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Full Length Article

Association of Plant Pathogenic Fungi and *Tylenchulus semipenetrans* with Declining Citrus Orchards of Punjab, Pakistan

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Abstract

Decline is a serious threat to citrus industry of Pakistan. During 2016–17, a survey was conducted in twelve major citrus growing areas of Punjab, Pakistan including Bhalwal, Sillanwali, Kot-Momin, Sargodha, Shahpur, Khushab, Faisalabad, Toba, Gojra, Mianwali, Multan and Jhang to measure the incidence of citrus decline. Five orchards were selected randomly at each location. The maximum disease incidence (90%) was observed in Khushab and the minimum in Sillanwali (47%). For the isolation of nematodes, four subsamples were collected and mixed to form a composite sample from each tree. *Fusarium solani*, *F. oxysporum* and *F. semitectum* were found to be associated with declining citrus plants. *F. solani* incidence was the highest in Shahpur (73%) while the lowest (38%) from Gojra. The highest frequency of *F. oxysporum* (42%) and *F. semitectum* (66%) was recorded in Khushab and Mianwali, respectively. The maximum number of females nematodes per gram of roots were calculated from Jhang (648) followed by Shahpur (617). The highest population of second stage juveniles from per gram roots was recorded from Jhang (6483) while it was 1541 100 g^{-1} of soil in Shahpur. The varying frequency of fungal and nematode populations is helpful to determine the association of these pathogens with declining citrus trees in Punjab Province, Pakistan and the correct knowledge and identification of associated pathogens will help to formulate effective management strategy for citrus decline.

Keywords: Decline; *Fusarium solani*; *Fusarium oxysporum; Tylenchulus semipenetrans*; Juveniles

Introduction

Citrus is one of the most important fruit crops belonging to family Rutaceae and is widely cultivated under tropical and sub-tropical environmental conditions (Maheswari *et al*. 2023). In Pakistan, citrus is most widely cultivated fruit with a great export value (Ahmad *et al.* 2018). Citrus trees are affected by different pests and pathogens throughout the world. Economically the most important and well-known diseases in Pakistan are citrus greening, wither tip, canker, slow and quick decline (Rajput *et al.* 2020).

Among all the citrus diseases, decline is the most destructive disease known to be present in all citrus growing areas of Pakistan that results in decrease of its average life span. Symptoms including general sickness, chlorotic leaves, twigs drying and premature leaf fall, reduced fruit size, sudden/gradual dieback of the plants (Ali *et al*. 2023). It is the universal problem throughout the world and almost one million citrus trees were damaged due to decline in Brazil (Hajeri and Yokomi 2023). Average life of citrus in Pakistan rarely exceeds 25 years due to decline (Naqvi *et al.* 2022**)** whereas in developed countries it ranges from 50 to 100 years by adopting good agricultural practices. Citrus trees are prone to many soil-borne pathogens including nematode and fungal species throughout their life span (Kumar and Arthurs 2021). Among nematodes, citrus nematode (*Tylenchulus semipenetrans*) and burrowing nematode (*Radopholus similis*) are economically the most important and well-known species whereas some other species including sting nematode (*Belonolaimus longicaudatus*) and lesion nematodes *Pratylenchys brachyurus* have also been reported. Different fungal pathogens including *Phytophthora parasitica*, *P. citrophthora*, *P. palmivora* (Mehrotra 2011), *F. solani*, *F. oxysporum*, *Lasiodiplodia theobromae*, and *Phomopsis citri* were found to be responsible for causing decline, dieback, gummosis, wilt and root diseases in citrus (Kim *et al*. 2015).

Several soil and environmental factors including high and low temperature, humidity, soil type, texture and

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salinity may also be associated with citrus decline. These soil factors in addition to oxygen concentration, carbon dioxide concentration; root exudates affect the movement and survival of nematodes (Fujimoto *et al*. 2009). Deposition of salts in many parts of the world is found to be damaging factor for citrus crop as it is more sensitive to excess of salts (Soltabayeva *et al*. 2021).

Little work has been done in Pakistan about the synergism of different pathogens with declining citrus orchards. Therefore, it is dire need to investigate the cause of citrus decline so that sustainable and eco-friendly management practices should be adopted for the solution of this problem. For this purpose, a survey of major citrus growing areas of Punjab Province, Pakistan including Bhalwal, Sillanwali, Kotmomin, Sargodha, Shahpur, Khushab, Faisalabad, Toba Tek Singh, Gojra, Mianwali and Multan were performed to assess disease prevalence, incidence and various species of nematodes and pathogenic fungi associated with declining citrus plants. Information regarding the actual cause of citrus decline will be helpful in minimizing the yield losses by adopting proper management practices in specific location and creating awareness among farmer community about citrus decline.

Materials and Methods

Survey and distribution of disease

A comprehensive survey was carried out during two consecutive years 2016 and 2017 in the major citrus growing areas of Punjab including Bhalwal, Sillanwali, Kot-Momin, Sargodha, Shahpur, Khushab, Faisalabad, Toba Tek Singh, Gojra, Mianwali and Multan (Fig. 1 and Table 1). Five orchards were randomly selected to find out the disease prevalence in each locality whereas 10 trees from each orchard were inspected to calculate the disease incidence. Five trees were selected randomly at each location to collect the samples for the isolation of fungal pathogens and nematode population.

The following formulas were used to calculate the disease prevalence and disease incidence (Rehman *et al*. 2012).

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

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

However, disease severity was calculated by applying visual rating scale from 0–5 given by Kazmi *et al*. (2005). According to this scale 0 (healthy plants), 1 (1–10% decline), 2 (11–20%), 3 (21–30%), 4 (31–50%), 5 (above 50%)

Sampling and isolation of fungal pathogens

Diseased samples were taken from stem and roots of declining citrus plants for the isolation of fungal pathogens. Five samples were taken from each orchard, were cut into small pieces (4–5 mm), disinfected with 1% sodium hypochlorite solution for 2 min and rinsed three times with distilled water. The small pieces were then shifted to Petri dishes containing potato dextrose agar (PDA) amended with 100 µg mL^{-1} streptomycin and were placed in an incubator at 25°C for 5 days. Thereafter the grown fungal culture on plates were purified and identified on the basis of their morphological characters. For the isolation of *Phytophthora* spp. 4–5 pieces of diseased specimen were transferred into Petri plates containing V-8 agar comprising of eight vegetables modified by Pimaricin (10 mg L^{-1}), Ampicillin (150 g) and Rifampicin (10 mg L^{-1}). The plates were then placed in the dark at 23°C. The growth of fungal species was examined under stereomicroscope. Relative density of each fungus colonized with different pieces from culture plates was determined by using the following formula:

$$\text{Relative density of fungal species (\%)} = \frac{\text{No. of pieces colonized by pathogen}}{\text{Total number of pieces}} \times 100$$

Sampling and isolation of nematodes

Soil and feeder root samples were taken at the depth of 4-6 inch under the canopy of tree for nematode isolation. Four subsamples from each tree were taken and mixed thoroughly in polythene bags to form a composite sample of 1000 g and were labeled to avoid mixing of samples. The samples were then brought into the Laboratory of Plant Pathology, University of Agriculture, Faisalabad and were stored in refrigerator at 10°C to keep them fresh for further experiments. Isolation of nematodes from soil was performed by using Whitehead and Hemming tray method (Whitehead and Hemming 1965) whereas from roots nematodes were isolated by using Baermann funnel technique (McKenry and Roberts 1985). Citrus nematodes at J_2 stage were recorded from soil and root samples; however, number of females were counted from per gram of roots and data was recorded. Relative density of nematodes was measured by applying the following formula (Safdar *et al*. 2010).

$$\text{Relative density of nematode (\%)} = \frac{\text{No. of samples with } \textit{T. semipenetrans}}{\text{Total number of samples}} \times 100$$

Statistical analysis

The experimental data was subjected to statistical analysis using Statistix (Ver. 8.1) software. The design was completely randomized and the treatment means for each experiment were compared by LSD test at p=0.05.

Results

The disease prevalence calculated according to data recorded revealed that in Sargodha, Shahpur and Multan it was 100%, which showed that all the orchards observed

Table 1: Locations selected for survey in this study

Sr. No.	Location	Latitude	Altitude
1	Bhalwal	32.275363	72.903070
2	Sillanwali	31.829303	72.536946
3	Kot-Momin	32.190028	73.025769
4	Sargodha	32.089652	72.680676
5	Shahpur	32.286693	72.428378
6	Khushab	32.295870	72.348227
7	Faisalabad	31.416670	73.083330
8	Toba	32.574367	72.820961
9	Gojra	31.151976	72.679386
10	Mianwali	32.584759	71.535594
11	Multan	30.162047	71.525448
12	Jhang	31.280285	72.327385

Fig. 1: A map of the surveyed locations at different regions of Pakistan
The specific locations are highlighted in the map

were affected by decline whereas in Toba Tek Singh, Gojra, Khushab and Jhang 90% prevalence was recorded. Prevalence in Bhalawl, Kot Momin and Faisalabad was 80% followed by Sillanwali (70%). The minimum disease prevalence of 60% was recorded from Mianwali. The survey report indicated that disease incidence of decline was maximum in Khushab (90%) followed by Multan (90%), Shahpur (87%), Jhang (85%), Toba Tek Singh (83%), Sargodha (80%), Gojra (79%), Faisalabad (77%), Kot-Momin (73%), Bhalwal (68%) and Sillanwali (47%). However, the minimum incidence of 39% was recorded from Mianwali. However, mean disease severity was found maximum in Shahpur (3%) followed by Multan (2.4%), Sargodha (2.4%), Faisalabad (2.1%), Khushab (1.9%), Jhang (1.8%), Kot-Momin (1.6%), Toba Tek Singh (1.3%), Gojra (1%), Bhalwal (0.7%), Mianwali (0.7%), while minimum disease severity (0.6%) was observed in Sillanwali (Table 2).

Fungal pathogens associated with citrus trees

For the isolation of fungal pathogens root samples were collected from declining citrus plants during visit of citrus orchards of Punjab Province, Pakistan. It was observed that *Fusarium* species (*F. solani*, *F. oxysporum*) were most frequently associated with roots of declining plants as compared to other fungal species. However, there occurrence was variable among each of the locations visited. *F. solani* was most frequently isolated from root samples and its distribution was recorded maximum from

Table 2: Mean disease prevalence, incidence and severity of major citrus growing areas of Punjab

Locations	Disease prevalence (%)	Disease incidence (%)	Disease severity
Bhalwal	80	68.47	0.7
Sillanwali	70	46.63	0.6
Kotmomin	80	72.56	1.6
Sargodha	100	80.33	2.37
Shahpur	100	87.26	2.5
Khushab	90	91.24	1.9
Faisalabad	80	76.9	2.08
Toba	90	83.34	1.27
Gojra	90	79.04	1
Mianwali	60	39.06	0.7
Multan	100	90.39	2.38
Jhang	90	84.68	1.78

Table 3: Incidence of fungal pathogens associated with declining citrus plants, 2016-17

Location	*F. solani*	*F. oxysporum*	*F. semitectum*	*Phytophthora*	*Penicillium*	*Aspergillus*
Bhalwal	++++	++	_	+	_	+
Sillanwali	+++	++	+	+	_	+
Kotmomin	+++	++	_	+	+	_
Sargodha	++++	++	+	++	+	_
Shahpur	+++++	++	_	+	_	_
Khushab	++++	+++	_	+	+	+
Faisalabad	++++	+++	_	_	_	_
Gojra	++	++	_	+	+	_
Mianwali	++++	++	_	+	_	+
Multan	++++	+++	_	_	_	_
Jhang	++	++	+	_	_	+

+ sign indicates the disease incidence
+* 1-10%, ++*11-30%, +++*31-50%, ++++*51-70%, +++++*Above 70%

Table 4: Citrus nematode (*T. semipenetrans*) population in Province Punjab, Pakistan

Locations	No. of females g^{-1} of roots	No. of juveniles g^{-1} of roots	No. of juveniles 100 mL^{-1} of soil
Bhalwal	537 d	4457 e	1419 c
Sillanwali	463 g	4177 f	1371 d
Kotmomin	483 f	5327 c	1487 b
Sargodha	448 h	3241 h	1254 f
Shahpur	617 b	5983 b	1541 a
Khushab	513 e	4878 d	1108 g
Faisalabad	592 c	3684 g	1083 g
Toba	328 j	2937 j	836 i
Gojra	309 k	2317 k	743 j
Mianwali	379 i	3051 i	1018 h
Multan	293 l	1843 l	674 k
Jhang	648 a	6483 a	1329 e

Means sharing common letters in columns do not differ significantly at 5% level of significance by LSD test

Shahpur (72.66%) and Khushab (67.87%) whereas its lowest distribution was noticed in Gojra (38%) and Toba Tek Singh (34%). However, *F. oxysporum* and *Phytophthora* were the second and third most frequently isolated fungal species from root samples followed by *Aspergillus*, *Penicillium* and *F. semitectum* species. The association of fungal microorganisms varied significantly between different locations. So, the comparison of observed fungal species was also made at each location which is shown as in Table 3.

Association of *T. semipenetrans* with declining citrus plans

For the assessment of nematode population associated with infected citrus orchards number of females per gram of roots, number of juveniles per gram of roots and number of juveniles per 100 g of soil were calculated. The maximum number of females per gram of roots was recorded from Jhang (648) followed by Shahpur (617) while minimum was found in Multan (293). However, number of second stage juveniles (J_2) per gram of roots were the highest in Jhang (6483) followed by Shahpur (5983) whereas lowest J_2 per gram roots were counted from Multan (1843). Similarly, number of J2 of citrus nematode (*T. semipenetrans*) per 100 g of soil was also estimated and was noticed maximum from Shahpur (1541) followed by Kot Momin (1487) while minimum J_2 population 100 g^{-1} of soil was calculated from Multan (674) as shown in Table 4.

Discussion

The results of the following survey revealed that citrus decline is widely present throughout the Punjab Province and becoming a severe hazard day by day for citrus industry of Pakistan. The disease has complex etiology and numerous biotic and abiotic factors have been reported to be associated with it in various studies from several countries. Different scientists considered that phytoplasma and some other bacteria are responsible for citrus decline (Arif *et al*. 2005) while some were in opinion that it is due to citrus tristeza virus (Bertaccini *et al*. 2014). Besides these biotic factors some other factors such as temperature, soil moisture, salinity, pH, human activities including orchard management and intercropping, irrigation also have great influence in the development of citrus decline symptoms.

In this study, it was found that disease incidence and severity were high at Multan, Sargodha, Shahpur and Khushab. The reason of high incidence and severity might be the old citrus plantations and intercropping of barley, berseem and wheat. Flood irrigation method being practiced is responsible for disease spread. However, the situation regarding orchard management was much better in Sillanwali, Kot-Momin, Bhalwal and Mianwali. The results of our study indicated that prevalence, incidence and severity were maximum in Sargodha, Shahpur, Multan and Khushab while it was lower in Mianwali, Kot-Momin, Bhalwal and Sillanwali where better orchard management practices were followed.

Soil salinity, iron composition, soil compaction, total soluble salts, carbonates and bicarbonates are main factors which directly influence plant health. As high carbonates and bicarbonates reduces the iron in plants (Franzen and Richardson 2000). Similarly, soil salinity causes poor root growth which ultimately leads towards the iron deficiency and cause chlorosis in plants. The increase in salinity/sodicity might be due to the use of brackish water or salty range of Khura in Shahpur and Sahiwal (Zaka *et al*. 2004). Mane *et al.* (2011) conducted survey from different locations including Sargodha, NiBhalwal, Shahpur, Sillanwali, Kotmomin and Sahiwal and observed that about 40.6% of all the soils were salt affected which contain saline (3.9%), saline sodic (7.8%), and sodic (28.9%). Maximum salt affected samples were observed from Sahiwal (63.4%) followed by Shahpur (53.3%) and Sillanwali (50%). Citrus trees are more sensitive towards excess quantity of salts present in the soil (Srivastava and Ram 2000). High salt concentrations and increase moisture level make soil more compact and create hardpan of soil as a result of which root growth is restricted and plant growth is reduced. During our survey maximum disease incidence in Shahpur and Khushab might be the result of these soil edaphic factors of salinity and sodicity.

Among the fungal pathogens, *L. theobromae*, *L. hormozganensis*, *Phytophthora* spp. (*P. nicotiana*, *P. citrophthora*, *P. citricola*, *P. palmivora*), *F. solani* and *Neoscytalidium dimidiatum* were most commonly reported to cause symptoms of citrus decline (Safdar *et al.* 2010; Rehman *et al.* 2012; Al-Sadi *et al*. 2014). According to this survey *F. solani* was predominant species followed by *F. oxysporum*, *Phytophthora* spp. *F. semitectum* and other *Fusarium* species from all of the orchards in Province. *Fusarium* species have been reported to cause reduction in crop yield along with production of mycotoxins around the globe (Akhtar *et al.* 2020; Fatima *et al*. 2023; Naqvi *et al.* 2023). Among other pathogens *Fusarium* species are considered as the most important vascular pathogens of numerous plant species as well as citrus (Bashir and Tahira 2012; Rehman *et al.* 2012). *Fusarium* spp. have also been found in association with *Dalbergia sissoo* decline (Haq *et al*. 2021; Malik *et al.* 2024). Association of *N. dimidiatum* with re-grafted orchards of citrus on sour orange rootstock was also noticed from Italy (Polizzi *et al.* 2009).

Soil-borne fungi are more sensitive to the soil physico-chemical characteristics (Kanwal *et al*. 2017). Soils with more clay and dust particles have better potential to retain nutrients and provide favorable environment for microbes (Ciric *et al*. 2012). As there is high level of organic matter in Shahpur, Kot-Momin, Sargodha, Bhalwal and Sillanwali is high and fungal population found higher in these locations. Organic matter contents make layer on soil surface and microbial community developed under that layer. Similarly, soil texture of Shahpur, Sargodha, Sillanwali, Bhalwal, Kot-Momin have soil texture sandy loam and clay soils which are favorable for the growth of fungal species.

The most prominent effect of salinity has been observed to increase the egg production several folds in all the rootstocks evaluated. It was noticed that due to salinity nematode populations and fecundity enhanced and all the rootstocks lack the salt tolerance (Kiptoo *et al.* 2022). The current survey indicated the presence of citrus nematode (*T. semipenetrans*) at each location visited but its frequency was highest at Shahpur and Jhang. The results of present survey regarding citrus nematode populations are similar with many previous studies (Parvez *et al.* 2003; Iqbal *et al*. 2006). The highest level of nematode population from Shahpur showed that salt concentration and nematode population are directly associated with each other.

T. semipenetrns can be found in all kind of soil textures but more damage occurs in poorly drained soil having 2–3% organic matter contents (Zaki *et al*. 2012). The maximum organic matter contents were observed from Shahpur (2.51) followed by Kot-Momin (1.61%), Sargodha (1.60%), Bhalwal (1.47%) and Sillanwali (1.24%) (Kausar *et al*. 2016). From the results of our study, it is evident that there is positive correlation between soil organic matter and nematode population in Shahpur, where there is high level of organic matter contents. While in Bhalwal another reason of high infestation of nematode might be due to old citrus plantation. Similarly, temperature also influence nematode development and increase in population was observed from 20 to 3°C while optimum temperature for

maximum development was observed 25°C. As the temperature of Multan and Mianwali remains high as compared to other locations and nematode population was also found lower at these locations which shows that high temperature has negative impact on nematode development. Similarly, soils of Sargodha and Faisalabad are sandy loam and clay loam which retain moisture contents for long period of time and allow the movement and reproduction of nematodes. While in Multan the soils are sandy and become dry in short time and are not favorable for nematode development as compared to the soils of District Sargodha.

Conclusion

It is concluded that citrus decline is present throughout the major citrus growing areas of province Punjab, which is an alarming situation. However, frequency of different fungal pathogens and citrus nematode (*T. semipenetrans*) varies at different regions. Nematode populations were observed maximum from Shahpur region which contain saline soils. The results of current study revealed that the distribution of different pathogens associated with declining citrus plants from various regions having distinct climatic and soil characteristics which are helpful for selecting proper management practices at each location. The present survey indicated the actual picture of pathogens associated with citrus decline under changing soil and environmental conditions.

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Author Contributions

WAK performed the research, SAK supervised the research, AM, WA, ARK gave the concept and performed the analysis of results, WAK and AM wrote the manuscript, GMQD and TM performed the formal analysis and statistical analysis of results, All authors have critically reviewed the manuscript.

Conflicts of Interest

All authors declare no conflicts of interest

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable in this paper

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