Subtropical Plant Science. 45:11-17. 1992

Effects of Mild Isolates of Citrus Tristeza Virus on the Development of Tristeza Decline

Mario A. Rocha-Peña[1], Richard F. Lee, and C.L. Niblett

Citrus Research and Education Center, University of Florida. Lake Alfred, Florida 88350

Additional index words: Cross-protection, ELISA, monoclonal antibodies, strain discrimination, virus isolates.

ABSTRACT

Four mild isolates of citrus tristeza virus (CTV) (Tlla, T26, T30, T55a) were evaluated under greenhouse conditions for their ability to cross protect against the decline induced by a severe (T66a) challenge isolate in two susceptible scion/rootstock combinations. DAS-ELISA with ploYclonal antisera was used to determine the total antigen titer in plants inoculated with mild isolates and those challenged with the severe isolate. The severe strain specific MCA-13 monoclonal antibody was used in DAS-indirect ELISA for detection and quantification of the severe isolate in mixed infections. Plants pre-infected with T26, T30, and T55a mild isolates, and further challenged with the severe isolate, gave typically lower (MCA-13 OD_{405}) values for the T66a isolate than the unprotected challenged control plants. Isolate T26 and T55a reduced decline index scores and number of dead plants as compared with uninoculated challenged control treatments. The highest number of dead plants were obtained with T11a. The T30 mild isolate provided intermediate decline index scores and numbers of dead plants. The preliminary evaluation of mild isolates for their ability to provide cross-protection can be accomplished under controlled greenhouse conditions in a relatively short time of 18-24 months.

RESUMEN

Se evaluaron bajo condiciones de invernadero cuatro razas débiles del virus tristeza de los cítricos (VTC) (T11a, T26, T30, T55a) con fines de protección cruzada en contra de una raza severa (T66a) retadora en dos combinaciones cultivar/portainjerto susceptibles. Se utilizó la prueba DAS-ELISA con antisueros policlonales para determinar el antígeno total del VTC en las plantas inoculadas con razas débiles del virus y en aquéllas pre-infectadas con las razas débiles y subsecuentemente retadas con la raza severa. Se utilizó el anticuerpo monoclonal MCA-13 específico para razas severas en pruebas de DAS-ELISA indirectas para la detección y cuantificación de la raza T66a retadora en infecciones mixtas. Las plantas pre-infectadas con las razas T26, T30 y T55 débiles y subsecuentement inoculadas con la raza T66a retadora, dieron los valores menores de densidad óptica (DO_{405}/MCA-13) en DAS-ELISA indirecta que las plantas testigo sin protección, pero inoculadas con la raza severa. Las plantas pre-inoculadas con las razas T26 y T55a débiles mostraron los menores índices de declinamiento y número de plantas muertas en comparación con las plantas testigo sin retar. Con la raza T11a se obtuvieron los valores mayores de índice de declinamiento y mayor número de plantas muertas. La raza T30 débil mostró valores intermedios de protección. La evaluación preliminar de razas del VTC débiles con fines de protección cruzada se puede llevar a cabo bajo condiciones de invernadero en un tiempo relativamente corto de 18-24 meses.

Citrus tristeza virus (CTV), a member of the closterovirus group, is distributed worldwide and causes the most economically important viral disease of citrus (Bar-Joseph and Lee, 1989; Bar-Joseph *et al.* 1989). CTV occurs naturally with a diversity of isolates or strains which may differ greatly in their biological properties, such as symptomatology in different citrus hosts (Garnsey *et al.* 1987; McClean, 1974) and aphid transmissibility (Yokomi and Damgsteet, 1991; Yokomi and Garnsey, 1987). The most common damage caused by CTV is the decline induced in scions grafted onto sour orange (*Citrus aurantium* L.) rootstock, with the subsequent death of the infected plants. Some CTV isolates may cause severe stem pitting on some orange [*C. sinensis* (L.) Osb.] and/or grapefruit (*C. paradisi* L.) scions regardless of the rootstock, resulting in smaller fruit, loss of production, and debilitation of the tree. Likewise, there are some mild CTV isolates that do not cause any apparent effects on infected plants, even on trees grafted onto sour orange rootstock (Bar-Joseph *et al.* 1989; Lee and Rocha-Peña, 1991).

The use of mild strain cross-protection (Fulton, 1986; Lee *et al.* 1987), as a control strategy to reduce losses due to citrus tristeza virus has been used commercially to control severe stem pitting isolates of CTV on Pera sweet orange in Brazil (Costa and Müller, 1980; Müller, 1980), and is a part of South Africa's citrus cultivar improvement program to reduce CTV-induced stem pitting on grapefruit (DeLange *et al.* 1981; Garney and Lee, 1988). Relatively little work has been done to evaluate the potential of cross-protection against the CTV-induced decline (CTV-ID) on plants grafted onto sour orange, as most countries abandon sour orange as a rootstock when CTV-ID isolates become prevalent. However, experiments conducted in Australia (Thornton *et al.* 1980), the United States (Wallace and Drake, 1976; Yokomi *et al.* 1991), and Japan (Miyakawa, 1987) indicate that cross-protection against CTV-ID isolates may be possible.

CTV has been widespread in Florida for many years and CTV-ID has occurred in localized areas (Garnsey and Jackson, 1975; Norman *et al.* 1961). However, it had not caused major losses

[1]Campo Experimental General Terán. INIFAP/SARH. Aparto Postal #3. General Terán, Nuevo León 67400. México.

because most of the citrus acreage had been propagated on CTV-tolerant rootstocks, and because of the prevalence of mild CTV isolates which did not seriously affect trees grafted on sour orange rootstock (Brlansky *et al.* 1986; Lee *et al.* 1987). In the 1980's extensive acreage with plants propagated on sour orange was planted throughout the state. Severe dwarfing of young trees, as well as, large scale outbreaks of CTV-ID have appeared in many plantations, particularly in southern Florida, an area previously not affected by CTV. Losses have exceeded 50% in some plantings (Brlansky *et al.* 1986). The popular use of sour orange despite tristeza, along with natural prevalence of mild CTV isolates, provides the opportunity to evaluate cross-protection as an alternative control strategy for CTV-ID. The objectives of this research were to evaluate naturally occurring mild CTV isolates from Florida for their ability to cross protect in two susceptible scion/rootstock combinations and to develop a methodology to detect the presence of protecting and challenge CTV isolates in mixed infections.

MATERIALS AND METHODS

Virus isolates and donor hosts. Five naturally occurring Florida CTV isolates collected from field grown sweet orange or grapefruit trees grafted onto sour orange were used in these experiments after transmission by *Aphis gossypii* Glover. The very mild properties of the T11a, T26, T30 and T55a isolates have been described (Garnsey *et al.* 1987; Lee, 1984; Yokomi and Garnsey, 1987; Yokomi *et al.* 1987). The severe T66a challenge isolate causes strong vein clearing, stunting and stem pitting in Mexican lime seedlings [*Citrus aurantifolia* (Christm.) Swingle] and severe decline in sweet orange/sour orange combinations (Garnsey *et al.* 1987; Yokomi *et al.* 1987). The CTV isolates were propagated in either *C. excelsa* Wester, Madam Vinous sweet orange or Mexican lime plants and maintained in a greenhouse with mean minimum and maximum temperatures of 21 and 38°C. Inoculum source tissue from donor hosts was evaluated by serological indexing (see below) to confirm the presence of CTV before being used as inoculum.

Inoculation of CTV isolates and receptor hosts. One-year-old Valencia sweet orange plants budded on either sour orange or *C. macrophylla* Wester (a biotype of sour orange) rootstocks, were graft-inoculated with each of the CTV mild isolates using three leaf pieces or blind buds per plant (Garnsey and Whidden, 1970; Garnsey *et al.* 1987). Inoculum tissue was sealed firmly into the receptor stems with plastic grafting tape. Three weeks later the grafting tape was removed and the plants were evaluated for survival of grafted tissue and reinoculated if the grafted tissue had not survived. After verifying by serological indexing that infection by mild isolates had taken place, a minimum of four inoculum pieces of T66a infected tissue were used to challenge the test plants, and they were reinoculated if at least two inoculum pieces were not alive 21 days post-challenge. Surviving inoculum tissue was left in place for the duration of the experiment. Inoculated receptor plants were grown in a commercial potting mixture (Pro-mix BX) in five liter plastic containers, and fertilized with a mixture of NPK (20-10-20) every other week. Pest and disease management included the application of 0.300 g active ingredient (a.i.)/plant of aldicarb (Temik) and 0.86 g a.i. /L soil drench of metalaxyl (Ridomil) twice a year. The experiment was conducted in a greenhouse with mean minimum and maximum temperatures of 21 and 38°C. At least 15 plants were inoculated for each CTV isolate combination.

Serological tests. CTV infection and relative antigen titer of inoculated plants were determined throughout the study by the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Bar-Joseph *et al.* 1979), using polyclonal antiserum (PCA) No. 1053 prepared against whole, unfixed virions of CTV T26 (R.F. Lee, unpublished). The severe CTV T66a was detected in the challenged plants by DAS-indirect ELISA using the MCA-13 severe strain specific monoclonal antibody. This antibody does not react with mild CTV strains (Permar *et al.* 1990). For efficiency, the serological tests were performed with both scion/rootstock combinations at the same time.

Routinely, 0.5 g of bark, petioles and midribs of new, fully expanded tissue were finely chopped with a razor blade and ground, using a Tekmar Tissumizer, in 5 ml of phosphate buffered saline (PBS) - Tween + polyvinyl pyrrolidone [PBS = 8 mM Na_2HPO_4, 14 mM KH_2PO_4, 15 mM NaCl, pH 7.4, (+ 0.1% Tween 20 + 2% polyvinyl pyrrolidone (PVP-40 Sigma)]. Unless stated otherwise, 200 microliter samples were used per well of the microtiter plates and three washings with PBS-Tween (phosphate buffered saline + 0.1% Tween 20) were performed between steps. The immunoglobulins (IgG) present in the whole CTV antiserum were purified by the Protein A-Sepharose affinity method (Miller and Stone, 1978). A portion of purified immunoglobulins were conjugated to alkaline phosphatase by the glutaraldehyde method (Clark *et al.* 1986). Polystyrene Immulon II microtiter plates (Dynatech Laboratories) were coated with 2.0 µg/ml of purified IgG in carbonate buffer (0.015 M $NaHCO_3$, 0.03 M $NaCO_3$, pH 9.6) and incubated for 6 hr at 37°C. Antigen samples were added to the wells and incubated for 18 hr at 5°C. Enzyme conjugate was used at a dilution of 1:1,000 in conjugate buffer (PBS-Tween + 2% polyvinyl pyrrolidone + 0.2% bovine serum albumin) and incubated for 6 hr at 37°C. The reaction with one mg/ml of p-nitrophenyl phosphate (Sigma) in 10% triethanolamine, pH 9.8, was measured after 120 min. at 405 nm (OD_{405}) with a Labinstruments model EAR 400 AT ELISA plate reader spectrophotometer. Samples were considered postive when OD_{405} values were higher than 0.100 or three times the mean of healthy controls, whichever was greater.

For DAS-indirect ELISA, the microtiter plates were first coated with IgG from antiserum No. 1053. Antigen samples were added as described for DAS-ELISA. The MCA-13 strain specific monoclonal antibody (hereby MCA-13), as ascites fluid, was added at a dilution of 1:5,000 (v/v) in conjugate buffer and incubated 4 hr at 37°C. After washing, goat anti-mouse IgG labeled with alkaline phosphatase (Promega) at a dilution of 1:7,500 (v/v) in conjugate buffer was added and incubated for 2 hr at 37°C. The enzyme reaction was carried out as for DAS-ELISA.

For all serological tests, two replications were used per sample. Positive controls included four mild isolates (T11a, T26, T30, and T55a) and one severe (T66a) CTV isolate. Negative controls included extraction buffer, and similar buffer extracts from healthy *C. excelsa* and Valencia sweet orange plants. A standard curve prepared with purified CTV T26 virions diluted to OD_{260} values of 0.04, 0.02, 0.01, 0.005, 0.0025, 0.0012, 0.0006, and 0.0003 diluted in buffer extract of healthy *C. excelsa* was used to estimate the relative antigen concentration of test samples.

Detrimental effects of mild isolates and evaluation of cross-protection. To test the effect of each CTV isolate on the inoculated plants and the ability of the mild isolates to cross protect against the T66a challenge isolate, evaluations were made at five and ten months after the challenge inoculation with the T66a isolate. A decline index was assigned for each plant. The parameters scored were stem diameter, plant growth, and foliage symptoms for decline. Each parameter was visually rated from 0 (minimum) to 3 (maximum), for a maximum cumulative score of 9 for each plant. A high decline index sometimes was accompanied by plant death. The decline index for each treatment was the average of the cumulative scores for all plants in that treatment. Phloem necrosis was evaluated by cutting a bark flap at the bud union and the plant tissue was examined with a hand lens for browning.

RESULTS AND DISCUSSION

Antigen titer of mild and severe CTV isolates. The antigen titer expressed as optical density (OD_{405}) values for both scion/rootstock combinations are summarized in Tables 1 and 2. Valencia/sour orange plants inoculated with mild isolates but unchallenged with T66a gave OD_{405} values between 0.091 and 0.145 when analyzed by DAS-ELISA. The OD_{405} values for corresponding uninoculated healthy control plants averaged 0.039. The same treatments, including the healthy controls gave values in the range of 0.011-0.025 when analyzed by DAS-indirect ELISA. Treatments pre-inoculated with mild isolates and further challenged with T66a gave OD_{405} values in the range of 0.130-0.217 and 0.145-0.189 by DAS-ELISA and DAS-indirect ELISA, respectively. The control plants uninoculated with mild isolates byt challenged with T66a, gave values of 0.174 and 0.214 when tested by DAS-ELISA and DAS-indirect ELISA, respectively.

The Valencia/*macrophylla* plants inoculated with the mild isolates and unchallenged with T66a, gave OD_{405} values between 0.092 and 0.164 with PCA. The value for the corresponding uninoculated healthy control plants was 0.040. The same treatments, including the healthy controls gave values in the range of 0.018-0.037 when analyzed with MCA-13. Treatments pre-inoculated with mild isolates and challenged with T66a gave values in the range of 0.185-0.311 with PCA and 0.104-0.305 with MCA-13. The control plants uninoculated with mild isolates by challenged with T66a gave values of 0.194 with PCA and 0.251 with MCA-13 (Table 2). The T30 isolate was not evaluated in this portion of the experiment. The plants preinoculated with the T11a isolate were not protected and declined and died before the serological evaluation was made five months after inoculation (Table 2).

From the standard curve prepared with purified T26 virions (Fig. 1), it was estimated that an OD_{405} value of 0.638 was approximately equivalent to μg/ml of CTV antigen, assuming an extinction coefficient of 2.0 (Gonsalves *et at.* 1978). Therefore, there was an average of 3.1 μg of CTV antigen per every 100 mg of tissue for each 0.100 OD_{405} value in the test samples.

The results obtained with both scion/rootstock combinations, when plants pre-inoculated with mild isolates and further challenged with the T66a severe isolate, were analyzed in DAS-indirect ELISA (Tables 1 and 2), suggest that the mild isolates likely prevented or reduced to some extent the multiplication of the T66a challenge isolate. In this situation, even though the differences were not statistically significant, there was a trend mostly of the T26 and T30 isolates to give the lowest, but CTV positive OD_{405} values of all treatments evaluated. These results provided further evidence of the usefullness of the MCA-13 monoclonal antibody to detect selectively the presence of severe CTV isolates

Table 1. Relative antigen titer of citrus tristeza virus (CTV) mild isolates in Valencia/sour orange plants unchallenged and challenged by T66a severe isolate.

CTV isolate	Unchallenged[1]		Challenged[1,2]	
	OD_{405}[3]		OD_{405}	
	Polyclonal	MCA-13	Polyclonal	MCA-13
T11a	0.101[4]/ab[5]/	0.024 a	0.217 a	0.175 a
T26	0.123 ab	0.025 a	0.130 a	0.145 a
T30	0.091 ab	0.018 ab	0.212 a	0.189 a
T55a	0.145 a	0.014 b	0.194 a	0.181 a
Control Plants	0.039 b	0.011 b	0.174 a	0.214 a

1 One-year old plants were graft inoculated with the indicated mild CTV isolates by placing leaf pieces under bark flaps on the stem.
2 After verifying infection with mild isolates by DAS-ELISA with polyclonal antibodies, the challenged plants were graft inoculated with the T66a severe isolate.
3 Optical density at 405 nm (OD_{405}) was measured after 120 min of reaction.
4 Serological detection was carried out by DAS-ELISA with polyclonal antisera and DAS-indirect ELISA with MCA-13 severe strain specific monoclonal antibody. Tests were done six months after inoculation. OD_{405} readings are the average of duplicate assays of the corresponding plants in Table 3.
5 Numbers in the same column followed by different letters are statistically different by Duncan's test (P 0.05).

in mixed infections. The MCA-13 has been previously used in other studies to evaluate the presence of severe isolates in field cross-protection experiments (Rocha-Pena *et al.* 1991; Yokomi *et al.* 1991). The use of MCA-13 in DAS-indirect ELISA to determine the OD_{405} readings in the cross-protection experiments may provide a measurable parameter to estimate the ability of mild CTV isolates to prevent the establishment of severe challenge isolates. The routine use of CTV polyclonal antisera to verify the infection of the pre-inoculated plants before exposing them to the challenge, should be mandatory in any program related with cross-protection. There have been some reports of the lack of efficiency of some mild CTV isolates in cross-protection experiments (Costa and Muller, 1980; Muller *et al.* 1988); however, this phenomenon could be due to an absence of pre-infection by the mild isolates rather than a lack of protection.

In general, the OD_{405} values for DA-ELISA and DAS-indirect ELISA in both scion/rootstock combinations (Tables 1 and 2) can be considered relatively low, as compared to other research works (Bar-Joseph *et al.* 1979; Brlansky *et al.* 1986; Permar *et al.* 1990) where serological tests have been performed with CTV anatisera. This could be due to the warm temperatures prevalent in the greenhouse where the experiment was conducted, which likely

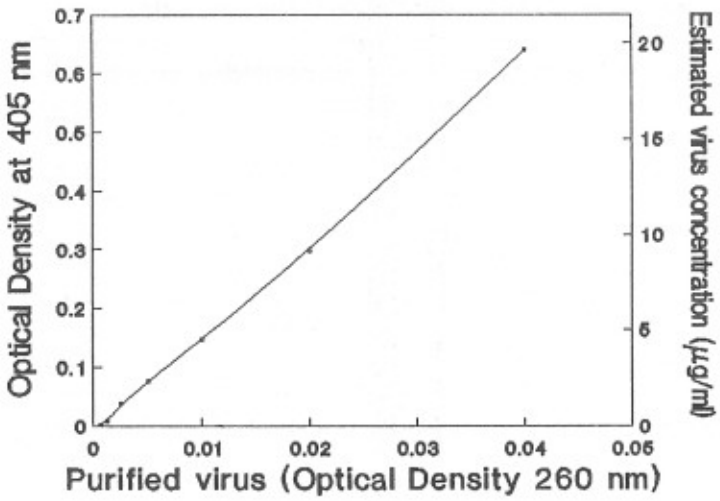

Figure 1. Plot of purified citrus tristeza virus (CTV) virions against optical density. Purified CTV T26 virions giving the desired optical density at 260 nm (OD_{260}) were mixed with a buffer extract containing bark of healthy *Citrus excelsa* (0.5g) ground in 5.0 ml of phosphate buffered saline, pH 7.6, + 0.05% Tween + 2% polyvinyl pyrrolidone. DAS-ELISA was performed as described in Materials and Methods. An extinction coefficient of 2.0 was assumed (Gonsalves *et al.* 1987) to estimate the relative virus concentration.

Table 2. Relative antigen titer of citrus tristeza virus (CTV) mild isolates in Valencia/macrophylla plants unchallenged and challenged by the T66a severe isolate.

CTV Isolate	Unchallenged[1]		Challenged[1,2]	
	OD_{405}[3]		OD_{405}	
	Polyclonal	MCA-13	Polyclonal	MCA-13
T11a	0.092[4]a[5]	0.026 a	–[6]	—
T26	0.132 a	0.030 a	0.185 a	0.104 a
T55a	0.164 a	0.014 a	0.311 a	0.305 a
Control Plants	0.040 a	0.018 a	0.194 a	0.251 a

1 One-year-old plants were graft inoculated with the indicated mild CTV isolates by placing leaf pieces under bark flaps on the stem.
2 After verifying infection with mild isolates by DAS-ELISA with polyclonal antibodies, the challenged plants were graft inoculated with the T66a severe isolate.
3 Optical density at 405 nm (OD_{405}) was measured after 120 min of reaction.
4 Serological detection was carried out by DAS-ELISA with polyclonal antisera and DAS-indirect ELISA with MCA-13 severe strain specific monoclonal antibody. Tests were done six months after inoculation. OD_{405} readings are the average of duplicate assays of the corresponding plants in Table 4.
5 Numbers in the same column followed by different letters are statistically different by Duncan's test (P 0.05).
6 - = severely diseased plants died before serological evaluation.

did not favor a high virus titer in the inoculated plants. In addition to this, all the serological tests in the whole experiment were performed at the same time, and no uniform new flush was available for some plants at the time of tissue harvest.

Detrimental effects of mild isolates and evaluation of cross-protection. The effect of the CTV mild isolates on performance of inoculated plants of both scion/rootstock combination was negligible. The decline indexes for the healthy uninoculated control plants were nearly equal to or greater than those inoculated only with mild isolates (Tables 3 and 4). In regard to Valencia/sour orange plants pre-inoculated with mild isolates and challenged with the T66a severe isolate, the lowest decline index (2.6) and lowest number of dead plants (0/5) were obtained with the T26 isolate. Whereas, the highest decline index (6.3) and highest number of dead plants (3/8) were obtained with the T11a isolate. A decline index of 4.0 and 1/6 dead plants were scored with the uninoculated and challenged control plants (Table 3). With Valencia/*macrophylla* plants pre-inoculated with mild isolates and challenged with the severe isolate, the T26 and T55a isolates obtained the lowest number of dead plants (0/5 and 0/2,

respectively). Whereas, the highest number of dead plants (3/3) were observed with the T11a. A decline of index of 6.8 and 3/6 dead plants were scored with the uninoculated and challenged control plants (Table 4). Phloem necrosis at the bud union was not observed in any treatment either at five or ten months after the challenge inoculation.

in concordance with the lowest OD_{405} values when these treatments were analyzed with the MCA-13 monoclonal antibody in DAS-indirect ELISA (Tables 1 and 2). The recent report (Van Vuuren *et al.* 1991) of the field ability of the T26 isolate to cross-protect in plants on sour orange rootstock, supports this conclusion.

Table 3. Effect of citrus tristeza virus (CTV) mild isolates on the development of the CTV decline syndrome in Valencia/sour orange plants unchallenged and challenged by the T66a severe isolate.

	Unchallenged[1]		Challenged[1, 2]		
CTV isolate	No. of plants	Decline index[3]	No. of plants	Decline index	No. of dead plants 10 months after challenge
T11a	4	0.0	8	6.3	3/8
T26	4	1.5	5	2.6	0/5
T30	5	1.0	4	4.2	1/4
T55a	5	0.0	7	5.5	2/7
Control plants	5	1.4	6	4.0	1/6

1 One year old plants were graft inoculated with leaf pieces with the indicated mild CTV isolates placed under bark flaps on the stem.
2 After verifying infection with mild isolates by DAS-ELISA with polyclonal antibodies, the challenged plants were graft inoculated with a T66a severe isolate.
3 Decline index is the average per treatment of the cumulative score given by visual readings on three criteria: stem diameter, growth reduction, and foliage decline symptoms, where 0 = healthy vigorous plant to 3 = severely diseased. Minimum index = 0, maximum index = 9. A high decline index sometimes was accompanied by plant death.

Of all treatments evaluated, the T26 isolate gave consistenly the lowest decline index scores and lowest number of dead plants as compared with the uninocuclated challenged control plants (Tables 3 and 4). These results suggest that mild isolates, especially the T26 isolate, may have prevented the multiplication of the challenge severe isolate to some extent, thus providing protection against the development of the CTV-ID syndrome. This was

Of special interest was the high decline index scores and the number of dead plants in those treatments pre-inoculated with T11a and further challenged with T66a. It seemed that the combination of T11a and T66a produced a more severe reaction on the challenged plants than that caused by T66a alone in the unprotected control plants. The lack of ability of T11a to cross protect has been previously reported (Yokomi *et al.* 1987).

Table 4. Effect of citrus tristeza virus (CTV) mild isolates on the development of the CTV decline syndrome in Valencia/*macrophylla* plants unchallenged and challenged by the T66a severe isolate.

	Unchallenged[1]		Challenged[1, 2]		
CTV isolate	No. of plants	Decline index[3]	No. of plants	Decline index	No. of dead plants 10 months after challenge
T11a	1	2.0	3	9.0	3/3
T26	4	1.7	5	4.2	0/5
T55a	2	2.0	2	2.2	0/2
Control plants	5	2.2	6	6.8	3/6

1 One year old plants were graft inoculated with leaf pieces with the indicated mild CTV isolates placed under bark flaps on the stem.
2 After verifying infection with mild isolates by DAS-ELISA with polyclonal antibodies, the challenged plants were graft inoculated with a T66a severe isolate.
3 Decline index is the average per treatment of the cumulative score given by visual readings on three criteria: stem diameter, growth reduction, and foliage decline symptoms, where 0 = healthy vigorous plant to 3 = severely diseased. Minimum index = 0, maximum index = 9. A high decline index sometimes was accompanied by plant death.

The relatively low decline index scores and low occurrence of dead plants on both scion/rootstock combinations in the unprotected control plants challenged with T66a indicate that under greenhouse conditions, the use of one single challenge isolate might not be sufficient to obtain an appropriate rate of decline in a short time basis. It is well documented that CTV occurs naturally as mixtures of isolates or strains with diverse biological properties (Garnsey *et al.* 1987, McClean, 1974). The T66a severe isolate was originally isolated from an infected field source, and subsequently aphid transmitted to avoid contamination with other viruses (Garnsey *et at.* 1987; Yokomi and Garnsey, 1987). It is possible that part of the original decline components from the field source could have been lost in the subsequent aphid transmissions. To overcome this possibility, it may be advisable in the future to use a mixture of several severe isolates as a challenge to enhance the possibility of obtaining an appropriate occurrence of decline under greenhouse conditions.

Cross-protection using mild virus isolates as a strategy to reduce losses due to CTV has been used in Brazil (Costa and Müller 1980; Müller, 1980; Müller *et al.* 1988), South Africa (DeLange *et al.* 1981; Garnsey and Lee, 1988), Japan (Ieki, 1989), India (Barlaraman and Ramakrishnan, 1980), and Australia (Broadbent, *et al.* 1991), against stem-pitting isolates either in orange, grapefruit, and/or acid lime. Several approaches have been reported for the evaluation of mild isolates under greenhouse conditions. However, these approaches have mostly addressed the cross-protection effects of mild isolates against stem pitting, and have included only the host reaction of Mexican lime, sweet orange, or grapefruit seedlings (Roistacher *et al.* 1987, 1988; Van Vuuren and Moll, 1987). Another approach, where the challenge inoculations are made by using aphid vectors to screen mild isolates (Yokomi *et al.* 1987) has not been extensively used.

The results of this work provide further evidence that the cross-protection against the CTV-induced decline of sweet orange grafted either on sour orange or *C. macrophylla* may be possible; the preliminary evaluation of mild isolates under greenhouse conditions can be made in a relatively short time (18-24 months); and the severe challenge isolate can be detected and spectrophotometrically measured by using the MCA-13 strain specific specific monoclonal antibody. The report of Miyakawa (1987) about the feasibility or cross-protection on sweet/sour orange combinations supports these conclusions.

The methodology described here provides the following advantages; 1) It allows the comparative evaluation of both the virus isolates and inoculated hosts under uniform greenhouse conditions; 2) It avoids the risks that represent the threat of recurrent freezes especially in Florida, the lack of an appropriate natural challenge pressure (efficient vector or severe isolate), and the effect of some other devastating diseases (i.e. greening or blight) that can hamper a reliable evaluation of cross-protection experiments under field conditions.

Some limitations in the methodology can be visualized. The use of leaf piece grafts is not always a highly efficient means to transmit CTV from the donor propagation hosts to the receptor test plants (Rocha-Peña, 1990; Rocha-Peña *et al.* 1991). At least three inoculations with mild isolates were made before a minimum of 3 or 4 plants in most of the treatments were infected with mild isolates, as determined by DAS-ELISA. Some treatments, such as Valencia/*macrophylla* were not evaluated because of the lack of replications. This leaves the possibility that a lack of transmissibility by leaf piece grafts of the CTV challenge isolate can be interpreted erroneously as a protecting effect by mild isolates. On the other hand, the inoculum tissue with the severe isolate is left in place to enhance the probability of graft-transmission in the challenged plants. This would supply a permanent source of the severe isolate against the mild isolates which may provide a stronger challenge pressure when compared to natural conditions. If this occurs, mild isolates with the potential ability to protect under natural challenge conditions could be underestimated or overlooked.

ACKNOWLEDGEMENTS

This work was supported in part by USDA Specific Cooperative Agreement 58-43YK-0008 and by The Florida Citrus Production Managers' Association. Support by Consejo Nacional de Ciencia y Tecnologia and Instituto Nacional de Investigaciones Forestales y Agropecuarias, Mexico, for the senior author is also acknowledged. The authors thank Drs. S.M. Garnsey and T.A. Permar of the U.S.D.A, Orlando, Florida for providing the MCA-13 monoclonal antibody. The technical assistance of N. Berger, T. Nguyen, S. Jackson, and J. Zellers, and the advise of M. Ahnger with statistics, are appreciated.

Agricultural Experiment Station Journal Series No. R-02143.

LITERATURE CITED

Balaraman, K. and Ramakrishnan, K. 1980. Strain variation and cross-protection in citrus tristeza virus. pp. 60-68, in: Calavan, E.C., Garnsey, S.M., and Timmer, L.W. (eds). Proc. 8th Conf. Intern. Organ. Citrus Virol. Riverside, California.

Bar-Joseph, M. and Lee, R.F. 1989. Citrus tristeza virus. Description of Plant Viruses No. 353 (No. 33 revised). Commonwealth Mycological Institute/Association of Applied Biologists. Kew Surrey, England. 7p.

Bar-Joseph, M., Garnsey, S.M., Gonsalves, D., Moscovitz, M., Purcifull, D.E., Clark, M.F. and Loebenstein, G. 1979. The use of enzyme-linked immunosorbent assay for the detection of citrus tristeza virus. Phytopathology 69: 190-194.

Bar-Joseph, M., Marcus, R., and Lee, R.F. 1989. The continuous challenge of citrus tristeza virus control. Ann. Rev. Phytopathol. 27: 292-316.

Brlansky, R.H., Pelosi, R.R., Garnsey, S.M., Youtsey, C.O., Lee, R.F., Yokomi, R.K., and Sonoda, R.M. 1986. Tristeza quick decline epidemic in South Florida. Proc. Florida State Hort. Soc. 99:66-69.

Broadbent, P., Bevington, K.B., and Coote, B.G. 1991. Control of stem pitting of grapefruit in Australia by mild strain protection. pp. 64-70, in: Brlansky, R.H., Lee, R.F., and Timmer, L.W. (eds). Proc. 11th Conf. Intern. Organ. Citrus Virol. Riverside, California.

Clark, M.F., Lister, R.M., and Bar-Joseph, M. 1986. ELISA techniques. Methods in Enzymology. 118:742-767.

Costa, A.S. and Müller, G.W. 1980. Tristeza control by cross protection: A U.S.-Brazil cooperative success. Plant Dis. 64:538-541

DeLange, J.H., Van Vuuren, S.P., and Bredell, G.S. 1981. Groeipuntenting suiwer sitrusklone vir die superplantskema van virusse. Subtropica 2 (5) :11-16.

Fulton, R.W. 1986. Practices and precautions in the use of cross-protection for plant virus disease control. Ann. Rev. Phytopathol. 24:67-81.

Garnsey, S.M. and Jackson, J.L. 1975. A destructive outbreak of tristeza in central Florida. Proc. Florida State Hort. Soc. 88:65-69.

Garnsey, S.M. and Lee, R.F. 1988. Tristeza. pp. 48-50, in: Compendium of Citrus Diseases. Whiteside, J.O., Garnsey, S.M., and Timmer, L.W. (ed). APS Press. 80 p.

Garnsey, S.M. and Whidden, R. 1970. A rapid technique for making leaf tissue grafts to transmit citrus viruses. Plant Dis. Rep. 54:907-908.

Garnsey, S.M., Gumpf, D.J., Roistacher, C.N. Cirverolo, E.L., Lee, R.F., and Yokomi, R.K. 1987. Toward a standardized evaluation of the biological properties of citrus tristeza virus. Phytophylactica 19:151-157.

Gonsalves, D., Purcifull, D.E., and Garnsey, S.M. 1978. Purification and serology of citrus tristeza virus. Phytopathology 68:553-559.

Ieki, H. 1989. The use of cross-protection with mild strains of citrus tristeza virus (CTV) to control stem pitting disease of citrus in Japan. pp.8-14. FFTC Extension Bulletin No. 284. Taiwan, Republic of China.

Lee, R.F. 1984. Use of double stranded RNAs to diagnose citrus tristeza virus strains. Proc. Florida State Hort. Soc. 97:53-56.

Lee, R.F. and Rocha-Peña, M.A. 1991. Citrus Tristeza Virus. pp. 226-249., in: Mukhapadhyay, A.N., Chaube, H.S., Kumar, J. and Singh, U.S. (eds). Plant Diseases of International Importance III. Prentice Hall. New Jersey.

Lee, R.F., Brlansky, R.H., Garnsey, S.M. and Yokomi, R.K. 1987. Traits of citrus tristeza virus important for mild strain cross-protection of citrus: The Florida approach. Phytophylactica 19:215-218.

McClean, A.P.D. 1974. The tristeza virus complex. pp.59-66, in: Weathers, L.G. and Cohen, M. (eds). Proc. 6th Conf. Intern. Organ. Citrus Virol. Riverside, California.

Miller, T.J. and Stone, H.O. 1978. The rapid isolation of ribonuclease-free immunoglobulin G by Protein A-Sepharose affinity chromatography. Journal of Immunological Methods 4:111-125.

Miyakawa, T. 1987. Protection against citrus tristeza seedling yellows infection in citrus by pre-inoculation with stem pitting isolates. Phytophylactica 19:193-196.

Müller, G.W. 1980. Use of mild strains of citrus tristeza virus (CTV) to re-establish commercial production of "Pera" sweet orange in Sao Paulo, Brazil. Proc. Florida State Hort. Soc. 93:62-64.

Müller, G.W., Costa, A.S. Castro, S.L., and Guirado, N. 1988. Results from preimmunization tests to control the Capao Bonito strain of tristeza. pp. 82-85, in: Timmer, L.W., Garnsey, S.M., and Navarro, L. (eds). Proc. 10th Conf. Intern. Organ. Citrus Virol. Riverside, California.

Norman, G., Price, W.C., Grant, T.J., and Burnett, H. 1961. Ten years of tristeza in Florida. Proc. Florida State Hort. Soc. 74:107-111.

Permar, T.A., Garnsey, S.M., Gumpf, D.J., and Lee, R.F. 1990. A monoclonal antibody that discriminates strains of citrus tristeza virus. Phytopathology 80:224-228.

Rocha-Peña, M.A. 1990. Citrus tristeza virus: cross-protection, graft transmission, and serology. Ph.D. Dissertation. University of Florida. Gainesville. 104 p.

Rocha-Peña, M.A., Lee, R.F., Permar, T.A., Yokomi, R.K., and Garnsey, S.M. 1991. 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. pp. 93-102, in: Brlansky, R.H., Lee, R.F., and Timmer, L.W. (eds). Proc. 11th Conf. Intern. Organ. Citrus Virol. Riverside, California.

Roistacher, C.N., Dodds, J.A., and Bash, J.A. 1987. Means of obtaining and testing protective strains of seedling yellows and stem pitting tristeza virus: A preliminary report. Phytophylactica 19:199-203.

Roistacher, C.N., Dodds, J.A., and Bash, J.A. 1988. Cross protection against citrus tristeza seedling yellows and stem pitting viruses by protective isolates developed in greenhouse plants. pp. 91-100, in: Timmer, L.W., Garnsey, S.M., and Navarro, L. (eds). Proc. 10th Conf. Intern. Organ. Citrus Virol. Riverside, California.

Thornton, I.R., Emmett, R.W. and Stubbs, L.L. 1980. A further report on the grapefruit tristeza preimmunization trial at Mildura, Victoria. pp. 51-53., in: Calavan, E.C., Garnsey, S.M., and Timmer, L.W. (eds). Proc. 8th Conf. Intern. Organ. Citrus Virol. Riverside, California.

Van Vuuren, S.P. and Moll, J.N. 1987. Glasshouse evaluation of citrus tristeza virus isolates. Phytophylactica 19:219-221.

Van Vuuren, S.P., Collins, R.P., and Da Graca, J.V. 1991. The performance of exotic citrus tristeza virus isolates as preimmunizating agents for sweet orange on sour orange rootstock under natural disease pressure in South Africa. pp. 60-63, in: Brlansky, R.H., Lee, R.F., and Timmer, L.W. (eds). Proc. 11th Conf. Intern. Organ. Citrus Virol. Riverside, California.

Wallace, J.M. and Drake. 1976. Progress report of studies in California on preimmunization against tristeza in budded citrus. pp. 58-62, in: Calavan, E.C. (ed). Proc. 7th Conf. Intern. Organ. Citrus Virol. Riverside, California.

Yokomi, R.K. and Damgsteet, V.D. 1991. Comparison of citrus tristeza virus isolates transmission efficiency between *Toxoptera citricidus* and *Aphis gossypii*. pp. 319, in: Proceedings Aphid-Plant Interactions: Populations and Molecules. Oklahoma State University. Stillwater, Oklahoma.

Yokomi, R.K. and Garnsey, S.M., 1987. Transmission of citrus tristeza virus by *Aphis gossypii* and *Aphis citricola* in Florida. Phytophylactica 19:169-172.

Yokomi, R.K. and Garnsey, S.M., Lee, R.F., and Cohen, M. 1987. Use of insect vectors to screen for protecting effects of mild citrus tristeza virus isolates in Florida. Phytophylactica 19:183-185.

Yokomi, R.K., Garnsey, S.M., Permar, T.A., Lee, R.F. and Youtsey, C.O. 1991. Natural spread of severe citrus tristeza virus isolates in citrus preinfected with mild CTV isolates. pp. 86-92, in: Brlansky, R.H., Lee, R.F., and Timmer, L.W. (eds). Proc. 11th Conf. Intern. Organ. Citrus Virol. Riverside, California.