

Evaluation of Pathogenicity of Symbiotic Bacteria of Entomopathogenic Nematodes against the Larvae of Tobacco Caterpillar, *Spodoptera litura* F. (Lepidoptera; Noctuidae)

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Authors' contributions

This work was carried out in collaboration between both authors. Author SA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SA and MKS managed the analyses of the study and the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

The tobacco caterpillar, *Spodoptera litura* is a polyphagous pest with attacking almost 290 host plants species globally causing a wide range of yield losses to the agricultural crops. In this investigation symbiotic bacteria of entomopathogenic nematodes were evaluated for their insecticidal activity against larvae of *S. litura* under lab conditions. The intact cell suspension and cell-free extract were screened for their bacterial pathogenicity and the results showed mortality of larvae after 24h of post treatment. The percent mortality treated with intact cell suspension was significantly higher in larvae treated with EPB3 strain with 90% which was on par with the reference strain *Bacillus thuringiensis* after 72h of post treatment. There is no significant difference among the larval mortality treated with cell-free supernatant. The virulence test was carried out to find out the LD₅₀ and LT₅₀ against the larvae and the results showed that EPB3 and *B. thuringiensis* required 10⁴ CFU for 50% lethality of larvae with a media lethal time of 24h and 22h, respectively when treated with cell suspension. In case of larvae treated with cell-free extract, LD₅₀ value with 10⁵-10⁶

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CFU with a median lethal time ranging from 33-45h. The results showed that the isolates EPB3 (*Xenorhabdus nematophilus*) is comparatively more virulent than other *Photorhabdus* isolates against the test insect *S. litura*. Further these symbiotic bacteria can be screened for their biocontrol efficiency under greenhouse and field conditions and can be developed as potential biopesticides against the insect pests.

Keywords: *Spodoptera litura*; symbiotic bacteria; bacterial pathogenicity; lethality; LD₅₀ and biopesticides.

1. INTRODUCTION

Spodoptera litura Fabricius (Lepidoptera: Noctuidae) an economically important insect pests worldwide, especially in the tropics, temperate zones, and other warm environments. Larvae voraciously feed on the leaves of the host plants resulting in skeletonized leaves. Controlling these insect pests currently depends mainly on chemical insecticides. However, outbreaks of this pest and field control failures have been documented frequently in Asia, mainly due to insecticide resistance [1-3]. Although they can be controlled by insecticides, there have been instance where they have developed resistance.

Biological control methods to reduce reliance on chemical insecticide applications are continuing to be developed [4]. Though *Bacillus thuringiensis* is used as a one of the biocontrol agent against the control of these larvae, there have been a development of resistance in the larvae for the different cry proteins which are major toxic proteins that helps in the mortality. Another biocontrol agent was entomopathogenic nematodes (EPN) (Heterorhabditidae and Steinernematidae) have been commercially available [5]. Entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis*, and their symbiotic bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp., respectively) are lethal endo-parasites of soil-borne insects. They are found in soils as non-feeding infective juveniles (IJs of EPN). These IJs enter the insect host through natural body openings or through the host cuticle, then penetrate into the host haemocoel, and release their associated bacteria that kill the host within 24 - 48 h of infection [6-7]. Several species of EPNs have been used commercially for biological control of insect pests and they are harmless to vertebrates and plants [5].

The toxin complexes (Tcs) produced by these symbiotic bacteria are orally active toxins that are displayed on the outer surface of the bacterium [8]. They require three components (A, B and C)

for full toxicity and one A component has been successfully expressed in transgenic Arabidopsis to confer insect resistance. One such group of Tc's, the PirAB binary toxins [9] have oral activity against mosquitoes and some caterpillar pests. Other toxins such as makes caterpillars floppy (Mcf) [10] and proteins encoded by the *Photorhabdus* virulence cassettes (PVCs) [11] only show injectable activity. Mcf1 promotes apoptosis in a wide range of cells and appears to mimic mammalian BH3 domain-only proteins in the mitochondrion [12]. They are virulent to a wide range of insects including Lepidoptera, Coleoptera, Hemiptera and Diptera [13]. With this background the present investigation was carried out to test the bacterial pathogenicity of symbiotic bacteria against the serious insect pest that feeds voraciously on both agricultural and horticultural crops, *Spodoptera litura*.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Culture Conditions

The four new isolates (EPB1, EPB4, EPB8 and EPB9) belongs to *Photorhabdus luminescence* isolated from nematode *Heterorhabditis bacteriophora* and one isolate (EPB3) of *Xenorhabdus nematophila* isolated from nematode *Steinernema* sp. were used in the study were presented in the Table S1 [14]. *Bacillus thuringiensis* was used as a reference strain was obtained from Biofertilizers lab, Department of Agricultural Microbiology, GKVK, Bangalore. Bacterial cultures were incubated at 28 ± 2 °C in the dark for 72h in a BOD incubator.

2.2 Preparation of Intact cell Suspension and Cell-Free Supernatant

A single bacterial colony of each isolate was streaked on NBTA medium and incubated at 28 ± 2 °C in the dark for 72h in a BOD incubator. The a single colony was transferred and grown in 25 ml LB broth and shaken at 180 rpm for 24h. The concentration of the intact cell suspension was adjusted to 10⁸ CFU/ml using phosphate

buffered saline solution. To prepare cell-free supernatant, 1 ml intact cell suspension was centrifuged at 5000 rpm for 5 min. The supernatant was then filtered using a 0.22-mm syringe filter. The flow-through was used as the cell-free supernatant [15].

2.3 Screening of symbiotic Bacteria of EPN for Bacterial Pathogenicity against *Spodoptera litura*

The tobacco caterpillar, *Spodoptera litura* larvae was obtained from National Bureau of Agriculturally Insect Resources (NBAIR), Hebbala, Bangalore and rearing was done in Department of Agricultural Microbiology, GKVK, UASB, Bangalore under conditions were: temperature $25 \pm 1^\circ \text{C}$, a photoperiod of 12 hour light and relative humidity $70 \pm 5\%$. The larvae were kept in capped plastic boxes having small holes in it for aeration. Castor leaves were provided as diet in the boxes for their survival. Isolates of symbiotic bacteria were sub-cultured in Luria broth and incubated at 28°C for 48h in a shaker cum BOD incubator at 180 rpm. A fully grown symbiotic bacterial culture were centrifuged for separation of cell pellet and supernatant. The cell pellet was taken and cells were dissolved in phosphate buffered saline solution and similarly cell supernatant was diluted for treatment. Castor leaves were treated with different concentrations of intact suspension and cell-free extract by leaf dip method [16] and mortality of insects was recorded at regular interval of 6h, 12h, 18h, 24h, 36h, 48h, 72h and 96h [17]. Percentage mortality is recorded from the average of ten larvae for each treatment and were replicated thrice.

2.4 Virulence assays of Symbiotic Bacteria of EPN Against *Spodoptera litura*

The virulence assays were carried out *in vitro* in sterile polystyrene boxes (HiMedia Laboratories, Mumbai, India) as per the procedure given by Kumar *et al.* [18]. A day old cultures were centrifuged and the cell pellet was taken and cells were dissolved in sterilized distilled water. The bacterial cultures were transferred to the polystyrene boxes at different concentrations ranging from 10^2 to 10^{10} CFU/ml. Each concentration of symbiotic bacterial isolate was replicated 10 times, and the experiment was independently repeated thrice. Similarly the experiment was also carried out with cell supernatant. The boxes were kept in a

Tupperware box and incubated at $25 \pm 1^\circ \text{C}$. Insect mortality was recorded every 12h till 100% insect mortality or pupation, whichever was earlier. Mortality data were used to calculate median lethal concentrations (LC_{50}) and median lethal time (LT_{50}) values.

2.5 Statistical Analysis

The percent mortality of larval data in percentage were transformed to arcsine values and analyzed. All the data were subjected to statistical analysis using analysis of variance (ANOVA) [19] at $p < 0.05$ level, further the treatment means were statically differentiated by performing Duncan's Multiple Range Test (DMRT) at $p < 0.05$ level. Statistically differentiated means were denoted by different alphabets. For all the above analysis, the software, DSAASTAT [20] was used. The LC_{50} values were calculated by Probit analysis [21] function using the software IBM SPSS Statistics v 20 (IBM Corp. Armonk, NY, USA). The LT_{50} values were calculated from the survival curves prepared by Kaplan-Meier survival analysis. The survival curves were compared using the log-rank test using GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla, CA, USA) statistical software. The log-rank test calculates the chi-square value (χ^2) for each event time for each group and sums the results which are used to derive the ultimate chi-square to compare the full curves of each group [22].

3. RESULTS AND DISCUSSION

Spodoptera litura is a polyphagous pest in tropical countries covers at least 290 host plants causing huge damage to vegetable and field crops. Larvae voraciously feed on the leaves of the host plants resulting in skeletonized leaves. Although they can be controlled by insecticides, there have been instance where they have developed resistance. Hence the researchers are directed towards the biological control of *S. litura*. In present study of the symbiotic bacteria were tested for bacterial pathogenicity against the larvae of *S. litura* to find the effective isolate among the five bacteria that can work efficiently as biocontrol agent.

3.1 Screening of Insecticidal Activity of Symbiotic Bacteria of EPNs Against Larvae of *Spodoptera litura*

Intact cell suspension and cell-free supernatant of symbiotic bacterial isolates of EPNs were tested for mortality against larvae of *S. litura* and

presented in the Table S2 and Fig. 1A. After 12-24 h of post-treatment with intact cell suspension, all bacterial isolates had caused lethality in the larvae. Among the isolates EPB3 had recorded highest larval mortality (93%) after 72h of treatment equivalent to *Bacillus thuringiensis* (95%) and differed significantly from other symbiotic bacterial isolates. All the other isolates intact cell suspension had recorded mortality in the range of 75-85% at 72h of post treatment. Similar trend of mortality was also observed in the larvae treated with the cell-free supernatant (Table S3 and Fig. 1B). After 48 h of post-treatment there was no significant difference in larvae mortality. Among the five isolates EPB1 (75%) shown highest mortality followed by EPB4 (72%) and EPB3 (70%).

In one of the previous study conducted by Kulkarni *et al.* [23] reported infectivity and virulence of EPN, *H. indica* on teak skeletonizer, *Eutectona machaeralis*. They have done bioassay studies by exposure of early last instar larvae of the teak skeletonizer to two bioassay conditions; filter paper bioassay (3, 5, 10, 20 and 30 IJs/ larva) and leaf treatment (30, 60, 100, 200 and 300 IJs/ larva) to determine susceptibility and critical doses. *E. machaeralis* larvae showed 35.29% mortality at lowest dose 3 IJs larvae and 100% mortality was obtained at the highest dose of 30 IJs larvae. They also showed ten times more doses, *i.e.* above 30 IJs larva were required to cause larval mortality when larvae were exposed to leaf treatment experiment using Potter's Tower. LC₅₀, LC₉₀, LT₅₀ and LT₉₀ values for *H. indica* in filter paper bioassay (4.57, 12.02 and 30.20, 54.95, respectively) and leaf treatment method (54.37, 114.50 and 40.62, 122.70, respectively) were calculated. They have concluded that doses above 100 IJs larva may be required for managing the pest by leaf treatment.

In one of the study conducted to know the effect of bacterial-EPN complex and symbiotic bacteria alone on *Spodoptera littoralis* had concluded that the larvae injected directly with the symbiotic bacteria alone namely *X. bovienii* and *X. nematophila* (10⁸ CFU/larva) caused septicemia after 24h of post infection and killed 90% of larvae after 36h, whereas the larvae injected with bacterial-nematode complex has started mortality after 36h of post injection and attained 90% of mortality after 5 days of infection [24]. In our study also the direct application of symbiotic bacterial isolates had killed ~90% of population in between 36-72 h after application due to the

direct action of toxic complexes and other secondary metabolites on the larval gut causing septicemia after the entry of the bacterial cells into the gut of the insect larvae.

3.2 Virulence test of Symbiotic Bacteria of EPNs Against *Spodoptera Litura*

Media lethal dose (LD₅₀) value represents the dose of the symbiotic bacteria to kill the half of the larval population. LD₅₀ and LD₉₀ was calculated and presented in the Table 1. Among the entomopathogenic bacterial intact cell suspension, *Bacillus thuringiensis* had recorded lowest dose of LD₅₀ and LD₉₀ of 8.95x10⁴ and 5.84x10⁶ CFU/ml, respectively followed by EPB3 with LD₅₀ and LD₉₀ of 6.55x10⁴ and 5.11x10⁷ CFU/ml respectively. EPB1, EPB4, EPB8 and EPB9 had recorded mortality with LD₅₀ and LD₉₀ in the range of 10⁵ CFU/ml. The larvae treated with the cell-free crude extract had recorded LD50 and LD90 values in range of 10⁵ to 10⁶ CFU/ml and 10⁹ to 10¹² CFU/ml, respectively.

LT₅₀ values represent the time taken for the fifty percent mortality of larval population treated with entomopathogenic bacteria and the results were shown in Table 2 and Fig. 2. The larvae treated with intact cell suspension of symbiotic bacterial isolates had 22 to 34h for median lethality of population and among them *Bacillus thuringiensis* (*Bt*) had recorded LT₅₀ of 22h followed by EPB3 with LT50 of 24h. Similarly, the larvae treated with the cell-free supernatant had taken 33-45h for fifty percent of mortality and among the isolates *Bt* had recorded LT₅₀ of 33h followed by EPB3 and EPB1 with a median lethal time of 34h and 36h, respectively.

The bacterial pathogenicity of these symbiotic bacteria, *Photorhabdus* and *Xenorhabdus* would be attributed mainly due to the production of potential virulence factors like protease, lipase, hemolysins, and insecticidal toxins including the toxin complexes (Tcs) and Makes caterpillars floppy (Mcf) toxins, *Photorhabdus* insect-related (Pir) toxins, and *Photorhabdus* virulence cassettes (PVCs) [25-26].

Nunez-Valdez *et al.* [27] evaluated the impact of bacterial rhabduscin synthesis on bacterial virulence and phenol oxidase inhibition in a *Spodoptera* model. The rhabduscin cluster of the entomopathogenic bacterium *Xenorhabdus nematophila* was not necessary for virulence in the larvae of *Spodoptera littoralis* and *Spodoptera frugiperda*. Bacteria with mutations

Table 1. Median lethal dose (LD₅₀) of symbiotic bacterial of EPN against *Spodoptera litura*

S. No	Bacteria	Intact cells		Cell-free extract	
		LD ₅₀	χ ²	LD ₅₀	χ ²
1.	EPB1	4.56X10 ⁵	9.183	6.84x10 ⁶	16.889
2.	EPB3	6.55X10 ⁴	2.06	4.52x10 ⁵	11.633
3.	EPB4	4.80X10 ⁵	7.858	6.28X10 ⁶	9.106
4.	EPB8	6.94X10 ⁵	11.631	5.85X10 ⁶	11767
5.	EPB9	6.56X10 ⁵	3.305	4.34X10 ⁶	11.646
6.	<i>Bacillus thuringiensis</i>	8.95X10 ⁴	8.992	5.98X10 ⁵	9.875

Note: The LC₅₀ values were calculated by probit analysis using IBM SPSS v 20.0. The LC₅₀ value was considered significantly different if the 95% confidence interval (CI) values did not overlap with the CI values of other EPNs

Table 2. Median Lethal time (LT₅₀) of symbiotic bacterial of EPN against *Spodoptera litura*

S. No	Bacteria	Intact cells		Cell-free extract	
		LT ₅₀	χ ²	LT ₅₀	χ ²
1.	EPB1	26	17.90	36	6.58
2.	EPB3	24	17.85	34	8.47
3.	EPB4	32	17.91	38	10.98
4.	EPB8	28	17.93	39	6.65
5.	EPB9	33	17.70	44	7.18
6.	<i>Bacillus thuringiensis</i>	22	18.73	33	6.47

Note: The LT₅₀ values were calculated from the Kaplan-Meier survival curves. The survival curves were compared using the log-rank test χ² (chi-square) value at P < 0.05

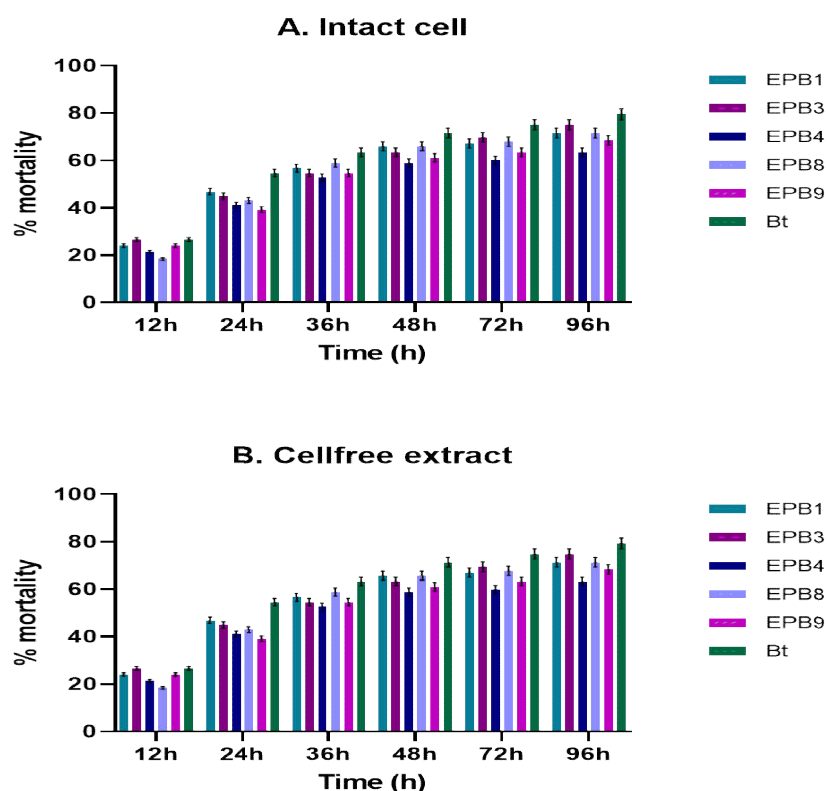


Fig. 1. Percent mortality of symbiotic bacterial isolates at different time intervals against *Spodoptera litura*. Error bars represent the SeM value

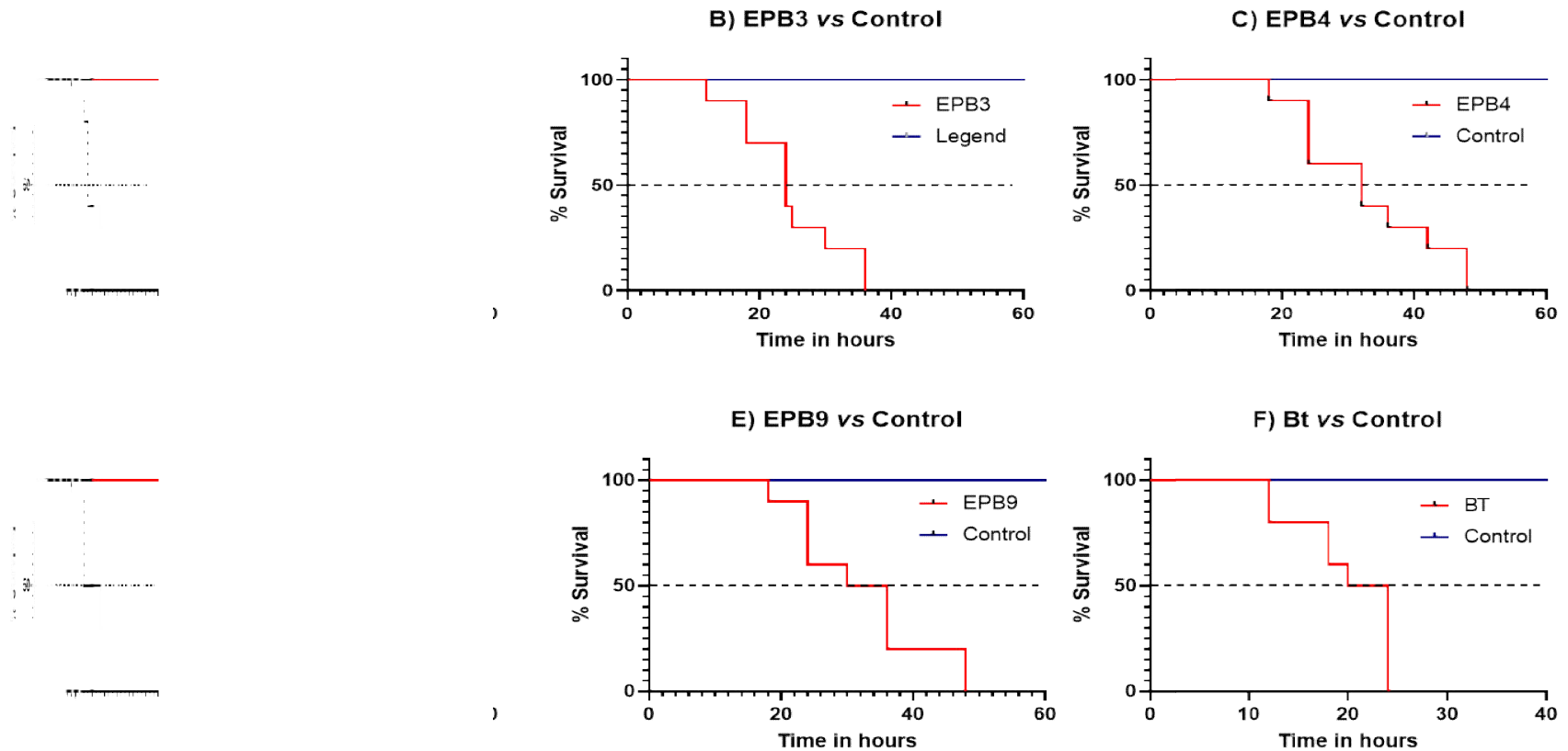


Fig. 2. Kaplan-Meier survival curves of symbiotic bacterial isolates (A) EPB1 (B) EPB3 (C) EPB4 (D) EPB8 and (E) EPB9 against *Spodoptera litura*. Blue line shows un-inoculated control, red line shows the EPB treatment. The survival curves were compared using log-rank test; all EPBs were significantly different from control ($P < 0.0001$). Three biological repeats with 10 larvae per replicate were used for survival analysis. For all graphs, X-axis – time in hours, Y-axis - % survival of the insect

affecting the rhabduscin synthesis cluster ($\Delta isnAB$ and ΔGT mutants) were as virulent as the wild-type strain. The authors confirmed that the *X. nematophila* rhabduscin cluster is required for the inhibition of *S. frugiperda* phenol oxidase activity. The inhibition of phenol oxidase activity by *X. poinarii* culture supernatants was restored by expression of the *X. poinarii* rhabduscin cluster under the control of an inducible P_{tet} promoter, consistent with recent pseudogenization. This study paves the way for advances in our understanding of the virulence of several Entomopathogenic bacteria in non-model insects, such as the new invasive *S. frugiperda* species in Africa.

The enhancement of pathogenicity of *Baculovirus* was increased two to tenfold when attenuated with the addition of *Xenorhabdus nematophilus* against *Plutella xylostella* and *Spodoptera exigua*. The treatment of DBM larvae with the combination of both the baculovirus and *Xenorhabdus nematophilus* recorded more than 40% of mortality within 24 h of post infection which might be due to the synergistic pathogenicity resulted due to the independent targets in the insect host [28]. The *Xenorhabdus* sp. was used in Thailand against the mosquito larvae to test the biocontrol efficiency and the findings has revealed that *Aedes* sp. started to die after 24 h of treatment with cell culture and showed the highest mortality rate of 87%-99% after 96 h of exposure to *Xenorhabdus stockiae*. The mortality rate of *Aedes* sp. was between 82%-96% at 96 h after post treatment with *Xenorhabdus indica* [29].

Sadekuzzaman *et al.* [30] reported that nodule formation is suppressed by RNA interference against *S. exigua* PLA₂ gene (*SeiPLA₂*) expression, supporting the suggestion that immunosuppression by inhibition of PLA₂ activity is the primary factor in the pathogenesis of *Xenorhabdus/Photorhabdus*. Various secondary metabolites of *Photorhabdus* and *Xenorhabdus* have been identified and proposed as virulence factors from their bacterial genomes. Shi and Bode [31] demonstrated a correlation between bacterial virulence and the intensity of immunosuppression by the bacteria. Immunosuppression is induced by the lack of eicosanoids due to inhibition of PLA₂ enzyme activity during *Xenorhabdus* and *Photorhabdus*.

The virulence of nine *Xenorhabdus bovienii* strains in *Galleria mellonella* and *Spodoptera littoralis* were characterized by comparative

genomic analysis and found that two strains were highly virulent and three expressed attenuated insect virulence in tested insect hosts. They speculated that the type VI secretion system (T6SS) in *X. bovienii* may be another addition to the armory of antibacterial mechanisms expressed by these bacteria in insect host by outcompeting the microbial competitors [32].

4. CONCLUSION

The present investigation revealed the role of symbiotic bacteria of EPNs in biocontrol of one of the major insect pest *Spodoptera litura*. Both *Photorhabdus* and *Xenorhabdus* showed mortality against the tobacco caterpillar when treated with both intact cell culture and cell-free supernatant contain toxic metabolites that are lethal. Among the five isolates used in the study, EPB3 (*Xenorhabdus nematophila*) was virulent compared to the other bacterial isolates with lowest LC₅₀ and LT₅₀ values followed by EPB1 (*Photorhabdus luminescence*). Further the isolates were screened for biocontrol efficiency under greenhouse and field conditions to develop a potential biopesticides against the insect pests of agricultural and horticultural crops.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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