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Unveiling the biocidal power and reproductive potential: *Steinernema anantnagense* and its impact on controlling apple leaf roller, *Rhopobota naevana* (Hubner) (Lepidoptera: Tortricidae)

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Abstract
The study on biocidal efficacy and reproductive potential of a native strain of entomopathogenic nematode, *Steinernema anantnagense* against apple leaf roller, *Rhopobota naevana* under laboratory conditions was carried out at Division of Entomology, FoA, Wadura, SKUAST- Kashmir, India. The EPN isolate was found effective against the insect pest. Mean percent mortality of 3rd, 4th and 5th instar of *Rhopobota naevana* was 100 percent at the inoculum levels of 200 IJs/grub at 72 hours after inoculation. Percent mortality increased with rise in inoculum levels and exposure time. Median lethal concentration (LC^{50}) values of the EPN decreased with the increase in time interval. Calculated value of median lethal time (LT^{50}) was directly proportional to the size of the larva. Time consumed by EPN to cause mortality to 3rd instar larva was least followed by 4th and 5th instar larva at all the evaluated time intervals. Production of infective juveniles was directly proportional to the size of the host. However, average population of *Steinernema anantnagense* produced in 5th instar was highest and was followed by 4th and 3rd instar larvae at all the four inoculum levels ie., 50, 100, 150 and 200 IJs per larva.

Keywords: Biocidal efficacy, Entomopathogenic nematode, LC_{50}, LT_{50}, *Rhopobota naevana*, reproductive potential, *Steinernema* sp.

Introduction
The apple (*Malus domestica* Borkh) is acknowledged as one of the earliest cultivated fruit crops, with its origin traced to the Caucasus Mountains in southwestern Asia (Janick, 2005) [11]. Widely cultivated in temperate regions globally, it is a lucrative crop. In India, apple cultivation is concentrated in Jammu and Kashmir, Himachal Pradesh, Uttarakhand, and certain areas of Arunachal Pradesh, with Jammu and Kashmir contributing 60% to the country's total apple production. One of the significant challenges faced by apple cultivation, particularly in the Kashmir valley, is the damage caused by the leaf roller insect, *Rhopobota naevana*. This polyphagous insect, known for feeding on various host plants, poses a threat during early spring when its eggs hatch. The larvae predominantly target terminal foliage, weaving the terminals together and causing extensive harm by destroying buds and severely skeletonizing leaves. This damage manifests as a burnt appearance at the tree's top. Traditionally, apple growers in Kashmir have relied on chemical insecticides to manage the apple leaf roller. However, the widespread use of chemical agents raises environmental concerns, including groundwater contamination, pesticide resistance, pest resurgence, residues in food products, toxicity hazards, and the depletion of biodiversity among beneficial natural enemies. Consequently, there is a pressing need for an alternative, eco-friendly pest management approach that ensures the safety of non-target organisms.
Entomopathogenic nematodes (EPNs), belonging to the Steinernematidae and Heterorhabditidae families, serve as lethal parasites targeting insect pests. These nematodes offer a safe and environmentally friendly pest management solution, exhibiting no harm to humans, other vertebrates, or non-target organisms, and are easily applicable (Askary and Abd-Elgawad, 2017) [2]. The host range of EPNs varies among species, with documented infections in over 200 insect species across different orders (Woodring and Kaya, 1988) [22]. Symbiotic relationships exist between *Steinernema* and *Heterorhabditis* nematodes and the bacteria Xenorhabdus and Photorhabdus, respectively (Boemare *et al*., 1993) [8].

Infective juveniles (IJs) of these nematodes enter the insect body through natural openings such as the mouth, anus, spiracles, or intersegmental membrane of the cuticle (Bedding and Molyneux, 1982 [7]; Peters and Ehlers, 1994 [14]). Upon entering the haemocoel, they release bacteria, which rapidly multiply in the haemolymph, causing septicemia and leading to the host's demise within 24-48 hours (Adams and Nyugenaskery, 2002) [1]. The symbiotic bacteria produce a diverse array of toxins and hydrolytic exoenzymes, facilitating the bioconversion of insect larvae into a nutrient-rich solution ideal for nematode development. Native strains of EPNs have demonstrated superior performance against local insect pests compared to exotic or non-indigenous strains, attributed to their adaptation to local soil and environmental conditions. Additionally, native EPNs exhibit higher virulence and recycling potential within the host (Istkhar and Aasha, 2016) [10].

Materials and Methods

Collection of *Rhopobota naevana* larva

The larvae of *Rhopobota naevana* were obtained from the experimental apple nursery plots located at the Faculty of Agriculture, Wadura, Sopore campus of Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir (SKUAST-K). The purpose of this collection was to assess the bioefficacy of a local entomopathogenic nematode strain. Larvae were exclusively sourced from plots devoid of any pesticide application.

Preparation of nematode culture

For the nematode culture preparation, the native entomopathogenic nematode (EPN) strain employed in this study was *Steinernema anantnagense*. Isolated from the soils of Kashmir valley, India, and identified according to specified methods, *S. anantnagense* was cultured using fully grown larvae of the Greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae). In 20 cm diameter petri dishes lined with filter paper, ten 5th instar larvae of *G. mellonella* were individually inoculated with approximately 1 x 10^3 infective juveniles (IJs) of the EPN strain in 0.5 ml of sterilized distilled water, following the protocol by Kaya and Stock (1997) [12]. The dishes were covered with an inverted petri bottom and stored in a BOD incubator (Dutky *et al*., 1964) [9]. After 2-3 days, nematode-infected deceased larvae were transferred to a modified White trap (White, 1927) [21]. Infective juveniles emerging from *G. mellonella* larvae were collected three times a week until production declined. Following 7–10 days, IJs emerging from the insect cadaver were collected, allowing them to settle in clean beakers for one hour. The supernatant was discarded, and to resuspend the IJs, sterilized distilled water was added, followed by a repetition of the decanting process three times. After treatment with 0.05 percent formaldehyde, the clean suspension was rinsed with distilled water and resuspended in distilled water at a concentration of approximately 1 × 10^3 IJs/ml. This nematode suspension was stored in tissue culture flasks (250 ml) in a BOD incubator at 10 ± 1 °C.

Nematode efficacy against apple leaf roller, *Rhopobota naevana*

Bioassays were conducted on 3rd, 4th and 5th instar larvae of *R. naevana* for testing the efficacy of the EPN species. Individuals of each larval instar of similar size and weight were selected. Six-well plates were used to carry out the experiment. Each well was lined with double-layered Whatman filter paper No. 1. Infective Juveniles of the species used in the experiments were seven days old only. Four concentrations: 50, 100, 150 and 200 IJs of EPN strain suspended separately in 350-µl distilled water and sprayed evenly onto the filter papers (Askary *et al*., 2023) [5]. Treatments with only distilled water were kept as control. For each concentration of IJs, 12 individuals of *R. naevana* of a single instar having similar size and weight were used and arranged as a single larva/ well (n = 12). Plates were covered with their respective lids and kept in a BOD incubator at 20 ± 2 °C in the dark. The observations of the experiment were taken at five intervals i.e. 24, 48, 72, 96 and 120 hours to record the mortality of the larva.

Reproductive potential of EPN in insect cadaver

To record the reproductive potential of EPN strain, White traps were observed daily under a stereoscopic microscope for the emergence of IJs from the cadaver of *R. naevana* in each White trap and were collected daily and counted until nematode emergence stops. Thus, the number of IJs produced per cadaver was determined.

Statistical analysis

Larval mortality caused by the EPN strain was analyzed statistically using SPSS software. LC^{50} (Lethal concentration 50) and LT^{50} (Lethal time 50) values were calculated at 50 percent confidence limit.

Results

Susceptibility of *Rhopobota naevana* to nematode strain

All the three larval instars (3rd, 4th and 5th) of *R. naevana* were susceptible to EPN strain tested. However, major differences were noticed in nematode pathogenesis across the EPN strain both in terms of virulence (power to kill) and efficacy (time to kill). Time consumed by nematode to cause larval mortality increased with the increase in the size of larvae. At lower inoculum level of nematode, it requires more time to kill the larva of a particular instar, whereas at higher inoculum level it took lesser time to kill the larva. In terms of virulence, insect mortalities within 24 -120 hours post-inoculation varied with respect to size of larval instar of insect pest. At 24 hours interval, *S. anantnagence* applied @ 50, 100, 150 and 200 IJs/ larva caused 16.67, 25.00, 33.33, and 41.67 percent mortality, respectively to 3rd instar larvae of *R. naevana* and they were statistically significant ($p \leq 0.05$) from each other (Table 1). At 48 hours interval, percent mortality in the same instar was 33.33, 41.67, 50.00 and 66.67 when inoculated @ 50, 100, 150 and 200 IJs/ larva. The larval mortality of 3rd instar larvae was recorded hundred percent with the application of 150 and 200 IJs/ larva at time interval of 72 hours. Hundred percent mortality in the same instar was found at 96 hours when treated with 50 and 100 IJs per larva. In case of 4th instar, 8.33, 16.67, 25.00 and 33.33 percent mortality was found at 24 hours inoculation interval when treated with the inoculum levels of 50, 100, 150 and 200 IJs per larva respectively. At 48 hours interval, percent mortality in the same instar was 25.00, 33.33, 50.00 and 58.33 when inoculated with 50, 100, 150 and 200 IJs per larva respectively. Hundred percent mortality was calculated in 4th instar at 72 hours when treated with 200 IJs per larva. At 96 hours interval, 100 percent mortality was observed in the same instar when inoculated @

50, 100 and 150 IJs/ larva. In 5th instar of *R. naevana*, 0.00, 8.33, 8.33 and 16.67 percent mortality was found at 24 hours inoculation interval when treated with the inoculum levels of 50, 100, 150 and 200 IJs per larva respectively. At 48 hours interval, percent mortality in the same instar was 16.67, 25.00, 33.33 and 41.67 when inoculated with 50, 100, 150 and 200 IJs per larva respectively. Hundred percent mortality was calculated in 5th instar at 72 hours when treated with 200 IJs per larva. At 96 hours interval, 100 percent mortality was observed in the same instar when inoculated @ 150 IJs/ larva and 100 percent mortality was observed at 120 hours interval in the same instar when treated with 50 and 100 IJs/ larva (Table 1).

Table 1: Efficacy of *Steinernema anantnagense* against different larval instars of apple leaf roller, *Rhopobota naevana* under laboratory conditions

IJs/larva	3rd Instar Larva					Sub Mean	4th Instar Larva					Sub Mean	5th Instar Larva					Sub Mean	Factor Mean	
	Percent mortality (Hours after treatment)						Percent mortality (Hours after treatment)						Percent mortality (Hours after treatment)						IJs/larva	Time
	24	48	72	96	120		24	48	72	96	120		24	48	72	96	120			
50	16.67 (24.09)	33.33 (35.00)	58.33 (49.42)	100 (90.00)	100 (90.00)	61.67 (57.70)	8.33 (10.00)	25.00 (30.01)	41.67 (40.52)	100 (90.00)	100 (90.00)	55.00 (52.10)	0.00 (4.05)	16.67 (24.09)	41.67 (40.52)	58.33 (49.42)	100 (90.00)	43.33 (41.61)	53.33 (50.47)	15.55 (19.26)
100	25.00 (30.01)	41.67 (40.52)	66.67 (53.95)	100 (90.00)	100 (90.00)	66.67 (60.89)	16.67 (24.09)	33.33 (35.00)	58.33 (49.42)	100 (90.00)	100 (90.00)	61.67 (57.70)	8.33 (10.00)	25.00 (30.01)	50.00 (45.57)	66.67 (53.95)	100 (90.00)	50.00 (45.90)	59.44 (54.83)	31.66 (31.78)
150	33.33 (35.00)	50.00 (45.57)	100 (90.00)	100 (90.00)	100 (90.00)	76.67 (70.11)	25.00 (30.01)	50.00 (45.57)	66.67 (53.95)	100 (90.00)	100 (90.00)	68.33 (61.90)	8.33 (10.00)	33.33 (35.00)	58.33 (49.42)	100 (90.00)	100 (90.00)	60.00 (54.88)	68.33 (62.29)	57.77 (51.51)
200	41.67 (40.52)	66.67 (53.95)	100 (90.00)	100 (90.00)	100 (90.00)	81.67 (72.89)	33.33 (35.00)	58.33 (49.42)	100 (90.00)	100 (90.00)	100 (90.00)	78.33 (70.80)	16.67 (24.09)	41.67 (40.52)	100 (90.00)	100 (90.00)	100 (90.00)	71.67 (66.92)	77.22 (70.20)	81.66 (74.00)
Control	0.00 (4.05)	0.00 (4.05)	8.33 (10.00)	8.33 (10.00)	8.33 (10.00)	4.99 (7.62)	0.00 (4.05)	0.00 (4.05)	8.33 (10.00)	8.33 (10.00)	8.33 (10.00)	4.99 (7.62)	0.00 (4.05)	0.00 (4.05)	8.33 (10.00)	8.33 (10.00)	8.33 (10.00)	4.99 (7.62)	4.99 (7.62)	81.66 (74.00)
Mean	23.33 (26.73)	38.33 (35.81)	66.66 (58.67)	81.66 (74.00)	81.66 (74.00)	58.33 (53.84)	16.66 (20.63)	33.33 (32.81)	55.00 (48.77)	81.66 (74.00)	81.66 (74.00)	53.66 (50.04)	6.66 (10.43)	23.33 (26.73)	51.66 (47.10)	66.66 (58.67)	81.66 (74.00)	45.99 (43.38)		
CD (p<0.05)	Treatments (T) =0.06 Time (Ti) =0.06 Instar (I) =0.047 Treatments*Time (T*Ti) =0.136 Treatments*Instar (T*I) =0.105 Time*Instar (Ti*I) =0.105 Treatments*Time*Instar (T*Ti*I) =0.235																			

Values in parentheses are arc sine transformed values. Each figure is mean of 12 replications

Median lethal concentration

Calculated median lethal concentration (LC^{50}) varied between the different larval instars of *R. naevana* used in the experiment. However, it is common for the EPN strain that with the increase in the size of larva, the number of infective juveniles required to kill 50 percent test population of the larva also increases. LC^{50} values of *S. anantnagense* was found highest at 24 hours and lowest at 96 hours in case of all the three larval instars of *R. naevana*. 100 percent mortality was found at 96 hours interval so there was no need to go to 120 hours interval. At 24 hours, *S. anantnagense* required 211.33, 251.74 and 309.35 IJs to kill the 50 percent test population of 3rd, 4th and 5th instar larvae, respectively (Table 2 and Fig 1). At 48 hours post inoculation, 140.51, 158.34 and 211.33 IJs of *S. anantnagense* killed 50 percent population of 3rd, 4th and 5th instar larvae of *R. naevana*, respectively. 57.74, 86.60 and 118.15 IJs of *S. anantnagense* killed 50 percent population of 3rd, 4th and 5th instar larvae of *R. naevana* at 72 hours post inoculation respectively. At 96 hours, only 10.70 IJs of *S. anantnagense* were required to cause 50 percent mortality of all the three larval instars i.e, 3rd, 4th and 5th of the test insect.

Table 2: Median lethal concentration (LC_{50}) of *Steinernema anantnagense* against different larval instars of leaf roller, *Rhopobota naevana* at different time intervals

Rhopobota naevana	Median Lethal concentration (*IJs/ larva)			
	Post nematode inoculation interval (hours)			
	24	48	72	96
3rd Instar	211.33	140.51	57.74	10.70
4th Instar	251.74	158.34	86.60	10.70
5th Instar	309.35	211.33	118.15	10.70

* Infective juveniles

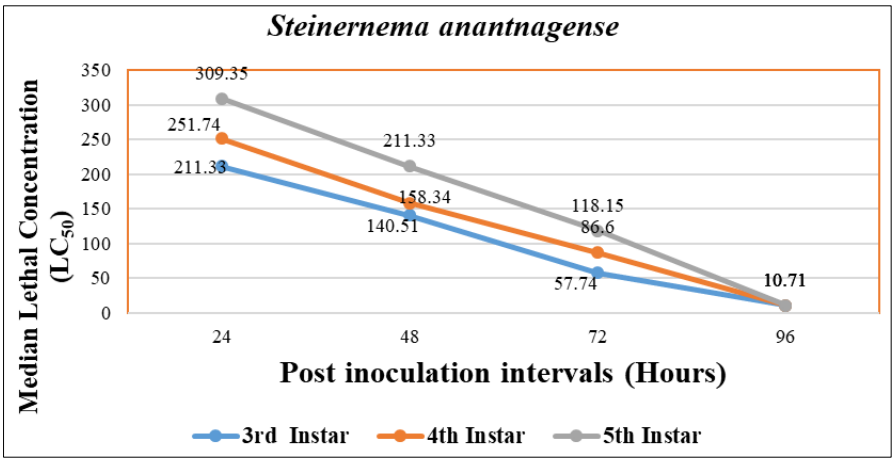

Fig 1: Median lethal concentration (LC_{50}) of *Steinernema anantnagense* against different larval instars of apple leaf roller, *Rhopobota naevana* at different nematode concentrations

Median lethal time

Median lethal time (LT^{50}) required for 50 percent mortality of 3rd, 4th and 5th instar larvae of *R. naevana*, results are presented in Table 3 and Fig. 2. The results revealed that with the increase in the level of infective juveniles per larva, there was a significant decrease in time required to kill the 50 percent population of the test insect. The lowest level of 50 IJs of *S. anantnagense* required about 86, 94 and 102 hours for causing death to 50 percent population of 3rd, 4th and 5th instar larvae, respectively. 70, 83 and 91 hours are needed for causing death to 50 percent population of 3rd, 4th and 5th instar larvae, respectively at an inoculum level of 100 IJs. In 3rd, 4th and 5th instar larvae, 62, 74 and 80 hours are needed for 50 percent mortality at 150 IJs of *S. anantnagense* and the maximum of 200 IJs required 50, 61 and 72 hours for causing mortality to 3rd, 4th and 5th instar larvae, of *R. naevana* respectively.

Table 3: Median lethal time (LT_{50}) of *Steinernema anantnagense* against different larval instars of apple leaf roller, *Rhopobota naevana* at different nematode concentration

Rhopobota naevana	Median Lethal Time (hours)			
	Number of nematodes (*IJs/ larva)			
	50	100	150	200
3rd Instar	86.00	70.00	62.00	50.00
4th Instar	94.00	83.00	74.00	61.00
5th Instar	102.0	91.00	80.00	72.00

* Infective juveniles

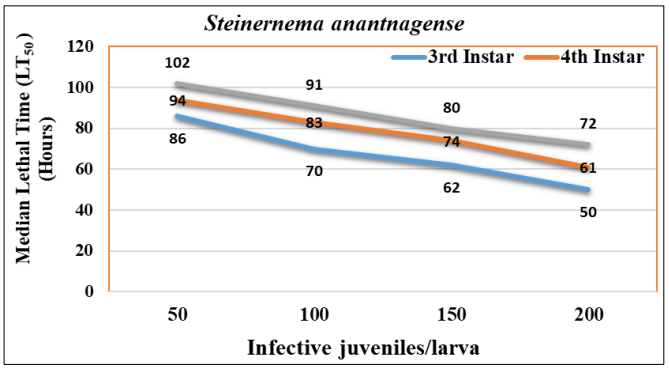

Fig 2: Median lethal time (LT_{50}) of *Steinernema anantnagense* against different larval instars of apple leaf roller, *Rhopobota naevana* at different nematode concentrations

Nematode reproductive potential

The nematode progeny obtained from the different larval instars of *R. naevana* greatly varied (Fig 3). The number of progeny produced by *S. anantnagense* inoculated @ 50 IJs/ larva, when applied to 3rd, 4th and 5th instar larva of *R. naevana*, produced 0.15 x 10^5, 0.18 x 10^5 and 0.21 x 10^5 IJs respectively. 0.13 x 10^5, 0.15 x 10^5 and 0.23 x 10^5 IJs were produced by 3rd, 4th and 5th instar larva of *R. naevana* respectively, when inoculated @ 100 IJs/ larva. Similarly, IJs were produced in all the three instars of *R. naevana* at the inoculum levels of 150 and 200 IJs per larva also. The highest number of nematode progeny was recorded from 5th instar larvae of *R. naevana*, at all the inoculum levels.

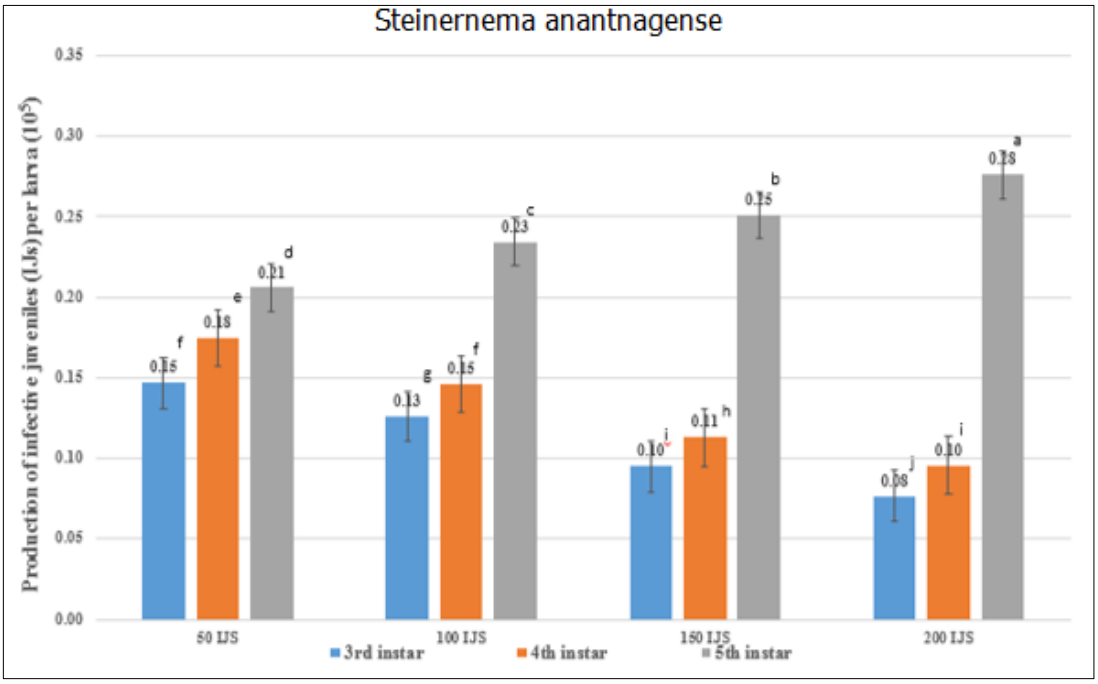

Fig 3: Reproductive capacity (10^5) of *Steinernema anantnagense* in 3rd, 4th and 5th instar of *Rhopobota naevana* at different inoculum levels

Discussion

The study focused on the interaction between *Rhopobota naevana* and the entomopathogenic nematode (EPN) strain *Steinernema anantnagense*. Existing literature strongly supports the efficacy of EPNs in causing mortality in various Lepidoptera insect pests. All three larval instars (3rd, 4th, and 5th) of *R. naevana* were found to be susceptible to the tested EPN strain. The time required by the nematode to induce larval mortality increased as the size of the larvae increased. Lower nematode inoculum levels necessitated more time to kill larvae of a specific instar, while higher inoculum levels expedited larval mortality. This finding aligns with reports indicating significant variability in the efficacy of different EPN species or strains in controlling specific insect pests. In laboratory conditions, Be´lair *et al.* (1999) [6] observed that concentrations of 25 IJs/plate of *S. riobrave* 335, *S. feltiae* UK, *S. carpocapsae* All, and *S. glaseri* 326 resulted in 85, 55, 45, and 8 percent mortality, respectively, of *Choristineura*

rosaceana third instar larvae of Tortricidae. Similar trends were observed with both EPN isolates at inoculum levels of 100 and 150 IJs. These results corroborate findings from other studies where LT_{50} values of *S. glaseri, S. thermophilum*, and *Heterorhabditis indica* exhibited differences at the same inoculum level against larvae of *Pieris brasssicae* (Lalramliana and Yadav, 2009) [13]. Askary and Ahmad (2020) [4] assessed different native isolates of EPN against the oriental armyworm, *Mythimna separate*, and observed variations among the nematode isolates. Ratnasinghe and Hague (1997) [18] studied the efficacy of *S. carpocapsae, S. riobravis, and S. feltiae* against different stages of *P. xylostella* in the laboratory, noting that LT_{50} was less than 3 hours for all three nematodes tested, with *S. carpocapsae* proving most effective, causing mortality after a 6 hour exposure. Pathogenic variability in nematode isolates has been reported by various researchers (Peters and Ehlers, 1994 [14]; Shapiro-Ilan *et al*., 2003 [20]; Askary *et al*., 2023) [5]. Several researchers have reported variations in progeny production among different entomopathogenic nematode (EPN) isolates within insect host cadavers. Shakeela and Hussaini (2006) [19] documented the extraction of 4.65×10^5 and 5.35×10^5 infective juveniles (IJs) of *S. abbasi* and *H. indica*, respectively, from a single larva of the greater wax moth, *Galleria mellonella*. The rate of EPN multiplication within the host cadaver is subject to variations based on factors such as isolates, species, host susceptibility, host size, invasion rates, and environmental conditions like temperature and humidity (Rahoo *et al*., 2016a, b; Rahoo *et al*., 2017; Askary *et al*., 2018; Askary and Ahmad, 2020) [17, 15, 16, 3, 4].

Conclusion

The native EPN strain, *Steinernema anantnagense* showed the greater potential in terms of causing mortality and multiplying within the host, *R. naevana*. However, further studies need to be set to establish the performance of these nematode strains under field conditions before their final recommendation.

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