Research Article

Entomopathogenic Nematodes Survey and Identification from Different Districts of Khyber Pakhtunkhwa, Pakistan

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Abstract | A survey was undertaken in six districts of Khyber Pakhtunkhwa i-e Chitral, Kohat, Haripur, Mardan, Swat, and Peshawar to collect entomopathogenic nematodes, Insect bait technique was used for Entomopathogenic nematodes collection. For this experiment, *Galleria mellonella* larvae were reared in the laboratory. When samples soil were collected from different regions of Khyber Pakhtunkhwa for nematodes collection *G. mellonella* larvae were left in sampling jar and were collected back after 2-3 days' post application. Dead larvae were then put on the white trap and EPNs has then collected afterward. It is concluded on the basis of results that EPNs is positively recorded in grassy land, tomato field, forest land and poplar trees land in six selected districts of Chitral, Kohat, Haripur, Mardan, Swat, and Peshawar of Khyber Pakhtunkhwa. Furthermore, the EPNs is positively investigated to maximum level in Chitral, Peshawar, Mardan, Haripur, Swat, and Kohat respectively. The EPNs positive sample indication is directed that the environment of these districts are appropriate for EPNs presence.

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Introduction

An underground soil system is more abundant in living organisms as compared to above ground soil systems and nematodes serve as a diverse component of this system (Yeates et al., 1993; Giller, 1996). Nematodes are a diverse group of organisms that are known to have a significant role in different functions in soil like bacteria transporting and mineralization (Lawton et al., 1996; Ferris et al., 1998). Although nematodes are diverse group with important functions within a soil still a very little study is available on by ecologist. However, nematodes that parasitize vertebrate hosts got more researched and consideration (Strong et al., 1996; Preisser and Strong, 2004; Jaffee and Strong, 2005). A major difficulty that is normally facing with working on these organisims are recognizing them on the basis of morphological characters up to species level. To study biology and population of nematodes in detail, an alternative method is required for quantification and identification of these organisms. Entomopathogenic nematodes (families Steinernematidae and Heterorhabditidae) are nematodes group that are vital both economically and ecologically. These are fatal parasites of a wide-ranging insect pests. A significant role played by these nematodes in regulating webs of soil food (Kristan and Hammond, 2004; Cattadori and Hudson, 2005; Mougeot et al., 2005; Grewal and Peters, 2005). EPNs serve as biological insecticides, mass-produced *in vitro* and sold around the world for controlling root weevil in the USA, almost 25,0 00 ha of citrus are treated annual-

ly with Entomopathogenic nematodes.(Gaugler and Han, 2002).

Entomopathogenic nematodes have a different life cycle as compared to other parasitic organisams. Entomopathogenic nematodes release a specific type of bacteria inside the host body that cause death of the cadaver. The nematodes enter its host body through different means like mouth, anus or spiracles and release this bacterium which replicate inside the cadaver. The nematodes feed upon cadaver, reproduced there and remain there until the food become limiting and nematode find new host for itself (Hominick and Gaugler, 2002). Ecological preferences of Entomopathogenic nematodes will help in their host preferences in spite of their commercial exploitation to use it as biological control. For example, nematode species used to control woodland insect pest found especially in coastal soils just become possible due to the information about their habitat preferences. The 40 different species of this group of nematodes are cosmopolitan in nature, having been found in a diversity of habitats, except from Antarctica (Han and Ehlers, 2000). Still, habitat preference literature not sure yet for Entomopathogenic nematodes, especially from studies pre-1995 (Mracek, 2006), so it is requirement of day to do more surveys for correct identifications with large sizes of samples (Hasan et al., 2009).

The two main groups of Entomopathogenic nematodes identified so for i.e. the Steinemematids and the Heterorhabditis. Both genera belong to the order Rhabditida. Genus Steinernema considered as the major Entomopathogenic nematode group used for biological control. 38 species of genus Steinernema and another 8 species belong to Heterorhabditis reported highly parasitic on lepidopterous and coleopterous insect larvae (Hominick et al., 1997). Due to the presence of molecular methods available now days, non-skilled nematologist can also do profitable surveys that might give quantitative data (density of population) and qualitative (absences/ presence) relate to particular nematodes of interest in field of identification. Several molecular methods have developed by molecular biologists working with numerous organisms to detect and quantify populations. One technique that mostly in used is real-time PCR (Hominick et al., 1997; Stock et al., 1999; Sturhan and Ruess, 1999; Mracek et al., 2005). Habitat allowed the presence of different species in different areas. *Steinernema affine* (Bovien) and *S. kraussei* (Steiner) both species are known to be found in Scotland (Gwynn and Richardson, 1996; Spiridonov et al., 2004) and thought to favor different habitats (Ferre, 1992; Clementi et al., 1993; Foley et al., 1993; Higuchi et al., 1993; Cross, 1995; Brunborg et al., 2004; Atkins et al., 2005). Keeping in view the present study was conducted to find out the occurrence of EPNs in the selected districts of Khyber Pakhtunkhwa.

Materials and Methods

Rearing of host, Galleria mellonella

Galleria mellonella (L.) was reared on an artificial diet. Artificial diet was prepared by mixing 300 ml liquid honey, 400 ml glycerol, 200 ml milk powder, 200 g whole-meal coarse flour, 100 g dried brewer's yeast, 100 g wheat germ and 400 g bran. The diet was prepared in an open sterilized container and then transferred to a plastic container. The larvae obtained from Agriculture Research Institute Tarnab, Peshawar was placed in a rearing chamber on this artificial diet already prepared along with Crumpled paper towels or corrugated cardboard was added to the larval container. The female moths were ovipositing in the folds of paper towel and resulting larvae was started feeding on artificial diet. Then late instar larvae or pupa was harvested and placed into a second container. Late instar larva was allowed to pupate or pupa to emerge as adults. Then adults were mated and females were do laying eggs again. The eggs were then shifted to fresh food and the life cycle was continued (Ellis et al., 2013).

Rearing of entomopathogenic nematode

The EPNs were reared Lindegren et al. (1993). A 9 cm Petri dish lined with Whatmann's no.1 filter paper was sterilized in an autoclave at 121° C, 15 lbs/ inch². It was cooled downed for 20 minutes at room temperature. Suspension of Infective Juvenile (IJ) EPNs (500 IJs/ ml) was dropped thoroughly over the filter paper. Ten of the 5 - 6th instars of *G. mellonella* was placed on Petri dish and incubated at 20±5°C for 48 hours at the dark situation. *G. mellonella* cadavers were then transferred to modified»white traps« (White, 1929) which was consisted of a glass Petri dish (9 cm in diameter) filled with distilled water to a depth of 0.5 cm. The bottom of an inverted Petri dish (3 cm in diameter) was placed in the bigger Petri dish. A sheet of filter paper was placed on the smaller Petri dish allowing the edge of the paper to

come in a contact with the distillated water. The dead larvae were placed on the filter paper and incubated at room temperature until all the nematode progeny had emerged and moved down into the water of the bigger Petri dish. Then the IJs of EPNs was harvested after two weeks and EPNs suspension was then kept in tissue culture flask at 10°C in an incubator.

Survey of EPNs from different areas of Khyber Pakhtunkhwa province

Collection of soil samples: It is known that Entomopathogenic nematodes distribution normally depends on temperature and precipitation and is closely related to vegetation type and presence of insect hosts. On the basis of above-mentioned facts survey sites were selected as i.e. Peshawar, Mardan, Swat, Chitral, Haripur and Kohat. Soil samples were collected from cultivated and non-cultivated areas as grassland and forest land of above-mentioned areas to increase chances of EPNs presence.

A hand shovel was used to collect soil samples. Each soil sample was comprised of 6-15 sub-samples taken from same location almost 10 m away from each other. Total of 108 soil samples were collected and checked for EPNs presence from different cultivated and non-cultivated areas of Khyber Pakhtunkhwa province during 2015-2016. Each time after sampling the shovel was water rinsed and air dried to reduce chances of contamination.The soil sample was then mixed and half of each sample was then used for extraction of Entomopathogenic nematodes (Campos-Herrera et al., 2011; El Borai et al., 2012).

Nematode isolation and propagation

Insect baiting technique was adopted for isolation of EPNs from samples of soil, used by Bedding and Akhurst (1975). Five last instar *Galleria mellonella* (L.) larvae were placed in an empty jam glass jar containing moistened soil obtained from one of the samples and stored at room temperature (25 ± 2°C) for 2 weeks. The jars were checked every two days after 48 hours of placing the larvae. Dead larvae from each container were placed on White traps to collect emerging IJ or J2. After collection, the nematodes were then pooled and used to infect new fresh *G.mellonella* larvae to check their pathogenicity.

Statistical analysis

The collected data were analysed through SPSS software (version 16.0) for the presence of EPNs positive samples.

Results and Discussion

Field crops and EPNs status in six selected districts of Khyber Pakhtunkhwa province presented in Table 1. These field crops included apple orchards, cabbage, carrot, maize crops, peach orchard, pine tree forest, poplar tree land, potato, spinach, sugarcane, tobacco and wheat. The soil samples from 20 cm depth were collected from 6 different locations of field crops. However, maximum 76.90% soil samples were recorded which showed negative availability of Entomopathogenic nematodes (EPNs) in different field crops. These negative EPNs reported field crops were consisting of 100% apple orchard, cabbage, carrot, maize crops, peach orchard, pine trees forest, poplar tree land, potato crops, spinach, sugarcane, tobacco field and wheat crops. Similarly, minimum 23.10 % soil sample were investigated EPNs positive. These negative EPNs reported field crops were consisting of 100% apple orchard, cabbage, carrot, maize crops, peach orchard, pine trees forest, poplar tree land, potato crops, spinach, sugarcane, tobacco field and wheat crops.. Afterward, the rearing procedure and molecular studies applied for investigation of EPNs in soil samples. Finally, the positively EPNs were recorded 42.85, 42.85, 25 and 16.67% in grassy land, tomato field, forest land and poplar trees land respectively (Table 2). The results from six locations of Province KP indicated that 88.90, 83.30, 83.30, 77.80, 77.80 and 50% soil samples were negatively investigated EPNs respectively (Table 3). Similarly, 50, 22.20, 22.20, 16.70, 16.70 and 11.10% positively EPNs were investigated in six different regions soil of KP province respectively (Table 3). Moreover, soil samples from six different districts were collected from forest land field, grass land and tomato field. In these fields soil 100 and 50% EPNs were investigated positively in tomato field and grassy field, followed by Peshawar district and soil samples were collected from cabbage, carrot, grassy, maize crop, peach orchard, spinach crop and sugarcane field. These soil samples were investigated maximum 80% EPNs positively recorded in grassy field. Similarly, Soil samples from apple orchard, cabbage, forest land, grassy land and tomato field were collected from district Swat. Meanwhile, soil sample from cabbage, grassy land, polar tree land, sugarcane and wheat crop were collected from district Mardan. 50 and 16.70% positive EPNs were investigated from the soil samples of grassy land and poplar tree land. Afterwards, soil samples from grassy land, maize crop, pine tree forest

land and tobacco crop were collected from district Haripur. Positively 50% EPNs was recorded from these soil samples in grassy land field. Finally, 25% positive EPNs were investigated in grassy land field collected from Kohat district different soil samples of Kohat (Table 3).

Table 1: *Entomopathogenic nematodes (EPNs) status in different vegetation's of six selected districts of Khyber Pakhtunkhwa Province.*

Vegetation	EPNs status		Total no. of sampling
	Positive (%)	Negative (%)	
Apple orchard	0	100 (3)	3
Cabbage field	0	100 (8)	8
Carrot field	0	100 (3)	3
Forest land	25 (3)	75 (9)	12
Grassy land	42.9 (18)	57.1 (24)	42
Maize crop	0	100 (14)	14
Peach orchard	0	100 (1)	1
Pine trees forest	0	100 (4)	4
Poplar trees land	16.7 (1)	83.3 (5)	6
Potato crop	0	100 (1)	1
Spinach field	0	100 (1)	1
Sugarcane crop	0	100 (4)	4
Tobacco crop	0	100 (1)	1
Tomato field	42.9 (3)	57.1 (4)	7
Wheat crop	0	100 (1)	1
Total	23.1 (25)	76.9 (83)	108

Table 2: *District wise presence of entomopathogenic nematodes (EPNs).*

District	EPNs status		Total no. of samples
	Positive (%)	Negative (%)	
Chitral	50 (9)	50 (9)	18
Peshawar	22.2 (4)	77.8 (14)	18
Swat	16.7 (3)	83.3 (15)	18
Mardan	22.2 (4)	77.8 (14)	18
Haripur	16.7 (3)	83.3 (15)	18
Kohat	11.1 (2)	88.9 (16)	18
Total	23.1 (25)	76.9 (83)	108

The present research field survey has been conducted to find out native Entomopathogenic nematodes species in various districts of Khyber Pakhtunkhwa. Even though international surveys already were undertaken provide valuable data on EPNs distribution (Garcı´a del Pino and Palomo, 1994; Kary et al., 2009; Edgington et al., 2010; Khatri-Chhetri et al., 2010; Ma et al., 2010), species habitat preferences are still poorly understood. The present study aimed at understanding the natural occurrence of EPNs in

Table 3: *District wise EPNs status in different vegetation's.*

Districts	EPNs status		Total no. of sampling
	Positive (%)	Negative (%)	
Chitral			
Forest land	0	100 (3)	03
Grassy land	50 (6)	50 (6)	12
Tomato field	100 (3)	0	03
Total	50 (9)	50 (9)	18
Peshawar			
Cabbage field	0	100 (2)	02
Carrot field	0	100 (3)	03
Grassy land	(4) 80	20 (1)	05
Maize crop	0	100 (4)	04
Peach orchard	0	100 (1)	01
Spinach crop	0	100 (1)	01
Sugarcane crop	0	100 (2)	02
Total	22.2 (4)	77.8 (14)	18
Swat			
Apple orchard	0	100 (3)	03
Cabbage field	0	100 (3)	03
Forest land	100 (3)	0	03
Grassy land	0	100 (5)	05
Tomato field	0	100 (4)	04
Total	16.7 (3)	83.3 (15)	18
Mardan			
Cabbage field	0	3 (100)	03
Grassy land	50 (3)	50 (3)	06
Poplar trees land	16.7 (1)	83.3 (5)	06
Sugarcane crop	0	100 (2)	02
Wheat crop	0	100 (1)	01
Total	22.2 (4)	77.8 (14)	18
Haripur			
Grassy land	50 (3)	50 (3)	06
Maize crop	0	100(7)	07
Pine trees forestland	0	100 (4)	04
Tobacco crop	0	100 (1)	01
Total	16.7 (3)	83.3 (15)	18
Kohat			
Forest land	0	100 (6)	06
Grassy land	25 (2)	75 (6)	08
Maize crop	0	100 (3)	03
Potato crop	0	100 (1)	01
Total	11.10 (2)	88.9 (16)	18

KP-Province, representing the most systematic and extensive survey made for the first time in the province to evaluate indigenous species of EPNs. It is because, these species are effective more than exotic. Similarly, the novel species are suitable for commercial exploitation and certainly practicing as a management tool in the IPM technique. Surely, these novel species are an adjustment with natural environmental conditions. Another research of Choo et al. (1995) also surveyed to identify effective EPNs in various areas to find out the native species and strains to further explore it for commercial use.

The current study reports-first time EPNs occurrence in various districts of Khyber Pakhtunkhwa province of Pakistan. During the surveys, genus *Heterorhabditis* and *Steinernema* were recorded. At least two Steinernematids (IJ to develop in male and female) must invade before reproduction can occur while one *Heterorhabditis* is sufficient to multiply after an invasion. Similarly, the researchers Hazir et al. (2003a) find out Steinernematidae Spp (S. *feltiae*, *S. affine,* and an unidentified *Steinernema* sp.) with *H. bacteriophora* sp from Turkey. The results of the present research are confirming the presence of *H. bacteriophora* in Chitral and Haripur districts of Khyber Pakhtunkhwa province. Furthermore, the researchers Susurluk et al. (2002) isolated *H. bacteriophora* and unidentified species along with S. *feltiae* from Ankara during their research study. Furthermore, *S. feltiae* species has been recovered from the coast of the Black Sea by the researchers during their research field survey from Ankara, Turkey.

This field survey was conducted to check different vegetation for EPNs presence in various location of Khyber Pakhtunkhwa province. Therefore, various vegetations types such as grassy land, forest tree land, tomato crop, and poplar tree land were used for soil samples collection and this vegetation reports positive results in the research area. The same results were identified by other researchers (Eivazian et al., 2009) in the north-west mountainous area of Iran. Furthermore, the results of Eivazian et al., 2009 indicated that the highest recovery rate was recorded in Grassland followed by alfalfa field, orchards, cropland and then vegetable in the mountain area of Iran. Moreover, cotton field, fields of potato, poplar, fallow and sunflower area were totally absent for nematodes in the mountain area of Iran. Their study finds out that isolates *of H. bacteriophora* found out from alfalfa fields and grasslands (41.2%, and 35.3%). Similarly, *S. carpocapsae* was isolated from an orchard and *S. bicornutum* found from an alfalfa field. *H. bacteriophora* were isolated from 4 various habitats of 17 places (grassland, vegetable, orchard, and alfalfa) and along-with some climate differences as Iran north-west parts, annual temperature of mountainous area range from -20 to 39°C with a temperate-cold climate. Hence, it is possible that the relevant isolates might have diverse traits of ecology. The EPNs mostly recovered from grassland, orchard and alfalfa fields where chemical control usage was almost equal to none which make it cleared that region of agriculture through high chemical to control a pest, input has an EPNs with lower recovery rate. Furthermore, chemical pesticides should directly or indirectly impact on EPNs to reduce its host abundance. Afterward, in the north-west regions of Iran, *H. bacteriophora* was widely distributed entomopathogenic nematode followed by *S. feltiae* species which was distributed worldwide (Parkman et al., 1994; Adams et al., 2006). Dispersal events and climate changes, as well as those related to activities of human beings, could be a strong source of Entomopathogenic nematodes distribution worldwide.

The *Heterorhabditis* juveniles disperse actively and passively, both horizontally and vertically (Hominick et al., 1996). Passively, they may be dispersed through Insects, human, soil, wind and rain. The texture of soil, suitable host's availability and vegetation are factors that have been implicated in influencing pattern of local distribution (Smart and Nguyen, 1994).

Total sampling size consisted of one hundred and eight soil samples collected from various regions of Pakistan in which only 25 soil samples (23%) showed positive results. Probably, low positive results may be because of the only choice of insect *G. mellonella* for baiting and it may not be the suitable host for every species of EPNs. Similar low results were also recorded by two other scientists. Probably, the temperature is also a considerable factor. Baiting at room temperature is standard for EPNs in the laboratory. Not only our results but scientists in different regions of the world also found low positive results for EPNs (Choo et al., 1995; Rosa et al., 2000; Hazir et al., 2003a). Meanwhile, *Steinernema* sp found in temperate regions of the world while *H. bacteriophora* distributed in continental and Mediterranean climates (Glazer et al., 1991; Iraki et al., 2000; Salama and Abd-

Elgawad, 2001). These findings support the results of the present research study. Similarly, *S. feltiae* distributed widely in temperate regions, whereas *H. bacteriophora* usually present in areas with the Mediterranean and continental weathers (Adams et al., 2006). *Heterorhabditis* sp. found the foremost genus of EPNs in Mediterranean countries of the Middle East like Egypt and West Bank Palestinian Territory (Hominick, 2002). Moreover, *S. carpocapsae* recorded a universal spreading, however, sometimes in northern and central Europe recorded at low frequency. There is rising evidence of nematode species preferences for certain habitats. *Steinernema* sp. was found mostly in grasslands, and absent virtually in forests (Adams et al., 2006). *Heterorhabditis* sp. firstly described in the Czech Republic as indigenous. Therefore, it was not recovered by Mracekˇ and Becvar, 2000 although; they did sampling intensively in appropriate habitats, including different localities. In an extensive survey in Germany, *S. carpocapsae* was found at only one of different 1193 sites (Sturhan, 1999). The rate of recovery of this study is supporting the present research field survey. This observation has led to the hypothesis that *Heterorhabditis* sp. evolved from marine nematodes and Steinernematids from terrestrial nematodes (Poinar, 1983). One other relevant study on habitat preference of *Heterorhabditis* is that of (Stuart and Gaugler, 1997). In their New Jersey survey, they found that *Heterorhabditis* were abundant equally in weedy and turf habitats, but were absent from closed canopy forest.

Heterorhabditis genus was recorded as the most widely distributed genera in EPNs and mostly recovered from uncultivated grassland (Yoshida et al., 1998). Similarly, *H. indica* was isolated from fourteen sites in the subtropical to warm temperate coastal regions. Diverse families/genera/species/genera of these parasites found in various ecosystems/habitats depending on their hosts'insect (Hominick et al., 1997). It has been reported *Heterorhabditis* sp are in warmer, tropical conditions while, *Steinernematids* are found in cooler, temperate regions (Mracek, 2006). Furthermore, *H. indica* occurred in various vegetation's such pine forest, grassland on the side of cropland, in a park with turf grasses and a beach site grasses in Ireland. The results of various research studies showed the same finding of our study where *Heterorhabditis* sp. was recovered from uncultivated grass and forest land of Chitral and Swat district of Khyber Pakhtunkhwa province.

Conclusions and Recommendations

It is concluded on the basis of results that EPNs is positively recorded in grassy land, tomato field, forest land and poplar trees land in six selected districts of Khyber Pakhtunkhwa. Certainly, the soil and host of these fields are suitable for the availability of EPNs. Furthermore, the EPNs positively investigated in maximum from Chitral, Peshawar, Mardan, Haripur, Swat, and Kohat respectively. The EPNs positive sample indicated that environment of these districts are appropriate for the presence EPNs. Afterward, the Chitral district records EPNs in grassy land and tomato fields, in Peshawar district EPNs were found in grassy land. While from Swat district EPNs was recorded in forest land. Similarly, Mardan district records showed that EPNs was present in grassy land and poplar tree land. Finally, Haripur and Kohat district records EPNs positivity in the grassy land. Surely, the environment of Khyber Pakhtunkhwa districts favorable for EPNs presence in different soil and hosts i.e. grassy land, tomato fields, forest land and poplar trees land.

On the basis of above-mentioned conclusion, it is recommended that Khyber Pakhtunkhwa province soil is enriched in EPNs. An extensive survey should be done in EPNs positive sampling areas in different vegetation's and soil type for better results and species.

Novelty Statement

This is a first study which indicates occurence and identification of entomopathogenic nematodes from differnt districts of Khyber Pakhtunkhwa, Pakistan.

Author's Contributions

ZH and ARS conceived and designed the experiments. ZH performed the experiments. FA analyzed the data. ZH, ARS and FA wrote the paper.

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