Evaluation of Recently Selected Mild Isolates of *Citrus tristeza virus* for Cross Protection of Hamlin Sweet Orange on Smooth Flat Seville Rootstock

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ABSTRACT. *Citrus tristeza virus* (CTV) isolates were collected from surviving trees in 1996-1997 from areas in Florida having a high incidence of decline on sour orange rootstock, and preliminary evaluations were made of their cross-protecting ability. Six isolates were selected for additional evaluation, and were compared for cross protection with five mild isolates previously evaluated (T11, T26, T30, T49, and T55) using two severe Florida isolates for the challenge. One severe isolate causes moderate, and the other mild stem pitting in sweet orange; both cause quick decline in trees on sour orange rootstock. Under glasshouse conditions, Hamlin sweet orange plants grafted onto smooth flat Seville rootstock were inoculated with all the mild isolates. After 7 mo, half of the plants in each treatment were challenged by grafting with both of the severe isolates. After graft take was confirmed, all plants were trained to one shoot. The shoots were harvested 6, 9, 13, 16, 20 and 29 mo post-challenge (MPC), and shoot weight, shoot length and leaf area were measured for each plant. Plants were tested on three occasions with double antibody sandwich indirect ELISA using polyclonal antibody G-604 and monoclonal antibody MCA-13 to detect broad-spectrum and severe strains of CTV, respectively. Stem pitting symptoms and increase in trunk diameter were evaluated at the end of the experiment (29 MPC). None of the mild strains increased the measured parameters when compared to the challenge-only control treatment. However, some challenged mild isolates took longer to show significant growth reduction as compared to the non-challenged control, indicating potential cross-protecting ability. Four of the recently selected mild isolates demonstrated better cross protection potential than the previously selected mild strains under the conditions of this evaluation.

Index words. Citrus tristeza virus, mild strain cross protection, serological detection.

Mild strain cross protection (MSCP) was defined by McKinney in 1929 as the phenomenon which occurs when a mild isolate of a virus prevents or delays the symptom development of a second, more severe isolate of the same virus when inoculated into the same plant (15). MSCP has been useful to help identify strains of the same virus, such as psorosis A and psorosis B (26), and has been used commercially with a number of crops (9). It has been a useful management strategy to maintain production of Pera sweet orange in Brazil (1, 7) and grapefruit in South Africa (28) and Australia (3) where severe stem-pitting strains of *Citrus tristeza virus* (CTV) limit yield and fruit quality and shorten the productive life of unprotected trees. All registered citrus trees in South Africa are propagated with a mild strain of CTV; the isolate used for cross protection depends on the cultivar (van-Vuuren, pers. comm.).

Florida situation. The brown citrus aphid, *Toxoptera citricida* Kirk, was found in Florida in November 1995, and has expanded its geographical range about 150 miles (240 km)/year (11). At the beginning of 2000, it was estimated that 14% of the sweet oranges and 42% of the grapefruit in Florida were on sour orange rootstock (5). From our observations, losses of trees on sour orange rootstock due to tristeza decline accelerate the second year following establishment of *T. citricida*. Epidemic losses on sour orange rootstock due to CTV have occurred the past 2 yr because of the stress of severe droughts in the spring of 2000 and 2001.

A state-wide survey using ELISA for detection of mild and decline strains of CTV was conducted prior to introduction of *T. citricida*. Addi-

tionally, selected samples were biologically indexed on Duncan grapefruit and Madam Vinous sweet orange (4). This systematic sampling of 20% of the commercial groves in Florida showed that decline strains of CTV, as detected by MCA13 (19), ranged in incidence from 7 to 23% in the five major growing areas. In residential areas the incidence of decline strains ranged from 1.8 to 6%. Also, biological indexing indicated that some trees from residential areas harbored CTV strains which would cause moderate to severe stem pitting on Duncan grapefruit and/or mild stem pitting on sweet orange, but no stem pitting was seen in the field. Further evidence that stem pitting strains of CTV may already be present in Florida in commercial areas has been provided from single-aphid transmissions using *T. citricida*. Nine out of 42 single-aphid transmitted sub-isolates from a typical Florida decline isolate, T66, caused stem pitting on Pineapple sweet orange (27). Single-aphid transmissions with the *T. citricida* from other Florida isolates have resulted in finding decline isolates among the resultant sub-isolates (2). Thus, Florida may lose the remainder of the trees on sour orange rootstock, and then face spread of CTV strains of increasing severity which might cause stem pitting of orange and grapefruit scions, regardless of the rootstock. This occurred in Venezuela from 1976 to 1995 following the establishment of *T. citricida* (23).

The selection of mild isolates useful for MSCP has been empirical as little is known about the mechanism of cross-protection of CTV in citrus (12, 20). At the 14th Conference of the IOCV we reported the use of molecular probes to aid in the selection of mild isolates useful for MSCP (18). We now report the results of a greenhouse evaluation of six mild isolates selected using the protocol described by Ochoa et al. (18), comparing their cross-protection potential with previously selected mild isolates (21, 24, 32). The challenge isolates used for this experiment were chosen to represent typical decline isolates which cause stem pitting in indicator plants that are present in Florida and which are likely to spread. The scion-rootstock combination selected for evaluation was one which may become popular in Florida in the future: Hamlin sweet orange on smooth flat Seville rootstock. Hamlin is a vigorous sweet orange which comprises about 23% of the annual propagations in the state (http://doacs.state.fl.us/~pi/budwood/cbrbhome.html), while smooth flat Seville is a CTV-tolerant rootstock producing fruit quality similar to that of sour orange (6).

MATERIALS AND METHODS

Selection of mild isolates. The procedure used to select the "new" mild CTV isolates has been described in detail by Ochoa et al. (18). Briefly, budwood was collected from surviving trees in fields where CTV was causing mass death of trees on sour orange rootstock. A budchip from each stick of budwood was inoculated into six to eight grapefruit and/or sweet orange budlings on sour orange rootstock in the greenhouse to establish an *in planta* culture. When the plants were ELISA-positive for CTV, two or three of the infected budlings were challenge-inoculated with several very severe decline and stem pitting isolates collected from Florida. If the plants continued to grow and develop new flush, the challenged plants were thrown away, and the remaining *in planta* cultures, established from the original field isolate, were then used as source plants for single-aphid transmissions with *T. citricida*.

The resulting sub-isolates were tested with molecular methods to determine if mild, severe, or a mixture of mild and severe isolates of CTV were present (18). After the molecular screening, the apparent

mild-only isolates were grafted into Mexican lime, Duncan grapefruit, and pineapple sweet orange to ensure that the isolates were mild in these hosts. We selected six sub-isolates obtained from isolates originally collected from the field in 1996-1997, which appeared to contain only mild strains of CTV: 97-1-5-1, St. Cloud Bk 12 2-17 SO-1, 96-8 Babb, 97-1-6-p1, 97-1-7-p1, and 97-1-2-p2. Since these sub-isolates had been aphid transmitted, they should only contain CTV, although other aphid transmitted citrus viruses could be present. Five previously selected and tested mild isolates were used for comparison: T26, T30, T49, T55a and T11a.

T26 has been shown to protect Valencia sweet orange on sour orange and *C. macrophylla* rootstocks against the quick decline isolate T66a in Florida under greenhouse conditions (24). T55a, an aphid-transmitted sub-isolate of T55, was able to protect Valencia sweet orange on *C. macrophylla* rootstock against T66a (24). T30 protects against quick decline isolate T36, but not so well against T66 under greenhouse conditions (31). The combination of isolates T26 and T30 was shown to protect well against T36-type Florida quick decline isolates (24, 32). T49 is reported to provide protection against decline in sweet orange on sour orange rootstock (24, 30). T11a is a mild isolate, but previous research has indicated it does not cross-protect against CTV decline, and may often interact to produce symptoms more severe than the those of the severe challenge isolate alone (24).

Severe isolates. The challenge isolates used were FL-169, a quick decline isolate causing mild stem pitting in sweet orange (4), and T-68, a quick decline isolate causing moderate stem pitting on sweet orange (17). These Florida isolates may represent severe isolates which can be expected to appear in the Florida industry in the next decade.

Inoculations. All CTV isolates were maintained in a greenhouse having 18-23/30-35°C night and day temperatures, respectively, in Madam Vinous sweet orange; this host can be more consistently infected upon graft-transmission (25). Three leaf pieces from each source plant were inoculated on each of ten Hamlin plants grafted on smooth flat Seville rootstock (25). At this time, 20 plants were selected for use in no-mild control treatments. Graft take was evaluated after 3 weeks and plants were re-grafted if needed with three more leaf pieces from the original source plant, and graft take was recorded 3 weeks later. At this time at least one leaf piece on each plant remained alive. Three months later, plants were tested for the presence of CTV using a direct tissue blot immunoassay (DTBI) (8) and polyclonal antiserum G-604 (14). Three months after the DTBI, five plants from each mild treatment were challenged by grafting one bark patch from the T68 source plant and one from the FL-169 source plant. Each bark patch was about 5 × 10 mm. This dual-challenge method was used to obtain symptom development in a shorter time than when using a single isolate for challenge (24, 25). The remaining five plants in each mild-treatment remained unchallenged. The 20 control plants were divided into four treatments: unchallenged, dually-challenged, challenged with T68 only, and challenged with FL169 only. The graft take from the challenge inoculations was recorded after three weeks and new grafts were made where one or both grafts did not survive. After three more weeks, graft take was recorded, and at this point, both severe isolate grafts as well as at least one mild isolate graft were alive on all plants. Challenge grafts were left in place to create a continuous infection source (13).

Measurements. Plant growth was measured from 3 mo post-chal-

lenge (3 MPC) until 29 MPC. At 3 MPC, plants were cut back and trained to one shoot. At 6, 9, 13, 16, 20 and 29 MPC shoots were cut back above the first leaf from the previous cutback and allowed to grow one new flush from the remaining node. Weight, length and leaf area of the removed part of the stem was measured.

At 3, 6, 9, 16, and 29 MPC, the harvested shoots were tested using double antibody sandwich indirect (DASI)-ELISA (22) using polyclonal antiserum G-604 for broad spectrum detection (14) and monoclonal antibody MCA-13 for detection of severe strains (19).

Stem-pitting symptoms were evaluated by counting the number of pits in the proximal 10 cm of the harvested flush at 20 and 29 MPC. At the end of the experiment (29 MPC), the remaining tissues from the shoots grown after 3 MPC were cut, and the bark was peeled. Stem-pitting on the older tissue between where the plants were cut back at 3 MPC and 29 MPC was rated using a scale of 0 (no pits) to 4 (countless pits with visible growth-reduction of the flush).

Initial trunk diameter (0 MPC) and final trunk diameter (29 MPC) at 1 cm above the bud union were measured. A mark was made on the stem with a waterproof pen to indicate the exact position and orientation of the measuring caliper. Relative trunk diameter growth was calculated as the difference between initial and final trunk diameter, divided by the initial diameter. Also, after removing the stem between the 3 and 29 MPC cutbacks to determine the amount of stem pitting, the stem diameter of the xylem tissue (peeled stem) at the proximal end of the stem where the 3 MPC flush was removed was measured.

Experimental layout. The experiment was set up using a randomized block design. Initial plant size and location in the greenhouse were expected to have an effect on growth, therefore blocks were designed to distribute plant sizes from large in block 1 to small in block 5. Treatment effect was evaluated using ANOVA with a confidence level of 90%. Individual treatment effects on shoot weight, shoot length, leaf area and number of pits were evaluated using the Duncan multiple range test, with a confidence level of 90%. Shoot weight, shoot length and leaf area were accumulated over the entire duration of the experiment and evaluated in the same fashion.

Treatment effect on stem pitting was evaluated using ANOVA after performing a square root transformation of the stem pit count and average stem pit rating. Because of the high number of zero-values in the unchallenged treatments, only data from the challenged treatments were analyzed.

Evaluation of results. Detrimental effects of the mild isolates on plant growth were examined by comparing growth data of unchallenged mild isolate treatments with the healthy, uninoculated treatment. The effect of the challenge isolates on plant growth was evaluated by comparing growth parameters between the challenged and the healthy uninoculated controls. The MSCP effect of the mild isolates was evaluated by comparing growth of the challenged mild isolate plants with the challenged healthy treatment and with the mild-only treatments.

RESULTS

Growth parameters. An overview of significant differences in growth between treatments is given in Table 1. Significant treatment effects at 90% confidence were found on all harvest dates except for 13 MPC. Significant reductions in growth as compared to the respective reference treatments are indicated in Table 1. Table 2 contains the mean growth parameters for 29 MPC and for the cumulative data,

TABLE 1
SUMMARY OF SIGNIFICANT TREATMENT EFFECTS OF *CITRUS TRISTEZA VIRUS* ISOLATES ON GROWTH[z]

	6 MPC[y]			9 MPC			13 MPC			16 MPC			20 MPC			29 MPC			Cum.[x]			Diameters			
	w[w]	l[v]	a[u]	w	l	a	w	l	a	w	l	a	w	l	a	w	l	a	w	l	a	0	29	Rel[t]	Sht[s]
Treatment effect (ANOVA)	X		X	X	X	X				X	X	X	X		X	X	X	X	X	X	X		X		X
Effect of mild on growth																									
T30/T26 (1)																									
T49 (2)																									
T55 (3)																									
97-1-5-1 (6)															X										
St Cloud Bk 12 2-17 SO-1 (7)																									
96-8 Babb (8)																									
97-1-6 p1 (9)						X							X		X										
97-1-7 p1 (10)															X										
97-1-2 p2 (11)																									
T11a (12)																									
Controls																									
Dual challenge													X		X	X	X			X					
T-68													X		X	X	X	X		X	X				
FL-169																									

[z]An 'x' indicates significantly less growth as compared to the control treatment. In the case of "Effect of mild (isolates) on growth" and "Controls", the healthy unchallenged treatment is used as the control. In the case of the "Effect of the challenge (isolate)", the corresponding mild-unchallenged treatment is used as the control.
[y]MPC = months post challenge.
[x]Cum. = cumulative data from 6 MPC, 9 MPC, 13 MPC, 16 MPC, 20 MPC, and 29 MPC.
[w]W = weight of flush removed.
[v]L = length of flush removed.
[u]A = leaf area of leaves on the removed flush.
[t]rel = relative diameter of the trunk. This measurement was the final trunk diameter minus the beginning trunk diameter, divided by the beginning trunk diameter.
[s]sht = the final diameter of the flush immediately below the site where the first flush had been removed at 6 MPC.

TABLE 1 (CONTINUED)
SUMMARY OF SIGNIFICANT TREATMENT EFFECTS OF *CITRUS TRISTEZA VIRUS* ISOLATES ON GROWTH[z]

	6 MPC[y]			9 MPC			13 MPC			16 MPC			20 MPC			29 MPC			Cum.[x]			Diameters			
	w[w]	l[v]	a[u]	w	l	a	w	l	a	w	l	a	w	l	a	w	l	a	w	l	a	0	29	Rel[t]	Sht[s]
Effect of Challenge																									
T30/T26 (1)	X			X	X	X				X	X	X	X		X	X	X	X	X	X	X				X
T49 (2)	X			X		X				X	X	X	X		X	X		X	X	X	X		X		
T55 (3)				X	X	X				X	X	X	X		X	X	X	X	X	X	X		X		X
97-1-5-1 (6)																	X								
St Cloud Bk 12 2-17 SO-1 (7)	X		X	X	X	X				X	X	X	X		X	X		X	X	X	X				X
96-8 Babb (8)													X		X	X	X			X					X
97-1-6 p1 (9)																X		X							
97-1-7 p1 (10)																X		X							X
97-1-2 p2 (11)													X		X										
T11a (12)				X		X				X	X	X	X		X	X	X	X	X	X	X				

[z]An 'x' indicates significantly less growth as compared to the control treatment. In the case of "Effect of mild (isolates) on growth" and "Controls", the healthy unchallenged treatment is used as the control. In the case of the "Effect of the challenge (isolate)", the corresponding mild-unchallenged treatment is used as the control.
[y]MPC = months post challenge.
[x]Cum. = cumulative data from 6 MPC, 9 MPC, 13 MPC, 16 MPC, 20 MPC, and 29 MPC.
[w]W = weight of flush removed.
[v]L = length of flush removed.
[u]A = leaf area of leaves on the removed flush.
[t]rel = relative diameter of the trunk. This measurement was the final trunk diameter minus the beginning trunk diameter, divided by the beginning trunk diameter.
[s]sht = the final diameter of the flush immediately below the site where the first flush had been removed at 6 MPC.

TABLE 2
MEAN GROWTH PARAMETERS FOR 29 MONTHS POST CHALLENGE (MPC) AND FOR THE CUMULATIVE DATA, INCLUDING TRUNK AND STEM DIAMETERS AT 29 MPC FOR MILD ISOLATES OF *CITRUS TRISTEZA VIRUS*

		29 MPC				Cumulative			Stem diameters			
Mild isolate	Challenge isolate(s)	Weight (cm)	Area (cm^3)	Length (cm)	Pits/ 10 cm	Weight (cm)	Area (cm^3)	Length (cm)	0 MPC	29 MPC	Relative diameter growth[z]	29 MPC, wood diam., base of first flush
		P = 0.0001*				P = 0.0001*			P = 0.5075*	P = 0.0540*	P = 0.7143*	P = 0.0001*
T26/T30	-	88 (abc)	1653 (abc)	94.0 (ab)	0 (d)	292.1 (ab)	6688 (ab)	308.8 (a)	0.64	1.15 (bcd)	1.03	0.79 (abc)
	T68/FL169	31.2 (h)	902 (efg)	38.4 (ghi)	40 (bc)	107.3 (h)	2965 (h)	145.3 (gh)	0.64	1.10 (cd)	0.81	0.50 (h)
T49	-	104.9 (a)	1914 (a)	75.4 (bcd)	0 (d)	330.5 (a)	7103 (a)	303.7 (a)	0.68	1.31 (a)	1.11	0.80 (abc)
	T68/FL169	52.7 (efgh)	1239 (bcdefg)	59.8 (cdefgh)	40 (bc)	169.5 (efgh)	4353 (defgh)	202.7 (defgh)	0.54	1.11 (cd)	1.36	0.67 (cdefg)
T55	-	79.1 (abcd)	1817 (ab)	70.4 (bcdef)	0 (d)	273.7 (abc)	6484 (ab)	285.0 (abc)	0.65	1.21 (abc)	1.00	0.78 (abc)
	T68/FL169	27.6 (h)	682 (g)	28.2 (i)	28 (bcd)	121.8 (gh)	3241 (gh)	137.5 (h)	0.60	0.99 (d)	0.74	0.55 (gh)
Healthy	-	70.1 (bcde)	1587 (abcd)	69.8 (bcdef)	0 (d)	252.4 (abcde)	6237 (abc)	285.2 (abc)	0.57	1.14 (bcd)	1.11	0.75 (abcd)
	T68/FL169	43.2 (fgh)	1053 (cdefg)	33.6 (hi)	49 (b)	176.9 (efgh)	4512 cdefgh)	183.5 (efgh)	0.58	1.13 (bcd)	1.04	0.66 (cdefg)
	T68	39 (gh)	731 (g)	41.0 (ghi)	100 (a)	187.7 (defgh)	3999 (fgh)	162.9 (fgh)	0.70	1.20 (abc)	0.81	0.73 (abcde)
	FL169	85.5 (abc)	1810 (ab)	75.8 (bcd)	0 (d)	263.5 (abcd)	5703 (abcdef)	240.0 (abcdef)	0.55	1.20 (abc)	1.49	0.80 (abc)
97-1-5-1	-	87.4 (abc)	1693 (ab)	82.0 (bc)	0 (d)	224.2 (bcdef)	4979 (bcdefg)	243.8 (abcde)	0.62	1.14 (bcd)	0.94	0.75 (abcd)
	T68/FL169	61.8 (abc)	1401 (abcdef)	51.6 (defghi)	24 (bcd)	175.3 (efgh)	4197 (efgh)	179.4 (efgh)	0.67	1.18 (abc)	1.06	0.67 (cdefg)
St Cloud Bk	-	80.8 (abc)	1587 (abcd)	75.6 (bcd)	0 (d)	266.5 (abcd)	6200 (abc)	295.4 (ab)	0.69	1.20 (abc)	0.87	0.83 (ab)
12 2-17 SO-1	T68/FL169	32.9 (h)	849 (efg)	50.0 (defghi)	23 (bcd)	113.2 (gh)	3120 (h)	208.4 (cdefgh)	0.56	1.09 (cd)	0.96	0.57 (fgh)
96-8 Babb 2	-	85.5 (abc)	1863 (ab)	110.8 (a)	0 (d)	251.1 (abcde)	5903 (abcde)	294.0 (ab)	0.69	1.28 (ab)	1.04	0.86 (a)
	T68/FL169	51.4 (efgh)	1278 (bcdefg)	48.0 (defghi)	43 (bc)	174.3 (efgh)	4215 (efgh)	174.7 (efgh)	0.65	1.20 (abc)	0.94	0.66 (cdefg)
97-1-6 p1	-	63.5 (bcdefg)	1374 (abcdef)	64.8 (cdefg)	0 (d)	187.4 (defgh)	4531 (cdefgh)	209.7 (cdefgh)	0.61	1.14 (bcd)	0.96	0.70 (bcdef)

[z]The relative growth in trunk diameter was determined by measuring at 29 MPC, subtracting the beginning trunk diameter, then dividing by the beginning trunk diameter.
*These values represent the P-values found by analysis of variance. Letters within each column indicate significant differences between treatments within the same column using the Duncan multiple range test at a confidence level of 90%. Duncan tests were not performed in columns where P > 0.1000.

TABLE 2 (CONTINUED)
MEAN GROWTH PARAMETERS FOR 29 MONTHS POST CHALLENGE (MPC) AND FOR THE CUMULATIVE DATA, INCLUDING TRUNK AND STEM DIAMETERS AT 29 MPC FOR MILD ISOLATES OF *CITRUS TRISTEZA VIRUS*

		29 MPC				Cumulative			Stem diameters			
Mild isolate	Challenge isolate(s)	Weight (cm)	Area (cm^3)	Length (cm)	Pits/ 10 cm	Weight (cm)	Area (cm^3)	Length (cm)	0 MPC	29 MPC	Relative diameter growth[z]	29 MPC, wood diam., base of first flush
		P = 0.0001*				P = 0.0001*			P = 0.5075*	P = 0.0540*	P = 0.7143*	P = 0.0001*
	T68/FL169	30.6 (h)	707 (g)	43.2 (fghi)	16 (cd)	122.4 (gh)	3002 (h)	155.0 (gh)	0.61	1.09 (cd)	1.14	0.60 (efgh)
97-1-7 p1	-	84.3 (abc)	1844 (ab)	70.8 (bcdef)	0 (d)	219.2 (bcdef)	5304 (bcdef)	222.2 (bcdefg)	0.57	1.13 (bcd)	1.12	0.78 (abc)
	T68/FL169	42.9 (fgh)	1017 (defg)	44.0 (efghi)	25 (bcd)	155.4 (fgh)	3996 (fgh)	177.9 (efgh)	0.62	1.12 (bcd)	0.89	0.61 (defgh)
97-1-2 p2	-	67.7 (fgh)	1543 (abcd)	71.4 (bcde)	0 (d)	197.7 (cdefg)	4986 (bcdefg)	245.2 (abcde)	0.59	1.06 (cd)	0.93	0.72 (abcde)
	T68/FL169	53.9 (defgh)	1245 (bcdefg)	52.0 (defghi)	28 (bcd)	158.1 (fgh)	3942 (fgh)	183.9 (efgh)	0.59	1.07 (cd)	0.91	0.61 (defgh)
T11a	-	89.9 (ab)	1447 (abcde)	76.2 (bcd)	0 (d)	274.7 (abc)	6086 (abcd)	274.8 (abcd)	0.57	1.19 (abc)	1.14	0.80 (abc)
	T68/FL169	29.1 (h)	795 (fg)	32.8 (hi)	24 (bcd)	128.6 (gh)	3081 (h)	167.5 (efgh)	0.56	1.12 (cd)	1.12	0.71 (bcdef)

[z]The relative growth in trunk diameter was determined by measuring at 29 MPC, subtracting the beginning trunk diameter, then dividing by the beginning trunk diameter.
*These values represent the P-values found by analysis of variance. Letters within each column indicate significant differences between treatments within the same column using the Duncan multiple range test at a confidence level of 90%. Duncan tests were not performed in columns where P > 0.1000.

and the trunk and stem diameter measurements taken at the end of the experiment.

In general, growth of the plants infected with the mild isolates was not significantly different from the growth of uninoculated plants, except for some parameters at 20 MPC, when leaf area was significantly reduced by isolates 97-1-5-1; 97-1-6 p1 and 97-1-2 p2. Isolate 97-1-6 p1 also significantly reduced the leaf area at 9 MPC and weight of removed flush at 20 MPC (Table 1). These significant reductions in growth were not present at 29 MPC or when the cumulative growth data were analyzed.

Effect of challenge isolates. Significant growth reduction caused by the combined challenge isolates, compared to growth of healthy plants, was not present until 20 and 29 MPC. Isolate T68, by itself, significantly reduced shoot weight and leaf area at both 20 and 29 MPC, and also significantly reduced shoot length at 29 MPC. Severe isolate FL169 caused no significant reduction in growth. The dual challenge treatment (T-68 plus FL-169) caused significant growth reduction similar of that of T68 alone. The cumulative data also show significant growth reduction caused by the T68 and dual severe-isolate infection, but not by FL169 infection. It is interesting to note that the stem pitting of T68 was significantly less when dually inoculated with FL-169, an isolate that causes mild sweet orange stem pitting, than when inoculated alone (Table 2).

Cross-protection effects. Significant growth reduction in mild-inoculated, challenged treatments, compared to the corresponding mild-only treatment started at 6 MPC for the T26/T30 combination, T49 and the St. Cloud Bk 12 2-17 SO-1 mild isolates. At 9 MPC these effects continued in these isolates, and significant growth reduction was noticed also with the T55 and T11a isolates. This trend continued through 16 MPC. At 20 MPC, significantly less growth was found in the 96-8-Babb challenge, compared to its corresponding mild-only control.

Isolates 97-1-5-1, 97-1-6 p1, 97-1-7 p1 and 97-1-2 p2 occasionally produced significant growth reductions with the challenge treatment, mostly at later dates (20 and 29 MPC). Statistical analysis of the cumulated growth data show an overall significant growth reduction caused by the challenge for mild isolates T11a, T26/T30, T49, T55, St. Cloud Bk 12 2-17 SO-1, and 96-8 Babb, but not for 97-1-5-1, 97-1-6-p1, 97-1-7-p1 and 97-1-2-p2 (Table 2).

Trunk diameters. Significant treatment effects on trunk diameter were found only at 29 MPC and with proximal stem diameters of the peeled first flush at 29 MPC, but not in the relative trunk diameter growth (Table 2). Only challenged T49 and T55 treatments showed significant diameter reduction, compared to their corresponding unchallenged mild treatments. Peeled first flush diameter was significantly less in the challenged T26/T30, T55, St Cloud Bk 12 2-17 SO-1, 96-8 Babb and 97-1-7 p1 isolates. The severe challenge isolates did not have a significant effect on the stem diameter.

Serological assays. Antibody MCA13, which reacts selectively with severe strains of CTV (19), should detect the presence of the challenge isolate, as it does not react with any of the mild isolates used in this experiment. DTBI performed three months after inoculation of the mild isolates showed a general infection rate of 62%. Average optical density values (OD_{405}) for tests at 3, 6, 9, 16 and 29 MPC, with their statistical analysis, are summarized in Table 3. No treatment effect was found in the MCA13 ELISA at 3 MPC (Table 3), and only 18% of the challenged plants were MCA13-positive at this time (Table 4). At 6 MPC 89% of the challenged plants were MCA-13-positive, and this percentage increased to and stabilized

TABLE 3
MEAN ELISA VALUES FOR ALL PLANTS IN THE TREATMENT INDICATED AT THE DIFFERENT MONTHS POST *CITRUS TRISTEZA VIRUS* CHALLENGE (MPC) INDICATED

	3 MPC		6 MPC		9 MPC		16 MPC		29 MPC	
	Poly[z]	Mono[y]	Poly[z]	Mono[y]	Poly[z]	Mono[y]	Poly[z]	Mono[y]	Poly[z]	Mono[y]
Treatment	0.0007*[x]	0.4582*[x]	0.0001*[x]	0.0023*[x]	0.0001*[x]	0.0001*[x]	0.0006*[x]	0.0001*[x]	0.0001*[x]	0.0001*[x]
T30-T26	1.948 (ab)	0.136	0.141 (def)	0.012 (c)	0.435 (de)	0.020 (c)	0.916 (abcd)	0.059 (e)	0.476 (d)	0.017 (f)
T30-T26/Challenge	1.550 (abcd)	0.572	0.505 (a)	0.394 (ab)	0.989 (c)	0.681 (b)	1.307 (ab)	2.328 (ab)	0.774 (ab)	0.564 (abc)
T49	0.828 (cde)	0.118	0.100 (def)	0.009 (c)	0.400 (de)	0.043 (c)	0.717 (abcd)	0.047 (e)	0.461 (d)	0.020 (f)
T49/Challenge	1.791 (abc)	0.135	0.550 (a)	0.393 (ab)	1.384 (a)	0.964 (a)	0.853 (abcd)	1.454 (bc)	0.830 (a)	0.486 (abcd)
T55	1.037 (bcde)	0.125	0.148 (def)	0.008 (c)	0.273 (ef)	0.010 (c)	0.435 (cde)	0.022 (e)	0.537 (d)	0.020 (f)
T55/Challenge	2.547 (a)	1.737	0.528 (a)	0.568 (a)	1.211 (abc)	0.783 (ab)	0.804 (abcd)	1.585 (bc)	0.843 (a)	0.564 (abc)
Healthy	0.156 (e)	0.120	0.027 (f)	0.011 (c)	0.009 (f)	0.012 (c)	0.044 (e)	0.017 (e)	0.037 (e)	0.012 (f)
Dual Challenge	0.449 (de)	0.791	0.488 (a)	0.157 (bc)	1.089 (abc)	0.791 (ab)	1.357 (a)	2.548 (a)	0.836 (a)	0.630 (a)
T68	0.152 (e)	0.118	0.292 (bc)	0.127 (bc)	0.981 (c)	0.638 (b)	0.879 (abcd)	1.639 (bc)	0.616 (bcd)	0.447 (bcd)
FL 169	0.147 (e)	0.113	0.199 (cd)	0.088 (bc)	0.602 (d)	0.218 (c)	0.806 (abcd)	0.510 (de)	0.552 (cd)	0.287 (e)
97-1-5-1	1.440 (bcd)	0.128	0.216 (cd)	0.013 (c)	0.438 (de)	0.024 (c)	0.764 (abcd)	0.061 (e)	0.443 (d)	0.017 (f)
97-1-5-1/Challenge	1.272 (bcd)	0.126	0.467 (a)	0.203 (bc)	1.073 (abc)	0.647 (b)	1.285 (ab)	2.076 (abc)	0.610 (bcd)	0.388 (de)
St Cloud Bk 12	0.593 (de)	0.120	0.044 (ef)	0.017 (c)	0.015 (f)	0.007 (c)	0.036 (e)	0.024 (e)	0.011 (e)	0.011 (f)
St Cloud Bk 12/Challenge	0.449 (de)	0.803	0.490 (a)	0.517 (a)	1.048 (bc)	0.688 (b)	0.957 (abcd)	1.936 (abc)	0.784 (a)	0.556 (abc)
96-8 Babb 2	1.197 (bcde)	0.128	0.157 (cdef)	0.008 (c)	0.535 (de)	0.018 (c)	0.812 (abcd)	0.056 (e)	0.515 (d)	0.020 (f)
96-8 Babb 2/Challenge	0.869 (bcde)	0.404	0.517 (a)	0.185 (bc)	1.150 (abc)	0.692 (b)	1.103 (ab)	1.826 (abc)	0.756 (ab)	0.514 (bcd)
97-1-6 p1	1.206 (bcde)	0.137	0.107 (def)	0.014 (c)	0.394 (de)	0.091 (c)	0.320 (de)	0.024 (e)	0.535 (d)	0.024 (f)
97-1-6 p1/Challenge	1.158 (bcde)	0.129	0.482 (a)	0.161 (bc)	0.927 (c)	0.649 (b)	1.381 (a)	1.972 (abc)	0.760 (ab)	0.520 (abcd)
97-1-7 p1	1.970 (ab)	0.128	0.192 (cd)	0.019 (c)	0.406 (de)	0.021 (c)	0.686 (bcd)	0.043 (e)	0.470 (d)	0.021 (f)
97-1-7 p1/Challenge	1.789 (abc)	0.312	0.426 (a)	0.129 (bc)	1.322 (ab)	0.850 (ab)	0.828 (abcd)	1.263 (cd)	0.850 (a)	0.579 (ab)
97-1-2 p2	0.979 (bcde)	0.123	0.190 (cde)	0.010 (c)	0.464 (de)	0.017 (c)	0.960 (abcd)	0.118 (e)	0.536 (d)	0.027 (f)
97-1-2 p2/Challenge	1.858 (abc)	0.196	0.402 (ab)	0.112 (bc)	1.204 (abc)	0.672 (b)	1.042 (abc)	1.448 (bc)	0.710 (abc)	0.428 (cd)
T11a	1.338 (bcd)	0.122	0.121 (def)	0.008 (c)	0.359 (de)	0.012 (c)	0.684 (bcd)	0.035 (e)	0.471 (d)	0.021 (f)
T11a/Challenge	1.248 (bcde)	0.843	0.466 (a)	0.127 (bc)	1.096 (abc)	0.638 (b)	1.031 (abc)	1.195 (cd)	0.878 (a)	0.617 (a)

[z]Poly = polyclonal ELISA test for detecting all CTV isolates.
[y]Mono = monoclonal antibody MCA13 used for selective detection of severe CTV isolates (19).
[x]Values marked with * represent the P-values found by analysis of variance. Letters within each column indicate significant differences between treatments within the same column using the Duncan multiple range test at a confidence level of 90%. Duncan tests were not performed in columns where P > 0.1000.

TABLE 4
NUMBER OF PLANTS RATED AS *CITRUS TRISTEZA VIRUS* (CTV)-POSITIVE USING POLYCLONAL AND MONOCLONAL ELISA TO DETECT ALL ISOLATES OR SEVERE ISOLATES ONLY, RESPECTIVELY, AT THE DIFFERENT TEST DATES

	Polyclonal[z]		Monoclonal[y]	
3 MPC[x]	91/108[w]	84%	11/61[w]	18%
6 MPC	102/114	89%	58/65	89%
9 MPC	109/113	96%	60/64	94%
16 MPC	94/98	96%	46/49	94%
29 MPC	104/110	95%	59/63	94%

[z]Polyclonal = Using polyclonal antibodies for detection of all isolates of CTV.
[y]Monoclonal = Using monoclonal antibody MCA13 which selectively detects decline isolates of CTV (19).
[x]MPC = months post challenge.
[w]Number of plants found positive for CTV/total number of plants tested. The number of plants tested varied among test dates depending on the availability of suitable tissue. A maximum of 96% of plants expected to be polyclonal positive, and 94% of plants expected to be MCA13 positive actually tested as such.

at 94% at 9 MPC and later test dates (Table 4). No correlation was found between the ELISA values and the growth data analysis summarized in Table 1.

Of the 100 plants inoculated with mild isolates and expected to be positive to the polyclonal ELISA before challenge, 62 were positive. However, 100% of the grafts on these plants were alive at that time. Although all mild-inoculation grafts for St Cloud Bk 12 2-17 SO-1 survived throughout the experiment, none of the plants were positive in the DTBI test done 3 mo after grafting. After the challenge inoculations, only 7 out of 24 tests in the mild-only control were found positive by polyclonal ELISA.

Symptoms. Only 9 of the 60 plants inoculated with T-68 showed stem pitting at 20 MPC, and 52 of 60 at 29 MPC. Analysis of variance showed some treatment effect ($P = 0.117$) on stem pit counts at 29 MPC. Only the challenged mild isolate 97-1-6-p1 had significantly fewer pits than the challenge-only control (Table 2). Dually challenged isolates T55, 97-1-5-1, St. Cloud Bk 12 2-17 SO-1, 97-1-6 p1, 97-1-7-p1, 97-1-2-p2 and T11a had significantly fewer pits than the healthy treatment challenged with T68 alone; however the healthy treatment with dual challenge also had significantly fewer pits than plants inoculated with T68 alone (Table 2). No significant differences between challenged treatments were found in stem pit ratings.

No correlation was found between the number of pits and the growth parameters at 29 MPC. However, some correlation was found between average stem pitting ratings and area, length and weight at 29 MPC and cumulative area, length, and shoot weight (Table 5).

DISCUSSION

MSCP using properly selected mild isolates is a valuable management tool to continue citrus production in the presence of severe strains of CTV which cause decline on sour orange and stem pitting of scions. Over the previous two decades, we have selected five mild isolates from Florida which appear to slow the rate of decline on sour orange in Florida (24, 32). When evaluated for MSCP in South Africa (29), isolate T30 slowed the rate of decline of sweet orange on sour orange rootstock although the trees were dwarfed, and in Brazil (30) grapefruit trees on sour orange rootstock were stunted,

TABLE 5
CORRELATION BETWEEN THE NUMBER OF STEM PITS/10 CM AND THE GROWTH PARAMETERS INDICATED AT 29 MO POST *CITRUS TRISTEZA VIRUS* CHALLENGE AND WITH THE GROWTH PARAMETERS CUMULATIVELY FROM ALL EVALUATIONS

Growth parameter	Formula	R^2	P-value
Area	1686 - 270.8 x	0.39	<0.0001
Length	77.2 - 12.9 x	0.40	<0.0001
Weight	82.5 - 16.1 x	0.55	<0.0001
Cumulated area	5862.3 - 844.4 x	0.43	<0.0001
Cumulated length	267.1 - 36.8 x	0.43	<0.0001
Cumulated weight	254.2 - 42.0 x	0.45	<0.0001

but decline was delayed and fruit size remained good. Because the efficiency of MSCP of CTV is strain-specific and host-specific (12), we undertook the collection of additional mild isolates in 1996-1997 before mild strains became masked by severe strains due to increased spread of CTV by *T. citricida*. While a long-term objective for management of CTV is the development of CTV-resistant scions and rootstocks by genetic engineering, it will probably be two decades before any useful result is available to growers. Therefore, MSCP will continue to play a valuable role in sustaining citrus production in Florida, and continued selection will be necessary to make sure growers have mild strains available for cross protection.

Selection of mild isolates useful for MSCP has been an empirical process traditionally, often taking 12-15 yr (7, 12). Recently we reported on an expedited selection procedure (18). This involved selection of isolates from surviving trees on sour orange rootstock, establishment of the isolates *in planta,* and preliminary graft challenge. If the challenged plants continued to grow for 2-3 flushes, unchallenged cultures of the isolate were used as a source for single-aphid transmissions using *T. citricida*. The resultant sub-isolates were screened by molecular probes to determine if they were composed of mild strains, severe strains, or a mixture of both. Isolates or sub-isolates consisting only of mild strains were then selected for greenhouse evaluation of their cross protecting ability.

Within 5 yr we have completed the above selection procedure and greenhouse evaluation and have identified four mild isolates (97-1-5-1, 97-1-6 p1, 97-1-7 p1, and 97-1-2 p2) which appear to have better cross protection potential than the previous mild isolates extensively used and tested in Florida. Of course, the new isolates need to be tested further with other scion/rootstock combinations and under field conditions, but we now have four isolates to evaluate in further field tests with a degree of confidence that they are mild-only strains of CTV rather than having to establish a very large planting with several hundred isolates using the empirical method. The tree to tree movement of the CTV isolates can be controlled in the greenhouse trials, while this has often confused results obtained based on empirical results of field testing only.

We wished to create a very severe challenge condition in order to get measurable results quickly (10). Thus we chose to use two severe challenge strains and left the challenge inoculum in place on the plant as suggested by Rocha-Peña et al. (24) and Lee and Niblett (13). We anticipate a less severe challenge by aphids under field conditions and think the MSCP isolates may perform better in the field than in the greenhouse tests where an optimal temperature for CTV was maintained and where the challenge was very severe.

The milder challenge isolate FL-169 may have given some protection or diluted the biological activity of the more severe challenge isolate, T-68. Evidence of this can be seen when comparing the stem pitting occurring in healthy, T-68 only, FL-169 only and dual inoculation of T-68 and FL-169 (Table 2). FL-169 caused mild stem pitting in Madam Vinous sweet orange (4); however, in this test, no stem pitting was seen in Hamlin sweet orange.

Although all inoculation grafts survived for several months and long enough to transmit CTV, a number of inoculated plants did not become ELISA-positive, for example the St. Cloud Bk 12 2-17 SO-1 mild isolate (Table 6). These plants were consistently ELISA-negative, confirming that in some instances CTV does not pass through the graft (25). The fact that some plants were ELISA-positive at one time of testing and negative at other times, and in other instances, some plants were not ELISA-positive at any time even though the grafts were still alive, suggests poor transmissibility from

TABLE 6
NUMBER OF PLANTS TESTED POSITIVE FOR *CITRUS TRISTEZA VIRUS* (CTV) BY ELISA / NUMBERS TESTED

		3 MPC[z]		6 MPC		9 MPC		16 MPC		29 MPC	
Treatment	Challenge	Poly[y]	Mono[x]	Poly	Mono	Poly	Mono	Poly	Mono	Poly	Mono
T30/T26	-	4/4	0/4	5/5	0/5	5/5	0/5	1/2	0/5	5/5	0/2
	Dual	5/5	1/5	5/5	5/5	5/5	5/5	3/5	3/5	5/5	5/5
T49	-	5/5	0/5	5/5	0/5	5/5	1/5	2/3	0/5	5/5	0/5
	Dual	5/5	0/5	5/5	5/5	5/5	5/5	4/4	4/4	5/5	5/5
T55	-	4/4	0/4	4/5	0/5	5/5	0/5	3/5	0/5	4/4	0/4
	Dual	3/3	2/3	5/5	5/5	5/5	5/5	2/4	3/5	5/5	5/5
Healthy	-	0/5	0/5	0/4	0/4	0/5	0/5	0/5	0/5	0/5	0/5
	Dual	2/5	1/5	5/5	5/5	5/5	5/5	4/4	5/5	5/5	5/5
	T68	0/5	0/5	4/5	3/5	4/5	4/5	3/5	3/5	4/5	4/5
	FL169	0/5	0/5	4/5	1/5	5/5	3/5	4/4	3/5	4/5	3/5
97-1-5-1	-	5/5	0/5	5/5	0/5	5/5	0/5	4/5	0/5	5/5	0/5
	Dual	5/5	0/5	5/5	4/5	5/5	5/5	4/4	5/5	5/5	5/5
St Cloud Bk 12 2-17 SO-1	-	2/5	0/5	3/5	0/5	2/5	0/5	0/4	0/5	0/5	0/5
	Dual	3/4	1/4	5/5	5/5	5/5	5/5	3/3	5/5	5/5	5/5
96-8 Babb	-	5/5	0/5	5/5	0/5	4/5	0/5	3/4	0/5	4/4	0/4
	Dual	5/5	2/5	5/5	5/5	4/4	4/4	4/4	5/5	5/5	5/5
97-1-6 p1	-	5/5	0/5	5/5	0/5	5/5	2/5	2/4	0/5	5/5	0/5
	Dual	5/5	0/5	5/5	5/5	5/5	4/5	2/3	2/3	5/5	5/5
97-1-7 p1	-	5/5	0/5	5/5	0/5	5/5	0/5	5/5	0/5	5/5	0/5
	Dual	5/5	1/5	5/5	3/5	5/5	5/5	4/5	4/5	5/5	5/5
97-1-2 p2	-	5/5	0/5	5/5	0/5	5/5	0/5	3/4	0/5	5/5	0/5
	Dual	4/4	0/5	5/5	4/5	5/5	5/5	3/4	4/5	4/4	3/4
T11a	-	4/4	0/4	3/4	0/4	5/5	0/5	1/2	0/5	5/5	0/5
	Dual	5/5	2/5	5/5	3/5	5/5	5/5	3/3	4/4	4/4	4/4

[z]MPC = month post challenge.
[y]Poly = polyclonal antibodies used for ELISA which detect all isolates of CTV.
[x]Mono = Monoclonal antibody MCA13 was used for ELISA which selectively detects severe isolates of CTV (19).

the graft, and perhaps at times low, undetectable virus titer in the plant. Rocha-Peña et al. (25) have shown that the transmissibility of CTV from the graft is a property of the specific virus isolate. High graft-transmissibility is a very important trait of an effective cross-protecting mild isolate (12).

It should be stressed that MSCP is not the same as long-term virus resistance. Rather, MSCP provides a management tool which will prolong acceptable levels of production (13). The selection and evaluation of MSCP strains should be continuous, because the severe strains in an area are constantly changing as a result of vector transmission. MSCP research may not be possible in the future in Florida because of the lack of research support caused by too-optimistic promises from scientists using genetic engineering approaches.

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