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Associations among fungi, bacteria, and phytoplasma in trees suffering citrus decline in Punjab

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A survey was conducted in major citrus growing areas of Punjab during 2006-07 to investigate the association among different pathogens, which can cause citrus decline. The random sampling was carried out during survey to observe the fungal, bacterial and phytoplasmal symptoms on citrus trees. *Fusarium* spp incidence was recorded very high as 100% in Jhang and Faisalabad, while in Sargodha was 25%. *Phytophthora* and *Diplodia* spp was only found in Sargodha with varying severities as 5% and 8.33% respectively. Where as *Nattrassia* spp was found 17.24% in Sahiwal but no incidence was observed in Faisalabad, Jhnag and Kasur. *Xanthomonas compestris* pv *citri* was recorded 50% in Sahiwal where as in Faisalabad, Toba Tek Singh and Kasur was found free of this bacteria. Phytoplasmal disease of citrus (*Spiroplsma citri*) were found to be 27.58% in Sahiwal while it was not encountered in Jhang district. The current study deals with the investigation and association among fungi, bacteria and phytoplasma in trees suffering citrus decline in Punjab.

Keywords: *Phytophthora* and *Diplodia* spp

Introduction

Citrus is most valuable fruit crop grown all over the world including Pakistan. It has commercial importance on world level and is cultivated more than 125 countries within 35° latitude north or south of the equator. (Duncan and Cohn, 1990). Citrus fruit has importance because of high nutritional value and rich source of vitamin C in combination with sugar, amino acids, organic acids and minerals like calcium and magnesium in abundance. (Niaz *et al*., 2004).

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Citrus occupies top position in the fruit crops grown in Pakistan and ranks second in the world after grapes on the basis of production. The total area under citrus cultivation in Pakistan was 183. 8 thousand hectares with a production of 1943.7 thousand tones during 2004-05. Citrus fruit contributes 34.9% of all fruit crops grown in Pakistan. The area under citrus cultivation in province of Punjab 94.6% of the total citrus area cultivated in the country followed by Sindh, NWFP and Baluchistan cultivating 2.2%. 2.3% and 0.7% respectively, where as citrus fruit production in Punjab 96.3% of the total produce followed by 1.4%, 1.8%, 0.3% in Sindh, NWFP and Baluchistan respectively (Anonymous, 2005).

With the increase in population, the demand for citrus fruit is increasing all over the world. Climatic conditions and soils of Pakistan particularly of the province of Punjab are most suitable for growing citrus.

Citrus production is facing problems like biotic and abiotic stresses. Among abiotic stress there are two categories which are related to soil i.e. salinity, nutrient toxicity/ deficiency, compaction/ hardpan in subsurface and other is irrigation problem including water logging and water stress. Where as biotic factor concern there are four major categories that are cultural (pruning/ shading, excessive bearing, root stock-scion incompatibility and union creasing), physiological (hormonal imbalance, alteration “in” “on” and “off” year cycle), entomological (blackfly, psylla, bark eating caterpillar, leaf minor, aphids, mealy bug and mites) and pathological (fungal, bacterial, viral and phytoplasmal diseases). (Srivastava and Shyam Singh, 2009). Citrus production is vulnerable to numerous constraint and citrus decline has been recognized a serious problem around the world. Martin and Joseph (1946) reported that soil fungi were responsible to certain extent for citrus decline in United States.

Citrus decline was reported first time in Brazil during 1999 and is becoming a serious threat to citrus cultivation. (Renato *et al*. 2003)

The major diseases of citrus in Pakistan, with emphasis on citrus greening disease (unknown causal agent), citrus canker (*Xanthomonas campestris pv. citri* [X. *axonopodis pv. citri*]), citrus wither tip (*Colletotrichum gloeosporioides* [*Glomerella cingulata*]), citrus slow decline (*Tylenchulus semipenetrans*), and citrus quick decline (*Fusarium solani*). The symptoms of these diseases as well as their incidence and severity in citrus plants in Pakistan are described (Hafeez, 1985).

Citrus production losses due to *Phytophthora* spp are damping off in seed bed, root and crown rot in the nurseries, foot rot and gummosis, brown, and fibrous root rot in the groves are very common. Foot rot results from infection of bark near ground level producing lesions on the trunk or crown roots that can girdle and kill the tree. In bearing groves fibrous root rot damage causes tree

decline and yield losses. Most important species are *Phytophthora nicotianae* and *P. citrophthora*. (J.H Grahim and L.W. Timmer, 2011). Where as other fungi most important species are Fusarium oxysporium and Fusarium solani, (Faisalabad symposium), *Natrassia magnifera* was also reported causing decline in shade trees (Jamali and Banihashemi, 2010).

For an assessment of the factor responsible for the decline in citrus production, in citrus growing areas of Punjab comprising of Sargodha, Sahiwal, Toba Tek Singh, Faisalabad, Jhang and Kasur (Pattoki) were surveyed during the 2006-07. During the survey observations were made on , age , general condition and health of the orchards, type of soil, intercropping, disease symptoms and type of citrus fruit being grown. It has been observed during the survey that Citrus orchards in different districts have a number of citrus varieties being grown in them. Incidence of fungal, bacterial and phytoplasmal pathogen was assessed visually by identification of typical citrus canker symptoms on leaves and fruit of citrus.

Materials and methods

Declining trees show variety of symptoms including gummosis, foot rot, canker and greening, slow and quick decline. Random sampling was done on the basis of symptoms on citrus trees. The diseased samples were properly labeled, handled and brought in to laboratories of Crop Diseases Research Program (CDRP), National Agricultural Research Center, Islamabad, for further examination.

Isolation of fungi

Diseased leaves, stem parts and roots were cut into small pieces and surface sterilized with 1% sodium hypochloride solution for one minute and then rinsing in sterilized distilled water. The samples were placed on sterile filter paper to absorb excessive chlorox. The samples were thereafter shifted on PDA plates and incubated at 22-25 ºC for seven days. For detection and isolation of *Phytophthora* Rye A agar medium were used (60gm rye seed were soaked in distilled for 24hrs, take supernatant and put in refrigerator at 4°C, add distilled water and placed in hot water bath at 60C for 2hr and then blended for 2 minutes, filtered through muslin cloth and mix it with already taken supernatant, combine with 15g agar and 20g sucrose. The volume was then adjusted to 1 liter and autoclaved at 120°C, 15 psi for 15 minutes).The Rye A agar petri plates were incubated at 20°C and 80% relative humidity. The growth was regularly examined under stereomicroscope.

Identification of fungi

For the identification of fungi slides were prepared using lactophenol solution or cotton blue and observed under the microscope at 10x and 40x. Fungi were identified as previously described. (Gilman, 1945; Barnett, 1960; and Domsch *et al*., 1980).

Fungi were isolated and purified by picking up pieces of mycelium and transferring on the pure cultures on PDA and Rye A Agar medium slants and stored in a refrigerator to be used in future for pathogenicity tests and screening of root stocks of citrus.

Isolation of bacteria

Diseased leaf pieces (2x7 mm) were surface sterilized with 1% Clorox and transferred to Petri plates containing Yeast-Extract-Caleium-Carbonate (YDC) agar medium and incubated at 25 -27 ^{o}C for 72 hrs. (Wilson *et al*. 1967).

Isolation and identification of spiroplasma

Spiroplasma causing symptoms of stunted growth, dense foliage, yellowing and varied chlorotic patterns on leaves in citrus tree was confirmed by the Phytoplasmal DNA Amplification and Polymerase Chain Reaction using following procedure.

Phytoplasmal DNAextraction

0.1 gram of leaf sample was ground in 0.5 ml hot (65^{o}C) Cetyl trimethyl ammonium bromide (CTAB) buffer and incubated at 65^{o}C for 30 minutes. Equal volume of chloroform and isoamyl alcohol (24:1) was added and suspension centrifuged at 4000rpm for 10 minutes. Aqueous (upper) layer as removed to a clean tube. Two third volume of cold isopropanol and 1/10 volume of 3M sodium acetate was added to the aqueous layer and left over night at -20^{o}C. Next day the mixture was spun at 10,000 rpm for 30 minutes and supernatant poured off. Pellet was dried in vacuum desiccators. Pellet was resuspended in 0.5 µl TE buffer and transferred to microfuge tube (Daire *et al*., 1997).

DNA Amplification

Phytoplasmal DNA was amplified by the polymerase chain reaction (PCR) using the universal primer pairs derived from highly conserved ribosomal sequences and priming the 5' end of the 16s rRNA gene respectively, amplifying a 500 BP fragment, PCR and RFLP analysis performed (Marcone *et al*., 2000).

Sequence of the primers

Forward Primer: 5'-ACGAAAGCGTGGGGAGCAAA-3'
Reverse Primer: 5'-GAAGTCGAGTTGCAGACTTC-3'

Polymerase chain reaction

Extracted DNA of citrus plants with typical symptoms of phytoplasma was taken. The 50µl of reaction mixture contained 3µl of extracted DNA, 1µl of each primer, 8µl of dNTPs, 0.5µl of Taq polymerase, 5µl of 10x PCR buffer, 3ul of 25mM $MgCl_2$ and 29.5µl of (double distilled deionized) ddH_2O were prepared. The Gene Amp PCR system 2400 (Perkin Elmer) were used. During first cycle denaturation was carried out at 94°C for 5 minutes, annealing at 50 °C for 1 minute and extension was conducted at 72°C for 3 minutes. After that 35 cycles were conducted at 94°C denaturation temperature for 1 minute, 52°C annealing for 1 minute and 72°C extension for 1 minute. Post PCR extension was carried out at 72°C for 10 minutes and finally was stored at 4^oC.

RFLP analysis of PCR products

For identification of phytoplasmas, bands of the appropriate size (0.5 kb), amplified using PCR, were digested with restriction enzymes AluI, and according to instructions provided by the manufacturer, at 37°C for two hours. The digested products were then separated by electrophoresis on a 1% electrophoretic gel and on 5%polyacrylamide gel, stained with ethidium bromide.

Gel electrophoresis

Electrophoresis of the amplified PCR products was carried on a 0.8% horizontal agarose gel in Tris borate EDTA (TBE) buffer at 100-200 volt and

current of 50 mA for one and a half hours. The gel was visualized under UV transilluminator after staining with ethidium bromide (EtBr).

Results

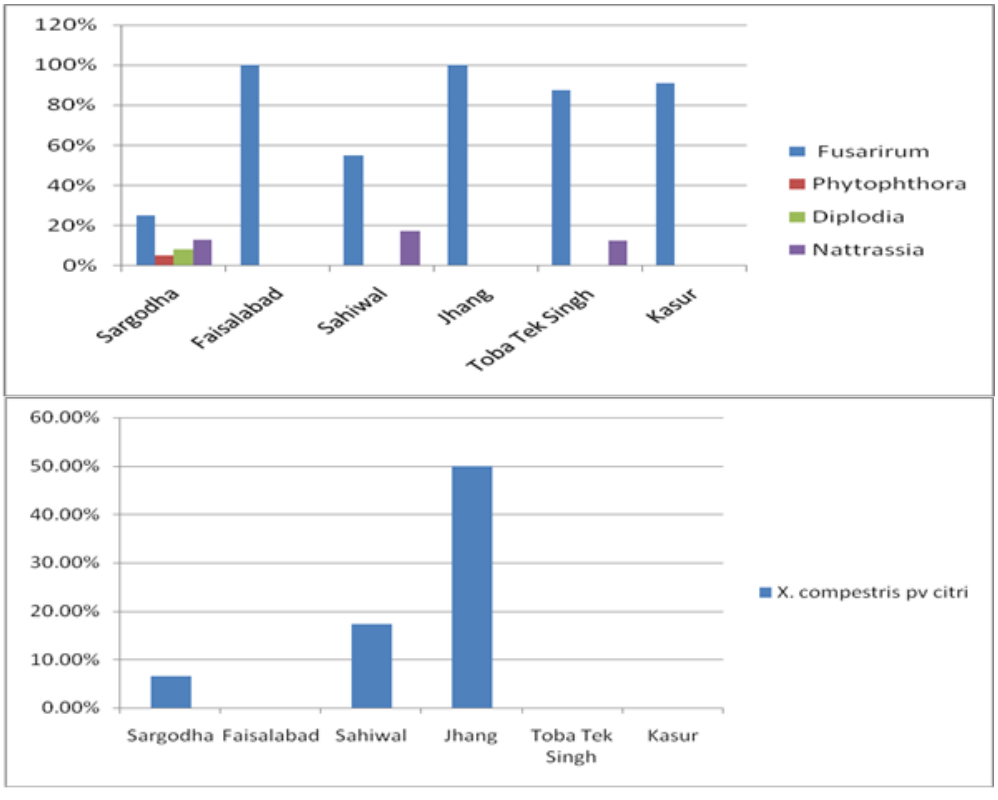
The present study showed that pathogenic fungi which were commonly found in citrus growing areas of Punjab is *Fusarium* spp, *(Fusarium oxysporium* and *Fusarium solani), Phytophthora spp (Phytophthora citricola and bataticola), Diplodia* and *Nattrassia spp* with varying level of frequencies. It was observed that *Fusarium* spp was present in all citrus growing areas with very high incidence 100% in Faisalabd and Jhang to low as 25% in Sargodha district. Where as *Phytophthora* and *Diplodia* spp was only found in Sargodha with incidence 5% and 8.33%, respectively. The incidence of *Nattrassia* fungus was recorded not very high which was 17.24%, 13% and 12.5% in Sahiwal, Sargodha and Toba Tek Singh while it was not present in Faisalabad, Jhang and Kasur. The presence of causal organism of citrus canker, *Xanthomonas compestris* pv *citri* was observed as high as 50% in Jhang and 6.6% in Sargodha, but it was not recorded in Faisalbad, Toba Tek Singh and Kasur. In district Jhnag, Kinnow is widely grown and is most affected by citrus canker. The *Spiroplasma citri* was found high as 27.58%, 25%, 15.62% Sargodha and Toba Tek Singh while it was not present in Jhang district. (Table 1).

Association among these microorganisms with respect to locations

Only Fusarium spp and Spiroplasm citri ware observed in Faisalabad and Kasur, there was no other microorganism was found in these locations. *Phytophthora spp* and *DiplodiaI* spp was not present in any location except Sargodha. In Sargodha all pathogenic fungi, bacteria and phytoplasma are present, while in Faisalabd, Sahiwal, Toba Tek Singh and Kasur pathogenic fungi and phytoplasma are observed except in Jhnag, there is no apparent symptoms of phytoplasma were found, but severely affected by citrus canker. In general all citrus growing areas in Punjab were found affected by variety of microorganisms including fungi, bacteria, and phytoplasma, among all citrus growing areas Sargodha was found most affected district by all these microorganisms in Punjab, where as least one was Kasur.

Table 1. Incidence of Fungi, Bacteria and Phytoplasma in different citrus growing areas

Pathogen	Location with sample infested/ total no sample along with incidence %											
	Sargodha		Faisalabad		Sahiwal		Jhang		Toba Tek Singh		Kasur	
Fusarium spp	15/60	25%	16/16	100%	16/29	55.17	04/04	100 %	28/32	87.5%	10/11	91%
Phytophthora Spp	03/60	5%	00/16	0%	11/29	0%	00/04	0%	00/32	0%	00/11	0%
Diplodia spp	05/60	8.33%	00/16	0%	00/29	0%	00/04	0%	00/32	0%	00/11	0%
Nattrassia spp	08/60	13%	00/16	0%	05/29	17.24 %	00/04	0%	04/32	12.5%	00/11	0%
Xanthomonas compestris pv *citri*	04/60	6.6%	00/16	0%	05/29	17.24 %	02/04	50%	00/32	0%	00/11	0%
Spiroplasma citri	15/60	25%	02/16	12.5%	08/29	27.58 %	00/04	0%	05/32	15.62 %	01/11	9%



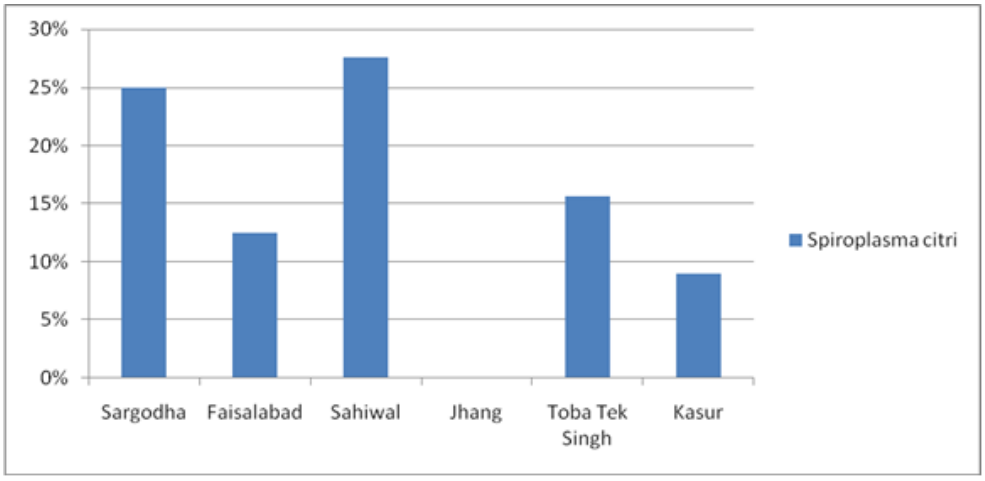


Fig. 1. Incidence of fungal, bacterial and phytoplasma in major citrus growing areas of Punjab

Table 2. Varieties of citrus grown at different locations of citrus growing areas

S.no	Disricts	No of locations for sampling	Varieties
1	SARGODHA	60	Feutrlls, Grape Friut, Kinnow , Malta, Musambi.
2	FAISALABAD	16	Grapefruit, Lemon, Kinnow (China Sweet) Malta, Musambi, Sweet Orange
3	SAHIWAL	29	Feutrlls, GrapeFriut, Kinnow, Lemon Malta, Mitha, Mussambi
4	JHANG	04	Kinnow.
5	TOBA TEK SINGH	32	Feutrlls Kinnow, Musambi, Orange.
6	KASUR	11	Grapefruit, Kinnow, Lemon, Musambi.

Disscussion

Citrus decline in major citrus growing areas of Punjab is being investigated through a multidisciplinary approach. Percentage of declining trees is range from 0-20% with severity ranging from slightly declining trees to severely declined trees. Only a few trees were observed that had suddenly declined due to quick decline. Symptoms that are apparent range from deficiency symptoms to fungal, bacterial, spiroplasmal, viral and nematode diseases and disorders.

From results it is also clear that among fungal pathogen *Phytophthora* and *Diplodia* spp are not citrus decline causing pathogens. As *Phytophthora* and

Fusarium are soil borne in nature and may pose a serious threat to the future cultivation of citrus orchards in Punjab.

However, we could not find any strong relation of these microorganisms with specific varieties except Kinnow, which was effected seriously by all fungal, bacterial and phytoplasmal pathogens and is most important citrus cultivar grown in Punjab. Sahi *et al.* (2007) reported that Kinnow variety is more affected by citrus canker disease.

Khanzada *et al* (2009) reported that a nematode, *Tylechulus semipenetarns*, causing slow decline of citrus, were also present in major citrus growing orchards in Punjab. Citrus Tristeza Virus was also observed in citrus declining trees in citrus orchard at National Agricultural Research Centre, Islamabad, Pakistan. The presence of this virus was confirmed by using serological techniques, Enyzme Linked Immunosorbant Assay (ELISA). (Rashad Ali, unpublished data). Presumably, these pathogens alone and/ or in a combination are causing significant losses in major citrus growing areas of Punjab.

There are number of problems associated with the management of the citrus orchards in Punjab, ranging from improper fertilization to shortage of water and inappropriate irrigation, inter cropping, pruning and non judicial use of pesticides which has resulted in a complex situation that has ultimately led to the development of slow to quick decline of citrus trees.

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