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Isolation and mass production of native isolates of entomopathogenic nematodes from Anand (Gujarat), India

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Abstract
The survey was conducted in Anand city and its adjoining area during *Kharif*-2021 to isolate native entomopathogenic nematodes (EPNs). A total of 94 soil samples were collected from fruit plants, perennial trees and fallow land, out of them two samples were found positive for EPNs, one from the campus of the Bhikhabhai Jivabhai Vanijya Mahavidhyalaya (Palm) and the second one from Atmiya Vidhya Dham (fallow land). The frequency of occurrence of these nematodes was 2.13 per cent. Based on primary identification up to the genera level, the first isolate was identified as *Heterorhabditis* sp. (Anand H-I). and the second was *Steinernema* sp. (Anand St-I). Both isolates were mass multiplied on six different artificial media. The maximum multiplication of *Heterorhabditis* sp. (Anand H-I) and *Steinernema* sp. (Anand St-I) was observed on Wout's medium followed by Soya flour medium for *Heterorhabditis* sp. (Anand H-I) and Bengal gram medium for *Steinernema* sp. (Anand St-I).

Keywords: Entomopathogenic nematodes, EPNs, survey, isolation, mass production, *Heterorhabditis* sp. and *Steinernema* sp.

Introduction

Entomopathogenic nematodes (EPNs) are parasites of insects belonging to the families Steinernamatidae and Heterorhabditidae. The infective juveniles of these families are free-living, non-feeding and have the ability to search out their hosts. This group of nematodes is characterized by carrying specific symbiotic bacteria of the genus *Xenorhabdus* or *Photorhabdus* in their intestine which play an important role in the pathogenicity of the nematode-bacteria complex to insect hosts and the subsequent reproduction of the nematodes in the hosts. They have the potential for long-term establishment in the soil through the recycling of infected insect larvae. The EPNs possess unique attributes like complex nematode-bacterium mutualistic symbiosis, broad host range and their amenability to be cultured in *in-vivo* and *in-vitro* on large scale, making them very suitable to be used as a biocontrol agent. EPNs are currently used as bio-pesticides for controlling several important insect pests worldwide (Shapiro-Ilan *et al*., 2002) [13]. EPNs-based pesticides are nowadays largely marketed in western countries in comparison to other organisms, apart from the bacteria *Bacillus thuringiensis,* but the survival and adaptability of EPNs are influenced by environmental factors. Due to unfavorable climatic conditions, exotic EPN strains may not be well adapted to local climates and their efficacy might be reduced. Therefore, isolation of native EPNs strain and mass multiplication is very important. This study aims to isolate and *in vitro* mass multiply the native strain of EPNs isolated from Anand city.

Materials and Methods

Rearing of bait insect, *Corcyra cephalonica*: In the present study, Rice moth *Corcyra cephalonica* larvae was used for the mass culturing of EPN isolates. Rice moth *C. cephalonica* larvae were obtained from AICRP on Biological Control of Crop Pests, ICAR unit-9, AAU, Anand. The larvae were reared on artificial diet containing following composition sorghum whole grain – 2.5 kg, broken sorghum grain – 0.5 kg, Streptomycin – 0.5 g and yeast powder – 1.0 g.

Collection and baiting of soil samples: A total of 94 soil samples were collected from different localities of Anand city and its adjoining area from fruit plants, perennial trees and fallow land from the depth of 10-15 cm during *Kharif* 2021 as per the methodology described by Kaya and Stock (1997) [7].

Approximately 500 g soil of each sample was placed in plastic container and baited with five larvae of *C. cephalonica*. The containers were covered tightly with lids containing small holes to facilitate gaseous exchange and kept at room temperature (25-30 ºC). The larval mortality was recorded daily for up to 10 days. The *Steinernema* spp. infected C. cephalonica larvae became flaccid and their colour changed from white to orange/ yellow/ brown and in the case of *Heterorhabditis* spp. infected larvae, the colour was changed from white to brownish red or brick red that showed faint luminescence in the dark. Dead larvae were washed thrice with sterile distilled water, dried on filter paper and transferred over a white trap for extraction of the EPNs.

Isolation and extraction of EPNs by white trap method: Dead larvae from each sample were incubated on a modified White trap (White, 1927) [15]. The EPNs that emerged out from the cadaver were collected in a beaker using sterile distilled water. The collected EPNs were rinsed thrice with sterile distilled water, allowed to settle at the bottom of the beaker and the supernatant suspension was separated out. This process was repeated 4-5 times for obtaining clear nematode suspension.

***In vitro* Mass multiplication of isolated EPNs**

Preparation of artificial diet and inoculation of EPNs

Infective juveniles of *Heterorhabditis* sp. (Anand H-I) and *Steinernema* sp. (Anand St-I) which were recovered from *C. cephalonica* using White trap were *in vitro* mass multiplied on six different artificial media. All the ingredients were mixed as per the composition mentioned in Table 1**.** Each media was placed in a conical flask. Inside the flask, small pieces of sponge were placed to absorb media liquid. The conical flasks were then closed and sterilized using an autoclave at 121 °C for 15 min. Fresh IJs of both isolated EPNs extracted from the infected insect cadavers were inoculated aseptically @ 1000 IJs/f1ask. Care was taken not to shake the flasks during incubation.

Harvesting and counting of EPNs: EPNs multiplied on different media were extracted after 15 days of inoculation. Coated sponge media in a flask was transferred on a facial tissue paper which was spread over a 20 mesh aluminum wire mesh support. The flasks were washed thoroughly and washing were also added to the media. The wire mesh support was then kept over a Petri dish filled with water so that a thin film of water touched the material over the tissue paper. The nematodes settled in a Petri dish were collected after 24 hrs and transferred to a 500 ml beaker. The nematode suspension in the flask was allowed to settle and the supernatant was decanted. This process was repeated several times to obtain clear nematode suspension. The nematode yield from each medium harvested was expressed in terms of the number of IJs/f1ask and counted by using the counting and estimation method.

Table 1: Composition of different artificial media

Medium I	:	Egg yolk (1 g) + soya bean flour (1 g) + gelatin (0.2 g) + distilled water (30 ml)
Medium II	:	Egg yolk (1 g) + gram flour (1 g) + gelatin (0.2 g) + distilled water (30 ml)
Medium III	:	Egg white (1 g) + wheat flour (1 g) + agar-agar (0.2 g) + distilled water (30 ml)
Medium IV	:	Nutrient broth (0.88 g) + yeast extract (0.32 g) + corn flour (14.4 g) + sesamum oil (10.4 g) + distilled water (60 ml)
Medium V	:	Nutrient broth (0.88 g) + yeast extract (0.32 g) + gram flour (14.4 g) + sesamum oil (10.4 g) + distilled water (60 ml)
Medium VI (Wout's medium)	:	Nutrient broth (0.44 g) + yeast extract (0.16 g) + soyabean flour (7.2 g) + corn oil (5.2 g) + distilled water (27 ml)

Result and Discussion

Isolation of native EPNs

A total, of 94 soil samples were collected from the different localities of Anand city and its adjoining area from perennial trees, fruit plants and fallow land. Out of these, 70 soil samples were collected from the rhizosphere perennial trees, eight from the fruit plants and 16 from fallow lands. The result indicated that out of the total collected 94 samples, EPNs were found to be positive from two soil samples. The frequency of occurrence of these nematodes was 2.13 per cent (Table 2). These native EPNs were isolated from the campus of the Bhikhabhai Jivabhai Vanijya Mahavidhyalaya (Palm) and Atmiya Vidhya Dham (fallow land) (Table 3). Based on colour of the cadaver, primary identification was done. The brackish-red colour was observed in *C. cephalonica* cadavers infected by the isolate Anand H-I collected from Bhikhabhai Jivabhai Vanijya Mahavidhyalaya (Palm) and creamy-white in isolate Anand St-I from Atmiya Vidhya Dham (fallow land). The isolated EPNs, Anand H-I and Anand St-I belong to genera *Heterorhabditis* and *Steinernema*, respectively. The result of the present investigation confirms the finding of Lephoto and Gray (2021) [8], who reported that, out of the total collected 80 soil samples, EPNs were recovered from only one soil sample. Similarly, Amuri and Devi (2020) [1] found one soil sample to be positive for EPNs out of the total 200 collected soil samples. The present investigation showed 2.13 per cent frequency of occurrence for EPNs which is similar in line with the findings of Gowda *et al.* (2020) [4] who recorded 2.3 per cent. Moreover, Maru *et al.* (2007) [9] reported that out of the total collected 97 samples, *Steinernema* sp. and *Heterorhabditis* sp. were recorded only in six samples collected from the rhizosphere of wheat, rose, guava and forest trees. Further, Devi *et al.* (2017) [3] stated that EPNs were isolated from five samples out of 100 soil samples. These earlier findings are partially supported by the present result. Similarly, Chand *et al.* (2016) [2] also collected 105 soil samples, and out of them, three samples were found positive. Prajapati and Maru (2022) [11] also collected 103 soil samples from different locations of Anand agricultural university campus, Anand, out of these three samples were found positive and were collected from three different locations *i.e.*, Horticultural farm (Mango), the Veterinary College Garden and the International Agri-Business Management College and they found very low (2.9%) frequency of occurrence.

In contrast, Kasi *et al.* (2021) [6] recorded the occurrence of *Steinernema* and *Heterorhabditis*, 27.03 and 27.45 per cent, respectively. Similarly, Nthenga *et al*. (2021) [10] concluded that out of 76 soil samples, 39 samples (51.32%) were found to be positive for EPNs, showing a higher frequency of occurrence as compared to the present investigation. The discrepancy in findings might be due to differences in sampling site, soil type, habitat and host insect availability.

Table 2: Soil sample collected from different places

Sr. No.	Soil samples collected from	No. of soil samples collected	No. of soil sample found positive	Frequency of occurrence (%)
1.	Perennial crops	70	1	1.43
2.	Fruit crops	07	0	0
3.	Fallow land	16	1	6.25
Total		94	2	2.13

Table 3: Samples possessing entomopathogenic nematodes

Sr. No.	Habitat	Area of Sampling	Latitude-Longitude	Genera identified
1.	Bottle palm	Bhikhabhai Jivabhai vanijya mahavidhyalay	22.554506, 72.926566	*Heterorhabditis* sp. (Anand H-I)
2.	Fallow land	Atmiya Vidhya Dham (AVD)	22.557486, 72.919006	*Steinernema* sp. (Anand St-I)

In vitro Mass multiplication of EPNs:

The experimental result revealed that among both the species, *Steinernema* sp. (Anand St-I) was multiplied on all the tested six media, whereas *Heterorhabditis* sp. (Anand H-I) failed to multiply on three media *viz.,* medium I (egg yolk + soya bean flour + gelatin), medium II (egg yolk + gram flour + gelatin) and medium III (egg white + wheat flour + agar-agar). The data (Table 3) revealed that the maximum multiplication of both the isolates *Heterorhabditis* sp. (Anand H-I) (2.82×10^5) and *Steinernema* sp. (Anand St-I) (2.45×10^5) was observed on medium VI containing soya bean flour and corn oil. The medium IV containing corn flour and sesamum oil recorded a higher yield of *Heterorhabditis* sp. (Anand H-I) (1.99×10^5) as compared to *Steinernema* sp. (Anand St-I) (1.05×10^5) whereas, on medium V supplemented with gram flour and sesamum oil *Steinernema* sp. (Anand St-I) yielded higher than *Heterorhabditis* sp. (Anand H-I). The lower yield of *Steinernema* sp. (Anand St-I) was recorded on medium I (0.24×10^5) followed by medium II (0.19×10^5) and medium III (0.13×10^5) as compared to other media. The result of the present study falls in line with earlier reports made by Wouts (1981) for *H. heliothidis,* Hussaini *et al.* (2002) [5] for *H. indica* and Umamaheshvari *et al.* (2008) [14] for both *H. indica* and *S. siamkayai.* They reported that Wout's medium was best suitable for the multiplication of EPNs. However, the yield of IJs were varied, the possible reason for that was the difference in the inoculation rate of IJs, harvesting period and other cultural conditions. Seenivasan (2017) [12] reported that the media composition of wheat flour medium, egg yolk medium and modified egg yolk medium were not suitable for the growth of *Photorhabdus* spp. associated with *H. bacteriophora* strain KKMH1. A similar reason may be responsible for no multiplication of *Heterorhabditis* sp. in the present experiment.

Table 3: *In vitro* mass multiplication of isolated EPNs

Tr. No.	Treatments	No. of IJs ($\times 10^5$) per flask	
		***Heterorhabditis* sp. (Anand H-I)**	***Steinernema* sp. (Anand St-I)**
T_1	Medium I	0 (0)	4.38 (0.24)
T_2	Medium II	0 (0)	4.29 (0.19)
T_3	Medium III	0 (0)	4.10 (0.13)
T_4	Medium IV	5.30 (1.99)	5.02 (1.05)
T_5	Medium V	5.17 (1.49)	5.26 (1.82)
T_6	Medium VI	5.45 (2.82)	5.39 (2.45)
S.Em ±		0.03	0.03
C.D. (0.05)		0.10	0.10
C.V. %		2.47	1.45

Note: Figures in parentheses are retransformed values and those outside are log (X+1) transformed values

Conclusions

The present investigation revealed that out of the total collected 94 soil samples from Anand city, two samples were found positive and identified as *Heterorhabditis* sp. (Anand H-I) and *Steinernema* sp. (Anand St-I). Both the isolated EPNs, Anand H-I and Anand St-I were multiplied maximum on medium VI containing soya bean flour and corn oil.

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