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Occurrence, Identification and Mass Culturing of Native Isolates of Entomopathogenic Nematodes (EPNs)

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ABSTRACT: Many rhabditid nematodes like Metarhabditis belonging to the bacteriophage group are considered to be necromenic associates of insects and used to be facultative entomopathogenic nematodes (EPNs). In the present study, for isolation of EPNs, a total of 280 soil samples were collected from different agricultural localities in three district of Haryana during 2019-2020. Out of them, 83 soil samples contained EPNs with 29.6 per cent frequency of occurrence. The insect pathogenicity of nematodes was tested by "*Galleria mellonella* baiting" method. On the basis of morphological and morphometric characterization, these isolates were identified as *Metarhabditis amsactae*, (Nematoda: Rhabditidae). *Metarhabditis amsactae* was characterized by acuticular striations relatively fine in male. Reproductive system monorchic, testis single reflexed. Spicules paired and symmetrical, ventrally arcuate. Gubernaculum well-developed, oval shaped. Bursa leptoderan with eight pairs of genital papillae. Third-stage juvenile ensheathed in cuticle of second stage juveniles. Sheath free anteriorly in third-stage juveniles, firmly bound to the posterior region of the body. These nematodes can be mass culturing *in vivo* and *in vitro*, and safely used against various insect pests of different crops and vegetables.

Keywords: Entomopathogenic nematodes, *Metarhabditis amsactae*, *Galleria mellonella*, frequency, mass culturing.

INTRODUCTION

Entomopathogenic nematodes (EPNs) are those nematodes that have a facultative or obligate parasitic association with insects. Parasitic associations with insects have been described from 23 nematode families. Seven of these families contain species that have potential for biological control of insects (Koppenhofer and Kaya 2001). The most commonly found EPN species belong to the families Allantonematidae, Mermithidae, Steinernematidae and Heterorhabditidae. The EPNs in the families of Steinernematidae and Heterorhabditidae (Kaya and Moon 1978; Woodring and Kaya 1988) are potential virulent agents because of their symbiotic association with bacteria *Xenorhabdus* spp. (Steinernematids) and *Photorhabdus* spp. (Heterorhabditids), respectively (Kaya, 1990). These bacteria are gram negative, non-spore formers, having four resistant stages, generally non-pathogenic when ingested by a host and are also incapable of penetrating a host and kill the host insect by causing septicemia within 24-48 h. EPNs have ubiquitous distribution (Hominick *et al*., 1996) and their presence in cultivated areas is more frequent followed by forest ecosystems. Occurrence and distribution of EPNs has been recorded in different local geographical locations (Canhilal *et al.*, 2017; Kalita *et al.*, 2019). Surveys for EPNs have been conducted in South Africa, Ethiopia, Southeast Asia, Indo-Malaysian region and tropical areas as well as parts of Nigeria (Aliyu *et al*., 2015). *H. indica* was recorded from India by Poinar *et al*. (1992). Since then, various species of *Steinernema* have been recorded, of which some are new to the science (Ganguly and Singh 2000; Prasad *et al.*, 2001). Genus Metarhabditis (rhabditids) was described in 2004 with type species *M. andrassyana* (Tahseen *et al.*, 2004). Sudhaus (2011) revised the family rhabditidae in which many species were transferred from and into different genera. Some species from Rhabditis (*R. rainai* Carta and Osbrink 2005; *R. costai* and *R. freitasi* Martins Júnior (1985) and other from *Oscheius* (*O. amsactae* Ali *et al.*, 2011) were transferred to *Metarhabditis.* Species of the genus *Oscheius* was synonymized with *M. amsactae* (*O. ciceri*, *O. hussaini* Shaheen *et al*. (2011); *O. gingeri* Pervez *et al.* (2013). In the last three decades, many EPNs have been isolated in various habitats all over the world, revealing hundreds of new isolates and the list of many new species (Hominick, 2002) continues to increase every year. To date, around 100 valid species of *Steinernema* and 21 species of *Heterorhabditis* have been identified from different countries of the world (Bhat *et al*., 2020) and commercialized for use in

biological control (Shapiro-Ilan *et al*., 2018). Apart from *Heterorhabditis* and *Steinernema*, recently, several species from *Oscheius* were confirmed as EPNs (Ye *et al*., 2018; Castro-Ortega *et al*., 2020). The life cycle and development of EPNs takes approximately 1-3 week depending on the EPN species, host and environmental conditions (Campos-Herrara *et al.*, 2015) and varies among species to species and strain to strain (Hazir *et al.,* 2001). Generally, EPNs have been recovered from soil samples using insect baiting trap method (Bedding and Akhurst 1975). These nematodes can easily be mass multiplied *in vivo* and *in vitro* production systems.

METHODOLOGY

Present studies were carried out under laboratory conditions in the Department of Nematology, Chaudhary Charan Singh Haryana Agricultural University, Hisar. For isolation of EPNs, surveys of different agricultural areas were carried out during 2019 to 2020. Sampling was done by taking soil samples from five to six random sites at the depth of 8 to 10 cm and all the five subsamples were bulked to make composite sample of about 500 g with proper labeled code. The soil samples were moistened by using distilled water after remove any type of debris to facilitate the movement of nematodes.

Maintenance of Greater wax moth (*Galleria mellonella*). Nematodes were cultured on larva of Greater wax moth, *G. mellonella* (Woodring and Kaya 1988). The culture of Greater wax moth was maintained on artificial diet (Plate-1) under laboratory conditions consisting of following ingredients.

— Wheat flour : 100 g
— Corn flour : 200 g
— Wheat bran : 100 g
— Milk powder : 100 g
— Yeast extract powder : 50 g
— Honey : 175 ml
— Glycerin : 175 ml

All the above ingredients were mixed thoroughly in a bowl. Then, honey and glycerin were added in separate container which were added to the flour mixture and mixed thoroughly. Healthy larvae of *G. mellonella* were released in the rearing jars which were placed in an incubator at 25 ± 1ºC, containing diet and allowed to continue their life cycle and development (Plate-1, A). Later on, pupae of *G. mellonella* were separated from the diet and were kept separately in plastic containers for emergence of adults. The adults were collected daily and transferred into another glass jar for laying eggs on a white card board with folded paper strips to provide sites for egg laying (Plate-1, B). The jars were again kept in a BOD incubator at 25±1ºC. Cotton dipped in honey solution was kept as food for adults of *G. mellonella*.

Plate 1. Larvae of *Galleria mellonella* reared on artificial diet (A) and eggs of *G. mellonella* laid on plastic trays (B), isolation of EPNs from soil using *G. mellonella* larvae as bait (C) and isolation of EPNs from cadaver of *G. mellonella* by white trap method (D)

Bioassay for occurrence of EPNs. The insect pathogenicity of nematodes was tested by "*Galleria mellonella* baiting" method (Bedding and Akhurst 1975). The soil samples were mixed properly and 8-10 larvae of forth instars of *G. mellonella* were placed in 250 ml jam bottles (Plate-1, C) which were then filled with collected soil up to the brim. The jam bottles were inverted upside- down in the laboratory to recover EPNs. The *G. mellonella* larvae were infected with the EPNs, present in soil samples and the jam bottles were checked on 2nd, 4th, 6th, 8th and 10th day, if any larva was found dead, then dead larvae (cadaver) were removed from soil and washed with double distilled water to remove soil particles and disinfected with 0.1 per cent sodium hypochlorite before being placed on White traps (White, 1927) to obtain emerging infective juveniles (IJs) while live larvae were placed in same soil in jam bottle. The cadavers were placed on the moist filter paper on watch glass in Petri plates to come in contact with the water (Plate-1, D). The cadavers were incubated at 25± 2 ºC in an incubator and check daily for emergence of third stage juveniles from the cadavers until all nematode progeny emergence ceased after 15-20 day and moved down into the water in the Petri plate. The harvested EPNs were sterilized with 0.1 per cent sodium hypochlorite and washed with double distilled water, released in tissue culture flask with distilled water to a depth of one cm at 16 ºC for further experimentation.

Identification of EPNs bymorphometric observations. One ml nematode suspensions containing 200 IJs were used to inoculate the larvae of *G. mellonella* for isolation of 1st and 2nd generation males and females. After two days of mortality of *G. mellonella* larvae, cadavers were dissected in Ringer's solution (Woodring and Kaya 1988) to extract first generation males and females. Some of the cadavers were kept for further extraction of 2nd generation male and females. The first and second generation males and females and third stage IJs were heat-killed in Ringer's solution and fixed in (Triethanolamine- 2 ml + 37 % Formalin- 8 ml + sterile distilled water- 90 ml) TAF

(Courtney *et al*., 1955). The fixed nematodes were infiltrated in glycerol by processing them in Seinhorst's solution I and II (Seinhorst, 1959). Cleared specimens were mounted in dehydrated glycerine using glass wool. Observations were taken by phase contrast microscope from Department of pathology, CCS HAU, Hisar.

Following morphometric characters were used for identification

L = Body length
MBD = Maximum body diameter
EP = Distance from anterior end to excretory pore
ES = Pharynx length
T = Tail length
ABD = Anal body diameter
a = L/MBD
b = L/ES
c = L/T
c' = ABD/T
V = Distance from anterior end to vagina
SL = Spicule length
GL = Gubernaculum length
D % = EP/ES × 100
E % = EP/T × 100
SW % = SL/ABD × 100
GS % = GL/SL × 100

***In vivo* culturing of EPNs isolates and their storage.** Pure culture of individual isolates of EPNs was maintained on late instar larvae of *G. mellonella* under laboratory conditions. The IJs were extracted from white trap method, washed well with 0.05 per cent formaldehyde and stored in thin layer of distilled water in tissue culture flasks. The nematode numbers were kept about 10,000 IJs/ml of distilled water in flasks. IJs in tissue culture flasks were stored in BOD at 16 ^{o}C that served as inoculum for further experiments.

***In vitro* culturing of EPNs using Wout's medium-I.** Two most virulent strains obtained from *in vivo* culturing, were selected for *in vitro* culturing. Prepared nutrient agar (35 g) and nutrient broth (13 g) were weighed and mixed in one litre distilled water separately, gently heated by stirring uniformly. The medium was poured in conical flasks plugged with cotton and autoclaved at 121.6 ^{o}C for 15 to 20 minutes. The ingredients of Wout's medium-I *viz.,* nutrient broth- 0.88 g, yeast extract- 0.32 g, corn flour - 14.4 g and groundnut oil - 10.4 g and distilled water - 60 ml were weighed according to the requirement and mixed in distilled water. The medium was gently heated and stirred uniformly. Sponge material (foam) was cut into small pieces (1x1 cm^3), washed thoroughly with distilled water 2 to 3 times and dried at room temperature. Medium was poured on the sponge, so that it was coated uniformly onto the sponge. Six to eight media coated sponges were put in 250 ml conical flasks. The mouth of the flasks were cleaned to remove the adhered medium and plugged tightly with non absorbent cotton. These flasks were autoclaved at 121.6 ^{o}C for 30 minutes. After autoclaving, the flasks were allowed to cool before use.

Isolation of symbiotic bacteria from the haemolymph of infected *G. mellonella*. Symbiotic bacteria were isolated from the haemolymph of *G. mellonella* larvae (Kaya and Stock, 1997). Under a laminar flow sterile bench, last instar larvae of *G. mellonella* were placed in a Petri dish with moist filter paper and inoculated with surface sterilized IJs separately for each nematode strain (100 IJs per larva). The cadaver was dipped in absolute ethanol, ignited and immediately plunged into sterile water. Using the sterile injection needles the cadaver was carefully opened from the ventral side. Then using a sterile inoculation loop, the haemolymph of the cadaver was slightly touched and transferred to the nutrient agar plates by streaking in a zig zag manner near the burner. The Petri plates were then properly labelled, put in polythene cover and kept at 25 to 28± 1^{o}C for 48 h in BOD incubator.

Multiplication of bacteria in nutrient broth and surface sterilization of IJs. In the laminar flow chamber, the single colony of bacterium in the nutrient agar was gently touched with sterile inoculating loop and transferred to the nutrient broth in 250 ml flasks. The flasks inoculated with the bacterium, were wrapped with black paper, kept on the mechanical shaker for continuously stirring at room temperature for 24 to 48 h. After 48 h bacterium culture were inoculated to the media in flasks at 0.5 ml per flask, kept in dark at 28± 1^{o}C in a BOD incubator. The IJs were surface sterilized by adding one ml 0.25 per cent (w/v) Hymaine (methylbenzethonium chloride) to the suspension and washed with sterile water 2 to 3 times in the laminar air flow chamber. With a sterile micropipette, most of the supernatant was removed without disturbing the IJs at the bottom of the tube. Each step was started with enough IJs to compensate for some loss during the process. At the final step the surface sterilized IJs (2000 IJs/ ml) were collected in a beaker with sterile distilled water.

Inoculation of IJs on media and their harvesting. The nematodes were inoculated in flasks @ 2000 IJs per flask (250 ml) under aseptic conditions. The cotton plug flasks were wrapped with black paper to ensure dark conditions and incubated at 25± 1 ^{o}C for 30 and 60 days. The flasks were checked for contamination on every alternate day. Each treatment was replicated four times. After the incubation for 30 and 60 days at 25± 1 ^{o}C, the IJs were extracted from the Wout's medium I by pouring out the coated sponge media in 400 mesh sieve placed in the water filled basin for 1 h. The IJs were collected from the basin and concentrated in a beaker. The nematodes were counted under stereoscopic microscope by the dilution method and the number of IJs in each flask was recorded.

RESULTS AND DISCUSSION

The main objectives of the present investigations were to isolation and identify native EPN species with excellent strains so that they may be used for successful bio-control of various insect pests in different parts of Haryana. Three districts *viz*., Palwal, Mewat and Hisar

were intended to obtain a total of 280 soil samples that represented various soil types and irrigation systems. These samples were then processed to the soil baiting technique with *G. mellonella*, a widely preferred host for isolating EPNs from soil. Out of 280 soil samples, 83 (29.6 %) were found to have *G. mellonella* infestations with EPNs, according to the data. The highest frequency of occurrence of EPNs (67.5 %) was recorded in Ber orchard of horticulture research farm, CCS HAU, Hisar. In the present study, the infestation in ber orchard was more where the possibilities of soil borne insects are more. Samples collected from ber orchard in Palwal and Mewat districts showed low frequency of occurrence of EPNs *i.e.* 44.5 and 40 per cent, respectively. This low recovery rate is congruent with several previous studies on EPNs recovery in different surveys worldwide (Caoili *et al.*, 2018; Kour *et al*., 2020). This also is supported by Kumar *et al*., 2021 that collected 313 soil samples from different districts of Haryana during 2018-2021, 99 samples (31.6 %) were found to be positive for the EPNs. Maximum frequencies of occurrence of EPNs was found in ber orchards followed by sugarcane, wheat and cluster been, as only 71.1, 37.5, 35.7 and 35.3 percent, respectively.

Table 1: Occurrence of entomopathogenic nematodes (EPNs).

Sr. No.	Crops	Location	District	Sample Collected	Sample contained EPNs	Per cent Frequency of occurrence
1.	Guava	Horticulture research farm, CCS HAU	Hisar	9	0	0
2.	Guava	Mewat	Mewat	10	04	40
3.	Ber	Horticulture research farm, CCS HAU	Hisar	37	25	67.5
4.	Ber	Palwal	Palwal	09	04	44.5
5.	Ber	Sonkh	Mewat	10	04	40
6.	Sapota	Horticulture research farm, CCS HAU	Hisar	12	0	0
7.	Citrus	Horticulture research farm, CCS HAU	Hisar	15	03	20
8.	Citrus	Arya nagar	Hisar	08	01	12.5
9.	Citrus	Balsamand	Hisar	08	02	16.6
10.	Citrus	Malapur	Hisar	10	0	0
11.	Citrus	Ludas	Hisar	10	0	0
12.	Grapes	Horticulture research farm, CCS HAU	Hisar	10	0	0
13.	Cotton	Plant breeding research farm, CCS HAU	Hisar	15	05	33.4
14.	Cotton	Punanha	Mewat	15	05	33.4
15.	Cotton	Hathin	Palwal	15	07	46.7
16.	Forest area	Forest area, CCS HAU	Hisar	18	02	11.1
17.	Cactus	Botanical garden, CCS HAU	Hisar	12	02	16.7
18.	Rose	Nematology research farm, CCS HAU	Hisar	10	0	0
19.	Sesame	Plant breeding research farm, CCS HAU	Hisar	10	0	0
20.	Okra	Nematology research farm, CCS HAU	Hisar	20	13	65
21.	Pigeon pea	Punanha	Mewat	09	02	22.3
22.	Brinjal	Punanha	Mewat	06	02	33.4
23.	Cluster bean	Punanha	Mewat	07	02	28.5
	Total			**280**	**83**	**29.7**

Out of 51 samples collected from citrus, only six samples (11.6 %) showed presence of EPNs. Ten soil samples collected from guava orchards in Mewat district, of which four samples contained EPNs. The frequency of occurrence of EPNs in cotton field was 33.4 per cent in both Hisar and Mewat districts which were less than Palwal district *i.e.* 46.7 per cent. The frequency of occurrence of EPNs in forest area and cactus was very low *i.e.* 11.1 and 16.7 per cent, respectively. Similarly, Kalita *et al.* (2019) conducted a survey in Assam and found eight soil samples were positive for EPNs with frequency of four per cent from cultivated and uncultivated fields. Soil samples recorded two per cent *Steinernema* spp., one per cent of *Heterorhabditis* spp. and one per cent of *Oscheius* spp.

In present study the occurrence of EPNs was found more in perennial crops than in annual crops. This is due to more insect pest buildup in undisturbed soil environment areas. In field crops, frequency of occurrence of EPNs was 65 per cent in okra followed by 46.7, 33.4, 28.5 and 22.3 per cent in cotton, brinjal, cluster bean and pigeon pea, respectively. Fifty one soil samples *viz.*, sapota, grapes, sesame, rose and twenty soil samples of citrus from Ludas and Malapur did not show presence of EPNs. Dichusa *et al.* (2021) also reported similar findings about occurrence of EPNs in different ecosystems and found that only five samples were positive to EPNs with 10 percent frequency. Only one EPN comes under the genera *Heterorhabditis* and other four comes under the genera *Metarhabditis* and *Oscheius*. Analysis of D2D3 segments of the 28S rDNA confirmed high sequence similarity to *Heterorhabditis indica*, *Metarhabditis rainai*, *Oscheius insectivora*, and *Oscheius* sp. Gowda *et al*. (2020) also recovered 3 soil samples containing EPNs out of 130 samples during a survey of Uttar Pradesh, India from 2016-2017 with frequency of 2.3 percent. Out of 200 soil samples collected from 40 soil samples in Thialand and found the prevalence of EPNs was 8.0 per cent (Ardpairin *et al*., 2020).

Identification of native strains of entomopathogenic nematodes (EPNs). After isolation, identification is an important part of the study on EPNs. Colour of cadaver gives some clue for identification of these nematodes upto generic level. Morphological and morphometric methods are used for their identification. The EPNs used in present studies were identified as *Metarhabditis amsactae*. Genus *Metarhabditis* was proposed Tahseen *et al*. (2004) with type species *Metarhabditis andrassyana*. This genus *Metarhabditis* differes from *Heterorhabditis* in having metastegostom with knobbed setose denticles and eight pairs of genital papillae on bursa. Identification of six isolates *viz.*, HAR-St-I, HAR-St-II, HAR-St-III, HAR-Ht-I, HAR-Ht-II and HAR-Ht-III, which were selected during culturing on *G. mellonella* larvae in laboratory, were identified as *Metarhabditis amsactae* Sudhaus (2011) on the basis of morphological and morphometric characters. The morphological and morphometric characters of strains HAR-St-II and HAR-Ht-III (Hisar populations) are given in Table 2.

Descriptions of *Metarhabditis amsactae*. Female (Plate 2): Body mostly straight, rarely arcuate. Both outer and inner layers of cuticle striated with striations 1–1.2 µm wide varying with body regions. Lip region almost continuous. Stoma rhabditoid type. Pharynx with cylindrical to weakly swollen corpus, narrow and long isthmus. Nerve ring encircling the anterior half of pharynx. Excretory pore located at pharyngeal length, (112.4- 167.3 µm in strain HAR-St- II and 113.6-145.9 µm in strain –HAR-Ht- III) variable in position ranging from middle of isthmus. Excretory duct distally cuticularized for a length of 8–10 µm. Cardia conoid, Intestinal lumen wide. Reproductive system amphidelphic-didelphic type. Ovaries moderately developed, dorsally reflexed, distal end not reaching to vulval level, anterior ovary slightly larger. Vagina thick-walled, often cuticularized. Position of vulva from anterior end was 382.6-589.6 µm in strain HAR-St- II and 384.2-512.6 µm in strain HAR-Ht- III. Tail elongate conoid. Tail length of both strains were 69.7 - 102.4 µm.

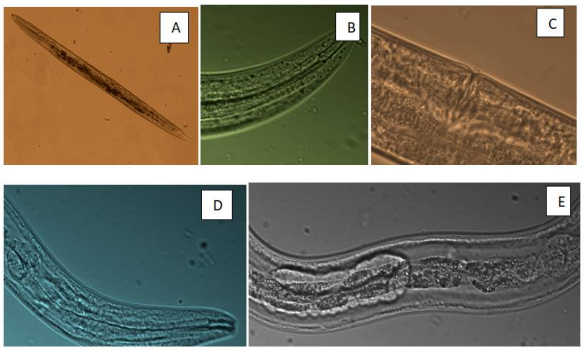

Plate 2. A- Entire female body of *Metarhabditis amsactae*, strain HAR-St-II, B- anterior portion, C- vulval region, D-excretory pore and E.- reflexed ovary.

Male (Plate -3): Similar to female except smaller in size (589.4 -778.7 µm of HAR-St- II and 671.3 -908.5 µm of HAR-Ht- III). Cuticular striations relatively fine. Reproductive system monorchic, testis single reflexed. Spicules paired and symmetrical, ventrally arcuate. Gubernaculum well-developed, oval shaped. Tail conoid with posterior two-thirds abruptly tapering and reduced. Bursa leptoderan, with eight pairs of genital papillae, with genital papillae-1 and genital papillae-2 spaced, precloacal, genital papillae-3 slightly posterior to cloaca in most specimens, pairs genital papillae 4 to 6 located at conoid part of tail and genital papillae-7 to 8 located at posterior part of the bursa.

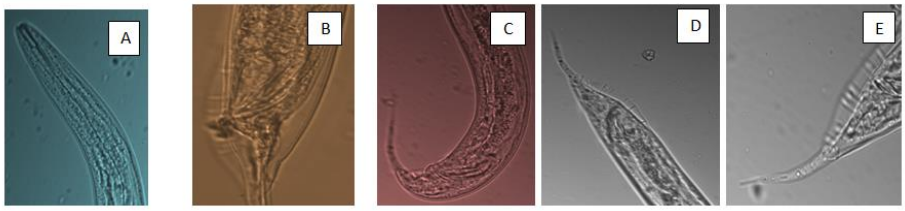

Plate 3. Male of *Metarhabditis amsactae*, strain HAR-St-II A- oesophageal region showing excretory pore, B gubernaculum, C- spicules, D- genital papillae with bursa and E- tail region.

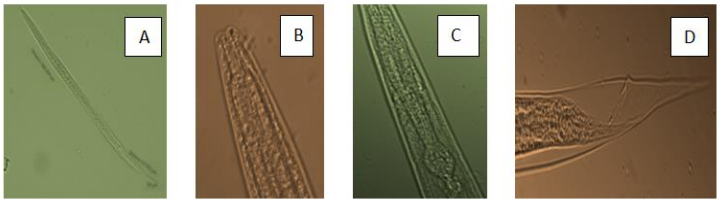

Plate 4. A- entire body of *Metarhabditis amsactae* juvenile, strain HAR-St-II, B- anterior region, C- esophageal region and D- posterior portion of juvenile.

Infective Juveniles (Plate- 4): L= 389.6 - 596.2 µm in HAR-St-II and 311.6- 496.3 µm in HAR-Ht-III. Third-stage juveniles ensheathed in cuticle of second stage juveniles. Sheath free anteriorly in third-stage juveniles, firmly bound to the posterior region of the body. Cuticle with transverse striae. Lip region smooth; Stoma tubular with closed opening. Nerve ring and excretory pore located at isthmus level. Value of c' 0.1-0.2 in HAR-St-II and 0.2- 0.3 in HAR-Ht-III. Tail conoid shape with 39.5-57 µm length which was similar to population of Bhat *et al.* (2020) *i.e.* 48-58 µm.

Table 2: Morphometric characters of Hisar populations of *Metarhabditis amsactae*.

Characters	*Metarhabditis amsactae* strain HAR- St- II (Hisar population)			*Metarhabditis amsactae* strain HAR- Ht- III (Hisar population)		
	Male (10)	Amphidelphic Female (10)	Infective juveniles (10)	Male (10)	Amphidelphic Female (10)	Infective juveniles (10)
L	681.8 ± 64.8 (589.4 -778.7)	921.9 ± 141.8 (721.0 -1146.8)	504.2 ± 60.6 (389.6 - 596.2)	798.2 ±87.9 (671.3 - 908.5)	903.1 ± 98.1 (769.7- 1025.3)	411.1 ± 53.4 (311.6- 496.3)
MBD	42.5 ± 2.7 (38.1 -46.0)	66.4 ± 13.3 (44.8 - 81.7)	25.0 ± 3.9 (17.9 - 30.7)	42.3 ± 8.0 (31.1- 55.3)	50.6 ± 8.2 (39.0- 62.3)	24.0 ± 3.9 (18.1- 29.4)
EP	104.5 ± 1.6 (102.0 -107.0)	136.3 ± 19.6 (112.4 -167.3)	97.2 ± 5.3 (90.3 - 105.7)	121.9 ± 7.6 (111.5- 125.4)	128.8 ± 11.0 (113.6- 145.9)	92.0 ± 9.8 (67.9- 102.4)
ES	124.3 ± 6.5 (105.1-143.7)	177.0 ± 14.4 (154.3 -196.4)	125.0 ± 4.9 (115.3 -131.2)	140.3± 17.5 (123.3- 170.6)	162.5 ± 10.2 (145.1-175.7)	101.2 ± 8.6 (90.0- 110.8)
T	45.8 ± 16.4 (35.1 -69.7)	85.6 ± 10.3 (69.7 -102.4)	115.6 ± 11.3 (95.6 -119.6)	33.0 ± 11.7 (30.8- 57.9)	88.4 ± 12.7 (69.7- 102.4)	50.5 ±5.0 (39.5- 57.0)
ABD	26.2 ± 2.0 (23.5 -29.7)	22.7 ± 4.5 (16.4 -29.1)	13.9 ± 2.3 (9.0 -16.3)	23.9 ± 3.3 (18.6- 28.0)	19.5 ± 1.2 (18.0- 21.3)	13.8 ± 1.3 (11.9- 16.0)
SL	39.4 ± 2.8 (35.4 -44.4)	-	-	52.2 ± 5.6 (39.3- 58.8)	-	-
GL	17.7 ± 1.9 (15.3 -21.6)	-	-	23.4 ± 5.3 (11.7- 30.0)	-	-
VA	-	473.6 ± 74.4 (382.6 -589.6)	-	-	436.4 ± 49.2 (384.2- 512.6)	-
A	15.9 ± 0.5 (15.3 -16.9)	14.0 ± 1.1 (12.6 -16.1)	20.2 ± 0.8 (19.1 -21.6)	19.2 ± 1.9 (16.4- 22.3)	18.0 ± 1.1 (16.5- 19.7)	17.4 ± 2.9 (11.0- 21.4)
B	7.2 ± 0.3 (6.8 -7.6)	5.2 ± 0.4 (4.7 -5.8)	4.0 ± 0.3 (3.3-4.5)	5.7 ± 0.5 (5.1- 6.6)	5.5 ± 0.3 (5.2- 5.8)	4.1 ± 0.4 (3.4- 4.6)
C	16.3 ± 4.6 (11.2 -23.5)	10.7 ± 0.4 (10.2 -11.3)	4.3± 0.1 (4.0-4.6)	26.4 ± 7.8 (13.9-40.8)	10.3 ± 0.5 (9.6- 11.1)	8.2 ± 0.9 (7.3- 10.0)
c'	0.6 ± 0.2 (0.4 -0.9)	3.8 ± 0.3 (3.4 -4.3)	0.1 ± 0.0 (0.1 -0.2)	0.8 ±0.3 (0.3-1.3)	4.5 ± 0.4 (3.9- 5.2)	0.3 ± 0.0 (0.2- 0.3)
D%	111.3 ± 6.0 (101.8 -118.4)	76.7 ± 5.1 (71.7 -85.2)	77.7 ± 1.7 (75.2 - 80.5)	87.6 ±7.4 (79.0- 98.6)	79.2 ± 2.2 (76.7- 83.0)	90.9 ± 6.6 (74.7- 97.7)
E%	257.0 ± 91.3 (153.6 -406.0)	158.8 ± 5.2 (151.1 -165.4)	84.4 ± 4.6 (80.9 -94.4)	404.8 ± 119.5 (210.5- 596.6)	147.0 ± 11.0 (135.8- 165.2)	182.2 ± 11.5 (169.3- 203.0)
SW%	150.1 ± 1.5 (147.9 -151.9	-	-	220.3 ± 26.8 (189.0- 297.7	-	-
GS%	44.8 ± 1.6 (43.0 - 48.6)	-	-	44.2 ± 6.7 (29.8- 52.9)	-	-

Values in the parentheses show range, measurement are in µm, mean ± SD

***In vitro* mass culturing of *Metarhabditis amsactae*.** *Metarhabditis amsactae* strains HAR-St-II and HAR-Ht-III were inoculated on Wout's medium –I for mass culturing. The populations of IJs were recorded after 30 and 60 days of inoculation which have been shown in Table 3. Results indicated that mean number of IJs of strain HAR-St-II were significantly higher (77,740.2 IJs/ flask) than strain HAR-Ht-III (71451.8 IJs/ flask). Interaction between strains and observation time was found to be non significant. In Wout's medium-I, multiplication of strain HAR-St-II was more than strain HAR-Ht-III, both after 30 and 60 days. Multiplication of both strains of *M. amsactae* was higher after 30 days (77740.2 IJs of HAR-St-II and 71451.8 IJs of HAR-Ht-III) than after 60 days (23416 IJs of HAR-St-II and 19331.3 IJs of HAR-Ht-III).This is also supported by Kranti and Narendra (2018),that found two local isolates of entomopathogenic nematodes from Haryana namely *Steinernema abbasi* and *Heterorhabdus indica* were mass multiplied on eight culture media and harvested at two different time intervals i.e. at 30 days and 60 days interval. Of the eight media tested, *S.*

abbasi and *H. indica* both multiplied maximum on modified dog biscuit medium followed by Wout's medium. Similarly, results of Leite *et al.* (2017) indicated that increasing nutrient concentration levels was detrimental to nematode production of *Steinernema feltiae*. It is because media containing agar (0.4 % and 0.6 %) increased nematode yields when cultures were grown at low agitation speed. When IJs were used as the inoculum, 0.2 per cent agar also enhanced recovery and nematode yield at the higher agitation speed.

Table 3: Number of IJs of *Metarhabditis amsactae* strain HAR-St-II and HAR-Ht-III produced on Wout's medium-I (Mean of four replications).

Observation Period	Strain HAR-St-II	Strain HAR-Ht-III	Mean
30 day	77740.2 (278.8)	71451.8 (267.3)	273.1
60 day	23416 (152.9)	19331.2 (139.1)	146.0
Mean	215.9	203.2	
C. D. at 5 %	observation period: (3.9), strains: (3.9) and observation period x strains: (NS)		

Values in parentheses are square root transformations, initial inoculum: 2000 IJs/ flask

CONCLUSIONS

On the basis of results obtained in present investigations, it may be concluded that 83 samples contained EPNs, out of 280 soil samples collected from different localities with frequency of occurrence 29.6 per cent. Maximum samples were collected from ber in which frequency of occurrence of EPNs was recorded 58.9 per cent. Fifty one soil samples collected from sapota, grapes, sesame and rose did not show presence of EPNs. Six isolates (strains) *viz.*, HAR-St-I, HAR-St-II, HAR-St-III, HAR-Ht-I, HAR-Ht-II and HAR-Ht-III were selected from the EPNs isolated andidentified as *Metarhabditis amsactae* on the basis of morphological and morphometric characterization. In Wout's medium-I, mass culturing of strain HAR-St-II was more than strain HAR-Ht-III, both after 30 and 60 days. Multiplication of both strains of *M. amsactae* was higher after 30 days than after 60 days.

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Conflict of Interest. None.

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