Serological Reactivity in Citrus Tristeza Virus Strains in India

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ABSTRACT. In 1977 and 1983, cross protection experiments in Key lime were undertaken at Bangalore and Tirupati by preinoculating plants with mild CTV strains. In 1992 the status of various trees in these experiments was examined by ELISA, using polyclonal (PAb) and monoclonal (MAb) antibodies. All the tested trees were found to react uniformly with PAb and two MAbs 3DF1, which reacts with most CTV isolates, and MCA13, which commonly reacts only with severe CTV isolates. In a limited survey for CTV in several Indian states it was found that only seven out of the 721 field samples were CTV-infected and reacted with both MAbs. These observations indicate that the epitope recognised by MCA13 is very common in Indian isolates.

Index words: MAbs, cross protection, citrus tristeza virus, virus strains.

Citrus tristeza virus (CTV) has been reported from various states of India (1,4,9,13). Different strains of CTV were identified in India on the basis of either vector specificity or reaction in differential hosts and designated as mild or severe strains (6,2,5). Among the seven aphid species reported as vectors of CTV (6), *Toxoptera citricidus* has been found to be the most efficient vector. Balaraman and Ramakrishnan (2) identified mild and severe strains of CTV on the basis of host-pathogen interaction using a set of *Citrus* species. In a cross protection experiment they inoculated Key lime plants with *i*) a mild CTV strain, *ii*) a mild + a severe strain, *iii*) a severe strain alone, and *iv*) uninoculated healthy control and transplanted these plants in the field for observations at the Indian Institute of Horticultural Research at Bangalore. A similar experiment was also conducted at Tirupati by the Citrus Improvement Project using locally identified mild and a severe strains of CTV on the basis of reaction to differential hosts (11). More recently monoclonal antibodies (MAbs) have been developed for quick detection of CTV (10,14,15) and one of them seems to discriminate mild and severe strains of the virus in Florida (10). Since we have been receiving reports that the cross protection experiment at Bangalore failed after a few years in the field, whereas no such information was received from Tirupati experiment, it was decided to survey them and test individual trees for their pattern of reaction with a set of strain-discriminating MAbs. Similarly, field samples were tested from various states of India to assess CTV incidence and variability of strains. The results of these studies are reported here.

MATERIALS AND METHODS

Collection of samples. Ten fully expanded young leaves of new growth were sampled from individual trees of both the cross protection experiments at Bangalore and Tirupati. In addition. samples were analysed from citrus orchards/nurseries in Maharashtra, Karnataka, Andhra Pradesh, Punjab, Delhi, Haryana and Uttar Pradesh to assess the incidence of CTV and the presence of different strains. Fourteen isolates of CTV collected from different parts of the country are being maintained and used for strain differentiation at Delhi. These and eight more maintained at Pune which were collected from Maharashtra were included for serological characterization.

Antibodies. Polyclonal antisera (PAbs # 1052 specific to CTV, courtesy Dr. S. M. Garnsey, Florida, USA) and an antiserum to Indian CTV designated as CTV-P were used. IgG was purified using DEAE-Sephacel column chromatography and adjusted to a final con-

centration of 1.0 mg/ml (O.D. 280 = 1.4). MAb MCA 13 which discriminates certain severe CTV isolates (10) also was obtained from Florida. MAbs 3DF1 and 3CA5 were provided by Dr. M. Cambra, IVIA, Spain. These MAbs are known to recognise most CTV strains (14,15).

DAS-ELISA. Double antibody sandwich ELISA (3,7) was used to determine the presence of CTV. Test samples were prepared by homogenizing small pieces of midribs from leaves in 20 volume of phosphate buffered saline, pH 7.4, containing 0.5% Tween (PBST) using a tissuemizer. Dynatech ELISA plates were coated with 200 µl/well of 1 µg/ml #1052 PAb in carbonate buffer, pH 9.6, and incubated at 37 C for 3 hr. Plates were washed thrice in PBST and 200 µl of sample extract were loaded in each well. The antigen coated plates were incubated over night at 4-8 C, washed with PBST, and 200 µl/well of homologous IgG conjugated with alkaline phosphatase (AP) at a 1:4000 dilution in PBST containing 1/40 (v/v) of healthy plant extract. This extract was prepared by homogenizing 1 g of healthy Mosambi sweet orange leaf lamina in 4 ml of 0.05M Tris, pH 7.4, and clarifying at 5000 *g* for 10 min. After 3 hr at 37 C the plates were washed with PBST and developed with 200 µl/well of the substrate solution (0.6 mg/ml *p*- nitrophenyl phosphate in diethanolamine buffer, pH 9.8). The optical density (OD 405) was determined after 15 and 45 min in a Dynatech Mini ELISA reader (manual) adjusted to zero with the buffer control. Samples were considered positive for CTV when they gave ELISA values at least three times higher than the healthy control.

DAS-Indirect ELISA. This system was used (10) to determine the presence of mild and severe strain of CTV in plants of cross protection experiments at Bangalore and Tirupati and also in samples obtained from other locations. To conduct these experiments, the plates were coated with antigen samples added in two plates as described above. MAbs 3 DF1, 3 CA5 and MCA 13 diluted 1:10,000 in PBST were added to individual plates. After incubation and washing as for DAS-ELISA, 200 µl/well, of goat anti-mouse (GAM) IgG conjugated with AP was added at a 1:4000 dilution in PBST containing 1% normal rabbit serum. The plates were incubated for 3 hr at 37 C and developed as for DAS-ELISA.

SSEM. Serologically specific electron microscopy was performed to confirm the results of a few positive and negative samples evaluated by ELISA. The carbon coated grids were floated on CTV PAb IgG (1 µg/ml) for 30 min and then for 15 min in a drop of buffer chopped samples. Then the grids were washed with double distilled water and stained with 2% uranyl acetate and viewed in an electron microscope (8).

RESULTS

Results of ELISA tests on field samples are summarized in Table 1. All the isolates maintained in the glasshouse at Pune and at New Delhi reacted positively in DAS and in DAS-I ELISA using MAbs 3DFI and MCA13. Indexing of field trees revealed that only two out of 22 Mosambi samples from New Delhi, and five out of 25 Malta samples from Ludhiana were CTV positive, and all reacted in DAS-I ELISA with both 3DF1 and MCA13 MAbs. The remaining 714 samples tested did not react in any of the ELISA tests. It was remarkable that among the different cultivars tested CTV was present only in sweet orange trees.

Analysis of the trees of the cross protection experiment at Bangalore (Table 2) revealed that no matter if they were uninoculated controls or they had been inoculated with the mild strain, the severe strain, or both of them, all trees sampled reacted with the three MAbs and gave similar ELISA values. The uninoculated control from the glasshouse gave negative reaction and the mlid control T-30 (from Florida) did not react with MCA-13.

In the cross protection experiment at Tirupati (Table 3) the uninoculated

TABLE 1
CITRUS TRISTEZA VIRUS (CTV) INDEXING OF GLASSHOUSE AND FIELD SAMPLES USING ELISA WITH POLYCLONAL AND MONOCLONAL ANTIBODIES

		No. of samples reacting positively/ total no. of samples tested		
		DAS-ELISA with (PAb)	DAS-I-ELISA (with MAbs	
Location	Host		3DFI	MCA-13
Pune[z] (Maharashtra)	Kagzi lime	8/8	8/8	8/8
	Mosambi Swo[w]	4/4	4/4	4/4
New Delhi[z]	Kagzi lime	14/14	14/14	14/14
New Delhi[y]	Kinnow mandarin	0/65	0/65	0/65
	Mosambi Swo	2/22	2/22	2/22
	Kagzi Kalan (acid lime)	0/40	0/40	0/40
Ludhiana[y] (Punjab)	Kinnow	0/25	0/25	0/25
	Malta Swo	5/25	5/25	5/25
	Lime	0/17	0/17	0/17
Abohar[x] (Punjab)	Kinnow	0/180	0/180	0/180
Modipuram[x] (Uttar Pradesh)	Kagzi Kalan	0/197	0/197	0/197
Karnal (Haryana)	Kagzi Kalan	0/150	0/150	0/150

[z]glasshouse isolate,
[y]field trees on rough lemon rootstock
[x]nursery plants budded on rough lemon
[w]Swo = sweet orange.

control trees did not react with any of the MAbs even after ten years of exposure to natural infection in the field even though *T. citricidus* was found colonizing these trees. All the trees inoculated with the mild strain, the severe, or with both, reacted with MAbs 3DF1 and MCA13.

For further confirmation of the above results four ELISA positive and eight ELISA negative samples were screened by SSEM technique. CTV particles were trapped with PAbs from ELISA positive samples whereas no particle was trapped by ELISA negative samples.

TABLE 2
ELISA REACTIONS WITH MONOCLONAL ANTIBODIES OF LIME TREES IN A CROSS PROTECTION EXPERIMENT AT BANGALORE

Trees inoculated with:	Total trees indexed	DAS-I ELISA with MAbs[z]		
		3DF1	3CA5	MCA 13
Mild strain	6	0.78	0.90	1.14
Severe strain	6	0.92	1.03	1.19
Mild + Severe strains	6	0.80	1.06	1.16
Uninoculated control	6	0.52	0.78	1.07
Healthy control (glasshouse)	–	0.00	0.00	0.04
Buffer	–	0.00	0.00	0.00
Control T-36 (Severe, Florida)	–	1.13	1.30	1.26
Control T-30 (Mild, Florida)	–	1.26	1.17	0.00

[z]ELISA values are the average of two replications. Zero was adjusted with buffer.

TABLE 3
ELISA REACTION WITH TWO MONOCLONAL ANTIBODIES OF LIME TREES IN A CROSS PROTECTION EXPERIMENT AT TIRUPATI

Trees inoculated with:	No. of trees indexed	DAS-I ELISA with MAbs[z]	
		3DF1	MCA13
Mild strain	6	1.06	1.01
Severe strain	6	1.05	1.39
Mild + Severe strains	6	0.85	0.81
Uninoculated control	6	0.00	0.00
Healthy control (glasshouse)	–	0.00	0.00
Buffer	–	0.00	0.00
Control T-36 (Severe, Florida)	–	1.04	1.35
Control T-30 (Mild, Florida)	–	0.92	0.00

[z]ELISA values are the average of two replications. Zero was adjusted with buffer.

DISCUSSION

CTV infection was detected in all uninoculated conlrol trees of the cross protection experiment at Bangalore. This is an indication that CTV is spreading in this region. *T. citricidus* was found colonizing these trees. Samples collected from both mild or severe or challenge inoculated trees reacted with the three MAbs. Since MCA13 recognized CTV from all these samples but it did not react with the control T-30, it appears that mild strains used in this experiment could not be discriminated by MCA13 or that a severe strain, reacting with MCA13 would have infected preinoculated trees by aphid vector.

The results obtained from the cross protection experiment at Tirupati also revealed that the CTV strains used in this experiment could not be differentiated by MAbs 3DF1 and MCA13. The finding that uninoculated Key lime trees, remained free from CTV infection in spite of the presence of *T. citricidus*, might indicate low transmissibility of CTV strains in the area including those used in the cross protection experiment. It should be expected that the trees preinoculated with the mild strain were not superinoculated by aphids with the severe strain. This would support previous suggestion that some mild strain in India do react with MCA13 and, therefore, this MAb should not be used to distinguish mild and severe strains. Further studies on these strains and *T. citricidus* should help to establish the reason for the lack of spread of CTV at Tirupati.

Our survey showed the presence of CTV in Pune (Maharashtra), Ludhiana (Punjab) and Delhi. However, nursery plants indexed from Abohar (Punjab), Modipuram (U.P.) and Karnal (Haryana) did not show the presence of CTV infection. The efficient vector of CTV - *T. citricidus* is not present in the North Indian plains and CTV spread in this area is negligible. ther vectors, though present, are very inefficient, therefore CTV spread could be checked through eradication and use of certified budwood. Additional studies on biological properties and reaction with new MAbs would be necessary to determine the range of variability among the Indian CTV isolates. It will, therefore, be necessary to prepare more monoclonals to CTV with Indian isolates to confirm the strain variations in the country. It is also evident that in north India where *T. citricidus* is not present, CTV spread is mainly through propagation of contaminated budwood.

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