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Molecular Identification of Entomopathogenic Nematode Isolate and its Virulence to White Grub, *Leucopholis burmeisteri* (Coleoptera: Scarabaeidae)

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Abstract

Entomopathogenic nematodes (EPNs) carrying symbiotic bacteria represent one of the best non chemical strategies for pest management. Isolation and identification of native isolates in the field are necessary for successful control of insect pests due to their better adoption in local environment. An native isolate of EPN was identified as *Steinernema carpocapsae* recovered from soil rhizosphere of coconut (*Cocos nucifera* L.) plantations based upon molecular resolution of 16s rRNA and COXII genes. The virulence of the identified nematode isolate was evaluated on white grub (*Leucopholis burmeisteri)* neonate larvae and found highly virulent at nematode density of 700 Infective Juveniles (IJs) / larvae caused 100% mortality within 4-5 days of post inoculation and 73.3% mortality of early 2nd instar grubs within 6-8 days of post inoculation at density of 6000 IJs/larva (P=0.05). In laboratory assays the identified isolate demonstrated virulence to white grub indicates its potential as a biological control agent and could be potentially incorporated in integrated pest management programmes.

Keywords

Entomopathogenic nematodes; *Steinernema*; Coconut; *Leucopholis burmeisteri*; 16s rRNA; Virulence

Introduction

Entomopathogenic nematodes (EPNs: genera *Steinernema* Travassos and *Heterorhabditis* Poinar) are bio control agents that infect a wide range of soil insects [1]. EPNs have a mutualistic symbiosis with bacteria, Steinernematids are associated with *Xenorhabdus* spp. and Heterorhabditis are associated with *Photorhabdus* spp. [2,3]. Infective Juveniles (IJs), the only free-living stage, enter hosts through natural openings (mouth, anus, and spiracles). After entering the host's hemocoel, nematodes release their bacterial symbionts, which are primarily responsible for killing the host usually within 24 to 48 hours.

At least dozen of the 100 EPN species described have been commercialized for use in biological control in abroad [4]. Major EPN species being used for biological control presently are *Steinernema carpocapsae*, *S. feltiae*, *S. riobrave*, *S. scaptersci*, *S. glaseri*, *Heterorhabditis bacteriophora*, *H. megidis* [5]. Among these EPNs, *S. carpocapsae* has widely proven to be the most effective nematode species in China [6]. *S. carpocpasae* A24 strain has been used effectively against the soil-dwelling stage of the peach fruit moth, *Carposina niponensis*. Walsingham, which is a serious pest of apples in china [7,8]. Another species of Steinernematids *S. glaseri* use against white grubs and to complement *S. carpocapsae* (Bio safe) in management of turf grass pests in USA [9].

In India these nematodes have been investigated against many agricultural insect pests [10], including plantation insect pests [11,12]. Cardamom root grub as reported to be successfully managed by using indigenous EPN in Kerala [13]. Globally there are more than 40 recognized EPN species within these two families [14]. However, from Indian soils only four species have been described [15]. So far, more than 40 indigenous strains of *Steinernema* have been isolated from Indian soils [16]. In this respect, and with particular reference to entomopathogenic nematodes, the isolation of indigenous species and/or populations provides a valuable resource not only from a biodiversity perspective but also from a more applicable standpoint [17]. Indigenous EPN may more suitable for inundative release against local insect pests because of adaptation to local climate and other population regulators [18]. However, information on indigenous EPNs especially in plantation crops pest (white grubs) management in India is still limited.

White grubs are the soil inhabiting root-feeding larvae of scarab beetles (Coleoptera: Scarabaeidae). They cause significant damage to many agricultural and horticultural crops, including plantation crops like coconut and arecanut [19,20,21]. The extent of damage caused by white grubs solely depends upon the species involved, the numbers present and host crop. In India, white grub is one of the five pests of national importance [22]. In many crops, white grubs cause losses to the extent of 40-80% [23], 100% losses in arecanut [24]. Presently, chemical insecticides (e.g. phorate and chlorpyrifos) [21] are the preferred options for management of white grubs. But increased awareness on the side effects caused by indiscriminate use of chemical pesticides and global concern regarding possible traces of pesticides in exports had made to shift from chemical intensive management to alternative control strategies. Therefore, farmers are looking for effective, ecologically and economically viable alternative for the control of this pest.

There are several target pests in plantation crops that can be controlled with entomopathogenic nematodes [12]. A recent survey on indigenous EPN in coconut and arecanut indicates opportunity for discovery of new nematodes strains and species adapted to local environmental conditions and pests of plantation crops [25,26]. When indigenous isolates are discovered, their identification is not always straightforward and they lack morphological variations and thus many researchers have turned to molecular techniques, based on the Polymerase Chain Reaction (PCR), small subunit ribosomal RNA (rRNA) and potentially highly variable regions and found useful for differentiating the species and strains of *Steinernema* [27,28]. Besides, mitochondrial cytochrome oxidase subunit I (COI or COII) also successfully used as DNA barcodes for species identification

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[29,30]. An attempt was therefore made in this investigation to identify collected indigenous EPN species at molecular level, as well as to study their virulence against white grub larvae collected from the coconut ecosystem.

Materials and Methods

Geography of the study site

The soil samples were collected from coconut and arecanut plantations ecosystem having good vegetation coverage where cohabitating EPNs were previously recovered both Steinernematids and Heterorhabditis. The sampling was carried out during June to December 2013 on a random basis. The site is located at 11° 18' to 12° 48' N latitude and 74° 52' to 75° 26' E longitude. It has an average elevation of 10.7 m above mean sea level (MSL). The mean annual rainfall is 3500 mm and mean maximum and minimum temperature is 36°C and 22°C respectively. The climate of the region is warm, humid and tropical.

Nematode isolation and storage

Totally 117 soil samples were collected from the rhizosphere of different crops *viz*., coconut, arecanut and intercrops such as black pepper, banana, citrus, cocoa, cinnamon, mango etc. in coconut and arecanut cropping system during post monsoon season from Kasaragod during 2013. Each soil sample was of nearly 500 g and was taken from 0-12 cm depth. Information on locality, soil type, rainfall and habitat were noted for each sample. EPN were recovered from soil samples using the insect baiting method described by Bedding and Akhurst [31]. Insect baits (three last instar *G. mellonella* larvae were placed in 250 ml plastic containers. (four containers/sample) with moistened soil obtained from each sample. Containers were covered with a lid turned upside down and kept at room temperature (28 ± 2°C) [17]. The dead larvae were washed twice in sterile distilled water and placed in modified white trap [32] to collect infective juveniles from death larvae. The suspension which was acquired in this way, was used for artificial infection of the larvae of *G. mellonella* at 24-27°C [33]. The recovered IJs were kept in vented tissue culture flask at 15°C until use. After storage at 15°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.

Identification of EPN isolates

The collected *Steinernema* isolates were identified as *S. carpocapsae* by studying the standard key morphometric traits for IJs and first generation males suggested by Stock [34] and Hunt [35]. To confirm this, species level identification was done by molecular approach [27,36] and was compared to those available in Gene Bank.

DNA extraction and PCR

DNA isolation: Total genomic DNA was isolated with DNeasy blood and tissue kit (QIAGEN) as per the manufacturer's instructions. Total 200 µl of nematode sample containing, 500-1000 IJs was used for DNA extraction in a 2 ml eppendorf reaction tube. After extraction, isolated DNA was stored at -20°C until use. The quality and quantity of the extracted DNA was assessed using spectrophotometer and agarose gel electrophoresis.

PCR amplification: The primers SCCYTFI and R1 were used to amplify the mitochondrial region located between the 3' region of the COII and the 5' end region of the 16s rRNA. Primers were designed based on the cytochrome oxidase domain of *S. carpocapsae* from the already available data in the public database. The obtained primers were sourced from SIGMA-ALDRICH custom DNA Oligos. The amplification of the cytochrome oxidase (COII) region was carried out in a reaction volume of 25 µl mixture containing 25 ng of DNA, 200 µM dNTPs (Fermentas), 0.4 µM each forward and reverse primers, 0.5U Taq DNA polymerase (Fermentas) and 10x Taq DNA polymerase reaction buffer with 20 mM $Mgcl_2$ (Fermentas) with PCR cycling conditions of 94°C for 1 min, followed by 35 cycles of 94°C for 3 min, 55°C for 1 min and 72°C for 1 minute with a final extension at 72°C for 10 minute. All PCR amplifications were carried out using a BIORAD Thermo cycler. From the amplified PCR products (Figure 1), an aliquot of 3 µl was stained with EDTA. The mixture was then loaded on 2% (W/v) agarose gel (Lonza, USA) in 1X TAE buffer before the gel was run at 100V for 60 minute and observed under UV transilluminator (BIORAD). The approximate band size were scored by comparing them with a molecular ladder.

Purification, cloning and sequencing of the amplicons: Amplicons of the expected sizes (~970 bp) were excised and eluted from the gel using QIA-quick PCR purification Kit (QIAGEN). Samples that amplified consistently using SCCYT primers were cloned using Ins-TA clone ™ (Fermentas) PCR cloning Kit. The cloned fragments confirmed by colony PCR and were sent for sequencing commercially. Single representative clone from the corresponding samples of band size were subjected to plasmid isolation using Gene-JET plasmid Miniprep kit (Fermentas, Life sciences) and sent for sequencing in both directions using the same amplification primers of cytochrome oxidase gene. Where a single clear PCR band was obtained, direct sequencing of purified PCR products was performed with a terminator cycle sequencing ready reaction kit (Big Dye; PerkinElmer Applied Biosystems), Scigenom, Kochi, India. Isolated raw sequences were checked and edited manually Bio-Edit v 7.0.9 [37] and assembled using CAP3 sequence assembler. Consensus sequences obtained were compared and subjected to phylogenetic analysis.

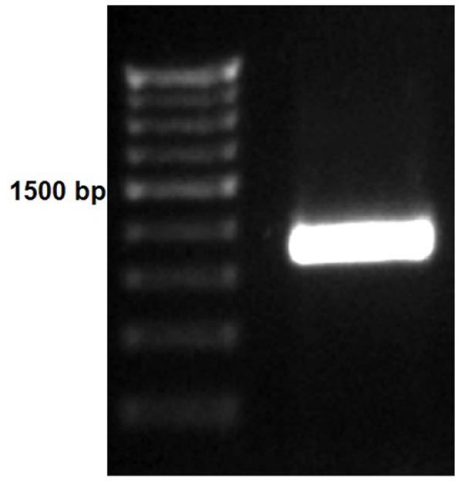

Figure 1: PCR amplification of cytochrome oxidase II (*COXII*) of *Steinernema sp.*

Phylogenetic analysis

Sequences of the ribosomal and ITS region of *Steinernema* species have been used by different authors in taxonomic and phylogenetic studies [38]. In the present study, consensus sequences obtained for COII gene regions and those retrieved from BLASTN hits aligned over the same length in Clustal W (with gap opening penalty for multiple alignments of 10 and extension of 0.2) and MUSCLE (with gap opening penalty for multiple alignments of -12 and extension of -1) using MEGA v 5.0 [39]. This was done to reveal regions of similarity and dissimilarity between the sequences. COII data sets were analyzed and phylogenetic tree was constructed based on NJ-method available in the MEGA software. All phylograms were constructed using 1000 bootstrap replicates to assess their support for each clade or phylogenetic branching [40]. Thorough pair wise sequence comparisons, missing data and alignment gaps were eliminated.

Entomopathogenic nematodes against white grub

Collection of white grub larvae: Early and 2nd late instar larvae of white grub, *L. burmeisteri* were used in virulence study. The larvae were freshly collected from white grub infested arecanut fields and maintained at 28 ± 2°C and sweet potato were supplied as food material during the experiment period.

Virulence: The identified isolate, *Steinernema carpocapsae* were evaluated against white grub larvae. Nematodes were cultured in parallel in last instar *G. mellonella* based on procedures described by Kaya and Stock [41]. Virulence assays were conducted in plastic containers using procedures described by Shapiro [42] and Shapiro-Ilan [43]. Plastic containers (4×6×6 cm) were filled with 80 g of a sterile sandy soils (sand fraction very high 87.2-95.4%, silt 0.2-7.8% and clay 0.6-10.8%) pH:5.5, organic matter:0.46) collected from ICAR-CPCRI Research Farm. Nematodes were pipetted in 2ml water onto the soil surface of each container and final moisture level 10% w/w. Six replicates of five larvae were used (100, 300, 500 and 700 IJ/ neonate larvae). And (500, 1000, 3000 and 6000 IJ/ early second instar larvae). Instead of IJs, 2 mL of sterile water was used as control. The number of white grub larvae dying due to infection by EPN was counted and the percentage larval death was computed. Mortality counts were taken after three days from the day of treatment up to ten days. The number of dead larvae was determined by observing larval movement. The dead white grub larvae were checked daily for the emergence of IJs from the cadavers. The virulence of IJs emerging from the dead white grub larvae was verified by inoculating the IJs to the later larval instar stages of wax moth, *G. mellonella*. Differences in mean percentage larval mortality among treatments were detected through analysis of variance (using arcsine transformed means) and LSD (SAS, P<0.05).

Results and Discussion

Isolation of EPNs from soil

Entomopathogenic nematodes belonging to *Steinernema* sp., were recorded in 4 (3.41%) of collected samples. The pH of the positive samples ranged from 5.7-6.2. The nematodes are mainly present in red sandy soils as revealed by mortality of wax moth larvae due to EPN infection. The recovery rate of nematodes in present study was higher than earlier reports by Boag [44] in Scotland (2.20%), Ozer [45] in Turkey (4.72%). Similarly in India, the occurrence of EPN, *Steinernema* sp. was recorded in seven per cent soil samples and *Heterorhabditis* sp. in 11% soil samples collected from the rhizosphere of coconut and clove [25]. The positive samples were recovered from sandy soils. Soil types play an important role in occurrence of entomopathogenic nematodes. Steinernematids were found to occur more in sandy soils in Sweden [46] and sandy to loam sand in Belgium [47]. Lorena in Costa Rica [48] observed the, Steinernematids in sandy to sandy loam soils of acidic pH 5.7. Many other workers in India have reported average occurrence of Steinernematids in red soils, sandy and loam soils. Rajkumar [49] showed that out of 105 soil samples collected from Rajasthan, only 5 (4.76%) were found to be positive for Steinernematids and Heterorhabditis. Subsequently, Parihar [50] undertook another survey in Rajasthan and reported the presence of EPNs in 8 samples out of 477 samples (1.68%) studied.

Identification of EPN

Based on the analysis of their COXII nucleotide sequences and their morphometric traits, the nematode isolates recovered were identified as *Steinernema carpocapsae***.** COXII nucleotide sequences obtained were compared using BLAST [51] against known sequences in Gene Bank and it was found that all sequences generated using these primers showed close matches to *S. carpocapsae* with maximum 80% similarity (Accession No. gbAF192995) (Figures 1 and 2). Similar confirmations were recorded from Kerala regions of India [52]. The occurrences of four species of *Steinernema* were also recorded in the agro-ecosystems of India [16]. The present study is in support of earlier records.

Entomopathogenicity

Results indicated the isolated EPNs were pathogenic to different stages of white grub but percentage mortality was varied. Caused 100 per cent mortality to neonate's larvae of white grub within 3-4 days and 60% mortality was observed in early second instar white grub larvae within 6-8 days. Mortality rates were increased with the increasing number of IJs and exposure period. Reproduction of nematodes and emergence of new IJs were observed in white grub larvae parasitized. We can conclude that isolated nematodes are having virulence regarding infectivity, development and reproduction of nematodes on white grub larvae. Similar studies in this regard were conducted by Jagadeesh Patil [53] and they reported that *S. carpocapsae* inoculated @ 5000IJs/larvae observed 75% mortality of white grub larvae at 14 days after treatment. The current experiment reveals that to attain 100% mortality the dosage of EPN should be 700 IJs/early instar larvae and to attain 73.3% mortality requires 6000 IJs/early second instar larvae. Complete mortality was attained when larvae were exposed to more number of days (P<0.05) (Table 1).

The variation in mortality percentage within different stages of larvae was observed by earlier workers [54,55, 56] in different strains of entomopathogenic nematodes in foliage feeding and soil pest. Dose-response bio-efficacy tests indicated that, a positive relationship

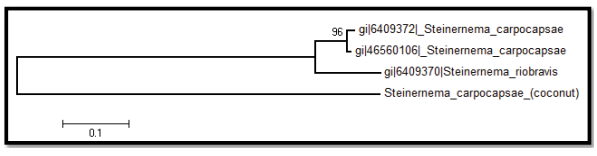

Figure 2: Phylogenetic relationship of *S. carpocapsae* with S. riobravis (NJ-method) using MEGA 5.0. Bootstrap support for each clade is indicated at the nodes.

Table 1: Mortality of different stages *L. burmeisteri* larvae following exposure to different dose of *S. carpocapsae.*

Insect stage	IJs /larvae	Percentage mortality
Early instar(Neonate)	0 (control)	3.3 (4.4^{d})
	100	46.6 (43.0^{c})
	300	83.3 (72.5^{b})
	500	100 (90.0^{a})
	700	100 (90.0^{a})
Early 2nd instar	0(control)	0(0.0^{d})
	500	30(30.4^{c})
	1000	43.3(40.9^{cb})
	3000	60(51.3^{ab})
	6000	73.33(61.7^{a})

Legend: Figures within a column followed by different letters are significantly different at P = 0.05, by DMRT: Figures in the parenthesis are angular transformed values

existed between nematode density and grubs mortality. As the dosage level increased from 100 to 6000 IJs/larva, progressive increase in mortality percent was observed. The larval mortality was increased with increase in exposure time. In India, prospects and status of EPN for the biological control of insects pest has been reported by several authors [57,58]. However, few reports are available till date on the effects of EPN on white grub, infecting the arecanut and coconut palms. With the identified potential of the EPN indicated the scope for utilization of EPN as a bio-control agent for white grub management.

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