

INCIDENCE OF CITRUS GREENING DISEASE IN PAKISTAN

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ABSTRACT

Citrus Greening Disease (CGD) is one of the most important and severe diseases of citrus in Punjab and NWFP. Surveys conducted during 1997 in these provinces revealed the presence of citrus greening disease and its psyllid vector. Typical symptoms (yellow veins) of citrus greening caused severe leaf drop and trunk dieback with death of some trees. Leaf mottling was the prominent characteristic for field diagnosis of this malady. Variability in the disease severity and incidence was observed in citrus cultivars at different locations. Diagnostic tests using light microscope of thin sections stained with phenolic thionin and orange G combination elucidated abnormality in infected phloem tissues. Presence of fastidious prokaryotes, Bacteria Like Organism (BLO) was observed in infected tissues.

Keywords: Citrus greening disease, fastidious prokaryotes, Bacteria like organism, differential staining.

INTRODUCTION

Citrus greening disease (CGD) is one of the most severe diseases of citrus. It has a large geographic distribution. Citrus greening disease is a major cause of production and tree losses in Asia and Africa, two of the main citrus growing areas of the world (Graca, 1991 and Jagoueix *et al.*, 1996).

Plants affected with citrus greening disease (CGD) are generally stunted, have sparse yellow foliage, and show twig dieback (Catara *et al.*, 1988; Khan, 1989). At early stages of infection symptoms are seen only on one part of the canopy, hence the name of the disease yellow shoot (in China) (Su and Chen, 1990). The most characteristic symptom is leaf mottle (normal-green patches on a pale green background). The symptom is conspicuous in sweet orange than mandarin leaves. Leaf mottling is the best foliar symptom for the diagnosis of greening. Fruits on severely affected trees are small, lopsided and poorly colored, remaining dull green, hence the name greening given to the disease. Occasionally, color development on affected fruits starts at the peduncular end rather than at the stylar end as it is the case in normal fruits. This symptom is referred to as color inversion or "red nose." Variable numbers of aborted seeds are present in fruits of affected sweet orange and mandarin tree. The etiology of greening disease of citrus has been remained the subject of a protracted debate. Initial reports suggested a virus as the causative agent (Hoffmeyer and Oberholzer, 1948). Mycoplasma organisms (Lafleche and Bone, 1970) were next thought. Hull, (1972) claimed it as a

bacterium and Garnier *et al.*, (1984) proved it a gram negative bacterium. Greening is caused by an uncultured phloem-restricted bacterium, which was also characterized by Jagoueix *et al.*, (1996). According to these authors liberobacters from Asia and Africa belong to two different candidatus species: *Liberobacter asiaticum* and *L. africanum*. The purpose of this article was to record the incidence of citrus greening disease in Punjab and NWFP and its confirmation through differential staining technique.

MATERIALS AND METHODS

Two surveys of citrus orchards were conducted in Punjab (Okara, Sahiwal, Samundari, Faisalabad, Cheniot, Jhang, Sargodha, Mandi Bahuddin, and NWFP (Peshawar) in February-March and March-April, 1997, respectively. Five plants from each orchard and 1-3 orchards from each locality were checked. Citrus greening incidence was recorded as orchards infected/total orchards checked. Leaves showing mottle were collected during survey. Leaves from trees without greening symptoms were also sampled, some from normal symptom-less tree, others showed zinc deficiency symptoms. The disease rating was done according to the following scale:

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|---|--|
| 0 | No symptoms |
| 1 | Greening symptoms on upto 25% of the twig and leaves |
| 2 | 26-50% canopy symptoms |
| 3 | 51-75% canopy symptoms |
| 4 | More than 75% showing dieback |

Trees in stages 1 and 2 were considered slightly damaged, those rated 3 were moderately damaged and those in stage 4 were severely damaged.

Isolations: The leaf pieces were dipped into 95% ethanol for 3 minutes. The leaf samples from individual trees were kept in plastic bags at 4 °C, no longer than 5 days, before use. Leaves were washed and rubbed under tap water, and wiped dry with filter paper. The leaf midribs with about 1 mm of the leaf blade on each side were removed with a scalpel and then triturated in pestle and mortar in 0.01 M phosphate buffer solution (PBS). The pieces were then immersed in a 1.0% Sodium hypochlorite solution for 2-5 min and rinsed 2-3 times with sterile water to remove any residual Sodium hypochlorite. The infected leaf midrib ground in 1-5 ml of 0.01 M PBS. Titration of infected parts using a mortar and pestle was an effective means of extracting fastidious bacteria. The extract was smeared on nutrient agar or other bacteriological media. Several ten-fold dilution series of the extracts were prepared and smeared on nutrient agar, Yeast extract dextrose CaCO₃ Agar (YDCA), Peptone Sucrose Agar (PSA), Malt Extract Agar (MEA), and Potato Dextrose Agar (PDA). The Gram staining was also done for this bacterium.

Electron Microscopy: Sweet orange (*Citrus sinensis*) leaves showing yellow veins (typical mottling) from trees with dieback and fruits showing small size, poor color were collected for this study. They were kept in plastic bags. Leaf midrib and veins showing symptoms of greening were chopped into 1-2 mm pieces then triturated in pestle and mortar in 0.01 M PBS.

The copper grids coated with carbon were loaded with samples after three washing with sterile distilled water (SDW) and stained with 2% of uranyl acetate washed thrice with SDW and desiccated for one hour, then observed under scanning electron microscope.

Differential staining: Infected leaves midribs and veins were fixed in FAA (Formalin acid alcohol) solution, (formalin 13 cc, glacial acetic acid 5cc and 50% ethyl alcohol 200 cc) and the following procedure was followed:

Dehydration and embedding

1. 70% Ethanol (or 50% ethanol depending upon Ethanol concentration).
2. 85% Ethanol 1 hour

3. Ethanol + Butanol Mixture 1 1 hour
4. Ethanol + Butanol Mixture 2 1 hour
5. Ethanol + Butanol Mixtures 3 1 hour
6. Butanol 1 hour
7. Butanol Approx. 12 hour
8. Butanol + Paraplast Overnight (in an oven)
9. Paraplast only. At least a few days (paraplast should be replaced by pure paraplast).

Preparation of Ethanol-Butanol mixture:

Total volume 500 ml, 80% Ethanol 325 ml (Ethanol 260 ml + H₂O 65 ml) = Butanol 175 ml). 90% Ethanol 225 ml (Ethanol 202.5 + H₂O 22.5 ml) + Butanol 275 ml). 100% Ethanol 125 ml + Butanol 125 ml (Personel communication with Phytobacteriologist, Dr. Kaku, NIAR Japan).

Staining

1. Dip the slide in Xylene 20 min
2. Dip the slide in Xylene 15 min
3. Dip the slide in Xylene + Ethanol (99%) 15 min
4. Dip the slide in Ethanol 100% 15 min
5. Dip the slide in Ethanol 85% 5 min
6. Dip the slide in Ethanol 70% 5 min
7. Dip the slide in Ethanol 50% 5 min
8. Dip the slide in Ethanol phenol + thionin (100 ml or 5% phenol + 0.1 g thionin) 1 hr
9. Rinse in H₂O
10. Dip a few times Ethanol 50%
11. Dip a few times Ethanol 70%
12. Dip a few times Ethanol 85%
13. Dip a few times Ethanol 100%
14. Counter staining with orange G (saturated) 1 min
15. Rinse in 100% Ethanol
16. Rinse in 100% Ethanol
17. Rinse in Ethanol + Xylene mixture (1 = 1)
18. Rinse in Xylene
19. Rinse in Xylene

RESULTS

Prevalence of Citrus Greening Disease : Of the 183 locations visited in 10 geographical areas 68% had citrus greening disease (CGD) symptoms (Table 1). Sargodha area showed 62% CGD, while the Okara

and Sahiwal 20-27%. The maximum number of locations 90% having CGD were observed in Peshawar areas while minimum number of locations had CGD in Chinot Area (16%).

Table 1. Prevalence of citrus greening at different locations in Punjab and NWFP.

Area	Total Locations Surveyed	Location showing Number	CGD
Okara	5	1	20
Sahiwal	11	3	27
Samundari	17	9	52
Faisalabad	26	15	57
Cheniot	6	1	16
Jhang	36	19	52
Sargodha	32	20	62
Mandi Bahauddin	9	6	66
Peshawar	41	37	90
	183	126	68

Orchard wise prevalence of CGD in Punjab and NWFP is given in Table 2. In orchards at Faisalabad, Sargodha and Mandi Bahauddin showed 60-62% prevalence of CGD, while at Peshawar showed 86%. It was observed that 57% orchards in Punjab and NWFP had prevalence of CGD.

Table 2. Orchard wise prevalence of citrus greening in Punjab and NWFP.

Area	Total Orchard Surveyed	Orchard showing Number	CGD
Okara	10	2	20
Sahiwal	15	3	20
Samundari	18	9	50
Faisalabad	30	18	60
Cheniot	6	1	16
Jhang	37	20	54
Sargodha	35	22	62
Mandi Bahauddin	10	6	60
Peshawar	43	37	86
Total	204	118	57

Incidence and Severity of Citrus Greening Disease:

Area wise incidence and severity is given in Table 3. The highest incidence and severity of CGD was observed in Peshawar areas (4) where as Okara and Sahiwal, Chinot and Jhang areas showed 1, while Faisalabad and Sargodha 2-3 on rating scale (0-4).

Table 3. Incidence and severity of citrus greening disease in Punjab and NWFP.

Area	Incidence (%)		Severity (Scale 0-4)
	Range	Mean	
Okara	0-4	1	0-1
Sahiwal	0-3.3	0.5	0-1
Samundari	0-5	2.3	0-2
Faisalabad	0-6.6	3.4	0-2
Cheniot	0-6	2.5	0-1
Jhang	0-11	3.5	0-1
Sargodha	0-14	4.5	0-3
Mandi Bahauddin	0-8	3.1	0-2
Peshawar	0-17	5.3	0-4

Cultivar wise incidence and severity is shown in table 4. The highest incidence (90% of CGD was observed in Malta with severity 4, while the lowest in Mitha, and lemon having the mean incidence 2-15% with severity 0-1.

Table 4. Susceptibility of different citrus fruit types to citrus greening disease.

Types	Incidence (%)		Severity (Scale 0-4)
	Range	Mean	
Kinow	0-50	22	0-1
Malta	0-90	40	0-4
Musambi	0-70	25	0-3
Grape fruit	0-70	15	0-2
Mitha	0-25	10	0-1
Lemon	0-10	2	0-1

Isolation of Citrus Greening Disease Organism:

Leaves with typical mottling symptoms were used for isolation of the bacterium. No growth was observed on PDA, PSA, Malt extract and Yeast Dextrose Calcium Carbonate Agar (YDCA). Very small, round and white colonies were observed on nutrient agar. The isolated organism gave gram-negative reaction. But completion of Koch's postulates could not be established with this bacterium.

Electron Microscopy: The girds prepared with typical sample showed very few round shaped bacteria of 100-150 nm diameter under scanning electron microscope.

Differential Staining: Thin section prepared with microtomy and stained with phenolic thionin and orange G combination elucidated abnormality in

infected phloem tissue (shrinkage of tissue and cells filled with BLO) observed under the light microscope.

DISCUSSION

The incidence of citrus greening disease a psyllid borne bacterial malady is becoming very high in citrus orchard in Punjab and NWFP. It has a disappointing surprise to find much of the citrus variety collection of the experiment station at Peshawar showing greening. Since part of the fruits program is to propagate trees for distribution to farmers, the growing nursery stock was examined and many nursery trees were found infected. This was not surprising since buds were taken directly from the experiment station orchard. The greening vector *Diaphorina citri* (Aubert, 1991) was abundant in the variety orchard, in the nursery, and on ornamental sour orange hedges, which also showed typical greening symptoms for sour orange. The fact that some of the older citrus orchards both in the Rio Chinari and Swat valleys, the nursery stocks for which were reported to have been propagated at Peshawar, were healthy whereas younger ones were diseased indicating that greening in the experiment station planting at Peshawar may be of recent origin. The older orchards may have been planted with stock propagated prior to introduction of greening. Greening was undoubtedly introduced in citrus brought to Peshawar from other parts of Pakistan or perhaps from India where the disease is well established.

Variability in citrus cultivars response to citrus greening was noticed. Greening symptoms were more severe in sweet orange (*Citrus sinensis*) (Catara *et al.*, 1988). Particularly less severe on Kinnow and grapefruits, and least on Sweet lemon. In Taiwan (Miyakawa, 1980) India (Nariani *et al.*, 1973) the Philippines (Gonzales, 1989) sweet orange and mandarin were the most susceptible while lemon, sour orange and grape fruit were more tolerant. In India the rough lemon and sweet lime were tolerant. In Australia the greening like disease was most severe on grapefruit and sour orange (Fraser, 1978). Our results corroborate with respect to cultivar response to citrus greening with other researchers (Verma *et al.*, 1993; Swai *et al.*, 1992; Whittle, 1992; Aubert, 1990; Graca, 1991; Kapur *et al.*, 1988, Kapur and Sohi, 1989), in most of the cases small differences may be due to root stock and environment. In our survey in Punjab and NWFP, in most of the orchards interveinal and veinal chlorosis (yellowing) was observed on leaves. Interveinal yellowing was observed (2-3) from Lahore to Sahiwal on Kinnow, 91-2) from Sahiwal to Faisalabad, while veinal yellowing was 0-1 on these

routes. From Faisalabad to Mandi Bahuddin interveinal chlorosis was 1-2 and veinal chlorosis was 1-3 in Malta. On the route from Rawalpindi to Khanpur interveinal chlorosis was 1-2 and veinal chlorosis was 2-4 in red blood. Isolations were made by crushing the infected tissues from samples collected from Punjab and NWFP. Bacteria could not be isolated by clearing the debris with centrifugation. However, very few fastidious prokaryotes like BLO were observed under electron microscope. Gram staining results from infected leaves extracts gave the similar results like others (Garnier *et al.*, 1984; Garnier, 1984). Diagnostic tests using light microscopy of thin sections stained with phenolic thionin and orange G combination elucidated abnormality in infected phloem tissues. Presence of bacteria was observed in the infected tissue. Infected phloem tissue showed red patches with Safranin stain. A quicker laboratory test is always desirable, and since the causal bacterium has not been isolated on artificial media in the world, so advance molecular biological techniques (Vilchevanoux *et al.*, 1992; Jagoueix *et al.*, 1996 and Nakashima *et al.*, 1996) is the only solution for the detection of citrus greening organism. For accurate detection and characterization of greening BLO, polymerase Chain Reaction (PCR) will be used, provided necessary diagnostic chemicals could be arranged. Further studies for detection of the bacterium, transmission, management of the disease with biological agent will be done to develop integrated management package for citrus greening.

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