Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2019; 7(6): 493-498

Received: 13-09-2019
Accepted: 15-10-2019

Devindrappa
Division of Crop Protection
ICAR- Indian Institute of Pulses Research, Kanpur, Uttar Pradesh, India

Sanjay M Bandi
Division of Crop Protection
ICAR- Indian Institute of Pulses Research, Kanpur, Uttar Pradesh, India

Bansa Singh
Division of Crop Protection
ICAR- Indian Institute of Pulses Research, Kanpur, Uttar Pradesh, India

Krishna Kumar
Division of Crop Protection
ICAR- Indian Institute of Pulses Research, Kanpur, Uttar Pradesh, India

Corresponding Author:
Devindrappa
Division of Crop Protection
ICAR- Indian Institute of Pulses Research, Kanpur, Uttar Pradesh, India

Identification and parasitic potential of *Steinernema siamkayai* against soil-dwelling stage of *Helicoverpa armigera* (Hub.) under laboratory condition

Devindrappa, Sanjay M Bandi, Bansa Singh and Krishna Kumar

Abstract
Entomopathogenic Nematodes (EPNs) belonging to genera *Heterorhabditis* and *Steinernema* have been known for their bio-control potential against soil inhabiting insects or their life stages present in soil. In the present study, we identified five isolates of EPN's from different crop rhizospheres of Uttar Pradesh through *Galleria mellonella* (L.). Insect Baiting Technique and White's Trap Method. Morphological and morphometric studies of Infective Juveniles (IJs) and first generation male and females revealed the isolate belonged to the genus *Steinernema* of the family *Steinernematidae.* The species level identity of isolates was confirmed by sequencing of ITS-rDNA region. Our isolate showed maximum homology with ITS sequences of *Steinernema siamkayai* available in the NCBI GeneBank. Based on the morphological and morphometric, and molecular analysis, the nematode isolates were identified as *S. siamkayai.* The parasitic potential of *S. siamkayai* was tested against the soil dwelling stage, pupae of *Helicoverpa armigera* (Hub.) and results demonstrated that *S. siamkayai* was found to reduce / inhibit 60 to 90 per cent of adult emergence from pupae present in soil at varied numbers of IJ concentration. This study reports identification and entomopathogenicity of *S. siamkayai* against soil dwelling pupal stage of *H. armigera.*

Keywords: Entomopathogenic nematodes (EPNs), *Steinernema siamkayai Galleria mellonella, Helicoverpa armigera,* ITS-rDNA

Introduction
Entomopathogenic Nematodes (EPN's) *Steinernema* spp. and *Heterorhabditis* spp. belonging to the families, Steinernematidae and Heterorhabditidae have been reported as potential biological control agents for the suppression of soil-inhabiting insects and their life stages. EPNs, *Steinernema* spp. and Heterorhabditis spp possess species-specific symbiotic bacteria of the genus *Xenorhabdus* and *Photorhabdus* [2, 4]. Isolating indigenous EPN population is an important step to attain the effective biological control of soil inhabiting insects and their life stages due to their better adaption to prevailing parameters of local environment [29]. Temperature and host availability are thought to be an important factors in determining their distribution in soil habitat [31]. Their occurrence and distribution has been reported from varied local geographical locations **[24]**. In the last three decades, many EPNs have been isolated in various habitats all over the world, revealing hundreds of new isolates and many new species **[26]**. Currently, over 80 species of *Steinernema* and 20 species of *Heterorhabditis* have been described (NCBI. 2015). Most of these EPNs were isolated from either North America or Europe **[10].** In India, they have been studied from all ecological zones [15]. These nematodes can easily be mass multiplied on *in vivo* and *in vitro* production systems.
Introduction of new EPN isolates as bio-control agents in particular region requires prior knowledge of their occurrence, distribution and proper identification of native species. Identification of new indigenous isolates is not always straight forward and they lack morphological variations, thus many researchers have relied on analysis of ITS-rDNA sequence, a potentially highly variable region found useful for differentiating the species and strains of *Steinernema* [32, 33]. Therefore we attempted to identify EPNs isolated from different crop rhizospheres of Uttar Pradesh and their efficacy was assessed against soil dwelling pupae of *Helicoverpa armigera* (Hub.).

Materials and methods

Geography of the soil sample survey site

The Uttar Pradesh is situated in the heart of Indo-Gangetic Plain, the average temperature varies in the plains from 3° to 4 °C in January, 43° to 45 °C in May. The climate of the region is warm, humid and varies from tropical to sub-tropical. There are three distinct seasons - winter from November to February, summer from March to June, and the rainy season from June to September. Total of 150 soil samples were collected randomly from different crop rhizospheres (Table 1) in different areas of Uttar Pradesh having good crop coverage. The sampling was carried out randomly and from each sample, approximately 500 g of soil was drawn from composite sample of 6-8 subsamples taken at 0- 15 cm depth in an area of approximately 25 m^2 with the help of hand showel.

Isolation and storage of Entomopathogenic nematodes (EPNs)

The EPNs were isolated by using the insect baiting method described by Bedding and Akhurst [3]. Moistened soil obtained from each sample was placed in 250 ml plastic container and was baited with last instar larvae of *Galleria mellonella* L. The dead larvae were washed in distilled water and placed in modified White's Trap [3] to collect infective juveniles (IJs) from dead larvae. The resultant nematode suspension was used for artificial infection of the larvae of *G. mellonella* at 24- 27 °C [20]. The recovered IJs were kept in vented tissue culture flask at 27 °C until use. After storage at 27 °C for one week, they were allowed to acclimatize at room temperature for 1h and their viability was checked by observation of movement under a stereomicroscope. They were repeatedly sub-cultured on larvae of *G. mellonella* to obtain large number of pure populations of infective juveniles (IJs) for subsequent studies.

Morphological and morphometric characterization of EPNs

The temporary and permanent mounts of isolates (IIPR 01, IIPR 02, IIPR 03, IIPR 04 and IIPR 05) of EPNs were prepared for morphological and morphometric study. Ten each Infective juveniles (IJs) and first generation males and females of each isolates were used for studying the standard key morphometric traits as suggested by Hunt (13). To confirm this, species level identification was done by molecular approach and was compared to those available in Gene Bank [13, 25].

Molecular Characterization of EPNs

DNA extraction and Polymerase Chain Reaction (PCR):

The genomic DNA was extracted from a single first generation female using a modified method of Joyce *et al.* [16], using Qiagen Blood and Tissue Kit. Nematodes were collected from White's Traps under sterile conditions of laminar flow, and placed in sterile 1.5 ml Eppendorf tubes. The nematodes were rinsed three times using approximately 1 ml distilled water per wash. After washing, DNA was extracted and stored at 4 ˚C.

PCR amplification: DNA suspension of 10 µl was added to a PCR reaction mixture containing 2.5µl 10X PCR buffer with $MgCl_2$, 0.5µl dNTP mixture (10mM each), 0.5µl (100pM/µl) of each primer, 0.3U Taq polymerase and 10.7µl double distilled water to make the volume of 25µl. The forward primer TW81 (5'- GTTTCCGTAGGTGAACCTGC- 3') and the reverse primer AB28 (5'- ATATGCTTAAGTTCAGCGGGT- 3') were used in the PCR reaction for amplification of the complete ITS. The amplification profile was carried out using a BioRad thermo-cycler, which was preheated at 94 ℃ for 5min followed by 35 cycles of 92 °C for 60s, 60 °C for 30s and 72 °C for 60s and then 72 °C for 10min. After DNA amplification, 5µl of product was visualized in 1.5% agarose gel. Amplified product was purified using a Qiagen Gel Purification Kit. DNA fragments were sequenced by Sanger's method (Eurofins Genomics India Pvt., Ltd., Bengaluru. India)

Pathogenic potential of EPN isolates against pupae of *H. armigera*

In order to maintain sufficient number of *H. armigera* pupae for the experiment, Culture of *H. armigera* were collected from infested field at New Research Centre and maintained at laboratory, Division of crop protection, ICAR-Indian Institute of Pulses Research, Kanpur. The pathogenic test of *S. siamkayai* isolates against pupae of *H. armigera* was carried out in laboratory. If we target the specific soil dwelling stage (pupae) of *H. armigera* and soil application potential EPN can suppress the next generation population, it lead to lower infestation of crops. Sterilized sandy loam soil weighing 100g was put in plastic sample container (60 mm dia.) and 20 ml sterilized distilled water was added to moisten the soil. Five concentrations of nematode (0, 500, 1000, 2000, 3000 IJs/container) were added in the soil having five pupae of *H. armigera*. Each concentration was replicated thrice. The emergence of adult was recorded every day up to 10 to 12 days and per cent suppression of adult emergence from soil was calculated.

Results

Survey for indigenous strains of EPNs in Uttar Pradesh (UP)

Total 150 soil samples collected during survey for presence of EPNs in five districts of Uttar Pradesh using insect baiting technique (3)) revealed quite low occurrence of EPNs in their natural habitats compared to other forms of nematodes. Out of a total 150 samples, EPNs were encountered in only 12 samples (Table1) therefore; the frequency of occurrence of EPNs was eight per cent only. The isolates were designated as IIPR 01 to IIPR 05.

Morphological and morphometric characterization of EPNs

Morphometric measurements of various taxonomic characters were studied under steriozoom microscope for individual population. The Isolates of EPNs were found to be *Steinernema* species on the basis of morphological characters of the adults which had rounded head, Rhabditoid esophagus, and distinct excretory pore opening, just at level of mid-esophagus. Females with amphidelphic ovaries, short muscular vagina and vulva located near mid body region. Males monarchic, testis reflexed; spicules were paired, symmetrical gubernaculum present. Infective juveniles having annulated cuticle, collapsed stoma, esophagus and intestine with bacterial pouch located at the beginning of the intestine (Fig. 1, 2 and 3). Based on morphological studies all five isolates were identified as *Steinernema siamkayai*. Morphometric measurements (minimum to maximum) observed in the present study showed the length of IJs 428-

480 µm, head to excretory pore 29-38 µm, tail length 28-43 µm, greatest width 17-24 µm, and esophagus length 90-108 µm (Table 2). In first generation females, total body length ranged from 1085-1189 µm, greatest body width 109-152 µm, head to excretory pore 48-64 µm, esophagus length 127-142 µm and tail length ranged from 21-32 µm (Table 3). Males of first generation curved posteriorly, J -shaped when heat killed, total lengths ranged from 1085-1185 µm, spicule length 74-81µm and gubernaculum length 47-61µm (Table 4). These morphological measurements matched with earlier described species of *Steinernema siamkayai*.

Molecular characterization

The isolates of *Steinernema* spp. yielded 1kb fragment upon PCR amplication with the ITS primers. Ribosomal DNA (ITS region of EPNs) sequences generated for the isolate were individually matched and aligned with sequences available in NCBI Gen Bank. Nematode DNA sequence deposited in Gen Bank under accession number MH260074.1, MH260075.1, MH260076.1, MH260077.1, MH260078.1 were compared with sequences of *Steinernema* spp. available in Gen Bank. The BLAST search analysis showed maximum identity with *Steinernema siamkayai*.

Phylogenetic analysis

The sequences of the ITS-rDNA region of the EPNs were used to study the phylogenetic relationships between the *Steinernema siamkayai* and known *Steinernema* spp. The sequence was aligned by BioEdit and phylogenetic analysis of sequence data done using the Neighbour-Joining method by using MEGA 6.0. Sequenced data revealed that, more variability among the interspecies. The ITS regions were much more variable and provided most of the base differences for species diagnosis [27]. The isolates IIPR03, IIPR04 were aligned clearly and without gap under same clade, whereas isolates IIPR05, IIPR01 and IIPR02 aligned with other clades in phylogenetic tree. (Fig.4)

Pathogenic potential of EPN isolates against pupae of *H. armigera*

S. siamkayai suspensions were found pathogenic to pupae of *H. armigera* in all varied concentrations except control under laboratory condition. Irrespective of the nematode concentrations tested, the low mortality was recorded at lower nematode concentration which increased with increase in concentration. However, *S. Siamkayai* was capable of reducing 60 to 90 per cent pupal emergence from soil in different dosage level of EPN. (Fig. 5).

Discussion

In the present study, we found the occurrence of EPNs in regions of Uttar Pradesh where there was evidence of continuous breeding of host insect pests in cryptic habitat. The probable reason for low occurrence of EPNs in the collected samples (8%) was due to climatic factors of particular region. Our findings supported by the studies of Ehlers and Peters [5] who reported temperature as one of the major climatic factors regulating temporal and spatial distribution of EPNs. Low occurrence EPNs from the region might be due to prevalence of high atmospheric temperature, low relative humidity and long dry spell, resulting in the suppression of growth and development of the EPNs in the soil. Present results were further supported by Gaugler [9], who reported that IJs of EPNs were very sensitive to environmental extremes such as temperature and relative humidity. Hence, high atmospheric temperature and long dry spell during summer season in Uttar Pradesh may be influencing the spatial distribution of EPNs.

Our results of morphological and morphometric analysis of isolates were closely resembled the original description of *Steinernema siamkayai* measurements of different stages of *Steinernema* species based on their length of IJs which was less than 600µm and 428-480µm in the present study, Head to excretory pore 31-365µm and Tail length 28-41µm (Table 2). The first generation of male curved posteriorly, J -shaped when heat killed and first generation of male, total length average was 1,135 µm whereas 1085-1215µm in the present study, spicule 74-81µm and gubernaculum 49-65µm (Table 4), and narrow, thus these observations are closely agreed with the original description of *S. siamkayai* by Stock *et al.* [34]. The accurate diagnosis of these EPN isolates was supported by incorporating molecular characterization. DNA sequence and phylogenetic analysis of entomopathogenic nematodes provided additional criteria for identifying and delimiting species within *Steinernema* and analyses of ITS rDNA sequences also have been used to reconstruct phylogenetic relationships and delimit nematode species with similar morphology [1, 17, 25]. Sequences of the internal transcribed spacer (ITS) regions of *Steinernema* species have been used by different authors [27, 28] in taxonomic and phylogenetic studies. In the present results showed that, isolated EPNs ITS region sequences were matched and aligned with *S. siamkayai*, when checked with the BLAST searches analysis with available sequences in NCBI GenBank these and sequenced data were deposited under accession number MH260074.1, MH260075.1, MH260076.1, MH260077.1, MH260078.1, in the GenBank. Sequenced data had close relationship with *S. Siamkayai* and, *S. abbasi*. Sequence analysis of ITS and 18S rRNA gene well supported the study of Hussaini *et al*., [14]. Identification and description of some Indian populations of *Steinernema* species, in the present study, *Steinernema* species were recovered from different crop rhizospheres of Uttar Pradesh. This is in line with different *Steinernema* spp. reported from India [7] which was confirmed by Khan and Haque [21] who reported that, *Steinernema* species *was* the predominant species in Western Uttar Pradesh. *Steinernema siamkayai* was found to be pathogenic to *H. armigera* pupae at all nematode concentrations; however, the dose mortality relationship was not linear. Decrease in pathogenicity with age of larvae and pupae of *Pseudaletia unipuncta* Haworth (Lepidoptera: Noctuidae) to *S. Carpocapsae* was also reported by Medeiros *et al*., (2000).

Table 1: Details of Entomopathogenic nematodes isolates collected from different locations

Isolate code	Geographical origin	Crop habitat	GPS coordinates		EPN Species	Soil texture
			Longitude (N)	Latitude (E)		
IIPR01	IIPR main farm, Kanpur	Chickpea	26°48.89'	080°27.383'	*Steinernema sp.*	Sandy loam
IIPR02	IIPR NRF, Kanpur	Field pea	26°52.24'	080°24.981	*Steinernema sp.*	Sandy clay
IIPR03	CSAU Vegetable farm,	Field pea	26°49.802'	080°27.179	*Steinernema sp.*	Sandy loam

	Kanpur					
IIPR04	Mallapura	Cowpea	27°31.329'	080°48.720'	*Steinernema sp.*	Sandy loam
IIPR05	Bhawanipur	Banana	26°29.629'	080°16.398'	*Steinernema sp.*	Clay soil

Table 2: Morphometric characters of infective juveniles (IJs) of *S. siamkayai* (n=10) (Mean and Range, all measurements in µm)

Characters	Entomopathogenic Nematode isolates and their NCBI GenBank Accession no.				
	IIPR01	IIPR02	IIPR03	IIPR04	IIPR05
	MH260074	MH260075	MH260076	MH260077	MH260078
Total body length (L)	450.2 (440-460)	460.5 (428-470)	475.3 (470-482)	490.5 (445 - 480)	470.4 (430 -480)
Greatest width (W)	20.1 (17-22)	21.5 (19.24)	20.2 (17-22)	21.2 (18 - 22)	21.5 (18 - 22)
Excretory pore (EP)	31.2 (31-36)	37.2 (30-37)	34.5 (33-36)	35.5 (35 - 36)	35.5 (31 - 36)
Esophagus length (LS)	98.2 (95-102)	105.3 (93-108)	102.2 (90-106)	106.2 (91 - 108)	103.2 (91 -105)
Tail length (TL)	36.2 (32-43)	40.2 (28-40)	40.5 (34-40)	42.2 (32 - 42)	41.5 (32 - 41)

Table 3: Morphometric characters of first generation females of *S. siamkayai* (n=10) (Mean and Range, all measurements in µm)

Characters	Entomopathogenic Nematode isolates and their NCBI Gen Bank Accession no.				
	IIPR01	IIPR02	IIPR03	IIPR04	IIPR05
	MH260074	MH260075	MH260076	MH260077	MH260078
Total body length (L)	1140.5 (1101-1189)	1130.3 (1130-1215)	1141.5 (1092-1193)	1135.4 (1090-1190)	1130.8 (1085-1189)
Greatest width (W)	128.5 (111 -152)	126.2 (122 - 153)	129.8 (121 - 149)	130.5 (111- 144)	128.5 (109- 138)
Excretory pore (EP)	50.6 (48 - 61)	58.2 (50-64)	60.2 (49 - 60)	59.2 (48- 60)	55.2 (48 - 60)
Esophagus length (LS)	134.5 (127 -142)	134.5 (132 - 138)	136.5 (128 - 142)	138.5 (132 - 145)	136.5 (132-138)
Tail length (TL)	24.5 (21 - 32)	26.2 (26 - 32)	22.9 (21 - 32)	25.9 (21 - 33)	23.1 (22 - 30)

Table 4: Morphometric characters of first generation male of *S. siamkayai* (n=10) (Mean and Range, all measurements in µm)

Characters	Entomopathogenic Nematode isolates and their NCBI GenBank Accession no.				
	IIPR01 (MH260074)	IIPR02 (MH260075)	IIPR03 (MH260076)	IIPR04 (MH260077)	IIPR05 (MH260078)
Total body length (L)	1139.5 (1101-1189)	1132.5 (1130-1215)	1142.5 (1092-1193)	1132.4 (1090-1190)	1142.8 (1085-1189)
Greatest width (W)	124.2 (111 -152)	124.3 (122 - 153)	122.8 (121 - 149)	129.8 (111- 144)	123.5 (109- 138)
Excretory pore (EP)	55.6 (48 - 61)	57.2 (50-64)	58.5 (49 - 60)	59.2 (48- 60)	54.4 (48 - 60)
Esophagus length (LS)	136.5 (127 -142)	137.5 (132 - 138)	135.5 (128 - 142)	137.5 (132 - 145)	135.5 (132-138)
Tail length (TL)	25.1 (21 - 32)	25.2 (26 - 32)	24.9 (21 - 32)	24.9 (21 - 33)	24.1 (22 - 30)
Spicule length (SPL)	77.1 (76- 81)	76.9 (76 - 80)	74.5 (75 -78)	76.2 (74 -79)	74.5 (74 - 80)
Gubernaculum (GuL	54.5 (49-61)	55.5 (50-65)	55.2 (49-63)	55.5 (51-62)	55.4 (51-60)

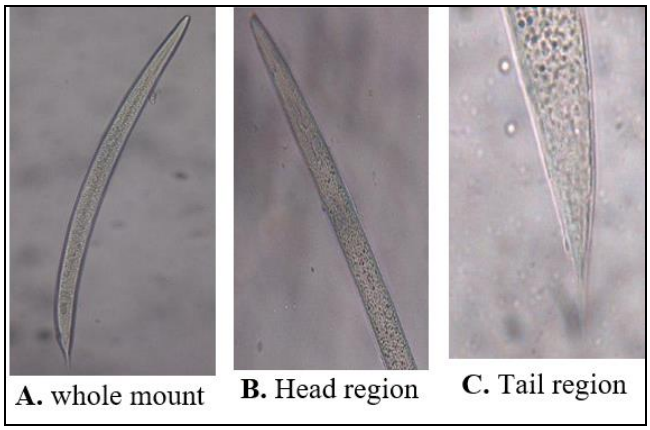

Fig 1: Morphology of Infective Juveniles (IJs) of *Steinernema siamkayai*

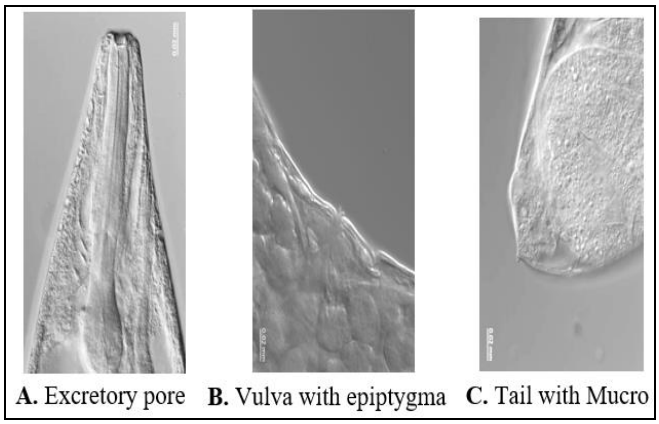

Fig 2: Morphology of first generation female

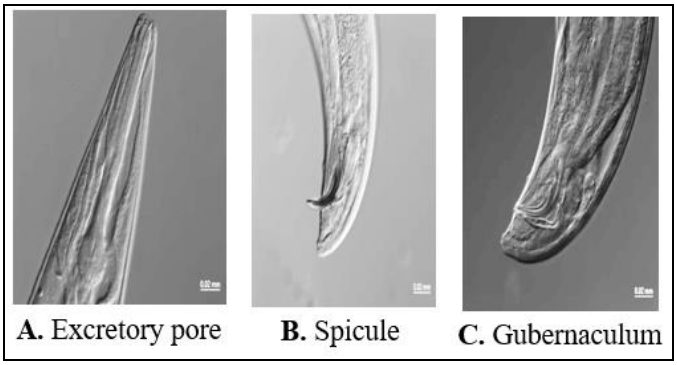

Fig 3: Morphology of first generation male

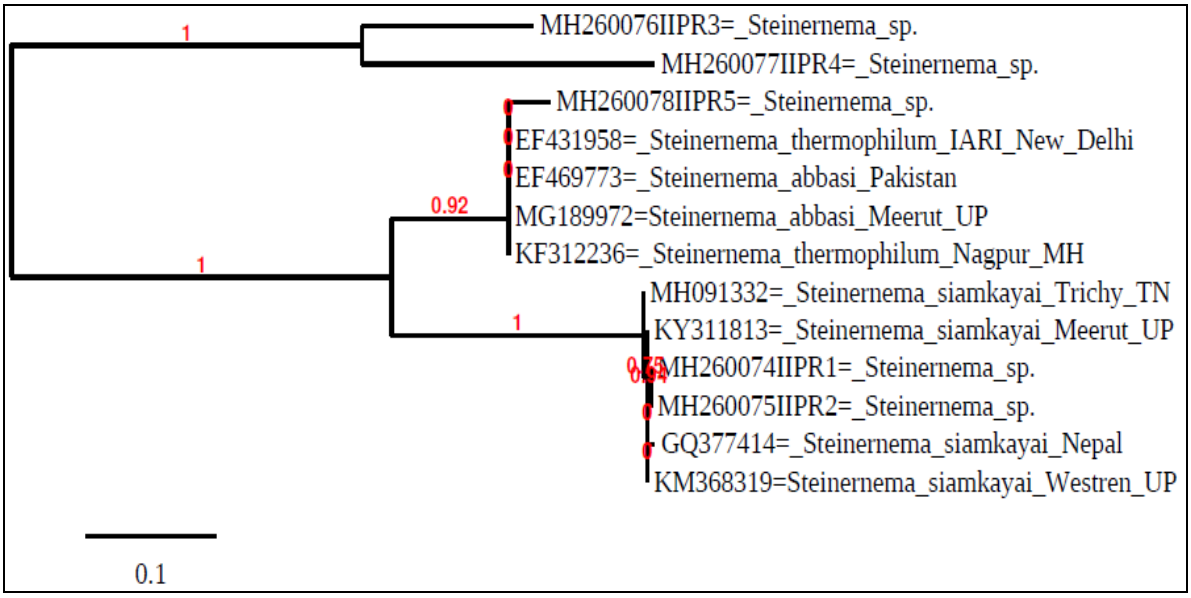

Fig 4: Phylogenetic relationships of *Steinernema* species based on ITS rDNA regions.

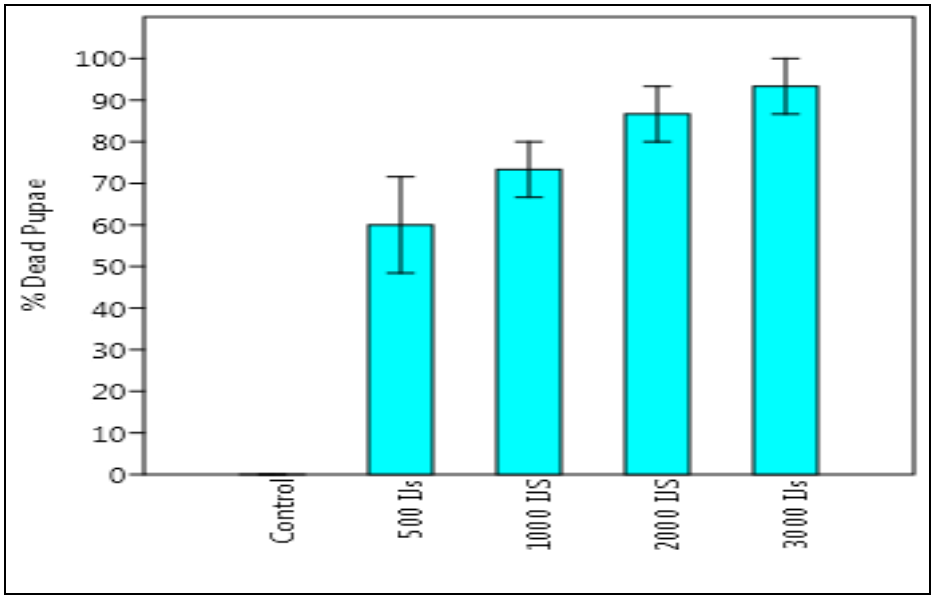

Fig 5: Pathogenic potential of *S. siamkayai* against pupae of *H. armigera*

Conclusion

On the basis of results obtained in the present investigation, it may be concluded that, the native isolates of EPNs are better adapted to the local agro-climatic conditions. The search for local EPN isolates through systematic survey, identification and taxonomic characterization are the first critical step in building an effective biological management strategy. The isolated EPNs species could be potentially useful in integrated pest management (IPM) program of *H. armigera* to develop new commercial strains adapted to the local environment.

Acknowledgement

The authors express their gratitude to the Director, ICAR-Indian Institute of Pulses Research (IIPR), for providing all the facilities and Indian Council of Agricultural Research, New Delhi for the financial support.

References

1. Adams BJ, Burnell AM, Powers TO. A phylogenetic analysis of Heterorhabditis (Nematoda: Rhabditidae) based on internal transcribed spacer 1 DNA sequence data. Journal of Nematology. 1998; 30:22-29.
2. Akhurst RJ. Morphological and functional dimorphism in *Xenorhabdus* spp. bacteria symbiotically associated with the insect pathogenic nematodes Neoplectana and Heterorhabditis. Journal of General Microbiology. 1998. 121:303-309.
3. Bedding RA, Akhurst RJ. A simple technique for the detection of insect parasitic nematodes in soil. Nematologica, 1975; 21:109-110.
4. Boemare NE, Laumond C, Mauléon H. The nematode-bacterium complexes: biology, life cycle, and vertebrate safety. Biocontrol Science and Technology. 1996, 333-345.
5. Ehlers RU, Peters A. Entomopathogenic nematodes in biological control: feasibility, perspectives and possible risks. In: Biological Control: Benefits and Risks, Hokkanen, H. M.T. and Lynch J.M. (Eds.), Cambridge, UK Cambridge University Press, 1996, 119-135.
6. Ganguly S, Singh LK. *Steinernema thermophilum* sp. n. (Rhabditida: Steinernematidae) from India. International Journal Nematology. 2000; 10:183-191.

7. Ganguly S. Taxonomy of entomopathogenic nematodes and work done in India. Hussaini SS, Rabindra RJ and Nagesh M. (eds.). In: Current Status of Research on Entomopathogenic nematodes in PDBC, Bangalore, ICAR. 2003, 69-108.
8. Gaugler R, Han R. Production technology. In: Gaugler R (ed.), Entomopathogenic nematology. Wallingford, UK, CAB International. 2002, 289-310
9. Gaugler R. Biological control potential of neoaplectanid nematodes. Journal of Nematology. 1981; 13:241-249.
10. Grewal PS, Peters A. Formulation and quality. In: Nematodes as biocontrol agents, Grewal, P.S., Ehlers, R.U. and Shapiro, I.D. (eds.), Wallingford: CABI Publishing, 2005, 79-90.
11. Hominick WM, Reid AP, Bohan DA, Briscoe BR. Entomopathogenic nematodes - biodiversity, geographical distribution and the convention on biological diversity. Biocontrol Science Technology. 1996; 6:317-331.
12. Hominick WM. Biogeography. In: Entomopathogenic Nematology, Gaugler, R. (ed.), CABI Publishing, London, UK, 2002, 115-144.
13. Hunt DJ. Overview of taxonomy and systematics. Entomopathogenic Nematodes: Systematics, Phylogeny and Bacterial Symbionts. Nematology Monographs and Perspectives, 2007. Brill, Leiden-Boston, USA.
14. Hussaini SS, Ansari, MA, Ahmad W, Subbotin SA. Identification of some Indian populations of *Steinernema* species (Nematoda) by RFLP analysis of ITS region of rDNA. International Journal of Nematology. 2001; 11:73-76.
15. Hussaini SS. Enhanced storage stability of an entomopathogenic nematode in India. In: Current Status of Research on Entomopathogenic Nematodes in India, Hussaini, SS, Rabindra RJ, Nagesh, M. (eds.), PDBC, Banglore, 2003, 27-68.
16. Joyce SA, Burnell AM, Powers TO. Characterisation of Heterorhabditis isolates by PCR amplification of segments of mtDNA and rDNA genes. Journal of Nematology. 1994; 26:260-270.
17. Kaplan DT, Thomas WK, Frisse LM, Sarah JL, Stanton JM, Speijer PR *et al*. Phylogenetic analysis of geographically diverse *Radopholus similis* via rDNA sequence reveals a monomorphic motif. Journal of Nematology. 2000; 32:134-142.
18. Karimi J, Kharazi-Pakdel A, Yoshiga T, Koohi-Habibi M. Introduction of *Steinernema carpocapsae* Weiser, 1955 (Rhabditida: Steinernematidae) from natural population of white grub, *Polyphylla olivieri* (Coleoptera: Melolonthidae) from Iran. Journal of Agricultural Faculty of Uludag University. 2010; 24(1):47-54.
19. Kaya HK, Gaugler R. Entomopathogenic nematodes. Annual Review of Entomology. 1993; 38:181-206.
20. Kaya HK, Stock SP. Techniques in insect Nematology. In: Lacey LA (ed.), Manual of techniques in insect pathology. San Diego, CA, Academic Press. 1997, 281-324
21. Khan MR, Haque Z. Occurrence of entomopathogenic nematodes in districts of Western Uttar Pradesh, India. Indian journal of nematology. 2010; 40**(**2):264-266.
22. Lalramliana, Yadav AK. Occurrence of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Meghalaya, NE India. Science Vision. 2010; 10(3):89-100.
23. Medeiros J, Rosa JS, Tavares J, Simões N. Susceptibility of *Pseudaletia unipuncta* (Lepidoptera: Noctuidae) to entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) isolated in the Azores: effect of nematode strain and host age. Journal of Economic Entomology. 2000. 93:1403-1408.
24. Mwaniki SW, Nderitu JH, Olubayo F, Kimenju JW, Nguyen K. Factors influencing the occurrence of entomopathogenic nematodes in the Central Rift Valley Region of Kenya. African Journal of Ecology. 2008; 46:79-84.
25. Nadler SA, Adams BJ, Lyons ET, DeLong RL, Melin SR. Molecular and morphometric evidence for separate species of Uncinaria (Nematoda: Ancyclostomatidae) in California sea lions and northern fur seals: Hypothesis testing supplants verification. Journal of Parasitology. 2000; 86:1099-1106.
26. NCBI. NCBI taxonomy database. National Center for Biotechnology Information, U. S. National Library of Medicine, Bethesda, M.D. USA. Retrieved from May 2015, from http://www.ncbi.nlm.nih.gov/taxonomy.
27. Nguyen KB, Duncan LW. Steinernema diaprepesi n. sp. (Rhabditida: Steinernematidae), a parasite of the citrus root weevil, *Diaprepes abbreviates* (L) (Coleoptera: Curculionidae). Journal of Nematology. 2002; 32:159-170.
28. Nguyen KB, Maruniak J, Adams BJ. Diagnostic and phylogenetic utility of the rDNA internal transcribed spacer sequences of Steinernema. Journal of Nematology. 2001; 33:73-82.
29. Nikdel M, Niknam G. Morphological and molecular characterization of a new isolate of entomopathogenic nematode, *Steinernema feltiae* (*Filipjev*) from Arasbaran forests, Iran, Journal of Asia-Pacific Biodiversity, doi: 10.1016/j.japb.2015.04.008
30. Prasad GS, Ranganath HR, Singh PK. Occurrence of the entomopathogenic nematode in parts of south Andamans. Current Science. 2001; 80:501-502
31. Raja RK, Sivaramakrishnan S, Hazir S. Ecological characterization of *Steinernema siamkayai* (Rhabditida: Steinernematidae), a warm adapted entomopathogenic nematode isolate from India. Bio Control 2011; 56:789 798
32. Rajkumar Rachana KE, Sabana AA, Shahin S, Subaharan K. Molecular identification of Entomopathogenic nematodes of North Kerala. Abstract of papers of National Seminar on Applications of Bioinformatics in Agriculture at CPCRI, Kasaragod.2013.
33. Reid AP, Hominick WM. Restriction fragment length polymorphisms within the ribosomal DNA repeat unit from a British entomopathogenic nematode (Steinernematidae) and its potential for species identification. *Parasitology*, 1992; 105:317-323.
34. Stock SP, Somsook V, Reid AP. *Steinernema siamkaya*in. sp. (Rhabditida): Steinernematidae), an entomopathogenic nematode from Thailand. Systematic Parasitology. 1998; 41:105-113.