

## Occurrence, distribution, and diversity of plant-parasitic nematodes associated with citrus plants

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### Abstract

A survey was conducted to assess the community analysis of phyto-nematode associated with citrus plants in major citrus growing regions of Himachal Pradesh, India. Total of 110 sample were collected randomly from four districts. Nematodes were extracted and identified to the genus level based on their morphological characteristics. Nematode communities were analyzed by absolute frequency, relative frequency, relative density, and prominence value in different localities. Our study revealed the presence of saprophytic, and seven genera of plant parasitic nematodes from the collected samples. *Meloidogyne* spp., *Paratylenchus* spp., *Criconeema* spp., *Rotylenchulus* spp., *Helicotylenchus* spp., *Haplolaimus* spp., and *Tylenchulus semipenetrans* were observed in the surveyed orchards. Amongst the plant parasitic nematodes, *T. semipenetrans* was highly abundant (100%) followed *Meloidogyne* spp., (69 %), by *Helicotylenchus* spp., (58%), *Pratylenchus* spp., (45%), *Criconeema* (27%), *Hoplolaimus* spp., (15%) and *Rotylenchulus* spp., (14%). The highest population density of *Tylenchulus semipenetrans* was reported in Una, followed by Sirmaur, and Kangra district, while it was least prevalent in Hamirpur. The results provided valuable information on the community structure of nematodes from major citrus-growing region of Himachal Pradesh. Moreover, this data can be used as a preventive measure before plant parasitic nematode incidence results in greater losses.

**Keywords:** Citrus, Plant parasitic nematodes, infestation, distribution, prevalence

### Introduction

Citrus crop is widely cultivated around the globe, particularly in tropical and subtropical regions. Brazil, India, and China are the main producers followed by the United States of America (Seminaro *et al.*, 2023) [21]. The citrus fruit (Family: Rutaceae) is one of the most significant fruit crop in India after mangoes and bananas with an annual harvest of 4.79 million tonnes from about 0.62 million acres of cultivated land (Nagachandrabose *et al.*, 2021) [14]. A number of citrus plants, such as orange (*Citrus sinensis*), mandarin (*Citrus reticulata*), grapefruit (*Citrus paradisi*), and lemon (*Citrus limon*) are economically important (Bozbuga *et al.*, 2022) [4]. Citrus fruits are rich in carbohydrates (sucrose, glucose, fructose) dietary fiber, which can reduce inflammation, improve gastrointestinal function, provide vascular protection, and prevent diseases such as diabetes, cancer, and neurological disorders. They are also important sources of bioactive chemicals and vitamin C, which are essential for improving human health because of their antioxidant qualities (Saeid & Ahmed, 2021) [19]. However, like in any agriculture sector citrus is attacked by many pests and diseases which significantly affect their production.

Several biotic and abiotic factors are responsible for limiting the growth, production, and survival of citrus plants. Among the biological factors affecting the quality and quantity of plants, plant parasitic nematodes significantly hinder citrus-production, resulting in substantial yield losses (Verdejo-Lucas & McKenry, 2004) [22]. Citrus plants host numerous microscopic organisms known as plant parasitic nematodes (PPNs). Various nematode species inhabit the rhizosphere, including ectoparasite, endoparasite, and semi-endoparasites, which inflicts considerable damage to citrus crops (Kumar & Das, 2019 [11]; Zalpuri *et al.*, 2013 [23];

Zoubi *et al.*, 2022) [24]. Various species including citrus nematode *Tylenchulus semipenetrans*, the spiral nematode *Helicotylenchus*, the sheath nematode *Hemicycliophora*, the lance nematode *Hoplolaimus*, the needle nematode *Longidorus*, lesion nematode *Pratylenchus*, burrowing nematode *Radopholus*, stubby root nematode *Trichodorus* the stunt nematode *Tylenchorhynchus*, dagger nematode *Xiphinema* and reniform nematode *Rotylenchulus reniformis*, *Criconemoides* and root-knot *Meloidogyne* were reported from citrus orchard considered as responsible for citrus die back disease (Abd-Elgawad, 2014 [1]; Zoubi *et al.*, 2022) [24]. The citrus nematode, *T. semipenetrans* (Cobb, 1913) (Tylenchida: Tylenchulidae), is one of the PPNs that significantly damage citrus farms all over the world (Bozbuga *et al.*, 2022) [4]. The initial report originated from California in 1912, followed by subsequent documentation of its distribution throughout the citrus-cultivated regions worldwide (Duncan, 2005) [8]. The initial report in India was made by Siddiqui from Aligarh, Uttar Pradesh (Kumar & Das, 2019) [11]. Infestation by *T. semipenetrans* leads to a progressive decline and dieback of plants depending on the age and health of the plant, the rootstock's susceptibility, and the nematode population density (Ravichandra & Ravichandra, 2014) [18]. Citrus plants that have been infected with nematodes exhibit decreased vigour, chlorosis of leaf, dieback, leaf loss, and decreased fruit quality and quantity (Cohn, 1964) [6]. Several studies have reported that the yield losses cause by the presence of this nematode range from 10-30% of total crop losses (Verdejo-Lucas & McKenry, 2004) [22]. In India, over 90% of citrus orchards and nurseries are affected by this nematode, leading to substantial economic losses (Nagachandrabose *et al.*, 2022) [15].

Prevalence and distribution of the PPNs community in a region are essential factors for the development of sustainable management strategies. Limited work has been conducted on the assessment of PPNs in citrus plants in Himachal Pradesh. The present work was conducted to determine the community analysis of PPNs associated with citrus rhizosphere which enhances the understanding of the distribution and prevalence of PPNs, which has significant implications for the proper design of nematode control measures.

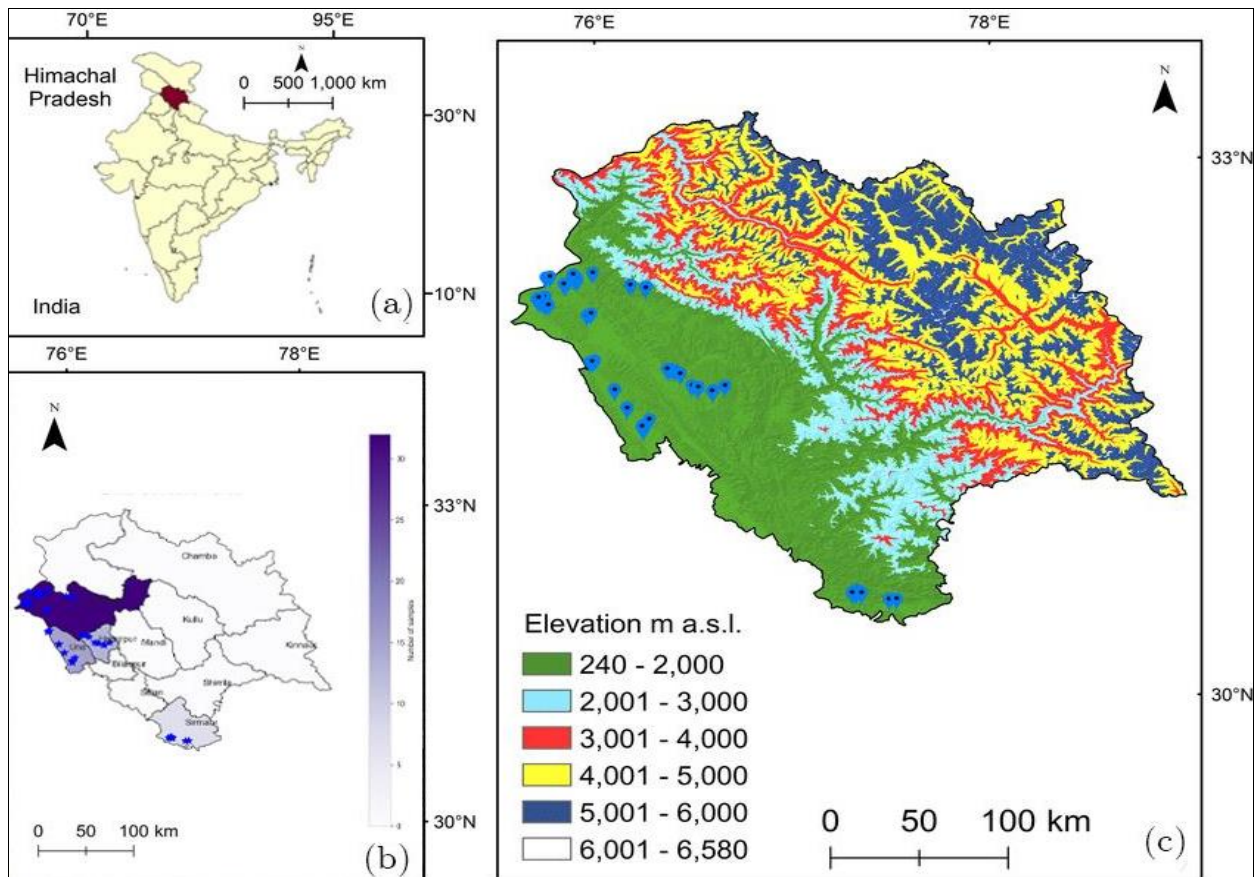
**Table 1:** Distribution of sample analyzed by prospecting area and citrus rootstocks.

Area of sample collection	Number of samples	Root-stock variety
Kangra	46	Citrus limon, Citrus reticulata, Citrus sinensis, Citrus pseudolimon
Una	26	Citrus limon, Citrus reticulata, Citrus sinensis
Hamirpur	23	Citrus limon, Citrus reticulata
Sirmaur	15	Citrus limon, Citrus reticulata, Citrus pseudolimon

**Material and Methods**

**1. Survey and sampling**

Sampling was conducted in four districts including Kangra, Hamirpur, Sirmaur, and Una during 2021-2022 in the prominent citrus-growing areas of Himachal Pradesh (Fig. 1). The experimental sites start at 32° 17' 26.677"N - 30° 29' 56.194"N to 75° 41' 13.957"E-77° 28' 32.141"E. A total of 110 soil and root samples were gathered from the rhizosphere of various citrus plants, at a depth ranging from 15-30 cm as mentioned in Table 1. Each soil sample consisted of 1 kg of soil and feeder roots, a mixture of five soil sub-samples (A sample taken from corner of the field and one from the center) collected in a zigzag path. Samples were collected in a plastic bag to avoid loss of water and stored in a BOD incubator at 4 °C to avoid the decay of the specimens with proper labeling as date of collection, place, and number of trees in an orchard. Samples were processed within a week after the collection.



**Fig 1:** Map of the surveyed citrus fields of Himachal Pradesh.

**2. Nematode extraction and identification**

Nematodes were isolated from samples using Cobb’s (1918) [5] sieving and decantation method, then further processed with a modified version of Baermann’s (1917) funnel technique (Cobb, 1918 [5]; Schindler, 1961) [20]. The nematodes were extracted from each soil (200cc) following the modified Baermann method. The roots of each sample were rinsed with water to remove any debris, then chopped into small pieces (1-2 cm) of which 10 g used for nematodes extraction. The nematodes after extraction were stored for processing. The nematode specimens thus obtained were

subjected to hot water treatment for killing or fixed in 4% formalin-formaldehyde. The nematodes were transferred in Seinhorst’s solution-I (96% ethanol-20 ml, glycerin-1 ml, distilled water-79 ml) in a cavity block. The cavity block was partly covered and put in a glass vessel containing one-tenth of its volume of 96% ethanol and the glass vessel was placed in an oven at 40° C for 12 hours. The cavity block was filled with Seinhorst’s Solution-II (Glycerine-5 parts + 96% ethanol-95 parts) was partly covered and placed in an oven at 40° C for 4 hours. Finally, the nematodes were left in the pure glycerol overnight in the oven at 40° C. The

nematodes were placed in glycerol and mounted on glass slides. The nematodes were identified under the inverted microscope (Olympus) by using morphological characteristics in based on the approach outlined by (Inserra *et al.*, 1988 <sup>[10]</sup>; Mekete *et al.*, 2012) <sup>[13]</sup>.

### 3. Community Assessment of Nematodes

Nematode occurrence and diversity were determined by calculating the density, prevalence, and mean intensity by following the Norton’s formula (Norton, 1978) <sup>[16]</sup>. In order to describe the structure of the nematode community, a number of parameters were used. These parameters included Absolute frequency, Relative frequency, Absolute density, and Relative density, as well as Prominence value.

$$\text{Mean intensity} = \frac{\text{No. of a particular nematode species in the Positive Sample}}{\text{number of positive sample}} \times 100$$

$$\text{Absolute frequency} = \frac{\text{No. of sample containing species}}{\text{No. of samples collected}} \times 100$$

$$\text{Relative frequency} = \frac{\text{Frequency of species}}{\text{Sum of frequencies of all species}} \times 100$$

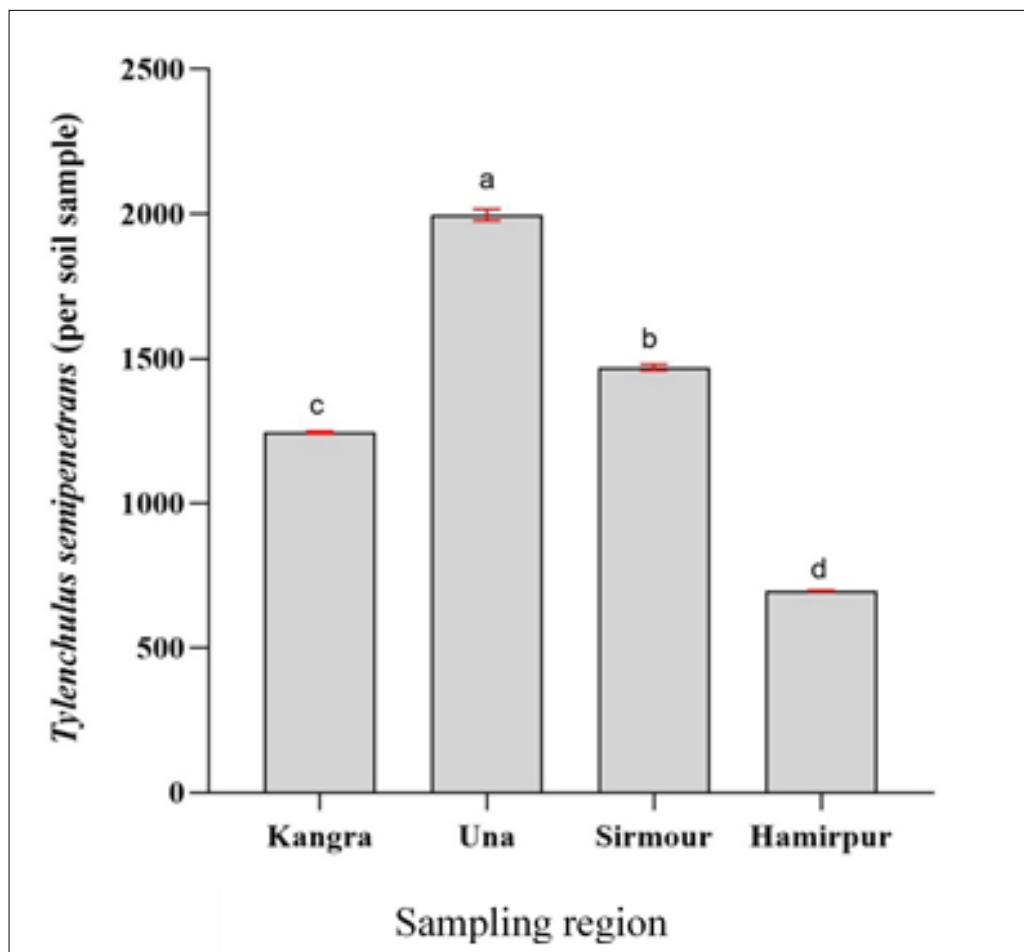
$$\text{Absolute density} = \frac{\text{Number of individual of species}}{\text{Volume or mass or unit of sample}} \times 100$$

$$\text{Relative density} = \frac{\text{Number of individual of species}}{\text{Total number of individuals of all species}} \times 100$$

$$\text{Prominence value} = \text{Relative Density} \sqrt{\text{Relative frequency}}$$

### Result

A large number of PPNs were found to be associated with citrus plants. Seven genera of PPNs, sand aprophytic were identified based on their morphology from the collected samples. The statistical data on the mean intensity, density, and prevalence for the area under study are displayed in Table 2. *Tylenchulus semipenetrans*, *Helicotylenchus* spp., *Pratylenchus* spp., *Criconema* spp., *Melidogyne* spp., were detected in all citrus cultivated regions surveyed. The samples were highly infested with *Tylenchulus semipenetrans*. The major PPNs found in samples were *T. semipenetrans* and *Helicotylenchus* spp. with a high prominence value of up to 426.58 and 30.91 respectively. *T. semipenetrans* was found in all the surveyed fields where citrus species (*C. reticulata*, *C. pseudolimon*, *C. sinensis*, and *C. limon*) were planted in Kangra, Una, Sirmaur, and Hamirpur districts in Himachal Pradesh. Amongst the plant parasitic nematodes, *T. semipenetrans* was highly abundant (100%) followed *Melidogyne* spp., (69 %), by *Helicotylenchus* spp., (58%), *Pratylenchus* spp., (45%), *Criconema* (27%), *Hoplolaimus* spp., (15%) and *Rotylenchulus* spp., (14%). Indeed, *T. semipenetrans* was highly prevalent in Una, Sirmaur, and Kangra, while it was least in Hamirpur. Furthermore, *T. semipenetrans* had the highest overall density in Una with 1992.92 individuals per soil sample (Fig. 2).



**Fig 2.** Population densities of *Tylenchulus semipenetrans* (per soil sample) in the main citrus growing regions in Himachal Pradesh.

**Table 2:** Community analysis of plant parasitic and saprophytic nematodes infecting citrus plants in (200cc soil) from a major citrus growing area of Himachal Pradesh.

S.No.	Nematode Species	Mean Abundance	Absolute Frequency	Relative frequency	Absolute density	Relative Density	Prominence value
1.	<i>Tylenchulus semipenetrans</i>	2676.86	100	27.77	1338.43	80.95	426.58
2.	<i>Helicotylenchus</i> spp.,	254.50	58.18	16.16	127.25	7.69	30.91
3.	<i>Meloidogyne</i> spp.,	231.74	69.09	19.19	115.87	7.00	30.66
4.	<i>Pratylenchus</i> spp.,	49.33	45.45	12.62	24.66	1.49	5.29
5.	<i>Criconeema</i> spp.,	38.45	27.27	7.57	19.22	1.16	3.19
6.	<i>Rotylenchulus</i> spp.,	13.62	14.54	4.03	6.81	0.41	0.83
7.	<i>Hoplolaimus</i> spp.,	25.16	15.45	4.29	12.58	0.76	1.57
8.	Saprophytic	16.93	30	8.33	8.46	0.51	1.47

## Discussion

In order to gain a clear insight into the distribution of PPNs linked to citrus trees in Himachal Pradesh, comprehensive survey were carried out across the major citrus growing regions. On the basis of morphological characteristics, saprophytic, free-living, and seven genera of plant parasitic nematodes were identified. *Tylenchulus semipenetrans* was the predominant PPN identified in all investigated citrus orchards. These results are in line with those findings by Nasir *et al.*, (2021) who reported the presence of *T. semipenetrans* from all the surveyed orchards. Similar to our result 100% of citrus orchards are infested by *T. semipenetrans* where citrus species (*C. reticulata*, *C. paradisi*, *C. sinensis*, and *C. limon*) were planted in the Mediterranean region of Turkey (Bozbuga *et al.*, 2022) [4]. Furthermore, the presence of *T. semipenetrans* was observed throughout the citrus orchards in both the Nile Delta and middle Egypt (El-Banhawy *et al.*, 2006) [9]. Moreover, *T. semipenetrans* was the predominant nematode species identified in citrus root seedlings and demonstrated the highest distribution across samples collected from several nurseries in Egypt (Radwan & Fatma, 2003) [17]. The percentage occurrence of *T. semipenetrans* from three different governates in Egypt was reported from 88.88% to 77.77% (Bakr *et al.*, 2011) [3]. *T. semipenetrans* had occurrence rate of 100% in both soil and roots in the orchards of Nakhathalguri, Tinsukia district and the presence of *Helicotylenchus* was observed in most of the orchards surveyed (Mahanta *et al.*, 2018) [12]. In northern Egypt, nine PPN genera were found to be associated with citrus plants studied. The citrus nematode, *Tylenchulus semipenetrans*, was the most prevalent, (frequency of occurrence =100%) of the surveyed locations, followed by (*Trichodorus* spp.) stubby root nematodes and (*Pratylenchus* spp.) lesion nematodes (Abu Habib *et al.*, 2020) [2]. Five major genera of plant-parasitic nematodes—*Tylenchulus semipenetrans*, *Pratylenchus* spp., *Hoplolaimus* spp., *Helicotylenchus* spp., and *Criconemoides* spp., were found to be prevalent in Assam. The relative frequency, relative density, and prominence value of *Tylenchulus semipenetrans* were 13.15%, 6.25, and 40.50, respectively (Devi, 2017) [7]. The present finding of *T. semipenetrans* followed by *Helicotylenchus* spp., in a large number has also been observed in various citrus species grown at the citrus research station, Tinsukia, Assam (Kumar & Das, 2019) [11]. Whereas, *Xiphinema* and *Pratylenchus* species were reported to be prominent followed by *Hoplolaimus*, and *Tylenchulus semipenetrans* in various citrus growing regions of Jammu (Zalpuri *et al.*, 2013) [23]. Eleven genera

and ten species of PPNs were reported, with *Tylenchulus semipenetrans* (88%), *Helicotylenchus* spp. (75%), *Pratylenchus* spp. (47%), *Tylenchus* spp. (51%), and *Xiphinema* spp. (31%) exhibiting the highest prevalence. However, A study reported that PPNs distribution was influenced by soil physicochemical properties, including texture, pH, and mineral content, suggesting that both plant and soil factors significantly impact PPN communities in citrus orchards (Zoubi *et al.*, 2022) [24]. The findings emphasize the widespread occurrence of citrus nematode infestations and their potential impact on citrus production. Efforts to develop effective management strategies for controlling citrus nematodes are crucial to protect citrus orchards and ensure the sustainability of the citrus industry.

## Conclusion

This study highlights the presence of PPNs in major citrus-growing areas of Himachal Pradesh. It was observed that the orchards were highly infested with PPNs. Based on the morphological characteristics, saprophytic, free-living, and seven genera of plant parasitic nematodes were identified. Citrus nematode *T. semipenetrans* was the predominant nematode identified in all the studied samples. The highest population density of *Tylenchulus semipenetrans* was reported from Una, followed by Sirmaur, and Kangra district, while it was least prevalent in Hamirpur. In order to evaluate resilient approaches in citrus growing systems, plant-parasitic nematode variability, and community structures are essential indicators. These findings emphasize the need for effective management strategies to control citrus nematodes and prevent further damage to citrus orchards in Himachal Pradesh. Continued research and collaboration between researchers, farmers, and government agencies are essential to develop and implement management strategies effectively which can significantly reduce nematode populations and protect the sustainability of the citrus industry in the region.

## Author contributions

Jigmet Yangchan: original draft preparation, data analysis, curation, and validation. Kanika Choudhary: writing—review and editing, final draft preparation. Ruchika Kumari: visualization, writing—review, critical revision. Sunil Kumar: Supervision, Conceptualization, Curation, and Validation. All authors have read and agreed to the published version of the manuscript.

## Conflicts of Interest

The authors declare no conflict of interest

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