



Efficacy of two strains of Entomopathogenic nematodes (*Heterorhabditis indica* and *Steinernema carpocapsae*) against *Helicoverpa armigera* under laboratory conditions

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Abstract

Helicoverpa armigera is known to be an important agricultural pest for several crops due to its mobility, high production rate, and short generation durations. It mainly damages the economic portion of the plant (seeds, leaves, etc.). Efficacy of entomopathogenic nematodes (EPNs) against *H. armigera* organized as potential biocontrol agents, due to nontoxic effect on other living organisms and the ability to overcome targeted pests. The present study was designed to examine the susceptibility of *H. armigera* to the EPNs. Two species of EPNs (*Heterorhabditis indica* and *Steinernema carpocapsae*) were used against different larval stages (2nd, 3rd, and 4th) of *H. armigera* using different concentrations. Treatments were replicated five times in each separate petri plate with one larva. Data was analyzed at a 5% level of significance and subject to statistical analysis. The experimental results were organized with five treatments and five replications in Complete Randomized Design (CRD). The data has been reported on daily basis of time intervals (72 hours) of larval instars (2nd, 3rd, and 4th). The results showed that the maximum mortality in 2nd, 3rd, and 4th instar larvae of *H. armigera* after 72 hours were recorded 91.7, 87.6, and 78 %, respectively, when subjected to highest concentration of *H. indica* (250 IJsm1-1) as compared to *S. carpocapsae*. In conclusion, the study confirms that both EPN species are effective biocontrol agents against *H. armigera*, with efficacy increasing significantly at higher concentrations.

1. Introduction

American bollworm, *Helicoverpa armigera* Hübner (Lepidoptera; Noctuidae) is extremely polyphagous and considered the major insect pest of cotton crop, causes quantitative and qualitative damage to the crops which resulting yield loss. It is widely distributed throughout the world and mostly present in Chile, Pakistan, Netherland, China, Uruguay, and India (Fichetti et al., 2009; Murua et al., 2018). It was found to be a major pest of cotton, sorghum, maize, sunflower, and tomatoes. They damage the blooms, flowers, leaves and buds of cotton crops (Kary et al., 2012). *Helicoverpa armigera* prefers cotton, chickpea and okra for larval feeding and oviposition (Kakimoto et al.,

2003). This adaptability allows *H. armigera* to overcome both natural plant defenses and engineered resistance mechanisms, rendering many conventional and transgenic control strategies less effective over time (Lomate and Hivrale, 2011).

Helicoverpa armigera has a serious problem with pyrethroids resistance. Synthetic pesticides are used to control insect pest of Lepidoptera worldwide which has led to the growth of susceptibility to insecticides in several species (Bloem and Carpenter, 2001). Conventional chemical control methods have become increasingly ineffective against *H. armigera* due to widespread development of resistance to multiple insecticide classes,

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including pyrethroids, organophosphates, carbamates, organochlorines, and even *Bacillus thuringiensis* (Bt) toxins (Souza et al., 2017). Documented cases of resistance have led to frequent field failures and substantial economic losses across the Indian subcontinent. The pest's complex digestive physiology further complicates control efforts, as it possesses a sophisticated gut proteinase system with predominantly trypsin-like activity that can adapt to plant proteinase inhibitors by overexpressing inhibitor-insensitive proteinases or degrading the inhibitors themselves (Chamani et al., 2025).

Given these challenges, sustainable integrated pest management approaches incorporating biological control agents have gained significant research attention. Two species of Entomopathogenic nematodes (EPNs) related to families Steinernematidae and Heterorhabditidae are specially used to overcome and ultimately reduce the population of pests in specific crop (Divya and Sankar, 2009). The EPNs have a mutualistic symbiotic association with specific type of bacterial genus (the genus *Heterorhabditis* has mutualistic relation with bacterial genus *Photobacterium* and Steinernematids with genus *Xenorhabdus*) (Shapiro-Ilan et al., 2009). This combination forms a functional unit which is responsible for the severe pathogenicity against insect pests (Azizoglu et al., 2020). The EPNs have several life stages, but 3rd stage is more crucial for pest management, known as infective juveniles (IJs) stage (Eleftherianos et al., 2016). The other EPNs stages are non-feeding and free-living that can survive without host for two to several months. The physiology and morphology of EPNs allow them to adapt and seek new hosts.

The mode of action begins when the infective juveniles actively seek the host larvae, by using cues like chemical cues (Risser et al., 2016). Once appropriate host is located, the infective juveniles (IJs) reached and penetrates the insect body through natural openings (mouth, anus and spiracles), studies reported that some species of EPNs can penetrate the larval body directly through cuticle (Eleftherianos et al., 2016). After entering the IJs moved to hemocoel of the insect's body and releases mutualistic bacteria (Grewal, 2000), the bacteria's start multiplication inside the insect host and starts tissue breakdown, which killed the insect host within 24 to 72 hours due to septicemia (Torres-Barragan et al., 2011). These bacteria also generate wide types of toxins and antimicrobial substances that help to protect insect larval cadaver from being colonized by other competing microorganisms (Risser et al., 2016). Then IJs of EPNs feed on the host digested tissues and multiplying bacteria, maturing into adults and reproducing. One to three generations of EPNs can develop inside the protective environment of insect cadaver. After depletion of host's resources, the new generation of IJs emerges from the

cadaver, dispersing into soil to locate new hosts and continue the life cycle (Grewal, 2000).

Moreover, EPNs are cosmopolitan in distribution (lived throughout the world except Antarctica), lived in soil ecosystems and can collectively infect many insects, making them ubiquitous natural enemies in several agroecosystems (Risser et al., 2016). In Pakistan, survey studies proposed that several species of EPNs have identified such as *Steinernema pakistanense*, *S. asiaticum*, *S. abbasi*, *S. balochiense*, *S. siamkayai*, *Heterorhabditis indica* and *H. bacteriophora*, which are present in soil ecosystem of Pakistan (Khan et al., 2021; Javed et al., 2025).

This study addresses this knowledge gap by systematically evaluating the comparative efficacy of *H. indica* and *S. carpocapsae* against *H. armigera* under standardized laboratory conditions. The research specifically examines mortality rates of different larval stages to determine optimal application parameters for field implementation. Understanding the precise mechanisms and effectiveness of these nematode strains against *H. armigera* is critical for developing sustainable, resistance-proof biocontrol strategies that can reduce reliance on chemical insecticides. The findings from this laboratory investigation will provide foundational data for subsequent field trials and potential integration into comprehensive *H. armigera* management programs worldwide.

2. Materials and Methods

2.1 Culture of *Helicoverpa armigera*

Helicoverpa armigera laboratory strains were developed by prepared late instar larvae from various host plants. The larvae were collected in glass vials containing an artificial diet and transferred to the insect Biodiversity Laboratory, Department of Entomology, University of Agriculture Faisalabad. The larvae were reared on an artificial diet maintained in a controlled environment. The diet was poured into small cups approximately 5 ml per cup and left to cool and solidify ready for the addition of larvae. Larvae were reared at a temperature of 25±5°C with 75±5% relative humidity (r.h) under the specific hours e.g. 16 h: 8 h L: through an artificial diet. Small vials were used to collect pupae and put on petri plate. For adult emergence of pupae petri plate was placed in plastic containers followed by standard procedure. Open plastic containers covered with white netting were used for adults and top of the netting contained a food source having a cotton wool pad soaked in 10% (v/v) honey solution.

Eggs laden netting was replaced approximately every 48 hours and this process was repeated again and again until production of egg decreases or until no more eggs are needed. Smaller cubes were prepared by cutting netting and were poured in 250 ml plastic cups of round shape and sealed lid made of plastic and give them

controlled environment conditions until the hatching of eggs. At the very end of the process, the eggs were incubated for hatching. Generation of F1 neonate larvae Followed by standard procedure was used for bioassays.

2.2 Culturing of entomopathogenic nematodes

Rearing of the late instar larvae of *G. mellonella*, the bigger wax moth was done by using the two species, *H. indica* and *S. carpocapsae* of Entomopathogenic nematodes. Hives of bee that were infected with *G. mellonella* was obtained from different locations at the University of Agriculture, Faisalabad. Last instar larvae of *G. mellonella* were removed for a culture of nematodes. For the whole experiment two different species of EPNs *H. indica* and *S. carpocapsae* were used. Dead larvae color was used for identification, the grey color showed by *Steinernema* infected larvae and brick red color showed by *Heterorhabditis* genus larvae after death. Entomopathogenic nematodes with the help of the white trap method were collected from *G. mellonella* (White, 1927). Blackish, damaged and alive larvae were separated and removed. The White trap consisting of a clear plastic container 9 cm diameter×4.5 cm deep was used for extraction and collection of nematodes from infected larvae. An inverted Petri dish was taken in the container and filled with distilled sterilized water, about 1 cm deep.

Put a sheet of filter paper on the petri dish by allowing the filter paper edges to come into contact with water. After that dead larvae (2-3 per trap) are taken to the top of the petri dish on filter paper. With the help of a lid, the plastic container was closed then incubated at 20-27°C, so that infected juveniles begin to leave the dead larvae around 8-20 days after infection. Infective juveniles moved

own into the water with the aid of filter paper. They were collected regularly and there would not be any more juveniles in the cadaver. The IJs are collected from the container and then the container is refilled with sterilized distilled water for the next harvest. Water from IJs is moved to a 100 ml beaker.

Beakers are loaded with distilled water down to the rim to provide a simple suspension. Nematodes must be replenished with sterilized distillation water and repeat the process 3-4 times, or either suspension is removed. Nematodes are collected after that and Infective juveniles are kept at about 20°C. Nematode suspension was diluted to about 5,000 ml⁻¹. These kept in shallow clear plastics containers having lids with no more suspension up to 1 cm in depth to ensure that enough oxygen is available. Freshly hatched juveniles were kept for 3 months. All nematode culture was re-cultured every 3 months. Suspension of the nematode can dilute to around 5,000 ml⁻¹. These held lids with no more suspension up to 1 cm in depth in small, transparent plastics containers to ensure adequate oxygen is usable.

Freshly hatched juveniles are to be kept for three months. Only newly emerging Entomopathogenic nematodes (less than 2 weeks old) were used to investigate their effects on *H. armigera*'s different life stages. Two species of EPNs (*H. indica* and *S. Carpocapsae*) were used against different larval installations (2nd, 3rd, and 4th) and preparatory stage of *H. armigera*, using specific concentrations. Every procedure is repeated five times in each petri dish, with one larva (9 cm diameter).

2.3 Bioassay

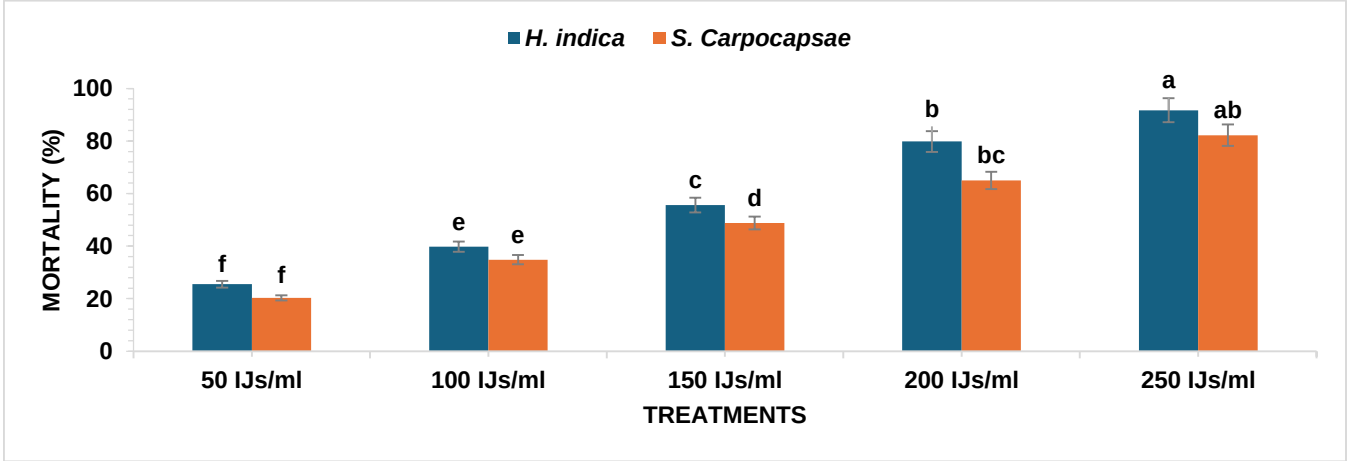


Figure 1. Mean percent mortality of 2nd instar larvae of *H. armigera* as affected by different concentrations of *H. indica* and *S. carpocapsae* after 72 hours of treatments. Bars with different lower-case letters are significantly different. LSD test at *p* ≤ 0.05. Vertical bars indicate SE. Where, IJs: Infective juveniles.

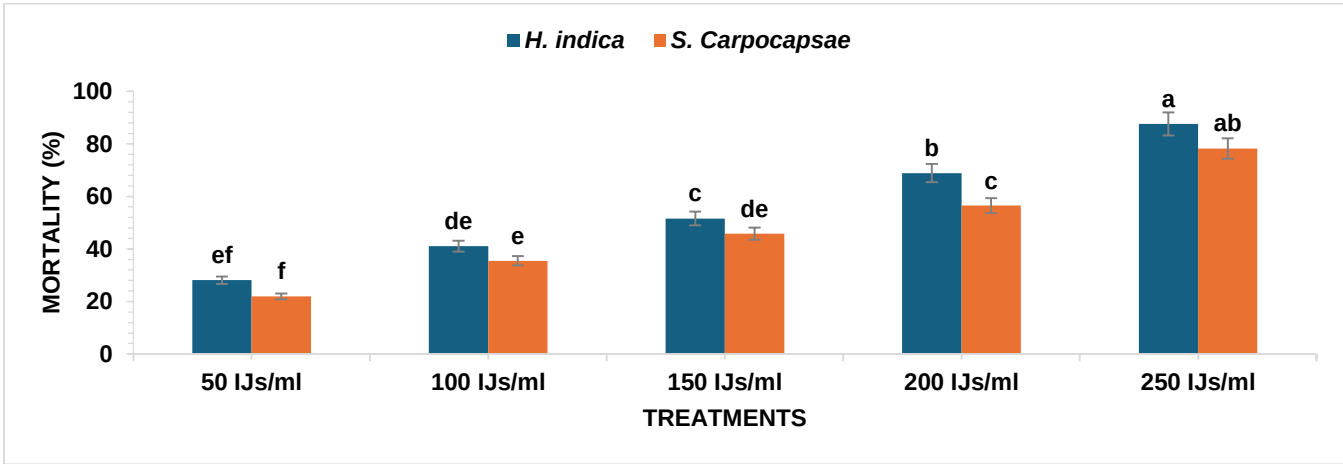


Figure 2. Mean percent mortality of 3rd instar larvae of *H. armigera* as affected by different concentrations of *H. indica* and *S. carpocapsae* after 72 hours of treatments. Bars with different lower-case letters are significantly different. LSD test at $p < 0.05$. Vertical bars indicate SE. Where, IJs: Infective juveniles.

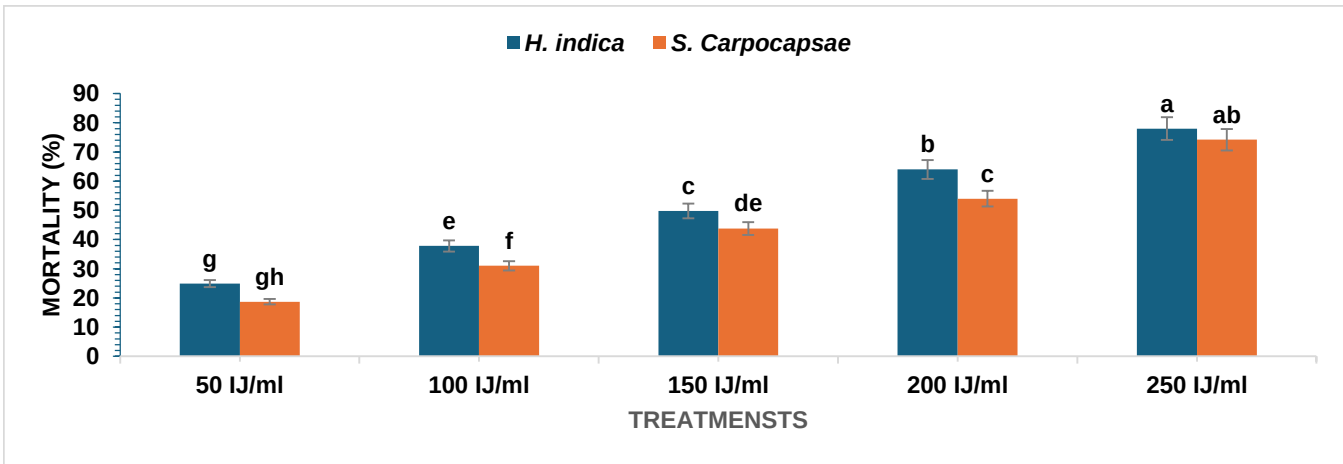


Figure 3. Mean percent mortality of 3rd instar larvae of *H. armigera* as affected by different concentrations of *H. indica* and *S. carpocapsae* after 72 hours of treatments. Bars with different lower-case letters are significantly different. LSD test at $p < 0.05$. Vertical bars indicate SE. Where, IJs: Infective juveniles.

Various EPN species *H. indica* and *S. carpocapsae* also maintained in the lab have been tested against different larval stage (2nd, 3rd, and 4th) of *H. armigera*. The larvae were placed in petri plate stuff with round filter paper contain 5 larvae in each petri plate at different doses of IJs (50, 100, 150, 250 IJS ml⁻¹ and, control). Data on larval mortality have been reported. Mortality data were collected at regular time intervals (24, 48 and, 72 hours) at 28°C and 92% r.h. Five individuals were treated with a specific nematode’s concentration in each treatment. They have been different combinations of treatments: 6 concentrations 0, 50, 100, 150, 200, and 250 4 instars 2nd, 3rd and, 4th with 3 replications under complete randomized design. Each

procedure was repeated five times. Data was recorded on mortality, multiplication and subject to statistical analysis at a 5% level of significance.

2.4 Statistical analysis

Data was recorded on mortality and subject to statistical analysis using Statistix V8.1 and means were further analyzed using LSD test at a 5% level of significance (Steel et al., 1997).

3. Results

3.1 Efficacy of *Heterorhabditis indica* and *Steinernema carpocapsae* on 2nd instar larvae of *Helicoverpa armigera*

Analysis of variance revealed that the different Infective juvenile (IJs) concentrations of both entomopathogenic nematodes *H. indica* and *S. carpocapsae* significantly affect 2nd larval instar of *H. armigera* after 72 hours of application. The results further showed that the tested treatments of different infective juvenile concentrations of *H. indica* and *S. carpocapsae* differed significantly ($p < 0.05$) from each other. Moreover, a significantly higher larval mortality of *H. armigera* was observed in case of higher IJs concentrations of *H. indica* as compared to *S. carpocapsae* and its lower doses (Figure 1). A clear dose dependent trend was recorded, with higher infective juvenile (IJs) concentrations produced significantly greater mortality ($p < 0.05$).

The results of the experiment exhibited that maximum mortality was observed when 2nd instar larvae of *H. armigera* were subjected to highest concentration of *H. indica* (250 IJs ml⁻¹) (91.7%) and for *S. carpocapsae* caused 82.21% mortality with same rate of treatment, followed by its 2nd highest concentration (200 IJsml⁻¹) (79.8%). The results further revealed that mortality gradually declined when the 2nd instar larvae of *H. armigera* were exposed to lower concentration of infective juvenile of *H. indica* and *S. carpocapsae* at the rate of 150 IJsml⁻¹ with mean mortality 55.60 and 48.81%, respectively. The significantly different minimum mean mortality of 2nd instar larvae of *H. armigera* was recorded in case of lowest infective juvenile concentration (50 IJsml⁻¹) of *H. indica* and *S. Carpocapsae* the mean mortality was recorded as 25.5% and 20.29%, respectively (Figure 1). Overall, mortality increased significantly with rise in concentrations of entomopathogenic nematodes both species and *H. indica* was generally more virulent under the conditions tested.

3.2 Efficacy of *Heterorhabditis indica* and *Steinernema carpocapsae* on 3rd instar larvae of *Helicoverpa armigera*

ANOVA exhibited that the tested treatments of different Infective juvenile (IJs) concentrations of both entomopathogenic nematodes *H. indica* and *S. carpocapsae* significantly affect 3rd instar of *H. armigera* after 72 hours of application. The results further showed that the tested treatments of different juvenile concentrations of *H. indica* and *S. carpocapsae* differed significantly ($p < 0.05$) from each other. Moreover, a significantly higher larval mortality of larvae was observed in case of higher concentrations of IJs of *H. indica* as compared to *S. carpocapsae* and its lower doses (Figure 1). A clear dose dependent trend was recorded, with higher infective juvenile (IJs) concentrations produced significantly greater mortality ($p < 0.05$).

The results indicate that mortality at 250 IJsml⁻¹ for *H. indica* differed significantly from most of other treatments, whereas *S. carpocapsae* at the same concentration showed partial overlap with adjacent groups. The higher treatment test (250 IJsml⁻¹) resulted in the maximum mortality of 3rd instar larvae of *H. armigera*, with

H. indica achieving 87.6% mortality and *S. carpocapsae* reaching 78.2%. At 150 IJsml⁻¹, the mean mortality was noticed as 51.6% for *H. indica* and 45.8% in case of *S. carpocapsae*. Statistically non-significant, the minimum mean percentage mortality was recorded as 28.1 and 22% after the treatment of *H. indica* and *S. carpocapsae* infective juveniles at the rate of 50 IJs ml⁻¹, respectively. Overall, all the tested concentrations, *H. indica* consistently produced greater mortality than *S. carpocapsae* demonstrating superior virulence (Figure 2).

3.3 Efficacy of *Heterorhabditis indica* and *Steinernema carpocapsae* on 4th instar larvae of *Helicoverpa armigera*

Analysis of variance revealed that all the tested treatments of different Infective juvenile (IJs) both entomopathogenic nematodes *H. indica* and *S. carpocapsae* significantly affect 4th larval instar of *H. armigera* after 72 hours of application. The results further showed that the tested treatments of different juvenile concentrations of *H. indica* and *S. carpocapsae* differed significantly ($p < 0.05$) from each other. A clear dose dependent trend was recorded, with higher infective juvenile (IJs) concentrations produced significantly greater mortality ($p < 0.05$) (Figure 3). The results of the experiment showed that significantly maximum mean mortality was observed when 4th instar larvae of *H. armigera* were subjected to highest concentration of *H. indica* (250 IJsml⁻¹) (78.0%) and for *S. carpocapsae* caused 72.2% mortality with same rate of treatment, followed by its 2nd highest concentration (200 IJsml⁻¹) (64.0 and 54.0%, respectively).

The results further revealed that mortality gradually declined when the 4th instar larvae of *H. armigera* were exposed to lower concentration of infective juvenile of *H. indica* and *S. carpocapsae* at the rate of 150 IJsml⁻¹ with mean mortality 49.8 and 43.8%, respectively. The significantly different minimum mean mortality of 4th instar larvae of *H. armigera* was recorded in case of lowest IJs concentration (50 IJsml⁻¹) of *H. indica* and *S. Carpocapsae* with mean mortality 24.9% and 18.7%, respectively (Figure 3). Overall, mortality increased significantly with rise in concentrations of entomopathogenic nematodes both species and *H. indica* was generally more virulent under the conditions tested.

3.4 LC₅₀ of EPNs for pathogenicity on 2nd instar larvae

The lethal concentration LC₅₀ of the two entomopathogenic nematodes species tested against *H. armigera* exhibited noticeable differences in their virulence. *Heterorhabditis indica* showed an LC₅₀ of 145.3 IJsml⁻¹ and *S. carpocapsae* showed an LC₅₀ of 167.7 IJsml⁻¹. The results clearly showed that *H. indica* relatively more virulent than *S. carpocapsae*, as evidence by its lower LC₅₀ value (Table 1).

3.5 LC₅₀ of EPNs for pathogenicity on 3rd instar larvae

The lethal concentration LC₅₀ of the two entomopathogenic nematodes species tested against *H. armigera* exhibited

Table 1. LC₅₀ of both entomopathogenic nematodes species against 2nd instar larvae of *Helicoverpa armigera*

Nematode species	95% CI (Lower–Upper)	Slope ± SE	χ ²	LC ₅₀ (IJsm ⁻¹)
<i>H. indica</i>	132–165	1.23 ± 0.15	2.34 (3)	145.3
<i>S. carpocapsae</i>	150–182	1.10 ± 0.12	1.98 (3)	165.7

Table 2. LC₅₀ of both entomopathogenic nematodes species against 3rd instar larvae of *Helicoverpa armigera*

Nematode species	95% CI (Lower–Upper)	Slope ± SE	χ ²	LC ₅₀ (IJsm ⁻¹)
<i>H. indica</i>	120.4–171.8	1.82 ± 0.24	3.61	148.5
<i>S. carpocapsae</i>	150.1–210.5	1.65 ± 0.21	2.94	176.7

Table 3. LC₅₀ of both entomopathogenic nematodes species against 3rd instar larvae of *Helicoverpa armigera*

Nematode species	95% CI (Lower–Upper)	Slope (± SE)	χ ²	LC ₅₀ (IJsm ⁻¹)
<i>H. indica</i>	148.2 – 201.7	1.24 ± 0.12	3.65	173.4
<i>S. carpocapsae</i>	165.0 – 218.9	1.05 ± 0.10	4.12	190.7

noticeable differences in their virulence. *Heterorhabditis indica* showed an LC₅₀ of 148.5 IJml⁻¹ and *S. carpocapsae* showed an LC₅₀ of 176.7 IJsm⁻¹. The results clearly showed that *H. indica* relatively more virulent than *S. carpocapsae*, as evidence by its lower LC₅₀ value (Table 2).

3.6 LC₅₀ of EPNs for pathogenicity on 4th instar larvae

The lethal concentration LC₅₀ of the two entomopathogenic nematodes species tested against *H. armigera* exhibited noticeable differences in their virulence. *Heterorhabditis indica* showed an LC₅₀ of 173.4 IJml⁻¹ and *S. carpocapsae* showed an LC₅₀ of 190.7 IJsm⁻¹. The results clearly showed that *H. indica* relatively more virulent than *S. carpocapsae*, as evidence by its lower LC₅₀ value (Table 3).

4. Discussion

The experiment was performed in biodiversity lab department of Entomology, University of Agriculture, Faisalabad. The experiment was conducted to check the efficacy of entomopathogenic nematodes as microbial control agent against *H. armigera*. Different concentrations (50, 100, 150, 200, and 250%) of EPNs species *H. indica* and *S. carpocapsae* were applied against different larval instars (2nd, 3rd, and 4th) of *H. armigera*. American bollworm mortality was tested by applying different concentrations after the time intervals (72 hours). Mortality was daily tested to analyze the capacity of EPNs for biocontrol. The discussion of the current study is as follows.

Helicoverpa armigera most vulnerable stages were 2nd and 3rd towards *H. indica* against *S. carpocapsae*. Both EPN species had strong pathogenicity against *H. armigera* larvae, and *H. indica* can cause high mortality. Different stages of *H. armigera* were vulnerable to EPNs due to increased concentrations of nematodes as 250 and 200 IJsm⁻¹ concentrations triggered higher mortality than concentrations of 150, 100, and 50 IJsm⁻¹. The present

study agreement with Kary et al. (2012) stated that in laboratory conditions, *H. indica* has more infectivity toward cotton bollworms than *S. carpocapsae* and *S. feltiae*. These results corroborate earlier studies reporting that *Heterorhabditis* spp. often outperform *Steinernema* spp. against lepidopteran larvae due to their rapid penetration ability and the higher virulence of the symbiotic bacterium *Photorhabdus* compared to *Xenorhabdus* (Hazir et al., 2004; Gaugler, 2018). The superior performance of *H. indica* in this study aligns with observations by Acharya et al. (2020), who also found *Heterorhabditis* isolates to be more aggressive against *H. armigera* larvae.

The declining mortality observed at lower concentrations (50-150 IJs ml⁻¹) is similarly consistent with previous research. Lower IJ availability reduces the probability of successful infection, resulting in reduced larval mortality (Lacey and Georgis, 2012). In the present study, minimum mortality (25.5% and 20.29% for *H. indica* and *S. carpocapsae*, respectively) occurred at 50 IJsm⁻¹, confirming that insufficient nematode density compromises control efficacy. Such trends have been extensively reported for EPN applications against various insect orders, including Lepidoptera (Acharya et al., 2020).

Overall, the present findings clearly indicate that *H. indica* exhibited higher virulence than *S. carpocapsae* under the tested laboratory conditions. Differences in pathogenicity between *Heterorhabditis* and *Steinernema* species have been widely documented and are often attributed to differences in foraging strategies, penetration behavior, and the pathogenicity of their symbiotic bacteria (Dillman et al., 2015; Gaugler, 2018). The sit-and-wait ambusher strategy of *S. carpocapsae* may limit its effectiveness against highly mobile larval hosts, whereas the cruiser behavior of *Heterorhabditis* species may enhance host encounter rates (Lewis et al., 2006). This ecological

difference may partially explain the higher mortality caused by *H. indica* in the present experiment.

5. Conclusion

In conclusion, the study confirms that both EPN species are effective biocontrol agents against *H. armigera*, with efficacy increasing significantly at higher concentrations. *H. indica* demonstrated greater virulence than *S. carpocapsae*, suggesting its stronger potential for integration into biological pest management programs targeting *H. armigera*. Further field evaluations are recommended to validate laboratory findings under variable environmental conditions.

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6. Acknowledgment

N/A

7. Conflict of Interest

The authors declare no conflict of interest.

8. Authors' Contribution

UL reared the culture of insects

JAB helped in collecting data and provided guidelines on bioassay setting and proof read the MS

MHS performed statistical analysis and prepared data tables

RM completed write up and finalized the MS

MA Maintained the culture of EPNs

SA Prepared figures and result write up

MM help in collecting and rearing population

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