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Evaluation of the efficacy of six indigenous isolates of entomopathogenic nematodes (*Steinernema* spp. and *Heterorhabditis* spp.) from Haryana, India

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Abstract: *Efficacy of six indigenous isolates of entomopathogenic nematodes (EPN) from Haryana, India, was evaluated against the fourth instar larvae of greater wax moth,* Galleria mellonella *Linnaeus (Lepidoptera : Pyralidae). Two isolates of* Steinernema siamkayai *and four isolates of* Heterorhabditis indica *were tested. The larvae of* G. mellonella *were found to be susceptible to all the nematode strains tested. However, the degree of susceptibility of* G. mellonella *larvae to infection varied among different nematode strains. The larvae of greater wax moth were exposed to 10, 20, 40, 60, 80, 100, 500 and 1000 infective juveniles (IJs) concentration of each nematode strain.* LC_{50} *and* LT_{50} *values were quantified. Among all the* Heterorhabditis indica *isolates, Rohtak isolate was the most virulent (*LC_{50}*29.22 IJs/ larvae at 48 h), whereas the Karnal isolate was the most virulent (*LC_{50}*15.33 IJs/larvae at 48 h) among all the* Steinernema siamkayai *isolates.*

Keywords: *Entomopathogenic nematodes,* Heterorhabditis , Steinernema, *bioassays, LC50, LT50, Haryana, India.*

INTRODUCTION

Application of biopesticides for the management of insect pests is environmentally safe and results in pesticide-free produce. Among biopesticides, entomopathogenic nematodes (EPNs) are promising agents for the control of various insect pests (Grewal *et al*., 2005). EPNs belonging the genera *Steinernema* and *Heterorhabditis* associate with pathogenic bacteria and are obligate pathogens of insects (Poinar, 1979). Occurrences of EPNs have been reported from many diverse climates throughout the world (Hominick *et al.* 1996). Since native species of EPNs are likely to be more adapted to the local climate, they may be more suitable for biocontrol of the target pests is a region. Initially exotic species/ strains of *S. carpocapsae, S. glaseri, S. feltiae,* and *H. bacteriophora* were imported by the researchers for initiating research on entomopathogenic nematodes in India. In many cases, these nematodes yielded inconsistent results in field trials, probably due to their poor adaptability to the local agro-climatic conditions. Because of varied geographic, climatic and weather conditions, India has a rich biodiversity base. Therefore, a number of nematode isolates from different parts of India have been reported (Ganguly, 2003). Two new species, *H. indica* (Poinar *et al*., 1992) from Tamil Nadu and *S. thermophilum* (Ganguly& Singh, 2000, 2003) from New Delhi are among the indigenous nematode isolates.

Other indigenous isolates belonging to different species are *H. bacteriophora* (Sivakumar *et al.,* 1989), *S. bicornutum* (Hussaini *et al*., 2001), *S. carpocapsae* (Hussaini *et al*., 2001), and *S. riobrave* (Ganguly *et al*., 2002). Some of the native populations of *Steinernema* were also identified by restriction fragment length polymorphism (RFLP) analysis and analysis of the PCR- amplified ITS- r DNA region using 17 restriction enzymes by Hussaini *et al.* (2001).

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These results showed that *S. abbasi* and *S. tami* were present in India. In addition, surveys have revealed natural occurrence of several species/strains of *Steinernema* and *Heterorhabditis* in Tamil Nadu (Bhaskaran *et al*., 1994), Kerala (Banu *et al.,* 1998), Andaman and Nicobar Islands (Prasad *et al.*, 2001) and Gujarat (Vyas, 2003). Recently, six EPN isolates were identified during a survey in search of indigenous EPNs in Haryana, India. The virulence of these indigenous strains of EPNs belonging to *Heterorhabditis indica* and *Steinernema siamkayai* against the fourth instar larva of greater wax moth, *Galleria mellonella* nematodes was evaluated under the laboratory conditions.

MATERIALS AND METHODS

Test insect: The greater wax moth, *G. mellonella*, was cultured in lab on the artificial diet under hygienic conditions at room temperature (27 ± 2^0C) and 60% ± 5% relative humidity (RH). The adult moths were supplied with 10% honey solution in mating jars. Folded Tissue paper was provided for egg laying in the jars.

Egg masses (each containing around 450-550 eggs) collected from mating jars were placed on freshly made artificial diet and the insect larvae were allowed to develop to the fourth stage that were used for bioassays.

Preparation of artificial diet: The artificial diet for culturing of Galleria mellonella larvae was prepared using the following constituents:

Part A: Corn flour- 4 parts, Wheat flour- 2 part,s Wheat bran – 2 parts, Milk powder- 2 parts, Yeast extract- 1 part.

Part B: Honey- 2 part, Glycerine- 2 part.

The ingredients of Part A were mixed thoroughly in a pan. The ingredients of Part B were mixed separately in a beaker. The mixture was slowly and continuously added to Part A and mixed thoroughly taking care that no lumps are formed as well the mixture is not too wet. The prepared diet was stored for at least one day before use.

Isolation of nematodes from soil: A total of 315 soil samples were collected from Kurukshetra, Panipat, Rohtak, Hisar and Jind districts during 2014-15. Relatively moist sites were chosen for soil sample collection. About 500 g of soil was collected from each sampling site at a depth of 5-15cm from the soil surface. Out of 315 soil samples, six (1.9%) were found positive for EPNs. Insect baiting technique using late instar larvae of *Galleria mellonella* was adopted for the isolation of EPNs from the soil samples (Bedding & Akhrust, 1975). Out of six positive samples, 4 samples were containing *Heterorhabditis indica* while rest two samples were containing *Steinernema siamkayai* (Table 1). Identification of EPN isolates was done on the basis of morphological and morphometric characteristics. The nematode isolates were cultured and maintained in the lab on *Galleria mellonella*, using standard methods (Kaya & Stock, 1997). Infective juveniles (IJs) were harvested using White's traps (White, 1927) and stored at 15^0C in the BOD incubator prior to being used in bioassays within the first week of emergence.

Nematode strains:

Virulence assays: The virulence assays were carried out *in vitro* in 12-well sterile polystyrene tissue culture plates. Two layered Whatman No.1 filter paper moistened by 100 µl sterile double-distilled water was used for lining each well of the culture plate. The required IJ concentrations were prepared by serial dilution of the stock solution containing 10,000 IJs/µl. The infective juveniles were placed in the wells of tissue culture plate on the filter paper at the rate of 10, 20, 40, 60, 80, 100, 500 and 1000 IJs per well. Distilled water without nematode IJ was added to the control wells. After 15 minutes of adding nematodes, a single test insect (*G. mellonella*) was placed to each well. Each concentration of IJs was replicated 10 times. The plates were incubated at 27 ± 2^0C in a BOD incubator. Insect mortality was recorded every 12 h till 100% insect mortality of *Galleria* larvae or pupation, whichever was earlier. Median lethal concentrations (LC_{50}) and median lethal time (LT_{50}) values were calculated based on mortality data.

Statistical analysis: Probit analysis (Finney, 1971) function using the software SPSS® Statistics

v 21 (IBM Corp. Armonk, NY, USA) was carried out to calculate LC_{50} values. Survival curves prepared by Kaplan-Meier survival analysis were used for calculating the LT_{50} values. The survival curves were compared using the log-rank test using GraphPad Prism 5.0 statistical software. The log-rank test calculates the chi-square value ($\div^2$) for each event time for each group and sums the results which are used to derive the ultimate chi-square to compare the full curves of each group (Rich *et al.,* 2010). The P-value is used to determine the significance of the chi-square value ($\div^2$). The P-value <0.05 denotes significantly different survival curves as compared to control.

RESULTS

In this study, *G. mellonella* larvae were found to be susceptible to all the six EPN isolates tested (Fig. 1 A, B & Fig. 2 A, B).

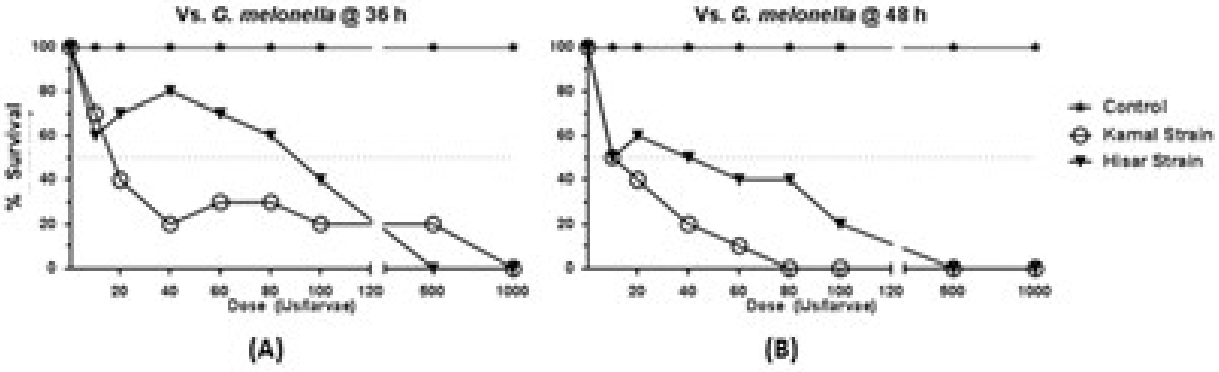

Fig.1: Dose response curves of fourth-instar larvae of *G. mellonella* at (A) 36 h, (B) 48 h upon infection by different concentrations of *Steinernema* IJs. X-axis represents the dose of IJs/ larvae and Y-axis shows the percent survival.

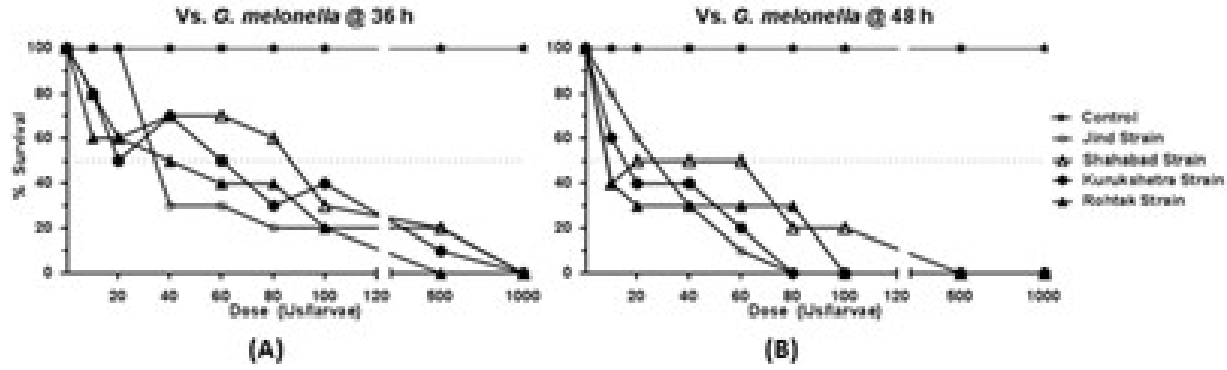

Fig.2: Dose response curves of fourth-instar larvae of *G. mellonella* at (A) 36 h, (B) 48 h upon infection by different concentrations of *Heterorhabditis* IJs. X-axis represents the dose of IJs/ larvae and Y-axis shows the percent survival.

Degree of susceptibility of *G. mellonella larvae* to EPN infection varied from strain to strain and the exposure time. All the EPN strains studied exhibited a positive correlation between the doses of infective juveniles and larval mortality time. Fourth instar larvae of *G. mellonella* suffered different levels of mortality with different doses of the six entomopathogenic nematode strains. Median lethal concentration (LC_{50}) at 36 and 48 h and the median lethal time (LT_{50}) for each concentration of the nematode IJs were calculated.

Table 1: Entomopathogenic nematode isolates used in this study

Genus	Isolates	Origin (locality/state)	Nematode identity
Steinernema	Karnal	Karnal, Haryana, India	*S. siamkayai*
Steinernema	Hisar	Hisar, Haryana, India	*-do-*
Heterorhabditis	Jind	Jind, Haryana, India	*Heterorhabditis indica*
Heterorhabditis	Shahabad	Shahabad, Haryana, India	*-do-*
Heterorhabditis	Kurukshetra	Kurukshetra, Haryana, India	*-do-*
Heterorhabditis	Rohtak	Rohtak, Haryana, India	*-do-*

Table 2: Median lethal concentration (LC_{50}) values of entomopathogenic nematode isolates against fourth-instar larvae of *Galleria mellonella*.

Strains	LC_{50}(36h)	LC_{50}(48h)
Steinernema sp.(Karnal isolate)	15.53(1.24-35.7)	12.48(3.8-19.9)
*Steinernema*sp.(Hisar isolate)	62.71(16.0-288.6)	25.5(8.4 - 46.2)
*Heterorhabditis*sp.(Jind isolate)	48.13(1.44 – 222.36)	22.12(14.38 – 30.12)
*Heterorhabditis*sp.(Shahbad isolate)	70.15(36.73 – 137.1)	18.18(3.25 – 36.1)
*Heterorhabditis*sp. (Kurukshetra isolate)	45.37(22.2 – 80.5)	16.37(6.3 – 25.5)
*Heterorhabditis*sp.(Rohtak isolate)	29.22(12.1 – 50.2)	9.1(0.28 – 21.3)

(Note: The LC_{50}values were calculated by Probit analysis using SPSS software. Numbers in parenthesis represent 95%confidence limits.)

Table 3: Median lethal time (LT_{50}) of entomopathogenic nematode isolates against the fourth-instar larvae of *Galleria mellonella*

Dose(IJs/larvae)	LT_{50}values					
	Steinernema (Karnal isolate)	*Steinernema* (Hisar isolate)	*Heterorhabditis* (Jind isolate)	*Heterorhabditis* (Shahbad isolate)	*Heterorhabditis* (Kurukshetra isolate)	*Heterorhabditis* (Rohtak isolate)
10	36	36	60	48	48	42
20	30	48	48	42	42	48
40	36	48	36	48	48	42
60	36	48	36	54	42	36
80	36	48	36	48	24	36
100	24	24	30	30	24	18
500	12	12	30	24	12	12
1000	12	12	18	18	12	12
χ^2(log-rank test)	48.5	65.2	63.9	66.3	69.1	63.4
P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

(Note: The LT_{50} values were calculated from the Kaplan –Meier survival curves. The survival curves were compared using the log-rank test χ^2 (chi-square) value at P=0.05).

The data revealed that after 36 h of infection, median lethal concentration (LC_{50}) of Karnal isolate of *Steinernema siamkayai* isolate (15.53 IJs/larva) was lower than Hisar isolate (62.71 IJs/larva) and at 48 h post infection, Karnal isolate was identified as the most virulent *Steinernema* isolate with LC_{50} 12.48 IJs/ larva, significantly lower than Hisar isolate (25.50 IJs/larva) (Figure 1(A) and 1(B), Table 2). LT_{50} analysis revealed that Karnal isolate was quicker in killing the insect with lower LT_{50} as compared to Hisar isolate (Table 3). Hence, among *Steinernema* isolates, Karnal strain was found to be most pathogenic on *G. mellonella*.

At 36-h post-infection, among the *Heterorhabditis indica* isolates, Rohtak isolate was identified as the most virulent with LC_{50}29.22 IJs/ larvae, significantly lower than all other tested *Heterorhabditis* isolates and at 48 h post-infection, the LC_{50} values for Rohatk isolate was again found significantly lower than other *Heterorhabditis* isolates (Figure 2(A) and 2(B), Table 2). The LT_{50} values for the *Heterorhabditis* isolates showed that the Rohtak isolate was the quickest in killing *G. mellonella* with the lowest LT_{50} values as compared to other *Heterorhabditis* isolates (Table 3), suggesting that it was the most virulent among all the tested *Heterorhabditis* isolates.

DISCUSSION

The aim of this study was to evaluate the efficacy of six indigenous isolates of EPNs (four isolates of *Heterorhabditis indica* and two isolates of *Steinernema siamkayai*) isolated from Haryana, India against the fourth instar larvae of greater wax moth. Dose and time required to cause insect mortality by each EPN isolate were used for determining virulence of each isolate. These bioassays have been used in many previous studies to evaluate the efficacy of EPNs against various insect pests (Bhatnagar *et al*., 2004; Phan *et al*., 2005). In the present study, *G. mellonella* larvae showed a high susceptibility to all the six tested nematodes. The EPN isolates showed different levels of virulence against *G. mellonella* as indicated by their LC50 and LT50 values. The dose of IJs applied and insect mortality showed a positive correlation for all the EPN strains studied. On the basis of LC_{50}, Rohtak isolate of *Heterorhabditis indica* and Karnal isolate of *Steinernema siamkayai* emerged out to be most effective.

The positive correlation between the dose of infective juveniles and host mortality has also been recorded in many previous studies (Glazer & Navon, 1990; Peters & Ehlers, 1994; Kumar *et al*., 2015). As shown in the present study, many insect hosts have also shown differences in the infectivity among nematode strains (Forschler & Nordin, 1988; Griffin *et al*., 1989). The pathogenicity of EPNs is a complex process. It depends upon many biotic and abiotic factors, like host invasion, penetration, reproduction, environmental conditions etc. (Kaya & Gaugler 1993). Hence, owing to one or other biotic or abiotic factors, different nematode species and isolates showed differential behavior in their pathogenicity against a specific insect host (Forschler & Nordin, 1988; Griffin *et al*., 1989). In many studies, host invasion and penetration ability of the nematode species has been found to be affecting virulence of EPNs (Gaugler, 1988; Lewis *et al*., 1992; Glazer *et al*., 2001).

Present study suggests that the virulence behavior of an EPN isolates from a collection of isolates/strains can be assessed using *G. mellonella* as suitable primary model insect. However, this hypothesis should be further validated by testing more number of different insect species

In conclusion, our findings demonstrate that all the six indigenous isolates of EPNs are virulent to *G. mellonella* larvae, however, Karnal isolate of *Steinernema siamkayai* and Rohtak isolate of *Heterorhabditis indica* show better efficacy compared to other isolates of *Steinernema siamkayai* and *Heterorhabditis indica*, respectively. It may, therefore, be concluded from this study that these EPN isolates may have good potential as biocontrol agents against crop pests.

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