass production technique is needed for these locally available EPN. These EPN have potential for use as biocontrol agents for the management of *S. litura* under local conditions and hence maybe used in integrated pest management for vegetable crops and flowers under protected structures.

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S. Vashisth, Y.S. Chandel, R.S. Chandel and M. Kalia. Патогенный и репродуктивный потенциал трех изолятов энтомопатогенных нематод из Гималаев и коммерческого изолята *Heterorhabditis indica* против *Spodoptera litura*.

Резюме. Гусениц *Spodoptera litura* заражали 10, 20, 30 и 40 инвазионными личинками (ИЛ) четырех видов энтомопатогенных нематод (ЭПН): *Heterorhabditis bacteriophora* (HRJ), *Heterorhabditis* sp. (HSG), *Heterorhabditis* sp. (HKM) и личинками из коммерческого препарата на основе *H. indica*. Смертность гусениц оценивали после 24, 48, 72 и 96 часов после внесения ИЛ. Все четыре изученных изолята нематол успешно заражали гусениц *S. litura*. Приемлемые уровни эффективности были получены при использовании изолята by *H. bacteriophora* (HRJ), который приводил к 91.7% смертности гусениц третьего возраста *S. litura* после 96 часов обработки. Коммерческий изолят *H. indica* показал 83.0% смертности гусениц. Все исследованные изоляты ЭПН были способны размножаться в теле зараженных гусениц и давать новых инвазионных личинок. Сделан вывод о пригодности всех трех гималайских изолятов, которые давали более 90% смертности гусениц третьего возраста *S. litura*. Наибольшая продукивность рамзножения нематод в гусеницах была достигнута при использовании *H. indica* ($26.3 \pm 3.76 \times 10^3$ ИЛ на гусеницу) и *H. bacteriophora* (HRJ) ($26.0 \pm 1.73 \times 10^3$ ИЛ на гусеницу) при дозе в 40 ИЛ на гусеницу.