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Biocontrol potential of entomopathogenic nematodes *Steinernema* and *Metarhabditis* against tobacco caterpillar, *Spodoptera litura* (Fabricius)

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Abstract
A survey was conducted in Thiruvananthapuram, Kollam and Pathanamthitta districts of Kerala during July, 2017 to isolate indigenous entomopathogenic nematodes (EPNs) from different crop habitats *viz.* vegetables, banana and coconut through random sampling and to determine the potential of indigenous EPNs in causing mortality of tobacco caterpillar, *Spodoptera litura* (Fabricius). A total of forty soil samples were collected from rhizosphere region of different crop habitats and three native isolates of EPNs were obtained. The infectivity of indigenous isolates to tobacco caterpillar was assessed at an inoculum level of 300 infective juveniles (IJs) and Isolate 2 showed highest mortality (29.99 per cent) and maximum emergence of IJs ($3.5x10^5$) at 24 h after treatment. Results of pathogenicity of native EPN isolates against tobacco caterpillar at inoculum levels of 10, 50, 100 and 200 IJs revealed that Isolate 2 @ 200 IJs recorded highest mortality of tobacco caterpillar (80.52 and 99.35 per cent at 60 and 72 HAT respectively). Based on morphological characters, Isolate 2 was identified as *Metarhabditis rainai* which can be exploited successfully for the management of tobacco caterpillar.

Keywords: Entomopathogenic nematodes, infective juveniles, tobacco caterpillar, *Metarhabditis rainai*

1. Introduction
Tobacco caterpillar, *Spodoptera litura* Fabricius is one among the serious insect pests of vegetable crops causing yield loss upto 45 per cent [1]. It also causes considerable yield loss in other economically important crops such as cotton, groundnut, tobacco, castor, and pulses [2]. The consumption of pesticides against tobacco caterpillar has been increasing in the country leading to problems like environmental pollution, pesticide resistance, pest resurgence and residue in feeds, food, soil and water. So it is utmost necessary to have a sustainable use of chemical pesticides in controlling these insect pests for maximizing the crop yield and meeting the increased demands of agricultural products and commercialization. Thus the demand for biocontrol agents has been increased to overcome these problems. One among the biocontrol agents are the Entomopathogenic nematodes (EPNs) which is gaining momentum.

EPNs are a group of nematodes causing death to insects. They belong to families Steinernematidae, Heterorhabditidae and Rhabditidae. The genus under these families include *Steinernema/Neosteinernema*, *Heterorhabditis* and *Rhabditis* (*Oscheius*) and have a symbiotic association with insect pathogenic bacteria belonging to the genera *Photorhabdus*, *Xenorhabdus* and *Serratia* respectively. These nematodes have a life cycle consisting of egg stage, four juvenile stages and adult stage. The 3rd stage juvenile known as infective juvenile (IJ) is responsible for causing mortality of insect pests. They enter through the insect's natural body openings, the mouth, anus or respiratory openings (spiracles) and then penetrate into the haemocoel from the gut. Once on reaching the insect's blood, IJ releases the highly specialized symbiotic bacterium. The bacteria then convert the internal contents of the insect into 'nutrient soup' for easy feeding and multiplication by the nematodes and produce a range of antibiotics [3] which prevents putrification of the cadaver. The death of the insect occurs usually within 24-72 hours. Hence the present study was conducted to test the pathogenicity of native EPN strains against tobacco caterpillar.

2. Materials and Methods
2.1 Isolation of indigenous entomopathogenic nematodes (EPNs)
A random sampling was conducted during 2017 in four districts of Kerala to isolate

indigenous strains of EPNs. Further investigation on screening of native EPNs against various test insects and its identification were carried out at Department of Nematology, College of Agriculture, Vellayani. A total of forty soil samples were collected from rhizosphere region of different crop habitats viz. vegetables, banana and coconut in each district. The soil samples were collected from fields having extensive cultivation of the respective crops. Soil samples and dead cadavers were collected from the rhizosphere of above mentioned crops. 5 to 10 cm of top soil from the base of the plant was removed and around 500 cc soil samples were collected from a depth of 10-30 cm. Samples were packed in polythene bags and maintained at refrigerated conditions in the laboratory for further processing. The soil was thoroughly mixed and half of each sample was used for extraction of EPNs.

2.2 Rearing of trap insect, rice moth (*Corcyra cephalonica*)

C. cephalonica was reared in the laboratory of Department of Nematology on artificial diet prepared with crushed maize. The crushed maize bought from a local market and was sterilized in an oven at 100ºC for 30 minutes in the laboratory. 100 g of the broken groundnut kernel was transferred to each basin and the contents were hand mixed. Dry yeast and wettable sulfur was added @ 5g per basin and were mixed thoroughly. Two hundred numbers of 3rd instar *C. cephalonica* larvae were collected from Biocontrol laboratory at Parottukonam and were released in 2.5 kg crushed maize in plastic containers covered with a piece of muslin cloth. The plastic containers were kept in room temperature in the laboratory of Department of Nematology. Fifth instar larvae were used for inoculating the EPNs and some of them were left to complete their life cycle so as to collect the emerging adults of rice moths. The moths after collection were transferred to separate oviposition containers for egg laying. The eggs collected from oviposition containers were put in fresh artificial diet for maintaining the culture of *C. cephalonica* larvae.

2.3 Isolation of indigenous EPN

The larvae of *C. cephalonica* were used to concentrate EPN from the soil samples. Fifth instar larvae reared in standard medium was used for trapping EPN [4]. The soil samples were homogenized before they were baited. Ten fifth instar larvae of *C. cephalonica* were released into the plastic container containing 200 g of soil sample. Baited samples were stored in dark at room temperature. Samples were monitored for mortality upto 12 days. Insect cadavers from each soil sample were taken out and examined for infection. Fifth day after baiting, soil from the plastic containers were emptied into a pan and observed for infection by the EPNs. Dead larvae were collected from the container and was surface-sterilized in 1.0% Sodium hypochlorite solution for 3 minutes, then washed three times in sterile distilled water [5] and placed in "White trap" [6] . Infective juveniles (IJs) of EPN started to emerge from sixth to tenth day after infection. The emerged IJs were collected in tissue culture flasks containing 0.1 per cent formaldehyde and kept in BOD incubator at 15ºC.

2.4 Screening of EPN under *in vitro* conditions

Egg masses of *S. litura* were collected from banana field and were kept in a plastic trough which was maintained inside an insect cage for 3-5 days. Hatched larvae were separated into a clean trough containing castor leaves and were covered with a piece of muslin cloth. 3rd instar larvae were transferred to the petridish lined with moist filter paper. Nematode suspension containing a concentration of 300 IJs/larva was inoculated into each petridish. The treatment was replicated five times along with a control in which sterile water was used. Banana leaves were provided as a food source in the petridish. The experiment was repeated for all the native EPNs isolated. Number of dead larvae was recorded at 24 HAT and was placed in the white trap for extraction of IJs. Nematodes that emerged from the cadavers were collected. Number of IJs emerged out of each cadaver was counted under stereozoom microscope using a counting dish and colour change in cadaver also recorded at 72 HAT.

2.5 Pathogenicity of EPNs

The indigenous isolates were evaluated for the pathogenicity against the test insect (tobacco caterpillar) in the laboratory and their effective doses were determined. The test insects were treated with different concentration of IJs (10, 50, 100 and 200 IJs/insect) and were incubated at room temperature. Each concentration of indigenous isolates was replicated four times. Mortality of the insects was recorded at 24, 36, 48, 60 and 72 HAT. The corrected mortality percentage was worked out by using Abbott's formula [7] and compared with that of standard chemical.

$$\text{Corrected mortality} = \frac{[T\text{-}C]}{100\text{-}C} \times 100$$

Where T- mortality in treatments C -mortality in control

The concentration *viz*., 10, 50, 100 and 200 IJs of three indigenous isolates were taken from the stock culture and the final quantity was made to 10 ml. Experiment was laid out in CRD with four replications. Treatment with sterile water was kept as control. As mentioned in 2.4 the experimental set up was made and larvae (10 Nos.) were released into the petri plate. Flubendiamide 39.35 SC 0.01% was the chemical check for the experiment. Mortality was noted at 24, 36, 48, 60 and 72 HAT. The cadavers were transferred to white trap and the emerging IJs were collected.

2.6 Morphological characterization

The adults of first and second generation were extracted from the insect haemocoel in Insect Ringer's solution. The first and second generation adults were dissected out at 2-4 and 4-5 days after inoculation (DAI) respectively. The recovered nematodes were later transferred to clean ringer's solution for further processing. The nematodes were fixed in hot 50% triethanolamine formalin (TAF) fixative [8]. After 24 hours, the specimens were transferred to 100% TAF and kept in room temperature (28±2 ºC) for a week. The fixed nematodes were first transferred to a small cavity block containing 20 parts of 95 per cent ethanol, 1 part glycerin and 79 parts water (Seinhorst I). The cavity block was placed in a desiccator saturated by 95% ethanol and was incubated at 35-40^{0}C for 12 hours. The specimens were transferred into another cavity block containing 5 parts glycerin and 95 parts of 70 per cent alcohol (Seinhorst II). The cavity block with Seinhorst 2 was placed in a dessicator having calcium chloride at room temperature for about 3-4 weeks. This leads to slow dehydration and the nematodes gets gradually processed in anhydrous glycerin [9]. The specimens were mounted on glass slides in a drop of 100 percent anhydrous glycerin and sealed using wax. Twenty specimens of each stage (infective juveniles, females, males and hermaphrodites) were observed.

2.7 Statistical analysis
The data generated from the experiments were subjected to analysis of variance (ANOVA) technique [10]. The variables which did not satisfy the basic assumptions of ANOVA were subjected to angular transformations and analysed.

3. Results and Discussion
A total of forty samples were collected from the rhizosphere of vegetables, banana and coconut grown in districts Thiruvanathapuram, Kollam, Pathanamthitta and Alappuzha districts by random sampling. Three isolates of entomopathogenic nematodes (EPN) were isolated by 'insect baiting technique' using rice moth larvae (*Corcyra cephalonica*). Isolate 1 was obtained from the sample collected from cowpea plant grown in College of Agriculture, Vellayani, Thiruvananthapuram. Isolate 2 was obtained from the sample collected from tomato plant grown in a multicropped field in Mylom, Kottarakara (Kollam). Isolate 3 was obtained from the banana rhizosphere in Kainidi area of Alappuzha district. The frequency of distribution of EPNs in Thiruvananthapuram, Kollam and Alappuzha was recorded as 10 per cent (Table 1). No EPN species were obtained from the samples collected from Pathanamthitta district. From the collected samples three native isolates were obtained. As part of survey conducted to isolate EPNs in Kerala, 430 soil samples were collected and 129 soil samples were found positive for EPN with 30 per cent frequency of occurrence. *Heterorhabditis indica* occurred in 128 samples and *Steinernema* sp. in only one sample with frequency of occurrence of 90 and 0.8 percent respectively [11]. A recent survey reported that out of 141 soil samples collected from Kollam, Pathanamthitta and Alappuzha, 13.5% were found positive for the presence of EPN. It was also reported that soil samples collected from plots of ICAR-CPCRI, Kayamkulam recorded 33.3% of total number of EPN isolates [12]. EPN isolates (10 Nos.) were obtained from banana rhizosphere in Vellayani [13]. The present study was in line with the findings of Sosamma, Anes and Remya which revealed the natural occurrence of EPN in Kerala. In this study also three isolates of EPN were obtained from Vellayani (Isolate 1), Mylom (Isolate 2) and Kainidi (Isolate 3) areas of Kerala with 10 per cent frequency of occurrence. So results of the study revealed that the native isolates of EPNs prevalent in Kerala can be utilized for the management of insect pests without introducing new strains.

Table 1: Distribution of indigenous isolates of entomopathogenic nematodes in different districts of Kerala

Name of Districts	Number of samples collected	Number of Native isolate obtained	Name of the place	Name of the crop	GPS Co-ordinates	Percentage frequency of occurrence
Thiruvananthapuram	10	1	COA,Vellayani Poonkulam	Cowpea	8.4410ºN 76.9891ºE	10
Kollam	10	1	Mylom, Kottarakara	Tomato	8.5490ºN 76.3877ºE	10
Pathanamthitta	10	0	-	-	-	0
Alappuzha	10	1	Kainadi, Kavalam	Banana	9.4925ºN 76.4703ºE	10

Table 2: Effect of different isolates on mortality of tobacco caterpillar and emergence of IJs

Treatments	Tobacco Caterpillar		
	%Mortality(24h)	Colour change	Emergence (IJs)
Isolate 1 @300 IJs	19.99 (26.56)	No change	$2.7x10^5$
Isolate 2 @300 IJs	29.99 (33.21)	Pinkish	$3.5x10^5$
Isolate 3 @300 IJs	19.99 (26.56)	No change	$0.9x10^5$
CD (0.05)	(4.38)	-	-

The three native EPNs isolates obtained through random soil sampling were subjected to screening for testing their pathogenicity against test insect, tobacco caterpillar. The test insects were treated with 300 IJs of native EPN isolates (Isolate 1 (Vellayani strain), Isolate 2 (Mylom strain), Isolate 3 (Kainidi strain) and their mortality percentage was recorded 24 hours after treatment (HAT). The emergence of IJs from infected cadavers and change in colour of the cadavers were observed. Maximum mortality of tobacco caterpillars was observed with Isolate 2 (29.99 per cent) followed by Isolate 1(19.99 per cent) and Isolate 3 (19.99 per cent) with 300 IJs per larvae at 24 HAT (Table 2). Isolate 2 showed the maximum emergence of IJs ($3.5x10^5$) compared to Isolate 1 ($2.7x10^5$) and Isolate 3 ($0.9x10^5$). The cadavers infected with Isolate 2 had pinkish colour whereas cadavers infected with Isolate 1 and 3 didn't show any colour change. The mortality percentage observed in this study was different from the findings of Gupta [14]. He reported a per cent mortality of 40-60 in tobacco caterpillars treated with 50-100IJs/larva applied as foliar spray. The results of emergence of IJs from tobacco caterpillar obtained in the study were similar to the observations of Pervez and Ali [15]. They observed that *in vivo* production of IJs on *S. litura* showed the maximum yield in *S. mushtaqi* ($0.79x10^5$ IJs/cadaver) followed by *S. seemae* ($0.72x10^5$ IJs/cadaver), *S. carpocapsae* ($0.67x10^5$ IJs/cadaver) and *S. masoodi* ($0.51x10^5$ IJs/cadaver).There was no report on colour change of the tobacco caterpillar infected with EPN. However *G. mellonela* infected with Steinernematids showed cream or ochre-grey colour whereas Heterorhabditids showed red or burgundy colour [16].

Table 3: Effect of different levels of Isolate 1 on the mortality of tobacco caterpillarat different time periods under laboratory conditions

Treatments	Corrected mortality percentage				
	24 h	36 h	48 h	60 h	72 h
10 IJ	2.57[c] (9.22)	9.44[d] (17.89)	19.48[d] (26.19)	29.75[e] (33.05)	39.89[e] (39.17)
50 IJ	14.64[b] (22.5)	24.83[c] (29.89)	39.89[c] (39.17)	50.00[d] (45)	57.52[d] (49.33)
100 IJ	20.00[b] (26.56)	37.44[b] (37.73)	57.52[b] (49.33)	65.19[c] (53.84)	73.20[c] (58.83)
200 IJ	22.37[b] (28.23)	42.48[b] (40.67)	62.56[b] (52.27)	75.17[b] (60.11)	85.36[b] (67.50)
Flubendiamide 39.35 SC	100.00[a] (90.00)	100.00[a] (90.00)	100.00[a] (90.00)	100.00[a] (90.00)	100.00[a] (90.00)
CD (0.05)	(8.225)	(9.348)	(5.971)	(6.745)	(8.001)

Figures in parenthesis are arc sine transformed values

Pathogenicity of native EPN isolates against *S. litura* were assessed with inoculum levels of 10, 50, 100 and 200 IJs at different time periods viz., 24, 36, 48, 60 and 72 HAT. The results presented in Table 3 showed statistically significant variation between different levels of isolates on mortality of test insects. Among the EPN isolates, Isolate 2 at 200 IJs recorded the maximum mortality of tobacco caterpillar (99.35 per cent) followed by Isolate 1 (Vellayani strain) (85.36 per cent) and Isolate 3 (Kainidi strain) (85.35 per cent) at 72 HAT. When the concentration of IJs was reduced to 100 IJs per larvae, maximum mortality was recorded by Isolate 2 (Mylom strain) with 92.53 per cent mortality followed by Isolate 3 (Kainidi strain) (80.00 per cent) and Isolate 1 (Vellayani strain) (73.20) at 72 HAT. Mortality percentage at 60 HAT with 200 IJs was maximum in Isolate 2 (Mylom strain) (80.52) followed by Isolate 1 (Vellayani strain) (75.17) and minimum in Isolate 3 (Kainidi strain) (65.08). Percentage mortality was found directly proportional to concentration of IJs and period of exposure. Several workers reported the efficacy of EPNs against *S. litura*. A study reported that sand column bioassay tests of *Heterorhabditis* sp. (HUDP-1 strain) against *S. litura* recorded mortality per cent ranging from 16.7 to 88.9 when treated with 25, 50, 75, 100, 125 and 150 IJs/caterpillar [17]. Another study reported 100 per cent mortality of 3-5 instar larvae of *S. litura* after 96 hours of treatment in laboratory conditions by a local isolate of *S. carpocapsae* [18]. Cent per cent mortality of *S. litura* at an inoculum level of 400 IJs of *S. carpocapsae* was produced on animal protein based media at 96 hours after exposure [19]. But the present study is pioneer in testing the pathogenicity of *Metarhabditis* sp. against *S. litura*. However, Padmakumari [19] reported *Rhabditis* sp. causing mortality of egg mass and neonate larvae of rice yellow stem borer at 500 IJs/larvae at 31 h.

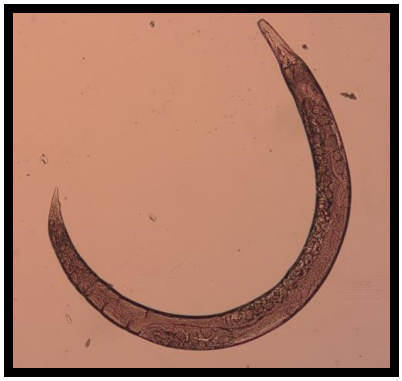

Plate 2: Lobed oesophageal glands

In this study among the three isolates, Isolate 2 (Mylom strain) performed best in killing tobacco caterpillar. So the morphological characters of Isolate 2 obtained from Mylom were studied. It was characterised by a hermaphroditic condition (Plate 1) in the first generation followed by males and female in the next generations. The stoma was tubular/ funnel shaped in hermaphrodites, males and females. Hermaphrodite had a cylindrical corpus throughout whereas the males and females had a typical "Rhabditoid oesophagous" with a cylindrical procorpus, undifferentiated metacorpus, distinct isthmus and pyriform shaped basal bulb with well-developed butterfly valve. They had lobed oesophageal glands extending over the intestine (Plate 2). Hermaphrodites had a conoid tail with post-anal swelling and pointed terminus. Females had a filiform tail and males had a short and pointed tail. Vulva was a tranverse slit with slightly protruding vulval lips and was seen near to the anal slit.

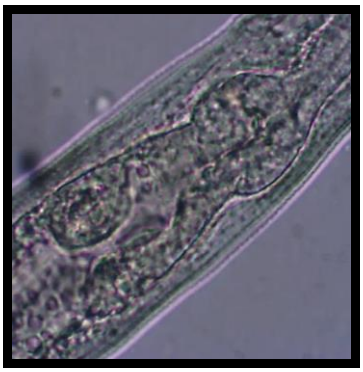

Plate 1: Hermaphrodite of *Metarhabditis rainai*

The male tail had a peloderan bursa with eight genital papillae. The spicules were paired and asymmetrical and slightly curved ventrally. Gubernaculum was slightly curved ventrally and had a length more than half the spicule length. Based on taxonomic keys Isolate 2 was identified as *Metarhabditis* sp. The isolate showed similarity to *Metarhabditis rainai* as this species alone showed hermaphroditism among the genus *Metarhaditis*. The biocontrol potential of *M. rainai* against tobacco caterpillar is reporting first time in this study.

4. Conclusion

Result of the study revealed the biocontrol potential of native strains of EPN against *S. litura*. Among the three native isolates, Isolate 2 identified as *M. rainai* proved to be the most potent strain. Soil application or foliar application of 200 IJs of *M. rainai* can be recommended in an IPM programme

without any harmful effect on the environment. The native isolate can also be used with other compatible biocontrol agents thus reducing waiting period for crop harvesting, minimizing the chance of development of resistance and most importantly reducing the toxic pesticide residue avoiding environmental pollution.

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