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OCCURRENCE AND DISTRIBUTION OF CITRUS TRISTEZA *CLOSTEROVIRUS* IN THE PUNJAB AND NWFP, PAKISTAN

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Abstract

Extensive surveys of the major citrus groves in Punjab (Faisalabad, Sahiwal, T.T. Singh, Sargodha and Bhalwal) and N.W.F.P (Haripur, Peshawar and Mardan) and ELISA tests revealed that CTV is prevalent and now is an emerging important disease. The citrus trees were found to be infected regardless of scion-rootstock combination. Maximum incidence of CTV was recorded at Bhalwal in the Punjab; 44.61% and 48.46% in 2006-07, and Mardan in N.W.F.P, 37.39% and 40.86% respectively. CTV infection was confirmed by DAS-ELISA in sweet orange, kinnow and grapefruit showing mean OD_{405nm} values of 0.60, 0.42 and 0.31 respectively. Similarly Mardan showed the highest infection with the mean ELISA values of 0.52, 0.34 and 0.20 in sweet orange, kinnow and grapefruit respectively. Among the varieties, sweet orange showed a significant increase of CTV in 2007 in Punjab as compared to other varieties. Twig portion of the kinnow in N. W. F. P was found to contain high concentration of CTV.

Introduction

Citrus is believed to have originated in Southeastern Asia, extending from Eastern Arabian to Philippines and from the Himalayas South to Indonesia or Australia (Davies & Albrigo, 1994). Citrus fruit contains appreciable amount of ascorbic acid, fair amount of vitamin A and B, as well as minerals, such as calcium, phosphorus and iron which add to its nutritive properties (Niaz *et al.,* 2004). Pakistan enjoys a well-known position among leading citrus producing countries of the world. Citrus is grown in Pakistan on an area of about 176.4,000 hectares with an annual production of 2,438 thousand tonnes (Anon., 2006).

Graft-transmissible virus and viral diseases are the major factors limiting citrus production throughout the world (Roistacher, 1998). Among these viruses, citrus tristeza *Closterovirus* (CTV) is one of the most destructive and widely distributed diseases in citrus growing areas of the world. A citrus tree once infected with the virus remains infected throughout its life and there is no way to free the plant from virus. CTV cause degeneration of cambium layers with the result that infected citrus trees start decline. CTV is a ssRNA *Closterovirus* with flexuous rod shaped particles approximately measuring 12 x 2,000 nm. CTV has many strains, which are complicated, vary greatly and range from mild isolates to severe stem pitting isolates (Su, 1998; Roberts *et al*., 2001). CTV infects all species, cultivars and hybrids of citrus, regardless of rootstock inducing mild or masking conditions to severe symptoms. It seriously infects sweet orange or other varieties when grafted on sour orange rootstock. It also induces disease in grapefruit, lemon, lime and calamondin.

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CTV is widely distributed to all citrus growing areas of the world. It has caused the death of millions of trees in Brazil, Spain and Argentina (Cambra *et al*., 2000; Mooney & Harty, 1992; Renfrow, 1996; Anon., 2004). In 1981, a total world loss of 50 million trees was estimated due to CTV and by 1991; CTV quick decline had destroyed 100 million trees in Argentina, Brazil, Spain, California, Venezuela and other areas (Bar-Joseph *et al*., 1981; Mooney *et al*., 1994). In Pakistan prevalence of CTV was confirmed through ELISA and electron microscopy, in Punjab and NWFP in citrus species (Catara *et al*., 1988, Anwar & Mirza, 1992). It can be a serious threat to citrus production in NWFP. CTV is naturally vectored by citrus brown aphid (*Toxoptera citricidus* Kirk.), which is fortunately not reported or found from Pakistan. However there are important aphid species such as *Toxoptera aurantii, Myzus persicae* and *Aphis gossypii*, which may be considered as putative vectors as no disease transmission have been made so far.

It was desirable to conduct extensive surveys of citrus orchards in Pakistan and collect data on the distribution of CTV in citrus cultivars, % disease incidence and confirm CTV infection through ELISA.

Materials and Methods

ELISA-based monitoring of CTV: These studies were carried out in the Institute of Horticultural Sciences and Department of Plant Pathology, University of Agriculture, Faisalabad over a protracted period from 2005-2008. Field surveys were, however, made at different places at appropriate time.

Selection of orchards: citrus groves were surveyed at five locations in the Punjab (Faisalabad, Sahiwal, Toba Tek Singh, Sargodha and Bhalwal) and three locations in N.W.F.P (Haripur, Mardan and Peshawar). The selection of orchards was based on four criteria i.e., i) they represent typically citrus growing area, ii) farmers follow traditional farming system, iii) have at least 200 living citrus trees excluding border trees and iv) are of 15-20 years of age. Trees were examined diagonally or in an 10x10m area at random (Hughes & Gottwald, 1998).

Collection of samples: Symptomology was the main criterion in the collection of citrus samples from the infected/suspected to be infected trees (Bos, 1970; Roistacher, 1991). Leaf, twig and bark tissue of sweet orange, kinnow and grapefruit was collected in sterilized polythene bags and stored in an ice box and then in a refrigerator at 4°C, until processed. Information was also collected on the source of nursery plants, age of trees and rootstock. Disease incidence was determined as:

$$\% \text{ Incidence of CTV} = \frac{\text{No. of ELISA positive plants}}{\text{No. of trees examined}} \times 100$$

ELISA test: All the samples were analysed by DAS-ELISA (Clark & Adams, 1977) using polyclonal antibodies (Accession No. 1102 CTV, Agdia, USA). Antibodies (1gG) of CTV were diluted in coating buffer at 1:200, and each well of ELISA plate was charged @ 200 µl/well. The coated plate was incubated at 4°C overnight and washed with PBST washing buffer 3 times at 5-minute intervals. Plants parts (leaves, twig bark and stem bark) were then chopped into small pieces and ground in the extraction buffer in a pestle and mortar using quartz sand. Extraction was filtered from the double layered

muslin cloth and samples were charged into the wells @ 200 µl. After incubation the plates at 4°C for overnight, it was washed as above, enzyme conjugate (IgG conjugated with Alkaline phosphatase) was diluted at 1:200 and 200 µl was added in each well. Substrate (p-nitrophenyl phosphate) was dissolved in substrate buffer @ 1 mg/ml, and 200 µl was added in each well. The plate was incubated at room temperature (25°C) for about one hour. The colour reaction was recorded visually as well as measured in microplate reader (ELx-Biotek 800) at 405 nm. The reaction was stopped by adding 50 µl of 3M Sodium hydroxide.

Statistical analysis: The data on CTV disease incidence and ELISA values were subjected to analysis of variance (ANOVA). Comparison of CTV incidence was done through Least Significant Difference (LSD) test at 5% level of probability (Steel & Torrie, 1997).

Results

Incidence of CTV in Punjab: The CTV incidence was calculated on the basis of ELISA positive samples collected from the 5 places viz., Faisalabad, Sahiwal, Toba Tek Singh, Sargodha and Bhalwal in Punjab. The highest incidence of 44.61% was found at Bhalwal followed by Sahiwal (28.46%) in 2006. Disease incidence at Faisalabad and Sargodha was at par with each other, 21.53% and 22.30%, respectively. The disease incidence in 2007 was relatively higher than the previous year where it was high (48.46%) at Bhalwal followed by Sahiwal (30%), Sargodha (24.61%) and Faisalabad (23.84%). The lowest incidence was recorded at Toba Tek Singh with 16.15% in 2006 and 16.92% in 2007 (Table 1).

Distribution of CTV in Punjab during 2006-2007: CTV infection was compared on the basis of ELISA values with respect to CTV infection and virus titre in plant parts during 2006-07. There was no significant difference in the virus titre in leaf and twig portions while virus titre was significantly lower in stem portion in 2007. ELISA reaction, showing the CTV distribution in both the years, was non-significant (Table 2).

Distribution of CTV in varieties at different places in Punjab: Highest CTV infection was recorded at Bhalwal in all citrus varieties. All the varieties i.e., Sweet orange, Kinnow and Grapefruit showed significantly higher incidence at Bhalwal with the mean incidence of 0.60, 0.42 and 0.31 respectively. The significantly lower incidence was found at T.T.Singh in case of sweet orange (0.34) and Kinnow (0.10), while CTV incidence in grapefruit was found significantly lower at Sargodha (Table 3). CTV infection in three varieties was not significantly different in 2006-2007. The virus titre was significantly different in the leaf, twig and stem portions of the sweet orange in both the years. Virus titre in leaf and twig was significantly lower in 2006 with the mean value of 0.32 and 0.32 respectively as compared to 2007, while virus titre was significantly higher in stem portion in 2006 as compared to 2007. There was no difference in the virus titre in the plant parts of the other two varieties like kinnow and grape fruit. Among the three varieties sweet orange showed the significant change in virus titre with subject to detection in all the plant parts. Virus titre in other two varieties was statistically at par with each other (Table 4).

Table 1. Incidence of CTV in Punjab during 2006-2007.

Places	2006		2007	
	No. of samples infected/examined	% Incidence	No. of samples infected/examined	% Incidence
Faisalabad	28/130	21.53	31/130	23.84
Sahiwal	37/130	28.46	39/130	30.00
T.T. Singh	21/130	16.15	22/130	16.92
Sargodha	29/130	22.30	32/130	24.61
Bhalwal	58/130	44.61	63/130	48.46

Table 2. Concentration of CTV in different plant parts during 2006-2007.

Year	Leaf	Twig	Stem	ELISA Reaction
2006	0.30 A	0.29A	0.31 A	0.27 A*
2007	0.31 A	0.30A	0.29 B	0.29 A
LSD	0.02	0.01	0.01	0.04

*****Mean values sharing similar letter in a column do not differ significantly at 5% level of probability as determined by LSD test.

Table 3. Distribution of CTV in varieties at different places as confirmed through ELISA (OD_{405nm}).

Places	Sweet orange	Kinnow	Grapefruit
Faisalabad	0.36 B*	0.21 BC	0.10 B
Sahiwal	0.47 AB	0.24 B	0.08 B
T.T. Singh	0.34 B	0.10 C	0.03 B
Sargodha	0.36 B	0.25 B	0.00 B
Bhalwal	0.60 A	0.42 A	0.31 A
LSD	0.13	0.11	0.10

*****Mean values sharing similar letter in a column do not differ significantly at 5% level of probability as determined by LSD test.

Table 4. Distribution of the CTV in different plant parts of three varieties during 2006-2007 as revealed by ELISA test.

Year	Sweet orange				Kinnow				Grapefruit			
	ELS**	Leaf	Twig	Stem	ELS	Leaf	Twig	Stem	ELS	Leaf	Twig	Stem
2006	0.40A*	0.32B	0.32B	0.37A	0.23A	0.30A	0.28A	0.28A	0.10A	0.25A	0.24A	0.25A
2007	0.44A	0.37A	0.33A	0.32B	0.25A	0.29A	0.30A	0.28A	0.11A	0.26A	0.25A	0.24A
LSD	0.08	0.03	0.02	0.03	0.07	0.03	0.03	0.02	0.06	0.02	0.02	0.02

*Mean values sharing similar letter in a column do not differ significantly at 5% level of probability as determined by LSD test.
**ELS = ELISA Reaction

Incidence of CTV in N.W.F.P during 2006-07: In N.W.F.P the highest disease incidence was found at Mardan with 37.39% and 40.86% during 2006-07 respectively. The lowest incidence was recorded at Peshawar with 23.47% in 2006 and 29.56% in 2007 (Table 5).

Distribution of CTV in N.W.F.P during 2006-2007: CTV infection based on ELISA reaction during 2006-2007 showed that there was no significant difference in two years. ELISA tests for the plant parts showed that virus titre was significantly different in the twig portion of the samples during 2006-07; it was higher in 2007 as compared to 2006. There was no significant difference in the virus titre in leaf and stem portions of the samples (Table 6).

Table 5. Incidence of CTV in N.W.F.P. during 2006-2007.

Places	2006		2007	
	No. of samples infected/examined	% Incidence	No. of samples infected/examined	% Incidence
Haripur	36/115	31.30	46/115	40.00
Mardan	43/115	37.39	47/115	40.86
Peshawar	27/115	23.47	34/115	29.56

Table 6. Reaction of different plant parts to CTV in ELISA test at 405 nm during 2006-2007 in NWFP.

Year	Leaf	Twig	Stem	ELISA reaction
2006	0.39 A	0.36 B	0.40 A	0.30 A*
2007	0.42 A	0.39 A	0.37 A	0.36 A
LSD	0.02	0.02	0.02	0.06

*****Mean values sharing similar letter in a column do not differ significantly at 5% level of probability as determined by LSD test.

Table 7. Distribution of CTV in varieties at different places in NWFP detected through ELISA.

Places	Sweet orange	Kinnow	Grapefruit
Haripur	0.50 A*	0.30 A	0.16 A
Mardan	0.52 A	0.34 A	0.20 A
Peshawar	0.35 B	0.26 A	0.10 A
LSD	0.13	0.18	0.19

*****Mean values sharing similar letter in a column do not differ significantly at 5% level of probability as determined by LSD test.

Table 8. Distribution of the CTV in different plant parts of three varieties during 2006-2007 as determined by ELISA.

Year	Sweet Orange				Kinnow				Grapefruit			
	ELS**	Leaf	Twig	Stem	ELS	Leaf	Twig	Stem	ELS	Leaf	Twig	Stem
2006	0.42 A*	0.45A	0.42A	0.46A	0.24A	0.36A	0.33B	0.36A	0.13A	0.33A	0.28A	0.30A
2007	0.49 A	0.48A	0.44A	0.44A	0.30A	0.38A	0.38A	0.33A	0.17A	0.37A	0.26A	0.27A
LSD	0.11	0.04	0.04	0.04	0.10	0.04	0.03	0.04	0.15	0.08	0.05	0.05

*Mean values sharing similar letter in a column do not differ significantly at 5% level of probability as determined by LSD test.
******ELS = ELISA Reaction

Distribution of CTV in varieties at different places in N.W.F.P: Distribution of CTV in different varieties at different places was found. The sweet orange was highly infected by CTV at Mardan and statistically at par with Haripur followed by Peshawar. Kinnow and grape fruit were infected by CTV at three places equally (Table 7). CTV incidence based on ELISA values was not significantly different among the three varieties during 2006-07. Virus titre in all the three plant parts like leaf, twig and stem of sweet orange and grapefruit was also compared with respect to years but found non-significant. Twig portion of the kinnow samples showed the significantly higher virus titre in 2007 as compared to 2006 (Table 8).

Discussion

Khan (1992) reported that in Pakistan more than 30 virus and virus like diseases of citrus are known to exist. Survey reports confirmed the presence of several virus and virus-like diseases in Pakistan including the serious threat by CTV and greening diseases. CTV was found to be common in citrus orchards and nurseries with average incidence of 27%. On grafting and mechanical inoculation symptoms of vein clearing and chlorosis in young leaves of *C. aurantium*, *C. limon* cv Eureka and *C. sinensis* were observed (Arif *et al.,* 2005). Cambra *et al*., (2000) conducted similar studies and discussed the incidence and epidemiology of the CTV in the Valencian community of Spain. Through the use of polyclonal antibodies (PCAB) in routine ELISA they reported the increase in incidence from 11% in 1989 to 53% in 1998. Our findings also confirm the previous work of Catara *et al.,* (1988) but they tested a limited number of samples, however it was confirmed through ELISA and electron microscopy where thread-like particles of CTV were observed under transmission electron microscope in the phloem of columella. Subsequently CTV was also confirmed through ELISA (Grimaldi & Catara, 1989). Later surveys for CTV and citrus greening disease in various districts of the Punjab ELISA tests and electron microscopic examination confirmed the presence of CTV in citrus cultivars e.g., Mosambi, blood red and pineapple sweet orange, out of which mosambi was the most affected (Catara *et al*., 1991).

Prevalence, incidence and disease severity index of any plant virus are important to determine the economic importance. Distribution and occurrence of CTV, based on ELISA showed that CTV is an important disease in Punjab and N.W.F.P. Incidence of CTV in both the provinces ranged between 16.15 to 48.46% and 23.47 to 40.86% in Punjab and N.W.F.P respectively, which can be alarming in this regard. Among the varieties sweet orange was severely affected by the CTV in both the provinces. The old plant plantation of the citrus in N.W.F.P could play a vital role in high incidence of CTV in this province. The samples collected from both places showed a similar reaction during the indexing through ELISA test therefore it can be concluded that the strain infecting the citrus trees in two provinces is same. Polyclonal antibodies used for the ELISA test could detect all the possible strains including severe to mild but they need to be differentiated. The results are in accordance with Anwar & Mirza (1992), who reported the presence of CTV in four districts of Punjab viz., Sahiwal, Sarogodha, Faisalabad and Sheikhupura. Infection varied from 7.14% to 18.18%. The highest infection (18.18%) was recorded in the Sahiwal district while the lowest (7.14%) was in Sheikhupura.

CTV could be detected in three main parts of trees i.e., leaves, twig and barks. During the studies it was found that different plant parts like leaves, twigs and stem portions of the samples showed the significant change during the two years. Anfoka *et al*., 2005 considered that the midribs and petioles were the best tissues for the detection through DAS-ELISA. Therefore, leaf portion was considered to be good sample for the ELISA test, but still variable amount of CTV can be due to uneven distribution of the virus in the different plant parts as well as the amount of virus titre in the plant parts. Viruses can be transferred within the plant due to two distinct processes: short distance and the long distance. Stem portion contained less titre as compared to upper portion. This was probably due to phloem-limited nature of the virus. Age of the tree, prevalent strain and the nature of the virus should be kept in mind during the diagnostic methods (Gafny *et al*., 1995). Variability in incidence of CTV at different places has also been

supported by the research conducted by the Shalintin *et al*., (1994) in Israel. They reported that the serological characterization of CTV that depends upon the CTV strains present in different areas and their reaction on the host plants. They also found that CTV strains were also not associated with antigenic variation of their coat protein. Further work is required to identify and differentiate strains of CTV prevalent in Pakistan through biological means. Spread of disease in the orchards may be attributed to the presence of some aphid vectors, which may be confirmed through disease transmission studies/virus-vector relationship.

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