Effect of infectivity concentration for Mass Propagation of *Heterorhabditis indica* in Different Host

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Abstract
Entomopathogenic nematodes (EPNs) are promising biological control agents against several insect pests. This work assessed the effectiveness of three EPN inoculation techniques: spread plate, immersion and shaking on five insect hosts: Bombyx mori larvae (BML), Bombyx mori pupae (BMP), Galleria mellonella larvae (GML), Philosamia ricini larvae (PRL) and Philosamia ricini pupae (PRP). The spread plate method consistently resulted in the highest mortality, with BML exhibiting ~72% mortality followed by BMP (~64%), GML (~66%), PRL (~54%) and PRP (~44%). Mortality was significantly lower in the immersion and shaking methods ($p < 0.0001$). A dose-dependent response was observed with EPN concentrations ranging from 50 to 1000 IJs/mL where PRL showed the steepest increase in mortality ($R^2 = 0.61$) and PRP showed the lowest susceptibility ($R^2 = 0.30$).

The emergence of infective juveniles also increased linearly with concentration, being highest in BML ($R^2 = 0.90$) and BMP ($R^2 = 0.87$) and lowest in PRP ($R^2 = 0.94$ but with lower absolute numbers). Control host GML maintained consistent mortality (~63–74%) and supported IJ emergence (~9–27), confirming its reliability as a baseline comparator. These findings highlight the influence of inoculation technique and host-specific susceptibility on EPN effectiveness, offering insights for optimizing biocontrol applications in integrated pest management programs.

Keywords: Entomopathogenic nematodes, *H. indica*, Pest management, Mortality percentage.

Introduction

Entomopathogenic nematodes (EPNs) are small, soil-dwelling roundworms that play a crucial role as biological control agents against a variety of soil-borne and foliar insect pests[9,10]. Among the recognized EPN species, *Heterorhabditis indica* has garnered significant attention due to its adaptability and high efficacy in targeting harmful insect pests[10,13]. These nematodes function as endoparasites, infecting insect hosts by penetrating their bodies and releasing symbiotic bacteria that ultimately cause mortality. Notably, *H. indica* has been reported to successfully infect over 200 different insect species across various taxa[14]. In agricultural pest management, *H. indica* serves as an effective alternative to chemical pesticides, offering an environmentally friendly approach in controlling insect pests in horticultural and field crops[11]. However, to fully harness its potential for large-scale pest control, it is essential to develop efficient mass production techniques. EPNs can be cultivated using two primary methods: *in vivo* (inside live insect hosts) and *in vitro* (using artificial media or bioreactors). The *in vivo* method is relatively simple and cost-effective, making it suitable for small-scale production while the *in vitro* method is more scalable and efficient for large-scale cultivation[6]. The success of these approaches largely depends on factors such as host selection and environmental conditions which directly influence nematode yield, virulence and overall quality[6].

This study explores the infectivity and mortality of *H. indica* in multiple test hosts including *Galleria mellonella* larvae, *Bombyx mori* larvae and pupae and *Philosamia ricini* larvae and pupae. Recent research suggests that alternative hosts such as silkworm species (*B. mori* and *P. ricini*), may serve as viable candidates for large-scale nematode production due to their high susceptibility to EPN infections[20]. In countries like India, where silkworm cultivation is already an established agricultural practice, leveraging *B. mori* for EPN propagation presents a sustainable and cost-effective solution. Silkworms not only support sericulture industries but also offer farmers an additional avenue for biological pest control, making this approach economically and practically viable.

For successful mass propagation of *H. indica*, infectivity concentration plays a critical role. For instance, infectivity concentration affects reproductive efficiency, requiring a balance to avoid low infection rates or resource competition. Optimizing infectivity concentration enhances nematode production for biocontrol applications. Considering the above-mentioned key parameters, the present study explores the impact of infectivity concentration on the mass propagation of *H. indica* in different hosts. Understanding these factors will help to refine mass production techniques, ensuring a consistent supply of high-quality nematodes for biocontrol applications. Standardization of inoculation methods and host selection will contribute to the commercialization of *H. indica* as a sustainable pest management solution.

By optimizing infectivity concentration, this study aims to enhance the efficiency of *H. indica* production, making it a viable and scalable alternative to chemical pesticides. These findings will play a crucial role in promoting EPN-based biocontrol strategies in agriculture and horticulture,

ultimately contributing to sustainable pest management and environmental conservation.

Material and Methods

Nematodes: A pure culture of the entomopathogenic nematode *Heterorhabditis indica*, maintained in the Nematology Laboratory of the Department of Entomology, NBAIR, Bangalore, Karnataka, India, was used for the study. The nematode was cultured and maintained as per the method described by Woodring and Kaya[18] and infective juveniles recovered from the White trap were stored in sterile water in a culture flask at 10 °C.

Host organism: The entomopathogenic nematode *Heterorhabditis indica* was tested on different insect hosts collected from various sources in this study. The larvae of *Galleria mellonella* (Lepidoptera: Galleriidae) were obtained from old stored honeycombs at the Department of Entomology, NBAIR, Bangalore. These larvae were then reared on an artificial diet following the method described by Singh[16]. The eggs of *Bombyx mori* (Lepidoptera: Bombycidae) were sourced from the Central Silk Board, Seed Production Centre, Vijayapura, Karnataka. The silkworms were reared on fresh mulberry leaves as per the standard protocols outlined by Dandin et al[5]. Additionally, *Philosamia ricini* larvae were procured from the Silk Board, Hosur, Karnataka and were directly used for the experiment. These hosts, representing different species of Lepidoptera, were used to assess the pathogenicity of *H. indica* under varying infective concentrations.

Inoculation by spread method: In the spread method of insect host inoculation, ten insect larvae or pupae were placed in a 90-mm Petri dish lined with Whatmann no. 1 filter paper. This setup consisted of 5 plates as one replicate and the experiment was repeated three times as three replications. Using a micropipette, 500 µl of tap water containing infective juveniles (*H. indica*) at a concentration of 200 IJs / 50 µl was added to each Petri dish. Subsequently, the post-inoculation method was implemented and a new test date was scheduled for the experiment.

Effect of concentrations of entomopathogenic nematodes on host mortality: Experiments on four distinct insect host species: BML, BMP, PRL and PRP, using the insect host GML as a control test organism were conducted to assess the pathogenicity of entomopathogenic nematodes (EPNs) in terms of mortality and emergence. For each test organism (BML, BMP, PRL and PRP), 15 Petri dishes were prepared per concentration, with each dish containing 10 insect larvae, resulting in a total of 150 larvae per concentration. These hosts were exposed to four concentrations of infective juveniles (IJs): 250, 500, 750 and 1000 IJs/mL. For the control host GML, nematode pathogenicity was assessed across five lower concentrations: 20, 40, 60, 80 and 100 IJs/mL. The Petri dishes were placed in sealed plastic containers (11 × 11 × 7.5 cm) lined with moist filter paper to preserve high humidity after inoculation. These containers were incubated at ambient room temperature. Mortality of larvae was observed at 24-hour intervals over a 4-day period. A larva was considered dead if it exhibited a change in body colour and failed to respond to physical prodding with forceps. Mortality percentages were calculated at each concentration level and used to assess the dose-dependent virulence of nematodes.

In parallel, nematode emergence was recorded from the cadavers of infected larvae. Emergence was assessed by observing the appearance of IJs from host cadavers after the incubation period. This was done to determine the ability of EPNs to complete their life cycle and to produce infective juveniles in different hosts across varying concentrations.

This experimental design allowed for a comprehensive evaluation of the dose-response relationship of EPN infection in test organisms compared to the control GML and provided insights into host suitability based on mortality and emergence data.

Determination of the mortality: Mortality was determined by colour change and the lack of host activity when prodded with a pair of tweezers. Further, the emergence rate was recorded under a stereo microscope.

Statistics: The data were arranged and statistical analysis was performed in R Studio (version 2024.12.0+467). One-way ANOVA and *t*-test (Bonferroni's method) were incorporated using the ggpubr function under the ggplot2 package in R.

Results

Infection and emergence of *Heterorhabditis indica* from test hosts: During the experimental period, phenotypic changes in *Heterorhabditis indica* infection and the development of the infection in a variety of insect hosts were observed (Figure 1). Clearly, the larvae exhibited symptoms of infection body color change and decreased movement which suggest effective penetration and activity of the nematodes (Figure 1A). Pupae became suitable microhabitats for nematode multiplication, producing the emergence of infective juveniles from host bodies after infection advanced (Figure 1B). The growing number of these juveniles in water traps (Figure 1C) further verified their reproductive success, thus enabling their great potential as environmentally benign pest control tools.

Effects of different inoculation methods on number of mortality and mortality percentage of different hosts: Total mortality seen in five insect hosts, BML, BMP, GML, PRL and PRP under three inoculation techniques such as immersion, shaking and spread is displayed in figure 2A. The spread plate inoculation method showed a significantly higher number of mortality cases in all the experimental test hosts compared to the immersion and shaking plate methods (Figure 2). In the BML host, the spread method resulted in the highest mortality (~36 larvae), followed by immersion

(~28 larvae) and shaking (~25 larvae), with a highly significant difference ($p < 0.0001$).

A similar trend was observed in BMP, where spread caused ~32 deaths, while immersion and shaking led to ~25 and ~23 deaths, respectively ($p < 0.0001$). For GML, spread resulted in ~33 mortalities, whereas immersion and shaking showed ~24 and ~23 deaths respectively ($p < 0.0001$). In PRL, mortality was relatively lower, with the spread method causing ~27 deaths, immersion ~21 and shaking ~21 ($p < 0.001$). The PRP host showed the least susceptibility, with spread resulting in ~22 deaths and both immersion and shaking yielding ~18 deaths ($p < 0.0001$).

The same trend occurred in the mortality percentage of different hosts, where the spread plate method was found to have significantly higher observations across all hosts compared to the immersion and shaking plate methods (Figure 2B). For instance, in BML, spread resulted in ~72% mortality, compared to ~56% for immersion and ~50% for shaking ($p < 0.0001$). BMP showed a similar pattern: spread at ~64%, immersion at ~50% and shaking at ~46% ($p < 0.0001$). GML had ~66% mortality under the spread method followed by ~48% (immersion) and ~46% (shaking) ($p < 0.0001$). In PRL, spread induced ~54% mortality, while immersion and shaking each accounted for ~42% ($p < 0.001$). PRP exhibited the lowest mortality percentages overall, with ~44% under spread and ~36% under both immersion and shaking ($p < 0.0001$). These results further reinforce the efficacy of the spread method in maximizing EPN-induced host mortality to reveal notable variation in host susceptibility, with BML and GML being the most affected and PRP the most resistant.

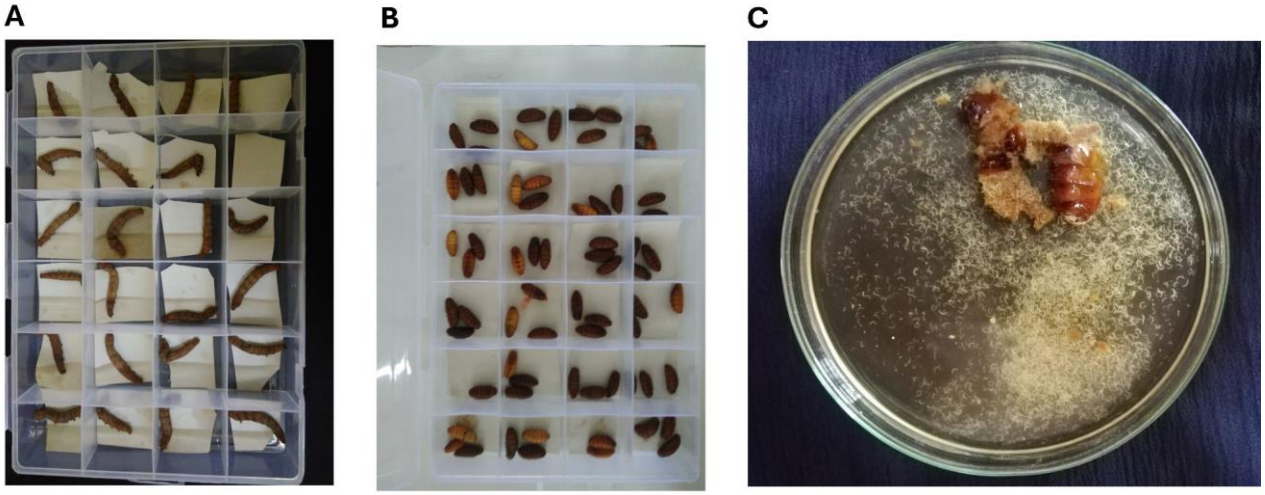

Fig. 1: Visual representation of entomopathogenic nematode (EPN) infection and development stages: (A) Manifestation of EPN infection on different test hosts, (B) Emergence of *Heterorhabditis indica* from infected hosts

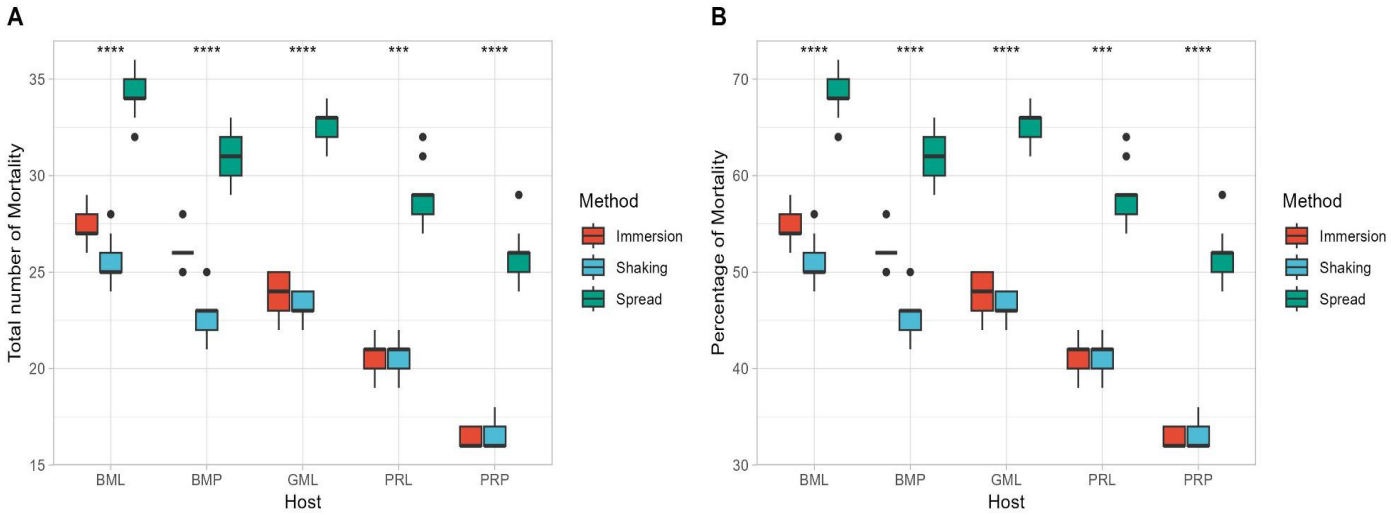

Fig. 2: Depicted the impact of different inoculation methods on *Heterorhabditis indica* infection across various insect hosts. (A) Total number of mortality (B) Mortality percentage of *H. indica* using immersion, shaking and spread methods across five hosts: *Bombyx mori* larvae (BML), *Bombyx mori* pupae (BMP), *Galleria mellonella* larvae (GML), *Philosamia ricini* larvae (PRL) and *Philosamia ricini* pupae (PRP).

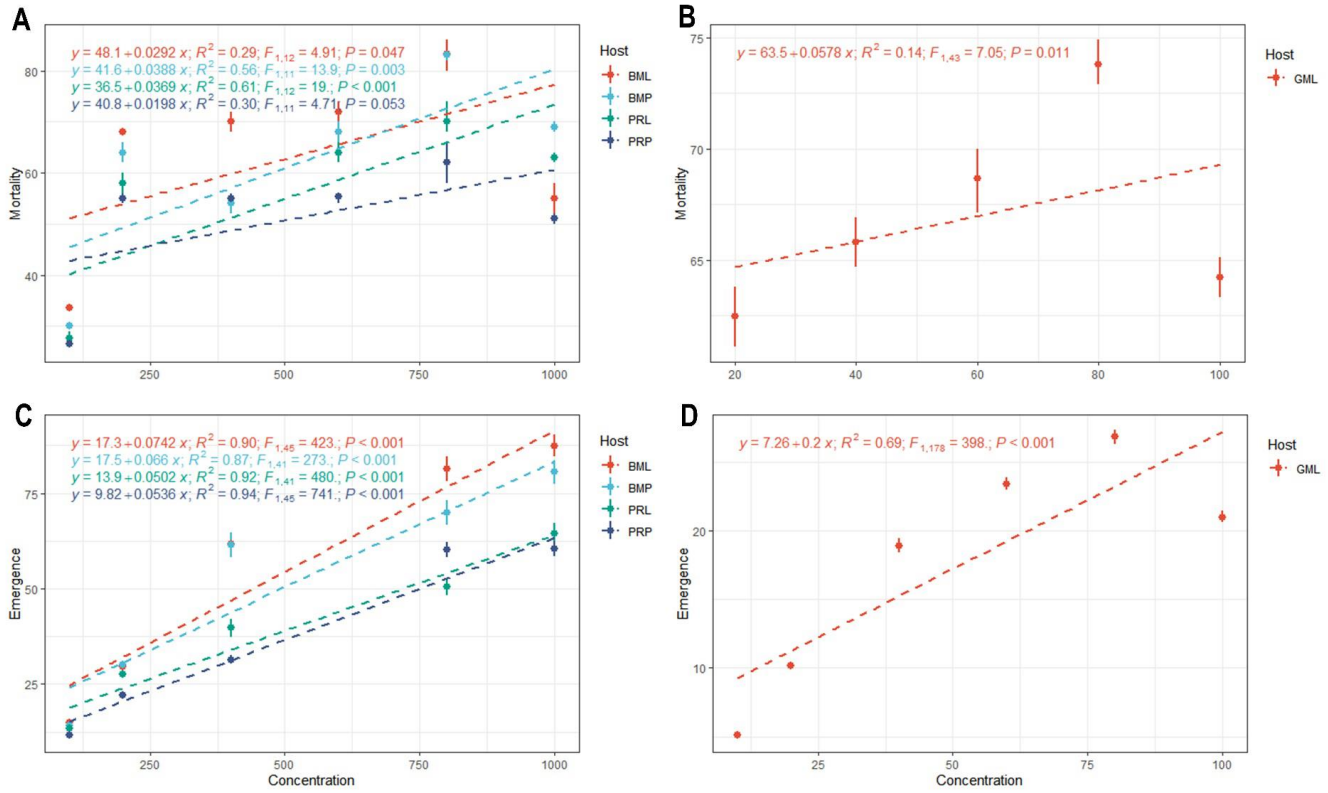

Fig. 3: Concentration-dependent mortality and emergence of *Heterorhabditis indica* in different insect hosts. (A) Mortality of four insect hosts—BML, BMP, PRL and PRP—with increasing concentrations of *H. indica*. (B) Mortality of GML across varying concentrations. (C) Emergence of *H. indica* from BML, BMP, PRL and PRP with increasing concentrations. (D) Emergence from GML larvae in response to different concentrations

Effect of entomopathogenic nematode concentration on host mortality and IJ emergence rate: The association between EPN concentration and the recorded mortality in four insect hosts BML, BMP, PRL and PRP is shown in figure 3A. Across all hosts, a positive correlation suggested a dose-dependent rise in mortality. Among the studied hosts, BML revealed a modest increase in mortality (y = 48.1 + 0.0292x, R^2 = 0.29, $F_{1,12}$ = 4.91, *p = 0.047*), with mortality rising from ~48% at the lowest dose to ~77% at the highest concentration (1000 IJs/mL). BMP showed a stronger response (y = 41.6 + 0.0388x, R^2 = 0.56, $F_{1,12}$ = 13.9, *p = 0.003*), with mortality increasing from ~42% to ~80%. PRL demonstrated the steepest slope and the highest model fit (y = 36.5 + 0.0396x, R^2 = 0.61, $F_{1,12}$ = 19, *p < 0.001*), with mortality increasing sharply from ~36% to ~76%. In contrast, PRP showed a weaker response (y = 40.8 + 0.0198x, R^2 = 0.30, $F_{1,11}$ = 4.71, *p = 0.053*) where mortality rose from ~41% to ~60%, indicating lower susceptibility (Figure 3A).

The mortality pattern of GML, which was the control test organism, is shown in figure 3B. There was a slight but statistically significant negative relationship (y = 63.5 - 0.0578x; R^2 = 0.14, $F_{1,43}$ = 7.05, *p = 0.011*). Ranging from ~63% to 74%, mortality remained constant across the investigated value range (20–100 IJs/mL). The decline in mortality at the highest concentration suggests that GML might be unaffected or adapted to the tested conditions. The low R^2 value and minimal trend imply that the control organism maintained baseline mortality, reinforcing its role as a reliable comparator for the experimental treatments.

Figure 3C presents emergence data (number of infective juveniles emerging per host) from BML, BMP, PRL and PRP in response to increasing EPN concentration. All hosts showed a strong linear increase in emergence. BML again showed the highest emergence (y = 17.3 + 0.0742x; R^2 = 0.90, $F_{1,45}$ = 423, *p < 0.001*), with emergence numbers rising from ~25 to ~90 individuals. BMP also supported high emergence (y = 17.5 + 0.066x; R^2 = 0.87, $F_{1,45}$ = 273, *p < 0.001*), followed by PRL (y = 13.9 + 0.0502x; R^2 = 0.92, $F_{1,41}$ = 480, *p < 0.001*). PRP again showed the lowest values, both in terms of slope and baseline (y = 9.82 + 0.0536x; R^2 = 0.94, $F_{1,45}$ = 741, *p < 0.001*), suggesting that it is the least conducive host for nematode reproduction. These results confirm that emergence rates are not only dose-dependent but also host-specific, with BML and BMP serving as better propagation platforms for EPNs compared to PRP.

Figure 3D displays the emergence data from the control host GML. A strong positive correlation was observed (y = 7.26 + 0.2x; R^2 = 0.69, $F_{1,78}$ = 398, *p < 0.001*), indicating a linear increase in emergence from ~9 to ~27 individuals over the tested concentration range. Interestingly, although GML was

used as a control, it still supported significant IJ emergence, indicating that it may naturally harbor or tolerate nematodes to some extent. Its linear emergence trend reinforces its utility as a baseline comparator for evaluating reproductive potential in other hosts.

Discussion

***In vivo* mass multiplication of *Heterorhabditis indica* for biocontrol development:** This study aimed to optimize the *in vivo* mass multiplication of entomopathogenic nematodes (EPNs), specifically *Heterorhabditis indica*, for their development as potent biocontrol agents. We evaluated the effects of different inoculation methods and concentrations on various host species, namely *Bombyx mori* larvae (BML), *Bombyx mori* pupae (BMP), *Galleria mellonella* larvae (GML), *Philosamia ricini* larvae (PRL) and *Philosamia ricini* pupae (PRP). Our findings highlight the complex relationships between nematodes and their hosts, revealing significant trends in infectivity and mortality dynamics.

Effect of inoculation methods on host mortality and mortality percentage: The present study highlights the significant impact of inoculation methods and nematode concentrations on the efficacy of entomopathogenic nematodes (EPNs) across diverse insect hosts. Among the methods tested, the spread plate technique consistently resulted in higher mortality rates compared to immersion and shaking methods. This increased efficacy may be due to better distribution of infective juveniles (IJs) and improved contact between nematodes and host surfaces, facilitating more effective host invasion[2,8].

In terms of host susceptibility, BML and GML showed the highest mortality rates whereas PRP exhibited the lowest. These differences can be attributed to variations in host physiology, immune defense mechanisms and surface characteristics such as cuticle thickness and chemical composition, all of which affect nematode penetration and colonization[3,19]. Such host-specific susceptibility is consistent with earlier findings emphasizing the role of both host and nematode species in determining infection success[15].

Impact of EPN concentration on host mortality and IJ emergence: A dose-dependent relationship between EPN concentration and host mortality was also evident. Higher concentrations led to significantly increased mortality across most insect hosts, supporting previous reports that a higher density of IJs improves the chances of successful infection and rapid host death[4,12]. However, the relatively stable mortality observed in GML, even at lower concentrations, suggests possible host-specific factors such as lower immune resistance or higher cuticle permeability that favor nematode invasion at even minimal dosages.

The emergence data align with mortality trends. BML and BMP recorded the highest number of emerging IJs (infective juveniles), indicating that they provide suitable internal environments for nematode development and reproduction. Conversely, PRP exhibited minimal emergence, reinforcing the notion that not all hosts support optimal nematode reproduction[1]. The quality of the host directly influences the reproductive output of EPNs, as nutrient availability and microbial competition in the host cadaver play critical roles in determining the number of progeny[7,17].

Conclusion

With BML and GML exhibiting the greatest vulnerability, this study shows that the spread plate inoculation approach is the most efficient strategy for maximizing entomopathogenic nematode (EPN)-induced mortality across several insect hosts. Increasing EPN concentrations also helped to increase host mortality and infective juvenile (IJ) emergence; BML and BMP were more favorable for EPN growth. Consistent mortality and notable IJ emergence in the control host GML confirmed its usefulness as a reliable comparator.

Future studies should emphasize optimizing EPN application techniques for field conditions, investigating host-nematode compatibility and determining the underlying physiological and immunological responses of resistant hosts such as PRP. Combining EPNs with additional biocontrol agents and assessing their efficacy under varying environmental conditions would help to enhance their practical applicability in sustainable pest control initiatives.

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