Research Article

# *In-Vitro* Pathogenicity of Entomopathogenic Nematodes Associated Symbiotic Bacteria and their Metabolites against Armyworm *Spodoptera litura* Fabricius

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**Abstract** | Entomopathogenic nematodes (EPNs) are effective biocontrol agents against different insect pests. However, very little work has been done in Pakistan on the symbiotic bacteria associated with EPNs and their metabolites. In this laboratory study, two bacterial isolates *i.e. Xenorhabdus* spp. associated with EPN *Steinernema glaseri* (Steiner) (Steinernematidae) and *Photorhabdus* spp. associated with EPN *Heterorhabditis bacteriophora* (Poinar) (Heterorhabditidae), were evaluated against armyworm *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) which is a destructive lepidopterous pest. These EPNs associated bacterial isolates were applied @ $4\times10^7$ CFUs/mL against different larval instars of *S. litura*. At day one post-treatment, maximum larval mortality (59.40%) was exhibited by *Xenorhabdus* spp. against 2nd instar larvae followed by 52.80,46.20 and 46.20% mortality of 5th, 3rd and 4th instar larvae, respectively. In case of *Photorhabdus* spp., maximum mortality (26.40%) was observed against 2nd instar larvae. In other bioassay, 40% suspensions of bacterial metabolites were evaluated against different larval instars of *S. litura* and were found significantly effective against all larval instars. Maximum average mortality (52.80%) was observed in *Xenorhabdus* spp. derived metabolites against 2nd instar larvae as compared to *Photorhabdus* spp. In both bioassays, mortality was increased along with the exposure time and reached 100% in all *S. litura* larval instars at fourth day post-treatment. Findings of this *in-vitro* study demonstrate that EPNs and their symbiotic bacterial isolates would be effective biorational control tools against *S. litura*.

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## Introduction

Armyworm *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) is one of the major insect pests attacking agricultural and horticultural crops worldwide including Pakistan. Due to its worldwide distribution and polyphagous nature, it is considered as the most devastating and economically important pest with a host range of more than 300 plant species (Qin *et al*., 2004; Abdullah *et al*., 2019). In Pakistan, *S. litura* infestations cause substantial losses to a wide array of agricultural, fruit and vegetable crops (Abdullah *et al*., 2019).

For many decades, synthetic insecticides are being employed as the primary tool for the management of insect pests including *S. litura* (Rehan *et al*., 2011). Extensive and recurrent use of synthetic insecticides has led to many ecological problems including environmental contaminations, eradication of beneficial fauna and human health hazards (Khan and Ahmad, 2019; Gomes *et al*., 2020). Moreover, many field populations of *S. litura* all over the world including Pakistan have attained resistance against the synthetic insecticides of almost all modes of actions or classes (Tong *et al*., 2013; Saleem *et al*., 2016).

This situation necessitates looking for alternate biorational pest control strategies such as biological control using different entomopathogenic microbes (Thakur *et al*., 2021; Ahmad *et al*., 2022). Many species and strains of entomopathogenic fungi, viruses and nematodes have been demonstrated effective against *S. litura* and other insect pests (Acharya *et al*., 2020; Ahmad *et al*., 2022; Batool *et al*., 2022). Entomopathogenic nematodes (EPNs) represent important biological control agents against insect pests. EPNs have the remarkable ability to search, locate and kill their hosts within few days and they can also penetrate the bark and pupation chambers (Brixey, 2000; Bhat *et al*., 2020). EPNs belonging to families Steinernematidae and Heterorhabditidae exhibit a mutualistic symbiotic relationship with bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively. These EPNs associated bacteria and their metabolites are highly virulent and pathogenic to a wide range of lepidopterous and coleopterous pests including *S. litura* (Mahar *et al*., 2004; Bish *et al*., 2015; Abbas *et al*., 2022; Ahmad *et al*., 2022; Tomar *et al*., 2022).

In Pakistan, very little work has been done so far on the characterization and evaluation of pathogenicity potential of bacteria associated with EPNs and their metabolites against different insect pests. Therefore, this laboratory work was aimed to evaluate the pathogenicity of two bacterial isolates, *i.e. Xenorhabdus* spp. associated with EPN *Steinernema glaseri* (Steiner) (Steinernematidae) and *Photorhabdus* spp. associated with EPN *Heterorhabditis bacteriophora* (Poinar) (Heterorhabditidae), against different larval instars of *S. litura*.

## Materials and Methods

*In-vivo production of EPNs using wax moth Galleria mellonella*

Both EPN species used in the study were reared on the late instar larvae of wax moth *G. mellonella*. The basic methods described by Poinar (1979) and summarized by Woodring and Kaya (1988) were employed by slight modifications for this *in-vivo* production of EPNs. Moth larvae were reared at controlled temperature of 15°C and remained viable for a period of 30 days. Pupating or dead larvae were being constantly removed during the storage.

*Rearing of wax moth*

Bee hives infested with *G. mellonella* were collected from the farm area of the University of Agriculture, Faisalabad. *G. mellonella* last instar larvae were separated for nematode culture. Only small sized larvae were left for moth emergence and egg-laying. Modified artificial diet was prepared by mixing rice, wheat, oat and maize porridge (20 g), yeast granules (50 g) in solution of 80 mL warm honey and (100 g) glycerol (Khan *et al*. 2016). This diet was provided to *G. mellonella* larvae incubated at 25°C. Larvae were taken out from the diet when they reached last instar stage and were used for storage and nematode isolation/multiplication. Wax moths laid eggs on hard folded paper sheets placed in the box where wax moth adults had already been shifted. Eggs were then collected and shifted on artificial diet.

*Extraction and storage of EPNs*

EPNs were isolated from the dead larvae of *G. mellonella* using modified White trap (White, 1927). Alive, putrid smelling and black colored (indication of contamination) larvae were discarded. Modified White trap for the collection of EPNs was comprised of a clear plastic container (9 cm diameter × 4.5

cm deep). An inverted Petri-dish (5.0 cm diameter × 3.5 cm deep) was placed in the container. The container was filled with sterilized distilled water up to a depth of 1.0 cm. A sheet of filter paper was placed on Petri-dish in such a way that the edge of the filter paper came in contact with water. Then dead larvae (2–4 per trap) were placed on the filter paper kept on Petri-dish and plastic container was closed with the lid followed by incubation at 20–27 ºC depending upon nematode species until infective juveniles (IJs) started to leave the cadaver 8 to 20 days after infection. IJs moving down through the filter paper into the water were harvested on daily basis until there was no recovery from cadaver. All IJs were collected and the container was rinsed and refilled with sterilized distilled water for next harvest. Water containing IJs was transferred to 100 mL beaker. Water was diluted by filling the distilled water up to the top of beaker to get clear suspension. Nematodes were then allowed to settle for about 30 min followed by siphoning of supernatant. Beakers were refilled with sterilized distilled water and this process was repeated 3–4 times till the suspensions became clear. Then nematodes were collected in clean transparent plastic pots. Date of harvesting of nematode species was recorded and IJs stored at about 10–15ºC. Dead larvae were identified on the basis of color development. Larvae infected by *Steinernema* exhibited grey coloration and by *Heterorhabditis* genus caused brick red coloration of dead larvae (Tabassum *et al*., 2005). Nematode suspensions were kept in clear shallow plastic containers provided with lids by diluting up to approximately 5,000/mL at a depth of 1.0 cm to ensure sufficient availability of oxygen. EPNs were stored at 10–15˚C. Nematodes produced *in-vivo* could be stored for three months. All nematode cultures were re-cultured after every 4 months constantly. The viability of nematodes was constantly assessed prior to the execution of experimentations. Air was provided by aerator being used in fish aquarium tanks to maintain availability of oxygen to the nematodes. Only freshly emerged EPNs (less than 2 weeks old) were used in experimentation.

*Isolation and multiplication of bacteria (Xenorhabdus spp. and Photorhabdus spp.)*

For this purpose, nutrient agar (NA), bromothymol blue and distilled water were mixed @ 37 g, 25 mg and 1000 mL, respectively in a media bottle and the mixture was autoclaved for 30 min at 120 lb pressure. The bottle was removed and 4 mL of 1.0% 2,3,5 triphenyl-tetrazolium chloride was added at 45˚C under sterile conditions. Blue black media was then distributed into Petri-dishes, followed by cooling and storage in a fridge at 4˚C. The media was cooled and used for multiplication of bacteria.

Isolation of *Photorhabdus* spp. and *Xenorhabdus* spp. was carried out using the methods described by Akhurst (1983). In brief, four last instar larvae of *G. mellonella* were infected with IJs of *S. glaseri, H. bacteriophora* and *H. indica* separately. The infected cadavers of *G. mellonella* were surface sterilized in 70% alcohol for 5 min after 48 h and were left to dry on a laminar flow bench. Cadavers were opened by the help of sterilized needles and care was taken to prevent any damage to gut epithelium. A droplet of the oozing haemolymph was taken using an inoculating needle and smeared onto NBTA (NA + 0.00 25% bromothymol blue + 0.00 4% triphenyl-tetrazolium chloride (Akhurst, 1980) agar. The plates were wrapped with Paraffin tape followed by incubation at 28˚C in dark conditions for 36 to 48 h. Single colonies were picked and smeared on NBTA agar until uniform colonies were obtained. To test the purity, the bacteria was inoculated and isolated from *G. mellonella* in the Koch's postulates.

Taxonomic test was conducted by maintaining the primary form of the symbiotic bacteria from these cultures by streaking on NA and NBTA (Akhurst, 1980). The Primary and secondary forms were identified on the basis of morphological characters and color development on NBTA. For taxonomic purpose only primary form of bacteria is utilized and the stock cultures of the bacteria were maintained on yeast extract/salts (YS) agar (Dye, 1968) at 12˚C and further sub-culturing was carried out on monthly basis. Gram staining was carried out by using 24 h old cultures and assessed for motility by microscopic examination. Bio-luminescence was assessed quantitatively by keeping under observation in a dark room for 10 min. Pathogenicity of lepidoptera was assessed by injecting 5 days old bacterial cultures (on NA) into the haemocoel of *G. mellonella* larvae under sterile conditions. A total of 100 cells suspended in normal saline were injected in each larva. Mortality rate was recorded after 24–48 h at 23˚C. Pure colonies of the bacteria *Photorhabdus* spp. and *Xenorhabdus* spp. were added to nutrient broth No. 2 and were kept in a mechanical shaker for 24–48 h at 28˚C and 150 rpm in the dark.

*Preparation and storage of cell and cell-free suspensions of bacteria*

Cell suspensions were multiplied in nutrient broth for 24–48 h and were calibrated on electro-photometer to 0.48 representing $4.0\times10^7$ cells/mL. The calibrated cell suspension was centrifuged at 2500 for 15 min to obtain cell free toxins. A pellet of cells was formed under the centrifuge bottle and the supernatant was filtered through a filter paper of 0.2 µm pore size using a syringe. It was labeled as 'S'. Subsequent dilutions (1.0, 10, 20 and 40%) were made by adding water to S.

*Storage of cells and metabolites from the bacteria*

Fresh bacterial cell culture (not older than 48 h) was used in all experiments. However, *Photorhabdus* spp. and *Xenorhabdus* spp. were stored over several months in order to study shelf life. Cells and their corresponding cell-free toxins adjusted to $4\times10^7$cells/mL and its dilutions were stored at cool temperature either in a laboratory bench or in a fridge.

*Target insect collection and rearing*

Egg masses of the target insect *S. litura* were collected from the field and hatched larvae were reared on cabbage leaves in plastic pots fitted with muslin net.

*Effect of isolated bacteria on larvae of S. litura*

Two bacterial isolates (*Xenorhabdus* and *Photorhabdus*) were used against different larval instars ($2^{nd}$, $3^{rd}$, $4^{th}$ and $5^{th}$) of *S. litura* at $4\times10^7$ concentration. Each treatment was replicated five times with three larvae in each Petri-dish (9 cm dia.). Data was recorded on mortality and was subjected to statistical analysis at 95% level of significance. Experiment was repeated twice.

*Effect of isolated bacterial metabolites on larvae of S. litura*

Bacterial metabolites isolates were used against different larval instars ($2^{nd}$, $3^{rd}$, $4^{th}$ and $5^{th}$) of *S. litura* at 40% concentration selected from the previous experiment. Each treatment was replicated five times with three larvae in each Petri-dish (9 cm dia.). Data were recorded on the larval mortality and were subjected to statistical analysis at 95% level of significance. Experiment was repeated twice.

## Results and Discussion

*Pathogenicity of isolated bacteria on different larval instars of S. litura*

Different concentrations of bacterial cells were significantly effective against all larval instars. Mortality was reached up to 100% in all larval instars of *S. litura* upon treatment with bacterial cell suspensions in 4 days. Highest morality (up to 59.40%) was found in case of *Xenorhabdus* spp. against $2^{nd}$ instar as compared to *Photorhabdus* spp. that caused 26.40% larval morality after $1^{st}$ day. Mortality was insignificant in $3^{rd}$ and $4^{th}$ larval instars (46.20%). In $5^{th}$ larval instar mortality was 52.80 and 19.80% in *Xenorhabdus* and *Photorhabdus* spp., respectively. Mortality was increased with the passage of time reaching up to 100% at $4^{th}$ day of bioassay. Minimum mortality was found in *Photorhabdus* spp. against $3^{rd}$ and $4^{th}$ larval instars (Table 1).

**Table 1:** *Effect of pathogenic bacteria isolated from EPNs on different larval instars of Spodoptera litura under laboratory conditions.*

| Bacteria | Larval instars | Larval mortality (%) | | | |
|---|---|---|---|---|---|
| | | After $1^{st}$ day | After $2^{nd}$ day | After $3^{rd}$ day | After $4^{th}$ day |
| *Xenorhabdus* | $2^{nd}$ | 59.40 $^{cd}$ | 100.00 $^{a}$ | 100.00 $^{a}$ | 100.00 $^{a}$ |
| | $3^{rd}$ | 46.20 $^{de}$ | 86.40 $^{ab}$ | 100.00 $^{a}$ | 100.00 $^{a}$ |
| | $4^{th}$ | 46.20 $^{de}$ | 86.40 $^{ab}$ | 100.00 $^{a}$ | 100.00 $^{a}$ |
| | $5^{th}$ | 52.80 $^{de}$ | 93.20 $^{a}$ | 100.00 $^{a}$ | 100.00 $^{a}$ |
| *Photorhabdus* | $2^{nd}$ | 26.40 $^{fg}$ | 52.80 $^{de}$ | 93.20 $^{a}$ | 100.00 $^{a}$ |
| | $3^{rd}$ | 6.60 $^{h}$ | 46.20 $^{de}$ | 72.80 $^{bc}$ | 100.00 $^{a}$ |
| | $4^{th}$ | 13.20 $^{gh}$ | 39.60 $^{ef}$ | 86.40 $^{ab}$ | 100.00 $^{a}$ |
| | $5^{th}$ | 19.80 $^{gh}$ | 52.80 $^{de}$ | 100.00 $^{a}$ | 100.00 $^{a}$ |
| Control | $2^{nd}$ | 0.00 $^{i}$ | 0.00 $^{i}$ | 0.00 $^{i}$ | 0.00 $^{i}$ |
| | $3^{rd}$ | 0.00 $^{i}$ | 0.00 $^{i}$ | 0.00 $^{i}$ | 0.00 $^{i}$ |
| | $4^{th}$ | 0.00 $^{i}$ | 0.00 $^{i}$ | 0.00 $^{i}$ | 0.00 $^{i}$ |
| | $5^{th}$ | 0.00 $^{i}$ | 0.00 $^{i}$ | 0.00 $^{i}$ | 0.00 $^{i}$ |
| LSD | 15.98 | | | | |

** = Means followed by the same letter are not significant from each other at P ≤ 0.05 according to least significant difference test.*

*Effect of isolated bacterial metabolites on different larval instars of S. litura*

Bacterial metabolites were significantly effective against all larval instars. Mortality was found to be 100% in all *S. litura* larval instars when treated with metabolites up to 4 days. Maximum mortality was observed in metabolites which were isolated from *Xenorhabdus* spp. against $2^{nd}$ larval instar which was 52.80% as compared to *Photorhabdus* spp. which was 26.40% after $1^{st}$ day. Mortality increased as time increased and reached up to 100%. Minimum mortality was observed in *Photorhabdus* spp. against $3^{rd}$ and $4^{th}$ larval instars (Table 2).

**Table 2:** *Effect of pathogenic bacterial isolates derived from EPNs on different larval instars of Spodoptera litura under laboratory conditions.*

| Bacteria | Larval instars | Larval mortality (%) | | | |
|---|---|---|---|---|---|
| | | After 1st day | After 2nd day | After 3rd day | After 4th day |
| *Xenorhabdus* | 2nd | 52.80 ef | 86.40 abc | 100.00 a | 100.00 a |
| | 3rd | 46.20 fg | 79.60 bcd | 100.00 a | 100.00 a |
| | 4th | 39.60 fgh | 72.80 cd | 100.00 a | 100.00 a |
| | 5th | 33.00 ghi | 66.00 de | 100.00 a | 100.00 a |
| *Photorhabdus* | 2nd | 26.40 hij | 52.80 ef | 100.00 a | 100.00 a |
| | 3rd | 19.80 ijk | 52.80 ef | 86.40 abc | 100.00 a |
| | 4th | 13.20 jk | 46.20 fg | 72.80 cd | 100.00 a |
| | 5th | 6.60 k | 39.60 fgh | 93.20 ab | 100.00 a |
| Control | 2nd | 0.00 l | 0.00 l | 0.00 l | 0.00 l |
| | 3rd | 0.00 l | 0.00 l | 0.00 l | 0.00 l |
| | 4th | 0.00 l | 0.00 l | 0.00 l | 0.00 l |
| | 5th | 0.00 l | 0.00 l | 0.00 l | 0.00 l |
| LSD | 15.47 | | | | |

** = Means followed by the same letter are not significant from each other at P ≤ 0.05 according to least significant difference test.*

The pathogenicity potential of symbiotic bacterial was evaluated in this study against larvae of *S. litura*. Result showed that bacteria *Xenorhabdus* spp. caused highest morality (up to 100 %) in all larval instars of *S. litura* upon treatment with bacterial suspension in 4 days bioassay. It is due to the reason that the bacteria incite damage to the haemocytes of lepidoptera leading disintegration of the fat body which is the main source of antimicrobial peptides (Dunphy and Bourchier, 1992; Dunphy, 1995; Bauer *et al*., 1998; Kenney *et al*. 2019). Similar trend was observed by Mahar *et al*. (2004) in diamondback moth (*Plutella xylostella*) by treating its different larval instars with broth cell and cell-free metabolites of *X. nematophila* isolated from EPN *S. carpocapsae*.

Cells of EPN *S. carpocapsae* showed pathogenic abilities against a wide range of potentially important insect pests due to symbiotic bacterium, *X. nematophila* (Poinar, 1979; Strauch and Ehlers, 1998; Vicente-Díez *et al*. 2021). Many studies have been conducted to study the effect of *Xenorhabdus* and *Photorhabdus* bacteria on the insect immunity system of lepidoptera, particularly of *G. mellonella* (Dunphy and Thurston, 1990; Forst *et al*., 1997; Salgado-Morales *et al*., 2019; Elbrense *et al*., 2021). The bacteria proliferate and kill the host within hours (Dunphy and Thurston, 1990; Dunphy and Bourchier, 1992). An outstanding characteristic of *Xenorhabdus* is its ability to counteract the insect immune system (Nielsen-LeRoux *et al*., 2012; Darsouei *et al*., 2019).

In the second bioassay, biocontrol potential of EPN-derived bacterial metabolites was evaluated against different larval instars of *S. litura*. Metabolites were isolated from both species of bacteria. Maximum mortality was observed in 2nd instar larvae. Metabolites isolated from *X. nematophila* were reported by many researchers as effective control means against many insect pests (Ensign *et al*., 2002; Mahar *et al*., 2004; Jan *et al*., 2008; Cevizci *et al*., 2020). The crystal proteins produced by *Bacillus thuringiensis* and *B. sphaericus*, the VIP toxins of *B. thuringiensis* and cholesterol oxidase of *Streptomyces* spp. are effective bacterial proteins all of which cause lysis of the midgut epithelium of the insect hosts (Bowen *et al*., 1998). Similar activities have been reported by *X. nematophila* and *P. luminescens*. The symbiotic bacteria from the 3rd stage IJs of EPNs secrete a wide variety of toxic metabolites which can be applied as insecticides (Ensign *et al*., 2002). Similar results were recorded by Jan *et al*. (2008) who demonstrated that the toxins and metabolites of bacterial symbionts (*X. nematophila*) isolated from EPN *S. carpocapsae* were found very effective against pupae of different insects.

## Conclusions and Recommendations

Overall study results demonstrate the effectiveness of bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. associated symbiotically with EPNs *H. bacteriophora* and *S. glaseri* and their metabolites against different larval instars of armyworm *S. litura*. Hence, these are recommended to be considered for the future development of microbially-derived biopesticidal tools for the management of *S. litura*.

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## Novelty Statement

This laboratory study proved the insecticidal potential of bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp.) and their metabolites associated with entomopathogenic nematodes *Heterorhabditis*

*bacteriophora* and *Steinernema glaseri* against the larvae of armyworm *Spodoptera litura* under laboratory conditions.

## Auhor's Contribution

**Hina Safdar**: Performed experiments, took data and wrote the first draft.
**Nazir Javed and Sajid Aleem Khan**: Planned and supervised the research project.
**Muhammad Zeeshan Majeed**: Performed statistical analysis and prepared results.
**Arif Mehmood**: Technically proofread the manuscript.
**Muhammad Arshad**: Provided technical suport and proofread the manuscript.

*Conflict of interest*
The authors have declared no conflict of interest.

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