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Exploring Citrus Nematodes and its Antagonists in Citrus Growing Areas of Punjab, Pakistan

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ABSTRACT

Citrus nematode (*Tylenchulus semipenetrans*) is a major threat to the citrus industry in Pakistan. During 2019-20, a survey was carried out for citrus nematode and its antagonists in citrus orchards (Both healthy and declining trees were samples) of Punjab, Pakistan. Bhalwal, Shahpur, Sillanwali, Kotmomin, Sargodha, Quaidabad, Jhang, and Rahim Yar Khan were surveyed for disease incidence, prevalence, and association of *T. semipenetrans*. Maximum disease prevalence (100%) was recorded in Rahim Yar Khan while minimum (20%) in Bhalwal and Kotmomin. Maximum slow decline with 80% field incidence was observed in Rahim Yar Khan while a minimum of 20% in Quaidabad. Soil and root samples were used for nematode extraction using Baerman's funnel method and the Whitehead Hemming tray method. A maximum number of J2s/100ml of soil were observed in soil samples taken from Rahim Yar Khan (1674) followed by Shahpur (1534), Qaidabad (1432), Sargodha (1347), and Bhalwal (1172). The highest number of nematode females per gram of root were recorded in Rahim Yar Khan (652) followed by Quaidabad (611), Bhalwal (490), Kotmomin (421), Sillanwali (387). Isolation of different fungal and bacterial antagonist isolates was also done by soil dilution plate technique using nutrient agar media and potato dextrose agar media. Identification of fungal antagonists was made on colony growth, color, and spore structure. Bacterial identification was also done on morphological characters and gram tests. *Trichoderma harzianum, Trichoderma viride, Trichoderma koningii Trichoderma atroviride, Pseudomonas fluorescens*, *Pseudomonas putida, Bacillus cereus* and *Bacillus subtilis* were found associated.

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INTRODUCTION

Citrus belongs to the family *Rutaceae* and subfamily *Aurantioideae* is one of the most important fruit crops of the world (Shah, 2004, Suganthi *et al.,* 2019). It is mostly grown in tropical and subtropical areas of the world. It is grown in almost 140 countries of the globe including Pakistan and stands first in area and production among the world's tree fruits (FAO, 2019). In Pakistan, citrus fruits are the most important fruit crops grown on an area of 160,000 hectares with a production of 1.5 MMT annually. Citrus is a rich source of vitamin C, sugar, and mineral with a significant amount of calcium and magnesium (Kadar, 2007).

Citrus production around the world is susceptible to challenges. In Pakistan, citrus production is almost 12-15 tons per hectare which is less as compared to developed countries. Many Kinds of pests and diseases are responsible for causing low yield. Citrus nematode (*Tylenchulus semipenetrans*) is a major issue in this regard as it is a leading cause of the citrus slow decline. It causes

severe losses by affecting the quality and quantity of fruit (Ahmad *et al.,* 2004, Safdar *et al.,* 2010). Persimmon, olive, and grapes act as alternate hosts for this nematode (Siddiqi, 1974). The development of new nematode races in tropical areas is also a matter of great concern in the current scenario. In the United States, in the core citrus growing regions i.e., California, Florida, Texas, and Arizona high citrus nematode infestation (50 to 90%) are reported (Heald and Bannon, 1987). In Pakistan, its infestation is up to 98.4% (Ahmed and Khan, 1999).

The citrus nematode is semi endoparasite which affects all citrus species by feeding deeper in cortical cells due to which the outer epidermis and cortex are sloughed out. As a result of feeding, trees lose their vigor and subsequently lead towards slow decline (Abd Elgawad *et al.,* 2010). Annually 30-50 % yield losses are caused by *T. semipenetrans* (Baines *et al*., 1962).

There is a dire need to take necessary steps for the management of citrus nematode to avoid yield losses. It is difficult to manage citrus nematode as no single method provides satisfactory control (Verdejo and McKenry, 2004). Nematicides had been extensively used against nematodes but these nematicides were found harmful to the ecosystem (Sharma and Pandey, 2009; Li *et al.,* 2011; Singh *et al.,* 2012). It is needed to adopt alternative eco-friendly strategies. Biocontrol through the use of microbial antagonists like *Trichoderma* spp. are economically and environmentally friendly approaches to decrease the plant-parasitic nematodes (Mukhtar *et al*., 2021). *Bacillus* and *Pseudomonas* have been reported for their nematicidal effects through lytic enzymes, toxic insecticidal crystal proteins, volatile compounds, or parasitism (Gamalero and Glick 2020). These bacteria also elicit a defense system and enhance resistance in plants through various mechanisms including competition for nutrients (especially iron) or through induced systemic resistance (Bakker *et al.,* 2007; Gamalero and Glick 2020).

Similarly, soil borne fungi from genera *Trichoderma* are well known for having antagonistic potential against nematodes (Mukhtar et al., 2021) by enhancement of resistance in the host against pathogens, direct parasitism, antibiosis, enzymatic hydrolysis, and competition for food and space (Sharon *et al.,* 2007; Howell, 2003; Harman *et al.,* 2004). Since bacteria, fungi, and nematode occur together in the rhizosphere, the toxic metabolites naturally produced by bacteria and fungi may be responsible for keeping the nematode population at a low level. Very little work has been done on indigenous antagonistic fungi and bacteria against citrus nematode. In the present study survey of the citrus orchards of citrus growing areas of Punjab, Pakistan was done to assess the disease prevalence, incidence, and disease index of citrus slow decline. Citrus nematodes were also quantitatively assessed in these areas. Isolation and identification of locally found potential fungal and bacterial antagonists were also carried out from the citrus rhizosphere.

MATERIALS AND METHODS

Survey *of T. semipenetrans*

During the year 2019-2020, a comprehensive survey of major citrus growing areas of the Punjab Province, Pakistan was conducted. These areas included Sargodha, Bhalwal, Shahpur, Sillanwali, Kotmomin, Quaidabad, Jhang, and Rahim Yar Khan. To find out disease prevalence 10 orchards were selected randomly at each location while 10 plants were selected at each orchard for calculating disease incidence.

Disease prevalence, incidence, and index were recorded by using the following formulas respectively,

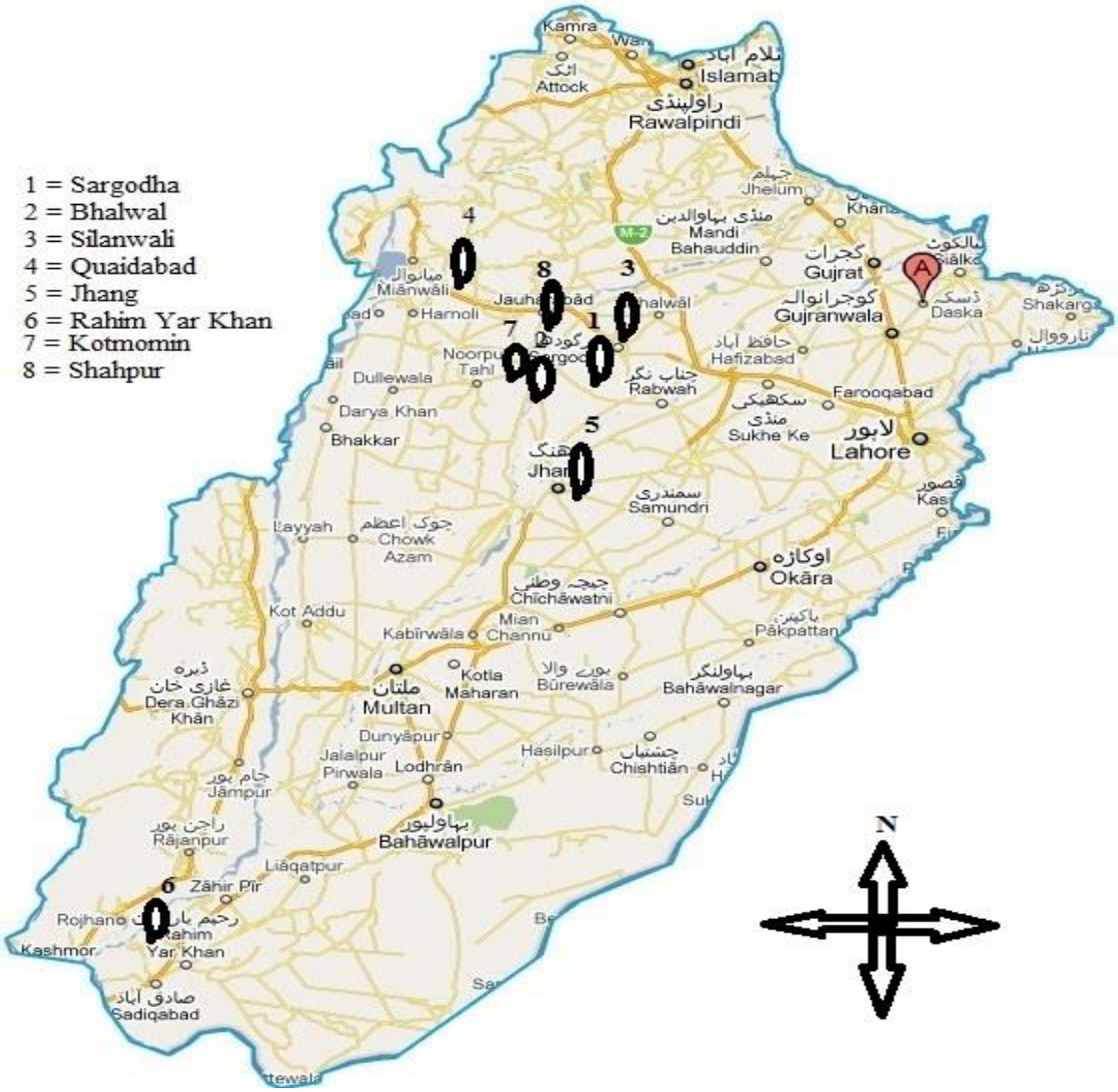
$$\text{Disease Prevalence (\%)} = \frac{\text{Number of infected orchards}}{\text{Total number of orchards}} \times 100$$

$$\text{Disease Incidence (\%)} = \frac{\text{Number of Plants declined}}{\text{Total number of Plants}} \times 100$$

$$\text{Disease Incidence (\%)} = \frac{0(n1) + 1(n2) + 2(n3) + 3(n4) + 4(n5) + 5(n6)}{N} \times \frac{100}{5}$$

(Zadoks and Schein 1979)

Where, n1=No. of trees in 0 rating, n2 =No. of trees in 1 rating, n3 =No. of trees in 2 rating, n4 =No. of trees in 3 rating, n5 =No. of trees in 4 rating, n6 =No. of trees in 5 rating, N = Total Number of trees.



Map of surveyed locations.

Collection of samples

To estimate the nematode population within soil and roots, samples were collected from different locations at a depth of 6-9 inches at a distance of 90-120 cm from the trunk under the canopy of the tree. From each orchard, ten trees were selected randomly, and four subsamples were taken from each tree mixed thoroughly to form a composite sample of 500 ml. Similarly, root samples were taken from tree roots (approximately 10 gram each sample). These samples were then mixed to make a composite sample. These samples were placed in polythene bags, labeled, brought into the laboratory, and placed at 15°C in the cool incubator.

Isolation and counting of nematodes from soil and root samples

Isolation of nematodes from soil and roots was performed by using different techniques like Whitehead and Hemming tray method (Whitehead and Hemming, 1965) and Baermanns funnel technique (McKenry and Roberts, (1985). In this method sample was placed in a bowl, mixed, and roots were separated from the soil. Coarse soil particles were ground to make the fine textured soil. With the help of a measuring cylinder 100 ml^3 soil sample was measured and spread on tissue paper on the perforated plastic dish. The plastic dish was fixed in a tray having water in it which was adjusted so that water just touches the tissue paper containing the soil

sample. Samples were covered with a plastic lid and placed for 48 hours in an incubator. After 48 hours' nematodes were settled down in the base of the tray. A suspension containing nematodes was poured into a beaker for counting the juveniles. (Whitehead and Hemming, 1965) Nematodes were allowed to settle in the bottom of the beaker and supernatant was discarded and the remaining solution was transferred to another beaker for further studies.

Nematodes from root samples were isolated by the Baermanns funnel technique (McKenry and Roberts, 1985). The distribution of nematode from roots and soil samples was calculated by using the following formula:

$$\text{Citrus nematode prevalence (\%)} = \frac{\text{Number of samples with } T.\ semipenetrans}{\text{Total number of samples}} \times 100$$

(Nasir *et al.,* 2021)

One ml aliquots were taken from nematode suspension in a Petri dish and counted under a stereomicroscope (Olympus SZ 61). The total population of nematodes was calculated by taking the means of three aliquots and multiplying them with total volume. The whole procedure was repeated three times.

Assessment of the number of females of *T. semipenetrans*

To assess the number of females of *T. semipenetrans*, feeder roots, 1 g from each sample were taken and washed with running tap water gently. After washing, roots were put into an airtight bottle having 200 ml of 0.5% sodium hypochlorite solution and were forcefully shaken for 4-6 minutes to remove soil attached with a gelatinous matrix of roots. Roots were then washed with tap water and acid fuchsin lactophenol staining was done (Byrd *et al.,* 1983). Washing with distilled water and glycerol (1:1) to remove excess stain and population of females was recorded under a stereoscope.

Identification of *T. semipenetrans*

Roots having stained nematodes were shredded with the help of a root shredder. A suspension containing stained female nematodes was placed in a beaker and a small amount of suspension was placed in counting plates. Juveniles were identified from fresh root samples under a stereoscope. After picking, nematodes were placed on slides having water drops and were flamed for 5-8 seconds to kill them. These were then covered with coverslips and identified under the microscope. Males were wormlike in shape and bodies were found vermiform, short slender with a small stylet. Spicules were observed curved and the tail was conically pointed. Basal rounded knobs were observed with a stylet. Females behind the neck portion were swollen. The anterior part of females was fixed in root tissues while the posterior region was out of roots. The posterior part of the females was also swollen with a thick cuticle containing a pointed post-vulvar section. Females were semi-endoparasites (Rashidifard *et al*., 2015).

Isolation of fungi and bacteria

The dilution plate technique was adopted for the isolation of mycoflora from the citrus rhizosphere (Johnson and Curl, 1972). The autoclaved PDA was poured into sterilized Petri plates and allowed to solidify overnight. One-gram soil was taken from each sample and 100 ml of autoclaved distilled water was added to each sample. Serial dilutions with concentrations up to $1x10^5$ were prepared. One ml from each dilution of fungi was poured on Potato dextrose agar (PDA) and incubated at 25±2°C for five days. The fungi were then purified and stored at 4ºC for experimental use.

Bacterial isolation was done on solidified nutrient agar (NA) medium. One ml from dilutions was shifted onto plates with the help of a sterilized pipette and the plates were then incubated at 30±2ºC. Bacterial colonies were purified by using the streak plate method. The purified bacteria were then stored at 4ºC for experimental use.

Isolated fungi and bacteria were identified based on morphological characteristics. Mycoflora was identified by comparing morphological features with those available at Faces of Fungi (FOF, 2021). The gram staining technique also proved helpful to identify bacteria. Gram staining greatly supports the microscopic identification of bacterial cultures up to a generic level. The suggestions of Schaad *et al*. (2001) were followed to perform gram staining. Protocols of Rachel (2021) were used for performing different biochemical tests for the identification of bacteria. Morphological features of bacteria from BacDive (2021) were used as a key for bacterial identification.

RESULTS

Disease prevalence

Disease prevalence is the ratio of the number of diseased

orchards to the number of total orchards sampled. For citrus slow decline and was calculated in different orchards of citrus growing areas of Bhalwal, Shahpur, Sillanwali, Kot Momin, Sargodha, Quaidabad, Jhang, and Rahim Yar Khan of Punjab Province as shown in Figure 1. Maximum disease prevalence (100%) was recorded in Rahim Yar Khan followed by Sargodha and Sillanwali (80%) and Shahpur and Quaidabad (60%). The minimum disease prevalence was recorded in Bhalwal and Kotmomin that showed (20%). Data on all visited places varied significantly when analyzed statistically at ($P \leq 0.05$).

Disease incidence

Diseases incidence is the number of diseased plants to the total number of plants observed. Maximum citrus slow decline incidence was observed in Rahim Yar Khan (80%) followed by Sargodha (74%), Sillanwali (62%), and Shahpur (48%). Jhang showed 44% incidence followed by Bhalwal, Kotmomin (36, 24 %). The minimum incidence of slow decline was recorded in Quaidabad (20%). Figure 2 shows that disease incidence was different significantly from each other at ($P \leq 0.05$).

Percent Disease Index

During the survey, it was indicated that Sargodha and Rahim Yar Khan have maximum PDI of 28.8% and 32.8% respectively while Sillanwali, Quaidabad, and Jhang have less PDI which was 18.6 %, 16.6 %, and 14 % respectively as shown in figure 3. Kotmomin and Bhalwal have minimum PDI which was 13.4%, 9.8%.

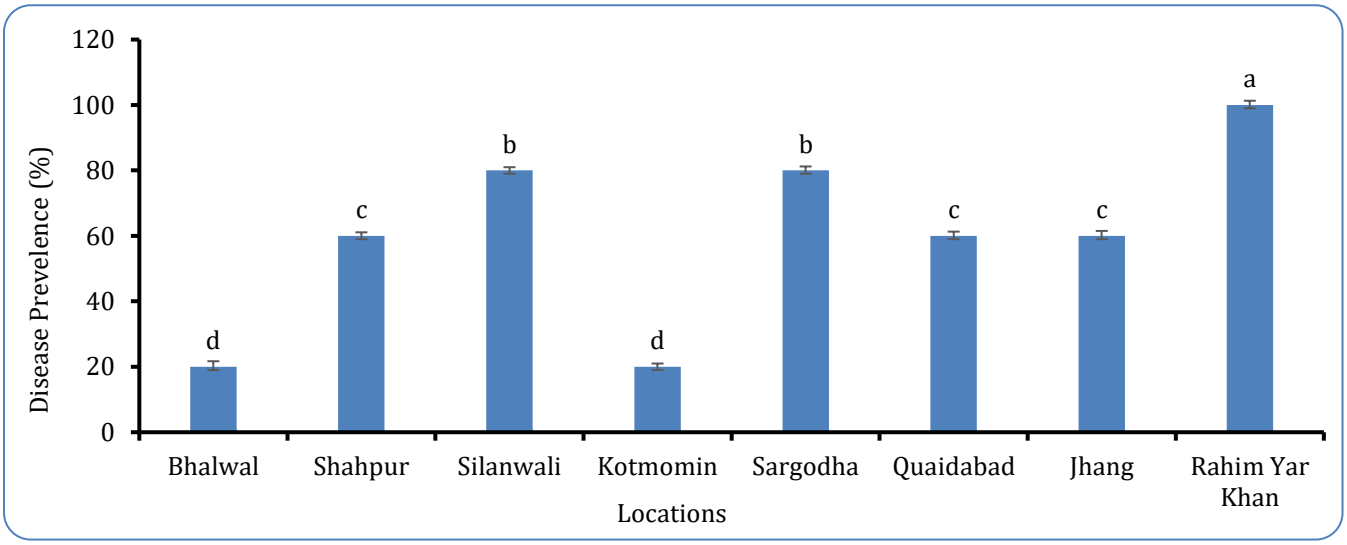


Figure 1. Disease prevalence of citrus slow decline during survey at different locations.

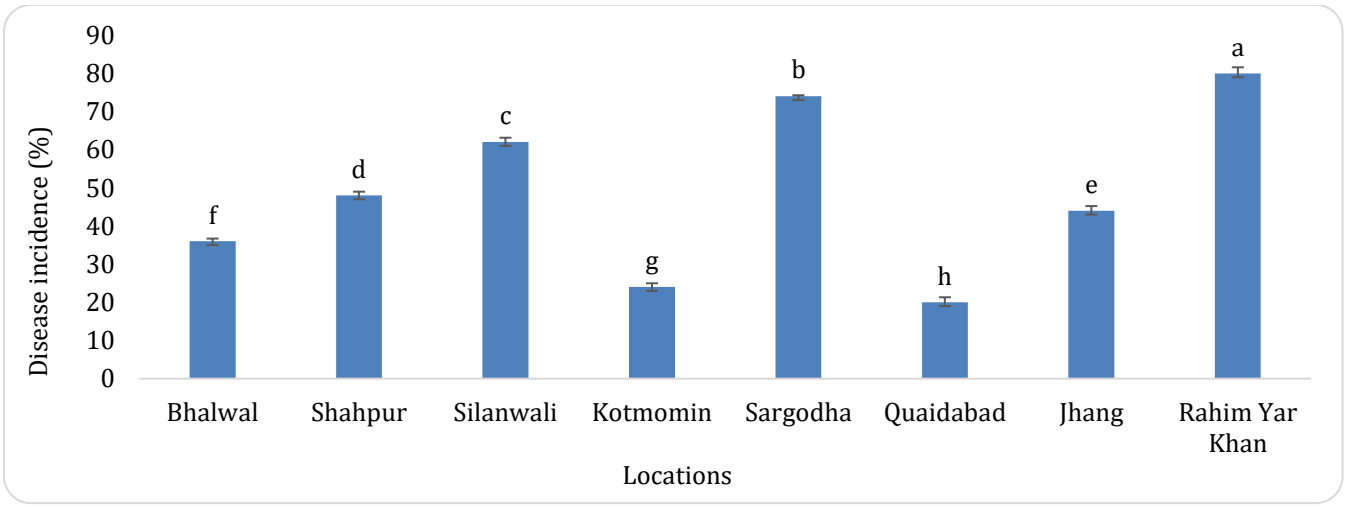


Figure 2. Disease Incidence of citrus slow decline during survey at different locations.

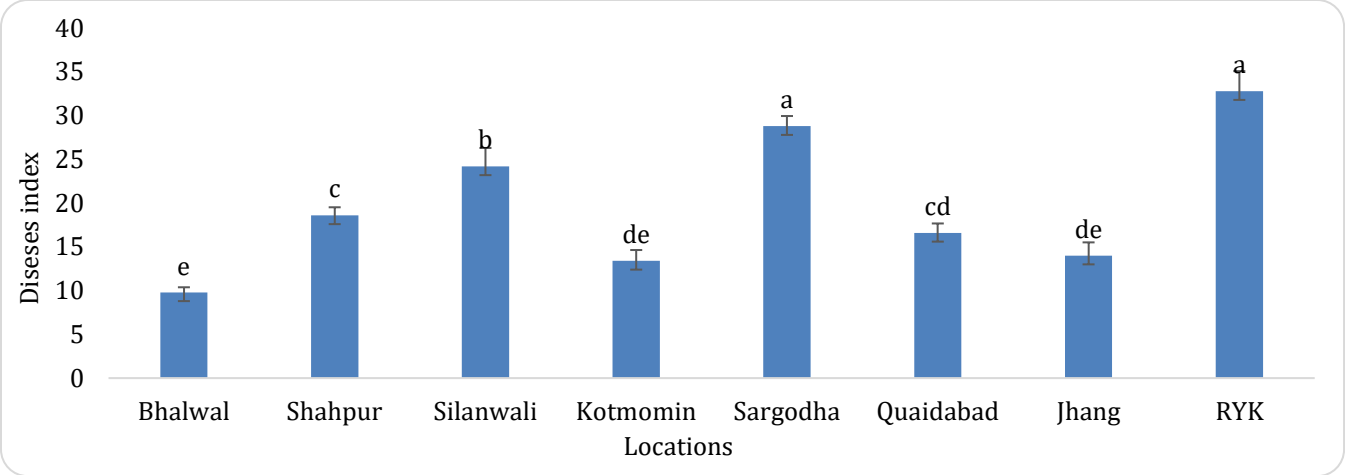


Figure 3. Percent disease severity index (PDI) of citrus slow decline at different locations during the survey.

Citrus nematode prevalence% from infected roots

The Survey of different locations indicated the maximum nematode frequency (100%) in Rahim Yar Khan, 95% in Shahpur. The frequency of *T. semipenetrans* was 85% in Sargodha and Quaidabad which was not significantly (P>0.05) different from each other. However, nematodes prevalence% in Kotmomin was recorded as 92%, Sillanwali 68% which was significantly *P*≤ 0.05 different from each other by using different extraction methods as shown in Figure 4.

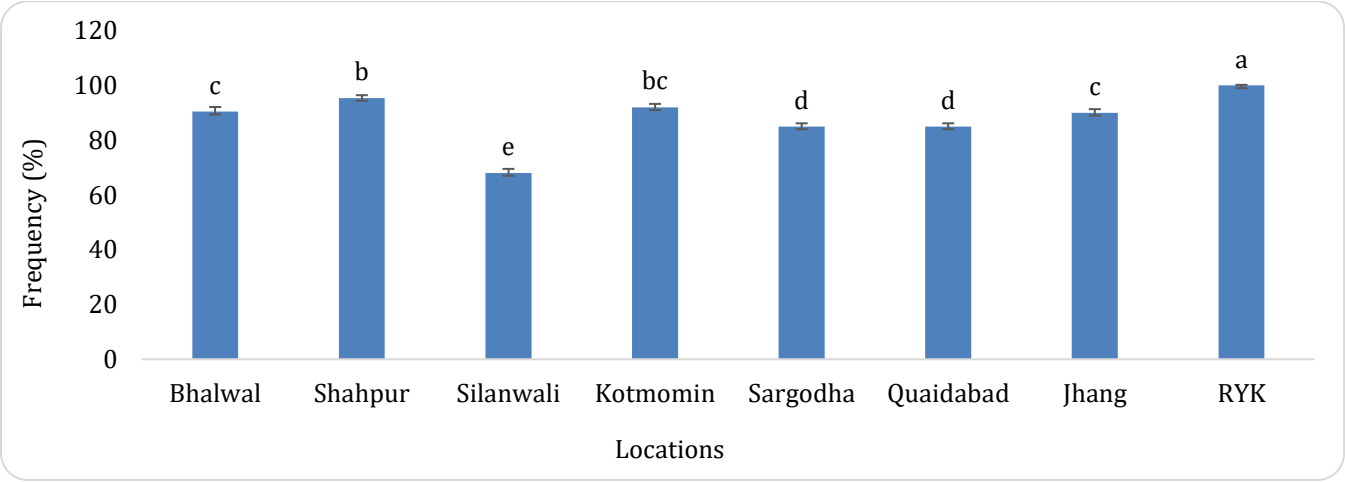


Figure 4. Citrus nematode prevalence (%) from different locations.

Number of Juvenile/100ml of soil

Results of the survey regarding J2s population at different locations revealed that the maximum number of 2nd stage juveniles (J2s/100ml) of soil were observed in Rahim Yar Khan (1674) followed by Shahpur (1534), Quaidabad (1432), Sargodha (1347) and Bhalwal (1172). Similarly, J2s of *T. semipenetrans* isolated from Kotmomin and Sillanwali were (1024), (956) respectively. The minimum number of J2s was collected from the soil sample taken from Jhang (805). Data showed a significant difference among values at (P≤ 0.05) as shown in figure 5.

Number of Juvenile/g of root

Root samples collected from district Rahim Yar Khan showed maximum J2s (5867) followed by Quaidabad (5692) and Shahpur (5465) which were not significantly (P>0.05) different from each other. Both Bhalwal showed (5043) and Sargodha (5128) which were not significantly (P>0.05) different from each other. The number of J2s isolated from Sillanwali (2984) and Kotmomin (3045) were not significantly (P>0.05) different from each other. The minimum number of J2s was calculated in Jhang (2124). Data showed a significant difference among

values at (P≤ 0.05) as shown in figure 6.

Number of female/g of root

The highest number of females were recorded in Rahim Yar Khan (652) followed by Quaidabad (611), Bhalwal (490), Kotmomin (421), Sillanwali (387). Data showed a significant difference among values at (P≤ 0.05) as shown in figure 6. The number of females was recorded in Shahpur (568) and Sargodha (545) (not significantly (P>0.05) different). A minimum number of females in Jhang (288) as shown in Figure7.

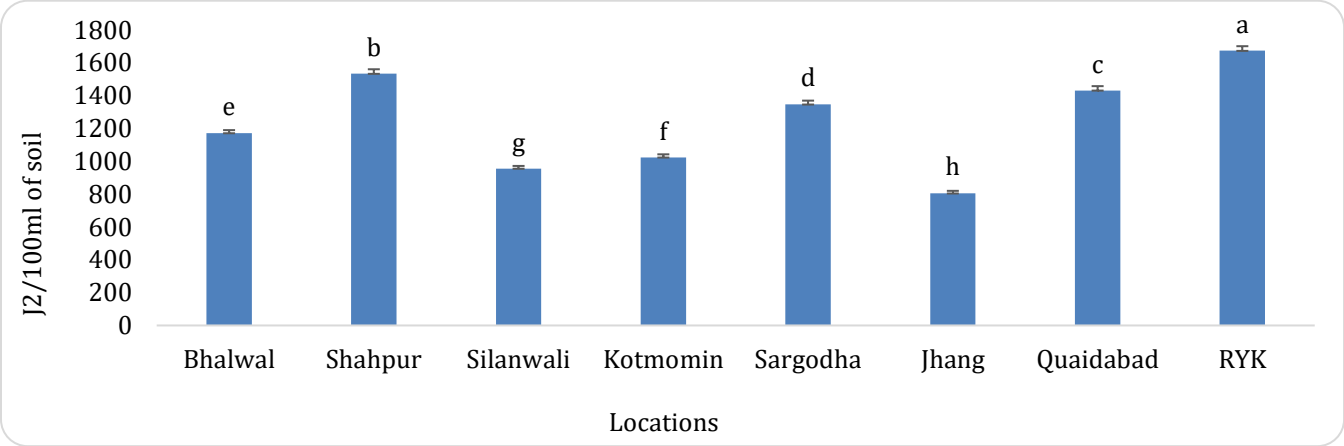


Figure 5. Population of J2 per 100ml of Soil at different locations.

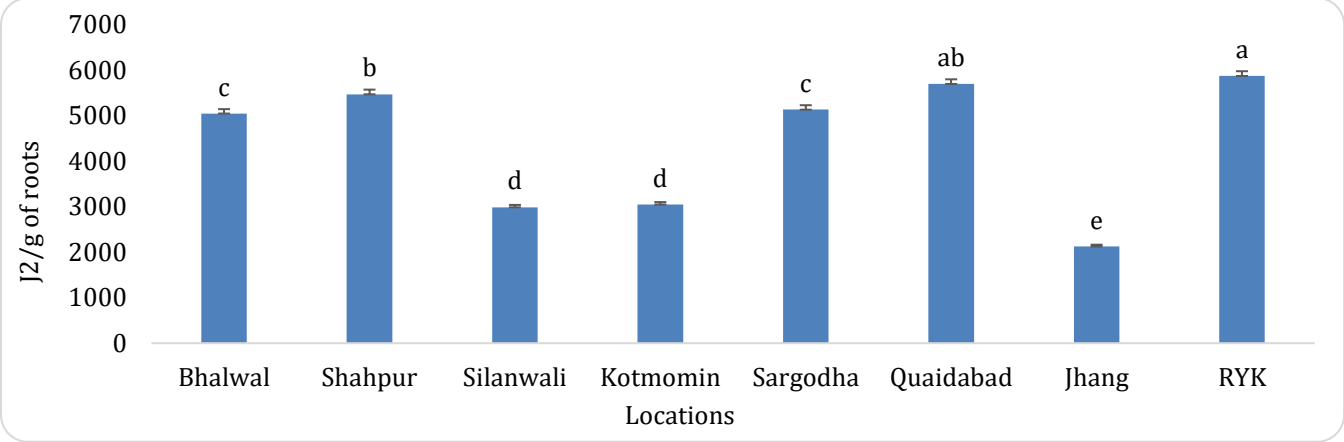


Figure 6. Population of J2 per gram of roots at different locations.

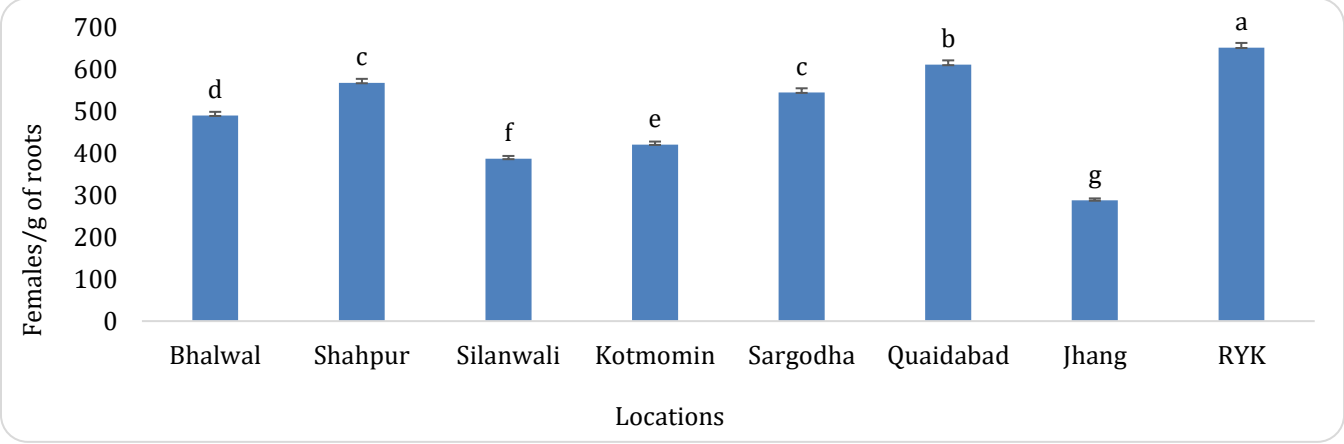


Figure 7. Population of females per gram of roots at different locations.

Potential Antagonistic Microorganisms Isolated from Citrus Growing areas of Punjab

The antagonistic microorganisms isolated from root zones of citrus in surveyed locations of Bhalwal, Shahpur, Silanwali, Kotmomin, Sargodha, Quaidabad, Jhang, and RYK with different frequencies. From Bhalwar, *Trichoderma harzianum*, *T. viride*, and *Bacillus subtilis* was recovered while a similar trend was observed in RYK. In Shahpur, *T. harzianum, T. koningii* bacterial species were recovered no fungal antagonists were recovered. *T. harzianum* was also recovered from Silanwali and Quaidabad. Similarly, *Bacillus subtilis* was recovered from the rhizisperic soils of Silanwali, Kotmomin, Sargodha, Jhang, and RYK. *P. fluorescens, B. cereus* and *P. putida* were recovered from Silanwali, Kotmomin, Sargodha, and Quaidabad respectively. Details of fungal and bacterial antagonists identified from the root zone of citrus orchards located in different districts of Punjab is shown in table 1 and 2.

Table 1. Fungal antagonists isolated and identified from different locations.

Antagonists	Bhalwal	Shahpur	Silanwali	Kotmomin	Sargodha	Quaidabad	Jhang	RYK
T. harzianum	+	+	+	-	-	+	-	+
T. viride	+	-	-	-	-	+	-	+
T. koningii	-	+	-	-	-	-	+	-
T. atroviride	-	-	+	-	-	-	-	-

Table 2. Fungal antagonists isolated and identified from different locations.

Antagonists	Bhalwal	Shahpur	Silanwali	Kotmomin	Sargodha	Quaidabad	Jhang	RYK
Bacillus subtilis	+	-	+	+	+	-	+	+
P. fluorescens	-	-	+	+	-	-	-	-
B. cereus	-	-	+	-	-	+	-	-
P. putida	-	-	-	+	-	-	-	-

DISCUSSION

Tylenchulus semipenetrans is a leading cause of the citrus slow decline. *T. semipenetrans* are found in all soil textures, but its damage is recorded maximum in comparatively shallower, poorly drained soils having organic matter 2-3%. It reduces the number of plants, fruit yield, and quality. The citrus slow decline occurs in the moderate temperature range because *T. semipenetrans* shows maximum population increase at the temperature of 20-31^{o}C while 25^{o}C is considered the optimum (Ducan and Joe. 1987). It is difficult to manage *T. semipenetrans* as no single tactic provides satisfactory management. Moreover, these nematodes interact with different pathogens and nutritional deficiencies, leading to a disease complex.

A comprehensive survey of citrus orchards was carried out for the assessment of disease incidence, prevalence, frequency, and disease severity in the major citrus growing areas like Bhalwal, Shahpur, Silanwali, Kotmomin, Sargodha, Quaidabad, Jhang, and Rahim Yar Khan of Punjab Pakistan. Similar surveys for the decline were also carried out by Parvez *et al.,* 2003; Al-Azzeh *et al.,* 2005 and Gene *et al.,* 2005 to isolate the citrus nematodes and to calculate the disease incidence. Results of the present study related to the nematode population are also in conformity with them as all of them gave showed the disease incidence levels between.

In the present survey, nematode population and female infection on roots varied in different areas. Several factors affected *T. semipenetrans* density and invasion, including soil texture, temperature, host variety, pH, soil depth, tree age, nutrients status, and moisture. It is among the most dominant soil borne pathogens of citrus orchards.

Maximum disease prevalence was recorded at 100% in Rahim Yar Khan. The minimum disease was recorded in Bhalwal and Kotmomin that showed 20% disease prevalence. Maximum disease incidence was observed as 80% in Rahim Yar Khan. The minimum incidence of slow decline was recorded in Quaidabad (20%). Maximum J2/100 ml of soil were observed in Rahim Yar Khan followed by Shahpur, Quaidabad, Sargodha, Bhalwal, Kotmomin, Sillanwali, and Jhang. The highest number of J2s per gram of roots were recorded in Rahim Yar Khan followed by Quaidabad, Shahpur, Sargodha, Bhalwal, Kotmomin,

Sillanwali, and Jhang. Females per gram of roots were recorded maximum in Rahim Yar Khan followed by Quaidabad, Shahpur, Sargodha, Bhalwal, Kotmomin, Sillanwali, and Jhang. In 1962 *Tylenchulus semipenetrans* were reported from Pakistan in Faisalabad (Brown, 1962). Results about the juvenile population of citrus nematodes in Punjab province are parallel to those of Ahmad and Khan, 1999; Iqbal *et al*., 2006 and Chohan *et al*., 2007.

Citrus nematode, results indicate that the population densities and frequencies of occurrence were different in the examined locations. Such differences may be due to different factors such as location, soil type, irrigation system, soil moisture, kind of rootstock, and agricultural practices as confirmed by Abd-Elgawad (1992), Ibrahim (1994).

The use of bioagents is an effective eco-friendly approach for the control of citrus nematode. So, these BCAs may also be suggested to the farming communities for profitable organic farming. The fungus *Paecilomyces lilacinus* and the bacterial genus, *Pseudomonas* are reported as effective bioagents against *T. semipenetrans*. These fungi and bacteria not only dropped the nematode populations but also increased the yield of citrus fruit (Hammam *et al*., 2016). The combined application of fungus *T. harzianum* and neem cakes is also reported to reduce the *T. semipenetrans* population (Parvatha *et al*., 1996).

Many workers have devoted their efforts for checking the regulatory effect of bacteria and fungi against plant parasitic nematodes. There are mainly two types of antagonism including direct antagonism and indirect antagonism. Direct antagonism includes hyperparasitism, lytic enzymes and antibiotic mediated suppressors along with other byproducts released by bioagents on other hand induced host defense and competition are linked with indirect antagonism. The results revealed that different fungal and bacterial antagonists were isolated from different locations. The fungal antagonist T. harzianum isolated from Bhalwal, Sillanwali, Shahpur, Quaidabad, Jhang and Rahim Yar Khan, T. viride was isolated from Bhalwal, Quaidabad and Rahim Yar Khan. Similarly, bacterial antagonists were isolated from different locations like P. flourescens was isolated from Sillanwali and Kotmomin, B. subtilis isolated from Bhalwal, Sillanwali, Kotmomin, Sargodha, Jhang, and Rahim Yar Khan.

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