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Efficacy of entomopathogenic nematodes in laboratory and field conditions of *Cicer arietinum* against cotton bollworm, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae)

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Abstract

Background The chickpea *Cicer arietinum* (Fabaceae) field has suffered significant economic losses due to the presence of cotton bollworm, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae). Attempts to control the larval population through chemical pesticides led to some reduction but also resulted in the development of resistance among the larvae. Consequently, the *H. armigera* population experienced a rapid resurgence in the field. Given the substantial losses caused by *Helicoverpa* larvae, there is now a growing interest in adopting eco-friendly methods for management. The application of biocontrol agents has emerged as a promising solution for effectively managing the infestation.

Results In the laboratory experiment, the entomopathogenic nematodes (EPNs) *Steinernema pakistanense* NNRC-NB.14, *S. balochiense* NNRC-NB.23, and *S. abbasi* NNRC-NB.33 were evaluated for their infectivity and mortality effects on mature cotton bollworm at various concentrations. The study demonstrated that the highest mortality rates were achieved at a concentration of 250 infective juveniles (IJs) per larva after 72 h. *Steinernema pakistanense* and *S. balochiense* exhibited higher mortality rates (95–98%) compared to *S. abbasi* (77%) at higher application concentrations. The field trial results reinforced the laboratory findings, demonstrating a significant decrease in *H. armigera* larval populations when employing EPN strains, particularly NNRC-NB.14 and NNRC-NB.33, compared to NNRC-NB.23. Despite the initial applications showing relatively low mortality percentages, the most effective larval control occurred after the third application of EPNs. The field trials revealed that, following the third spray, mortality percentages significantly increased from the initial range of 20–32% to a more substantial 70–90%. This cumulative impact highlights the importance of multiple applications in achieving optimal pest control.

Conclusion The findings of these studies highlight the promising potential of EPN as an eco-friendly and sustainable method for controlling the cotton bollworm. However, the successful implementation of EPN-based control strategies in agricultural systems requires careful consideration of factors such as nematode species selection, application methods, environmental conditions, and integration with other pest management practices. Further research is needed to optimize the efficacy and practicality of using EPN on a larger scale, addressing issues such as nematode production, formulation, and compatibility with existing farming practice.

Keywords Chickpea, *Helicoverpa armigera*, Eco-friendly methods, Pest management practices, Sustainable agriculture

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Background

Chickpea *Cicer arietinum* (Linnaeus Plantae: Fabaceae) is a highly popular vegetable in various regions of the world and serves as an essential protein source for vegetarian populations. Commonly known as gram, it holds significant importance as a pulse crop (Mabrouk and Belhadj 2012). India dominates chickpea production, accounting for 67.41%, followed by Australia (6.21%), Pakistan (5.73%), Turkey (3.86%), and Myanmar (3.74%) (FAOSTAT 2015). However, a significant challenge for chickpea growers is the cotton bollworm *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae), which inflicts up to 90% damage during the plant's vegetative growth to the pod formation stage. In an attempt to manage this problem, some growers resort to increased pesticide usage. Regrettably, the haphazard or incorrect usage of pesticides has resulted in residues within the food chain, the development of pesticide resistance, resurgence of pests, and adverse effects on non-target beneficial organisms and the environment (Patil et al. 2017).

To address this issue in an environmentally friendly way, beneficial nematodes are being explored as non-chemical alternatives for pest control. While commercially formulated beneficial nematodes are utilized for pest management in various crops worldwide, they still have relatively small niche markets (Lacey and Georgis 2012). Entomopathogenic nematodes (EPNs) of the Phylum Nematoda, belonging to the genera *Steinernema* and *Heterorhabditis*, play a crucial role as obligatory pathogens in nature. They have a unique association with mutualistic bacteria of the genera *Xenorhabdus* and *Photorhabdus* (Shapiro-Ilan et al. 2014) and are considered a successful example of biological pesticides. Nevertheless, due to the evident potential of these organisms in managing pests, there are expectations of their techniques and related research spreading further. EPNs have a widespread presence in natural habitats across the globe, making them valuable for developing EPN-based pest control methods in developing countries. In such regions, one can find EPNs species that are well-adapted to the local climatic and biotic conditions, making them a promising solution for pest management in their respective areas. The study aimed to evaluate the efficacy of EPN species against cotton bollworm under both laboratory and field conditions.

Methods

Culture of *Helicoverpa armigera*

The culturing of *H. armigera* was initiated within a laboratory setting, utilizing pupae sourced from the chickpea fields of Naseerabad, Balochistan (28.3° N latitude and 68.5° E longitude). Successive generations were systematically propagated for experimental purposes. The culture was sustained in a controlled environment, with a temperature kept at 25 ± 1 °C, a relative humidity of 70 ± 5%, and a photoperiod arranged as 14 h of light, followed by 10 h of darkness (LD 14:10). The laboratory cultivation of the cotton bollworm was conducted on a chickpea-based diet, as modified by Kalia et al. (2001).

Entomopathogenic nematodes

To recover EPN from the soil, the *Galleria* trap method suggested by Bedding and Akhurst (1975) was employed. Around 500 g of soil was collected from Naseerabad, Balochistan, near the roots of various vegetables, fruits, medicinal plants, and ornamental flowers, reaching a depth of 25 cm using a hand trowel. These soil samples were then individually placed in sanitized plastic pots (26 × 14 × 6 cm). Each pot was added with six larvae of the Greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae). The pots were covered with a perforated cap, inverted, and left to incubate at room temperature (25 ± 2 °C) for one week. Daily observations were made during this period. Dead larvae were removed and their bodies were washed with distilled water to eliminate soil particles. Subsequently, to isolate the third-stage juveniles, the lifeless larvae were transferred to the White trap method introduced by White (1927). For this purpose, small plastic containers were utilized and an inverted plastic cavity block (3.0 × 3.0 cm) with filter paper on top was placed at the bottom of each container, and distilled water was added up to 1 cm, protected with lids and kept at 28 ± 2 °C. The progeny of the nematodes moved into the adjacent water that developed from the lifeless larvae. Following the migration, on the fourth day of emergence infective juveniles (IJs) were gathered from the water for morphological observations. Approximately 20 male and female IJs were morphologically identified using the procedures outlined by Nguyen and Hunt (2007). The identification process was conducted under a compound microscope.

Laboratory trial

In the laboratory experiment, the goal was to compare the infectivity of three different extracted EPN strains against the last larval stage of the cotton bollworm. For each trial, two mature larvae were placed in a 90-mm-diameter Petri dish containing two filter papers. Each dish was then treated with three different concentrations of IJs (infective juveniles) suspension: 50 IJs/ml, 150 IJs/ml, and 200 IJs/ml. The Petri dishes were carefully sealed

with parafilm to maintain the experimental conditions and were placed in a climatic chamber set at 25 ± 1 °C and 80 ± 5% relative humidity. The control group received water only, without any nematodes. The experiment was repeated five times under the same controlled conditions but on different dates. Each trial was conducted in three replicates to ensure reliable results. To assess the effectiveness of the EPN strains, the corrected mortality of the larvae was recorded every 48 h. Additionally, after the larval death, their cadavers were observed under a stereomicroscope to check the presence of new progenies of nematodes.

Field trial

The cultivation of the chickpea variety "Lalrri" (Desi) commenced on November 28, 2022, under the care of farmer Hidayatullah Mengal in Naseerabad, Balochistan, Pakistan. Throughout the growth period, the mean temperature ranged between 26 ± 3 °C with relative humidity 27.13–33.9% during February and March, 2023. A controlled field experiment was carried out. Preceding the application of treatments, the existence of indigenous EPN populations in the fields was evaluated by baiting soil samples with *G. mellonella* larvae, following the procedure by Liu et al. (2009). *H. armigera* species was identified by following CABI compendium. The field experiment was performed in a chickpea field N 28° 30′ 46.50″, E 68° 11′ 3.72″ on March 02, 2023, at 5:00 pm, to determine which EPN species are effective against cotton bollworm in field conditions, temperature was 26 °C. The chickpea plants in the experiment field were 3 months old. Each plant covered an area of 6 inches (diameter = 12 inches), and experimental plot had an area of 15 m^2 (3 × 5m) with a 1m buffer space set between plots. The cotton bollworm species present in the experiment field was in a ratio of 4 to 5 larvae per plant, mainly in the first, second, and third instar with a ratio of 1:5: 2. The nematodes *S. pakistanense* (Shahina et al. 2001), *S. balochiense* (Shahina et al. 2015), and *S. abbasi* (Elawad et al. 1997) were administered in the form of liquid suspension containing 1% glycerin in the early morning. A dosage of 10^9 infective juveniles (IJs) per square meter was employed with backpack sprayer. Specifically, each square meter was treated with 1 L of the prepared nematode suspension. To maintain the integrity of the experimental area and to prevent the escape of treated cotton bollworm, protective nylon nets were erected around the designated microplots. The initial application acquired one week following the insect infestation. Subsequently, the second application was sprayed one week after the first, and the third application was administered one week after the second application. Water without IJs was set as a control. In the experiments, mortality was observed after 2 days of each spray. A similar volume of only water was used for the control experiment. The experimental setup included three replicates for each species independently and implemented through a complete randomized arrangement. Subsequently, the lifeless cotton bollworm was transported to the laboratory and positioned over the White trap, for the confirmation of juveniles' emergence and duly recorded.

Statistical analysis

Multifactor analysis of variance (ANOVA) was employed to analyze the statistical data, and subsequently Duncan's multiple range test was conducted (SAS Institute 2002). The efficacy of the treatment was determined using Abbott's formula.

The formula is as follows:

$$\text{Corrected Mortality} = \frac{\text{Observed Mortality in Treatment} - \text{Observed Mortality in Control}}{100 - \text{Observed Mortality in Control}} \times 100$$

Table 1 Status of entomopathogenic nematodes from Naseerabad, Balochistan

Sample code	Locality	Host	IJs length (µm)	EPNs species
NNRC-NB.14	Quba shir khan	*Psidium guajava*	667 ± 30 (649–716)	*S. pakistanense*
NNRC-NB.23	Dera Murad Jamali	*Cynodon dactylon*	415.5 ± 26.46 (330–485)	*S. balochiense*
NNRC-NB.33	Dera Murad Jamali	*Citrus sinensis*	595 ± 29.4 (522–615)	*S. abbasi*

Results

Entomopathogenic nematodes

The present surveys first time reported the presence of EPNs from Naseerabad, Balochistan, Pakistan. During this survey, only the genus *Steinernema* was identified, comprising three species of EPNs: *S. pakistanense* NNRC-NB.14, *S. balochiense* NNRC-NB.23 and *S. abbasi* NNRC-NB.33. Although these species were previously reported from Pakistan, the present survey revealed their presence in new geographical locations with some variations in their morphometrics. Key morphological characterization was made concerning the length of 3rd-stage infective juveniles (Table 1). These nematodes were cultured on the final larval instar of the greater wax moth, *G. mellonella*, following Dutky's method. To collect the infective juveniles (IJs) of each nematode species, White traps were utilized. The collected IJs were then individually stocked in Pyrex flasks containing 70 ml of distilled water and kept at 10 °C. These infective juveniles were stored for a period of 15 days to be used in the subsequent experiments.

Laboratory trial

In the laboratory experiment, isolates of *S. pakistanense* NNRC-NB.14, *S. balochiense* NNRC-NB.23, and *S. abbasi* NNRC-NB.33 were evaluated for infectivity and mortality of mature bollworm at different concentrations. The analysis of variance (ANOVA) revealed notable differences in the efficacy of nematode species against cotton bollworm (ANOVA $F=28$; df = 2; $P=0.05$). Additionally, there were significant variations in nematode concentrations (RCBD one-way ANOVA $F=23$; df = 2; $P=0.05$), and the interaction among the concentrations and three nematode species yielded prominent effects (ANOVA $F=27$; df = 2; $P<0.05$). The control treatment showed no response against cotton bollworm. Maximum mortality rates were achieved at concentrations of 250 IJs/ml after 72 h (Fig. 1). The results confirmed that the EPNs could confine the occurrence of *S. pakistanense* and *S. balochiense*, exhibiting higher mortality rates (95–98%) at high application concentrations than *S. abbasi* (77%). At the lowest concentration (150 IJs/ml), *S. pakistanense* caused 62% mortality, while *S. balochiense* and *S. abbasi* showed 45 and 30% mortality, respectively. Mortality rates of *H. armigera* increased depending on the concentrations. Nematode progenies reproduced in the dead cadaver of *H. armigera*, clearly seen when dead larvae were transferred to a vacant cavity block (Fig. 2).

Field trial

Despite recording mortality data following each spray, the initial application exhibited a relatively low mortality percentage. The highest larval control was noted after the third application of EPNs. The field trial results revealed a decrease in *H. armigera* larval populations when employing EPNs strains ($F=32.13$; df = 2; $P<0.001$). Despite recording mortality data after each spray, the initial application exhibited a relatively low mortality percentage. The most effective larval control occurred after the third application of EPNs (Fig. 2). Among the three EPNs species, the population of cotton bollworm was significantly reduced in the plots sprayed with NNRC-NB.14 ($F=50.13$; df = 2; $P<0.001$) and NNRC-NB.33 ($F=52$; df = 2; $P<0.001$) compared with NNRC-NB.33 (Fig. 3). After the first spray, mortality percentages ranged between 20 and 32%. After the third spray, mortality percentage significantly increased up to 70–90%. To validate

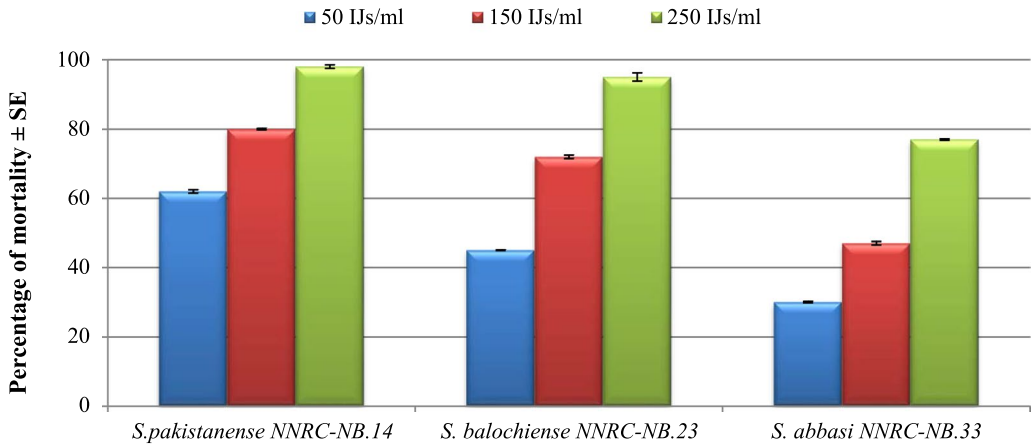

Fig. 1 *Helicoverpa armigera* mean mortality treated with three different species of entomopathogenic nematodes in laboratory trial depending on their concentration

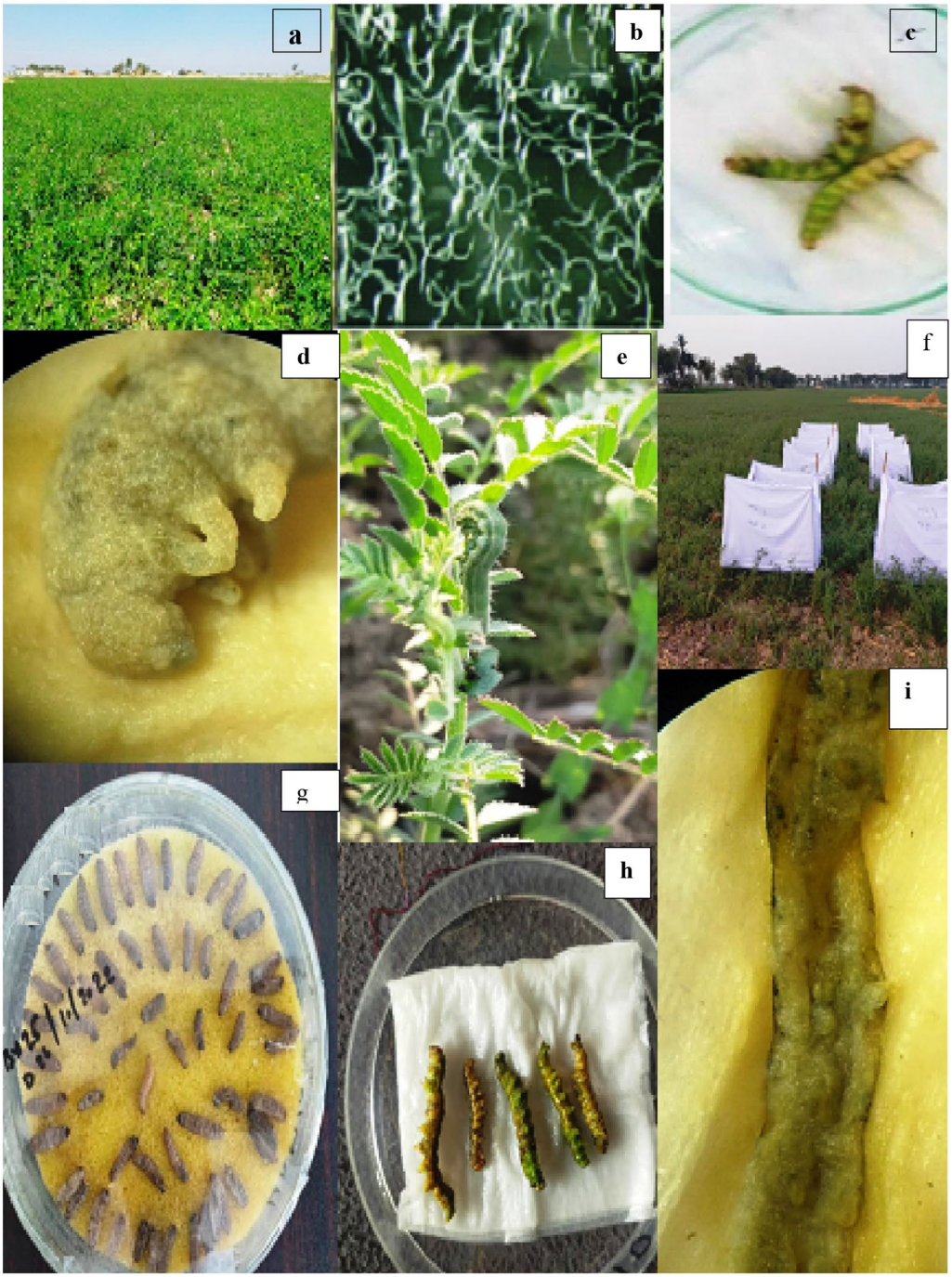

Fig. 2 Application of EPNs *against Helicoverpa armigera* in laboratory and field trial **a** Infected chickpea field, **b** harvesting of IJs for treatment, **c** laboratory trail, **d** emergence of IJs from dead cadaver, **e** population density for field trail, **f** controlled field, **g** infected *G. mellonella* for IJs application in field, **h** dead pod borer from field after IJs spray, **i** progenies emergence from pod borer

larval mortality caused by EPN species, the deceased cadavers were transported to the laboratory and placed over the White trap. The emergence of nematode juveniles was documented, revealing a significant population management in treated plots as comparison to the control.

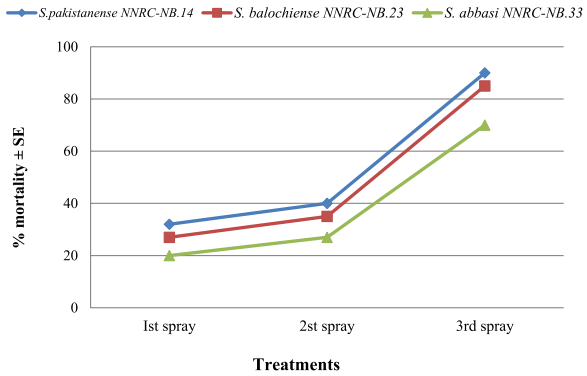

Fig. 3 *Helicoverpa armigera* mean mortality treated with three different spray events of entomopathogenic nematodes in field trial

Discussion

The observed efficacy of EPNs in controlling cotton bollworm, specifically *H. armigera*, aligns with findings from various studies focusing on nematode-based biocontrol strategies. Notably, the concentration-dependent effects witnessed in the present study, where higher nematode concentrations corresponded to increased mortality rates, corroborate similar observations in related research (Patil et al. 2017). Utilizing EPNs as biocontrol agents against agricultural pests, a critical requirement involves appropriately packaging infective juveniles (IJs) at an optimal temperature tailored to the specific needs of each species. This ensures that infective juveniles can endure with maximum shelf life (Lalramliana Yadav 2009).

In practical agricultural settings, our field trials echoed cumulative impacts reported in previous investigations, emphasizing the necessity of sustained biocontrol efforts. The repeated applications of EPNs led to a gradual reduction in *H. armigera* larval populations, supporting findings from studies exploring the practical application of specific nematode strains (Sharma et al. 2019; Hussain et al. 2014). Furthermore, our research contributes to the ongoing discourse on nematode-based pest management by highlighting the variation in mortality rates among nematode species. This features the importance of tailored selection based on the target pest and local environmental conditions, aligning with the findings of Prabhuraj et al. (2008).

After spraying *S. glaseri* (Steiner 1929) at a concentration of 200 IJs/ml on chickpea in pots, achieved a mortality rate of 24.6% for *H. armigera* after 6 days Patel and Vyas (1995). The bioefficacy study of GAU EPN 16 (*Heterorhabditis* sp.) against *H. armigera* on chickpea further contributes to the understanding of nematode-based pest control. The observed mortality rates of 50% on the 4th day, reaching a peak of 70.9% on the 6th day at a 2000 Us pot dose, and reductions in pod damage 49 and 37% for GAU EPN 3 *S. riobrave* (Cabanillas et al. 1994) and GAU EPN 16 highlight the potential practical application of specific nematode strains in the management of economically significant pests (Vyas et al. 2003). Prabhuraj et al. (2008) investigated a successful management strategy against chickpea pod borer, employing *Heterorhabditis indica* (Poinar et al.1992) (RCR) along with other entomopathogens. Compatibility studies reveal that *H. indica* can be effectively combined with other entomopathogens and botanicals. Laboratory bioassays and field trials are conducted over two consecutive years to identify the best combinations for controlling third and fourth instars of *H. armigera* larvae. The success of the management strategy against chickpea pod borer by *H. indica*, as identified in compatibility studies and laboratory bioassays, offers a promising avenue for integrated pest management. Patil et al. 2017) explore the bioefficacy of *H. indica* against *H. armigera*, focusing on different doses during the years 2018–2019. The study demonstrated that a concentration of 200 infective juveniles per ml per Petri dish induced quicker 100% mortality, with varying mortality rates observed across different larval instars and exposure times. The research concludes that the mortality percentage increased with extended exposure time. Investigation by Hussain et al. (2014) on the bioefficacy of locally isolated EPNs; *S. masoodi* (Ali et al. 2005), *S. carpocapsae* (Weiser 1955). Wouts et al. (1982), and *H. indica* further expands the repertoire of potential nematode-based pest management strategies.

Steinernema feltiae (Filipjev 1934) demonstrated significant lethality against *H. armigera* prepupae in soil. Injection of *S. feltiae* into the hemolymph of prepupae resulted in elevated plasma phenoloxidase activity, indicating an immune response triggered by the nematode. These findings underscore the potential of *S. feltiae* as an effective biological control agent against *H. armigera* infestations (Ebrahimi et al.2018).

Ten EPN isolates of *H. indica* successfully reproduced *on H. armigera* larvae, with isolates CICR-Su and CICR-SUB showing notably higher virulence. All ten EPN isolates successfully reproduced on *H. armigera* larvae, with isolates CICR-Su and CICR-SUB showing notably higher virulence (Gokte-Narkhedkar et al. 2019). Sharma et al. (2019) also conducted an experiment for successful control of gram pod borer using *Steinernema* spp. The study revealed a significant mean percent mortality of *H. armigera* with various *Steinernema* spp. strains and different inoculum levels under pot conditions. The maximum 88% mean percent mortality was observed with *Steinernema* spp. STUDR-1 at 2000 IJs per pot after 5 days of exposure. Laboratory bioassays and field experiments provided practical insights into the efficacy

of these locally isolated nematodes against legume pod borer infesting chickpea. In filed conditions after the onset of food storage within the cadaver, a multitude of EPNs emerged from the deceased insect's body, actively seeking new hosts (Kaplan et al. 2020). The effectiveness of biological control against *H. armigera* using EPNs was found to be influenced by both the specific nematode species and strains utilized and the dosage applied. When targeting *H. armigera*, augmenting the dosage from 1 to 3×10^9 IJs·hm^{-2} infective juveniles per hectare led to a decrease in *H. armigera* larvae and associated crop damage, consequently resulting in increased yield (Seenivasan 2022).

Our study results further emphasize the potential of EPNs as a crucial element within integrated pest management programs targeted at fostering sustainable agricultural practices.

Conclusions

The findings elucidated in this study represent a substantial advancement in our comprehension of the potential efficacy of entomopathogenic nematodes (EPNs) as a sustainable solution for pest management within agricultural ecosystems. Through a comprehensive examination of their performance, including concentration-dependent effects and cumulative impacts observed in field conditions, this research underscores the critical significance of precision and strategic formulation in the deployment of nematode-based biocontrol strategies. The demonstrated efficacy of EPNs in reducing pest populations, particularly when applied at optimal concentrations, highlights their potential as a valuable tool in integrated pest management (IPM) programs. By targeting specific pests while minimizing adverse effects on non-target organisms and the environment, EPNs offer a promising avenue for reducing reliance on conventional chemical pesticides and fostering environmentally sustainable agricultural practices. As we strive toward more ecologically sound and economically viable pest management solutions, EPNs emerge as a promising ally in our quest for a more resilient and harmonious agricultural landscape.

Abbreviations

EPNs	Entomopathogenic nematodes
IJs	Infective juveniles
spp	Species
ANOVA	Analysis of variance
RCBD	Randomized completely block design

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MA performed and wrote experiment; SJ analyzed the data, read, and approved the final manuscript; SM helps MA in experiment.

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Competing interests

We have no potential conflicts of interest.

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