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Characterization of entomopathogenic nematode, *Steinernema carpocapsae* from ginger (*Zingiber officinale* Rosc.) rhizosphere in India

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ABSTRACT

During a random survey, one isolate of entomopathogenic nematode from ginger rhizosphere was collected from Faizabad district of Uttar Pradesh (India). Morphological and morphometric studies identified the isolate as *Steinernema carpocapsae*. This was further confirmed by ITS-rDNA sequences analysis. Phylogenetic was constructed for studying relationship with known isolates. Pathogenic potential of the isolate of *S. carpocapsae* (IISR-EPN 06) on the larva of shoot borer (*Conogethes punctiferalis*), hairy caterpillar (*Euproctis* sp.) and greater wax moth (*Galleria mellonella*) was found under *in vitro* condition. Further this isolate displayed high virulence on above insect species. This study reported occurrence of a isolate of *S. carpocapsae* from ginger rhizosphere from India. This indigenous isolate could be investigated further for managing insect pests of ginger.

Keywords: Entomopathogenic nematodes, *Steinernema carpocapsae*, ginger, pathogenicity

Introduction

Ginger (*Zingiber officinale* Rosc.) production in India is sustained losses due to several reasons. Among them, one of the major constraints is insect pests, which causes significant yield losses (Devasahayam *et al.* 2012). The only effective method to manage this pest is the use of insecticides resulted pesticide residues in the produce affecting human health and also causing other ecological hazards. There has been a renewed interest in developing environment- friendly pest management schedules in agriculture. Entomopathogenic nematodes (EPNs) have received little attention by researchers though they have a great potential in reducing pest population, their role can be enhanced by little manipulation (Ali *et al.* 2005; Lorio *et al.* 2005). The biocontrol potential of EPNs can be exploited by isolating native EPNs tolerating local climatic condition. Some achievements have been documented for biocontrol of pests from several parts of the world (Hatting *et al.* 2009). Previous surveys revealed natural occurrence of several species/isolates of *Steinernema* and *Heterorhabditis* in Andman and Nicobar islands (Prasad *et al.* 2001), Gujarat (Vyas 2003), Kerala (Banu *et al.* 2004; 2005), New Delhi (Ganguly & Singh 2000), Tamil Nadu (Josephrajkumar & Sivakumar 1997), Meghalaya (Lalramliana & Yadav 2010) and Uttar Pradesh (Pervez & Ali 2007) of India. The study was carried out to identify of EPN isolated from ginger rhizosphere and to evaluate pathogenicity on larva of shoot borer, *Conogethes punctiferalis* (SBL), hairy caterpillar, *Euproctis* sp. (HCL) and greater wax moth, *Galleria mellonella* (GWML).

Materials and Methods

Collection of soil samples for detection of EPN

Soil samples were collected from ginger rhizosphere from Kumarganj (GPS $81^0 50^{'}$E $26^0 32^{'}$N), Faizabad districts (Uttar Pradesh). About 1 kg of soil sample was collected at a depth of 10-20cm using a hand trowel, each sample containing a composite from five random subsamples. Samples were placed in polyethylene bags to minimize dehydration, tag a label providing all necessary information and transported in to the laboratory. The hand trowel was sterilized by 70% ethanol before leaving the sampling site.

Extraction of EPN

EPN isolated from the soil using the insect baiting technique (Bedding & Akhurst 1975). About 250 g composite soil was placed in a plastic container and ten live greater wax-moth larva (GWML) used to bait the EPN. The soil sample was checked every day for 7 days for the presence of EPNs. On inspection of any dead GWML, they were placed in modified white trap (White 1927) for 2 weeks at room temperature (29±2 ^{0}C) for emergence of EPN. In case of the negative results, the isolation process was repeated two times for confirmation of the result. Emerged infective juveniles (IJs) were collected from White traps and used for infection on fresh GWML for production of EPN cultures.

Maintenance of EPN and insect cultures:

EPN cultured and maintained as per the procedure described by Kaya & Stock (1997). The IJs were surface sterilized in 0.1% Hyamine solution (Hussaini *et al.* 2000) and stored in distilled water in tissue culture flasks for identification. However, fresh nematode culture was used for bioassay studies. GWML reared on artificial diet as per the procedure described by David & Kurup (1988), while HCL and SBL procured from ginger fields of Indian Institute of Spices Research, Experimental Farm and farmers' field, district Kozhikode of Kerala. The larvae were sorted out and 3rd instar larva were used for the bioassay study.

Morphological characterization

Morphological and morphometric studies carried out based on 10 IJs and 10 first generation males (Hominick *et al*. 1997; Stock *et al*. 2000). Nematodes fixed and processed to dehydration following the method described by Seinhorst (1966). EPN isolated from the ginger rhizosphere was characterized and placed into similar species-groups using taxonomic criteria suggested by Stock & Kaya (1996) and Hominick *et al.* (1997).

Molecular characterization

Nematode-DNA was extracted following the protocol of Pastrick *et al*. (1995). Primers 18S (5' TTGATTACGTCCCTGCCCTTT 3') and 26S (5' TTTCACTCGCCG TTACTAAGG 3') as detailed by Vrain *et al*. (1992) were used for amplification of the ITS region of rDNA. After electrophoresis, the amplified products excised from 1% TAE buffered agarose gel using a QIA-quick PCR purification kit (QIAGEN), cloned and purified DNA sequenced at Xleres Biotechnology Pvt. Ltd., Bangalore.

Phylogenetic analysis

Phylogenetic tree was constructed with a Maximum Likelihood method (MLM). The evolutionary history was inferred by using the MLM based on the Tamura-Nei model (Tamura & Nei 1993). The tree with the highest log likelihood(-1022.2340) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree (s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+ Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 319 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*. 2013).

Entomopathogenicity

Pathogenicity of *S. carpocapsae* (IISR-EPN 06) on SBL, HCL and GWML was tested in Petriplate. For this, ten larva of tested insect were kept in Petriplate and 100 IJs of *S. carpocapsae* (IISR-EPN 06) were inoculated and their mortality was recorded at 24h interval. The experiment was conducted at room temperature (33±2^{0}C) and replicated ten times along with control. The mortality was calculated using following formula (Pervez & Ali 2009):

$$\text{Mortality (\%)} = D \times 100 / N$$

Where, D- Number of dead larvae; N – Total number of larvae

Results

Morphological characterization

Morphological characters of *Steinernema* sp. resembles that of "*carpocapsae*" group. Key diagnostic traits of the third-stage IJs and males were similar to a member of "*carpocapsae*" group. First generation males have small mucro at the end of the tail, yellow spicules with prominent capitulum, rostrum, vellum and a pointed terminus. The total length (infective juveniles (IJ) - 598.5µm & first generation male (FGM) - 1097 µm); width (IJ- 26.17 µm and FGM - 89.23 µm); EP (IJ- 43.71 & FGM- 61.69%), NR (IJ- 73.8 & FGM- 114.4%), ES (IJ- 131.8 & FGM- 141.6%); D (IJ- 33.1 & FGM- 44.4%); E (IJ- 82.1 & FGM- 217.7%) and tail length (IJ- 53.3 µm & FGM- 29.1 µm) observed were within the characteristics of the species (Nguyen & Smart 1995).

Molecular characterization

Nematode DNA sequence deposited in Gen Bank under accession number KM 212952 was compared to sequence of *Steinernema* spp. available in Gen Bank. The BLAST search indicated a 99% similarity in sequence of PCR product of our isolate with the isolates of *S. carpocapsae* available in the Genbank with accession number *viz*., KC571265,

GQ421605, HM140694, FJ860033, AY230164 and HQ406729. Other quite similar sequences were those of *S. eapokense* (AY487921) with 98%, *S. sasonense* (AY487919) with 97%, *S. siamkayai* (JN571085) and *S. tami* (AY171280) with 95% similarity. Some *Steinernema* spp. available in GenBank showed less than 95 % similarity.

Phylogenetic analysis

Phylogenetic analysis of ITS- rDNA sequence data placed this species in a clade with other isolates of *S. carpocapsae*. The ITS regions are much more variable and provide most of the base differences for species diagnosis (Nguyen *et al*. 2001). The isolates *S. carpocapsae* (IISR-EPN 06) aligned clearly and without gaps, with those of other *S. carpocapsae* isolates. In phylogram based on ITS sequences, *S. carpocapsae* (IISR-EPN 06) was close to *S. carpocapsae* (isolate IS 34) from Israel followed by *S. carpocapsae* (strain Caba 02) from Mexico (Fig. 1).

Entomopathogenicity

Results indicated *S. carpocapsae* (IISR-EPN 06) was found pathogenic to all tested insect pests; it caused cent percent mortality within 72h. The *S. carpocapsae* (IISR-EPN 06) isolate was found as quick killer; they start killing insect larvae within 24h. No mortality was found in control treatment (Fig. 2).

Discussion

The *S. carpocapsae* previously described as *Neoaplectana carpocapsae* from infected *Laspeyrasia pomonella* in Chehoslovakia (Weiser 1955) and later redescribed by Wouts *et al.* (1982). However, Karimi *et al.* (2010) summarized the occurrence of different isolates of *S. carpocapsae* across the world. PCR amplification of the ITS regions followed by DNA sequencing of the PCR product provided reliable data for diagnosing *Steinernema* species. However, the use of ITS region for species delimitation and phylogenetic reconstruction should be restricted to only those nucleotide positive for which character polarization and homology statements are robust. *S. carpocapsae* is well known species among the EPNs. The most important attributes include ease of mass production and ability to survive in a partially desiccated state allows them to store several months of room-temperature shelf-life. Rao & Manjunath (1966) used DD 136 (*S. carpocapsae*) for the control of insect pests of rice, sugarcane and apple. *S. carpocapsae* has been used against many insect pests like *Psendaletia separeta* and *Spodoptera litura* in pulses (Abdel-Razek *et al*. 2007; Pervez & Ali 2009), *Amsacta albisting* in groundnut (Bhaskaran *et al*. 1994), *Agrotis ipsilon* and *A. segatum* (Hussaini *et al*. 2000), *Athalia proxima* in mustard (Pervez *et al*. 2007), *Helicoverpa armigera* in pigeonpea and chickpea (Ali *et al*. 2008), and *Maruca vitrata* in pulses (Pervez 2012).

Our study revealed an EPN species identified as *S. carpocapsae* based on morphometrics and molecular characterization from ginger rhizosphere. The EPN isolate is capable of killing *G. mellonella*, *C. punctiferalis* and *Euproctis* sp. within 24-72h under laboratory conditions. This indigenous strain could be investigated further for the control insect pests of ginger in the similar ecological conditions of India.

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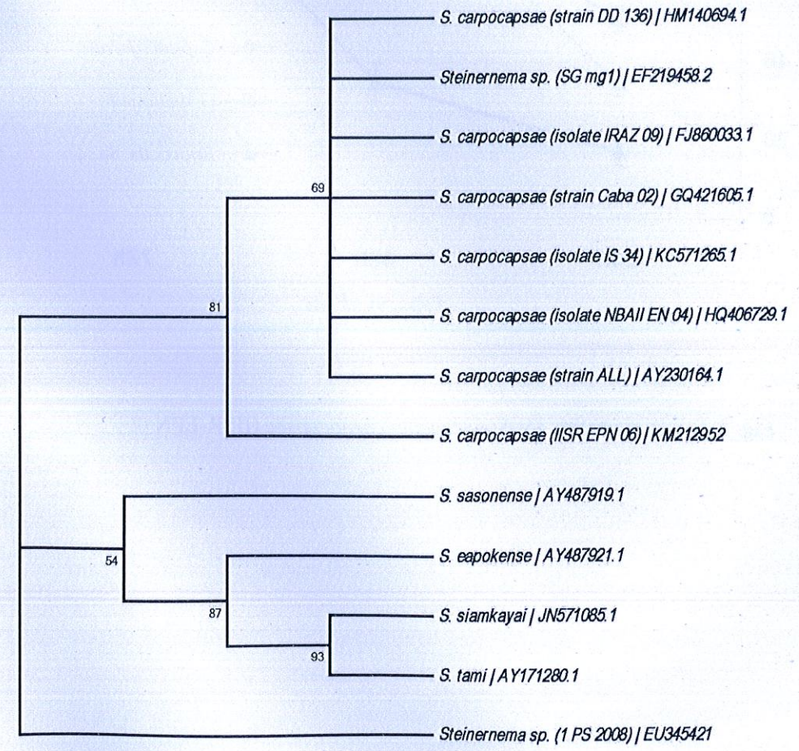

Fig 1. Phylogenetic relationship of *S. carpocapsae* (IISR-EPN 06) among closely related species based on the sequences of the ITS region.

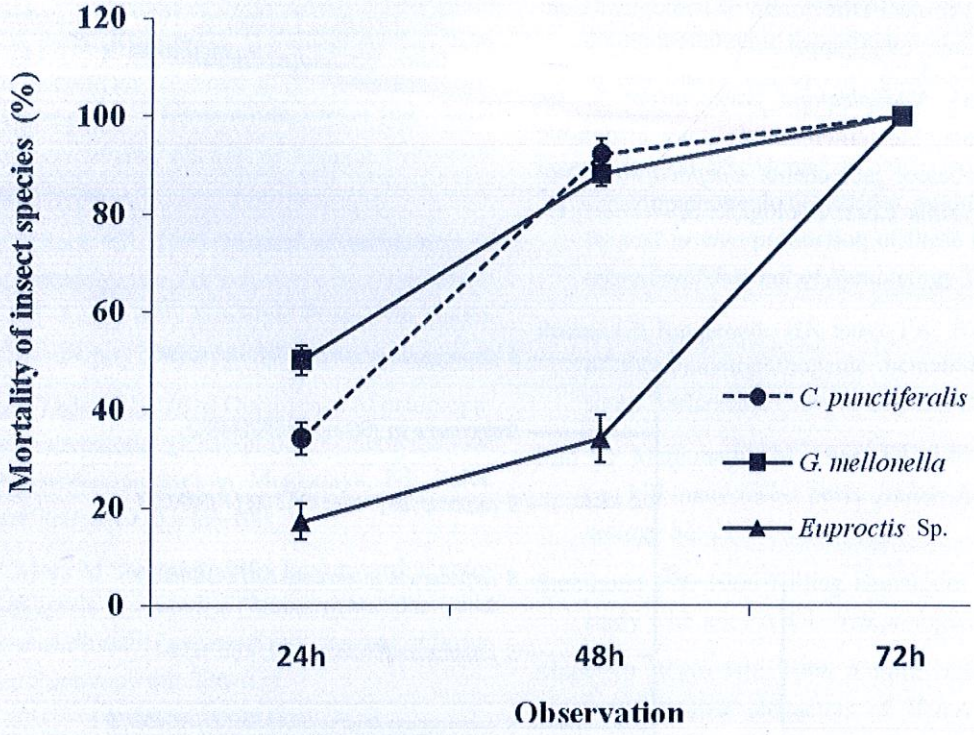

Fig 2. Insect mortality for *Steinernema carpocapsae* (IISR-EPN 06)