Natural Spread of Severe Citrus Tristeza Virus Isolates in Citrus Preinfected with Mild CTV Isolates

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ABSTRACT. Natural spread of severe citrus tristeza virus (CTV) isolates was monitored in a 2-ha, 306-tree plot near Dundee, Florida. This plot, planted in spring 1986, consisted of Hamlin orange and Redblush grapefruit trees on sour orange rootstock preinfected with fourteen different CTV mild isolates. The trees were surveyed for symptoms of CTV decline in November 1988, March 1989, and October 1989, and assayed by enzyme-linked immunosorbent assay (ELISA). A monoclonal antibody, CTV-MCA13, which reacts specifically to severe decline-inducing isolates of CTV, and a broad-spectrum monoclonal antibody, 3DF1, were used in double sandwich indirect assays. After 3 yr of natural challenge, 3DF1-based ELISA indicated that 10 of 18 (56%) Hamlin and four of 19 (21%) Redblush grapefruit control trees became infected with CTV; seven of these Hamlin and all Redblush trees naturally infected with CTV also reacted with CTV-MCA13, indicating that they were infected with a severe isolate. Infection levels of CTV severe isolates in preinfected Hamlin treatments ranged from 0 to 50% based on CTV-MCA13 ELISA, with two treatments free of severe isolates. In Redblush grapefruit, infection levels of CTV severe isolates ranged from 0 to 22% but 11 CTV mild isolate treatments remained free from severe CTV infection. Decline symptoms were observed in 34 Hamlin and six grapefruit trees and supported the CTV-MCA13 ELISA results. Although substantial differences occurred between Hamlin treatments, results were not statistically different due to the low number of replications and the distribution of severe CTV infections in the plots. However, the eleven mild isolates in Redblush grapefruit which were free of severe CTV were statistically better than the rest for cross protection ($P = .05$). CTV-MCA13 ELISA was effective in diagnosing infections by CTV-severe strains and could be used to predict decline in presymptomatic trees. These data suggest some mild isolates may influence the spatial and temporal patterns of CTV decline in the fields, especially in grapefruit.

Index words. monoclonal antibody, mild strain cross protection, ELISA, aphid vectors, tristeza control, epidemiology, quick decline.

Citrus tristeza virus (CTV) decline of sweet orange, grapefruit, and mandarin on sour orange rootstock was first noted in South Africa in the late 1890's (3). Since then, epidemics of CTV decline have destroyed countless millions of citrus trees on sour orange roots in South Africa, Brazil, Venezuela, Spain, and Southern California (2). Approximately 42,300 ha (more than 17.5 million trees) of citrus in Florida are currently on sour orange roots. Virtually all orange trees in Florida are infected with CTV because most scion trees used for propagation are CTV-infected (8) and natural spread of CTV by aphid vectors is common. In general, common Florida CTV field strains have been considered mild because most do not induce a severe decline, seedling yellows, or cause sweet orange or grapefruit stem pitting. Recent observations suggest that prevalence of more severe isolates is increasing. In 1975, an outbreak of CTV decline was reported in central Florida (7) and, more recently, a new epidemic of CTV decline was reported in groves in the flatwoods area of the east coast (Ft. Pierce area) and in southwest Florida (LaBelle-Immokalee) (5). In all cases, it appeared that infection by severe isolates was occurring in trees with pre-existing mild infection. In addition, CTV-induced stunting in young plantings on sour orange was observed (5). Yokomi and Garnsey (15) found some Florida CTV isolates, many of which are deline-inducing strains, were readily transmitted by *Aphis gossypii* Glover, and to a lesser degree by *Aphis spiraecola* Patch. This suggested that there is a growing reservoir of severe CTV strains on CTV-tolerant rootstocks and that CTV challenge pressure by such strains is increasing in Florida. A large proportion of the sour orange-rooted citrus in Florida, however, has not

declined and is still productive. It is not clear if these trees are actually protected, or that they simply have not been challenged by decline isolates. The purpose of this work was to study the protective ability of a number of Florida CTV mild isolates in sweet orange and grapefruit on sour rootstock against CTV decline under natural challenge conditions.

MATERIALS AND METHODS

CTV mild isolates. The fourteen CTV mild isolates used in this test are described in Table 1. The isolates were from two general categories: i) citrus budwood sources that become infected naturally with CTV mild isolates; and ii) CTV mild isolates collected from vigorous, sour orange-rooted sweet orange trees near trees showing severe CTV decline symptoms. A preliminary biological evaluation of these isolates was determined by graft inoculating young sweet on sour orange propagations with each isolate and evaluating stunting effects in the greenhouse (9). Test trees were propagated in April 1985 by grafting greenhouse-grown sour orange seedlings of a stem diameter of ca. 1.2 cm with virus-free buds from Hamlin sweet orange (H-1-4-1) and Redblush grapefruit (R-SF-58-39) and simultaneously inoculated by grafting a leaf piece from an inoculum source plant below the bud (9).

Field plot. A field site was selected near Dundee, Polk County, Florida because it had CTV decline isolates, natural challenge was substantial, and there was a high decline rate of grapefruit on sour orange rootstock (16). Trees were planted in spring 1986, in a 2-ha, 306-tree plot using a split-plot design with the mild isolates as the main plot and the variety as the subplot. The tree spacing was 9.1 x 9.1 m. Each treatment had nine or 10 single-tree replications.

Virus infection and survey. Extracts from young shoots of each tree were assayed in a direct, double antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) with polyclonal CTV antiserum 879 (4), to confirm infection by the mild CTV isolate before field planting. Trees were

TABLE 1
SOURCE OF FLORIDA CITRUS TRISTEZA VIRUS (CTV) MILD ISOLATES USED IN THE EXPERIMENT

Tristeza isolate[z]	Source[y]	Collection site
T1	Orig. Grant Mild (10)	Temple grove, Winter Garden
T4a	Etrog cit. SPB F/55-40	DPI[x] Indexing block, Winter Haven
T11a	Val. V-S-F/54-23	DPI Foundation grove, Davenport
T26	Val. V-S-F/37-10	DPI Foundation Grove, Davenport
T28	DD102bb	Grove, Ft. Pierce
T30	DPI scion	DPI Foundation grove, Davenport
T34	LB-1	Nursery, Avon Park
T35a	BA-41	Nursery, Haines City
T49	Navel N-15-28-1	RBT[w], Lake Gem
T50a	Hamlin 16-6-4	RBT, Zellwood
T55a	Val. V-136-53-4	RBT, Winter Garden
T56	Val. V-1-12-5	DPI Nursery, Dundee
T60a	FS219	Grove, Winter Garden
T61a	FS332	Grove, Pine Island (Oakland)
Healthy control[v]		Greenhouse

[z]CTV isolate code; lower case "a" following number designates an aphid-transmitted subisolate.
[y]Isolate source is as designated by authors and is mild based sweet on sour orange rootstock biological characterization (9).
[x]DPI = Div. of Plant Industry, Citrus Budwood Registration Office, Winter Haven.
[w]RBT = Registered Budwood Source Tree from a commercial nursery.
[v]Healthy controls were virus-free scion sources, Hamlin H-1-4-1 and Redblush grapefruit R-SF-58-39.

evaluated for symptoms of CTV decline on a scale from, 0 to 3, in November 1988, March 1989, and October 1989, where 0 = healthy and 3 = severely stunted or dead. Young shoots from test trees were collected during each inspection and assayed by ELISA using a severe strain-discriminating monoclonal antibody, CTV-MCA13 (12, 13) and a broad-spectrum monoclonal, 3DF1 (14) in DAS indirect assays to determine infection by natural CTV challenge isolates.

To determine level of natural challenge isolates near the plot, young shoots from approximately 35 mature nucellar Valencia sweet orange trees adjacent to the plot and 20 trees from a nearby Temple grove were sampled and assayed by CTV-MCA13 and 3DF1 ELISA.

Yield and data analysis. Fruit yield was evaluated after 3 yr and expressed as number of 41-kg boxes of fruit per tree. Statistical analysis of the data was done by GLM (SAS Institute, Raleigh, NC). Percentage data were converted by angle transformation before statistical analyses were performed.

RESULTS

CTV decline or stunting in the plot was first observed in 1988. CTV-MCA13 ELISA of all the Hamlin trees in November 1988, March 1989, and October 1989 indicated that CTV severe isolate infection levels was 12% (18 trees), 17% (26 trees), and 29% (44 trees), respectively (Table 2). By October 1989, 3DF1 ELISA indicated that 50% (nine of 18) healthy Hamlin controls had become CTV infected and 33% (6 trees) of these infections were by CTV severe isolates based on CTV-MCA13 reactions. Overall, 44 Hamlin trees tested positive with CTV-MCA13, and 34 had obvious stunting or decline symptoms or had died (Table 2). Twenty-nine of 135 (21%) trees preinfected with mild CTV isolates became coinfected with CTV severe isolates based on CTV-MCA13 ELISA. Infection levels by CTV severe isolates in 10 CTV mild isolate treatments were similar to that of the control (from 20-50%); whereas trees preinfected with T49 and T55a had no coinfections, and trees preinfected with T50a and T56 had one coinfection each in fall 1989. Although differences in severe CTV infection levels between treatments were large, they were not statistically different ($P = .05$).

Among the grapefruit trees, the incidence of CTV severe isolates in November 1988, March 1989, and October 1989 surveys was 4% (6 trees), 5% (7 trees), and 6% (9 trees), respectively (Table 2) as determined by CTV-MCA13 ELISA. Overall, these results indicated that 9 of 153 (6%) grapefruit trees became infected with CTV severe isolates, with five of these showing stunt or decline symptoms and one had died. Four of 18 (22%) noninoculated Redblush control trees became infected with CTV and all four reacted to CTV-MCA13. CTV-MCA13 ELISA results indicated that only five of 126 (4%) trees preinfected with CTV mild isolates became infected with CTV severe isolates; whereas eleven of 14 CTV mild treatments in Redblush were free from infection by severe CTV (Table 2). Extracts from one of 10 trees preinfected with T30 reacted with CTV-MCA13, whereas trees preinfected with T35a and T60a had two of nine trees, and two of 10 trees, respectively, reacting with CTV-MCA13. Statistical analysis of the CTV-MCA13 ELISA data indicated significant differences ($P = .05$) between the treatments with no coinfections and those with two or more coinfections.

All tissue samples collected from the nearby Temple grove tested positive by CTV-MCA13 ELISA. Analysis of the 35 mature Valencia trees adjacent to the test plot using 3DF1 ELISA indicated that all were naturally infected with CTV; and 33 of these also reacted with CTV-

TABLE 2
REACTION TO SEVERE-STRAIN DISCRIMINATING MONOCLONAL ANTIBODY, CTV-MCA13, DECLINE SYMPTOMS, AND YIELD OF HAMLIN AND REDBLUSH GRAPEFRUIT ON SOUR ORANGE ROOTSTOCK PEINFECTED WITH MILD CITRUS TRISTEZA VIRUS ISOLATES AFTER THREE YEARS OF NATURAL CHALLENGE NEAR DUNDEE, FLORIDA

Mild tristeza isolate	No. trees	CTV-MCA13[z] Nov 88	Mar 89	Oct 89	No. of declining trees[y]	Yield (boxes/tree[x])
Hamlin						
T1	10	1	1	3 ns[w]	3	1.25 ab[w]
T4a	10	0	2	3	2	1.22 ab
T11a	10	2	4	4	4	0.71 b
T26	9	1		3	2	1.03 ab
T28	10	2	2	4	3	0.73 b
T30	10	0	0	2	0	1.25 ab
T34	9	2	3	3	4	1.00 ab
T35a	10	3	3	5	5	0.90 ab
T49	9	0	0	0	0	1.03 ab
T50a	9	0	0	0	0	1.44 a
T55a	10	1	1	1	1	1.07 ab
T56	9	1	1	1	1	1.37 a
T60a	10	0	1	3	1	0.97 ab
T61a	10	2	2	5	3	1.30 a
HC[v]	18	3/6	5/8	7/10	5	1.06 ab
Total	153	18	26	44	34	
Redblush						
T1	10	0	0	0 a[w]	0	1.00 c
T4a	9	0	0	0 a	0	1.33 abc
T11a	10	0	0	0 a	0	1.05 c
T26	10	0	0	0 a	0	1.28 bc
T28	9	0	0	0 a	0	1.31 bc
T30	10	1	1	1 ab	1	1.28 bc
T34	10	0	0	0 a	0	1.63 abc
T35a	9	2	2	2 bc	2	1.11 c
T49	10	0	0	0 a	0	1.20 c
T50a	9	0	0	0 a	0	2.03 ab
T55a	10	0	0	0 a	0	2.10 a
T56	10	0	0	0 a	0	1.22 c
T60a	10	0	0	2 bc	0	1.60 abc
T61	9	0	0	0 a	0	0.83 c
HC[v]	19	3/4	4/4	4/4c	3	1.39 abc
Totals	153	6	7	9	6	

[z]Number of trees with positive reaction to severe-strain discriminating monocolonal antibody, CTV, MCA13.
[y]Decline based on a score of 2 or greater for visual symptoms of CTV decline (0 = healthy; 3 = severe). Only October 1989 data shown.
[x]Yield expressed as 41-kg boxes of fruit per tree.
[w]Mean separation by Duncan's multiple range test (P = .05); ns = not significantly different.
[v]Numerator is the number of trees positive for CTV-MCA13; denominator is the total number of trees infected by all CTV strains per sampling date.

MCA13, indicating that they were infected by CTV severe isolates.

In all but one case (Hamlin T34), CTV-MCA13 ELISA diagnosed the same or more infection by severe CTV than by visual inspection. The visual count in October 1989 was more closely related to the CTV-MCA13 result obtained 6 months earlier (March 1989). This was an indication that CTV-MCA13 ELISA with may be predictive of decline in presymptomatic trees.

Statistical differences were found in yields from the Hamlin and grapefruit plots (Table 2). In Hamlins, the

yield reduction was correlated (P = .05) to severe CTV infection when at least 40% of the trees became infected with CTV severe isolates. Yield reduction in grapefruit were correlated (P = .05) to tree vigor and not with the overall low level of infection by severe CTV.

A devastating freeze December 23-25, 1989, destroyed the entire plot and the experiment was terminated.

DISCUSSION

The lack of CTV severe isolate infection of Hamlin treatments preinfected with T49 and T55a and the low level of CTV severe isolate infection in T50a and T56 suggest that these isolates may be reducing or delaying infection by CTV severe isolates under test conditions. However, a combination of the low number of replications used, the distribution of CTV severe isolate infections in the Hamlin treatments, and the short duration of test prevented statistical confirmation that these mild CTV isolates may be effective in cross protection. The high level of infection by CTV severe isolates in the other 10 CTV mild treatments, however, indicated clearly that these isolates were not effective as CTV-protecting isolates.

In the case of grapefruit, 11 of 14 mild isolates had statistically lower levels of CTV severe isolate infection than the healthy controls. Overall, the comparatively low level of CTV severe isolates in grapefruit compared to that in Hamlin suggest that grapefruit is not as receptive to infection as sweet orange and may withstand greater natural challenge by CTV severe isolates. This may be due, in part, to the erratic distribution of CTV within the tree canopy of grapefruit compared to that in sweet orange (6, 11). Such poor systematic involvement of a new infection and preinfection by a CTV mild isolate in grapefruit may make establishment of a second isolate more difficult.

Yield from Hamlin trees was statistically reduced by severe CTV when infection level by severe CTV infection was at least 40%; whereas that from Redblush grapefruit was not related to severe CTV infection. Production data from 3-yr-old trees are quite preliminary and likely reflect effects from many horticultural factors, of which virus infection is but one. Fruit set may have already occurred before the effects of severe CTV infection were fully expressed. In addition, a freeze during blossom time in February 1989, could have affected yield figures. These data were included, however, because they showed that preinfection by a mild CTV isolate did not affect tree vigor or production. Since the plot was terminated in late December, 1989 due to a devastating freeze, long term yield evaluation was not possible. Infection by a severe decline-inducing CTV isolate in citrus tree on sour orange rootstock will either kill the tree or render it nonproductive. Many factors influence its incubation period before the tree begins to show CTV decline symptoms. In general, the incubation period is expected to be longer in large trees than in small trees. Some trees in our plot were showing obvious decline within 2 yr of planting.

Natural challenge by decline-inducing CTV isolates carried by aphid vectors at the experimental site near Dundee, FL, was severe. Virtually all trees surrounding the plot were propagated from nucellar sources and were planted virus-free on assorted rootstocks. These trees were approximately 15 yr old at the time of this study, and all had become naturally infected with CTV. The high percentage of samples from these trees that reacted to CTV-MCA13 indicated that they were infected by CTV isolates serologically distinct from the protecting sources used. Aphids, principally *A. spiraecola* and to a lesser degree, *A. gossypii*, were frequently observed on young flush in

the plot. Aphid numbers and species, however, were not monitored.

CTV-MCA13 detected CTV severe isolates in trees that were visually rated at least a 2 (on a 0-3 scale for decline) for CTV decline and further confirms that CTV-MCA13 can detect severe decline-inducing isolates in Florida. Extracts from a number of vigorous trees were also positive by CTV-MCA13. Most of these trees eventually developed decline symptoms. We assume that the rest of the presymptomatic trees would eventually show decline symptoms but the unexpected termination of the test precluded an accurate evaluation of CTV-MCA13 as a predictor of decline.

In summary, some mild Florida isolates in Redblush grapefruit and, to a lesser degree, Hamlin orange, may reduce or delay the infection of severe CTV isolates under natural field challenge. Such isolates may stabilize the incidence of CTV decline and increase the time required for the complete decimation of sour orange-rooted citrus in an area. Bar-Joseph (1) observed similar CTV-decline epidemics in Israel following a lag period of productive citriculture and suggested that this was due to cross-protection incompleteness. This incompleteness may be a function of challenge pressure. As a greater proportion of citrus is planted on CTV-tolerant or -resistant rootstocks and natural spread of CTV continues, the reservoir of severe CTV will increase and, in time, will probably place untenable pressure on sweet orange and grapefruit on sour orange rootstock regardless of preinfection by CTV mild isolates.

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