

Original Research Article

<https://doi.org/10.20546/ijcmas.2020.902.335>

Qualitative Toxicity Bioassay of Bacteria *Photorhabdus* Associated Symbiotically with Entomopathogenic Nematode *Heterorhabditis* against *Galleria mellonella*

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ABSTRACT

The bacteria *Photorhabdus* associated symbiotically with the entomopathogenic nematodes of genus *Heterorhabditis* is an effective killer of insect-pests belonging to a different order. The bacteria *Photorhabdus* encodes numerous toxins once inside the midgut of insects and cause rapid death by employing multiple toxicity mechanism. In this paper, we isolated the bacteria *Photorhabdus* associated with *Heterorhabditis* nematodes using a modified White trap and demonstrated a bioassay of toxicity against last instar larva of wax moth *Galleria mellonella*. The larva was injected with 25µl bacterial suspension inside the midgut by micro syringe at undiluted and diluted concentration. The bacteria was found quick in killing the larva with a medium lethal time 12hr post-injection at both of the concentration. The dead larva turned brick-red upon infestation by bacteria *Photorhabdus*. The bacteria perform three major tasks once inside the insect body, which includes the production of the digestive enzyme to convert the insect's tissue into a suitable growth medium. Later bacteria encode multiple toxin protein to kills it hosts and finally release several secondary antibiotic compounds to prevent the growth of any other microbe on the dead cadaver. The full potential of bacteria in crop protection schemes against insect-pests of crops is yet to be realized. The bacteria *Photorhabdus* have the full potential to be utilized as an alternative of already existing Bt crops once the biological pathways and genes involved in toxicity are understood.

Keywords

Entomopathogenic nematode, *Heterorhabditis*, *Photorhabdus*, *Galleria mellonella*, Bioassay

Article Info

Accepted:

20 January 2020

Available Online:

10 February 2020

Introduction

The biotic stress factors, particularly insect-pests, cause huge economic losses to the farmers. The annual crop losses due to the damaging insect-pests are predicted around 10.8% globally (Dhaliwal *et al.*, 2015). In India alone, crop yield losses are estimated at around 15.7% annually due to insect-pests.

(Dhaliwal *et al.*, 2015) To overcome the threats caused by insects to the crops, numerous cost-effective management tactics including the uses of pesticides have been employed in crop protection schemes. The excess uses of synthetic pesticides are led to environmental contamination and posing negative impacts upon aquatic and terrestrial biodiversity. In the modern-day agriculture

practices, the eco-safety and human health is the primer concern for crop protectionist to devise the alternative way for insect pest management. The uses of entomopathogenic nematodes provide an alternative solution to keep the insect-pests of crops below the economic threshold level.

These nematodes penetrate insects and employ a synergistic mechanism along with its mutualistic bacteria to kill insects by producing multiple toxin components. The major two genera of entomopathogenic nematodes namely *Heterorhabditis* and *Steinernema* carry mutualistic bacteria inside the anterior part of their alimentary canal. The bacteria *Photorhabdus* is associated with nematode *Heterorhabditis* (Boemare, 2002) and bacteria *Xenorhabdus* is associated with the nematode *Steinernema* (Bird and Akhurst, 1983).

The bacteria is regurgitated by the infective juveniles of these entomopathogenic nematodes inside the midgut of insect-pests. The particular bacteria *Photorhabdus* kills its host by employing multiple toxicity mechanisms to kill the insects. Unlike *Bt* toxin which is species-specific, the toxins of bacteria *Photorhabdus* exhibit broad-spectrum activities and kill insects belonging to different orders. The toxins produced by the bacteria *Photorhabdus* include Tc toxin, Pir toxin, Patox toxin, Pit toxins, Photox toxin, Pvc toxin, Pax toxins, and Mcf toxin, etc.

This array of toxins produced by the bacteria *Photorhabdus* helps in killing insect-pests quickly. There is huge potential lies with the application of this bacteria *Photorhabdus* against insect-pests of the crop as a whole, by mass culturing to isolate its toxins or by genetic insertion of the crop with *Photorhabdus* toxin genes against target insect-pests of economically valuable crops.

Materials and Methods

Collection of soil samples

The random sampling procedure was adopted to collect the soil samples from a different location around the NCR areas. The chances of getting nematode in a soil sample are below 30 percent. The soil sample was stored in the polythene bag and kept in the laboratory at 15°C temperature.

Isolation of entomopathogenic nematodes

The soil samples were processed to isolate entomopathogenic nematodes by insect baiting technique. Under this method, 5 to 6 last instar larva were added in 250ml capacity plastic containers filled with collected soil. Upon infectivity, by entomopathogenic nematodes, the dead cadaver turned to brick red, which indicates the presence of *Heterorhabditis* nematode.

Recovery of entomopathogenic nematodes from infected dead cadavers

The nematodes were recovered from the dead cadaver of *Galleria* by the modified White trap method. The nematodes begin to emerge from the dead cadaver after 8 to 1 days and migrate to the water containing the outer petri dish. The collected nematodes were stored in the tissue culture flask and incubated at 15°C temperature.

Sterilization of recovered entomopathogenic nematodes

To prevent any contamination of other microbes on the surface of collected nematodes, nematodes were sterilized. The nematodes suspension was filled in the 1ml Eppendorf tubes. The tubes were centrifuged at 10000rpm for 5 minutes to allow the settling of nematodes at the bottom of the

tube. The excess of supernatant was discarded, the pellet was dissolved in Ringer's solution and centrifuged at 10000 rpm for 5 minutes. The supernatant was discarded and the pellet was washed with 1 percent bleach to remove contaminants and centrifuged again. The sterilized nematode's pellet was rinsed with water for four to five times to remove the excess of bleach.

Isolation of and maintenance of bacteria *Photorhabdus* from infective juveniles

The sterilized infective juveniles were taken in a 1.5ml Eppendorf tube. The nematodes were crushed with a motorized tissue grinder until the formation of whitish suspension. The suspension of crush nematodes was streaked on the NBTA media plates for the growth of bacteria. The streaked plates of NBTA media were incubated at 28°C temperature.

Preparation of liquid culture of bacteria *Photorhabdus*

The liquid culture of bacteria was prepared by using Luria-Bertani broth medium. A single colony of bacterial culture was transferred into a 25 ml Falcon tube filled with 5ml of LB media. The Falcon tubes were incubated at 28°C for one day to allow the growth and multiplication of bacteria. The whole setup of toxicity bioassay experiment is visually represented in the figure-1.

Results and Discussion

Confirmation of the entomopathogenic nematode *Heterorhabditis*

The identity of isolated entomopathogenic nematodes by the modified White trap method was confirmed by observing the qualitative characteristics. Upon infestation, by entomopathogenic nematodes, the dead cadaver turned reddish in color post 48hr

incubation period. The body of the dead cadaver was intact and did not develop any putrefying odor. The characteristic brick red color is associated with the infestation of *Heterorhabditis* nematodes. The mutualistic bacteria associated with this nematodes encodes several antibiotic compounds, which prevent the attack of any other secondary microbes on dead cadaver which is the main reason for the absence of putrefying odor from a dead cadaver.

Confirmation of the entomopathogenic bacteria *Photorhabdus*

The NBTA plates were incubated at 28°C temperature which is an optimum growth temperature for the multiplication of bacteria *Photorhabdus*. Further, the identity of bacteria was confirmed by observing the changes in the color of NBTA media. In general, the color of NBTA media turns green or brownish green upon the consumption of medial components by bacteria. *Photorhabdus* produces multiple antibiotics, therefore, in general, it does not allow the growth or multiplication of any other microbes on the plates. The other characteristic feature associated with the bacteria *Photorhabdus* is the presence of bioluminescence which validates the true identity of these bacteria.

Toxicity assay of bacteria *Photorhabdus* against the 4th instar larva of *Galleria*

The toxicity of bacteria *Photorhabdus* were tested against the 4th instar larva of wax moth *Galleria mellonella*. The larva was injected with 25µl bacterial suspension through the last pair of thoracic legs by using a microsyringe. The larva was injected at undiluted and 10-1 dilution concentration. The injection of 25 µl of Luria broth to the larva was taken as a control. The experiment was done with 5 replicates and 3 repetitions.

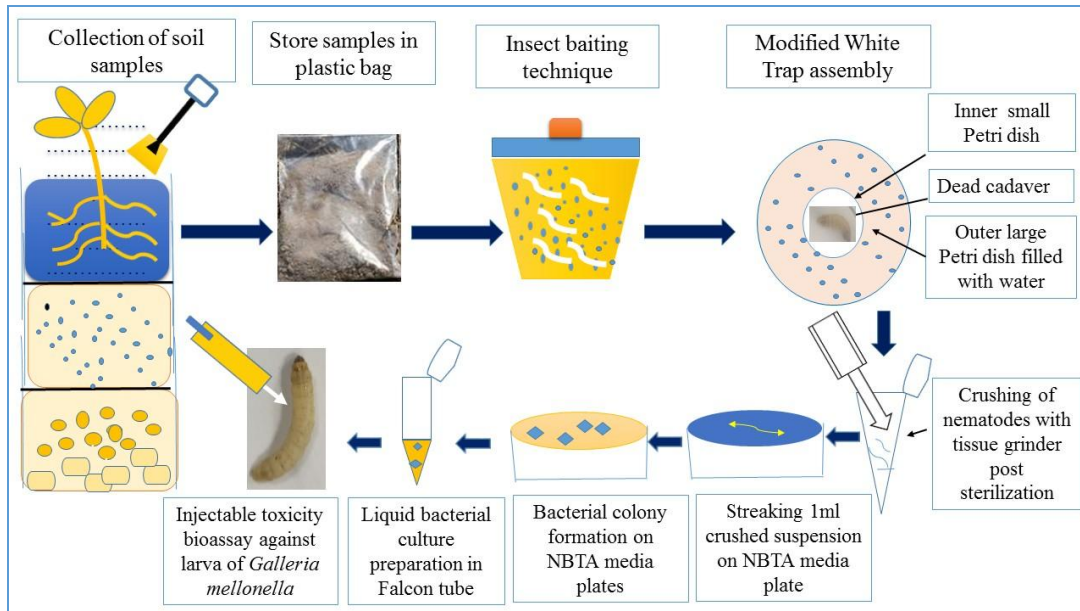


Figure.1 Visual experimentation setup for isolation of bacteria *Photorhabdus* and toxicity bioassay against *Galleria mellonella*

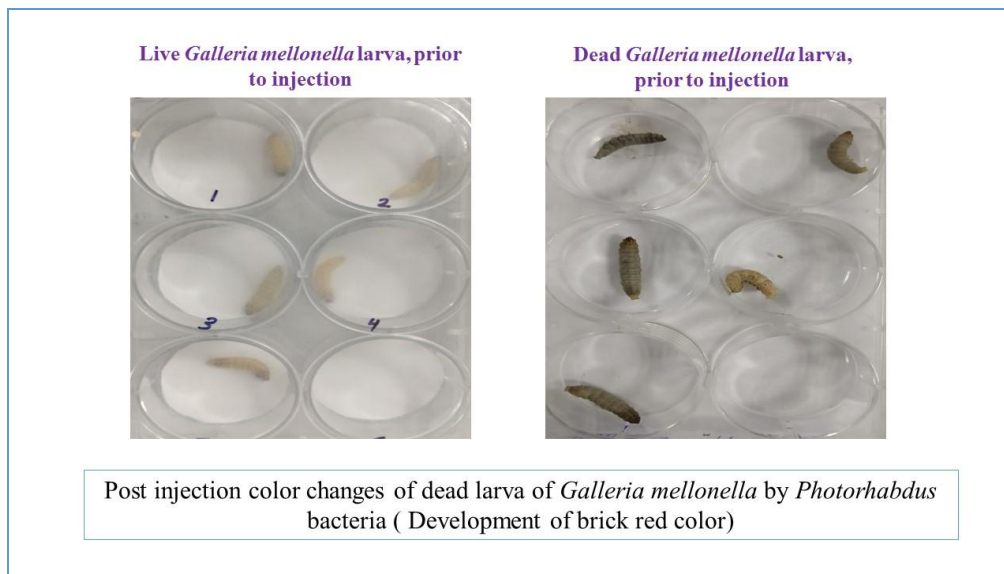


Figure.2 Injectable toxicity bioassay of bacteria *Photorhabdus* toxicity against *Galleria mellonella*

All the larva died 12hr post-infection on both the undiluted and undiluted concentration and no mortality was observed in control. The lethal time to kill the larva was 12h in both cases. In the figure-2, the color changes upon infection by bacteria *Photorhabdus* to larva of *Galleria mellonella* is depicted.

The scientific communities engaged in crop protection researches are strongly advocating the adaptation of alternative techniques then insecticides to manage insect-pests of crops. The aquatic and terrestrial fauna is under threat due to the excess uses of synthetic insecticides. Bio control management tactics

are needed to be identified and popularize to manage insect-pests.

Earlier Bt crops have proven their efficacy in the field against insects, but Bt toxins are species-specific and are reported to cause resurgence and resistant problems in insects. Alternatively the bacteria *Photorhabdus* and *Xenorhabdus* is found more virulent against insects and employs multiple toxicity mechanism to kill insects. In both of these entomopathogenic bacteria, *Photorhabdus* is found to encode multiple arrays of toxin then *Xenorhabdus* and its mutualistic *Heterorhabditis* are well adapted to tropical and subtropical parts of the world.

The bacteria *Photorhabdus* can be used against insects-pests of crops in two ways, either by mass culturing of bacteria to produce toxins or by genetic modification of crops to insert toxin genes against targeted insect-pests. The dedicated research work is required to understand the biological pathways involved in the toxicity mechanism played by bacteria *Photorhabdus*. Alteration of these pathways makes it efficient to the produced commercial formulation and helps in producing transgenic plants against insect-pests of economically valuable crops.

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How to cite this article:

Amit Ahuja, Ajay Singh Sindhu and Jagdish Yadav. 2020. Qualitative Toxicity Bioassay of Bacteria *Photorhabdus* Associated Symbiotically with Entomopathogenic Nematode *Heterorhabditis* against *Galleria mellonella*. *Int.J.Curr.Microbiol.App.Sci*. 9(02): 2942-2947. doi: <https://doi.org/10.20546/ijcmas.2020.902.335>