Vol. 44, No. 1, pp. 73-81 Indian Journal of Nematology June, 2014

Virulence of *Steinernema carpocapsae* and *Heterorhabditis indica* against Coconut Rhinoceros Beetle, *Oryctes rhinoceros* L. (Scarabaeidae: Coleoptera)

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Received on 31-05-2014 and Accepted on 06-06-2014

ABSTRACT: Biological control potential of two entomopathogenic nematodes, *Heterorhabditis indica* and *Steinernema carpocapsae*, was tested against neonate-, and third-instar grubs of *Orycuts rhinoceros* in laboratory and micro-plot experiments. The main aim of the study was to develop an efficient sustainable control method against the pest. With this we could develop a strategy of coconut production with the intention of diminishing or even preventing the appearance of pest resistance to insecticides.In the laboratory experiments, *S. carpocapsae* and *H. indica* were highly virulent to neonate grubs. Neonate grubs were susceptible to *S. carpocapsae* followed by *H. indica.* The virulence of the nematode species relative to each other differed greatly to neonate grubs but not to the 3rd instar grubs. In all the experiments,mortalityof rhinoceros grubs varied significantly among nematode dose and days after treatment. In comparison of neonate grubs mortality, 3rd instar grubs require more number of nematodes. The dosage and time mortality relationship of *S. carpocapsae* and *H. indica* against the neonate and 3rd instar stage of *O. rhinoceros* indicated that as the dosage increased the susceptibility also increased. The susceptibility of the developmental stages of *O. rhinoceros* differed greatly among tested concentrations of nematode species and time. Our observations, combined with those of previous studies on other nematode and white grub species, show that nematode virulence against rhinoceros grub developmental stages varies with time and nematode concentration.

Key words: Coconut palm, rhinoceros beetle, neonate, *Steinernema carpocapsae*, *Heterorhabditis indica*, mortality, vermicompost, developmental satges.

The rhinoceros beetle, *Oryctes rhinoceros* L. (Coleoptera: Scarabaeidae) is a serious pest of coconut throughout India and Southeast Asia (Nair *et al.*,1997). Eggs are laid in manure pits or other organic matter and hatch in 8-12 days. Larvae take another 82-207 days before entering an 8-13 day non feeding prepupal stage. Pupal stage lasts for 17-28 days. Adults remain in the pupal cell for 17-22 days before emerging and flying to palm crowns to feed. The beetles are active at night and hide in feeding or breeding sites during the day. Adults damage palms by boring into the centre of the crown and unopened fronds and spathes and this damage causes yield loss up to 10 per cent in production of nuts (Nair *et al*., 1997). The damage caused by rhinoceros beetle larvae provide entry points for lethal secondary attacks by the red palm weevil, *Rhynchophorus ferrugineus* and entry of fungal pathogens (Renou *et al*., 1998).

No single method had proved to be successful in suppressing the pest population. Current management of *O. rhinoceros* across the India relies upon the integration of mechanical, cultural, chemical methods, semiochemical approaches and use of bio-control agents such as *Baculovirus oryctes* and *Metarhizium anisoplae* are being practiced as bio- control measures. Any attempt to scale down the use of chemical insecticides is welcome considering and the safety to flora and fauna of the environment. Due to environmental and regulatory pressures, research toward developing alternative pest control measures are warranted (Tomerlin, 2000). Therefore, identifying bio-control agents that control the

soil-dwelling life stages of insects is of paramount importance for the development of successful biological control against *O. rhinoceros*. However, lacking complete research dataon efficacy of promising EPN strains against *O. rhinoceros* populations composed of different soil-dwelling development stages. Use of EPNs could offer an effective and safe alternatives to chemical insecticides (Friedman, 1990; Ehlers, 1996).

Entomopathogenic nematodes (EPNs) have potential as bio-control alternatives for *O. rhinoceros* suppression. EPNs in the families, Steinernematidae and Heterorhabditidae are lethal obligatory parasites of insects (Ishibashi & Choi, 1991), are found in soils throughout the world (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).

Growth and development of *O. rhinoceros* grubs mostly take place in manure/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 (Poinar, 1990). In this contrast two species of indigenous EPN strains, *Steinernema carpocapsae* and *Heterorhabditis indica* were evaluated against different stages of *Oryctes rhinoceros* larvae under laboratory condition.

MATERIALS AND METHODS

Nematodes: Two nematode species, *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. These nematodes were propagated in at room temperature on final instar wax moth, *Galleria mellonella* (L.) larvae (Kaya & Stock 1997). Nematode infective juveniles emerging from the *G. mellonella* larvae within 3 day 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 the experiments.

Insects: 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 with male and females 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 on. 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) used as incubator and containing a 3 cm thick layer of the same friable material used for oviposition. Then sufficient numbers of neonates (three day old having size of 2-3.5 cm and weight of 0.197-0.312 g) of rhinoceros grubs were used for the study.

The average size and weight 3rdinstar (8-10.5cm, 10-12.5g) grubs were collected from the vermicomposting units maintained at Central Plantation Crops Research Institute farmKasargod located at 12°302 N latitude and 75°02 E longitude at an altitude of 10.7 m above mean sea level. None of the vermicomposting units had been treated with any biococntrol agents during the previous year. Only apparently healthy grubs were used in the bioassays.

Efficacy of EPNs against neonates of *Oryctesrhinoceros* grubs

The experiment was conducted in 25-ml plastic cups (4.5 cm diam. 3 cm height; surface area: 15.9 cm^2)

containing 10g autoclaved semi-decomposed vermicompost (pH 5.98 and 52% moisture).A single neonatewas released into each cup,and grubs that didnot enter the vermicompost within 2 hour (h) were replaced. The virulence of *S. Carpocapsae* and *H. indica* to neonate grub was determined by pipetting 0.5 ml of distilled water containing 50, 100, 150, 200 and 250 IJs onto the semi-decomposed vermicompost surface of each cup. Control cups received same amount of water only. Themoisture content of the semi-decomposed vermicompostwas 12%. All cupswere covered loosely with lid containing to facilitate gaseous exchange and kept at room temperature at 25-30°C.Grubmortalitywas assessed at48, 72 and 96hours (h) after exposureand dead neonates werekept on White traps to observe nematode emergence from nematode-killedinsects. The experiment was arranged in a complete randomized design.Each treatment was replicated three times with 15cups/replicate (45cups/treatment)and repeated once.

Efficacy of EPNs against 3rd instar of *Oryctes rhinoceros* grubs

The experimentwas conducted in 250-ml plastic cups (5.5 cm diam. 7 cm height; surface area: 30 cm^2) containing 50g autoclaved semi-decomposed vermicompost (pH 5.98 and 52% moisture). A single 3rd instarreleased into each cup, and grubs that didnot enter the vermicompost within 2 h were replaced. The virulence of *S. carpocapsae* and *H. indica* to 3rd instar grub was determined by pipetting 1.5 ml of distilled water containing 25000, 50000, 100000, 200000 and 300000 IJs onto the semi-decomposed vermicompost surface of each cup. Control cups received same amount of water only. The moisture content of the semi-decomposed vermicompostwas 12%. All cups were covered loosely with lid containing to facilitate gaseous exchange and kept at room temperature at 25-30°C.Grubmortality was assessed at 5,8 or 10 DATand dead neonates werekept on White traps to observe nematode emergence from nematode-killedinsects. The experiment was arranged in a randomized completeblock design. Each treatment was replicated three times with 10 cups/replicate (30 cups/treatment) and repeated once.

Efficacy of EPNs against 3rd instar of *Oryctes rhinoceros* grubs in micro-plots.

To re-check *S. carpocapsae* and *H. indica* pathogenicity against 3rd instar *O. rhinoceros* in larger volume of vermicompost with wider area. We artificially constructed micro-plots sizeof 38x24x10 cm (L x B x H) by using 1cm thickness plastic sheet. Micro-plot was filled with 3 kg sterilized vermicompost (pH 5.98 and 52% moisture) with 10g of sterilized semi-decomposed coconut leaves as a food source for the larvae.Fifteen 3rd instar grubswere released into each micro-plot, and grubs that didnot enter the vermicompost within 2h were replaced with healthy ones.The virulence of *S. carpocapsae* and *H. indica* to 3rd instar larvae was determined by pipetting 150 ml of water containing 10 and 20 lakhs IJs on to the semi-decomposed vermicompostsurface ofeach micro-plot. Micro-plots were ensured proper gaseous exchange by covering muslin cloth tightly with rubber bands and kept at room temperature (25-30°C). Larval mortality was recorded at 5, 10 and15 DAT. At 10th day of observationanother 10g of sterilized semi-decomposed coconut leaves were incorporated in each micro-plot for continuous supply of food togrubs. Thedead cadavers of rhinoceroswere examined for the presence of EPN to ascertain the cause of death by placing over white trap. The experiment was arranged in a completerandomized design. Each treatment was replicated three times with 1 micro-plot/replicate (3 micro-plots/treatments).

RESULTS

Efficacy of EPNs against neonates of *Oryctes rhinoceros* grubs

Of the two nematode species tested, *S. carpocapsae* and *H. indica* were able to kill the neonate grubs of *Oryctes rhinoceros* in the 3 different exposure times in our study. *S. carpocapsae* caused the highestmortality to neonate grubs of rhinoceros at all three exposure times (Fig. 1). The per cent mortality (± standard error) following exposure to *S. carpocapsae*in concentrations of 50 to 250 IJs nematodes per host ranged from 6.7 ±1.3

to 40.0±1.3 after 48 h, from 44.4 ± 2.7 to 71.7 ± 2.7 after 72 h and from 75.6 ± 1.5 to100 after 96 h. The corresponding $LC_{50}$and LC_{90}values were 342, 75,22, 1879, 1493 and 136IJs per grub, respectively (Fig. 1and Table 1). With increasing concentrations of *H. indica*the mortality of neonate grubs showed a good response, even though we could not find 100% mortalityat any concentration or exposure times(Fig. 1). The LC_{50} and LC_{90} values after 48, 72 and 96 h calculated for H. indica were 394,116,28, 1670, 445 and 224 IJs per grubs, respectively.In general, the data suggested rhinoceros beetle grubs to be highly susceptible to two nematode species.

Analysis of variance showed that neonate grub mortalityof *O. rhinoceros* was significantly ($P< 0.05$) influenced by the concentration of nematode suspension (F= 270.89; df=5, 72; $P<0.0001$), nematode species (F= 17.03; df= 1, 72; $P<0.0001$), exposure times (F= 391.49; df= 2, 72; $P < 0.0001$), interaction between exposure times andconcentration of nematode suspension (F= 18.51; df= 10, 72; $P<0.0001$). Interaction between concentration of nematode suspension, nematode speciesand exposure times (F= 0.09; df= 10, 72; $P < 0.9998$), concentration of nematode suspension and nematode species (F= 0.81; df= 10, 72; $P < 0.5441$) and the interaction between nematode species and exposure times (F= 0.13; df= 10, 72; $P<0.8824$) did not havesignificant influence on the neonate grub mortality*O. rhinoceros*.There was no mortality in control treatment. In general percent mortalityof neonate grubs showed significantly ($P< 0.05$) more with increasing concentration of nematode suspension (Fig. 1).

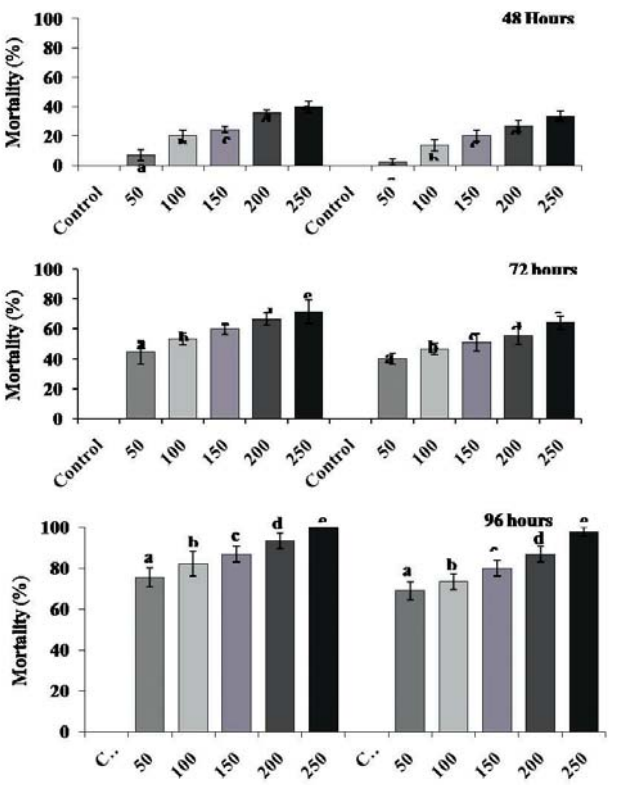

Fig. 1. Percent mortality (mean ± standard error) of neonate grubs of rhinoceros beetle at different concentrations of the entomopathogenic nematodes *Steinernema carpocapsae* and*Heterorhabditis indica* after exposure to48, 72 and 96 h. The grubs were individually exposed to nematodes in 25-ml cups with semi-decomposed vermicompost.There was no control mortality. Different letters on the top of error bars indicates statistically different values for different IJ's inoculation rates at (*P* Â 0.05) using Tukey'test. Error bars indicate standard error (n=3)

Efficacy of EPNs against 3rd instar of *Oryctes rhinoceros* grubs

Of the two nematode species tested, *S. carpocapsae* and *H. indica*, in general 3rd instar of *O. rhinoceros* grub percent mortality showed significantly ($P< 0.05$) more with increasing nematode concentration (Fig. 2). The effect of nematodes were found to be significant ($P< 0.05$) on grub percent mortality among the concentration of nematode suspension (F= 224.09; df= 5, 72; $P<0.0001$).The analysis of variance showed significantdifference for DAT(F= 59.08; df= 2, 72; $P<0.0001$). The analysis of variance revealed a significant two-way interaction between nematode concentration and DAT (F= 3.48; df= 2, 72; $P<0.0009$). Significant ($P< 0.05$) differences have been determined between exposure times as an average grubs mortality for the 5th, 8th and 10th day was 28.50, 37.18 and 46.70 %, respectively. While, significant differences have not been determined between nematode species. Irrespective of the DAT an average 38.46 and 36.46% grub mortality was recorded, when grubs were inoculated with *S. carpocapsae* and *H. indica*, respectively. There was no mortality in control treatment (Fig. 2).

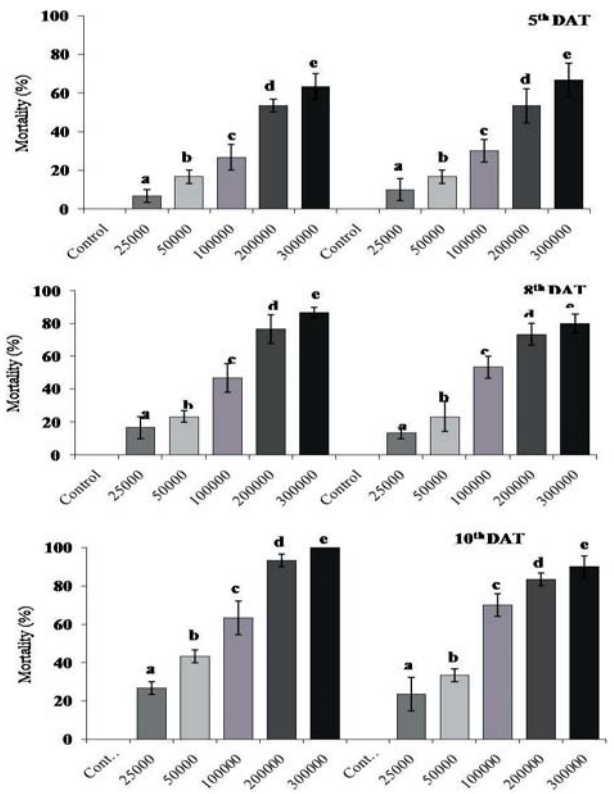

Fig. 2. Percent mortality (mean ± standard error) of 3rdinstars of rhinoceros grub species at different concentrations of the entomopathogenic nematodes *Steinernemacarpocapsae* and *Heterorhabditis indica* after 5, 8 and 10 days after treatment (DAT). The grubs were individually exposed to nematodes in 250-ml cups with semi-decomposed vermicompost. There was no control mortality. Different letters on the top of error bars indicates statistically different values for different IJ's inoculation rates at (*P* Â 0.05) using Tukey'test. Error bars indicate standard error (n=3)

Efficacy of EPNs against 3rd instar of *Oryctes rhinoceros* grubs in micro-plots.

Grub mortality varied significantly among observation DAT. Five days after treatment, the highest mortality 64.4%, was recordedin the highest dose (20 lakh IJs/ grub) of *S. carpocapsae*. Ten days aftertreatment, the highest mortality was with *S. carpocapsae* 82.2%, recorded at the highestdose. Fifteen days after treatment both species killed more than 90% of 3rd instar of *O. rhinoceros* grubs (*S. carpocapsae* 97.7% at 20 lakh IJs/grub; *H. indica*93.3% at 20 lakh IJs/ grub) (Fig. 3).

As expected, *O. rhinoceros* mortality increased with nematode concentration.Significant differences in mortality were found among the different concentrations tested (F= 1012.81; df= 2, 36; *P*<0.0001) and DAT(F= 91.76; df= 2, 36; *P*<0.0001), but there was no significant difference in mortality between two nematode species.There was a significant two-way interaction between nematode concentration and DAT on grub mortality (F= 28.76; df= 4, 36; *P*<0.0001).There was no mortality in control treatment (Fig. 3).

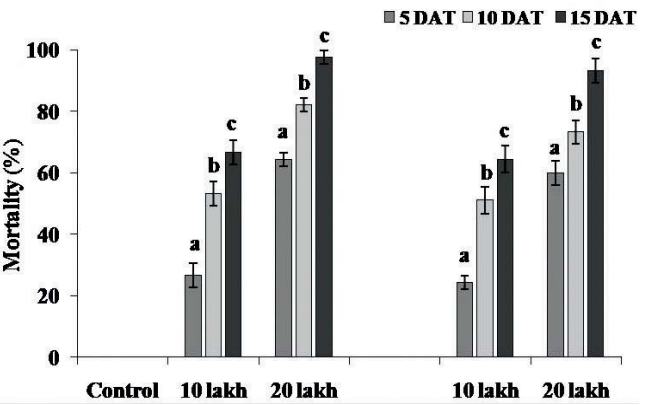

Fig. 3. Percent mortality (mean ± standard error) of 3rdinstars of rhinoceros grub in a micro-plot assay after exposure to two concentrations of the entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis indica* at5, 10 and 15 days after treatment (DAT). There was no control mortality. Different letters on the top of error bars indicates statistically different values for DAT at (*P* Â 0.05) using Tukey'test. Error bars indicate standard error (n=3)

DISCUSSION

The present investigation presents the first data on the susceptibility of rhinoceros grubs to *S. carpocapsae* and *H. indica*. The use of chemicals also disrupts the earthworm in the vermicomposting units, which is one of the major reasons for seeking alternative control measures. In contrast, EPNs are able to crawl into hiding places after their hosts. Vermicomposting units 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 (Poinar, 1990).

Both *S. carpocapsae* and *H. indica* were caused the moderate mortality in neonate grubs at 48 and 72 after exposure and *S. carpocapsae* had the lowest LC_{50} value at all exposure timeswhen compared to *H. indica*. The efficiency of an entomopathogenic nematode against a given host partlydepends on the host-finding ability and the penetration capability of theinfective juveniles (Ishibashi & Kondo, 1990; Peters & Ehlers, 1994).Our study clearly shows that the virulence of entomopathogenic nematodes is not affected by interactionsbetween nematode species and exposure times; i.e., the virulence of the nematode species is not differed relative to each other among hours after inoculation. Based on virulence capacity our results indicate both nematode species caused highest mortality with increasing concentration of nematodes and exposure times (Fig. 1). Host defence resulting in the encapsulation of entering nematodes (Peters, 1994) also affects susceptibility, but is often prevented by increasing nematode invasions (Peters & Ehlers, 1994). This may explain the increased mortality observed in neonate grubs for both nematode species at prolonged exposure times. Increasing numbers of infective juveniles can be expected to have found and entered the hosts (Epsky & Capinera, 1993).

Our study shows that susceptibility of *O. rhinoceros* to EPNs depends on the developmental stage of the insect; the effect, however, varies with the nematode species. Generally, *S. carpocapsae* and *H. indica* caused significantly higher mortality of neonate and third-instars. Neonate grubs were less susceptible to both the nematode species than were third instars.In comparing the efficacy of nematode species against rhinoceros grubs, we could find that nematode efficacy affected by rhinoceros grub larval stage. Previous studies have also demonstrated that the virulence of EPN depends on the larval stage of the host insect. For example, Kowalska (2000) reported the higher susceptibility of third-instars of the summer chafer, *Amphimallonsolstitiale* L. (Coleoptera: Scarabaeidae) to *S. glaseri* when compared with the second-instar. However, in some cases first or second-instars were found to be more susceptible than the third-instar (Deseö*et al*., 1990; Lee *et al*., 2002; Koppenhöfer & Fuzy, 2004). A decrease in susceptibility from *Anomalaorientalis* second to third instars has also been observed for *Heterorhabditis* sp. (Gyeongsan isolate), *S. carpocapsae*, *S. glaseri,* and *S. longicaudum* (Lee *et al*., 2002). Grewal et al. (2004) observed higher mortality of second instar than in third instar *P. japonica* with *H. bacteriophora* (GPS11 strain) (54–97 vs. 34%).

The greater capability of *S. carpocapsae* compared to *H. indica* in killing neonate grubs of rhinoceros beetle as demonstrated in our study might be explained by

Table 1. Mean number of nematodes required to cause 50% (LC_{50}) and 90% (LC_{90}) mortality in neonate grubs of *Oryctes rhinoceros*.

Nematode species	LC50	95% FL	LC90	95% FL	Slope ± SE	Goodness of fit test	
						X^2	$P > X^2$
48 hours' exposure							
S. carpocapsae	342	243-812	1879	798-21715	1.73 ± 0.43	16.06	<0.0001
H. indica	394	277-991	1670	755-16190	2.04 ± 0.51	16.04	<0.0001
72 hours' exposure							
S. carpocapsae	75	19-112	1493	525-33489	1.0 ± 0.34	8.16	0.0043
H. indica	116	35-221	4445	864-321478	0.81 ± 0.34	5.58	0.0181
96 hours' exposure							
S. carpocapsae	22	3-40	136	99-231	1.62 ± 0.44	13.14	0.0003
H. indica	28	4-49	224	156-574	1.42 ± 0.40	12.82	0.0003

foraging strategies of nematodes. Ambush foragers are more effective than cruise foragers at finding resources with high mobility. Neonate grubs of rhinoceros beetle are mobile, and ambush foraging strategy is likely to be the most effective for finding this host. In our assays, *S. capocapsae*, which exhibits a ambusher foraging strategy (Campbell & Gaugler, 1997; Grewal et al., 1994; Lewis, 2002), performed well as compared to *H. indica*. Whereas percent mortality of 3rd instar rhinoceros was not significantly influenced by the nematode species. However, Heterorhabditid IJs have an anterior "tooth-like structure" that could enable enhanced penetration of larval cuticle (Bedding & Molyneux, 1982). Heterorhabditids also generally search for their hosts by moving through the soil matrix with what is referred to as a "cruiser" foraging strategy whereas some steinernematid species (e.g., *S. carpocapsae*) are much less mobile and use an "ambusher" strategy (Campbell & Gaugler, 1997; Grewal *et al.*, 1994; Lewis, 2002). 3rd instar rhinoceros are immobile and stay in the manure/ vermicomposting pit for long periods, and a cruiser foraging strategy is likely to be the most effective for finding this host. In our assays, *S. capocapsae*, which exhibits a ambusher foraging strategy (Campbell &Gaugler, 1997; Grewal *et al.*, 1994; Lewis, 2002), performed well as compared to the heterorhabditids but *S. carpocapsae*, which has not proven to be effective against other beetle grubs in similar habitats, performed good. Thus, a difference in foraging strategy is unlikely to explain all of our results.

The higher concentrations proved to be more efficient in our experiment, however both the nematode species in the present research showed sufficient efficacy to neonate grubs at lower concentration doses. Based on our current findings, we conclude that the activity of EPNs is influenced more by the insect life stages than numbers of nematodes applied.The minor role of the nematode concentration can be explained by the fact that only a few invasive nematodes need to penetrate an insect host in order to kill it (Bednarek&Nowicki, 1986).

Our finding that two species of EPNs demonstrated the more or less similar results in neonate grubs at lower concentrations as with higher concentrations in 3rd instar grubs, gives these biocontrol agents in integrated agriculture better prospects from an economical point of usage, as the cost of plant protection is closely connected to the quantity of the applied EPNs.However, it is also important to note that results from laboratory tests are not always comparable to field testing (Cantelo&Nickle, 1992) as the functioning of EPNs in the open is influenced by an extensive list of factors. In one relevant study, the 100 % efficacy rate of *S. carpocapsae* in controlling Colorado potato beetle adults, pupae, and larvae in the laboratory manifested as only a 31 % reduction rate in this pest population when the test was repeated outdoors (Stewart *et al*., 1998). Some research has also shown that with proper application techniques and right timing as regards the insect developmental stage, we can reach almost the same results as with the use of insecticides (Schroer*et al*., 2005).Nematode applications may have to be timed accordingly for optimal efficacy.

ACKNOWLEDGEMENTS

The authors thank the Director, Central Plantation Crops Research Institute, Kasaragod for providing the research facilities and Indian Council of Agricultural Research, New Delhi for financial support.

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