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Manifestation and dispersal of entomopathogenic nematodes in orchard ecosystem with regards to soil characters in three different zones of Chhattisgarh state

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Abstract
Entomopathogenic nematodes (EPNs) are round worms from the genera Steinernematid and Heterorhabditid. They are ubiquitous in nature. Survey results of the present study show that 7 samples out of 800 collected samples (0.87%), were positive for EPN which were obtained from silty loam and loamy sand soil. This is an extensive first of its kind study on EPN survey throughout the state of Chhattisgarh. The EPN were recovered from 5 of the 24 surveyed districts namely Baloda Bazar (Ber crop), Mahasamund (Mango crop), Raipur (Sapota crop, Papaya and guava), Bijapur (Mango crop), Sukma (Mango crop). The organic matter estimate from EPN recovered soils were found to be in the range of 0.56- 1.06 % with pH of slight acidic to neutral (5.6-7.1). Water holding capacity of EPNs isolated soil was 32.05 - 38.87 % and bulk density was 0.96 – 1.27 g/cm^3 with moisture percentage of 4.26- 24 %. The present study shows that nematodes can be found in low moisture soil as well as high moisture soils. The results of molecular identification reveals that isolated EPN samples were identified as *Heterorhabditis indica*. EPNs are being investigated primarily for biocontrol purposes. There are many exotic EPN which had conflicting results in many field testing situations, most likely due to their low adaption to local agro-climatic conditions. Henceforth our quest was to look for an indigenous variety of EPN which can be used as a novel bio pesticide, our work mainly focuses on identification of local EPN strains from Chhattisgarh state which can be further used in Integrated Pest Management programme.

Keywords: Entomopathogenic nematode, *Heterorhabditis indica*, bio pesticide, integrated pest management

Introduction
Nematodes are roundworms that belong to the Phylum Nematoda (Kaya and Gaugler, 1993) [16]. Soil organisms include Entomopathogenic Nematodes from the genera Steinernematid and Heterorhabditid. They are found on every inhabited continent and in a broad variety of biologically different soil habitats, including farm fields, woodlands, grasslands, deserts, and even oceans and beaches. A mutualistic relationship exists between these nematodes and a bacterium (*Xenorhabdus* spp. and *Photorhabdus* spp. for Steinernematids and Heterorhabditids, respectively). The only stage that survives outside of the host is the non-feeding, third-stage infective juvenile (IJ). The IJ carries the symbiotic bacteria in its intestine. When the IJ finds a suitable host, it invades and penetrates to the host's hemocoel through natural openings (i.e., anus, mouth, or spiracles). The IJ then releases the symbiotic bacterium which kills the host within 48 h by septicemia. The bacterium produces antibiotics that prevent other microorganisms from colonizing the cadaver. In addition to serving as a food source for the nematode, the bacterium digests the host tissues, thereby providing suitable nutrients for nematode growth and development.
Rao and Manjunath, began research on EPNs in India in 1966, focusing on the biocontrol potentials of an exotic EPN species, *Steinernema carpocapsae* Weiser, and for the next two decades, the same trend continued, with various imported strains of EPNs being investigated primarily for biocontrol purposes. However, due to these strains' low adaptability to Indian sub-continent environmental conditions, the quest for indigenous strains that would suit the country's agro-climatic characteristics became imperative.

EPNs are currently being utilised to manage a variety of agricultural and horticultural insect pests in several countries. In India, researchers used exotic species/strains of *Steinernema carpocapsae*, *Steinernema glaseri*, *Steinernema feltiae*, and *Heterorhabditis bacteriophora* imported by researchers to undertake first EPN study (Kaya *et al*., 2006) [18]. These exotic EPN had conflicting results in many field testing situations, most likely due to their low adaption to local agro- climatic conditions. The age and habitat of EPNs, such as soil type, pesticide use, agricultural methods, and location, have an impact on their number (Mietkiewski *et al*., 1997; Chidawanyika *et al*., 2012; Usta, 2013) [20, 7, 27]. Multiple studies have been carried out over the world in search of EPNs to get new resources for biological management of insect pests (Bhat *et al*., 2020) [3].

Despite their recent increase in surveys to discover native EPN species, knowledge on the diversity of these nematodes and their associated bacterial species remains rather limited, especially in the third world countries. There is still a paucity of information on the diversity of entomopathogenic nematode species in Chhattisgarh, India and a need to find biological control agents for soil pests. Accordingly, we conducted an intensive survey throughout the state of Chhattisgarh for potential strains of entomopathogenic nematodes.

Materials and Methods

1. Collection of soil samples

Field surveys were conducted to identify native species and strains of EPNs. Soil samples were collected from orchard ecosystem from 24 districts in three zones of Chhattisgarh, namely Northern Hills, Plains and Bastar Plateau. Sub samples were taken from a depth of 20 cm, by using a hand spade, from underneath the canopy of ten trees, randomly at least 10 metres apart and 3 sub samples from a diameter of 1 metre around each tree were combined to form one composite sample of approximately 500 grams. In total, 800 soil samples were collected during entire survey period from September 2020 to March 2021 throughout the state of Chhattisgarh. The samples were collected in plastic zip lock pouches to prevent moisture loss and were brought to the laboratory for further processing. Information on sampling location, vegetation, geo location (longitude, latitude and altitude) were recorded. Samples which were collected for EPN isolation were processed within a time period of 4-5 days to ensure maximum recovery of EPN from soil unless present, if samples are kept as such for longer period without processing, it might result in loss of possible EPN species due to abiotic stresses like increased temperature and moisture loss.

2. Soil physical and chemical properties

To record physio-chemical properties (pH, Electrical Conductivity, Organic Carbon, Water Holding Capacity, Soil temperature, Soil moisture, Bulk Density and Soil Texture) from sampling sites, separate soil samples were collected for analysis of soil. Ten random samples in a zig zag pattern from each site i.e., from an area of 1 acre was collected from a depth of 20 cm with the help of hand spade, mixed properly by quadrate method and 1 composite sample of 1 kg was collected and brought to the laboratory for further analysis. Soil samples were processed at Referral Laboratory, Department of Soil Science and Chemistry, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur (C.G.).

3. Trapping and maintenance of Entomopathogenic Nematodes

3.1 Isolation of nematodes

Insect-baiting technique was used to isolate nematodes from soil samples (Bedding and Akhurst, 1975) [2]. Soil samples from various areas were divided and filled into 700 ml plastic containers. Five fifth instar larvae of the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyrallidae), were placed on the container after filling it with soil. The containers were then covered with a ventilated lid, turned upside down, and kept in the dark at 28±2 °C. During a 7-day period, samples were examined daily for dead insects. The cadavers which were suspected for EPN infection were rinsed in 70 % ethanol for few seconds for surface sterilization and then washed twice with distilled water to remove any dirt from it. The colour change and odour from the dead larvae were observed to test the presence of entomopathogenic nematodes and then placed on modified White traps (Kaya and Stock, 1997) [17] to collect infective juveniles. The White traps were incubated at 28±2 °C and examined under a stereo microscope until infective juveniles were observed in the water. Once infective juveniles emerged, they were collected from the trap on a day-to-day basis by pouring distilled water into 250 ml tissue culture flasks. All nematodes emerging from dead larvae in the same sample were grouped together and treated as a single isolate.

Culture of Entomopathogenic nematodes

To mass multiply EPNs, laboratory common host, Greater wax moth, *Galleria melonella* (L.) was used. It was reared on artificial diet as described by Singh (1994) at a constant temperature of 27 ± 2 °C and 65 ± 5% R.H. The fifth instar larvae were used for mass rearing of entomopathogenic nematode. In a petri dish, the nematode suspension was applied to a piece of Whatman no. #1 (1 mm) filter paper. Insect larvae were placed in a petri dish and was kept at room temperature. The infective juveniles started emerging from the cadaver 8-10 days post inoculation. The extracted nematode isolates were stored in vented culture flasks (250 ml) in distilled water, at 20 °C in BOD incubator and shaken weekly. Infective juveniles (IJs) were kept alive by recycling through wax moth larva to maintain the culture of nematodes and every 14 days the water of culture flasks was replaced using vacuum filtration unit.

4. Molecular identification of isolated Entomopathogenic Nematodes

The genomic DNA of seven isolates of EPN were extracted from the IJs by using Qiagen, DNeasy Blood and tissue kit (Catalog No.: 69504). The obtained genomic DNA was stored at –20 °C for future use. A PCR-based analysis and DNA sequencing was conducted to identify EPN species. Amplification of the nuclear rRNA gene cluster from isolates containing internal transcribed spacer ITS rRNA and D2-D3 of 28S rRNA gene was performed using two different primer pairs: TW81(f), AB28 (r), D2-D3 of 28S rRNA D2A: (f) D3B (r).

Results and Discussion

1. Collection of soil samples

The present study has documented the occurrence and distribution of EPN in 24 districts of Chhattisgarh state and the survey results showed that 7 samples out of 800 collected samples (0.87%), were positive for EPN representing

ecologically diverse type of soils. This is the first extensive study on EPN survey throughout the state of C.G. The EPN were recovered from 5 districts out of 24 surveyed districts namely Baloda Bazar (Ber crop), Mahasamund (Mango crop), Raipur (Sapota crop, Papaya and guava), Bijapur (Mango crop), Sukma (Mango crop) (Fig.1). Lalramliana and Kumar (2010) [19] recovered 89 (5.37%) positive EPN samples from ecologically diverse type of habitats. Rosa *et al*. (2000) [24] have summarized the rate of recovery of EPNs from various soil surveys conducted throughout the world. Most surveys showed their recovery rate from soils between 6 and 35%. Other surveys with 5% or less recovery of EPNs includes, 2% in Turkey by Hazir *et al*. (2003) [15] 2.20% in Scotland by Boag *et al*. (1992) [5] 3.8% in Northern Ireland by Blackshaw, (1988) [4] 4.6% in Korea by Choo *et al*. (1995) [8] 4.7% in Turkey by Ozer *et al*. (1995) [22] and 5% in Italy by Ehlers *et al*. (1991) [11].

Similarly Andalo *et al*. 2018 [1], isolated EPN from areas with a higher vegetation cover compared to the other areas, as pasture and maize. However, Griffin *et al*. (2000) [12] verified that *H. indica* Poinar, does not restrict its distribution as a function of the vegetation cover. Even Campos Herrera *et al*. (2016) [6] stated that the presence of entomopathogenic nematodes obtained in surveys performed in natural areas is considered high in relation to surveys conducted in crop areas. Earlier reports also suggest that cultivation practices can influence the occurrence of EPN and that they may be more prevalent in crop lands than in natural habitats because of pest outbreaks and sustained availability of different insect populations (Hazir *et al*., 2003) [15]. Since the nematodes isolated from native soil are efficient for controlling pests of this soil (Hazir *et al*., 2003) [15], one target of this study was the isolation of native species and strains of EPNs from the C.G soils. Hazir *et al*. (2003) [15] suggested that entomopathogenic nematodes could be more prevalent in crop areas rather than natural environment areas, depending on population peaks and availability of different insect populations.

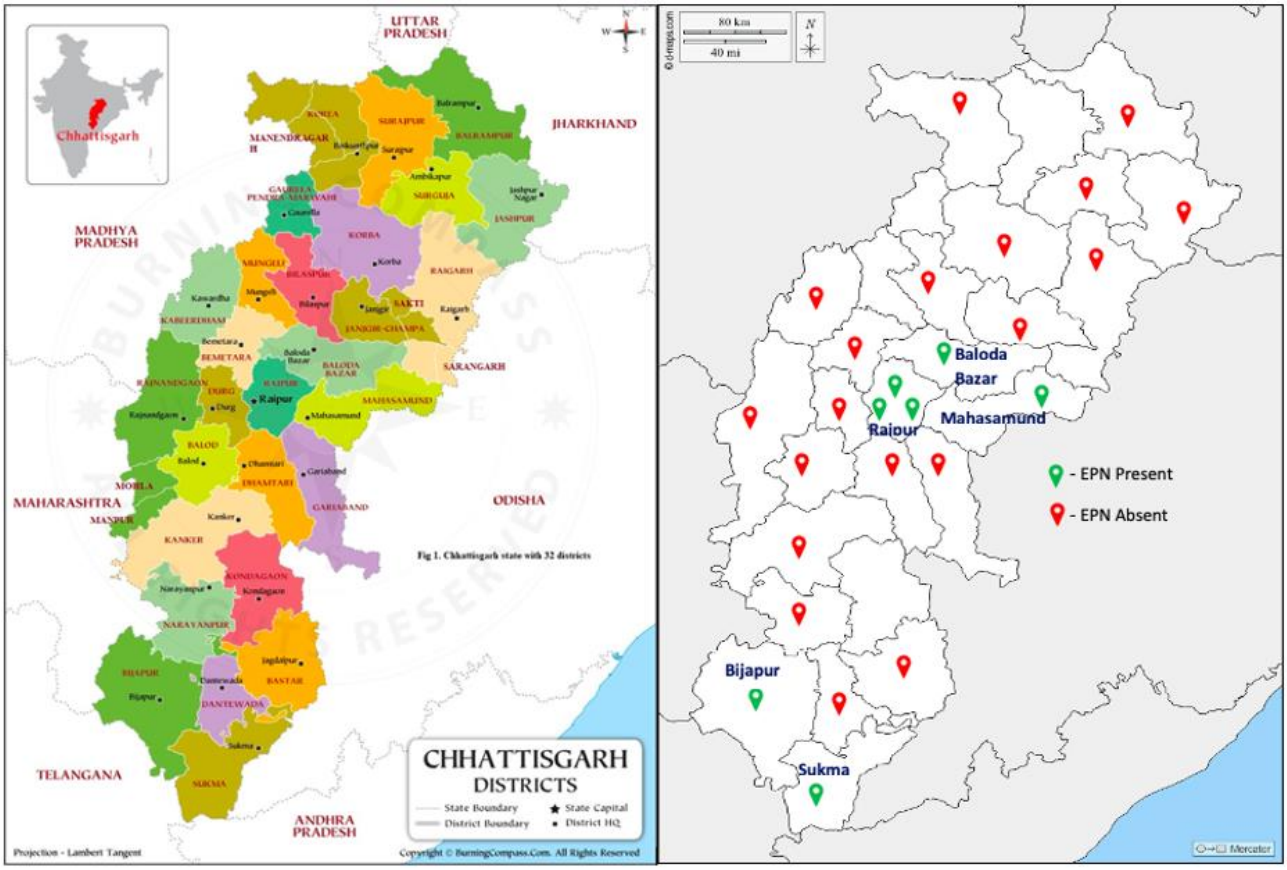

Fig 1: Collection of soil samples from 24 districts of C.G for EPN

Molecular identification of isolated Entomopathogenic Nematodes

The seven isolates whose sequences were obtained after sanger sequencing were blasted which resulted in *Heterorhabditis indica* species.

In agreement to our findings Yooyangket *et al*. (2018) [28] also detected *Heterorhabditis indica* using PCR diagnostic test at the infraspecific level, based on comparison of the partially edited nucleotide sequences (ITS and 28S rDNA) whereas Dhakal (2020) [9] has shown well resolved relationships between the clades only by using ITS rRNA gene sequences.

Soil characters

EPNs were recovered from silty loam and loamy sand soil, Choo *et al*. (1995) [8] reported more positive samples from sandy loam soils. They occurred in soils with electrical conductivity (EC) ranging between 0.12 and 0.39 dS/m with a pH range of 5.6-7.1 (acidic- neutral pH). Physicochemical tests determined that pH of soil level did not directly influence the presence of entomopathogenic nematodes: they were present in acid soil (pH<5.6) to neutral soil (pH>7.3) their presence in a wide range of pH soil levels (from 4.6 to 8) has also been established by Hara *et al*. (1991) [14], Griffin *et*

al. (1994) [13] and Stock *et al.* (1999) [26]. Other researchers have shown that the pH range of the soils from 4.5 to 5.7 was most considered tolerable by the EPNs (Nyasani *et al.*, 2008) [21]. The organic matter content was found to be in the range of 0.56- 1.06 % and this might be a reason for less recovery of EPN which was in accordance with the study of Dzięgielewska and Skwiercz (2018) [10] who found that low humus content (about 1– 5%) has less percentage recovery of nematodes whereas highest (36%) in the soil with moderate humus content (2–5%). Water holding capacity of EPNs collected soil was 32.05 - 38.87 % and bulk density was 0.96 – 1.27 g/cm3. An important factor for the migration of nematodes in soil is adequate soil moisture and in the present study the moisture content of the soil from EPN recovered sites varied from 4.26- 24 %. The present study has shown that nematodes can be found in low moisture soil as well as high moisture soils. Similar results were shown by Dzięgielewska and Skwiercz (2018) [10] who observed that lowest percentage of nematodes in soil samples was recorded in soils with extreme moisture levels: over 22% moisture content (25% of samples) and less than 1.53% moisture content (10% of samples). This shows that EPN can survive in low to high moisture content of soil although recovery of EPN depends on various other abiotic and biotic factors such as presence of insect population in the vicinity for its reproduction and survival whereas soil temperature of the EPN positive samples were more or less similar (20.23-26.3 ºC). There were no obvious trends associated with any of the soil characters and presence and absence of EPN.

Table 1: Characterization of the soil physico-chemical parameters and enumeration of entomopathogenic nematodes from different districts of C.G.

S. No	District	Sampling site	GPS location	Crop	pH	EC dS/m	OC (%)	WHC (%)	Soil Texture	Bulk density (g/cm3)	Moisture %	Soil Temperature (°C)
1.	Baloda Bazar	Horticulture Nursery, Hasda, Bhatapara	N21^{0}43'50.8584" E82^{0}2'27.0258" 236 m MSL	Ber	6.6	0.39	0.9	38.87	Silty Loam	1.14	24	20.83
2.	Mahasamund	Krishi Vigyan Kendra, Bhalesar	N21^{0}5'42.55764" E82^{0}3'50.53356"	Mango	6.46	0.2	0.57	32.05	Loamy Sand	1.2	4.26	20.23
3.	Raipur	PFDC Farm, College of Agriculture, IGKV	N21^{0}14'14.68788" E81^{0}42'51.29784"	Sapota	6.31	0.23	0.9	36.71	Silty Loam	1.19	21.69	23.3
4.	Raipur	PFDC Farm, College of Agriculture, IGKV	N21^{0}14'14.68788" E81^{0}42'51.29784"	Papaya	7.13	0.19	0.75	45.8	Silty Loam	1.21	11.05	26.8
5.	Raipur	PFDC Farm, College of Agriculture, IGKV	N21^{0}14'14.68788" E81^{0}42'51.29784"	Guava	6.42	0.2	0.79	37.01	Loamy Sand	0.96	14.41	24.6
6.	Bijapur	Krishi Vigyan Kendra, Bijapur	N18^{0}46'30.4662" E80^{0}52'52.23612" 260 m MSL	Mango	6.42	0.12	0.56	33.63	Silty Loam	1.27	6.37	24.63
7.	Sukma	Krishi Vigyan Kendra Farm, Sukma	N18^{0}23'3.71364" E81^{0}41'9.30624" 141 m MSL	Mango	5.6	0.29	1.06	36.36	Loamy Sand	1.22	7.86	26.3

Conclusion

This is a novel and first of its kind study where extensive survey of Entomopathogenic Nematodes was carried out in the state of Chhattisgarh. In this study, seven isolates of *Heterorhabditis indica* obtained were compared with soil properties and there was no particular trend in soil physio-chemical properties found from EPN recovered and non EPN soil samples. They were found from wide range of soil. Owing to their ability of not harming the natural enemies, they can be employed as a biocontrol agent in organic farming as indigenous strains provides a better alternative than exotic EPN species to manage insect pests. It is predictive from this study that orchards provide congenial abiotic conditions such as suitable moisture and temperature for the survival of EPNs and further surveys would help in finding more local strains of EPNs that can be utilised as a biopesticide and can be incorporated in Integrated Pest Management programme.

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