Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2020; 8(1): 1490-1493
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Received: 18-11-2019
Accepted: 22-12-2019

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Bioassay of infectivity of entomopathogenic nematode *Heterorhabditis* against *Galleria mellonella*

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Abstract
Entomopathogenic nematodes belonging to the families Steinernematidae and Heterorhabditidae are the natural killers of insect-pests of economically important crops. Entomopathogenic nematodes have a mutualistic association with bacteria and together they employ a synergistic mechanism to kill insect hosts by producing multiple toxins. In this paper, we have explained the protocol for the isolation of entomopathogenic nematodes from the soil and validated their infectivity against the larva of *Galleria mellonella*. Under this experimentation, an insect baiting technique was used to attract infective juveniles from the soil. The new progeny of nematodes were extracted by using the modified White trap methods. The infectivity was accessed by releasing five to six infective juveniles per larva kept in a small petri dish. The role of *Heterorhabditis* in infectivity was determined by brick red color development of dead larva, absence of putrefying odor and intactness of dead cadaver. Artificially improved strains of entomopathogenic nematodes can be included in crop protection after accessing their efficacy and compatibility with other components of integrated pest management tactics.

Keywords: Entomopathogenic nematode, *Heterorhabditis, Galleria mellonella*, bioassay

Introduction
Excess uses of insecticides are posing threat to the aquatic and terrestrial ecosystem (Pimentel, 1995) [1]. Entomopathogenic nematodes can be utilized as an effective alternative to these synthetic pesticides. The entomopathogenic nematodes of the genus *Heterorhabditis* (Poinar, 1976) [2], *Steinernema* (Travassos, 1927) [3] and *Neosteinernema* (Nguyen and Smart, 1994) [4] can kill insects belonging to different orders. Among these three genera, *Heterorhabditis* and *Steinernema* are efficiently exploited against insects as a biocontrol agent. The genus *Heterorhabditis* maintains mutualism with bacteria *Photorhabdus* and genus *Steinernema* is associated with the bacteria *Xenorhabdus* (Burnell and Stock, 2000) [5]. The bacteria secrets multiple insect-toxic protein toxins to kill the insects by causing septicemia. Upon reaching to the midgut of insects, these infective juveniles take two to three days to kill its host (Boemare *et al.*, 1996) [6]. These nematodes can be applied in the field with the traditional spraying equipment easily. These entomopathogenic nematodes have also found compatible with many commercial pesticides upon application in the field (Rovesti and Deseo, 1991) [7]. These nematodes are produced on its hosts via in vivo techniques or via in vitro techniques on solid or liquid culture media respectively (Ehlers *et al.*, 2001) [8]. Upon application, these nematodes face a number of the biotic and abiotic stresses in the field, which interfere with its proficient efficacy. The certain traits like heat tolerance, cold tolerance, sensation to ultraviolet light, persistence, desiccation, etc., determines its overall working fitness in the field (Gaugler,1988) [9]. In the past, artificial selection and hybridization tactics were employed in the trait improvement (Segal and Glazer, 1998) [10]. The major traits which affect the commercial production are related to host-seeking behavior and storage stability of entomopathogenic nematodes in a formulation. Once these traits are improved then the overall cost of production of a commercial formulation of entomopathogenic nematodes can be reduced. The most crop-damaging insect orders like Orthoptera, Coleoptera, Lepidoptera, and Hemiptera are the quick target of entomopathogenic nematodes (Gaugler, 2018) [11]. The potential of the utilization of entomopathogenic nematodes against the insect-pests of the various economically valuable crop is yet to be realized. The aim of the study to find the efficacy of entomopathogenic nematodes in causing toxicity against the insects.

Researches done in the past have proved the toxicity of *Heterorhabditis* nematodes against various insects belonging to different orders. The entomopathogenic nematodes belonging to a particular geographical region can manage or kills the insects effectively belonging to the same locality. In the future, these nematodes would be tested further for their infectivity against other important crop pests belonging to the same location.

Materials and Methods

Isolation of entomopathogenic nematodes from soil samples

The entomopathogenic nematodes were isolated by the bating technique using the last instar larva of wax moth *Galleria mellonella* as bait. The moist soil sample was collected from a depth of 10-15cm from the surface around the different location of NCR areas. The soil samples were cleaned to make it free from debris and stones. The moist soil samples were filled into 250ml a plastic container. Four to five Last instar larva of wax moth was released in the container. The container was covered with a punctured lid to facilitate smooth airflow inside the container. The plastic containers were incubated at room temperature in the laboratory. The regular observation was made to inspect the infectivity to larva by entomopathogenic nematodes. The dead cadavers were removed from the container after two to three days. The dead cadaver was of brick red color which indicates the infectivity of nematodes belonging to the genus *Heterorhabditis*.

Recovery of nematodes from dead cadavers by modified White Trap method

The entomopathogenic nematodes were isolated from the dead cadaver by the modified White trap method (Kaya and Stock 1997) [12]. In this method, a smaller Petri dish was kept inside a larger Petri dish. A circular strip of Whatman paper was laid inside the small Petri dish. The single dead cadaver was placed above the filter paper. The outer dish was filled with sterile water. The whole assembly of the White trap was incubated at room temperature. After the 7th day, the newly emerged infective juveniles were harvested by collecting water in a beaker from the outer Petri dish. The nematodes were allowed to settle down at the bottom beaker and excess of water was removed. The rinsed nematode was stored in a tissue culture flask of 250ml capacity and stored at 8 to 12^{o} C temperature in an incubator. The regular inspection was made to observe the life status of *Heterorhabditis* nematodes.

Results

Collection of soil sample by random sampling strategy

The soil samples were collected from different locations around the campus. The Dauer juveniles or infective juvenile is the only life-stage which can be isolated from the soil. As this stage is a non-feeding and wanders in the soil in search of an insect host. The appropriate sampling is an efficient task for the collection of entomopathogenic nematodes. The samples were collected in a polythene bag and kept moist and stored in the cool place as they need sufficient moisture for their activity and survival.

Entomopathogenic nematodes recovery from the collected soil samples

A sophisticated baiting technique procedure is followed to recover the entomopathogenic nematodes from soil samples. The soil samples were collected randomly from different locations and filled in a 250ml plastic container to perform bating tasks. In the soil, many other secondary pathogens are generally associated, which too can cause death to insects. The exact role of entomopathogenic nematodes in causing death to insects was determined by observing certain key features associated with these nematodes. Which includes the two to three days of infectivity period, absence of any putrefying odor and intactness of dead cadaver. Chromogenic changes of the cadaver upon infection determine the role of a particular genus of entomopathogenic nematodes. Here we got the brick red color of the dead cadaver, which determines the presence of *Heterorhabditis* nematodes. The whole setup of experiment is visually represented in the figure-1.

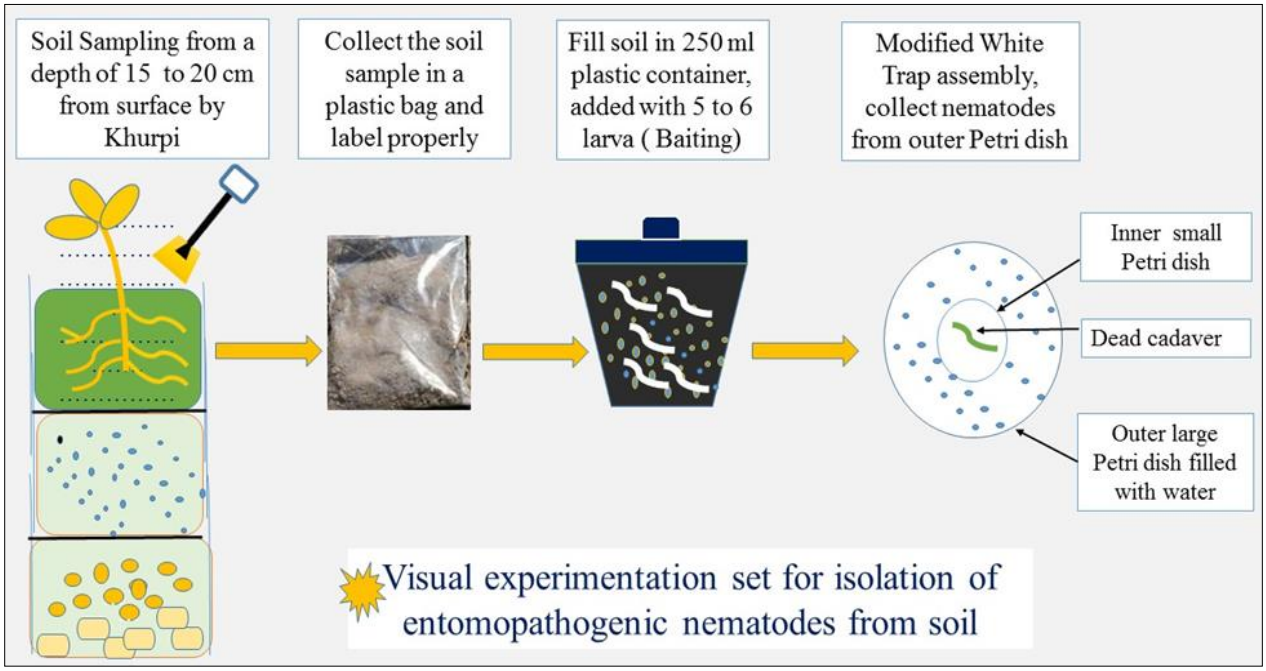

Fig 1: Visual representation of protocols adopted for isolation of entomopathogenic nematodes from soil

Bioassay of infectivity of entomopathogenic nematodes against test insect *Galleria mellonella*

To determine the infectivity of isolated infective juveniles, a bioassay against larva of wax moth was carried out. The fourth instar larva was kept inside a smaller petri dish and 5 to 6 freshly isolated infective juveniles were released. The nematodes caused death to larva within a period of 48 to 72 hours. The dead cadaver turned brick red which again validated the presence of *Heterorhabditis* nematodes. In our study, we have validated the infectivity of entomopathogenic nematodes by following standard protocols. The isolated infective juveniles were quick in killing and caused rapid mortality to the larva of wax moth *Galleria mellonella.* In the figure-2, the color changes upon infection by entomopathogenic nematodes to larva of *Galleria mellonella* is depicted.

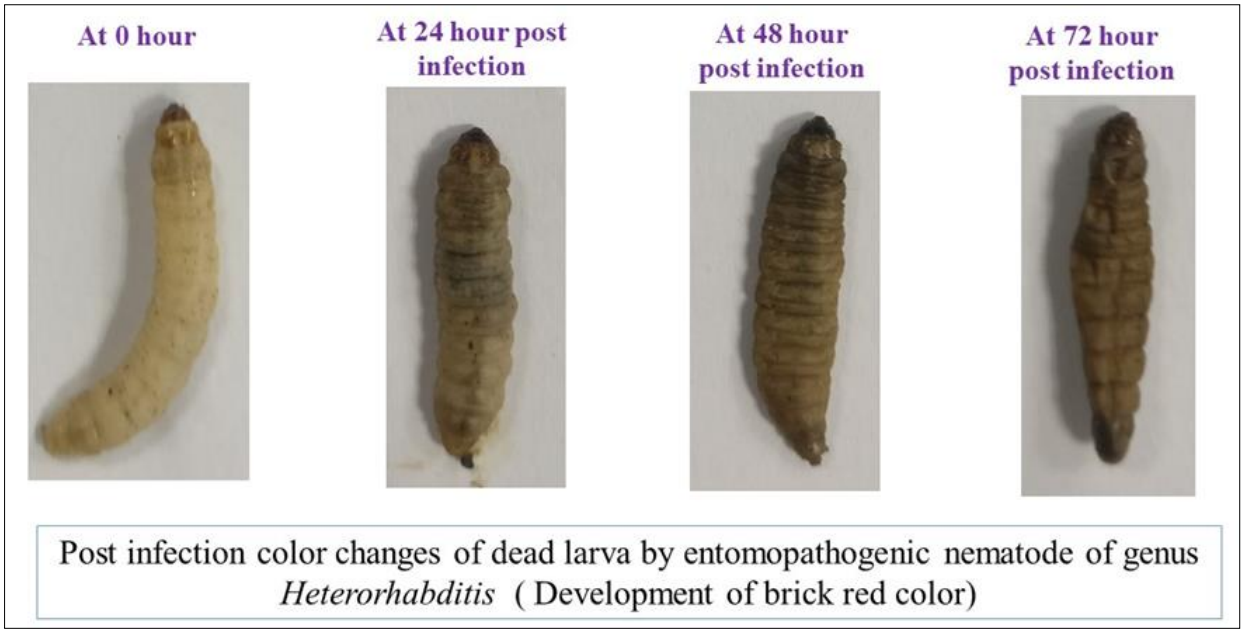

Fig 2: Changes in color development in dead cadaver infected by entomopathogenic nematode *Heterorhabditis*

Discussion

The entomopathogenic nematodes exhibit efficiency against insect pests of crops belonging to different orders. These nematodes are quick in killing and do not cause any harm to the ecosystem. The infective juvenile stages of these nematodes move freely in the soil in search of their insect host. Once they infect an insect host, they kill it quickly within a period of two to three days. This efficacy of infective juveniles is subjected to many abiotic and biotic stress factors (Stuart *et al*., 2015)) [13]. The successful utilization of these nematodes in integrated pest management schemes is dependent upon the virulence of a particular strain. Therefore, a preliminary bioassay of these nematodes is essential to find out the superiority in terms of host finding and infectivity. Once a superior strain is identified, it can further be improved by artificial hybridization and selection. In this paper, a visual of the whole experiment is created along with the step by step process of isolation of entomopathogenic nematodes from soil. It is inconvenient to find out directly infected insects from the soil. Therefore, baiting technique by using the larva of the wax moth is the most efficient way to isolates nematodes from the soil. To further enhance the multiplication of these nematodes in the laboratory, a modified version of the White trap can be used with proper maintenance. Enough number of soil samples are required to isolate entomopathogenic nematodes in the laboratory, as the chances of getting of nematodes in a sample are 20 to 30 percent only. The entomopathogenic nematodes are one of the best natural killers of insects, thus can be included in integrated pest management schemes. The entomopathogenic nematode belonging to the genus *Heterorhabditis* is more suited to the tropical and subtropical environment. The overall environmental fitness of these nematodes' fitness is determined by several traits including desiccation tolerance, temperature extreme tolerance, host finding, and infectivity behavior. The nematodes belonging to a particular location kills insects belong to the same native location effectively. Thus, the ongoing research aims to find out the particular isolates of nematode and to check out its efficacy against insects. Once the isolates are found superior or unique, it can further be improved for fitness determining traits for supporting effective insect-pests management strategies based on the usage of entomopathogenic nematodes.

Conclusion

Our findings have proven the efficacy of isolated *Heterorhabditis* against the test insect *Galleria mellonella.* The infectivity of this nematode would be tested further against insect-pests of economically valuable crops.

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