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Pathogenicity of *Heterorhabditis indica* against developmental stages of *Eudocima materna* L. (Lepidoptera, Erebidae)

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Abstract

Background: Virulence of the entomopathogenic nematode, *Heterorhabditis indica* against larvae (3rd, 4th and 5th larval instars) and pupae of citrus fruit piercing moth, *Eudocima materna* L. (Lepidoptera: Erebidae) was evaluated under laboratory conditions. The experiments included pathogenicity assessment of *H. indica* against larvae and pupae using a range of concentrations (10, 25, 50, 100 and 200 infective juveniles (IJs) larva^{-1} and pupa^{-1}) as well as evaluation of their reproductive potential on different larval instars with different concentrations (50, 100, 200, 400 and 600 IJs larva^{-1}).

Results: Application of increasing concentrations of IJs induced higher mortality on larval instars and pupae with mean mortality ranging from 26.6 to 100% (LC_{50}- 3rd (14.43 IJs larva^{-1}), 4th (17.08 IJs larva^{-1}), 5th (23.63 IJs larva^{-1})) and 10–70% (LC_{50}- 85.91 IJs pupa^{-1}) after 48 h post-exposure, respectively. *H. indica* successfully reproduced in the 3rd, 4th and 5th larval instars of *E. materna* and their offspring emerged from the cadavers. The highest reproduction was recorded in 5th instar larvae (1,082,855 IJs larva^{-1}) at 600 IJs larva^{-1} in *E. materna*.

Conclusion: The present study suggests that *H. indica* strain had the potential for biological control of *E. materna*.

Keywords: Biological control, Citrus fruit piercing moth, *Eudocima materna*, Entomopathogenic nematode, *Heterorhabditis indica*, Mortality

Background

Fruit piercing moths, *Eudocima* spp. (Lepidoptera: Erebidae), are considered as serious pests of fruit crops grown throughout the tropical and sub-tropical belt from Africa, Asia, Australia and to the Pacific Islands (Leong and Kueh 2011). The adult moths infest ripened fruits of various horticultural crops by piercing with its barbed proboscis which becomes an entry point for bacterial and fungal infections, finally causing rotting and premature dropping of infected fruits (Kamala Jayanthi et al. 2015). Damaged fruits are unmarketable and pose a threat of further pathogenic breakdown to healthy fruits in the packed lots (Fay and Halfpapp 2006). In India, 2 species, *Eudocima materna* and *E. fullonica* cause significant economic damage, in mandarins, sweet oranges and kinnow, particularly during August–December, with their peak activity during September–October (Rao and George 2018).

Adult moth lays oval-shaped and shiny pale green collared eggs on wild plants and weed hosts grown in and around citrus orchards (Shivankar and Singh 2000). Larvae are semi-loopers with stout appearance, feed on alternate weed hosts for about 4–5 weeks, progressing through 5 instars and pupate within a silken cocoon between webbed leaves and the leaves may stay on the tree or become desiccated and fall to the ground. The emergence of adult from the pupa takes place after 2–3 weeks. There are 2–3 generations in a year

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depending on the availability of weed hosts (Chhagan and McKenna 2019).

Insecticidal sprays, poison baits or the traps are traditional components of integrated citrus fruit piercing moth management strategies (Kamala Jayanthi et al. 2010). Since this pest species starts infesting citrus fruits at maturity/harvesting stage, application of chemical insecticides is not encouraged at this crop stage. Very few options are available for the management of this pest species even by using of poison baits or light traps, they are not much effective (Shivankar and Singh 2000). Thus, the use of entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* offers an eco-friendly alternative to chemicals and as biological control agents against different insect pests. EPNs have been reported to be pathogenic to larval and pupal stages of various groups of insect pests. Therefore, evaluation of the efficacy of different concentrations of *Heterorhabditis indica* against *E. materna* larvae (3rd, 4th and 5th instars) and pupae under laboratory conditions were aimed. Also, investigation of the reproductive potential of *H. indica* in *E. materna* larval instars was evaluated. *E. materna* complete its entire life cycle on wild plants and weed hosts which are generally grown on the bunds of the orchards and nearby localities; it is very imperative to carry out the field application of *H. indica* at these sites. In recent years, significant progress has been made in developing EPN formulations, especially for above ground applications such as combining EPNs with polymers or surfactants or sprayable gels (Shapiro-Ilan et al. 2012). The present work is the first investigation on using EPNs against fruit piercing moth larvae and pupae and provides some preliminary evidence for potential use of *H. indica* for biological control of *E. materna*.

Methods

Insect culture

The 1st and 2nd instar larvae of *E. materna* were initially collected from the weed host, *T. cordifolia* in and around the research farm (21° 08′ 44″ N 79° 01′ 32″ E) of ICAR-Central Citrus Research Institute, Nagpur, Maharashtra, India, during August, 2020. The larvae were reared on *T. cordifolia* leaves in a plastic tray covered with a muslin cloth at 25 ± 1 °C. The developed pupae were transferred to plastic containers (1000 ml) covered with a black muslin cloth. The newly emerged adults were transferred to a nylon cage (2 m × 2 m x 3 m size) at 25 ± 1 °C with 70% RH and a 12:12 h light/dark photoperiod; adults were fed with Nagpur mandarin fruits and a synthetic diet prepared by dipping of cotton balls in a honey solution (1(honey):1(water)) with vitamin-E capsules. The newly deposited eggs by adult female moths were collected with the help of a wet painting brush and carefully placed in plastic containers under lukewarm condition. Eggs were allowed to hatch and the newly emerged larvae were reared on *T. cordifolia* leaves for further development to various larval instars. Larvae were separated into 3rd, 4th and 5th instars based on their size, weight and head capsule width and subsequently used for efficacy and reproduction assays. For pupal bioassay, pupae were collected from the dried *T. cordifolia* leaves which were cultured in the lab. *E. materna* were successfully reared up to 5 generations (August 2020–January 2021) on weed host, *T. cordifolia* and larvae from second generation onwards were used for experimental purpose (Fig. 1). The average size (length (L), width (W)) of 3rd, 4th, 5th larval instars and pupae used were 21.24, 2.19; 33.85, 3.97; 51.84, 8.41 and 27.74, 9.61 mm, respectively, whereas the average weights were 112.13, 444.12, 1420.03 and 1330.02 mg, respectively.

EPN culture

EPN species, *Heterorhabditis indica*, was obtained from the Biological Control Centre, ICAR-IISR, Pravaranagar, Maharashtra, India. Infective juveniles (IJs) of *H. indica* were continuously propagated on last instar larvae of rice moth, *Corcyra cephalonica* (Kaya and Stock 1997). IJs harvested on a white trap (White 1927) were stored in sterile distilled water in tissue culture flasks at 15 °C for about one week before use. Only IJs with survival rates greater than 90% were used for relevant experiments.

Pathogenicity of *H. indica* against larval instars of *E. materna*

In order to examine the susceptibility of larval stages (3rd, 4th and 5th) of *E. materna*, IJs at concentrations 10, 25, 50, 100 and 200 IJs larva^{-1} were suspended in 150 µl of water and distributed evenly on the insect breeding dish (50 × 15 mm) lined with sterile filter paper previously moistened with 350 µl of sterile distilled water for each treatment. In control treatments, only 500 µl of distilled water was added to the dishes. Larvae of *E. materna* were then individually placed in the dishes and pieces of fresh *T. cordifolia* leaves were provided as food; then, the dishes were sealed with parafilm and incubated at 25 ± 1 °C. Ten individuals of each larval instar were used for each concentration and the experiment was conducted thrice ($n = 10$; $r = 3$). Larval mortalities were recorded after 24 and 48 h of treatment, followed by dissection of cadavers under the stereomicroscope for confirmation of nematode infection (Aryal et al. 2022).

Pupal susceptibility in a plastic container assay

To study the infectivity of *H. indica* to pupae of *E. materna*, different concentrations of IJs (10, 25, 50, 100 and 200 IJs pupa^{-1}) were suspended in 2.5 ml of distilled

Fig. 1 Life stages of *Eudocima materna* used in the study **a** 3rd instar, **b** 4th instar, **c** 5th instar, **d** pupa

water and distributed evenly in the 100 ml plastic containers (height: 7 cm; diameter: 5.5 cm; soil capacity: 100 cm^3) containing the mixture of 90 cm^3 autoclaved sand and soil (1:1). Distilled water alone was added to the Control treatments. The single pupa was placed at 1 cm depth in each container. The containers were closed and incubated at 24 ± 2 °C and 60 ± 5% RH. Each treatment contained 10 pupae and the assay was repeated once. The adult eclosion rate was examined on a daily basis for 15 days. The non-emerged pupae were collected and washed in distilled water and dissected in sterile distilled water under a stereomicroscope for confirmation of nematode infection.

Reproduction potential of *H. indica* in larval instars

The larval instars (3rd, 4th and 5th) of *E. materna* infected with IJs of *H. indica* at different concentrations (50, 100, 200, 400 and 600 IJs $larva^{-1}$) were selected for reproduction assay. A total of ten numbers of each larval instar for each IJ concentration were used in this assay. Five cadavers were selected randomly, rinsed with distilled water to remove nematode adhering to the body surface. The cadavers were individually transferred onto the white trap and incubated at 25 ± 1 °C. The IJs that emerged from each cadaver were collected fifteen days after treatment in centrifuge tubes separately and the concentration of IJs was determined by serial dilution method as described by Glazer and Lewis (2000).

Statistical analysis

To assess the number of IJs required for killing 50% lethal concentration (LC_{50}) and 90% (LC_{90}) of the various larval instars and pupae of *E. materna* after 48 h post-inoculation at 95% confidence intervals at $P \leq 0.05$, larval and pupal mortality resulted from *H. indica* and their concentrations was subjected to probit analysis (Finney 1971) using online tool OPSTAT. The mean reproduction rate of *H. indica* in different larval instars of *E. materna* at different concentrations was compared by Duncan's test at $P \leq 0.05$ using SPSS 21 software.

Results

Pathogenicity of *H. indica* at different concentrations against *E. materna* larval instars

Pathogenicity assessment indicated that *H. indica* was capable of infecting and killing different larval instars (3rd, 4th and 5th) of *E. materna* (Fig. 2). The larval mortality was influenced by 3rd, 4th and 5th instars as nematode concentrations increased with mean mortality ranging from 26.6–100%, when they treated with 10 to 200 IJs $larva^{-1}$ of the tested *H. indica* strain (Fig. 3). There were significant differences in mortality among different IJ concentrations on 3rd, 4th and 5th larval instars

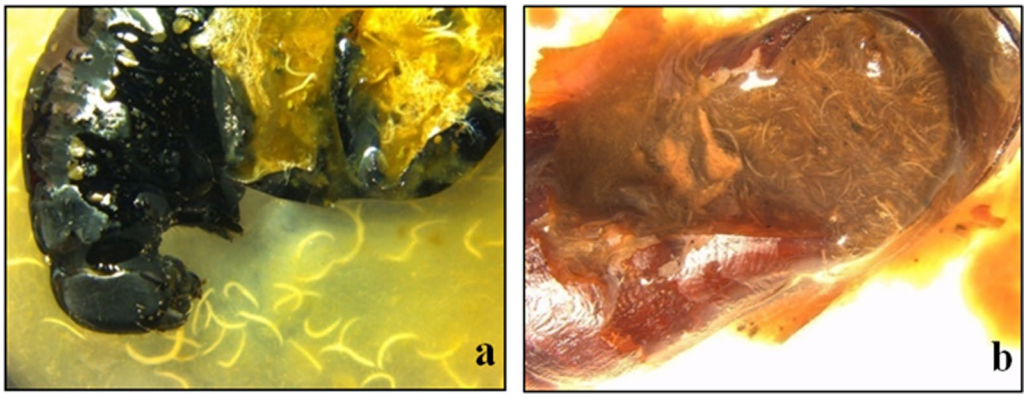

Fig. 2 *Heterorhabditis indica* invading the larva and pupa of *Eudocima materna* and multiplying within the hosts' bodies after treatment

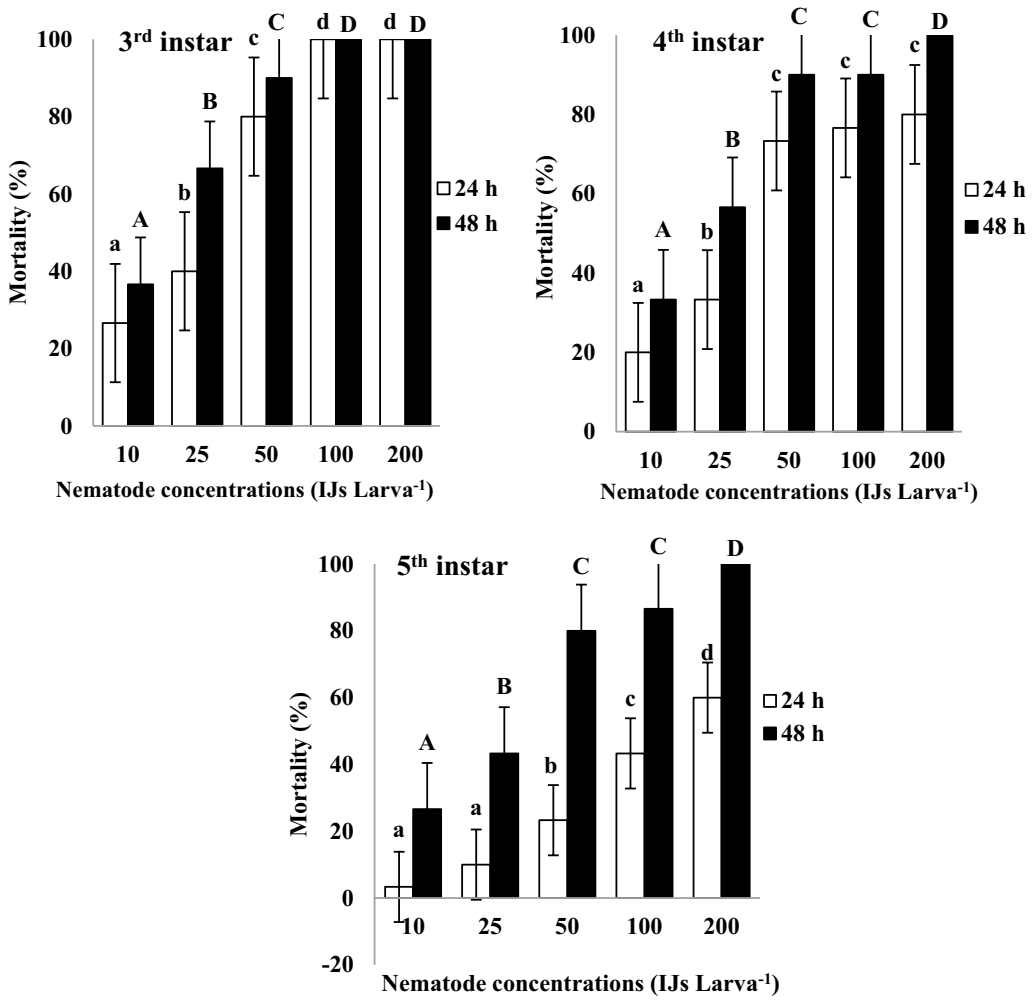

Fig. 3 Average percentage mortality of *Eudocima materna* larval instars (3rd, 4th and 5th) after exposure to *Heterorhabditis indica* at the rate of 10, 25, 50, 100 and 200 infective juveniles (IJs) per larval concentrations. Error bars indicate standard error of mean (SEM). Different lower case letters above bars indicate statistical significance among different IJ concentrations after 24 h of treatment. Different upper case letters above bars indicate statistical significance among different IJ concentrations after 48 h of treatment ($P \leq 0.05$, Duncan's test)

after 24 h ($F=526$, $df=4$, $P<0.001$; $F=116.66$, $df=4$, $P<0.001$; $F=83$, $df=4$, $P<0.001$) and 48 h ($F=165.75$, $df=4$, $P<0.001$; $F=176.75$, $df=4$, $P<0.001$; $F=143.6$, $df=4$; $P<0.001$) of treatment, respectively. However, 100% mortality of all the larval instars was observed after 48 h of treatment at highest nematode concentration (200 IJs larva^{-1}).

Obtained results showed that *H. indica* was able to kill different instars of *E. materna* with fewer IJs concentrations. Information on LC_{50} and LC_{90} together with upper and lower fiducial limits and Chi-square ($\chi 2$) for *H. indica* for different larval instars (3rd, 4th and 5th) are given in Table (1). The LC_{50} for *H. indica* was 14.43, 17.08 and 23.63 IJs larva^{-1} for 3rd, 4th and 5th larval instars after 48 h of incubation, respectively. In addition, the LC_{90} of *H. indica* for 3rd, 4th and 5th larval instars after 48 h was 59.56, 80.44, and 106.52 IJs larva^{-1}, respectively (Table 1).

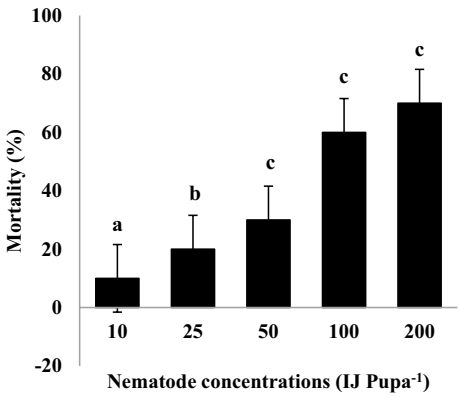

Fig. 4 Average percentage mortality of *Eudocima materna* pupae after exposure to *Heterorhabditis indica* at the rate of 10, 25, 50, 100 and 200 infective juveniles (IJs) per pupal concentrations. Error bars indicate standard error of mean (SEM). Different lower case letters above bars indicate statistical significance among different IJ concentrations after treatment

Pupal susceptibility to *H. indica*

Laboratory assay showed that pupae of *E. materna* appeared susceptible to *H. indica*. Insect mortality recorded with 1–3 days old infected pupae revealed significant differences and greater mortality (>60%) at concentrations of 100 and 200 IJs pupa^{-1} (Figs. 2, 4). Fully formed pupae were less susceptible to nematodes than 3rd, 4th and 5th instar larvae. >90% insect mortality was recorded when nematodes were applied on 3rd, 4th and 5th instar larvae, while insect mortality induced on 1–3 days old pupae showed a maximum 70% mortality at higher concentrations (200 IJs pupa^{-1}). The LC_{50} and LC_{90} of *H. indica* for pupae after 48 h of treatment were 85.91 and 617.54 IJs pupa^{-1}, respectively (Table 1).

Nematode reproductive potential on *E. materna* larval instars

The results indicate that *H. indica* successfully reproduced in the 3rd, 4th and 5th larval instars of *E. materna* and their offspring were emerged from the larval cadavers. Non-significant difference was detected among different concentrations of IJs inoculated to 4th instar larvae on the reproduction of *H. indica* ($F=1.16$; $df=4$; $P<0.356$). However, significant difference was observed between 50 to 200 IJs and 400–600 IJs concentrations inoculated to 3rd instar larvae on the reproduction of *H. indica* ($F=38.07$; $df=4$; $P<0.001$). Significant difference was also detected among the 5 IJ concentrations inoculated to 5th instar larvae on the reproduction of *H. indica* ($F=12.57$; $df=4$; $P<0.001$). The reproduction of *H. indica* in 3rd instar larvae was significantly lower than that of 4th and 5th instar larvae. Although the reproductive potential of *H. indica* in the larvae of *E. materna* significantly increased as the rate of IJs increased, the number of IJs per 5th instar larvae at the

Table 1 The calculated LC_{50} and LC_{90} values (IJs larva^{-1} or pupa^{-1}) for *Heterorhabditis indica* on *Eudocima materna* larvae and pupae in the laboratory bioassays

Larval instar/pupa	LC_{50} (95% CL)[a]	LC_{90} (95% CL)[a]	Slope ± SE[b]	Intercept ± SE[b]	χ^{2c}	*P* value[d]
3rd	14.43 (6.36–32.72)	59.56 (26.26–135.04)	2.08 ± 0.62	− 2.41 ± 0.92	0.47	0.03
4th	17.08 (7.36–39.65)	80.44 (34.65–186.73)	1.90 ± 0.55	− 2.34 ± 0.85	0.76	0.02
5th	23.63 (10.73–52.04)	106.52 (48.37–234.59)	1.96 ± 0.53	− 2.69 ± 0.84	0.64	0.02
Pupa	85.91 (32.99–223.75)	617.54 (237.12–1608.25)	1.49 ± 0.47	− 2.89 ± 0.85	0.37	0.03

[a] Concentrations are expressed in IJs/larva; [b]SE, standard error; [c]χ^2 of the slope; [d]*p* values represent the probability of the slope

higher concentration (1,082,855; 600 IJs larva^{-1}) was significantly higher than at the lower concentrations (Fig. 5).

Discussion

EPNs are successfully used in different parts of the world to control different insect pests. Susceptibility of EPNs to targeted insect pest can vary greatly among strains of the same species. Hence, an in vitro study on infectivity of EPNs is a prerequisite in a biocontrol program for a particular insect pest prior to field studies (Ricci et al. 1996). Based on the available literature scan and to the best of our knowledge, obtained data provide the first insight into the pathogenicity of EPNs against citrus fruit piercing moth larvae and pupae.

The present investigation showed that *H. indica* strain was highly virulent to *E. materna* larvae (3rd, 4th and 5th instars) and caused larval mortality under laboratory conditions. EPNs virulence was dependent on the larval stage of the host, *E. materna*. *H. indica* was highly virulent in 3rd instar larvae than the 4th and 5th larval instars. However, > 80% mortality was observed after 48 h of treatment in all the larval instars at 50 to 200 IJs concentrations. Several studies have shown that the larval stages of lepidopteran pests are susceptible to infection by different EPNs (Salari et al. 2021). Recently, Aryal et al. (2022) reported significant mortality of Queensland fruit fly (*Bactrocera tryoni*) larvae using *H. indica* strains (Hi.ECCH, Hi.HRN and Hi.HIE2) under in vitro conditions. Obtained results are consistent with the earlier studies and indicated that *H. indica* is highly virulent to larval stages of *E. materna*. Pupae of lepidopteran pests are also susceptible to EPN (Mhatre et al. 2020). Also, *H. indica* was highly lethal to *E. materna* pupae (70% mortality at 200 IJs pupa^{-1}). In similar studies, Yan et al. (2020) also reported that *H. indica* was highly virulent to lepidopteran pest, *Spodoptera litura* pupae. Recently, Aryal et al. (2022) reported the significant effect of *H. indica* strains (Hi.ECCH, Hi.HRN and Hi.HIE2) against pupae of *B. tryoni*. In contrast, Kaya and Hara (1981) reported that some lepidopteran insect pest pupae were less susceptible to EPNs. Similarly, Batalla-Carrera et al. (2010) reported low mortality (10%) in pupal stages of the tomato leaf miner, *Tuta absoluta*, whereas Kary et al (2019) reported no mortality in pupal stages of diamond back moth, *Plutella xylostella* with EPNs. From the present study, it can be concluded that the pupae of *E. materna* were susceptible to *H. indica*.

The reproductive potential of EPNs is an important feature for their persistence and pathogenicity against targeted insect pests (Blanco-Pérez et al. 2017). It not only causes insect pest mortality, but it also impacts the ability of EPNs to tackle the subsequent generations of the targeted pests (Patil et al. 2019). However, the reproduction rate of EPNs is influenced by different life stages of the host insects (Park et al. 2001). Obtained results indicated that the reproduction rate of *H. indica* was significantly dependent on the larval stage of *E. materna*. Specifically, the reproduction rate of *H. indica* was higher in 5th instar larvae than in 3rd and 4th larval instars. This result corroborates with the previous study that showed the reproduction rate of *H. indica* was higher in the later instars of *S. litura* (Acharya et al. 2020) and *H. bacteriophora* in *Heliothis virescens* (Gulzar et al. 2020).

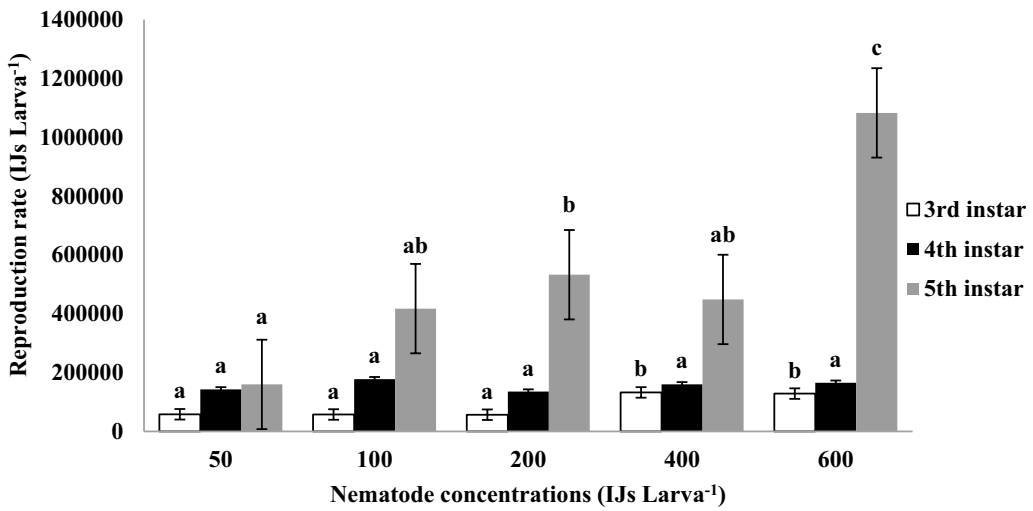

Fig. 5 Reproductive potential of *Heterorhabditis indica* in different larval instars of *Eudocima materna* within five different concentrations in a laboratory assay. Bars with different letters indicate significant differences among reproduction rates of each larval instar ($P \leq 0.05$, Duncan's test)

Furthermore, laboratory trials with diverse EPN strains are necessary to evaluate EPNs performance against the target pest, allowing the best performing strains to be selected for further field testing.

Conclusions

Efficacy of *H. indica* on larvae and pupae of *E. materna* was demonstrated under laboratory conditions. Given the difficulties and limitations in management of citrus fruit piercing moth in Citrus and other crops, the EPNs could be efficiently use against this pest species within the framework of biological control initiatives. Since the efficacy of *H. indica* on larvae and pupae *E. materna* under laboratory conditions was tested, its efficacy and mode of applications should also be tested further under field conditions for subsequent incorporation of EPNs in biological control programs. The information generated in this study would certainly be useful for better designing of management strategies for the management of fruit piercing moth in citrus and other crops.

Abbreviations

EPNs: Entomopathogenic nematodes; IJs: Infective juveniles; RH: Relative humidity.

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Author contributions

KKK designed the study, conducted the experiments, analysed the data and prepared the original manuscript. AG, GTB and YET provided research material and helped in reviewing and editing of the manuscript. DI and DPS assisted in experimental work. All authors read and approved the final manuscript.

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Availability of data and materials

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare no conflict of interest.

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