The Pharma Innovation Journal 2022; SP-11(7): 657-661

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; SP-11(7): 657-661

www.thepharmajournal.com
Received: 24-04-2022
Accepted: 30-05-2022

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Isolation of entomopathogenic nematodes, *Heterorhabditis bacteriophora* from vegetable growing areas of Assam

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Abstract
A survey was conducted for prevalence of entomopathogenic nematodes in vegetable growing areas of the district Jorhat, Assam. A total of 200 soil samples were collected from rhizosphere region of different vegetable crops. Soil samples were baited using *Galleria mellonella* larvae for the presence of steinernematid and heterorhabditid nematodes. Among the soil samples collected, five samples were tested positive for heterorhabditid isolate. From the morphological and morphometrical examinations, the isolate was identified as *Heterorhabditis bacteriophora*.

Keywords: Entomopathogenic nematodes, *Heterorhabditis* species, *Heterorhabditis bacteriophora*, morphological and morphometric studies

Introduction
Entomopathogenic nematodes (EPNs) area group of biological control agents that are characterized by their ability to search for hosts, safety to non target insects and environment. EPNs of the family Heterorhabditidae are obligate deadly insect parasites that spend part of their life cycle in hosts. The third stage of EPNs is referred to be the infective juveniles. Once the IJs penetrate the host, they release the bacteria that live symbiotically within the EPNs gut. Once released into the host, the bacteria multiply fastly and under optimal conditions causing host mortality within 24-48 h. This survey was conducted in different vegetable field of Jorhat district of Assam with the goal of finding EPN isolates and to identify the species present which are probably act as a biological control agents against important insect pests of vegetables.

Materials and Methods
Survey and sample collection: A total of 200 soil samples were collected from different vegetable fields of Jorhat district of Assam for the presence of entomopathogenic nematodes. From each field 10 numbers of soil samples were collected randomly. Soil samples collected from different vegetable fields includes cucumber, chilli, bhendi, brinial, cowpea, ridge gourd, cabbage, cauliflower, pumpkin, tomato, ivy gourd and carrot. Each soil sample was a composite of 5-20 random sub-samples taken in the same location. Information regarding date of sampling, standing crop in the field and soil type along with GPS (Global Positioning System) location was recorded. Samples were packed in polythene bags, and maintained at refrigerated conditions in the laboratory for further processing. The soil was thoroughly mixed on a plastic sheet and half of each sample was used for extraction of entomopathogenic nematodes (EPNs).

Extraction of entomopathogenic nematodes (EPNs) from soil samples
Nematode isolation: Entomopathogenic nematodes were isolated from soil samples taken during the month of June in 2017 from brinjal, bhendi, cucumber and chilly field using the *Galleria mellonella* L. (Lepidoptera: Pyralidae) baiting technique (Bedding and Akhurst, 1975) [1]. The adults of first and second generation found in the haemocoel of cadavers of *G. mellonella*; hence they were extracted by dissection in ringer's solution. The recovered nematodes were kept in clean ringer's solution for further processing.

Morphological and morphometric characterization

Morphology and morphometry

First and second generation adults and IJs were collected at random from infected insect larvae. Males and females were collected on the 4th and 8th days after inoculation of *G. mellonella* for the first and second generations, respectively, while IJs were collected within 2 days after emergence. For descriptive purposes, 20 specimens for each stage were fixed in TAF and processed to glycerin (Seinhorst, 1959) [11], and mounted into a small drop of glycerin. The cover slip was placed onto the glass slide. Morphological observations were made using light compound microscope (Magnus MLX) and phase contras microscope (Nikon Eclipse 50i). Morphometry was done with the help of inbuilt software of phase contrast microscope (Nikon DS-L1). The following characters were measured: total body length; maximum body diameter; anal body diameter; excretory pore position; distance from anterior end to nerve ring position; distance from anterior end to base of pharynx; gubernaculum length; spicule length. In addition to the deMan formula, the other characters studied were: D% (Distance from head to excretory pore/oesophageal length x 100), E% (Distance from Head to Excretory pore/tail length x 100), F% (Body width/tail length x 100). Morphological and morphometrical data of the isolates were compared with the original description of the type species.

Results and Discussion

During the survey of EPNs in Jorhat district of Assam, India, five nematode isolate belonging to the genus *Heterorhabditis bacteriophora* were recorded. Heterorhabditid isolate designated as EPN-H-J-1 and EPN-H-J-2 found from rhizosphere of brinjal and chilli respectively from ICR Farm, AAU, Jorhat. Heterorhabditid isolate designated as EPN-H-J-3, EPN-H-J-4 and EPN-H-J-5 were found from rhizosphere of chilli, cucumber, bhendi respectively from Experimental Farm, Deptt. of Horticulture, AAU, Jorhat. Morphological studies of the five heterorhabditid isolates were undertaken and it was found to be similar to each other. Therefore, detailed morphological and morphometrical studies of the isolate EPN-H-J-1 were undertaken and presented in Table 1 and Table 2. Morphological and morphometrical studies of different life stages (infective juveniles, adults of both generations) of EPN-H-J-1 revealed that it is closely resemble with *Heterorhabditis bacteriophora* (Poinar, 1976) [10] in most of the characters .The head of the third stage infective juvenile (IJ) bears dorsal tooth with mouth and anus are closed. Stoma appears as a closed chamber. The head is with sheath (cuticle of second-stage juvenile). Oesophagus and intestine are reduced. The excretory pore is posterior to nerve ring. The tail is long, pointed and covered with a sheath. The male of second generation had slightly round head. They possess a tubular stoma and pharynx with a cylindrical corpus. The isthmus is distinct with a globose basal bulb and prominent valve. The nerve ring surrounding the isthmus is located near the basal bulb. The excretory pore is located near the middle of the basal bulb. The reproductive structure is monarchic; anteriorly reflexed. The spicules are paired, symmetrical and separate, with prominent tips, slightly curved ventrally. The gubernaculums is flat and narrow, bursa peloderan, open, with nine pairs of genital papillae, tail pointed. The hermaphroditic female of first generation body curved ventrally like C- shaped when heat killed. Head region is slightly rounded, six lips are prominent. They possess a tubular stoma and pharynx with a cylindrical corpus. The isthmus is distinct and short. Nerve ring surrounding isthmus just anterior to basal bulb. Basal bulb often surrounded by anterior portion of intestine. Excretory pore is posterior to the basal bulb. Gonads amphidelphic and reflexed. Vulva near mid-body. Vulva protruding outward and functional for oviposition. Tail pointed. Tail is longer than anal body width, conoid with pointed terminus. Anal region is slightly protruding. The amphimictic female of second generation body curved ventrally like C- shaped when heat killed, smaller in size than hermaphroditic females. Head region is subconical, six lips are prominent. They possess a tubular stoma and pharynx with cylindrical corpus. The vulva does not protrude outward, surrounded by a hardened deposit. Anal region is slightly protruding.

The IJs of EPN-H-J-1 showed close similarity with *H. bacteriophora* with respect to head shape, ratio b, D% and E%, but exhibited minor differences from the type measurements by having lower tail length (76 vs. 91). The males of this isolate showed close similarity with *H. bacteriophora* with respect to head shape, anal body width, gubernaculums length but exhibited minor differences from the type measurements by having higher tail length (36 vs. 28), The hermaphroditic and amphimictic females of this isolate showed close similarity with *H. bacteriophora* with respect to head shape, tail length and vulval position but exhibited differences from the type measurements by having body length, lower body width and lower anal body width. The heterorhabditid isolate was similar to *H. bacteriophora* in original description with respect to third stage infective juvenile in character like greatest width; distance from anterior end to excretory pore; distance from anterior end to pharynx base; body width at anus; ratio a; ratio b; ratio c; D%; E%. However, the isolate showed variation in body length of IJs (vs.) and tail length (vs.). Variation also observed with respect to adult stage of both male and female generations in some characters like body length, position of pharynx, position of excretory pore, tail length, spicule length and gubernaculums length etc. Nguyen *et al*. (1995) [9] observed variation in body length, position of excretory pore, tail length and value of E% of *H. bacteriophora* in relation to time of harvest. It was observed that body length of infective juvenile was 605 µm (579 µm-634 µm) on 3rd day of harvest where as body length 565 µm (524 µm-604 µm) on 15th day of harvest. In the present investigation the third stage infective juveniles were obtained when they emerged from the cadavers after 7 to 10 days. Devi *et al*. (2016) [2] reported occurrence of *H. bacteriophora* from white grub infested areas of Majuli, Assam. They exhibited minor differences in morphometrical studies of *H. bacteriophora* from the type measurements by having higher body length of IJs (572 vs. 570), body width (26 vs. 24) and position of nerve ring (84 vs. 83). Poinar (1976) [10] isolated and described *H. bacteriophora* from Brecon, South Australia. The nematode was isolated from the body cavity of *Heliothis punctigera* Hall (Noctuidae: Lepidoptera). *H bacteriphora* is distributed in America, Southern and Central Europe, Australia and East Asia (Hominick *et al*., 1996) [4]. In Europe it has been reported from Spain, Italy, Moldova, Hungary, Southern France (Smits *et al*., 1991) [13], the Azores, Germany, Switzerland (Hominick, 2002) [4], South Russia (Ivanova *et al*., 2000) [7], the European part of Turkey (Hazir *et al*., 2003) [3] and Slovenia (Laznik *et al*., 2009) [8]. *H. bacteriophora* isolates were found in neutral (vertisol) or acidic (oxysol) soils in crop lands, orchards and woodland habitats in Guadeloupe (Grande Terrre, Basse Terre). *H. bacteriophora* was reported from India by Sivakumar *et al*. (1989) [12] and Hussaini *et al*. (2001) [6]

Table 1: Morphometrics of infective juvenile and second generation male of *Heterorhabditis* sp. (EPN-H-J-1) in comparison with original description of *Heterorhabditis bacteriophora*

Measurements in µm and in the form: mean± SD (range)

Character	*Heterorhabditis* sp. (EPN-H-J-1) (IJ) (n=20)	Type measurement of *H. bacteriophora* (IJ) (Poinar, 1976) [10] (n=15)	Type measurement of *H. bacteriophora* (IJ) (Poinar, 1990) (n=25)	*Heterorhabditis* sp. (EPN-H-J-1) (male) (n=20)	Type measurement of *H. bacteriophora* (IJ) (Poinar, 1976) [10] (n=15)
Body length(L)	537.5±52.2 (430-630)	570 (520-600)	558 (512-671)	851.6±58.6 (750-950)	820 (780-960)
Body width (W)	24.5±3.1 (15-30)	24 (21-31)	23 (18-31)	45.6±3.01 (40-51)	43 (38-46)
Anterior end to excretory pore (EP)	89.3±12.4 (73-115)	104 (94-109)	103 (87-110)	121.4±8.4 (105-135)	121 (114-130)
Anterior end to nerve ring (NR)	81.6±10.7 (68-104)	83 (81-88)	85 (72-93)	76.8±5 (70-85)	72 (65-81)
Anterior end to esophagus base (ES)	117.6±8.2 (100-130)	125 (119-130)	125 (100-139)	105.8±8.9 (95-121)	103 (99-105)
Testis reflection	-	-	-	76.8±8.3 (65-88)	79 (59-87)
Tail length (T)	76±10.9 (59-95)	91 (83-99)	98 (83-112)	36.4±2.6 (31-39)	28 (22-36)
Anal body width (ABW)	16.3±3.7 (9-22)	-	-	21.2±1.5 (19-24)	23 (22-25)
Ratio a= (L/W)	22±1.7 (19.3-27.5)	25 (17-30)	25 (17-30)	-	-
Spicule length (SL)	-	-	-	42.8±2.8 (39-48)	40 (36-44)
Gubernaculum length (GL)	-	-	-	22±3.01 (18-28)	20 (18-25)
D%= (EP/ES)×100	-	-	84 (76-92)	114.9±4.3 (110-125)	117
SW%=SL/ABW×100	-	-	-	201.9±11.5 (181.8-215)	174
GS%=GL/SL×100	-	-	-	51.2±3.9 (46.1-58.3)	50

Table 2: Morphometrics of Hermaphroditic and Amphimictic female of *Heterorhabditis* sp. (EPN-H-J-1) in comparison with original description of *Heterorhabditis bacteriophora*

Measurements in µm and in the form: mean± SD (range)

Character	*Heterorhabditis* sp. (EPN-H-J-1) Hermaphroditic female (n=20)	Type measurement of *H. bacteriophora* Hermaphroditic female (Poinar, 1976) [10] (n=15)	*Heterorhabditis* sp. (EPN-H-J-1) Amphimictic female (n=20)	Type measurement of *H. bacteriophora* Amphimictic female (Poinar, 1976) [10] (n=15)
Body length (L)	2094±287.9 (1640-2490)	4030 (3630-4390)	2412.5±168.6 (1490-2640)	3500 (3180-3850)
Body width (W)	165.7±10.08 (148-175)	165 (160-180)	151.8±9.7 (138-171)	190 (160-220)
Anterior end to excretory pore (EP)	154.9±24.3 (125-192)	209 (189-217)	153.6±19 (120-183)	192 (174-214)
Anterior end to nerve ring (NR)	132.1±11.4 (114-149)	126 (121-130)	111±4.9 (102-119)	103 (93-118)
Anterior end to esophagus base (ES)	172.3±15.8 (160-170)	197 (189-205)	-	-
Tail length (T)	80.8±5.2 (75-91)	90 (81-93)	82.4±3.1 (79-90)	82 (71-93)
Anal body width (ABW)	31.4±5.7 (22-38)	46 (40-53)	24.1±3.3 (20-32)	28 (22-31)
V%= distance from anterior end to vulva as percentage of length	49.8±1.6 (48.2-54.2)	44 (41-47)	48±1.2 (44-56)	47 (42-53)

Plate 1: Male of *H. bacteriophora* (40x)

Plate 2: Spicule of *H. bacteriophora* (100x)

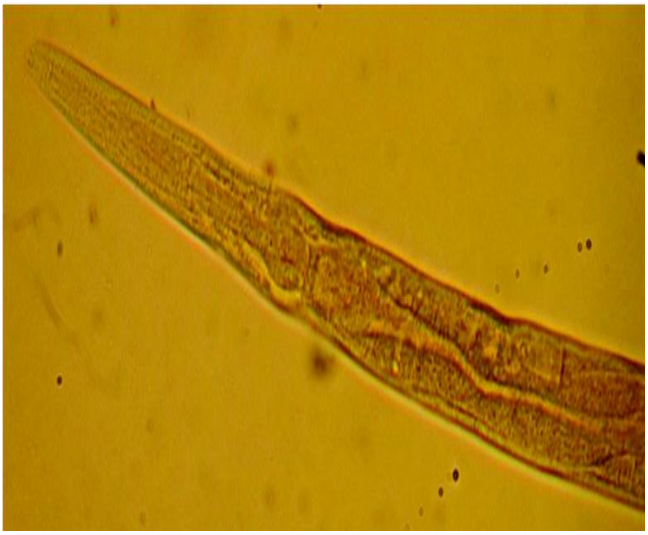

Plate 3: Anterior part of Amphimictic female of *Heterorhabditis bacteriophora* (100x)

Plate 4: Hermaphroditic female of *Heterorhabditis bacteriophora* (40x)

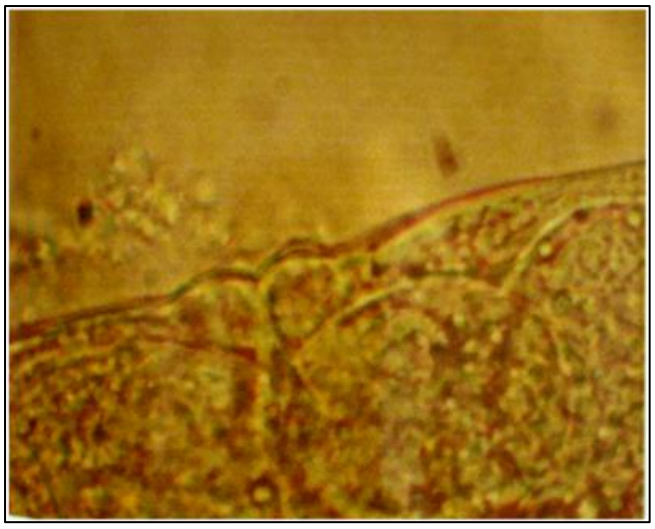

Plate 5: Vulva of Hermaphroditic female of *Heterorhabditis bacteriophora* (100x)

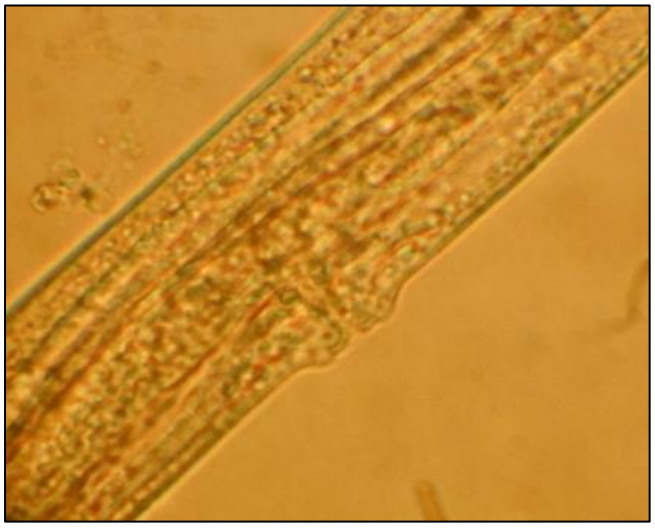

Plate 6: Vulva of amphimictic female of *Heterorhabditis bacteriophora* (100x)

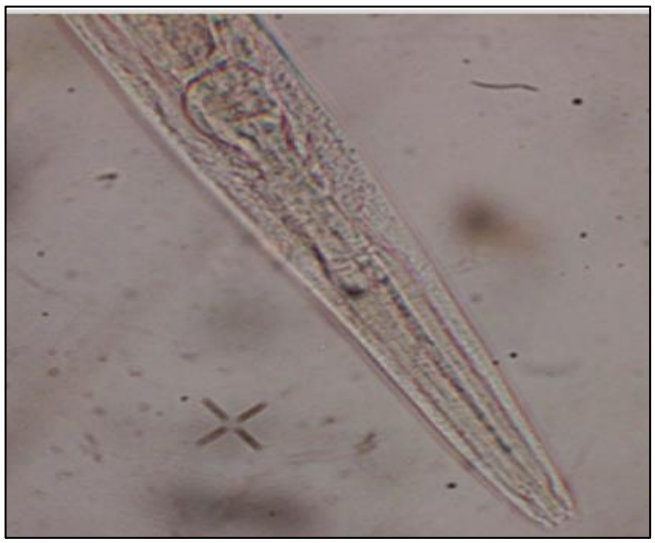

Plate 7: Oesophagus of Hermaphroditic female of *Heterorhabditis bacteriophora* (100x)

Plate 8: Infective juveniles of *Heterorhabditis bacteriophora* (40x)

Conclusion

Due to their potential as biological control agents, the correct identification of EPNs, is key for optimal management in pest control. Despite the fact that entomopathogenic nematodes were found only in five soil sample out of 100, the discovery of *Heterorhabditis bacteriophora* underscores the need for a more thorough assessment in Assam. Further research into the characterisation and host ranges of these EPN species is needed to investigate and confirm their potential use in insect pest biological control programmes.

Acknowledgement

The authors are grateful to the Head of the Department, Department of Nematology, AAU, Jorhat, Assam and Director of Post Graduate Studies, AAU, Jorhat, Assam for providing necessary laboratory facilities.

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