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Survival of Earthworms Exposed to the Entomopathogenic Nematodes *Steinernema carpocapsae* and *Heterorhabditis indica*

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ABSTRACT: Survival and infectivity of entomopathogenic nematodes (EPNs), *Steinernema carpocapsae* and *Heterorhabditis indica* were studied after passing the nematodes through the earthworm's, *Eudrilus eugeniae* gut. Infectivity was evaluated against first instar grubs of rhinoceros beetle, *Oryctes rhinoceros*. Both the species of EPNs had no deleterious effects on earthworms. *S. carpocapsae* and *H. indica* were recovered from the casts of earthworms. Infective juveniles (IJs) of both the EPNs species were *successfully transmitted by young and adults of E. eugeniae. M*ore than 50% of IJs recovered from the earthworm casts were viable and pathogenic to first instar grubs of *O. rhinoceros.* Although entomopathogenic nematodes had no deleterious effects on earthworms, their passage through *E. eugeniae* gut affected their mobility but not their virulence. Combined application of earthworms with entomopathogenic nematodes may enhance levels of inundative or inoculative biocontrol.

Key words: Interaction, *Heterorhabditis indica*, *Steinernema carpocapsae*, *Eudrilus eugeniae*, *Oryctes rhinoceros*

Earthworm (Annelida: Oligochaeta) are generally among the largest contributors to soil fauna biomass and are important for their contributions to organic matter decomposition and decomposition, nutrient mineralization soil profile development and soil macrospore development (Blair *et al*., 1995; Lee, 1985). Decomposition of organic matter under varying environmental conditions is a fundamental feature of terrestrial ecosystems. In vermicomposting, complex interactions between the organic waste, microorganisms, earthworms and other soil fauna animals result in the biooxidation and stabilization of wastes. A large variety of microorganisms and soil invertebrates proliferate and interact contributing to the cycle of matter in vermicomposting. Earthworms improve soil conditions (aeration, drainage and organic matter content) and they are able to change soil structure, move large amounts of soil and affect microfloral and faunal diversity (Brown, 1995; Doube and Brown, 1998). Many vertebrate and invertebrate animals can prey on earthworms and earthworm can survive as a host for a number of parasitic and pathogenic organisms (Curry, 1998). These include bacteria, fungi, protozoa, rotifers, platyheliminths, nematodes, mites and dipterous larvae (Edward and Bohlen, 1996). Entomopathogenic nematodes (EPNs) in the families, Steinernematidae and Heterorhabditidae (Rhabditida: Steinernematidae) lethal obligatory parasites of insects (Ishibashi and Choi, 1991), are found in soils throughout the world (Kaya and Gaugler, 1993; Kaya *et al*., 1993; Stuart *et al*., 2006) and are promising agents for control of soil dwelling insects (as soil application) as well as above ground insects (foliar spray) in cryptic habitats Arthurs *et al*., 2004; Shapiro-Ilan *et al.*, 2006). The only life stage typically found outside the host cadaver is the third-stage infective juvenile (IJ), a non-feeding, environmentally resistant "dauer" larva. The IJ exhibits species-specific foraging strategies that range from ambushing to cruising and actively responds to potential insect hosts, which it invades (Lewis *et al*., 2006). The aim of this study was to determine the pathogenicity of the EPNs, *Steinernema carpocapse* and *Heterorhabditis indica* against epigeic earthworm *Eudrilus eugeniae* (Oligochaeta: Lumbricidae) and to study whether *S. carpocapsae* and *H. indica* IJs are virulent against rhinoceros beetle, *Oryctes rhinoceros* (Coleoptera: Scarabaeidae) after their passage through *E. eugeniae* gut.

MATERIALS AND METHODS

S. carpocapsae and *H. indica* from the live nematode culture of the Department of Crop Protection, Central Plantation Crops Research Institute (CPCRI) Kasaragod, India, were used in this study. The nematodes were reared at 20°C in last instar larvae of wax moth, *Galleria mellonella* L. (Pyralidae), according to Woodring and Kaya (1988). The IJs emerging from the wax moth larval cadavers were collected in deionized water using a modified White's trap, and stored in darkness at 15°C (Kaya and Stock 1997). Nematode IJs emerging from the *G. mellonella* larvae within 3 d from the first day of emergence were collected and were kept in a tissue culture flask at 15°C. Nematode viability was 100%. Unless otherwise stated, IJs within a week of harvest were used in all experiments.

Adults of *O. rhinoceros* were originally collected by using pheromone traps from coconut planation infested with *O. rhinoceros* at CPCRI farm Kasargod, India located at 12°302 N latitude and 75°02 E longitude at an altitude of 10.7 m above mean sea level. After their catch, they were also confined inside rectangular plastic boxes (15 × 20 × 10 cm). These boxes were filled with a thick layer of 3-5 cm of friable material,collected from natural breeding sites, in the goal to create a middle like inside infested parts of palm trees where couples burrowed to copulate. These boxes were kept at room temperature for 7-8 days. Thereafter females were transferred to another set of boxes filled with friable material (semi-decomposed vermicompost and dry coconut petiole, of less than 3 mm in size). This material was mainly used for females which burrowed themselves inside to lay eggs. Boxes were daily monitored twice (in the morning and in the afternoon); during these operations content of each box was sieved separately to collect fresh eggs laid inside the substrate. Then, substrate was returned inside the box with female to continue its oviposition activity. Eggs collected in the same time, never directly handled, were transferred together, by group of 10 maximum, to small rectangular plastic boxes (5 × 10 × 5 cm) containing a 3 cm thick layer of the same friable material used for oviposition and were used as incubator. Then sufficient numbers of first instar of rhinoceros grubs were used for the study.

Eudrilus eugeniae was obtained from the vermicomposting units maintained at CPCRI, Kasaragod. Adult and young individuals were used in the bioassay.

Pathogenicity of EPNs against *Eudrilus eugeniae*

To collect the intestinal content before the assays, adult and young individuals of *E. eugeniae* were rinsed in tap water and placed on damp filter paper in 9 cm diameter Petri dishes for 24h at 18°C (Hartenstein *et al.,* 1981). The virulence of *S. carpocapsae* and *H. indica* against *E. eugeniae* were carried out adding 0, 1000, 3000, 5000, and 7000 IJs/earthworm on 5 g of sterilized semi-decomposed vermicompost substrate in a 5-cm diameter Petri dish. The assay consisted on 80 young and 80 adult individuals of *E. eugeniae* (two individuals per dish), using 80 Petri dishes, ones without nematodes were used as control. The Petri dishes were incubated at 22 ± 2°C for 24 h. After nematode treatment, the earthworms were carefully rinsed in distilled water. The earthworms were individually transferred to 4 cm x 1 cm diameter tubes containing 500 µl distilled water to obtain the first 24 h cast (cast I). Later, earthworms were again transferred to a new tube to obtain the second 24 h cast (cast II), and finally were transferred to Petri dishes with damp filter paper. The nematode transmission through the earthworm gut was assessed as an accumulative percentage of earthworms releasing IJs in their casts. The total number and percentage of mobile IJs in casts I and II were also recorded. The experiment was arranged in a complete randomized design. Each treatment was replicated four times and repeated once.

Pathogenicity of EPNs against first instar of *Oryctes rhinoceros* grubs

The experiment was conducted in 25-ml plastic cups (4.5 cm diameter. 3 cm height; surface area: 15.9 cm^2) containing 10 g autoclaved semi-decomposed vermicompost (pH 5.98 and 52% moisture). First instar grubs of same length (2 cm) and weight (0.27mg) were collected from the stock culture and single grub was released into each cup. Grub that did not enter the soil within 2 h were replaced. After passing through the earthworm gut, virulence of EPNs was assessed against Ist instar grubs of *O. rhinoceros* by pipetting 0.5 ml of

distilled water containing 0, 25, 50, 100 or 150 IJs/grub onto the semi-decomposed vermicompost surface of each cup. Control cups received same amount of water only. All cups were covered loosely with lid to facilitate gaseous exchange and kept at room temperature at 25-30°C. Larval mortality was assessed 72 h after treatment and dead First instar were kept on White's trap to observe nematode emergence from nematode-killed grubs. The experiment was arranged in a complete randomized design. Each treatment was replicated three times with 4 cups/replicate (12 cups/treatment and 36 grubs /nematode species) and repeated once.

Statistical analysis

Probit analysis was used to calculate LC_{50} and LC_{90} values (numbers of IJs/individual causing 50 % and 90 % mortality) and to calculate the respective 95% confidence intervals. The arcsine transformation was used to normalise the percentage nematode survival (after passing through earthworm gut) before an ANOVA was conducted. Analysis was undertaken on the transformed data and back transformed data only is presented. Nematode inoculation rates, nematode species, earthworm stages and casting time and their interactive effects on nematode transmission and their percent survival after passing through earthworm gut were subjected to a multifactor analysis. When ANOVA was significant, comparisons of relevant means were made using the Turkey's significance test values at the 5% level of significance. All statistical evaluations were performed using PROC. GLM (SAS software, version 9.3, SAS institute).

RESULTS AND DISCUSSION

Virulence of entomopathogenic nematodes against first instar grubs of rhinoceros beetle

LC_{50} values of two EPN species, *S. carpocapsae* and *H. indica* to first instar grubs of *O. rhinoceros* ranged from 60-70 IJs/grub respectively. Whereas the LC_{90} values for the *S. carpocapsae* and *H. indica* ranged from 114-140 IJs/grub respectively. These results indicate that *S. carpocapsae* and *H. indica* were effective in controlling first instar grubs of *O. rhinoceros* when applied to the semi-decomposed vermicompost surface. Patil *et al*., (2014). Also reported that *S. carpocapsae* and *H. indica* were highly virulent to neonate grubs. The LC_{50} and LC_{90} values calculated from the bioassay at 72 h after treatment are summarized in Table 1.

Virulence of *S. carpocapsae* and *H. indica* against *E. eugeniae*

No earthworm mortality was recorded when two EPN species *S. carpocapsae* and *H. indica* were inoculated @ 3000-7000 IJs/earthworm. These results revealed that *S. carpocapsae* and *H. indica* were not pathogenic to *E. eugeniae.* Although the biological bases of non-susceptibility of earthworms to EPNs are scarcely studied, others authors also observed the non-susceptibility of earthworms to *Steinernematids* (Capinera *et al.,* 1982; Nguyen and Smart, 1991; Shapiro *et al.,* 1993) and to the slug-parasitic nematode, *Phasmarhabditis hermafrodita* (Grewal and Grewal, 2003). No reports were available regarding the non-susceptibility of earthworm to *H. indica.* In this study we have documented the non-susceptibility of earthworms to *H. indica.*

Transmission of *S. carpocapsae* and *H. indica* by *E. eugeniae*

The EPNs, *S. carpocapsae* and *H. indica* were successfully transmitted by young and adults of *E. eugeniae.* Statistical differences were not observed within young and adult earthworms to the nematode species for cast I and cast II. However, the number of IJs observed in cast I was significantly higher than cast II (Fig. 1 and 2). These results indicate that the transmission of IJs by young and adult earthworms was significantly ($P < 0.05$) higher in the first 24h. These results are similar to previous reports by Campus-Herrera *et al.,* (2006).

The nematode transmission by both young and adults of *E. eugeniae* was significantly ($P < 0.05$) increased with increasing IJs inoculation rates to *E. eugeniae* (Fig. 1 and 2). Irrespective of the nematode species on an average 98 IJs were observed in cast I, when earthworms were inoculated with 7000 IJs/earthworm (Fig. 1). Out of 7000 IJs we could get only 148 IJs in both cast I and

Table 1. Mean number of nematodes required to cause 50% (LC_{50}) and 90% (LC_{90}) mortality of first instar grubs of rhinoceros beetle, *Oryctes rhinoceros* at 72 hours after treatment

Nematode species	LC50	95% FL	LC90	95% FL	Slope ± SE	Goodness of fit test	
						X^2	$P > X^2$
Sc	137	87-737	576	233-127371	2.05 ± 0.76	7.22	0.0072
Hi	183	107-6698	838	278-1216770	1.94 ± 0.80	5.83	0.0158

Sc=*Steinernema carpocapsae*, Hi=*Heterorhabditis indica*

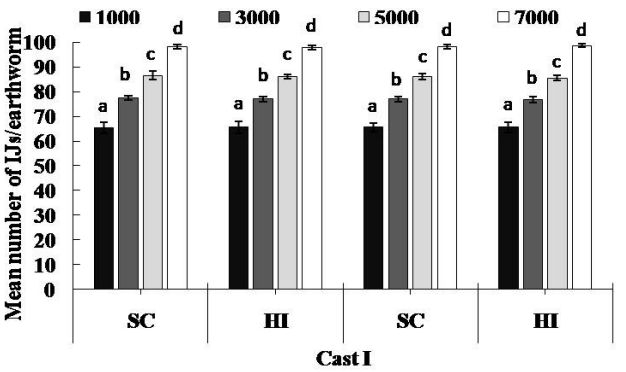

Fig. 1. Average number of *Steinernema carpocapsae* and *Heterorhabditis indica* IJ's transmitted by young and adults of *Eudrilus eugeniae* at 24 h post-exposure (cast I). Nematode inoculation rates, 1000, 3000, 5000 or 7000 IJs/earthworm. Different letters on the top of error bars indicates statistically different values for different IJ's inoculation rates at ($P < 0.05$) using Tukey' test. Error bars indicate standard error (n=4)

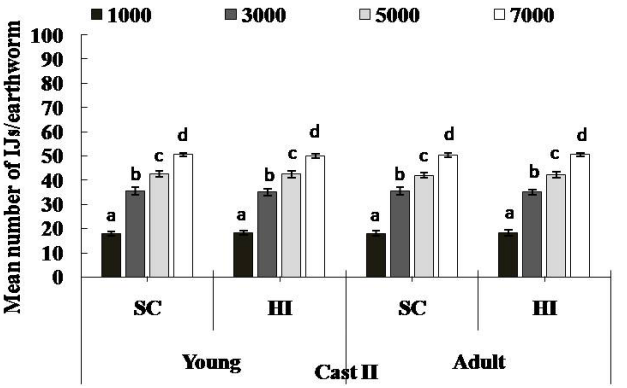

Fig 2. Average number of *Steinernema carpocapsae* and *Heterorhabditis indica* IJ's transmitted by young and adults of *Eudrilus eugeniae* at 48 h post-exposure (cast II). Nematode inoculation rates, 1000, 3000, 5000 or 7000 IJs/earthworm. Different letters on the top of error bars indicates statistically different values for different IJ's inoculation rates at ($P < 0.05$) using Tukey' test. Error bars indicate standard error (n=4)

cast II, remaining IJs might have carried through *E. eugeniae* surface that we have not recorded in this study. MacMillan *et al.,* (2009) reported that the increased dispersal of nematode, *Phasmarhabditis hermaphrodita* by earthworm, *Lumbricus terrestris* could be attributed to the nematode attachment to the earthworm surface. Similarly, Shapiro *et al.,* (1993) also reported that nematodes were found on the exterior surface of earthworms. Among the nematode species there was no significant difference in nematode transmission by *E. eugeniae.* The analysis of variance revealed a significant two-way interaction between cast and IJs inoculation rates ($P < 0.05$).

Infective juvenile's activity after passing through earthworm gut

In present investigation we found that the mobility of *S. carpocapsae* and *H. indica* IJs were reduced after passage through the *E. eugeniae* gut. On an average of 70% of nematodes were active after passage through *E. eugeniae gut* (Table 2). The remaining 30% reduction in survival of nematodes after passage through earthworm gut probably due to effect of digestive enzymes (Campus-Herrera *et al.,* 2006). Exact mechanism by which earthworms influence the nematodes remain unknown. Although some of them survive through the gut passage

Table 2. Percentage mobility of IJs of *Steinernema carpocapsae and Heterorhabditis indica* after passage through young and adults of *Eudrilus eugeniae*

Earthworm casts	IJs/earth-worm	Mobile IJs (%)				IJs/earthworm Mean
		Steinernema carpocapsae		*Heterorhabditis indica*		
		Young	Adult	Young	Adult	
I	1000	70.9 ± 0.1	70.6 ± 0.2	70.6 ± 0.2	70.6 ± 0.3	70.7
	3000	70.5 ± 0.2	70.4 ± 0.2	70.4 ± 0.3	70.7 ± 0.2	70.5
	5000	70.8 ± 0.5	70.7 ± 0.4	70.9 ± 0.6	70.8 ± 0.5	70.8
	7000	70.9 ± 0.3	70.7 ± 0.5	70.1 ± 0.1	70.8 ± 0.4	70.6
	Mean	70.77	70.6	70.5	70.72	
II	1000	70.8 ± 1.0	69.6 ± 1.4	69.8 ± 0.9	69.8 ± 0.7	70.0
	3000	70.4 ± 0.4	70.4 ± 0.7	70.7 ± 1.1	70.0 ± 0.3	70.3
	5000	70.6 ± 0.2	70.3 ±0.5	70.6 ± 0.7	70.4 ±0.6	70.4
	7000	70.3 ± 0.5	70.6 ±0.2	70.5 ±0.4	70.8 ±0.3	70.6
	Mean	70.52	70.22	70.4	70.25	
P value						
Earthworm (E)		0.5704	E*C	0.5534		
Cast (C)		0.1420	E*IJs	0.6845		
IJs/E (IJs)		0.7052	E*ES	0.5367		
EPN species (ES)		0.7524	C*IJs	0.7052		
E*C*IJs*ES		0.9998				

For each IJs/earthworm, the data are means of four replications ± SE.

(Double *et al.,* 1994; Moody *et al.,* 1996; Stephens *et al.,* 1995). Similarly, Shapiro *et al.,* (1993) also found the live nematodes in earthworm casts. We found nematodes pass through the earthworm's gut system, uninjured, emerging in the castings alive and active. The nematodes may be passed through the worm digestive system and still remain viable upon release (Shapiro *et al.,* 1995). Nematodes that pass unharmed through the earthworm gut or able to take advantage of or adapt to earthworm-induced changes in soil properties and processes may be dispersed by earthworms. Nematodes were recovered from the surface, interior, and casts of earthworms. Therefore, nematodes may have a phoretic association with earthworms (Shapiro *et al.,* 1995).

The nematode virulence against first instar grubs of *O. rhinoceros* was 100% even after passage through *E. eugeniae* gut. Shapiro *et al.,* (1993) reported that the nematodes found in the casts and debris of earthworm were able to infect and reproduce in the larvae of *G. mellonella.* In the case of plant parasitic species, this could lead to potential problem but for EPNs commonly used in insect pest biocontrol this may be beneficial (Shaprio *et al., 1993).* These results revealed that nematodes passage through *E. eugeniae* gut affected their mobility but not virulence. There is no significant interaction between earthworm, cast, nematode species and IJs inoculation rates (Table 2). Growth and development of *O. rhinoceros* grubs are mostly taking place in vermicomposting pits, such habitats are considered as a most favourable niche for enhancing the infectivity, survival persistence of EPNs, as these environments minimized nematode death from ultraviolet radiation and desiccation. These results suggested, it is good to combine EPNs and earthworms to achieve superior pest control.

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