

SCREENING ENTOMOPATHOGENIC NEMATODES FOR THEIR POTENTIAL AGAINST *DROSOPHILA MELANOGASTER*

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DOI: <https://doi.org/10.51193/IJAER.2026.12106>

Received: 03 Jan. 2026 / Accepted: 12 Jan. 2026 / Published: 22 Jan. 2026

ABSTRACT

Fruit flies are among the most destructive insect pests affecting horticultural crops globally. They cause significant losses in fruit and vegetable production by reducing both quality and yield. In a series of laboratory experiments conducted in the Khyber Pakhtunkhwa Province of Pakistan, the infectivity of six entomopathogenic nematode (EPN) species—*Steinernema carpocapsae*, *S. feltiae*, *S. kraussei*, *S. glaseri*, *Heterorhabditis indica*, and *H. bacteriophora*—was evaluated against the fruit fly *Drosophila melanogaster*. Following exposure, the larvae were dissected to detect the presence of nematodes. Among the tested species, *H. bacteriophora* and *S. feltiae* demonstrated the highest infection rates, with 97.6% and 85.1% respectively, indicating their strong potential as biological control agents. In contrast, *S. glaseri* was found to be the least effective against *D. melanogaster*. Based on adult fly emergence data, *H. bacteriophora* and *S. feltiae* were identified as the most virulent EPNs. The study also suggested that measuring adult fly emergence is a more reliable method for assessing EPN efficacy than larval dissection. Infections in the pupal stage were comparatively lower, with *S. feltiae* and *H. indica* causing 60% and 58% infection rates, respectively. These findings suggest that *D. melanogaster* is susceptible

to infection by *H. bacteriophora* and *S. feltiae*, highlighting their potential role as key components in integrated pest management (IPM) strategies for controlling fruit fly populations.

Keywords: Entomopathogenic nematodes, *Heterorhabditis bacteriophora*, *Steinernema carpocapsae*, fruit flies, management

INTRODUCTION

The tephritidae fruit flies are the most damaging insect pests in tropical and sub-tropical regions around the globe reducing the commercial value of fruits and vegetables (Grechi *et al.*, 2021). Fruit flies are two-winged insects of family Tephritidae in the order Diptera. This family comprises 481 genera containing 4352 species worldwide (Susanto *et al.*, 2017). The majority of the fruit flies are polyphagous in nature having a diverse host range, usually feeding on closely related host species. The majority of fruit flies have no economic importance and only 450 (~10%) are pests of fruits and vegetables. The plant parts which are damaged by fruit flies are fruit pulp and/or seed, flower, stems, buds, leaves and roots.

Using insect models to study pathogenic infection processes and host innate immune mechanisms offers several advantages over mammalian models. Insects are easy to rear in large numbers, and their small size simplifies procedures such as artificial infections and tissue extraction. Moreover, they share many physiological processes with mammals, making them valuable tools for identifying toxins and virulence factors—some of which are also required to subvert mammalian immune defenses (Vallet-Gely *et al.*, 2008; O’Callaghan & Vergunst, 2010).

Drosophila melanogaster has emerged as an ideal model organism for exploring host-pathogen interactions due to its well-established molecular and cellular toolkit (Limmer *et al.*, 2011). It is widely recognized as an excellent genetic and genomic model for investigating both host-pathogen dynamics and innate immune defense mechanisms (Apidianakis & Rahme, 2011). To date, most of the insights into the *Drosophila* immune response have been derived from studies involving bacterial, fungal, or viral pathogens (Glavis-Bloom *et al.*, 2012). However, immune reactions to insect-parasitic nematodes remain comparatively underexplored.

One such nematode, *Heterorhabditis bacteriophora*, exists in symbiosis with the entomopathogenic bacterium *Photorhabdus luminescens*, and together they are capable of invading and lethally infecting insect hosts. Notably, *Heterorhabditis* nematodes are also viable in the absence of their bacterial partner, indicating independent survival capabilities (Vlisidou *et al.*, 2012).

Class Secernentea of phylum Nematoda is one, of the five, very important order called Rhabditida. This order has fifteen families but two of them are of entomopathogenic in nature known as *Steinernematidae* and *Heterorhabditidae* (Dillman and Sternberg, 2012). Entomopathogenic

nematode (EPN) kills their host by entering into their body either through natural openings (anus, mouth or spiracles or making a hole by its tooth at a softer cuticular area like inter-segmental membrane (Castillo *et al.*, 2011; Kepenekci *et al.*, 2015).

The nematodes contain symbiotic bacterium of the genus *Xenorhabdus*. The bacterium has no free-living stage and is found only either in the nematode or in the body of infected host insect. When nematode enters into the body of the host insects, it releases mutualistic bacteria, which produces a toxin, resulted in the septicaemia and the insect die within 24-48 h (Bedding *et al.*, 1993; Campos *et al.*, 2016). Entomopathogenic nematodes have been used against soil pests such as white grubs, root weevils, rootworms, sciarid flies, cutworms, and armyworms (Hazır *et al.* 2003).

The free-living stage of both entomopathogenic nematode (EPN) genera is “infective juvenile” (IJ3). The difference in the biology of both EPN genera is; *Steinernematids* requires male and female to go into the insect body for reproduction, though only one infective juvenile of either sex has the capability to kill the insect (Bedding *et al.*, 1993) while *Heterorhabditis* develop into the hermaphrodite adult. The infective juveniles (with closed mouth and anus) enter into the insect body, moult into the J4s, which has an open anus and mouth. After few days, J4s molt into reproductive stage (*Steinernematidae* into both sexes and hermaphrodite adult in case of *Heterorhabditis*). They lay eggs, after hatching, which moult twice J1 and J2 and continue their life cycle to J3, J4 and reproductive stage or just come out of the insect body at J3 stage, in search of new host, if food resources are being depleted (Adams and Nguyen, 2002).

Fruit flies are one of the less evaluated insects. Although the soil dwelling stages in the biology of fruit fly (full fed larvae and pupae) give a good opportunity for entomopathogenic nematodes (Torrini *et al.*, 2017). Since EPN application has no health or environmental hazard, so it could be an important component of the fruit fly Integrated Pest Management (IPM) program. The present studies aimed to evaluate the infective capabilities of EPN for the infestation to fruit flies, with the intention that the results were postulate to the fruit flies of economic importance for field-testing.

MATERIALS AND METHODS

Nematode culture

The nematodes were cultured by infecting wax moth (*Galleria mellonella*) larvae supplied by the Live Foods, UK. The 9 cm plastic Petri dish was lined with filter paper, moistened by adding 1 mL tap water, and poured ≈500 nematodes with the help of micropipette. Ten active wax moth larvae were added to each Petri dish with different species of nematodes and sealed with a strip of Para film to preserve the moisture content, and incubated for 48 h at 20°C. The Petri dishes were removed from the incubator and dead larvae were transferred to “white trap” for the extraction of J3 (Fig.1). The white trap was made by placing lid of 5 cm plastic Petri dish upside down in a 300

mL plastic cup and a Whatman No.1 filter paper was placed over the Petri dish in the plastic cup. Twenty mL water was added to it and dead larvae were placed carefully on the raised platform on the filter paper. The nematodes developing in the body of wax moth moved out and swam into the water from the raised platform. The emerged J3 were then collected into 150 mL plastic cup everyday and stored at 10°C for future use. EPN of less than 10-day old were used in all studies.

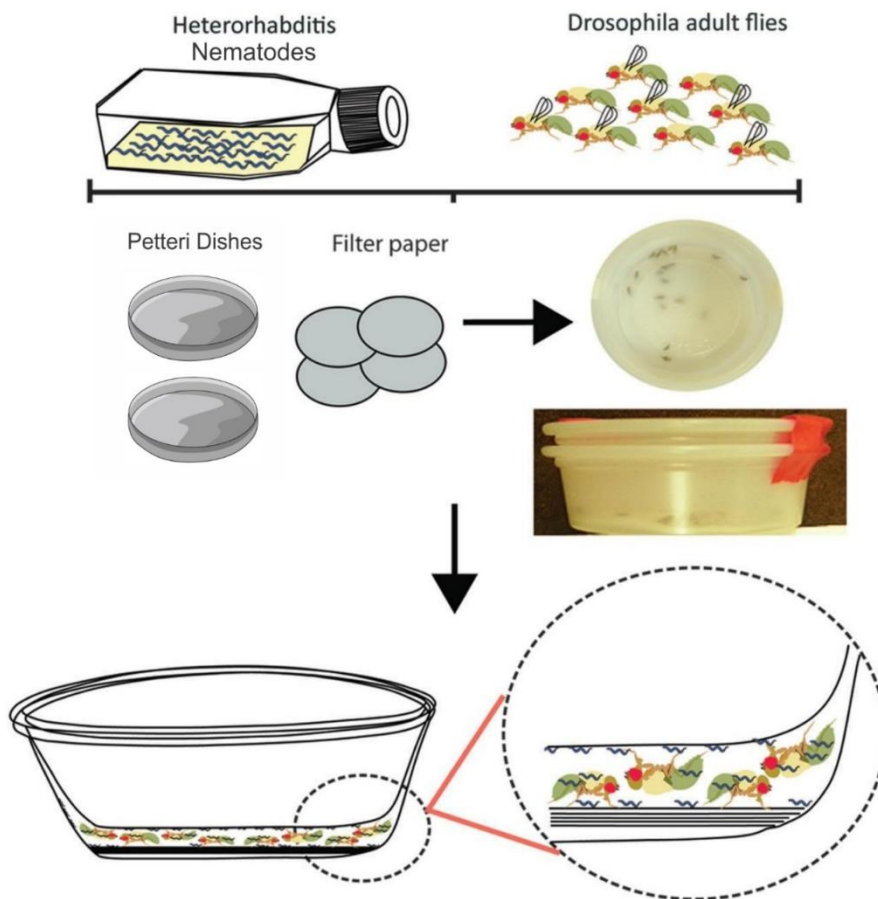


Figure 1: Flow Chart for infection to *Drosophila* flies with nematode. Nematodes infective Juveniles are kept in pre-sterile water in the flask.

Fruit fly culture

The mother culture of the fruit flies (*Drosophila melanogaster*) was received from the Global Live foods, West Yorkshire, UK and cultured in the lab in a 50 x 50 x 100 cm³ caddies on an artificial diet. Freshly prepared diet in a 150 mL cup were air cooled to room temperature and offered to fruit flies for 24 h. The cup was removed after one day with considerable numbers of fruit fly eggs in it. The cup was then placed in a separate chamber with no flies, for hatching and to get uniform-

age larvae for further use in experiments. After 8 days, the larvae were found crawling out of food (indicating the late last instar stages looking for suitable place to pupate). These full fed larvae were used for experiments (Fig.2).

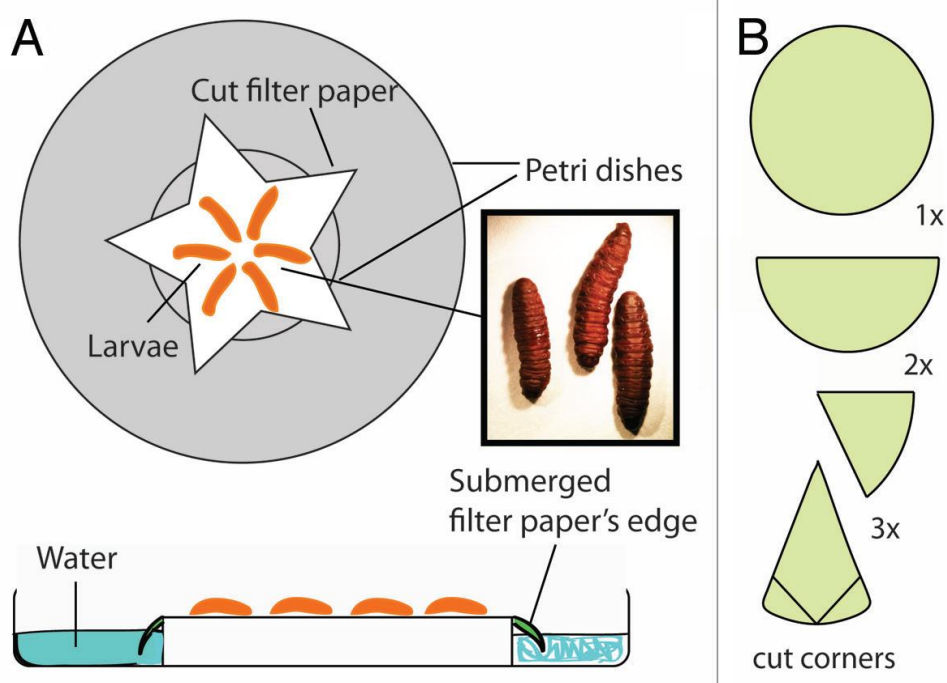


Figure 2: Presented fruit flies culture in the lab.

Efficiency of EPNs against fruit fly larvae based on dissection

All the nematode cultures were measured into a 1000 J3 per mL strength solution for the experiment. The 9-cm Petri dish, lined with Whatman No. 1 filter paper in the cover was used for the infection experiment. 0.5 mL of the solution containing nematodes (with approximately 500 J3) was added in each Petri dish using a micropipette. The solution was allowed to spread evenly on the surface of the filter paper. Ten fully fed fruit fly larvae of the apparently uniform size were collected with the help of fine insect handling forceps (Bioquip, Rancho Dominguez, CA, USA) from the mother culture ensuring the larvae may not get injured and were introduced into each Petri dish with different nematode species. After the introduction of nematodes, the Petri dishes were sealed using Para film and incubated for four days at 20°C. The Petri dishes were examined after four days to check if the larvae have turned into the pupae and recorded. All the larvae/pupae were dissected in the ringer's solution using a stereomicroscope to check the presence of nematodes in the insect body. The entomopathogenic nematodes used in these studies were, *S. carpocapsae*, *S. feltiae*, *S. kraussei*, *H. indica*, *H. bacteriophora* and *S. feltiae* (Shinfield strain). The infestation percentage was calculated for statistical analysis (Fig.3.).

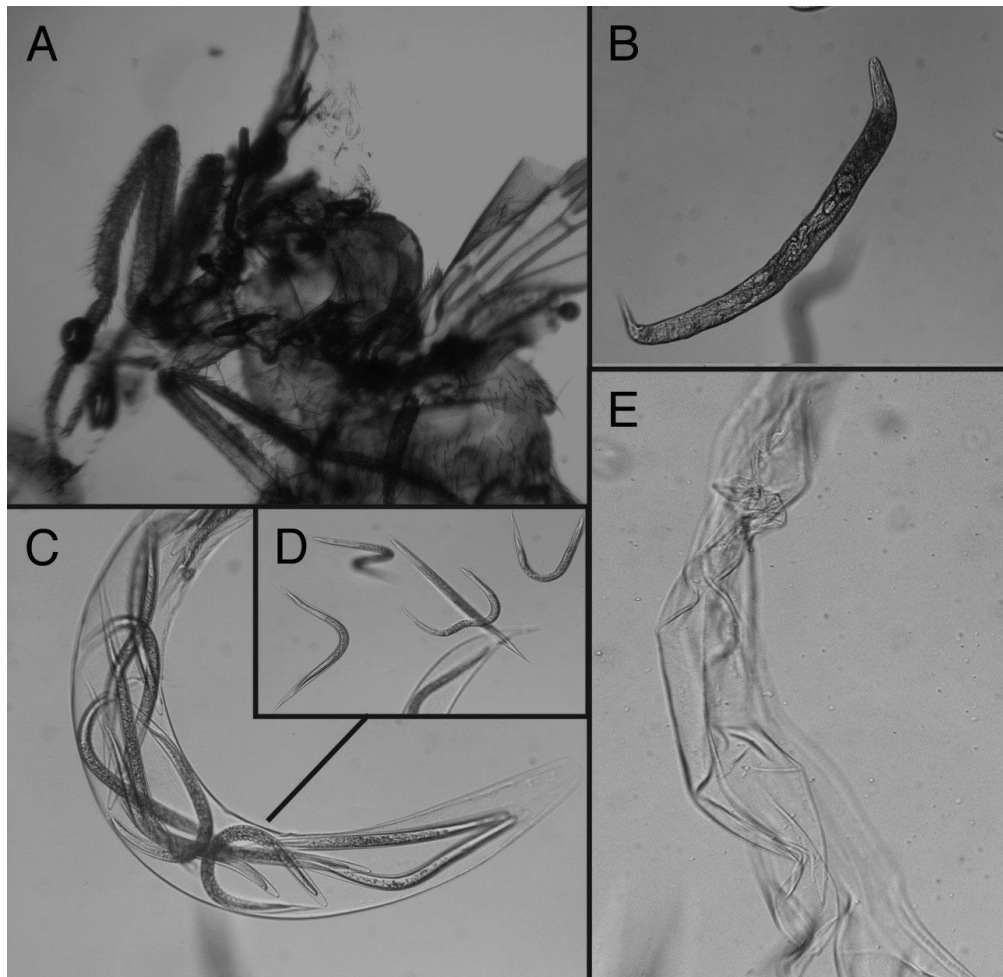


Figure 3: Presents nematodes development in *Drosophila* adult flies.

Efficiency of EPNs against fruit fly larvae based on adult emergence

In the second experiment, following the procedure described above, six cell plate (3.5 × 1 cm per cell) were padded with a Whatman No. 1 filter paper and 100 J3 in 0.1 mL water were added. Five last instar larvae were introduced in each cell. The experiment on each nematode species was replicated six times. The plates were covered and sealed with Para film to avoid any moisture loss and incubated at 20°C till the emergence of last fly. *S. carpocapsae*, *S. feltiae*, *S. kraussei*, *H. indica*, *H. bacteriophora* and *S. glaseri* were tested in this experiment. The observations were recorded on number of flies and nematode species emergence and converted into percent for statistical analysis.

Efficiency of EPNs against fruit fly pupae based on adult emergence

The pupal infection assessment experiment was conducted following the same procedure as described earlier. However, instead of infested larvae, pupae were tested for EPN infection. The last instar larvae were isolated into a Petri dish lined with moist filter paper, allowed to pupate at 20°C and only those pupae were used in the studies, which were pupated within the past 24 h. The nematodes used were *S. carpocapsae*, *S. feltiae*, *H. indica* and *S. glaseri*. Five pupae were added to one of the cells of a six-cell plate (measuring 3.5 x 1 cm each cell dimension) and incubated until the last fly emerged. Data on fly emergence were recorded as procedure described in the previous experiment.

Statistical analysis

The data generated in the experiment were analysed using the following model (Collins & Seeny, 1999).

$$y_i = \mu + T_i + \varepsilon + e_{ij}$$

for $i=1..t$;
 $j=1..r_i$

The LSD was calculated as:

$$\text{LSD} = t_c \sqrt{\frac{2Ea}{r}}$$

The Standard Error of Means were calculated as:

$$S \bar{x} = \frac{s}{\sqrt{n}}$$

The means were separated using Least Significance Difference Test at $\alpha=0.05$. All statistical analysis was done using MSTATC computer software.

RESULTS

Efficiency of EPNs against fruit fly larvae based in dissection

The infestation level was significantly higher in all nematodes than the control (Fig. 4). *Heterorhabditis bacteriophora* was significantly the most effective entomopathogenic nematode with 97.6% followed by *S. carpocapsae*, *H. indica* and *S. kraussei* having infection 67.60, 75.10 and 72.60, respectively. *S. feltiae* was least effective. However, all these species were found non-significantly different from each other. Both *Steinernema* and *Heterorhabditis* were equally

effective in infesting the fruit fly larvae with an infestation range of 62.6 to 85.10 and 75.10 to 97.60%, respectively.

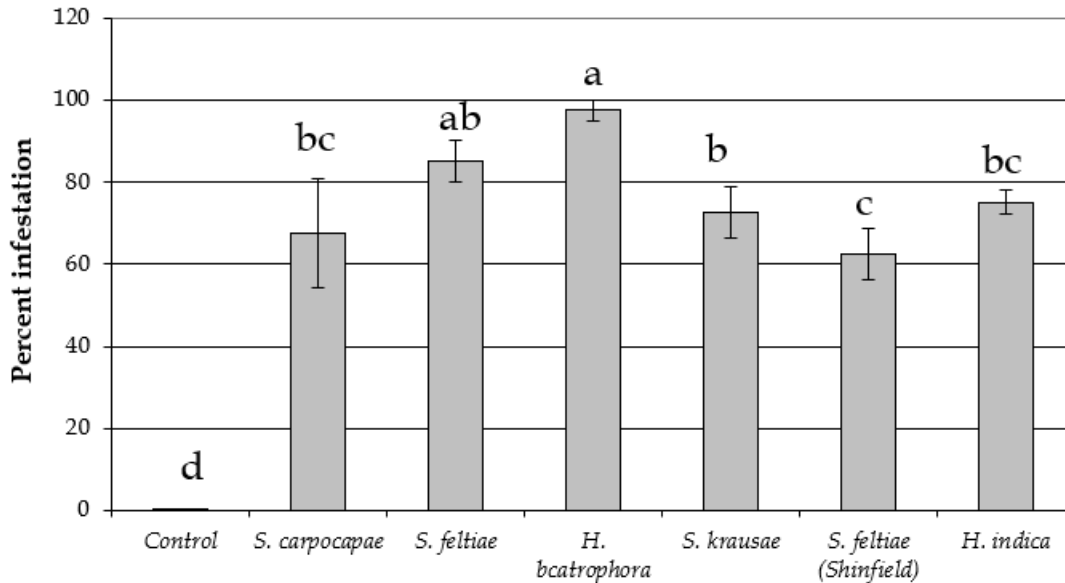


Figure 4: Percent infestation of different EPN species to full-fed larvae of fruit flies

As far as pupation is concerned, significantly more pupation (97.60%) was noted in the untreated check (Fig. 5) while, among the entomopathogenic nematode treatments, *S. feltiae* (72.60%) had significantly more pupation. All other nematode treatments pupated in significantly less percentage (ranged from 47.10 to 55.10) with a non-significant difference among them.

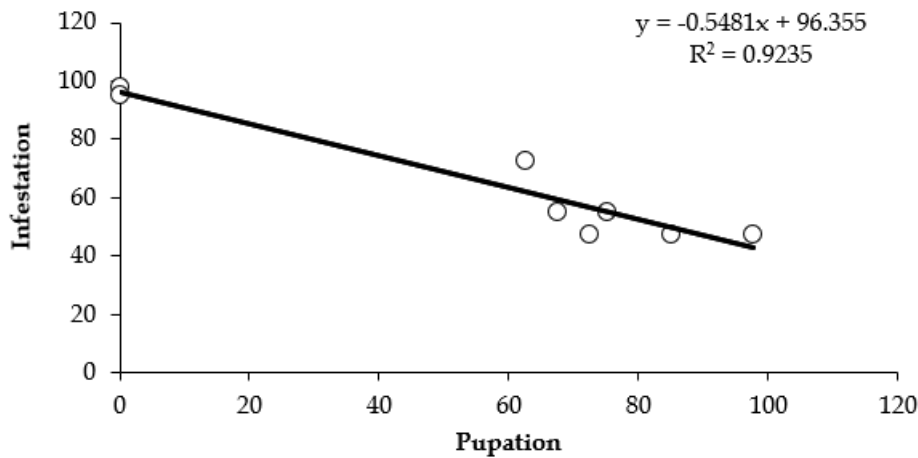


Figure 5: Relationship between percent EPN infestation and pupation of fruit flies

The relationship between the pupation of the infested larvae and the infestation level was significantly inversely proportional. The coefficient of correlation was estimated at 0.9255. All the nematode treatments behaved in a similar fashion as far as pupation is concerned.

Table 1: Percent adult emergence and percent infestation of last instar fruit fly larvae by different entomopathogenic nematodes.

EPN Species	% Fly emergence (Means ±SE)	% Infestation
<i>S. glaseri</i>	20.00±5.16b	80.00
<i>H. indica</i>	33.33±6.83b	66.67
<i>S. kraussei</i> ,	23.33±8.03b	76.67
<i>S. carpocapsae</i>	26.66±12.29b	73.34
<i>H. bacteriophora</i>	10.00±6.83b	90.00
<i>S. feltiae</i>	10.00±4.47b	90.00
Untreated control	96.67±3.33a	03.33
LSD _{0.05}	20.56	

Means followed by different letters are significant from each other at 5% level of probability using LSD test.

EPNs and fruit fly larvae based on adult emergence

The percent adult emergence from the nematode infested last-instar larvae was significantly higher compared with the control (Table 1). Among different nematode treatments, *H. bacteriophora* and *S. feltiae* were having the least adult emergence and had the highest infestation level. These were followed by *S. carpocapsae*, *S. kraussei* and *H. indica* with a statistical similar infestation level.

Table 2: Percent adult emergence and percent infestation of fruit fly pupae by different entomopathogenic nematodes.

EPN Species	% Fly emergence (Means \pm SE)	% Infestation
<i>S. feltiae</i>	40.56 \pm 6.035c	59.44
<i>S. glaseri</i>	88.335 \pm 5.693a	11.67
<i>S. carpocapsae</i>	68.41 \pm 10.768b	31.59
<i>H. indica</i>	42.46 \pm 3.860c	57.53
Untreated Control	94.44 \pm 3.514a	5.56
LSD _{0.05}	16.49	

Means followed by different letters are significant from each other at 5% level of probability using LSD test.

EPNs and fruit fly pupae based on adult emergence

While testing fruit fly pupae for infection by various entomopathogenic nematodes, *S. feltiae* was the most effective with 40.56 fly emergence and *H. indica* with 42.46 fly emergence, statistically at par with each other (Table 2). *Steinernema carpocapsae* had 68.41% fly emergence while highest fly emergence (88.33%) was in *S. glaseri* compared with as high as 94.44% in the untreated control, which was statistically at par with that of *S. glaseri*.

DISCUSSION

In the present study, *Heterorhabditis bacteriophora* and *Steinernema feltiae* exhibited the highest infectivity against *Drosophila melanogaster*, causing infection rates of 97.6% and 85.1%, respectively. These findings closely correspond with those of Sallam et al. (2024), who evaluated several entomopathogenic nematode (EPN) species and reported *H. bacteriophora* as the most effective against *Bactrocera zonata*, achieving 97.5% mortality. Similarly, our results demonstrate infection rates exceeding 97% for *H. bacteriophora*. Saleh et al. (2018) also reported high efficacy of *Heterorhabditis marelatus*, which outperformed a commercial *H. bacteriophora* strain and caused more than 77% adult mortality in *B. zonata*. In contrast to these studies, no significant differences in infestation levels were observed among EPN species from the two genera in our experiments.

Our results further revealed that the pre-pupal stage of *D. melanogaster* was the most susceptible to EPN infection. This finding differs from Mahmoud et al. (2016), who identified the adult stage of *B. zonata* as the most vulnerable to *S. feltiae*. The heightened activity of fruit fly larvae may increase their contact with EPNs, facilitating infection irrespective of the nematodes' foraging strategies (cruiser versus ambusher). This observation may explain why third-instar larvae of several fruit fly species are frequently reported as highly susceptible to EPN infection (Langford et al., 2014).

EPN efficacy is influenced by multiple factors, including host developmental stage, phased infectivity, ensheathment, nematode storage conditions, and production methods (Toledo et al., 2006). The relatively high infection rates observed in some pupal stages in earlier studies, which contrast with our findings, remain poorly understood. A strong inverse relationship between infestation level and pupation success was observed in this study, indicating that high nematode infection likely caused larval mortality prior to pupation. Supporting this interpretation, Usman et al. (2021) reported reduced adult emergence in *B. zonata* larvae and pupae treated with *H. bacteriophora* under semi-field conditions, confirming pupal susceptibility.

As pupation progresses, cuticle hardening may hinder nematode penetration. Additionally, Emekci et al. (2000) documented a sharp decline in respiratory rate during pupation—from 108 $\mu\text{L O}_2 \text{ insect}^{-1} \text{ h}^{-1}$ to 0.4 $\mu\text{L O}_2 \text{ insect}^{-1} \text{ h}^{-1}$ —which may reduce CO_2 emissions used by EPNs as host-location cues, thereby lowering infection rates. During dissections, several pupae exhibited no signs of adult development or nematode presence and contained only pale yellowish fluid, whereas untreated controls displayed clear adult formation at the same developmental stage.

Based on these observations, a fly emergence assay was conducted to test the hypothesis that the entry of a single *Steinernema* nematode into a larva may be insufficient to support reproduction. The results supported this hypothesis, showing a slight increase in larval infestation for *Steinernema* spp. when assessed via adult emergence. Conversely, infestation levels for *Heterorhabditis* spp. slightly declined, from 97.6% to 90% for *H. bacteriophora* and from 75.1% to 66.67% for *H. indica*. Overall, the fly emergence method proved to be a more efficient and cost-effective laboratory technique for assessing EPN infection.

In conclusion, our findings confirm that EPNs are highly pathogenic to fruit flies, particularly during soil-dwelling larval and pupal stages. These nematodes therefore represent promising biological control agents and could play a significant role in integrated pest management (IPM) programs by substantially reducing fruit fly survival.

CONCLUSION

Fruit flies (*Drosophila melanogaster*) are among the most destructive pests affecting fruits and vegetables worldwide. In the present study, six species of entomopathogenic nematodes (EPNs) were evaluated for their potential as biological control agents against this pest. Among the tested species, *Heterorhabditis bacteriophora* and *Steinernema feltiae* exhibited the highest infection rates (97.6% and 85.1%, respectively), demonstrating strong efficacy in suppressing *D. melanogaster*, whereas *S. glaseri* showed the lowest effectiveness. Adult emergence data further confirmed *H. bacteriophora* and *S. feltiae* as the most virulent EPN species. These findings indicate that *D. melanogaster* is highly susceptible to EPN infection, highlighting the potential of these nematodes as effective components of integrated pest management (IPM) strategies for fruit fly control. Future studies will employ this infection system to investigate gain- and loss-of-function traits in mutant strains of *D. melanogaster*, thereby improving our understanding of host susceptibility mechanisms.

NOVELTY STATEMENT

This study presents a novel and standardized laboratory protocol for the biological control of fruit flies (*Drosophila melanogaster*) using entomopathogenic nematodes. The protocol provides a robust platform for future investigations aimed at evaluating host–pathogen interactions, including the assessment of gain- and loss-of-function traits in mutant *D. melanogaster* strains.

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