Research Article

A Study on Population Diversity of Citrus Nematodes in District Sargodha

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Abstract | Citrus slow decline caused by *Tylenchulus semipenetrans* deteriorates the quality and quantity of citrus fruit and make it prone for the invasion of other microbes. A survey was carried out for the assessment of nematode population distribution in all 7 Tehsils of district Sargodha (Pakistan) viz: Sargodha, Silanwali, Sahiwal, Shahpur, Bhalwal, Kot Momin and Bhera. Population densities of nematode were assessed in different genera of citrus crop i.e. kinnow, sweet orange, feutral's early, grape fruit and sweet lime, both in healthy and declining orchards. Whitehead and Hemming tray method was used for the isolation of *T. semipenetrans* which showed that the population was low in declining orchards as compared to the population densities found in healthy orchards. Kinnow was the most infested by nematode with mean population 1161 while sweet orange was the least infested with 595 nematodes, in all localities. Maximum nematode population density in healthy and declining orchards was recorded in Kot Momin that was 2600 and 946 nematodes per 50 ml of soil, respectively. The lowest mean population was recorded in Silanwali with 1200 in healthy and 300 in declining orchards. The assessment of spatial distribution of nematode population would pave foe effective disease management strategies.

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Introduction

There are more than 30 fruits produced in Pakistan, this large fruit industry is dominated by citrus as it constitutes around 30% of the country's total fruit production. Punjab province is the highest producer of the country as 90% of citrus is produced in the province (Siddique and Garnevska, 2017).

Citrus belongs to family *Rutaceae* that includes organic product trees and bushes mandarins (*Citrus reticulate*), Grape natural product (*C. heaven*), lime (*C. aurantifolia*), lemon (*C. limonia*) sweet orange (*Citrus sinensis*), acrid orange (*C. aurantium*), wild orange (*C. macroptera*), citron (*C. medica*), calamondin (*C. mitis*), kaffir lime (*C. hystrix*), pummelo, (*C.grandis*), *Fortunella* spp. (Mukhopadhyay, 2004). The origin of citrus was viewed as north east of China and India yet its definite source isn't clear (Gmitter *et al.*, 1990). Citrus is the most valued fruit worldwide with more than 125 million tons yield, the climatic conditions of Pakistan are much favorable for its production (Rehman *et al.*, 2022). Citrus natural product has profoundly nutritive worth and significant for vitamin C. Its juice have a dietary source with stunning medical advantage that secure human body against malignancy, birth surrenders, ongoing issue liver, spleen, migraine issue, skin problem, and jumps ailments in view of having anti-oxidental potential (Kamboh, 2018). Optional metabolites from citrus utilized in modern requesting, blanching items, drugs planning and furthermore utilized in Chinese medication, for example, 'chen pi and ju pi' (Kuppusamy *et al.* 2016). Citrus is abundant in thiamine, riboflavin, pantothenic acid and nutritional fiber which are necessary for human health (Iqbal *et al.*, 2022).

Normal yield of citrus in Pakistan (11 t/ha) is low when compared with the average yields of various citrus producing countries like Brazil, USA and Turkey (22t, 26t and 27t/ha individually) (Khan *et al.*, 2022). Citrus yield is badly affected by numerous viral, fungal, nematodal and bacterial infections (Rao *et al.*, 2021). Citrus nematode (*Tylenchulus semipenetrans* Cobb.) is a serious and widespread pest of citrus crop all over the world. *T. semipenetrans* causes die back, gradual decline of trees and crop decrease in orchards. More than 80 cultivars are susceptible to this nematode (Karuri *et al.*, 2017). The citrus nematode is semi-endoparasite of fibrous roots of citrus, female penetrate anterior part into the cells of roots while the posterior remains outside of the roots, eggs are deposited on the gelatinous material secreted by female of citrus nematode (Olabiyi *et al.*, 2016). Yield losses caused by citrus nematode *T. semipenetrans* reported up to 10-30% losses and subjected to level of infestation, population density, soil properties, types of root stock, presence and absence of other soil borne pathogens and cultural practices (Abd-Elgawad, 2020). Citrus nematode not only harms the citrus plant but it also paves the way for onward invasion by other pathogens like *Phytophthora* and other fungi (Iftikhar *et al.*, 2023). Citrus nematode infestations have been reported from nearly all growing areas of Pakistan (Khanzada *et al.*, 2008). The extent of nematode population infestation varies with respect to soil conditions and micro-environment of the orchards (Kayani *et al.*, 2012). The effect of inoculum level on citrus growth and yield was assessed under controlled conditions in selected cultivars (Irshad *et al.*, 2012). Highly infested roots depict discoloration, more adhering soil particles making the roots prone to secondary invaders (Saeed *et al.*, 2019). Citrus slow decline is a promptly increasing problem in the orchards of Sargodha (Fateh *et al.*, 2017). This study was planned as there is no study of population diversity of nematodes in Sargodha was reported up till now. Nematodes population density distribution would be helpful in assessment of ecological disturbances, potential disease risk and implementation of suitable management tactics against nematodes.

Materials and Methods

The present research work was performed in Tissue culture and Plant Pathology laboratory of Department of Plant Pathology, College of Agriculture, University of Sargodha (Pakistan).

Orchard selection and collection of samples

A survey was conducted in 7 tehsils of District Sargodha (Shahpur, Bhalwal, Sargodha, Bhera. Sahiwal, Sargodha and Silanwali) for the selection of citrus orchards. Orchard selection was difficult because citrus orchards are present in a large numbers and each tehsil had almost more than 500 orchards so after every 3 km, 1 orchard was sampled and 10 orchards from every tehsil were selected for sampling on the basis of their condition as if they were healthy or declining. Selected orchards were tagged for the sampling purpose. 70 orchards were selected on the basis of their health status among which 60% orchards were those that were declining. X method was selected for sampling.

Soil sampling of different cultivars across various locations

From the selected orchards, samples were collected and their ages and varieties were noted, Kinnow, Sweet Orange, Feutrell's early, Grape fruit and Sweet Lime. Kinnow was the most planted variety

in district Sargodha due to its high prolific value due to high profit percentage because of export and high demand with in the country because of its sweet and delicious taste so sampling was done from 70 different locations in Sargodha district (Sargodha, Silanwali, Bhera, Sahiwal, Kot Momin, Shahpur and Bhalwal). Soil sampling was done from the selected orchards; soil arguer was used to dig soil from about 2.5ft away from the main stem of citrus tree and upper soil layer was removed and dug at 15-30 cm or when active fibrous roots appeared and from each tree 4 soil samples were collected around canopy of each citrus tree, and these samples were mixed gently and thoroughly till homogenized. About 1 kg of composite soil sample was collected in polythene bags and were marked for the identification and labeling purpose so 70 samples from each tehsil were collected. All of the collected soil sample were transferred carefully to laboratory and were incubated for the further process to conduct research.

Nematodes extractions

Juveniles extraction from soil: Soil samples collected from each tehsil were processed as the soil samples from 1kg polythene bag were homogenized manually, 50g of soil was weighed through the digital balance and these soil samples were used for the extraction of 2nd stage juveniles. These 2nd stage juveniles were extracted from 50g soil sample through the technique described by whitehead and Hemming also known as whitehead and hemming's tray method. As this method was the most convenient among other methods as modified apparatus was bought from the market and few trays were available so it was easy to process large number of samples. The soil samples of 50gram were placed in wide porous plates layered with tissue paper and placed on modified beaker containing water up to porous plate bottom. These plates were placed on a beaker that was filled with water up to a specific level i.e. so that the tissue of the tray got moisturized and got wet and these plates were incubated for 48 hours for migration of nematode from soil to water as nematodes are very fond of water and they move towards water from the soil and tissue papers are porous so nematodes can pass through the tissue papers easily and were incubated for almost 24-48 hours so that all nematodes can move from soil sample to water which was present in the beaker, after 48 hours porous plates and sample was reduced to 10-15ml concentration were removed and pipette was used to suck one ml of sample from the beaker and that sample was analyzed under the stereo microscope for observing the nematodes and to confirm their presence, after confirmation of the nematodes presence the sample was placed in a 12 wells plate for the population as 1mlof water sample was poured into each well of 12 well plate through pipette. And sample was counted in twelve wells and nematode populations were noted and average was taken of all twelve reading taken divided by number of wells counted and that population represented the one sample through the same method other samples were processed and populations were noted (Hooper *et al*. 2005).

Extraction of surface sterilized nematode

Fresh Soil sample were collected from citrus orchards and sterilized with 0.05% NaOCl solution for 2-3 minute. Juvenile were passed through 600 um mesh sieve and washed thoroughly with sterilizes water to eliminate the residual effect of NaOCl solution and collected in little sterilized water to increase population per ml water.

Measuring the incidence of citrus nematode

Suspensions of processed soil root sample were examined under inverted microscope in cell culture plate. The nematode suspension of 1ml from each processed sample were observed and counted in cell culture well in three repetitions. Numbers of nematodes was counted and their averages were calculated for 1 ml of suspension. Total Number of nematodes was calculated from representative sample by the given formula. Population density of nematodes was calculated by using this formula.

Total no of Nematode = No of nematode in ml of suspension × total volume of suspension

Morphological identification of citrus nematode

Citrus nematode was identified morphologically under digital camera fitted compound microscope. Freshly extracted healthy nematodes from soil sample were picked by micropipette and placed on sterilized glass slide and covered with glass cover. Gently heated the nematode with fire flame to stop the movement and observed under the microscope. Physical and anatomical feature of juvenile, male and female were observed at different magnifications. Firstly, the nematodes were observed at 4x then at 10x when it was confirmed that the nematodes have stylet were matching the citrus nematode morphological

features, they were magnified at 40x and observed carefully to study each of its part a slide was prepared where a droplet of water was placed on its surface and through micro pipette a nematode was picked and placed on the slide and slide cover was placed carefully as to ignore the air bubbles for clear results. After placing cover slide it was gently heated to stop the moment of nematode and not to burn the nematode, the nematode was carefully observed, its stylet and vulva and morphology was compared to its data base and was confirmed that it was *Tylenchulus semipenetrans* (Taylor and Netscher, 1974).

Statistical analysis

The collected data was analyzed using Statistix 8.1 software and analysis of variance (ANOVA) was used to compare the means at 5% probability in least significant difference test (LSD).

Results and Discussion

Distribution of T. semipenetrans (Cobb) in different areas of Sargodha district (Healthy orchards)

T. semipenetrans was present in most of citrus orchards sampled and population density of 2nd stage juvenile male in 50ml soil sample was ranged from 1320 to 2672. Kot Momin dominated the area wise population in healthy citrus orchard as mean population was recorded as 2672 per 50 ml soil sample followed by Shahpur and Bhera 2516 and 2159 respectively. However lowest population was recorded in Bhalwal (1320) per 50ml soil sample followed by Silanwali, Sargodha and Sahiwal (Figure 1). The present survey of citrus nematode updates the incidence of *T. semipenetrans* in different age of citrus orchards in Sargodha. Some orchards were found to be less infested with nematode in well managed condition. Numbers of nematodes per 50 ml of soil were varied in age difference orchards and in different genera. This suppression of nematode in citrus orchards was due to several factors like application of nematicides, soil solarization, cultural practices, healthy root stock, soil type and absence of fungal pathogen (Nita *et al.*, 2021). The nematode population varies with respect to different soils even in same agro-ecological zones (Jones *et al.*, 2013). Previous studies showed that soils with adequate drainage and high fertility level contain more abundant population of citrus nematodes (Renco *et al.*, 2020). The reason for containg higher number of nematodes in healthy soils may be the fertile clay content (Melakeberhan *et al.*, 2021). The higher nematode population is associated with high moisture contents in soil which favors the nematode activity (Nisa *et al.*, 2021). A number of extensive studies on citrus slow declined depicted that citrus nematode is the most damaging worm causing disturbance in citrus orchards and its population varies from field to field (Abu-Habib *et al.*, 2020). Fateh *et al.* (2017) recorded maximum disease incidence, severity and disease index from Tehsil Sargodha while minimum from Silanwali. The varied nematode population in different areas may also be attributed to change in soil temperature as it was recorded in a previous experiment that nematode population is directly proportional to the soil temperature i.e., increase or decrease with temperature (Saeed *et al.*, 2021). *Tylenchulus semipenetrans* affects the quantitative and qualitative traits of citrus and its efficacy varies according to soil conditions. This trend was recorded in different fields of Sargodha as soil type, pH and structure varies, nematode population distribution also varies (Abd-Elgawad, 2020). The variation in nematode population is attributed to the presence of other soil microflora and nutrient status as well. Nematode population tends to be decreased where other beneficial microbes are abundant and vice versa (Shokoohi and Duncan, 2018).

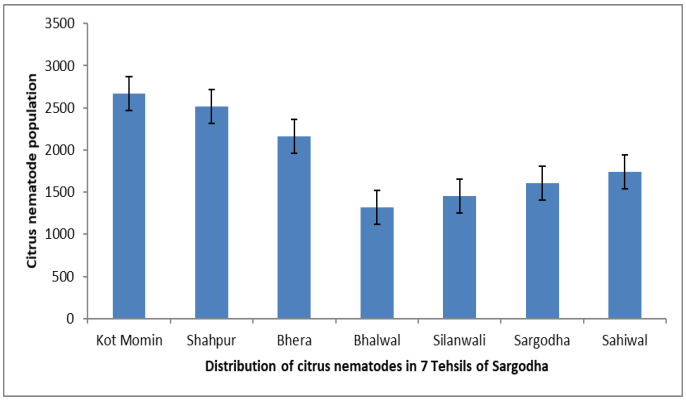

Figure 1: *Population dynamics of citrus nematodes in healthy orchards of district Sargodha.*

Distribution of T. semipenetrans (Cobb) in declining citrus orchards

T. semipenetrans was present in almost all of the citrus orchards that showed declining symptoms. Out of 250 samples collected, 97% were infested with *T. semipenetrans*. Population density of 2nd stage male juvenile in 50 ml soil solution was ranged from 352-946 per 50 g soil. The mean population density was recorded highest in Kot Momin (946 per 50ml soil) followed by Bhalwal (750) and Shahpur (637) per 50ml soil sample. Lowest mean population was

recorded in Silanwali (352 nematodes per 50ml soil sample) followed by Sargodha (403), Sahiwal (451) and Bhera (501) Figure 2. The lesser number of nematodes in declining orchards would be attributed to the low availability of nutrients and other necessary components in diseased plants, and to acquire more of these necessary components nematodes move towards healthy orchards (Benjlil *et al.*, 2020). A few researchers worked on the frequency of distribution of citrus nematodes on declining orchards and healthy orchards with the findings that orchards with high Fe, Ca and Na contents may contain more number of nematodes (Shanmugam *et al.*, 2021). According to Laasli *et al.* (2022) soil type also affects the nematode population as it halts and boost the nematode growth. Iqbal *et al.* (2007) observed significantly lower reproduction of nematodes in soils with more clay content as compared with soils having low clay percentage.

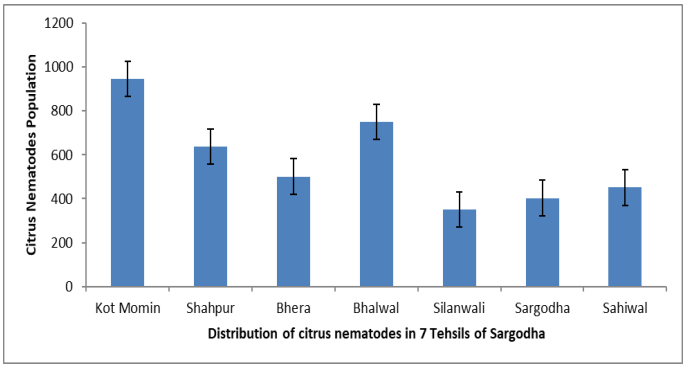

Figure 2: *Population dynamics of citrus nematodes in declining orchards of district Sargodha.*

Overall population density of citrus nematode in different cultivars

All samples collected from different cultivar of citrus orchards were assessed for their population density of nematode. Each orchards sample data pooled up and data was analyzed. The maximum number of mean population density of nematode was found in kinnow (1161) followed by Feutrell's early (950), Grapefruit (913) and sweet lime (877) while sweet orange showed lowest mean population density (433) per 50ml soil sample (Figure 3). Mahanta *et al.* (2018) stated that more number of citrus nematodes was recorded in khasi mandarin as compared to other citrus species. Deshmukh *et al.* (2016) determined the citrus nematode infestation in various types of citrus species viz. trifoliate orange and other hybrids. They concluded that different citrus species have varying number of nematode populations. The members of rutaceae family have their different level of resistance and susceptibility against the citrus nematodes which may also vary according to agro-ecological zones (Shokoohi and Duncan, 2018). Fateh *et al.* (2017) suggested a scale for disease index, a DI more than 15% indicates the alarming situation for a particular region. According to their findings the DI in Sargodha District ranges from 10-30%, which indicates strict actions have to be implemented in order to avoid from slow decline of citrus. The nematodes are attracted towards the root exudates of the plants, among citrus it varies from specie to specie i.e., each citrus cultivar has varied nematode population (Jaiman *et al.*, 2023).

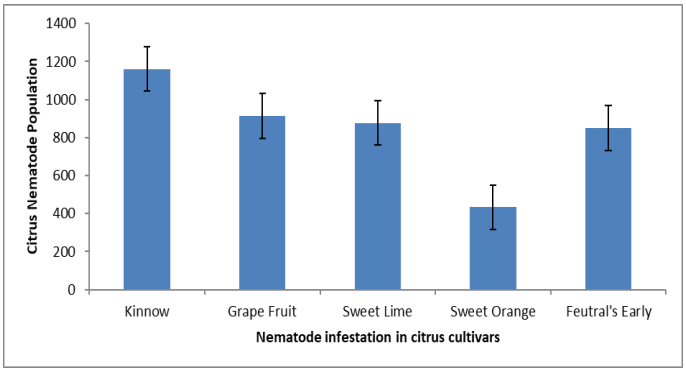

Figure 3: *Population dynamics of T. semipenetrans in different citrus cultivars.*

Population density of citrus nematode in Kinnow

All samples collected from kinnow cultivar of citrus orchards were assessed for their population density of nematode. Mean population density was calculated and results showed that maximum number of nematodes per 50ml soil were present in Sahiwal (1578) followed by Kot momin (1392) and Sargodha (1188) while minimum number of nematodes were recorded in Shahpur (438) followed by Bhalwal (588), Silanwali and Bhera per 50ml soil sample, respectively (Figure 4). Abobatta (2019) conducted a survey from citrus fields and recorded different population distribution of *T. semipenetrans* in Grapefruit, Lemon, Lime and Kinnow. Hammam *et al.* (2016) compiled a data recording citrus nematodes infestation in citrus growing areas of the world and concluded that nematode population differs in geographic regions even within the same species. Ennab (2016) found the nematode population is affected by soil type, pH level, nutrient status and soil moisture. Pandey *et al.* (2004) concluded that pH has significant influence on nematode population; the male and female nematodes have different behavior towards pH variation.

Population density of citrus nematode in sweet lime

All samples collected from Sweet lime cultivar of

citrus orchards were assessed for their population density of nematode. Mean population density was calculated and results showed that maximum number of nematodes per 50ml soil were present in Sargodha (2171) and minimum (348) in Bhera (Figure 5). Ghaderi *et al.* (2012) observed the reduced tree vigor in citrus trees due to severe attack of nematodes. Assessment of population densities in a particular area is necessary to document the possible extent of damage caused by citrus nematode (Saeed *et al.*, 2021).

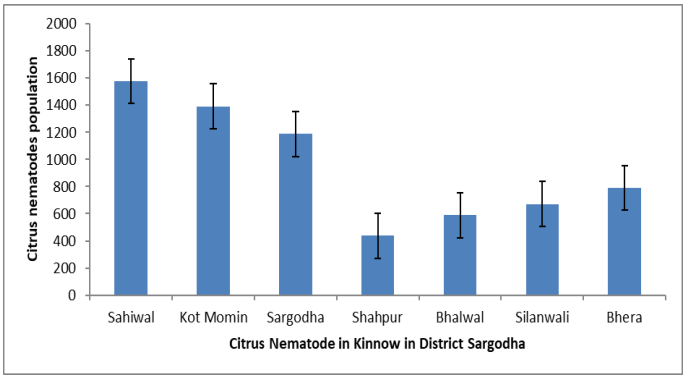

Figure 4: *Population dynamics of T. semipenetrans in Kinnow in Sargodha district.*

Population density of citrus nematode in Feutral's early

In case of Feutrals early, the mean population density was calculated and results showed that maximum number of nematodes per 50ml soil were present in Kot Momin (1656) and minimum (192) in Bhera (Figure 6). Rumiani *et al.* (2021) described that flooding facilitates the spread of nematodes in the orchards. Citrus nematode interrupts with root extension, root hair abnormalities, and absorption of nutrients, water and subsequent decrease in plant growth and vigor. Keeping in view the extent damages posed by citrus nematodes, farmers should opt integrated approach to save the orchards from this chronic menace (Mukhtar *et al.*, 2013).

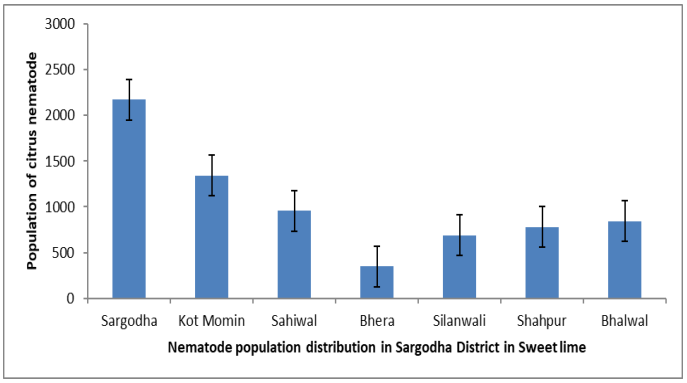

Figure 5: *Population dynamics of T. semipenetrans in Sweet Lime in Sargodha District.*

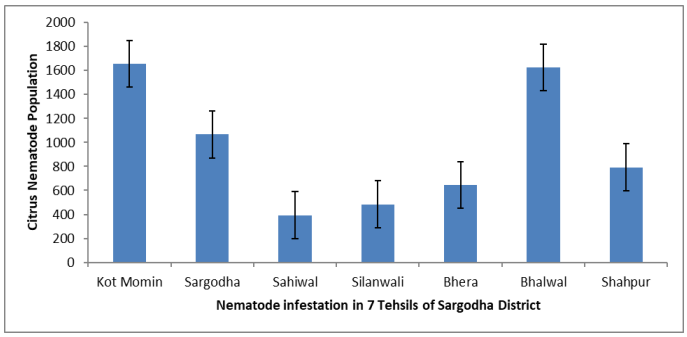

Figure 6: *Population dynamics of T. semipenetrans in Feutral's Early in Sargodha District.*

Population density of citrus nematode in grape fruit and sweet orange

Grapefruit was more affected in Kot Momin while sweet orange in Bhera while minimum infestation in case Grapefruit was recorded in Bhera and of Sweet orange in Kot Momin (Figures 7, 8). Araujo *et al.* (2019) determined the effect of nutrients and plant health on distribution of nematode population in various citrus species. Irshad *et al.* (2012) conducted an experiment under controlled conditions where after inoculation of the citrus plants were done with 2nd stage juveniles of citrus nematodes. It was observed that with increasing level of inoculum there was decrease in growth parameters of the inoculated plants. Citrus nematode population alters according to the microclimate of the crop species and surrounding cropping pattern (Ghaderi and Hesar, 2016).

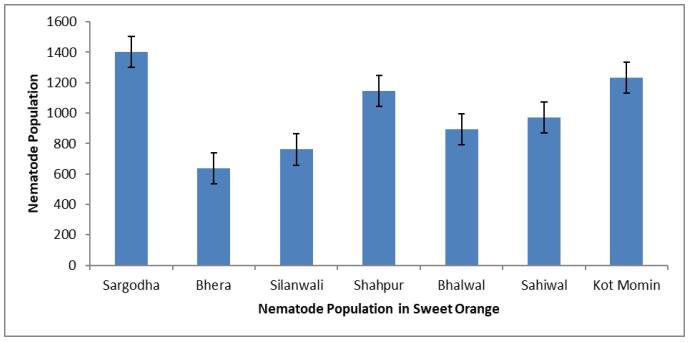

Figure 7: *Population dynamics of T. semipenetrans in sweet orange in Sargodha District.*

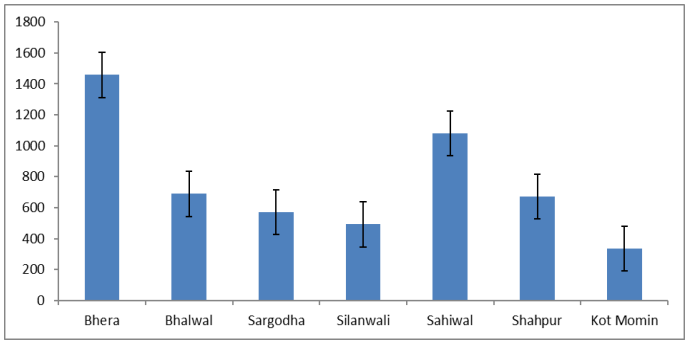

Figure 8: *Population dynamics of T. semipenetrans in Grapefruit in Sargodha district.*

Conclusions and Recommendations

It could be stated that nematode population varies with respected soil type, soil moisture, soil temperature and soil nutrient status. The results of present study would be helpful in sustainable and integrated management of citrus nematodes that is a potential threat for citrus orchards. Strict quarantine practices should be opted while planning for new orchards and farmers get acquainted with sanitation of orchards to intervene the survival phase of pathogen. The study is effective to understand the nematode population abundance in different geographical regions and to identify disease hotspots for risk assessment. There is need to expand this study on whole citrus family and inclusion of all major growing areas of Pakistan, so that a clear and concrete basis would be provided for authentic conclusions.

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Novelty Statement

Citrus nematode is a key ecological delinquent that has devastated the citrus production in different regions. In present experiment, the distribution of citrus nematode population was assessed in major citrus growing District Sargodha (Pakistan) for the first time that helped in plant health monitoring and nematode diversity.

Author's Contribution

Abdul Haseeb: Conducted research.
Yasir Iftikhar: Supervised research.
Muhammad Ahmad Zeshan: Helped in experiment layout.
Safdar Ali: Reviewed the manuscript.
Rana Binyamin: Data analysis.
Salman Ghuffar: Helped in data recording.
Muhammad Usman Ghani: Helped in improving discussions.

Conflict of interest
The authors have declared no conflict of interest.

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