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Pathogenicity of *Heterorhabditis* nematodes isolated from north-western Himalaya against the larvae of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae)

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Summary. The efficacy of three entomopathogenic nematodes (*Heterorhabditis* spp.), from north western Himalaya, India was studied against the diamondback moth, *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae), under laboratory conditions. The larvae were exposed to 10, 20, 30 and 40 infective juveniles (IJs) of each nematode species for different time periods and they were found to be susceptible to all the EPNs tested. However, the susceptibility of larvae to nematode infection varied according to the dosages of IJs and their exposure periods. The efficacy of these indigenous entomopathogenic nematodes was also evaluated against the commercially available entomopathogenic nematode *H. indica*. An indigenous isolate, *H. bacteriophora* (HRJ), along with the commercial isolate *H. indica* recorded 100.0% mortality of insect larvae in 96 h exposure time against third instar larvae of *P. xylostella*. However, it was noticed that with the advancement of larval stage its mortality rate reduces and vice versa with the exposure period. All the tested nematode species were also found to reproduce within the host and produced infective juveniles. In conclusion, the evidence obtained in this study suggests that all the three indigenous EPN species are virulent enough to produce 100% mortality of larvae of *P. xylostella*. These EPN species thus have potential for the management of *P. xylostella* under integrated management practices.

Résumé. Pathogénicité des nématodes *Heterorhabditis* isolés du nord-ouest de l'Himalaya contre les larves de *Plutella xylostella* (L.) (Lepidoptera : Plutellidae). L'efficacité de trois Nématodes entomopathogènes (*Heterorhabditis* spp.), du nord-ouest de l'Himalaya, en Inde, a été étudiée contre la Teigne des Crucifères, *Plutella xylostella* (Linnaeus, 1758), dans les conditions de laboratoire. Les larves ont été exposées à 10, 20, 30 et 40 juvéniles infectieux (JI) de chaque espèce de nématodes pendant différentes périodes. Elles ont été jugées sensibles à tous les nématodes entomopathogènes testés. Cependant, la sensibilité des larves à l'infection par les nématodes variait selon la doses de JI et le temps d'exposition. L'efficacité de ces nématodes entomopathogènes indigènes a également été évaluée par rapport à celle du nématode entomopathogène disponible dans le commerce, *H. indica*. Avec un temps d'exposition de 96 h des larves du troisième stade de *P. xylostella*, l'isolât indigène *H. bacteriophora*, ainsi que l'isolât commercial *H. indica*, ont entraîné une mortalité de 100% des larves d'insectes. Cependant, il a été remarqué qu'avec l'avancement de la phase larvaire, le taux de mortalité diminue et le temps d'exposition nécessaire augmente. Toutes les espèces de nématodes testées se sont également retrouvées dans l'hôte et ont produit des juvéniles infectieux. En conclusion, les preuves obtenues dans cette étude suggèrent que les trois espèces de nématodes entomopathogènes indigènes sont suffisamment virulentes pour entraîner 100% de mortalité des larves de *P. xylostella*. Ces espèces ont donc un intérêt potentiel pour la gestion de *P. xylostella* dans le cadre de pratiques agricoles intégrées.

http://www.zoobank.org/urn:lsid:zoobank.org:pub:4EB060AE-5499-4538-A376-C166F99A905C

Keywords: biological control; cole crops; diamondback moth; entomopathogenic nematodes

Himachal Pradesh is a mountainous state in north-western Himalaya located at 30°22′40″–33°12′40″N and 75°45′55″–79°04′22″E, with altitude ranging from 350 to 6975 m asl. There exists huge diversity in agroclimatic conditions, and the climate varies from hot to cold alpine across the state. Himachal Pradesh has recorded the highest growth rate in production of vegetables, fruits and floriculture crops (Sharma et al. 2003). The area under vegetable crops has increased from 23,000 ha in 1990–1991 to 68,865 ha in 2012–2013 with corresponding increase in production from 365,000 to 1,398,048 t, registering compound growth rates of 5.5% and 5.9% per annum, respectively (Anonymous 2014). Vegetable production in the state is characterized by small farmers growing high-value crops. Especially in the last decade, there has been a perceptible transition to cultivation of off-season vegetables under protected environment. Cabbage, cauliflower and cucurbits are three main off-season vegetable crops under protected structures in this region. Of the several insect pests damaging these crops, diamondback

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moth, *Plutella xylostella* (Linnaeus, 1758), is the most important pest of cruciferous vegetables of the family Brassicaceae, causing an average of 25–30% loss in vegetable production (Mehta et al. 2011). To maximize net returns, these vegetables are produced intensively, with indiscriminate use of pesticides in this region (Chandel et al. 2016). Several insecticides have been used indiscriminately for pest control on cole crops under field and greenhouse conditions, which has resulted in development of insect resistance to insecticides (Rao & Murthy 1999). Being high value crops, the introduction of biological pest control agents such as entomopathogenic nematodes (EPNs) has stimulated great interest worldwide on both above- and below-ground pests. Therefore, there is a need to use biocontrol agents for vegetable production to reduce pesticide residues in the crop produce.

Among the other entomopathogens, entomopathogenic nematodes (EPNs) are promising agents for the control of various insect pests (Grewal et al. 2005; Vashisth et al. 2013). EPNs of the genera *Steinernema* Travassos, 1927, and *Heterorhabditis* Poinar, 1976, are obligate pathogens of insects (Poinar 1979) that have been found in many diverse climates throughout the world (Hominick et al. 1996). They possess many of the attributes of an ideal biological control agent, including broad host range, high virulence, host seeking capability, ease of mass production, recycling ability, and are non-hazardous to the environment (Gaugler & Kaya 1990; Kaya & Gaugler 1993). Many species of EPNs belonging to both genera have been used with variable success as biological control agents against insects occupying different habitats (Gaugler 1988). However, most success has been achieved against soil-dwelling pests or pests in cryptic habitats (Williams & Walters 1999; Tomalak et al. 2005; Valle et al. 2008). Numerous studies have found that the susceptibility of different developmental stages of insect hosts shows great variations to different species or strains of EPNs (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). While developing a sustainable management strategy for any local insect pest, it is therefore always advisable to use the right indigenous EPN isolate, as it is more likely to be adapted to the local climatic conditions and host population. In addition, an ideal nematode species or isolate should also have good potential to recycle and propagate in insect host. Recently, surveys for isolation of EPNs were conducted (Vashisth et al. 2015), and virulent strains of *Heterorhabditis* spp. were baited out from soil samples brought from the temperate zone of Himachal Pradesh. In initial experiments, quick knockdown of larvae of *Corcyra cephalonica* (Stainton, 1866) and *Galleria melonella* (Linnaeus, 1758) was observed within 48 h post exposure. Therefore, the present studies were undertaken to study the bioefficacy of EPN species/strains from different regions in Himachal Pradesh for control of *Plutella xylostella*. The virulence of nematodes to diamondback moth larvae and their reproduction in insect host was studied under laboratory conditions.

Material and methods

The abbreviations used in the text are as follows: CSK HPKV, Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya; EPNs, entomopathogenic nematodes; IJs, infective juveniles; dia., diameter.

Nematode sources

Three EPNs originally isolated from Sangla (3215 m asl), Kamand (2421 m asl), and Rajgarh (2119 m asl) 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. For experimental study, the nematodes were reared in the laboratory on late instar larvae of *G. melonella* at 25°C, as described by Woodring & Kaya (1988). The IJs that emerged from wax moth larval cadavers were collected using modified White traps (Kaya & Stock 1997), and stored in darkness at 15°C in deionized water. Before being used for assay, the IJs were allowed to acclimatize for 1 h at room temperature and their viability was checked by observation of movements under a stereomicroscope.

Insect sources

Mass culture of *Plutella xylostella* was maintained on mustard seedlings and cabbage leaves. The adult moths were released in wooden cages (27 × 21 × 21 cm) fitted with glass on three sides and nylon netting on the back side. A small cotton swab saturated with 10% sugar solution was placed in each cage as food for the adults. Four-day-old mustard seedlings grown in small plastic pots were exposed overnight to adults for oviposition. Profuse egg laying was observed on mustard seedlings and eggs were allowed to hatch. Three-day old larvae were transferred to cabbage leaves kept in plastic jars (25 × 20 cm). Fresh leaves were provided regularly depending upon the requirement and the developing larvae shifted from old to fresh leaves at their own. The seedlings were exposed frequently depending upon the requirement of culture and larvae were maintained separately for each exposure date. The full fed larvae pupated on the lower surface of leaves in about 20 days and adults emerged in about 7–8 days. The culture was maintained in insectary at a constant temperature of 25 ± 1°C.

Larval mortality bioassay

The three local EPNs obtained during the survey along with commercially available formulation of *Heterorhabditis indica* Poinar, Karunakar & David, 1992 were tested for their efficacy against *P. xylostella* (Table 1). Larval mortality bioassays were carried out in Petri dishes (9.5 cm dia.) lined with double layer of Whatman No. 1 filter paper, following the methods of Kaya & Stock (1997). Nematodes in 1.0 ml of deionized water were added to the filter paper in concentrations of 10, 20, 30 and 40 IJs/larva. After 30 min, a single larva of *P. xylostella* was placed in each of the Petri dish. The dishes were sealed with parafilm and maintained in a climatic chamber at 27 ± 2°C in the dark. For each nematode species and concentration there were 10

Table 1. Details of entomopathogenic nematodes tested against different larval instars of *Plutella xylostella* (Linnaeus).

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

replicates and the experiment was repeated thrice. Untreated controls were identical to the treatment except that no IJs 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 body colour change of the cadaver to different reddish tones and production of bioluminescence (Grimont et al. 1984; Stock & Goodrich-Blair 2012), due to the presence of different species of symbiotic bacteria (*Photorhabdus*) (Stock 1993).

Reproduction of EPNs

Larvae of *Plutella xylostella* were exposed to 10, 20, 30 and 40 IJs concentrations of each EPN in separate Petri dishes and total number of IJs 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 deionized 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, till the emergence of IJs was stopped from insect cadavers and total number of IJs 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 IJs served as control.

Statistical analysis

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

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

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 $p < 0.05$.

Results

Heterorhaditis bacteriophora Poinar, 1976 (HRJ), *Heterorhaditis* sp. (HSG) and *Heterorhaditis* sp. (HKM) were isolated originally from Rajgarh (2119 m asl), Sangla (3215 m asl), and Kamand (2411 m asl) in north-western Himalaya during 2013–2014. These three isolates along with *H. indica* were evaluated for their virulence at different doses and time intervals required to kill the larvae of *Plutella xylostella*, and also on the basis of nematode's ability to propagate within the body of infected host and produce infective juveniles. The third instar larvae of *P. xylostella* were found to be susceptible to all the three tested local isolates of EPNs and *H. indica* which is commercially available for use in India. However, the degree of susceptibility of larvae to nematode infection varied in a dose-dependent manner. The time taken to produce kill, i.e. the exposure period, also influenced the mortality. The data revealed a positive correlation between the tested doses of infective juveniles and/or time with larval mortality, for all the tested local EPN isolates and *H. indica* (Table 2). The differences in per cent larval mortality of *P. xylostella* for each nematode species, related to the population species (S) ($F = 2.29$, $df = 3$, $p = 0.081$), exposure period (E) ($F = 350.82$, $df = 3$, $p = < 0.001$) and population number (P) ($F = 287.18$, $df = 3$, $p = < 0.001$) were observed to be highly significant. The interaction effect (S × P × E) was found to be non-significant. The mortality of third instar larvae of *P. xylostella* differed significantly in different EPN isolates/species. *H. indica* and *H. bacteriophora* (HRJ) caused maximum larval mortality (100.0%) after 96 h of exposure at a dose of 40 IJs/larva (Figure 1), whereas in *Heterorhabditis* sp. (HKM) and *Heterorhabditis* sp. (HSG), there was increase in mortality with further increase in exposure time beyond 96 h, and reaching 100% only after 120 h at 40 IJs/larva. Among the local isolates of EPNs, *H. bacteriophora* (HRJ) was the most virulent irrespective of exposure time. The efficacy of tested EPNs against fourth instar larvae of *P. xylostella* was significant across the dosages. Maximum mortality was produced by *H. bacteriophora* (HRJ) after 96 h of treatment (76.7%), followed by *H. indica* (72.5%) as shown in Figure 1(B). The data revealed significant differences in per cent larval mortality of *P. xylostella* for each nematode species: S ($F = 0.08$, $df = 3$, $p = 0.969$), E

Table 2. Values of correlation coefficient (r) between concentration/time with mortality of larvae of *Plutella xylostella* (Linnaeus).

	Value of correlation coefficient (r) for III and IV instar larvae			
	Concentration of IJs × mortality at 96 h		Time × mortality for 40 IJs/larva	
Species/strain	III	IV	III	IV
Heterorhabditis sp. (HSG)	0.9698	0.9845	0.9561	0.9927
Heterorhabditis sp. (HKM)	0.9811	0.8994	0.9826	0.9928
H. bacteriophora (HRJ)	0.9798	0.9778	0.9795	0.9891
H. indica	0.9927	0.9965	0.9984	0.9769

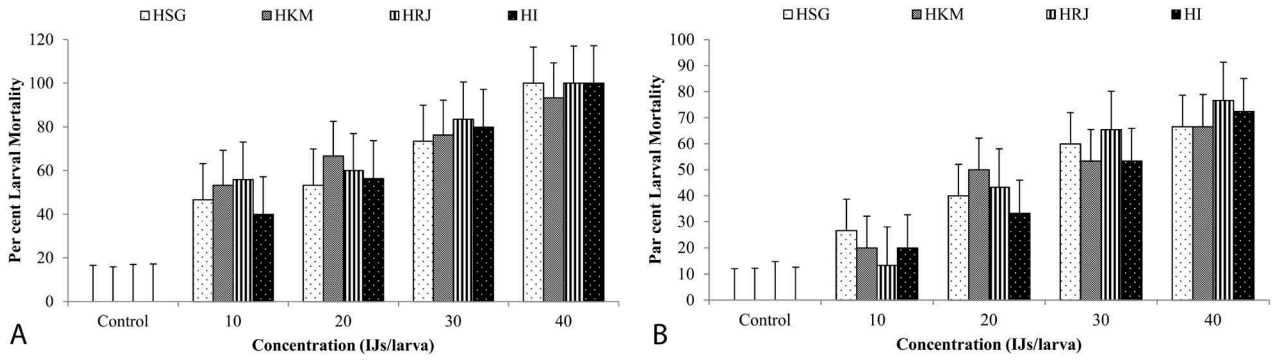

Figure 1. Percentage of mortality of *Plutella xylostella* (Linnaeus) larvae following exposure to different concentrations of infective juveniles (IJs) of nematodes under laboratory conditions after 96 hours of exposure. **A**, Third instar larvae; **B**, fourth instar larvae.

($F = 300.5$, $df = 3$, $p = < 0.001$) and P ($F = 156.89$, $df = 3$, $p = < 0.001$). The interaction effect (S × P × E) was found to be non-significant.

In order to study the reproduction of EPNs, the fourth instar larvae of *P. xylostella* were exposed to 10, 20, 30, and 40 IJs/larva for each nematode species. Following host mortality, the emerging IJs from host cadavers were collected and counted. It was observed that all four tested EPNs exhibited a successful host invasion as manifested by the change in coloration and production of bioluminescence. They further propagated in the insect larvae and production of first generation infective juveniles was clearly evident as shown in Figure 2(A–D). For all nematode species, a linear relationship was computed between the concentrations of IJs applied and total number of IJs produced per larva (Figure 2(A–D)). *H. indica* and *H. bacteriophora* (HRJ) produced significantly more number of infective juveniles per larva than the other two nematode species (Figure 2(A) and 2(D)). The maximum production of infective juveniles per larva for *H. indica* and *H. bacteriophora* (HRJ) was recorded to be $10.20 \pm 1.34 \times 10^3$ IJs/larva and $9.07 \pm 1.14 \times 10^3$ IJs/larva at a dose of 40 IJs/larva, respectively.

Among the four tested EPNs, the least progeny production was recorded for *Heterorhabditis* sp. (HKM). It increased linearly with an increase in concentration of IJs,

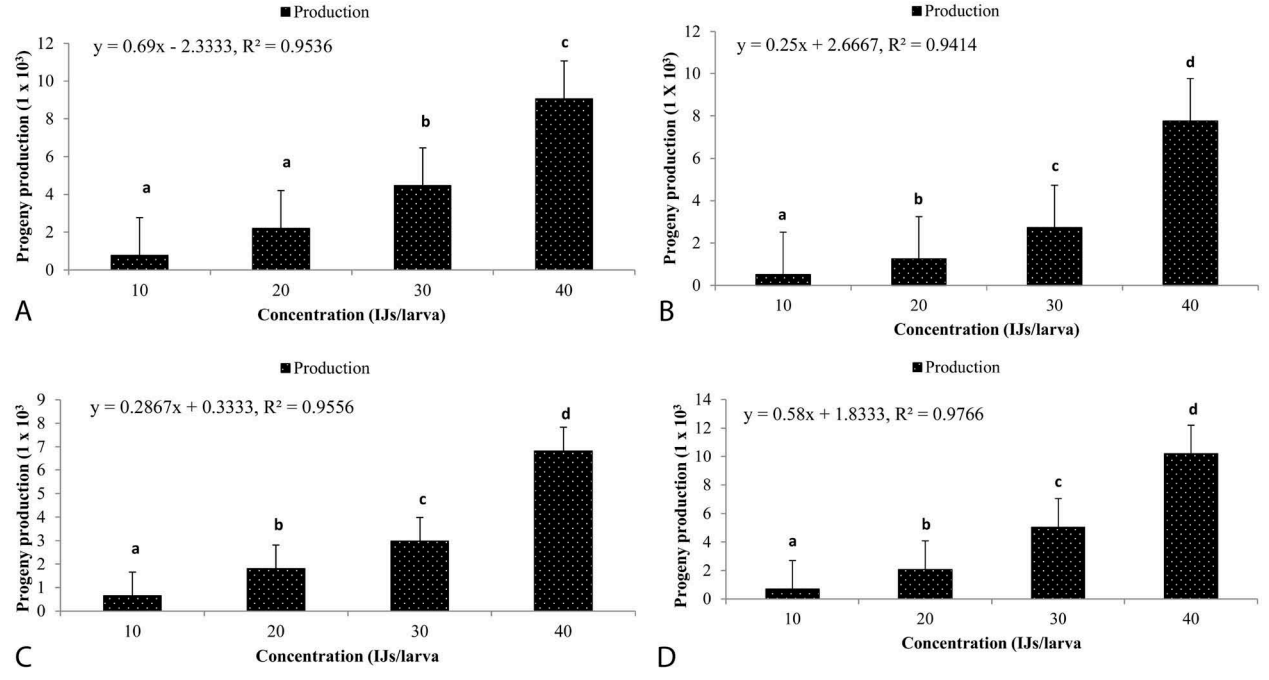

Figure 2. Production of first generation infective juveniles in *Plutella xylostella* (Linnaeus) larvae at different dosages of infective juveniles. **A**, *H. bacteriophora* (HRJ); **B**, *Heterorhabditis* sp. (HSG); **C**, *Heterorhabditis* sp. (HKM); **D**, *H. indica*. $^{**}p < 0.01\%$; means shown by the same letter are not significantly different ($p > 0.05$).

reaching to its maximum ($6.82 \pm 1.04 \times 10^3$ IJs/larva) at a concentration of 40 IJs/larva.

Discussion

The aim of this study was to evaluate the efficacy of EPNs isolated from higher hills of north-western Himalaya against different instar larvae of *Plutella xylostella*, which is a serious pest of cole crops in the north-western parts of India (Bhalla & Dubey 1986; Chauhan et al. 1997), and to compare their efficacy with *H. indica* which is normally recommended against wide range of insect pests in different crop ecosystems (Rishi & Prasad 2012; Vashisth et al. 2013). The *H. indica* which is available in market is indigenous to South India, which has a tropical climate (Prabhuraj & Shivaleela 2005), and is not very effective in north-western Himalaya, with temperate wet climatic conditions. Many species of EPNs have been used with variable success as biological control agents against insects occupying different habitats (Gaugler 1988). However, most success has been achieved against soil-dwelling pests or pests in cryptic habitats (Williams & Walters 1999; Tomalak et al. 2005; Valle et al. 2008). A total of four species/isolates belonging to genus *Heterorhabditis* were tested at doses ranging from 10 to 40 IJs ml^{-1}, and data on mortality were recorded after 24, 48, 72 and 96 h of treatment to find a dose producing desirable mortality, and increase in mortality at a given dose with time. Their ability to propagate within the body of infected host and produce infective juveniles was also studied. Several earlier workers have used these assays to adjudge the efficacy of EPNs against various insect pests (Sims et al. 1992; Ricci et al. 1996; Bhatnagar et al. 2004). In the present study, the larvae of *P. xylostella* were found to be susceptible to all the three tested local EPNs, but the degree of susceptibility of different strains/species varied significantly. There exists a positive correlation between the dose of IJs and host mortality for all tested EPNs. As far as the efficacy of local EPNs against *P. xylostella* is concerned, *H. bacteriophora* (HRJ) was found to be the most virulent species. The present findings on efficacy of EPNs against larvae of *P. xylostella* are in agreement with other insect hosts (Shinde & Singh 2000; Prasad et al. 2012). Hominick & Reid (1990) reported that, besides the intrinsic qualities of the species, efficacy of EPNs is greatly influenced by its dose. There are several similar previous reports by various workers which highlight a positive relationship between nematode concentration and host mortality (Forschler & Nordin 1988; Glazer & Navon 1990; Peters & Ehlers 1994). Differences in infectivity between nematode species/strains have also been documented for many other insect species (Forschler & Nordin 1988; Griffin et al. 1989; Simoes & Rosa 1996), and this variation in pathogenicity against a specific host insect can be attributed to several factors (Forschler & Nordin 1988; Griffin et al. 1989). Kaya & Gaugler (1993) reported that the pathogenicity of EPNs depends upon many biotic and abiotic factors like host invasion, penetration, reproduction, etc. Similarly, Vashisth et al. (2013) also reported that host invasion and penetration by EPNs influence their virulence. The investigations carried out by Gaugler (1988) and Lewis et al. (1992) on virulence of EPNs have also found it to be dependent on factors like host invasion and penetration ability of infective juveniles of nematodes.

Following the host mortality, the emerging IJs were collected from host cadavers and counted. The observations revealed that all three local species were able to invade and propagate within the host and produce infective juveniles. Reproduction and recycling of entomopathogenic nematodes in the host play an important role in their persistence in the soil, and also influence their overall effectiveness in pest control (Harlan et al. 1971; Georgis & Hague 1981). Knowledge about reproduction and recycling of nematodes is considered important in determining the time and dose of subsequent EPN application, which may be useful in reducing the cost of application (Loya & Hower 2003). The evidence obtained in the present study suggested that all tested species of nematodes were able to infect and propagate within the insect host and produce F_1 generation infective juveniles. All three tested indigenous strains/species of EPNs were virulent enough to produce 100% mortality against the larvae of *P. xylostella*; however, overall efficacy of *H. indica* was found to be significantly better. *H. bacteriophora* (HRJ) against third instar larvae was at par with *H. indica*. The present results indicate enough potential for *H. bacteriophora* (HRJ) isolated from Rajgarh having a desirable level of virulence against *P. xylostella*. In *H. indica*, all production and application technology has been thoroughly standardized, whereas for our local strains there is need for in-depth information on their behaviour and application technology to enhance their efficacy and help us understand the potential of these indigenous EPNs, especially *H. bacteriophora* for its success and commercialization against insect pests, and on cole crops.

Conclusion

It can be concluded that *Heterorhabditis indica* was more pathogenic than other species/strains of EPNs to the larvae of *Plutella xylostella*. The most susceptible stage was third instar larvae and optimum dose was 40 IJs/larva. A minimum of 96 h is required to produce mortality. The recently isolated EPN, *H. bacteriophora* (HRJ) is also promising, being the most pathogenic among the local isolates, giving at par mortality with *H. indica*, and this EPN has great potential as a candidate in biological control program of *P. xylostella*. Further studies are required to standardize its

mass production and application technology, which will enhance its insecticidal activity.

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