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Assessing the Efficiency of Inoculation Methods for Mass Propagation of *Heterorhabditis indica* in Various Hosts

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Highlights

- The highest infectivity percentage was recorded for the *ariPhilosamariicini* larvae and *Bombyx mori* larvae
- Mortality analysis confirmed the significant effect of inoculation method and host type on *H. indica* effectiveness
- The spread method induced the highest mortality for *H. indica*

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ABSTRACT

Mass cultivation for large-scale pest control is essential to harness the potential of *Heterorhabditis indica*. In the present study, the Mortality rate of different host and infectivity of *H. indica* were tested against several test hosts, namely *Galleria mellonella* larvae (GML), *Bombyx mori* larvae (BML), *Bombyx mori* pupae (BMP), *Philosamiaricini* larvae (PRL), and *Philosamiaricini* pupae (PRP). The highest constant infectivity percentage was recorded for the PRP and BML. At the 72-hour interval, the spread plate method showed the highest infectivity percentage for the PRP. It significantly decreased at the 96-hour interval due to possible temporal changes in nematode-host interactions. Mortality analysis confirmed the significant effect of inoculation method and host type on *H. indica* effectiveness. Notably, the immersion method showed the lowest infectivity percentages for BML and BMP at the 72-hour interval. The spread method induced the highest mortality for *H. indica*, emphasizing its initial efficacy. However, at the 96-hour interval, the spread method outperformed the other methods, achieving the highest mortality in most hosts. The present study findings will help develop cost-effective and sustainable strategies for the mass cultivation of entomopathogenic nematodes (*H. indica*), promoting their broader adoption as eco-friendly alternatives for pest management in agriculture.

INTRODUCTION

Soil-dwelling roundworms that are small in size are known as Entomopathogenic nematodes (EPNs) and are very effective as biological control agents against soil-borne and foliar pests (Abate et al., 2023). There are multiple EPNs recognized; however, *Heterorhabditis indica* has gained more attention due to its several beneficial characteristics, such as adaptability and ability to target noxious insect pests efficiently (Thube et al., 2023; Ghoneim and Hamadah, 2024). Despite their role as endoparasites, they can also enter the host of various species. In addition, they have been shown to infect more than two hundred distinct species of insect pests that belong to different taxa (Neira-Monsalve et al., 2019).

In addition, the bacterial genus *Photorhabdus* is mutually associated with *Heterorhabditis indica* species (Ghoneim and Hamadah, 2024). This association depends on the dauer juvenile (DJ), an infectious third-stage juvenile, serving as an infectious agent. These DJ stages, non-feeding and free-living, are typically found in the soil, where they seek out insect larvae as hosts (Somvanshi et al., 2016; Vlaar et al., 2021). Heterotrophic bacteria use their dorsal teeth to abrade the intersegmental membranes to infect insects. When it enters the hemocoel of the host, the nematode releases its symbiotic bacterium, *Photorhabdus luminescens*, which has been carried in the nematode's intestine (Ffrench-Constant et al., 2000). The nutrient-rich environment of the insect's hemolymph provides a favorable environment for bacteria to multiply and produce poisons and exoenzymes that kill the host to be killed in 24 to 48 hours. After the host dies, the cadaver reproduces for (at most, because it is parasitic) two to three generations of the nematode inside. As the cadaver's ability to provide nutrients decreases, nematode reproduction stops, and nematode dauer juveniles accumulate and become surface in the surrounding soil (Szelecz et al., 2016). In the absence of suitable hosts, these infectious stages can survive for months or more in the soil and then survive in the environment to await the availability of a new host. It has been demonstrated that *H. indica* can effectively control pests in large horticultural crop fields as a chemical substitute for environmentally benign insecticides; thereby, they can be helpful in integrated pest control (IPM) systems Kantor et al., 2022).

Mass cultivation of the nematode for large-scale pest control is essential for harnessing the potential of *H. indica* (Thube et al., 2023). EPNs can be produced using two primary techniques namely, *in vivo* (culturing inside live insect hosts) and *in vitro*

(artificial media or bioreactor systems). *The in vivo* method can be simple, cost-effective, and used for small-scale productions, while the *in vitro* method is more efficient and scalable which suitable for large-scale commercial productions (Zhen et al., 2018). However, the extent of success of these methods depends upon the types of inoculation and test insect hosts used, since these factors have considerable effects on nematode yield, virulence, and quality (Zhen et al., 2018).

In the present study, infectivity and mortality of *H. indica* against a broad range of test hosts, including *Galleria mellonella* larvae, *Bombyx mori* larvae and pupae and *Philosamariicini* larvae and pupae, were assessed. Nevertheless, more recent studies have shown the possibility of using alternative hosts, like silkworm species (*Bombyx mori* and *Philosamariicini*) with high susceptibility to entomopathogenic nematodes, as attractive potential candidates for large-scale nematode production (Mwaniki et al., 2013). Sustainable EPN multiplication thereby offers a unique opportunity with the silkworm, especially that of *Bombyx mori*, particularly in agricultural countries such as India, where silkworm cultivation is already within the agricultural framework. There has been a turn to essential quarantine measures in raising *B. mori*, which are suitable for both the practical and sustainable activity of silkworm farmers. Notably, *B. mori* can serve a dual purpose for farmers: When conditions favour the market for silk production, it can also be used as nematode propagation for biological pest control. This versatility offers a cost-effective, scalable solution for nematode production, which is consistent with existing farmer practices and contributes to a more economically viable solution.

By comparing these hosts, we aim to identify alternative, cost-effective, and readily available options for nematode mass production in India. Such findings will contribute to the standardization of *H. indica* inoculation methods, facilitating its broader use as an eco-friendly and sustainable solution for pest management in agriculture and horticulture. It is commercially sustainable only when we identify a suitable host and standardize different inoculation method. Standardization of Different inoculation methods for mass culturing of entomopathogenic nematode (*H. indica*) on different test host, by evaluating host infection levels during mass multiplication process. The trials were conducted by three different inoculation techniques Inoculation by Spreading, Inoculation by Shaking and Inoculation by immersion method. These findings will help identify cost-effective and sustainable strategies for EPN (*H. indica*) mass production, promoting their broader adoption as eco-friendly alternatives for pest management in agriculture.

1. Materials and Methods

2.1. Source of organism

The *Heterorhabditis indica* species and Galleria mellonella larvae (L.) (Lepidoptera: Galleriidae) (GML) were obtained from Nematology laboratory of Department of Entomology, NBAIR, Bangalore, Karnataka, India. Larvae and pupae of *Bombyx mori* (BML and BMP) procured from Central Silk Board, Seed Production Centre, Vijayapura, Karnataka, India. *Philosamiaricini* (PRL and PRP) were acquired from Central Silk Board, Hosur.

Method for post-inoculation

For three distinct inoculation procedures and for every host and nematode species combination, a total of 15 Petri plates containing 10 insect larvae each (N=150 larvae) were utilized. Following inoculation, Petri plates with inoculated hosts for each treatment were transferred 11 × 11 × 7.5 cm airtight plastic boxes, covered with damp filter paper, and allowed to incubate at 25 °C. For each treatment, the mortality percentage of the insect hosts was noted every 24 hours for a total of 4 days. Mortality was determined by color change and the lack of host activity when prodded with a couple of tweezers. The percentage of host infection in each treatment was calculated after 10 days. The existence of nematodes was confirmed through host dissection, followed by microscopic examination (Fig. 1).

2.2. Optimization of inoculation techniques

2.3.1. Inoculation by spread method

The spread method of insect host inoculation entails the placement of ten insect larvae/pupae in a 90-mm Petri dish that has been covered with Whatman filter paper (No. 1). For each replication 5 such plates were maintained. The experiment was repeated three times with 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 than new test date was scheduled for the experiment (Fig. 2).

2.3.2 *Inoculation by shaking*

A 250 ml beaker containing 150 insect larvae or pupae from each test host was used for the shaking method. A 1 ml nematode inoculum with 30,000 *Heterorhabditis indica* juveniles (200 IJs per host) was introduced. A lid was placed on the beaker and manually shaken for one minute to distribute the nematodes evenly on the insect bodies. Using tweezers, 10 insects were placed in a filter paper-lined 90-mm Petri plate. The post-inoculation technique was followed, and the experiment was repeated on a different date (Fig. 2).

2.3.3 Inoculation through immersion

For the immersion method, 150 larvae or pupae of each test host were immersed in 300 ml of distilled water that contained *H. indica* inoculum. In a 500-ml beaker with a suspension of *H. indica* that had 200 infective juveniles (IJs) per 50 µl, the hosts were submerged in the inoculum for 10 seconds. After immersion, the hosts were transferred to a sieve and briefly placed on a paper towel for 5 seconds to absorb any excess suspension. The insect hosts were subsequently transferred from the sieve using tweezers and then deposited in separate 90-mm Petri dishes lined with wet filter paper. The post-inoculation protocol was subsequently followed, and the experiment was repeated on a different test date (Fig. 2).

2.4 Statistics

The data was 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 by the ggpurb function under the ggplot package in R.

3. Results

3.1. Effects of different inoculation methods on infectivity percentage

The results of infectivity of *Heterorhabditis indica* in different inoculation methods across various hosts are presented in Figure 3. For the spread method, at 72 hours, the infectivity percentage was significantly higher (p< 0.0001) for *Philosamiaricini* pupae (PRP) (17.5%), followed by *Galleria mellonella* larvae (GML), *Bombyx mori* pupae (BMP) (12.4%), and *Bombyx mori* larvae (BML) (12.3%), with the lowest infectivity recorded for *Philosamiaricini* larvae (PRL) (11.2%). However, at 96 hours, the highest infectivity percentage (p< 0.001) was recorded for BMP (19.1%), followed by PRL (17.9%), BML (17.8%), PRP (9.1%), and the lowest value for GML (2.4%) (Fig.3).

For the shaking inoculation method, at 72 hours, the infectivity percentage was significantly higher (p< 0.01) for BML (13.5%), followed by BMP (12.2%), PRL (12.1%), GML (11.1%), and the lowest value for PRP (5.0%). Moreover, at 96 hours, the highest infectivity percentage (p< 0.001) was recorded for BML (11.3%), followed by BMP (10.1%). PRL and PRP had similar values (9.6%), while GML had the lowest infectivity percentage (1.1%) (Fig.3).

For the immersion method, at 72 hours, the highest infectivity percentage (p< 0.0001) was observed for BML (15.0%), followed by PRL (12.6%), BMP (12.5%), PRP (11.1%), and the lowest value for GML (7.7%). Furthermore, at 96 hours, BML recorded the highest infectivity percentage (13.2%), followed by BMP (12.6%), PRL (7.6%), PRP (5.9%), and the lowest value for GML (2.6%). These findings highlight significant differences in the infectivity of *Heterorhabditis indica* across different inoculation methods and host groups (Fig.3).

3.2. Effects of different inoculation methods on mortality percentage

3.2.1. In Bombyx mori Larvae (BML)

The mortality of the host due to *Heterorhabditis indica* across different inoculation methods for *Bombyx mori* larvae (BML) is presented in Figure 4. Significant variations were observed among the three methods: Immersion, Shaking, and Spread. Mortality trends were consistent across the 72- and 96-hour intervals.

At the 72-hour interval, the Immersion method showed the highest mortality (31%, $p< 0.001$, $r^2 = 75.5$), followed by the Shaking method (28.5%, $p< 0.001$, $r^2 = 86.0$), with the lowest value recorded in the Spread method (22%, $p< 0.001$, $r^2 = 99.0$). At the 96-hour interval, mortality was highest for the Spread method (35%), followed by the Immersion method (24.9%) and the Shaking method (24.8%) (Fig. 4).

3.2.2. In Bombyx mori Pupae (BMP)

For the *Bombyx mori* pupae (BMP) host, at the 72-hour interval, the highest mortality was observed in the Immersion method (25.3%, $p< 0.001$, $r^2 = 81.0$), followed closely by the Shaking method (25.0%, $p< 0.001$, $r^2 = 72.0$), and the lowest value was recorded in the Spread method (24.9%, $p< 0.001$, $r^2 = 89.0$). At the 96-hour interval, the Spread method exhibited the highest mortality (39%), followed by the Immersion method (26.5%) and the Shaking method (20.8%) (Fig. 4).

3.2.3. In Galleria mellonella Larvae (GML)

For the *Galleria mellonella* larvae (GML) host, at the 72-hour interval, the highest mortality was recorded in the Spread method (25%, $p = 0.09$, $r^2 < 0.01$), followed by the Shaking method (20.8%, $p = 0.8$, $r^2 < 0.01$), and the lowest value was observed in the Immersion method (19%, $p< 0.3$, $r^2 = 0.02$). At the 96-hour interval, the Immersion method recorded the highest mortality (6%), followed by the Spread method (4%), and the Shaking method (2.1%) (Fig. 4).

3.2.4. In Philosamiaricini Larvae (PRL)

For the *Philosamiaricini* larvae (PRL) host, at the 72-hour interval, the highest mortality was observed in the Immersion method (25%, $p< 0.001$, $r^2 = 60$), followed by the Shaking method (24.1%, $p< 0.001$, $r^2 = 67$), with the lowest value recorded in the Spread method (21%, $p< 0.001$, $r^2 = 89$). At the 96-hour interval, the Spread method recorded the highest mortality (37%), followed by the Shaking method (18%) and the Immersion method (17.2%) (Fig. 4).

3.2.5. In Philosamiaricini Pupae (PRP)

For the *Philosamiaricini* pupae (PRP) host, at the 72-hour interval, significantly higher mortality was observed in the Spread method (33.9%, $p< 0.001$, $r^2 = 48$), followed by the Immersion method (21.1%, $p< 0.001$, $r^2 = 49$), and the Shaking method (14.9%, $p< 0.001$, $r^2 = 86$). At the 96-hour interval, mortality was highest in the Spread method (19.3%), followed by the Shaking method (19.2%) and the Immersion method (11.1%) (Fig. 4).

DISCUSSION

This study was aimed the *in vivo* mass multiplication of Entomopathogenic nematode (*Heterorhabditis indica*) towards development of potent biocontrol agent. Standardization and validation of Infection protocol of Entomopathogenic nematode by spread method, shake method, and immersion method were studied on various hosts, namely *Bombyx mori larvae* (BML), *Bombyx mori pupae* (BMP), *Galleria mellonella larvae* (GML), *Philosamiaricini larvae* (PRL), and *Philosamiaricini pupae* (PRP) at 72-hour and 96-hour intervals. The findings showed the complex relationships between nematodes and their hosts by highlighting significant trends as well as host-specific and methodological implications on infectivity and mortality dynamics.

4.1. Different methods and hosts' infectivity percentage

The infectivity patterns indicated a strong dependence on both inoculation methods and host types. *Philosamiaricini pupae* (PRP) and *Bombyx mori larvae* (BML) showed consistently high infectivity percentages in all three experimental methods viz., Immersion, spread, and shaking. In the spread method, at the 72 -hour interval the PRP showed the highest infectivity percentage level. However, at the 96 hour interval this level significantly decreased. The possible reason behind this reduction could be due to the temporal changes in nematode-host interactions (Van et al., 2014). Moreover, in the case of *Bombyx mori pupae* (BMP) showed the opposite trend with respect to the PRP was observed, where the progressive and highest level of infectivity percentage was recorded at the 96 -hour interval at all three experimental methods. This observation suggests that in the long term the BMP may provide a more favourable microenvironment for the establishment of *H. indica* (Awais et al., 2021; Qin et al., 2022).

The noticeable variation was recorded in the infectivity level percentages for the shaking and immersion methods. For instance, at 72-hour intervals, the immersion approach showed the maximum infectivity with the BML host. However, this may have been reduced as a result of nematode desiccation or host resource exhaustion. Similarly, the shaking inoculation was more effective against BML and BMP hosts. Nevertheless, compared to the shaking method, the spread method's infectivity percentage at 96 hours was significantly decreased. Surprisingly, *Galleria mellonella*larvae (GML), the standard entomopathogenic nematode host model, were the most infected across all methods. Such findings, however, may represent a mode of possible resistance of this host to nematode spread or more unfavourable physical and biochemical conditions for nematode spread (El-Saadony et al., 2021; Zheng et al., 2021). Since most methods achieved consistently high infectivity percentages for GML and BML, they appear to be ideal target hosts for H. indica (Smith-Ávila et al., 2024).

4.2. Mortality percentage trends

Mortality analysis confirmed the significant effect of inoculation method and host type on *H. indica* effectiveness. Notably, the immersion method showed the lowest infectivity percentages for BML and BMP at the 72-hour interval. The spread method induced the highest mortality for *H. indica*, which emphasizes its initial efficacy. However, by 96 hours, the spread method outperformed the other methods in achieving the highest mortality in most hosts (Din, 2017; Wilber et al., 2020). The long-term efficacy of the spread method may arise from the gradual and continuous nematode exposure provided by this method. *Philosamiaricini larvae* (PRL) and *Philosamiaricini*pupae (PRP) showed opposite mortality dynamics. While PRP mortality in the spread method peaked at 72 hours, PRL showed a slow mortality response, with maximum values occurring at 96 hours. This temporal variation highlights the complex relationship between host life stages and nematode virulence (Tack et al., 2012; Iritani et al., 2019).

On the other hand, across all the experimental methods, GML consistently exhibited lower mortality, correlating with its suboptimal infectivity outcomes. This suggests a heightened resistance threshold in GML, thereby restricting its efficacy against H. indica. R^2 values indicated strong predictability and consistency in mortality outcomes, especially for the spread method in PRL and PRP, emphasizing the significance of host physiology in influencing nematode efficacy.

CONCLUSION

The present study findings indicate the importance of inoculation methods in improving the nematodes' infectivity and effectiveness. Our results showed that the spread method for mortality and infection rates demonstrates higher values at 96 hours after application, implying its effectiveness for field use, especially when the host susceptibility takes too long after the exposure. The contrasting outcomes among different hosts emphasize the importance of adapting the inoculation strategy depending on the biology of the host and the environmental factors. It will also be helpful to carry out biochemical and molecular studies that account for the patterns restricted to specific hosts observed in this study. Assessing the factors determining GML tolerance might also help remove barriers to tolerance as a model host. Additionally, optimizing inoculation parameters, such as nematode concentration and exposure duration, could significantly enhance the practical application of *H. indica* in integrated pest management systems. In conclusion, this study emphasizes the relative complexity surrounding the relationships between host and inoculation methods in determining the level of infectivity of H. indica. The findings may assist in formulating and deploying more efficient biological pest control methods for various pest management approaches. In conclusion, this study emphasizes the complex relationship between inoculation methods and the host in determining the infectivity and efficacy of *H. indica*. The findings may assist in formulating and deploying more efficient biological pest control methods for various pest management systems.

Declaration of Competing Interest

Authors have no competing interests to declare.

Data availability
Data will be made available on request.

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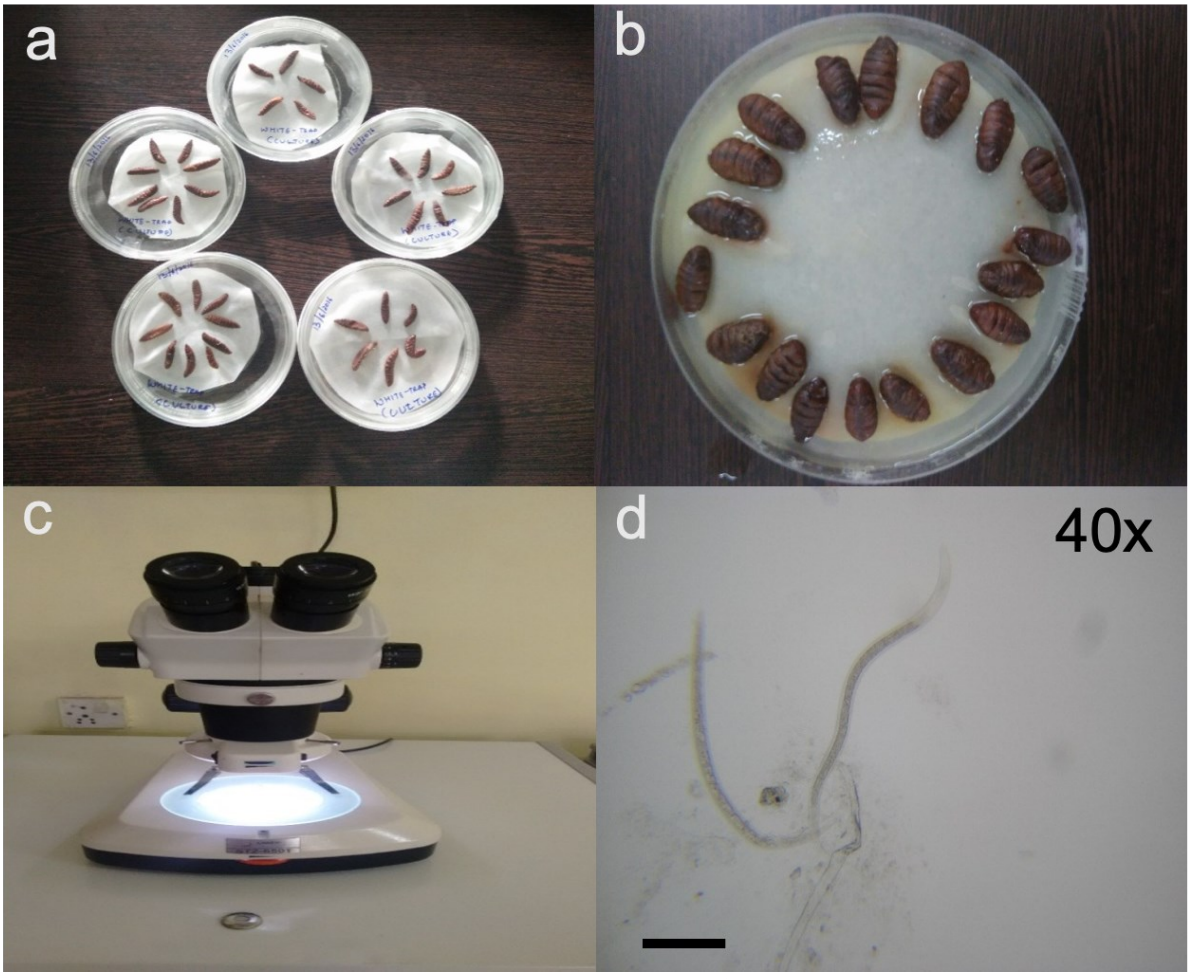

Fig 1: White Trap Method- Harvesting of Infective Juveniles (**a**) dead larvae (**b**)dead pupae. **(c)**Nematode observation under stereo microscope (**d**) Infective Juveniles under stereo microscope.

Fig 2: Inoculation by different methods. **(a)**Immersion method; **(b)** Inoculation by spread method; **(c)** Emergence of *H. indica* from the Host. **(d)** *P. richini* larvae and **(e)***B.mori* larvae.

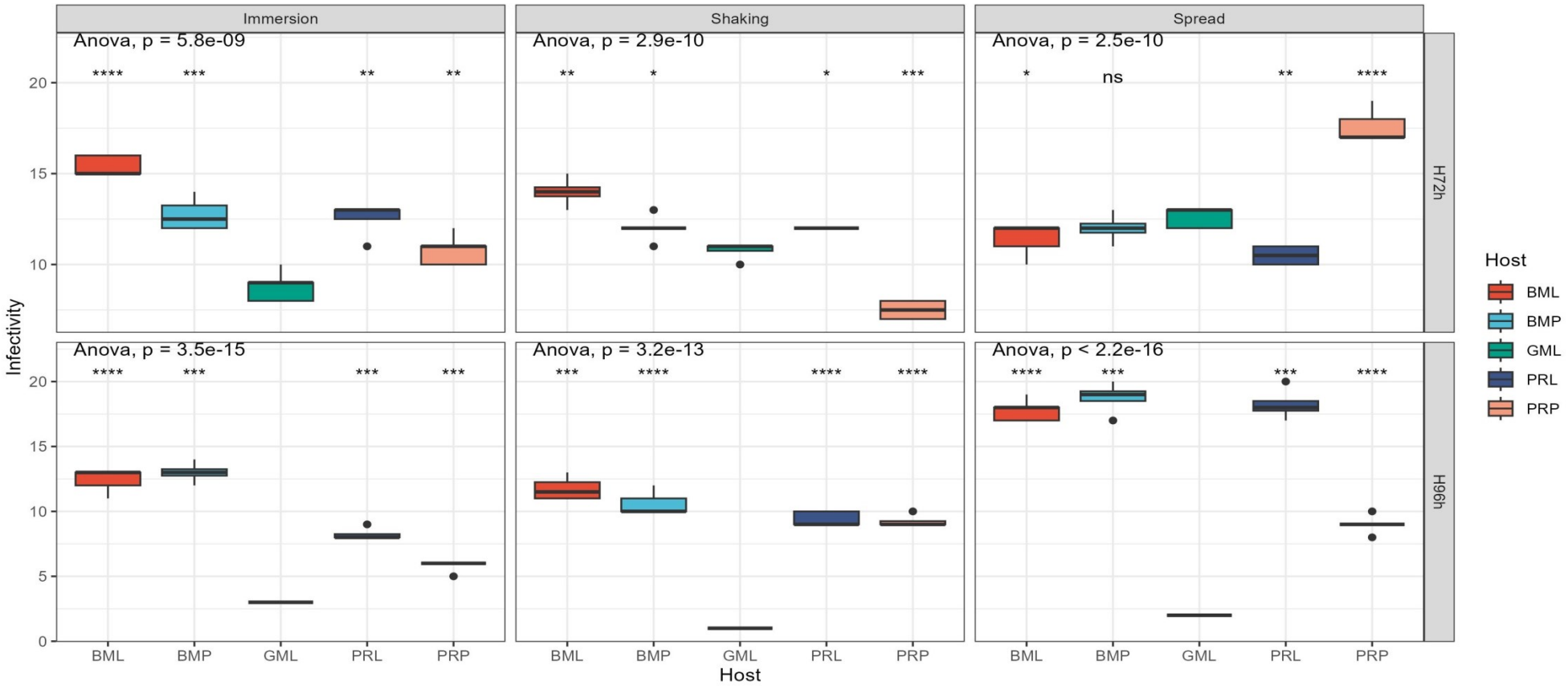

Fig 3: The infectivity box plot. Infectivity was plotted *Bombyx mori* larvae (BML), *Bombyx mori* pupae (BMP), Galleria*mellonella* larvae (GML), and *Philosamiaricini* larvae (PRL) and *Philosamiaricini* pupae (PRP) under Immersion, Shaking and spread method in 72 and 96 hours.

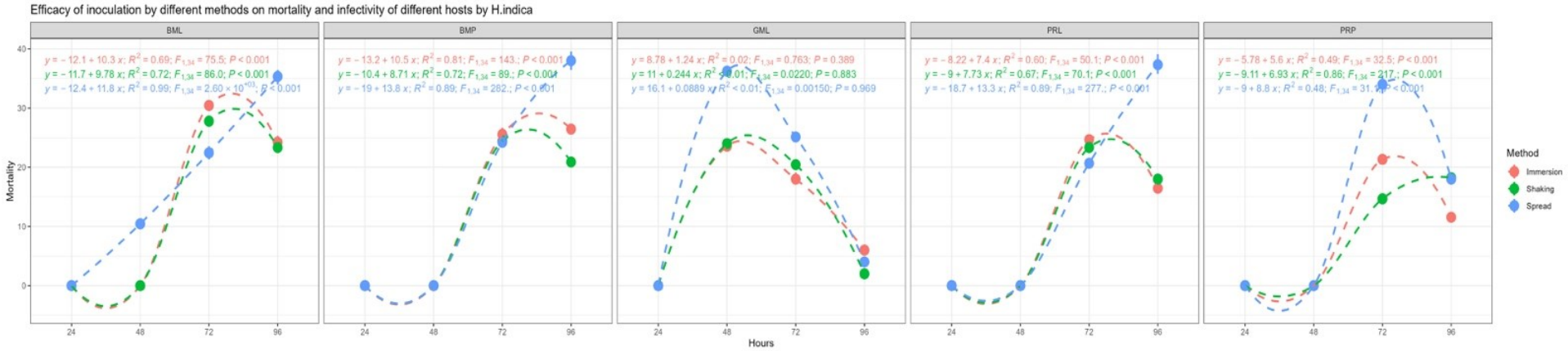

Fig 4: Mortality percentages of H. indica in different inoculation methods (Immersion, Shaking, and Spread) across five hosts: *Bombyx mori larvae* (BML), *Bombyx mori pupae* (BMP), *Galleria mellonella larvae* (GML), *Philosamiaricini larvae* (PRL), and *Philosamiaricini pupae* (PRP) at 72-hour and 96-hour intervals.