Use of Enzyme-Linked Immunosorbent and Dot-Immunobinding Assays To Evaluate Two Mild-Strain Cross Protection Experiments After Challenge with a Severe Citrus Tristeza Virus Isolate[1,2]

M. A. Rocha-Peña, R. F. Lee, T. A. Permar, R. K. Yokomi, and S. M. Garnsey

ABSTRACT. In two experiments citrus trees on sour orange rootstock were pre-inoculated with 14 mild citrus tristeza virus (CTV) isolates and were then graft or aphid challenged with a severe quick decline CTV isolate after the trees were established in the field. Double antibody sandwich ELISA (DAS-ELISA) with polyclonal antibodies was used to detect CTV infection, and DAS-indirect ELISA using MCA-13 monoclonal antibody was used to detect severe CTV isolates. All trees in the two experiments were surveyed in August and November, 1989. DAS-ELISA in the August survey showed all the pre-inoculated plants to be infected with CTV, and only one plant in the aphid challenged experiment was positive when assayed with DAS-indirect ELISA using the MCA-13 monoclonal antibody. In November, 1989, six additional plants were positive when assayed by DAS-indirect ELISA with MCA-13. Some originally healthy uninoculated control plants were found to be infected by DAS-ELISA but not by DAS-indirect ELISA, indicating that some natural spread of mild CTV isolates had occurred in both experiments. A dot-immunobinding assay was compared to DAS-ELISA and DAS-indirect ELISA and found to be more rapid and as sensitive in detection of CTV in crude preparations. The absence of reaction of the MCA-13 monoconal antibody in both DAS-indirect ELISA and dot-immunobinding assay on most of the plants previously challenged with the severe CTV isolate suggests that the mild CTV isolates may be providing cross protection against the severe challenge isolates.

Citrus tristeza virus (CTV) has been widespread in Florida for many years (5,15). However, until recently it had not caused major losses because much of the citrus acreage was propagated on CTV-tolerant rootstocks and mild CTV isolates were prevalent (2,12). After 1980 there was a significant increase (up to 31%) in the percentage of sour orange used as a rootstock for new plantings (C. O. Youtsey, personal communication). Sour orange as a rootstock induces high fruit quality, cold hardiness, and has tolerance to citrus blight, an endemic disease of unknown etiology (11). However, with increased plantings on sour orange, areas of CTV-induced quick decline and severe dwarfing have developed in some parts of Florida, particularly in the Southern Flatwoods where losses in some groves have exceeded 50% (2).

In 1985 and 1986, two field experiments were established to determine the feasibility of using mild CTV isolates naturally occurring in Florida to cross protect (12) against the quick decline syndrome on several susceptible scion/rootstock combinations. The trees in field experiments were challenged with a severe quick decline inducing isolate by aphid or graft inoculations. The objective of this research was to use the enzyme-linked immunosorbent (ELISA) and dot-immunobinding (DIBA) assays with polyclonal antisera and both polyspecific and strain specific monoclonal antibodies to determine the occurrence of severe CTV strains in those cross protection experiments after inoculation with the severe challenge isolate and to determine the incidence of CTV infection in the uninoculated control trees.

[1]Florida Agricultural Experiment Station Journal Series No. R-01442.

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MATERIALS AND METHODS

Cross protection experiments. Hamlin sweet orange and Red Blush grapefruit trees on sour orange rootstock previously pre-inoculated with 14 selected mild CTV isolates were transplanted to the field. All inoculated plants were verified by ELISA to be CTV infected before transplanting. The 14 mild CTV isolates tested are described in a companion paper (22). The T-66a challenge isolate is an aphid transmitted selection from a field selection from a tree undergoing quick decline in the Ft. Pierce area. Upon biological indexing (6), this isolate causes strong vein clearing, stunting and stem pitting in Mexican lime seedlings, severe decline on sweet-sour orange combinations, but no seedling yellows or stem pitting on grapefruit or sweet orange. One experiment of 288 plants with Hamlin scions, established in 1985, was inoculated with the T-66a severe CTV isolate by using *Aphis gossypii* 12 months after transplanting. The aphids were fed overnight on virus infected sweet orange donor plants and placed in cages (25 aphids/cage) following the procedure described by Yokomi, *et al.* (21). Four cages were placed on each tree. Twenty four hr later the cages were removed, aphid survival was recorded, and surviving aphids were killed with malathion.

A second experiment of 214 plants with Hamlin and Red Blush grapefruit scions, established in 1986, was graft challenged twice, with T-66a CTV infected *Citrus excelsa* tissue. In the fall of 1987, T-66a infected bark patches were inserted into a T-inverted section on the receptor trees and removed after six weeks. A second challenge was made in the same manner in the spring of 1988, with the bark patches remaining in the trees for seven weeks. Samples of infected *C. excelsa* donor tissue were graft inoculated to greenhouse grown plants to verify infectivity of inoculum.

Both experiments were arranged at a random block design and five replications per treatment were included.

Antisera used. Polyclonal antiserum #1053 specific for CTV was prepared previously in rabbits to whole, undegraded, and unfixed virus particles of CTV isolate T-26 (R. F. Lee, unpublished). Fraction G of immunoglobulins (IgG) from whole sera was purified by the Protein A-Sepharose affinity chromatography method (14) and adjusted at a final concentration of 1.0 mg/ml ($OD_{280} = 1.4$) (3). The 3DF1 mouse monoclonal antibody, which reacts with a broad spectrum of CTV isolates (19) was a gift of Dr. Pedro Moreno from Spain. The MCA-13 mouse monoclonal antibody is specific for severe CTV isolates and has been described previously (16).

Sample preparation. Fully expanded leaves at the basal end of the newest flushes were collected in August and November 1989 and stored at 5 C overnight. Midribs were excised, chopped and homogenized using a Tissumizer at a 1:20 (w/v) dilution in 0.05 M Tris, pH 8.0.

DAS-ELISA. The double antibody sandwich ELISA (DAS-ELISA) (1,3) was used to determine virus concentration in plants pre-inoculated with mild CTV isolates, and those pre-inoculated and further challenged with the severe T-66a CTV isolate. Two-hundred-µl samples were used; three washings with phosphate buffered saline, pH 7.4, containing 0.5% Tween 20 (PBS-Tween) were performed between steps. Immulon II microtiter plates (Dynatech Laboratories) were coated with 2 µg/ml of IgG from antiserum #1053 in carbonate buffer, pH 9.6, for 6 hr at 37C. Antigen samples were incubated overnight at 5C. IgG conjugated with alkaline phosphatase was used at a 1:1,000 dilution in conjugate buffer (PBS-Tween + 2% polyvinylpyrrolidone + 0.2% bovine serum albumin) mixed with homogenate of healthy plants for each ELISA plate (9:1 ratio of conjugate buffer to healthy homogenate) and incubated for 4 hr at 37 C. P-nitrophenyl phosphate (Sigma) was used as substrate at one mg/ml. The optical density at 405 nm (OD_{405}) was determined at 60, and 120 min

intervals using a Bio-Tek EL 307 ELISA plate spectrophotometer. Samples were considered positive when OD_{405} values were higher than 0.100 or three times the mean of healthy controls, whichever was greater.

DAS-Indirect ELISA. The double antibody sandwich indirect ELISA (DAS-indirect ELISA) (16) was used to determine the presence of the T-66a CTV isolate in both experiments. The plates were coated with polyclonal #1053 IgG, and antigen samples added as described for DAS-ELISA. The MCA-13 strain specific monoclonal antibody (16) was added at a 1:5,000 dilution in conjugate buffer and incubated overnight at 5C. After washing, goat anti-mouse IgG conjugated with alkaline phosphatase (Promega) was added at 1:7,500 dilution and incubated for 2 hr at 37 C. The enzyme reaction was carried out as for DAS-ELISA.

DIBA. The dot-immunobinding assay (DIBA) described by Hawkes, *et al.* (8) was adapted for citrus tristeza virus detection. Polyclonal IgG #1053 and the MCA-13 monoclonal antibody were used at 1.0, 0.5, 0.1 and 0.5 μg/ml. The Spanish 3DF1 monoclonal antibody was used at 1.0 and 2.0 μg/ml. All antibodies were diluted in 1% bovine serum albumin (BSA) in Tris buffered saline (TBS), pH 8.0. Commercial goat anti-rabbit IgG or goat anti-mouse IgG conjugated with alkaline phosphatase (Promega) were used at 1:7,500 dilution in 1% BSA in TBS, for samples assayed with either polyclonal or monoclonal antibodies, respectively. Nitrocellulose membranes {0.45 μm pore, (Micron Separations Inc.)} were cut at 3 X 10 cm, wet in TBS for 15-20 min, removed and blotted on paper filter for 5 min. Aliquots of 2 μl of antigen samples were spotted onto the membranes and let dry for 5 min. The membranes then were soaked for 60 min in 3% BSA in TBS and washed three times for 5 min each in TBS-Tween (TBS + 0.1% Tween 20). The membranes were incubated for 18 hr at room temperature with the appropriate antibody, and washed again with TBS-Tween. Membranes then were incubated for 2 hr at room temperature with appropriate goat anti-species IgG conjugated with alkaline phosphatase. After washing with TBS-Tween, the sites of antigen binding on the membranes were visualized by incubating the membranes in presence of 0.066 mg/ml nitro blue tetrazolium (NBT) and 0.033 mg/ml 5-bromo-4-chloro-3-indoyl phosphate (BCIP) (Promega) in reaction buffer (0.1 M Tris, 0.1 M sodium chloride, 0.05 M magnesium chloride, pH 9.4). The color reaction was stopped after 10-15 min by transferring the membranes to distilled water.

All serological assays were performed in duplicate. Mild (T-26, T-30, T-11a and/or T-55) and severe (T-36 and T-66a) CTV isolates from greenhouse grown plants were assayed as positive controls in every test. Negative controls included 0.05 M Tris, and sap from healthy *Citrus excelsa* plants.

RESULTS

In the first survey conducted on both field experiments in August 1989 using DAS-ELISA, all the pre-inoculated plants and those pre-inoculated and further challenged with T-66a were found infected with CTV. OD_{405} values for positive samples ranged from 0.145 to 1.999. Healthy controls showed values between 0.017 and 0.025.

The DAS-indirect ELISA using the MCA-13 strain specific monoclonal antibody gave a positive reaction (OD_{405} = 0.613) with only one plant (number 7-5) in the aphid challenged experiment established in 1985, and with the severe T-36 and T-66a CTV isolates (OD_{405} > 0.800) used as positive controls. None of the remaining plants that had been challenged in both experiments gave a positive reaction with the MCA-13 monoconal antibody. There were, however, 32 samples from both experiments which gave OD_{405} values close to 0.100, even when the positive controls with mild CTV iso-

lates gave values in the range of 0.030 after 120 min of enzyme reaction. Samples from these plants were collected and retested two weeks later, and again only one plant (number 7-5) was found to be infected by severe CTV strains by using the MCA 13 monoclonal antibody. Table 1 shows the results obtained by DAS-ELISA and DAS-indirect ELISA with some of those samples after 120 minutes of reaction. The plant number 7-5 and the T-36 and T-66a CTV severe isolates included as positive controls showed OD_{405} values of 0.501, 0.801 and 1.905, respectively, after 120 min reaction (Table 1). The plant number 7-6 also seemed to show a slight positive reaction (OD_{405} = 0.105) after 120 min of incubation. However, considering the background reaction showed by the healthy control (OD_{405} = 0.049), this sample would be still considered below the range for positive OD_{405} values. The CTV isolates T-11a and T-26 included as mild controls for the MCA-13 monoclonal antibody consistently gave OD_{405} values lower than 0.020, even after 2 hr of reaction with the substrate (Table 1).

Typical results obtained with DIBA are illustrated in Fig. 1. When the polyclonal #1053 IgG was used, a purple color developed on every sample on the nitrocellulose membranes (Fig. 1A), even on healthy controls and those field samples which had been given negative reaction on DAS-ELISA (Table 1). It was not possible to distinguish positive from negative samples using polyclonal #1053 IgG (Fig. 1A). These nonspecific reactions occurred within the first 1-2 min of reaction with the substrate solution. Attempts to prevent or minimize these nonspecific reactions, by cross absorbing the IgG #1053 with healthy sap, by using as low as 0.05 μg/ml of IgG dilution, or by adding 5 units/ml of heparin, to the incubation buffer (3) were unsuccessful.

When the 3DF1, the polyspecific monoclonal antibody, was used in DIBA, a purple color (Fig. 1B) developed within 1-15 minutes on the

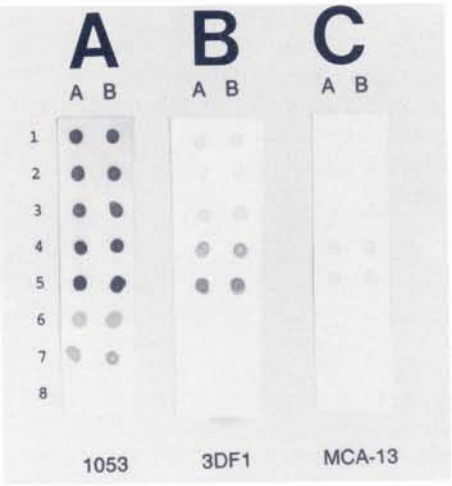

Fig. 1. Illustration of reactivity of CTV infected and healthy plants to (A) polyclonal antiserum #1053, (B) polyspecific monoclonal antibody 3DF1, and (C) strain specific monoclonal antibody MCA-13. Row 1 contains CTV isolate T-30 (mild), row 2 contains isolate T-26 (mild), row 3 contains isolate T-55a (mild), row 4 contains T-36 (severe), row 5 contains T-66a (severe), row 6 contains healthy *Citrus excelsa*, row 7 contains healthy Madam Vinous sweet orange, and row 8 contains TBS buffer. All plants are from the greenhouse. Columns A and B are duplicates of the same CTV isolate or healthy tissue.

positive controls T-30, T-26, T-55a, and T-66a CTV isolates. Use of 3DF1 gave a strong positive reaction to all the samples which had given positive reaction after 30 min on DAS-ELISA from the field samples. However, some of the samples which gave a positive reaction after 2 hr of enzyme reaction on DAS-ELISA (plant numbers 3-21, 4-28 and T-11a on Table 1) were not clearly detected by the 3DF1 monoclonal antibody. No background reaction was detected with Tris buffer or healthy control (Fig. 1B). It should be noted that the enzyme reaction of 3DF1 on the DIBA was stopped after 15 min because a purple precipitate started to appear on the membrane background.

When MCA-13, the severe strain specific monoclonal antibody, was used on DIBA, positive reactions occurred only with the T-36 and T-66a CTV positive controls (Fig. 1C) and with the plant code 7-5 (data not shown) which was found positive in DAS-indirect

TABLE 1
SUMMARY OF ENZYME-LINKED IMMUNOSORBENT ASSAYS (AUGUST 1989) OF CITRUS PLANTS PRE-INOCULATED AND PRE-INOCULATED AND CHALLENGED WITH A SEVERE CITRUS TRISTEZA VIRUS

	OD_{405} values[z]	
	Polyclonal #1053 DAS-ELISA	MCA-13 strain specific DAS-indirect ELISA
Plant number		
3-21	0.119/+[y]	0.056/–
4-3	0.577/+	0.048/–
4-9	0.075/–	0.016/–
4-26	0.561/+	0.034/–
4-28	0.101/–	0.020/–
7-5[x]	0.421/+	0.501/+
7-6	0.607/+	0.105/+
CTV isolate		
T-11a (mild)[w]	0.113/+	0.020/–
T-26 (mild)[w]	0.138/+	0.018/–
T-36 (severe)[w]	1.148/+	0.801/+
T-66a (severe)[w]	1.617/+	1.905/+
Healthy[w]	0.017	0.049

[z]Average of two replications per sample after 120 min reactions.
[y]Higher (+) or lower (–) than 0.100 or three times the mean of healthy control, whichever was greater.
[z]This plant was retested two weeks later and again found to be (+) with MCA-13 strain specific DAS-indirect ELISA.
[w]Greenhouse-grown control plants.

ELISA (plant number 7-5 on Table 1). A slight background with healthy controls was prevented when the MCA-13 was used at dilution of 0.1 to 0.05 μg/ml.

In the second survey of the aphid challenged experiment conducted in November 1989, six plants (numbers 2-36, 3-36, 4-36, 7-35, 8-2, and 8-35) were found positive when assayed with the MCA-13 strain specific monoclonal antibody in both DAS-indirect ELISA (Table 2) and DIBA (data not shown). None of these plants were positive in the previous summer survey, and furthermore, they had not been intentionally aphid inoculated with the T-66a CTV challenge isolate. Five of these plants (numbers 2-36, 3-36, 4-36, 7-35, and 8-35) were localized at the west edge of the experiment; whereas, plant number 8-2 was at the east edge of the experiment. None of the 144 remaining plants that were aphid challenged in spring of 1987, nor the plant number 7-5 found positive in the summer survey (Table 1), reacted positively with the MCA-13 monoclonal antibody by either DAS-indirect ELISA or DIBA.

When the whole aphid challenged experiment was assayed with the #1053 polyclonal IgG in DAS-ELISA, CTV infection was detected in all 144 plants pre-inoculated with mild CTV isolates. In addition, 25 of 32 of the originally healthy uninoculated control plants were also found to be CTV infected.

The graft challenged experiment was not surveyed in November 1989 because appropriate leaf tissue for sampling was not available at that time. Likewise, an early freeze in December 1989 prevented a later survey.

DISCUSSION

The results of this research provide further evidence of the usefulness the MCA-13 monoclonal antibody to detect severe CTV isolates, and introduces the use of the dot-immunobinding assay for CTV detection. When antibodies polystrain specific for all CTV strains are used in combination with the MCA-13 strain specific monoclonal antibody in comparative DAS-ELISA and DAS-indirect ELISA tests, respectively, differential detection of CTV isolates is possible. DAS-ELISA with polyclonal antisera has previously been used to evaluate virus titer on citrus plants (7,9,12). A high titer in a plant infected with a mild CTV isolate may be a relative estimate of the protecting ability of mild CTV isolates in cross protection experiments (12). However, polyclonal antibodies do not distinguish between severe and mild isolates. Since the MCA-13 monoclonal antibody reacts specifically with severe CTV isolates (16), its use provides a tool for evaluation of cross protection experiments.

In DAS-ELISA with polyclonal #1053 IgG positive reactions for some samples were not achieved unless reaction with the substrate was continued for 2 hr. This may reflect a low titer of the virus in those plants. Even after 2 hr reaction, the OD_{405} values of healthy controls were never higher than 0.025. These low background values were achieved by cross absorption of the enzyme conjugate with healthy sap prior to use. The low OD_{405} values found with the mild isolates, T-11a, T-26 and T-30 and the high values with the severe isolates T-66a and T-36 in both DAS-indirect ELISA (Tables 1 and 2), as well as in the DIBA (Fig. 1C), further demonstrated the high specificity of MCA-13 for severe CTV isolates.

The DIBA was rapid to perform and was as sensitive as DAS-ELISA in detecting CTV in crude preparations. But it was possible only with the 3DF1 monoclonal antibody. When #1053 polyclonal antibody was used, nonspecific reactions with healthy controls prevented differentiation between positive and negative samples. Non-specific reactions were reduced in other systems with the use of additives, such as heparin (4), or dextran sulphate (17). Other additives may prevent or minimize these nonspecific reactions with CTV #1053 polyclonal or other antibodies in the dot-immunobinding assay.

TABLE 2
SUMMARY OF ENZYME-LINKED IMMUNOSORBENT ASSAYS (NOVEMBER 1989) OF CITRUS PLANTS PRE-INOCULATED AND PRE-INOCULATED AND CHALLENGED WITH A SEVERE CITRUS TRISTEZA VIRUS

	OD_{405} values[z]	
	Polyclonal #1053 DAS-ELISA	MCA-13 strain specific DAS-indirect ELISA
Plant number		
2-36	0.629/+[y]	0.775/+
3-36	0.181/+	0.374/+
4-36	0.121/+	0.187/+
7-5	0.076/–	0.022/–
7-35	0.268/+	0.169/+
8-2	0.301/+	0.421/+
8-35	0.394/+	0.522/+
CTV isolate		
T-26 (mild)[x]	0.404/+	0.028/–
T-30 (mild)[x]	0.445/+–	0.017/–
T-36 (severe)[x]	0.528/+	0.720/+
T-66a (severe)[x]	0.357/+	0.267/+
Healthy[x]	0.009	0.002

[z]Average of two replications per sample after 120 min reactions.
[y]Higher (+) or lower (–) from 0.100 or three times the mean of healthy control, whichever was greater.
[x]Greenhouse-grown control plants.

In the November survey of the aphid challenged experiment, the positive reactions of MCA-13 with some non-inoculated plants may have been due to natural spread of a severe CTV isolate by aphids from adjoining citrus. The location of these plants at the edges of the experiment supports this explanation. DAS-indirect ELISA using MCA-13 has indicated severe CTV strains are present in citrus trees on CTV tolerant rootstocks on the immediate north side of both experiments (data not shown). The lack of reaction of these plants with MCA-13 in the first summer survey suggests that infection occurred between the surveys. Some uninoculated healthy control plants in both experiments also were found infected with CTV when assayed with #1053 polyclonal antibody in DAS-ELISA. These plants did not react positively with MCA-13 in either DAS-indirect ELISA or DIBA. This indicates that some natural spread of mild CTV isolates has occurred in both experiments. Similar results of natural spread of both mild and severe CTV isolates have been found in another field experiment on cross protection in Florida (22).

The plant number 7-5 which reacted positively with the MCA-13 monoclonal antibody in the first survey (August 1989) in the aphid challenge experiment, was not positive by either DAS-ELISA or DAS-indirect ELISA in the second survey (November 1989) using MCA-13. It should be noted that only hardened tissue was available from this plant at the time of the November survey, and a resultant low virus titer could explain the negative values. Unfortunately a hard freeze in December 1989 precluded further serological tests with that plant.

The lack of reaction of the MCA-13 monoclonal antibody in both DAS-indirect ELISA and dot-immunobinding assay on plants previously challenged with the severe CTV isolate in both experiments, as well as the absence of noticeable tristeza decline symptoms in the field, could reflect the ineffectiveness of the methods used to inoculate with the severe CTV isolate in both experiments. However, the aphid transmissibility of the T-66a challenge CTV isolate by *Aphis gossypii* is well documented (6,21). Furthermore, the donor plants for aphid feeding were verified by ELISA to be infected by CTV before the test, and the aphids were confined only to new flushes on the challenged plants. Why the aphid challenge inoculations were not more successful is unknown. It is possible that the cross protection worked in the aphid challenged experiment, and the mild CTV isolates prevented to some extent the establishment and replication of the inoculated severe CTV isolate. Little information is available on the distribution and prevalence of mixtures of CTV isolates inside the plant. With regard to the graft challenged experiment, previous studies on the transmission of CTV by grafting procedures have shown that a period of at least ten days of contact between grafted tissues is needed to obtain transmission of CTV to the receptor host (18,20). In the present study, even with two graft challenges in a 3-yr period, with the inoculum remaining in the challenged trees for at least six weeks, it appears that the severe CTV isolate was not established, at least at levels detectable by DAA-indirect ELISA. Furthermore, evidence from other studies indicates that *Citrus excelsa*, considered as an excellent propagation host for CTV purification (10,13), is apparently a poor donor host for graft transmission experiments (M. Rocha-Peña and R. F. Lee, unpublished). The efficiency of either graft or aphid inoculation methods could be investigated by further challenging trees in both experiments again and monitoring the establishment of the inoculated severe CTV isolate at shorter time intervals. This is now possible due to the availability of the MCA-13 monoclonal antibody.

ACKNOWLEDGEMENT

We thank R. H. Brlansky, C. O. Youtsey, and W. S. Castle for allowing

sample collection of trees included in the survey. This research was funded in part by the USDA Specific Cooperative Agreement LAL-03091 and the Florida High Technology and Industry Council in the USA, and by Consejo Nacional de Ciencia y Tecnologia (CONACyT) and Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP), Mexico.

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