A Survey of *Globodera Rostochiensis* on Potato Crops in Himachal Pradesh; Occurrence and Detection.

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Research Article

Keywords: Globodera, survey, soil sampling, plant parasitic nematode, potato crops, cyst nematode, percentage of occurrence.

Posted Date: September 13th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-770909/v1>

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Abstract

A survey was carried out to estimate the frequency and occurrence of potato cyst nematode (PCN) in potato cultivating fields in different districts of Himachal Pradesh. A total of 109 composite soil samples were collected from 5 districts of Himachal Pradesh, India. Nematode cysts were extracted from the soil using standard procedure. The population density and percentage of occurrence of cyst nematode were calculated. According to the results district, Mandi had the highest PCN cyst population density, and the percentage of occurrence is 100%. District Kangra had the least PCN cyst population density and the percentage of occurrence is 66.6%. This survey showed the occurrence *Globodera rostochiensis* district-wise. The collected cysts were identified by morphological and molecular techniques, up to species level based on nucleotide homology and phylogenetic analysis. According to morphometrics distance between the vulva and anus is very less (65.69 ± 22.2) μm . The outcome sequences are showed 95-99.05% similarity with sequence I.D KJ636272.1 from the Netherlands. This survey indicates that PCN *Globodera rostochiensis* were widely distributed in the potato cultivating fields of Himachal Pradesh. The present study helped determine the distribution of PCN in the soil and species dominance in particular areas. Hence, it helps design different strategies against PCN management.

Introduction

Potato cyst nematode (PCN) is highly adaptive and cause damage to potato plants. It consists of two highly specialized species *G. rostochiensis* and *G. pallida*. Both show successful and long association with their host plants. PCN has a special ability to survive during adverse conditions and has high reproductive potential. Their resistant behavior for their survival makes it difficult to eradicate from the soil once established. Studies reported the presence of *Globodera* spp. in several countries (Table 1) and cause damage to tuber crops (Okhi *et al.* 2018). Variation in virulence of *Globodera* is also found in Europe and South American population on different species of potato (Phillips and Trudgill, 1998). PCN follows the unique life cycle, a dead female hardened their body, convert in the form of a spheroid or rounded shape cyst and protect the eggs within. After hatching infective juveniles can disperse up to 1m in the soil. PCN infects plants by entering into the roots of the plants through the growing root tip, form feeding sites in the roots by modifying the root cells, nematode becomes sedentary. This infection leads to reduced root growth, a stunted shoot system, and poor productivity with ultimately leads to plant death (Mimee *et al.* 2015; Cotton *et al.* 2014). In the presence of the host plant, hatching can occur annually on an average 60-80% but about 20-30% of hatching can also take place in the absence of the host. The cyst can survive in the soil for the long period. PCN completed its life cycle 56-63 days both in the autumn and spring season (Ebrahimi *et al.* 2014). Sexual reproduction occurs in nematodes. Sex attractant pheromones are secreted by the females to attract males for mating, nematodes mate several times in their life span. Each female produces 200-500 eggs, after the death cuticle of the dead female convert into the cyst. Under unfavorable conditions, eggs remain in the dormant stage inside the cyst. After receiving a hatching stimulus in the form of chemicals released from the host root, hatching occurs. Juveniles released from the cyst. PCN eggs can remain inside the cyst in the dormant stage up to 30 years and are resistant to many nematicides. *Globodera* spp. hatching generally occurs at 10°C or above. *G. rostochiensis* eggs hatch between 5-29 °C temperature In favorable temperature conditions second-stage juveniles hatch from the cyst and escape from the cyst moves towards

the roots of the host plant (Kaczmarek *et al.* 2014). PCN can cause 33-54% potato tuber yield loss which may be considered a serious threat to potato cultivation. With the increase in the rate of infestation by PCN, it's urgent to formulate strategies for its management as well as it's very important to check further spread in the potato field (Seenivasan 2017).

This study was conducted to evaluate the population density and frequency of occurrence of potato cyst nematode (PCN) *G. rostochiensis* on potato crops in Himachal Pradesh.

Table 1: A short report of <i>Globodera</i> spp. in different countries		
Species	Country	Reference
G pallida	Japan	Okhi <i>et al.</i> 2018
G pallida	Peru	Ponce, 1977
G pallida	Spain	Ponce, 1977
G. pallida	India	Ganguly <i>et al.</i> 2010
G. pallida	United Kingdom	Kaczmarek <i>et al.</i> 2014
G. pallida	India	Seenivasan 2017
G. pallida	Panama	Ponce, 1977
G. pallida	USA	Skantar <i>et al.</i> 2011
G. rostochiensis	Russia	Subbotin <i>et al.</i> 2000
G. rostochiensis	India	Seenivasan 2017
G. rostochiensis	India	Ganguly <i>et al.</i> 2010
G. rostochiensis	USA	Skantar <i>et al.</i> 2011
G. rostochiensis	Bolivia	Ponce, 1977
G. rostochiensis	United Kingdom	Kaczmarek <i>et al.</i> 2014
G. rostochiensis	Guatemala	Ponce, 1977
G. sandveldensis	South Africa	Knoetze <i>et al.</i> 2017
G tabacum	Italy	Subbotin <i>et al.</i> 2011.
G. tabacum	USA	Skantar <i>et al.</i> 2011
G. mexicana	Mexico	Subbotin <i>et al.</i> 2011.

Material And Methods

Survey and soil sampling: Random survey was conducted in different districts of Himachal Pradesh during 2018-2021 where potato is cultivated in all three seasons i.e spring-summer and autumn year after year at

elevation 465m-7000m (1526ft-23000ft). For soil sampling two available methods were followed to collect samples from the fields:

1) By collecting soil samples around the plant rhizosphere and processing them into the laboratory. 2) By lifting plants gently and examining roots either in the field or in the laboratory for cyst/females. These methods have been used to detect the population of cysts in the soil. Cyst nematode host plants are available during a part of the year but highly resistant cysts remain available in the soil throughout the year. A soil sample should be taken randomly and in large amounts to achieve accuracy. A soil sample must be taken repeatedly from the same field to ensure the presence of a cyst. To achieve 90% of probability of detection of cyst nematode total sample size must be 6.9KG/ 0.33 hector best for comprising a minimum sample with the maximum probability of detecting cyst. The only aim of sampling is to achieve a high level of probability for getting cyst nematodes with a high level of accuracy (Wainer and Dinh 2021). A total of 109 soil samples were collected from 7 distt. The soil sample was collected from the root rhizosphere of the potato plant. The cysts were extracted from the soil by using 100 mesh sieve and 60 mesh sieve from 250ml of a soil sample by Fenwick method (Fenwick 1940). The cyst was hand-picked with the help of a sterilized brush and forceps under a stereo zoom microscope. The collected cyst was subjected to morphological and molecular characterization for identification up to species level.

Population density (P.D) and percentage of occurrence were calculated (Bakr *et al.* 2011)

P.D. = No. of nematodes / total no. of samples. % occurrence = No. of positive samples / total no. of samples ×100

Morphological identification: pure population of the collected cyst was subjected for morphological and morphometric identification under stereo zoom microscope and dissecting microscope. According to the morphological identification of PCN cyst shape, cyst color and the vulval cone is observed. Morphometrics of PCN was recorded according to the measurements of main diagnostic characteristics such as body length, body width, neck length, distance from vulval basin to anus, number of cuticular ridges between vulval basin, and anus and Granek's ratio.

Molecular characterization:

DNA extraction and polymerase chain reaction: Genomic DNA was isolated from a pure population of 100 mature cysts by using Qiagen Puregene buccal cell kit following manufacturer procedure and quantify in nanodrop spectrophotometer and agarose gel. This resulted in DNA is amplified in PCR. For the amplification of DNA following primers were used i) forward primer SSU18A (AAAGATTAAGCCATGCATG) ii) reverse primer SSU26R (CATTCTTGGCAAATGCTTTCG). For master mix of 25ul reaction was set up consisting of 10 µl of the DNA suspension was added to the PCR reaction mixture containing 0.2 mM dNTP's, 0.2 µM of each of the primer (reverse and forward) and 2.5 µl 10× buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂), 1 unit of Taq polymerase, and double distilled water to a final volume of 25 µl. The reaction conditions were: 94 °C for 5 min; 35 cycles of 94 °C for 1 minute; 52 °C for 1 minute 30 s; 68 °C for 2 min; 68 °C for 10 min Products (5 µL) were visualized on agarose gels stained with ethidium bromide (Floyd *et al.* 2002). By the

process of electrophoresis in Tris-Borate-EDTA (TBE) buffered 1.5% agarose gels with ethidium bromide the PCR products were separated.

Data analysis: Nonparametric tests were performed to analyze the difference among the cyst populations. The differences of cyst population among the various locations within a single district were accessed by Kruskal Wallis Test and pairwise comparison between the locations was made by Dunn's Multiple Comparison Test. For comparison of two groups, Mann Whitney test was performed. Also, the complete data of districts (Mandi, Chamba, Kullu, Kangra) was made in the same manner. GraphPad PRISM 5.0 was used to perform the analysis. Data for Una, Hamirpur, and Solan was not taken for analysis as no cyst was found in the soil of these regions.

Sequencing and phylogenetic analysis: PCR products were purified according to manufacturer's protocol and Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI Genbank database. Based on the maximum identity score first ten sequences were selected and aligned using the multiple alignment software programs Clustal W. the phylogenetic tree was constructed using MEGA 7.

Results And Discussion

In Himachal Pradesh, the population ranged between 245cyst/250cc soil. In Himachal Pradesh, the population of the cyst ranged from minimum to maximum. In the district, Mandi samples were collected from four different areas i.e Basunti, Rewalsar, Phulladhar, and Dhanotu among these maximum populations of the cyst were collected from Basunti and Phulladhar samples. In Distt Chamba samples were collected again from four different areas i.e Ahla, Banikhet, Salooni, and Saru. Out of these four areas maximum cysts were collected from Ahla area. The third Distt was Kullu, samples were collected from four different areas; Bajaura, Bandrol, Anni, and Bhuntar. The maximum cyst population was collected from Bajaura area. From Distt Kangra samples were collected from eight different areas i.e Talpura, Kangra, Jiya, Skoh, Pariharkhar, Shahpur, Malan, and Bagoda out this Jiya area has the maximum number of the cyst. Samples were collected from Distt Una, Hamirpur, and Solan, particularly these sites i.e Akrot farm, Daulatpur, Rampur, TiraSujanpur, Nadaun, Kandaghat, and Darlaghat didn't recover cyst.

Table 2: Showing the morphometrics of *Globoderarostochiensis* cyst in μm . ($\pm$ is mentioned as standard deviation).

In molecular characterization after sequencing, the resulted sequence were submitted to genbank NCBHI. Sequence ID MW577347 (HPPOCH) showing 99.05% similarity with KJ636272.1 from the Netherlands. Sequence ID MZ508279 (HPPOKL) showing 95.58% similarity with KJ636272.1 from the Netherlands. Sequence ID MZ508280 (HPPOMD) showing 99.05% similarity with KJ636272.1. Sequence I.D MZ518783 (HPPOPL) 98.58% similarity with KJ636272.1 from the Netherlands. All the nucleotide sequences of Himachal Pradesh showing similarities with the Netherlands.

Characters	Measurements in μm
Body length	613.08 $\pm$ 23.96
Body width	555.79 $\pm$ 36.04
Neck length	148.84 $\pm$ 38.46
Distance from vulval basin to anus	65.29 $\pm$ 22.20

Table 3. Population density and frequency of occurrence of PCN in various district of Himachal Pradesh in potato growing fields.

Distt	Location	Mean No. of cyst/ 250g of soil from different areas.						Sum	P.D	Occurrence
		1		2		3				
Mandi	Basunti	50	±1.63	48	±2.16	45	±1.63	143	47.67	100
	Rewalsar	10	±0.82	7	±1.70	12	±2.45	29	9.67	100
	Phulladhar	55	±3.74	49	±5.31	48	±1.89	152	50.67	100
	Dhanotu	5	±4.11	8	±4.19	5	±2.05	18	6	100
Chamba	Saru	0	±0.00	2	±1.63	0	±0.00	2	0.67	33.33
	Ahla	52	±2.16	50	±2.45	54	±2.49	156	52	100
	Banikhet	20	±5.10	18	±3.30	18	±6.48	56	18.67	100
	Salooni	5	±2.05	4	±1.70	5	±3.74	14	4.67	100
Kullu	Bajaura	48	±5.25	45	±4.08	50	±10.96	143	47.67	100
	Bandrol	4	±1.63	0	±0.00	0	±0.00	4	1.33	33.33
	Anni	25	±5.10	16	±3.09	21	±4.50	62	20.67	100
	Bhuntar	15	±2.45	14	±5.66	17	±2.16	46	15.33	100
	Talpura	0	±0.00	0	±0.00	0	±0.00	0	0	0
Kangra	Kangra	0	±0.00	0	±0.00	0	±0.00	0	0	0
	Jiya	45	±2.49	48	±2.49	0	±0.00	93	31	66.66
	Skoh	0	±0.00	0	±0.00	0	±0.00	0	0	0
	Parihar khar	3	±1.41	1	±0.82	0	±0.00	4	1.33	66.66
	Shahpur	0	±0.00	0	±0.00	0	±0.00	0	0	0
	Malan	0	±0.00	0	±0.00	0	±0.00	0	0	0
	Bagoda	0	±0.00	0	±0.00	0	±0.00	0	0	0
Una	Akrot farm	0	±0.00	0	±0.00	0	±0.00	0	0	0
	Daulatpur	0	±0.00	0	±0.00	0	±0.00	0	0	0
	Rampur	0	±0.00	0	±0.00	0	±0.00	0	0	0
Hamirpur	Tirasujanpur	0	±0.00	0	±0.00	0	±0.00	0	0	0
	Nadaun	0	±0.00	0	±0.00	0	±0.00	0	0	0
Solan	Kandaghat	0	±0.00	0	±0.00	0	±0.00	0	0	0
	Darlaghat	0	±0.00	0	±0.00	0	±0.00	0	0	0

P.D = cyst population density/total no. of samples, % occurrence = No. of positive samples / total no. of samples x 100; $\pm$ =standard deviation.

Result mentioned in the table.2 explains that the population density and % occurrence of PCN greatly differs from examined locations. The population density and % occurrence of PCN in Distt Mandi were highest and 100%. Whereas Distt Kangra showed the lowest population density and % occurrence. Soil samples collected from Distt Una, Hamirpur, and Solan were showed nil results from particular areas mentioned in the above table. Thus, data recorded during the survey conclude that Distt Mandi had the highest distribution of PCN and lowest in Distt Kangra. Such differences may occur due to location soil type, soil moisture, and irrigation system.

Statistical analysis of the cyst populations reveals some considerable results as given in Table 4 and 5. Table 4 comprehends the summary of P values for the test of differences between a different location within a district and all districts altogether. It can be seen that the cyst population was significantly different between the locations of district Mandi, Chamba, and Kullu. Also, the cyst populations between the districts (all locations altogether) are significantly different ($P < 0.05$). Figure 1-5 depicts the graphical variation of cyst population between the groups.

Pair-wise comparisons between all the groups in concern have been done and its results have been comprehended in table 5. Most of the pairs are not found to be different in the cyst population except a few as mentioned. District Kangra is found to be different from all other districts in the cyst population.

Table 4: with P value for comparison of locations within the district/All districts

Between locations of District	P Value	Significant? $P < 0.05$?	Test
Mandi	0.0261	Yes	Kruskal-Wallis
Chamba	0.0153	Yes	Kruskal-Wallis
Kullu	0.0108	Yes	Kruskal-Wallis
Kangra	0.0678	No	Kruskal-Wallis
All districts	<0.0001	Yes	Kruskal-Wallis

Table 5: with P value for pair wise comparison of locations/Districts

Locations in consideration	Significant? P < 0.05?
Mandi District	
Basunti vs Rewalsar	No
Basunti vs Phulladhar	No
Basunti vs Dhanotu	No
Rewalsar vs Phulladhar	No
Rewalsar vs Dhanotu	No
Phulladhar vs Dhanotu	Yes
Chamba District	
Saru vs Ahla	Yes
Saru vs Banikhet	No
Saru vs Salooni	No
Ahla vs Banikhet	No
Ahla vs Salooni	No
Banikhet vs Salooni	No
Kullu District	
Bajaura vs Bandrol	No
Bajaura vs Anni	No
Bajaura vs Bhuntar	No
Bajaura vs Talpura	Yes
Bandrol vs Anni	No
Bandrol vs Bhuntar	No
Bandrol vs Talpura	No
Anni vs Bhuntar	No
Anni vs Talpura	No
Bhuntar vs Talpura	No
Kangra District	
Kangra vs Jiya	No
Kangra vs Skoh	No
Kangra vs Parihar khar	No

Kangra vs Shahpur	No
Kangra vs Malan	No
Kangra vs Bagoda	No
Jiya vs Skoh	No
Jiya vs Parihar khar	No
Jiya vs Shahpur	No
Jiya vs Malan	No
Jiya vs Bagoda	No
Skoh vs Parihar khar	No
Skoh vs Shahpur	No
Skoh vs Malan	No
Skoh vs Bagoda	No
Parihar khar vs Shahpur	No
Parihar khar vs Malan	No
Parihar khar vs Bagoda	No
Shahpur vs Malan	No
Shahpur vs Bagoda	No
Malan vs Bagoda	No
All Districts	
Mandi vs Chamba	No
Mandi vs Kullu	No
Mandi vs Kangra	Yes
Chamba vs Kullu	No
Chamba vs Kangra	Yes
Kullu vs Kangra	No

Globodera spp. was first reported in 1961 from Nilgiris district, Tamil Nadu, India. Ganguli *et al.* 2010 reported two species *G. rostochiensis* and *G. pallida* from the potato breeding farms in Kufri, Shimla District of Himachal Pradesh. District Shimla, Kullu, Mandi, Chamba and Kangra showed the prevalence of PCN. District Shimla, Kullu, Mandi and Kangra showed 100% occurrence of PCN. Chandel *et al.* 2020 discussed the distribution of PCN in Himachal Pradesh, Jammu and Kashmir and Uttarakhand (northern hilly areas) India. Seenivasan *et al.* 2017 was conducted study for the distribution of PCN, *G. rostochiensis* and *G. pallida* in the potato fields of Kodaikanal hills, Tamil Nadu, India. Showed 36% of potato field is infested with

the mixed population of PCN. Due to the increase in the infestation of PCN on potato fields especially in potato breeding farms as results government of India has restricted the supply of potato seeds from the infested areas of these states. There is urgent need of controlling the infestation of PCN on potato. This survey may help in figure out the prevalence and distribution of PCN in Himachal Pradesh and also for planning effective strategies against PCN. However, the use of organophosphorus compounds has been shown to control nematodes but may lead to toxicity and regular use of organophosphorus compounds subside the microbial community and reduces soil fertility (Sidhu *et al.* 2019). In a study it was observed that even the use of carbendazim can be toxic to soil microorganisms (Singh *et al.* 2016) and that to nematode as well (Whitehead *et al.* 1985). Camacho *et al.* 2020 conducted the survey in Portugal during 2013-2019 and reported 28.6% population of *G. rostochiensis* and 49.4 % population of *G. pallida* in collected soil samples from different areas. Which finally conclude that *G. pallida* detected greater than *G. rostochiensis* in the different potato fields of Portugal. In the way survey of potato field resulted mixed PCN population. But out of two 67% were *G. pallida* and 8% were *G. rostochiensis* (Minnis *et al.* 2002). Infestation of *G. pallida* is more as compare to *G. rostochiensis* in England fields. Przetakiewicz 2013 reported the *G. rostochiensis* pathotypes Ro5 in Poland. *G. rostochiensis* and *G. pallida* are also found in island of newfoundland and *G. rostochiensis* are found in the potato field of Saanich Peninsula of Vancouver Island, British Columbia, Canada (Sun *et al.* 2007). In Columbia *G. pallida* is the predominant species in potato cultivating fields and recorded from new areas also. All municipalities area samples were recorded positive (Vallejo *et al.* 2021). In Ukraine PCN identified on morphological and molecular basis. *G. rostochiensis* is more prevalent than *G. pallida* (Pylypenko *et al.* 2005). As per discussion the prevalence of *Globodera* spp is very high and there is a urgent need of planning effective and sustainable strategies against PCN.

Declarations

- Ethics approval and consent to participate- Not applicable.
- Consent for publication- the data collected in this survey by ourselves and it is original data. Consent is yes.
- Availability of data and materials- Random survey was done in the potato growing fields of Himachal Pradesh, India and all the material used in this survey is used according to the methodology discussed in the manuscript.
- Competing interests- Not applicable.
- Funding- Not applicable
- Authors' contributions

Ashvika Pathania- Survey, data collection and preparation of manuscript.

Dr Joydeep Dutta – Editing and finalization of manuscript.

Dr Shashank Garg – Statistical analysis.

- Acknowledgements- The authors acknowledge the help for lab facilities provided by Lovely Professional University Phagwara Punjab, Sri Sai University Palampur Himachal Pradesh and Eurofins Pvt. Limited.

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Figures

Figure 1

Histogram for cyst population in Mandi District

Figure 2

Histogram for cyst population in Kullu District

Figure 3

Histogram for cyst population in Kangra District

Figure 4

Histogram for cyst population in Chamba District

Figure 5

Histogram for cyst population in All districts together