Distribution and Characterization of *Citrus tristeza virus* in South Florida Following Establishment of *Toxoptera citricida*

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ABSTRACT

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The incidence of *Citrus tristeza virus* (CTV) was found to increase significantly in southern Florida within 2 years after the establishment of its most efficient vector, *Toxoptera citricida* (Kirkaldy). Increased incidence of both mild and severe strains was documented, with the incidence of severe strains increasing more than mild strains. Molecular probes capable of differentiating mild, quick decline and various types of stem-pitting strains demonstrated that trees often were infected with more than one strain of CTV, with trees containing up to five different strains. Some CTV strains detected in the southeast urban corridor of Florida and in commercial groves in southwest Florida were found to react with probes specific for stem-pitting strains known from elsewhere in the world. The implications of the presence of these CTV strains in Florida and their possible presence in citrus budwood scion trees are discussed.

Additional keywords: brown citrus aphid

Citrus tristeza, caused by *Citrus tristeza closterovirus* (CTV), is the most destructive virus disease of citrus (16). Some strains of CTV cause very mild symptoms on sensitive indicators such as Mexican lime. Others cause decline and death of trees grafted on sour orange rootstock, and still others cause various types of pitting on the stems and branches of grapefruit and sweet orange (16). Both mild and decline strains of CTV have been documented in commercial citrus throughout

Florida, and stem-pitting strains have been documented in trees of Meyer lemon (*Citrus meyeri* Tan.) progeny of introductions into Florida from China (10,15). Molecular hybridization probes have been developed for Florida populations that are capable of detecting individual strains and differentiating mixtures of CTV strains (3,18), and there is a high correlation between the probe reactions and the biological activity of the CTV strains (27).

Several species of aphids are known to vector CTV, but the brown citrus aphid (BrCA), *Toxoptera citricida* (Kirkaldy), is known to be the most efficient vector (16,26). BrCA was discovered in Florida in the Miami/Ft. Lauderdale metropolitan area late in 1995. In less than 2 years, it had colonized Florida's major citrus production areas (13). Previously, severe and destructive outbreaks of CTV have occurred in Argentina, Brazil, South Africa, and Venezuela following the introduction of BrCA. Severe decline and stem-pitting strains of CTV already present in those countries were "sleeping" because they were transmitted inefficiently by local aphid species; however, when BrCA arrived, it was able to transmit those sleeping strains with disastrous results for the local citrus industries (23,24).

Prior to the appearance of BrCA in Florida, an extensive survey was done to determine incidence of CTV in the state (2). The survey provided a pre-BrCA baseline of CTV distribution in Florida. Additionally, a tree with an isolate causing severe stem-pitting in grapefruit was detected in a neighborhood in Delray Beach. It became infested with BrCA prior to removal. The objectives of this research were to determine whether the presence of BrCA had affected the incidence, distribution, and severity of CTV strains in Florida, and to determine whether the severe stem-pitting strain detected in Delray Beach (T3800) had been spread throughout the neighborhood by BrCA.

MATERIALS AND METHODS

Surveys and sampling. The initial surveys prior to the establishment of BrCA were performed over a period of time from January 1994 through March 1995 (2). The initial trees surveyed by Brown and Davison (2) were those that were monitored in fruit fly-trapping routes. BrCA was found in November 1995 and spread throughout the Florida peninsula at a rate of about 250 km/year (13). The surveys reported here were initiated approximately 2 years after the detection of BrCA in each area. Many of the trees sampled for CTV in 1994-95, before the BrCA arrived (2), had been removed when subsequent sampling was done. Because of this, nearby trees, especially those that were younger and planted after the 1994-95 survey, also were sampled. Samples in Kendall, Ft. Lauderdale, Delray Beach, and Palm Beach Gardens (Fig. 1) were taken from dooryard citrus in urban communities. They included a mixture of citrus cultivars. Samples were taken from sweet orange (*Citrus sinensis* (L.) Osbeck), sour orange (*Citrus aurantium* L.), grapefruit (*Citrus × paradisi* Macfad.), Meyer lemon (*Citrus meyeri* Tan.), lemon (*Citrus limon* (L.) Burm. f.), calamondin (*Citrofortunella microcarpa* (Bunge) Wijnands), kumquat (*Fortunella* spp.), key lime (*Citrus aurantifolia* (L.) Swingle), Persian lime (*Citrus aurantifolia* (Christm.) Swingle), and tangerine (*Citrus*

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reticulata Blanco). No trees were observed to have more than one scion grafted to the rootstock. Each sample was composed of four young flushes collected from around the tree. These were placed in plastic bags and transported on ice until the petiole or bark tissue could be removed. Petioles were dried prior to grinding for enzyme-linked immunosorbent assay (ELISA). Bark was chopped into fine pieces less than 0.5 mm in size, placed in 2-ml screw-top tubes, and frozen at -20°C until processed for ELISA or reverse-transcription polymerase chain reaction (RT-PCR).

Kendall. A small planting of 58 citrus trees (mixed experimental cultivars) was sampled in 1995. Of the original 58 plants, 52 remained and were sampled in 1997.

Ft. Lauderdale. In all, 88 citrus trees on Florida Department of Agriculture and Consumer Services, Division of Plant Industry (DPI) fruit fly trapline 307B (T49S-R42E) in northern Ft. Lauderdale (Fig. 1) were sampled initially over a period of

time between January 1994 and February 1995. A fruit fly trapline is a route taken by an inspector who examines fruit fly traps every 3 weeks. The traps are placed in fruiting trees, including citrus. The trees used by the inspectors are mapped and chosen for even distribution throughout the trapping district; therefore, use of the trapline was a convenient way to obtain a consistent dooryard sample. In 1997, 37 of the original trees were resampled, and 5 additional trees not included in the 1994-95 survey were sampled for a total of 42 plants.

Delray Beach. Thirty citrus trees on DPI fruit fly trapline 329B (S29-T46S-R43E) in Delray Beach (Fig. 1) were sampled in 1994 in the original survey. A host range analysis performed in conjunction with the original 1994-95 survey was analyzed in 1996 and revealed that a severe stem-pitting CTV strain (probably a Meyer lemon isolate) was present in a dooryard lemon tree (T3800; Table 1). This discovery coin-

cided with the initial colonization of the area by BrCA. BrCAs were recovered from the infected tree before it was removed (DPI sample no. E1996-2708). At the time of removal, several citrus trees in the same yard and several adjoining yards were sampled to see if the isolate had spread.

In 1998, 24 of the original 30 citrus trees on fruit fly trapline 329 B trees were resampled, along with 10 additional trees, for a total of 34 plants. Five of the original trees (D6, D7, D11, D13, and D20), and two additional trees (D35 and D36), were sampled in 2000 and tested with molecular probes and an ELISA that detects orange stem-pitting strains of CTV (19).

Leaf pieces of the seven plants sampled in 2000, along with two additional trees in the community of Palm Beach Gardens (Fig. 1), were grafted into Pineapple sweet orange and Duncan grapefruit seedlings for biological indexing. An epidemic of CTV decline was in progress in the upscale residential community of Palm Beach Gardens.

Immokalee. Sixty-three samples were taken from eight-by-eight-tree quadrants in two commercial sweet orange groves in the Immokalee area (Fig. 1). Neither grove had a prior known history of tristeza-induced decline, although one of the sites had many trees on sour orange rootstock. Previous surveys by Brown and Davison (2) indicated that CTV was widespread in the area.

Potted Mexican lime (*Citrus aurantifolia*) trap plants (49 total pots) were exposed for a month at a time in commercial groves in the Immokalee area. The pots were placed between trees along the rows in three different groves. After removal from the field, plants were treated with bifenthrin and imidacloprid to kill aphids. Plants were fertilized and held for two growth flushes prior to analysis for CTV.

A standard infectivity assay (22) was done with live BrCA collected from the field in groves near Immokalee on 28 and 29 December 1998. Aphids were collected from commercial groves in the Immokalee area and caged directly on small sweet orange indicator plants (one per plant). After 2 days, cages were removed and plants were sprayed with bifenthrin and treated with imidacloprid granules to kill the aphids. Plants were tested later by ELISA to determine number infected and to calculate the infectivity of field-collected BrCA.

ELISA. All plants were sampled for ELISA by picking four terminals per tree. If possible, terminals with relatively new growth (but fully expanded leaves) were selected. One petiole or leaf midvein per terminal was excised. Samples, comprised of four petioles or midveins, were processed using a tissue pulverizer (Model 4200; KLECO, Visalia, CA). Plants maintained in the Division of Plant Industry

Fig. 1. Outline map of peninsular Florida showing the locations of samples taken for *Citrus tristeza virus*, 1994-2000.

Quarantine Facility (Gainesville, FL) were used as positive controls. ELISA was performed using the double-antibody sandwich indirect (DAS-I) method as described by Garnsey and Cambra (5). Assays were run with "Taiwan mix," a cocktail of monoclonal antibodies (Taiwan) provided by Dr. S. M. Garnsey (9), which detects all strains of CTV, and with monoclonal antibody 13 antiserum (MCA 13) provided by Dr. T. Permar, Nokomis, Inc., which detects most strains of CTV that cause decline or stem-pitting in Florida. The assays followed the procedure of Ochoa et al. (20) with the exception that antimouse conjugate was used to detect Taiwan monoclonals. ELISA optical density at 405 nm (OD_{405nm}) readings that were greater than 2.0 times the values for healthy citrus extracts were considered positive. The ELISA used to detect orange stem-pitting strains (OSP-ELISA) was done exactly according to the protocol published by Nikolaeva et al. (19), using the polyclonal system. For the OSP-ELISA, samples that were considered positive were at least 10 times the OD_{405nm} values for the known non-stem-pitting isolates (T36 and T4) that were used as controls.

Molecular probes. Selected plants from each survey were chosen for molecular detection and characterization of the CTV strains present. The nucleic acid extraction, RT, amplification, and cloning of the capsid protein genes (CPGs) were performed

as described by Pappu et al. (21). To identify which strains of CTV were present in the samples, the PCR products were analyzed on agarose gels to detect the presence of the 672-bp CPG amplicons, which then were probed with the CTV strain group-specific probes as described previously (3,17,18,20; Table 2). The probes are short oligonucleotides labeled at the 5' end with biotin to react with a streptavidin conjugated alkaline phosphatase for detection of the hybridized amplicons by chemiluminescence. The unique sequences of the probes are the subject of U.S. Patent # 6,140,046.

Probe 0 contains a nucleotide sequence conserved in the CPGs of all known strains of CTV. Hence, it is a universal probe that hybridizes with all known strains of CTV. Probe I hybridizes with decline-inducing strains; probes II, III, IV and V hybridize with different groups of stem-pitting strains from various locations throughout the world; and probe VIII hybridizes with all mild strains. The type CTV strains of each probe and symptoms caused by the type strains are summarized in Table 2. Clones of the CPGs were amplified from selected samples and were sequenced to confirm the presence of the strains of CTV that were indicated by the strain group specific probes.

Biological indexing. Selected samples collected from Delray Beach and Palm Beach Gardens were grafted onto Pineap-

ple sweet orange and Duncan grapefruit seedlings by insertion of leaf pieces from the collected sample (about 2 by 10 mm) under flaps cut on the bark of the receptor plant. The plants were tested by ELISA after 2 months to verify the presence of CTV. Inoculated plants were maintained in a greenhouse with day and night temperatures of 30 to 35°C and 18 to 24°C, respectively, for 12 months. Plants then were evaluated for presence of stem pitting on a scale where 0 = no stem pitting, 1 = mild stem pitting, 2 = moderate stem pitting, and 3 = severe stem pitting (7).

RESULTS

Kendall samples. In the pre-BrCA sample of March 1995, two plants reacted positively with the Taiwan antiserum (3.4%) and no plants reacted positively with the MCA 13 antiserum (Table 3). In 1997, 18 of the remaining 52 plants (34.6%) reacted positively with the Taiwan antiserum. Of those 18 CTV-positive plants, 10 reacted positively with MCA 13 antiserum (19.2% of total plants; 55.6% of CTV positives). An additional three plants had weak positive readings. No plants reacted with MCA 13 antiserum that did not also react with the Taiwan antiserum. Both of the 1995 CTV-positive plants were missing or not detected at the later sampling date; thus, all 18 positives found in the second survey are assumed to be new infections.

Table 1. Comparison of biological indexing, molecular assays, and enzyme-linked immunosorbent assay (ELISA^a) results for *Citrus tristeza virus* (CTV) samples from Palm Beach County, FL, in 2000^a

Location, sample no.	ELISA	Probe 0	Probe I	Probe II	Probe III	Probe IV	Probe V	Probe VIII	Rating in sweet orange	Rating in grapefruit
PBG-1 ^b	Mild	NT ^c	NT	NT	NT	NT	NT	NT	0	0
PBG-2	MCA13+	NT	NT	NT	NT	NT	NT	NT	0	0
D6 ^d	Mild	1	0	0	0	0	0	4	0	0.5
D7	OSP+	2	2	0	2	0	0	1	0.5	1.0
D11	MCA13+	2	4	0	1	0	0	1	0	0.5
D13	OSP+	1	3	0	0	0	0	3	0	0
D20	OSP+	1	3	0	3	0	0	1	0.5	1.0
D35	MCA13+	1	1	0	0	0	0	2	0	NT
D36	OSP+	1	1	0	0	0	0	2	0.5	0
T3 ^e	OSP+	3	0	0	0	5	0	0	0.5	0
T66 ^e	MCA13+	3	5	0	0	0	0	0	0	0
T66H ^e	OSP+	3	0	0	4	0	0	0	1.5	0
T3800 ^e	MCA13+	4	0	0	0	0	4	1	0	3.0

^a Mild denotes reaction only with the Taiwan mix; MCA 13+ denotes reaction with monoclonal antibody 13, and OSP+ denotes a positive reaction with the orange stem-pitting detection ELISA (19).

^b Palm Beach Gardens.

^c NT = not tested.

^d Delray Beach.

^e Isolates used as controls. T3800 is the severe grapefruit stem-pitting isolate found in the 1994–95 surveys of Brown and Davison (2).

Table 2. Summary of the biological activity for the type strains of *Citrus tristeza virus* (CTV) detected by the different strain group-specific probes

Strain group-specific probe	Type strain	Symptoms induced by type strain
Probe 0	Universal	Recognizes all strains of CTV
Probe I	T36, T66	Decline on sour orange, seedling yellows
Probe II	B1, B53	Decline on sour orange, seedling yellows, stem pitting on grapefruit and sweet orange
Probe III	B165, B185	Decline on sour orange, seedling yellows, stem pitting on grapefruit and sweet orange
Probe IV	T3, B220	Decline on sour orange, seedling yellows, stem pitting on sweet orange
Probe V	B128, B249	Decline on sour orange, seedling yellows, stem pitting on grapefruit and sweet orange
Probe VIII	T26, T30, B188, B215	Mild strains from Florida and other parts of the world

Eight samples from the Kendall survey were analyzed with the molecular probes (Table 4). All samples reacted to probe 0, which detects all CTV. Two plants (K6 and K9) reacted with four additional different probes. These included probe I, which detects Florida decline strains; probe VIII, which detects mild strains; and probes III and V, which detect stem-pitting strains. Four samples (K25, K38, K42, and K51) reacted to two additional probes (probe I, which detects decline strains and probe VIII, which detects mild strains) in addition to probe 0, which detects all CTV. One of these samples (K42) also had a weak reaction to probe III, which detects stem-pitting strains. Two samples (K14

and K49) reacted only with probe I for decline strains in addition to the all-CTV-detecting probe 0. The molecular data for the 1997 Kendall samples corresponded very closely with the ELISA data. Of the samples tested by molecular probes, all that reacted positively with MCA 13 also reacted with probes that detect decline or stem-pitting strains of CTV.

Fort Lauderdale samples. In the 1994–95 survey, 11 of the 88 trees reacted positively with the Taiwan antiserum (12.5%), and two plants also reacted with the MCA 13 antiserum (2.3%; Table 3). In the 1997 survey, 13 of the 42 trees sampled reacted positively with the Taiwan antiserum (31.0%), and 3 trees reacted positively

with the MCA 13 antiserum (7.1%). As was the case at Kendall, no plants reacted positively with the MCA 13 antiserum that failed to react with the Taiwan antiserum.

Both trees that reacted positively with the MCA 13 antiserum and one of the trees that reacted positively with the Taiwan antiserum in the 1994–95 survey had been removed at the time of the 1997 survey. The five trees (FL109, FL119, FL122, FL124, and FL132) that reacted previously with the Taiwan antiserum in 1994–95 still gave positive reactions with the Taiwan antiserum in 1997. One of those samples also reacted positively with the MCA 13 antiserum. Its earlier reaction with the MCA 13 antiserum was borderline; there-

Table 3. Serological analyses of citrus samples collected in South Florida, 1994 to 1998

Location	Year	No. of plants tested	Positive reaction ^a			
			Taiwan antibody		MCA 13 antibody	
			Number	Percent	Number	Percent
Kendall	1995	58	2	3.4	0	0.0
	1997	52	18	34.6	10	19.2
Ft. Lauderdale	1994–95	88	11	12.5	2	2.3
	1997	42	13	31.0	3	7.1
Delray Beach	1994	30	7	23.3	1	3.3
	1998	34	21	61.8	10	29.4
Immokalee	1997	63	62	98.4	40	63.5

^a The Taiwan antiserum detects all *Citrus tristeza virus* (CTV). Monoclonal antibody 13 (MCA 13) detects most severe strains of CTV in Florida.

Table 4. Results of molecular analysis of *Citrus tristeza virus* (CTV) samples collected in South Florida, 1997 and 1998^a

Number	Location	ELISA (MCA 13)	Probes ^b						
			0	I	II	III	IV	V	VIII
K6	Kendall	+	5	5	0	2	0	4	3
K9	Kendall	+	5	5	0	2	0	4	3
K14	Kendall	+	5	5	0	0	0	0	0
K25	Kendall	+	5	4	0	0	0	0	4
K38	Kendall	+	5	4	0	0	0	0	5
K42	Kendall	+	5	4	0	1	0	0	5
K49	Kendall	+	5	3	0	0	0	0	0
K51	Kendall	+	5	3	0	0	0	0	5
FL101	Ft. Lauderdale	+	5	5	0	1	0	0	4
FL132	Ft. Lauderdale	?	5	5	0	1	0	4	5
FL133	Ft. Lauderdale	?	5	5	0	1	4	4	5
FL134	Ft. Lauderdale	?	5	5	0	2	0	4	4
FL138	Ft. Lauderdale	?	5	5	0	2	0	4	5
D1	Delray Beach	–	4	0	0	5	0	0	0
D2	Delray Beach	+	4	0	0	4	0	0	0
D6 ^c	Delray Beach	–	5/1	5/0	0	0	0	0	0/4
D7 ^{d,e}	Delray Beach	–	2	2	0	2	0	0	Tr
D8	Delray Beach	+	5	0	0	5	0	0	0
D9	Delray Beach	–	5	0	0	5	0	0	0
D10	Delray Beach	?	5	0	0	5	0	0	0
D11 ^c	Delray Beach	+	5/2	1/4	0	5/1	0	0	0/Tr
D12	Delray Beach	?	5	0	0	5	0	0	0
D13 ^{d,e}	Delray Beach	+	1	3	0	0	0	0	3
D15	Delray Beach	+	5	0	0	5	0	0	0
D16	Delray Beach	–	5	0	0	5	0	0	1
D20 ^{c,e}	Delray Beach	+	5/1	0/3	0	5/3	0	0	0/1
D32	Delray Beach	+	5	0	0	5	0	0	0
D35	Delray Beach	+	1	1	0	0	0	0	2
D36	Delray Beach	+	1	1	0	0	0	0	2

^a Reactions of each isolate to monoclonal antibody 13 (MCA 13), which detects most Florida severe CTV, and reactions to a battery of seven molecular probes are shown. Enzyme-linked immunosorbent assay (ELISA) notation: + = positive, – = negative, ? = questionable, borderline.

^b Type strains for each of the probes are: 0 (all CTV); I (quick decline), T36 and T66; II (stem-pitting), B1 and B53; III (stem-pitting), B165 and B185; IV (stem-pitting), T3 and B220; V (stem-pitting), B128 and B249; and VIII (mild), T26 and T30, and B188 and B215 (6). Tr = trace reaction.

^c Molecular testing in 1998 and 2000.

^d Molecular testing in 2000 only.

^e Positive by orange stem-pitting detection ELISA (19) in 2000.

fore, it is likely that it does not represent a new severe infection. The other two trees that reacted positively with the MCA 13 antiserum in 1997 were not sampled previously; therefore, their previous infection status is unknown. There were five trees that reacted positively with the Taiwan antiserum in 1997 that were CTV negative in the previous survey. These latter trees are considered to have new infections.

Five samples from the Ft. Lauderdale survey in 1997 were analyzed with the molecular probes (Table 4). All five samples reacted with probes 0, I, III, and VIII, demonstrating the presence of CTV, CTV decline strains, one group of CTV stem-pitting strains, and CTV mild strains, respectively. Samples FL132, FL133, FL134, and FL138 also reacted with probe V, which detects a second group of stem-pitting strains, and sample FL133 also reacted with probe IV, which detects a third group of stem-pitting strains. The molecular analysis of the Ft. Lauderdale samples did not correspond nearly as closely to the ELISA readings as did the analysis of the Kendall samples. Four of these samples (FL132, FL133, FL134, and FL138) had borderline MCA 13 reactions; however, they all contained two or more different severe strains of CTV.

Delray Beach samples. In the 1994 survey, 7 of the 30 plants (23.3%) reacted positively with the Taiwan antiserum, and only 1 (3.3% of the total plants) reacted positively with MCA 13 (Table 3). This single plant, a lemon, proved to have a severe grapefruit stem-pitting strain of CTV (T3800; Table 1). In 1996, five citrus trees in close proximity to the lemon with the severe stem-pitting strain were tested by ELISA, and three of these were tested by molecular probes. The severe stem-pitting isolate reacted with molecular probe V. None of the other three trees tested by molecular probes showed reactions to this probe. A fourth tree was ELISA negative. These results indicate that the stem-pitting isolate probably had not spread in the neighborhood by 1996, even though BrCA was present. In the 1998 survey, 21 of the 34 plants sampled (61.8%) reacted positively with the Taiwan antiserum, and 10 (29.4%) reacted positively with MCA 13 antiserum. An additional three plants had borderline reactions in the MCA 13 assay. The single MCA 13-positive plant in the 1994 survey that had the severe grapefruit stem-pitting isolate had been removed by the time of the 1998 survey. Thus, all the MCA 13-positive detections in 1998 are assumed to be new infections.

Thirteen plants from the Delray Beach survey in 1998 were tested further using molecular probes (Table 4). All reacted with probe 0, which detects all CTV. All except one of the plants (D6) also reacted with probe III, which detects certain stem-pitting strains of CTV. The plant that did

not react with probe III reacted to probe I, which detects decline strains. Two of the plants that reacted to probe III also reacted with probe I. There were no reactions to probe V, further suggesting that the severe stem-pitting strain, detected in 1996, had not spread to surrounding plants prior to removal of the tree. Only 6 of the 13 samples that reacted with probe III in 1998 had unequivocal MCA 13 reactions at that time.

Additional samples in 2000 indicated that one tree (D20) had acquired a decline strain and a mild strain, and one tree (D7) had acquired a decline strain and a stem-pitting strain in the intervening 2 years. D7 was CTV negative in the 1998 survey. In 2000, three plants (D7, D13, and D20) reacted positively to the orange stem-pitting detection ELISA. D7 had been ELISA negative in 1998. D13 and D20 were positive for CTV, but negative for MCA 13 antiserum in 1994–95. Both D7 and D20 were positive for MCA 13 in 1998.

Samples D6, D7, D11, D13, D20, D35, and D36 were biologically indexed on two Pineapple sweet orange and one Duncan grapefruit seedlings. Samples D6, D11, D13, and D35 did not cause stem pitting (0 rating) on Pineapple sweet orange, whereas samples D7, D20, and D36 caused mild (0.5 rating) stem pitting on Pineapple sweet orange seedlings. Samples D6 and D11 caused mild (0.5 rating) stem pitting on Duncan grapefruit, D7 and D20 caused mild (1.0 rating), and D13 and D36 did not cause stem pitting (0 rating). Neither isolate from Palm Beach Gardens caused stem-pitting in sweet orange or grapefruit.

Immokalee samples. All but 1 of the 63 samples (98.4%) taken from commercial groves in the Immokalee area reacted positively with the Taiwan antiserum (Table 3). Forty of the samples (63.5%) also reacted positively with MCA 13. There was evidence of a quick decline epidemic in progress at one of the sites. The area had not been sampled previously, but overall incidence of Florida severe strains of CTV (those that react positively with MCA 13) in the Gulf Citrus production area that includes Immokalee was about 23% in the 1994–95 survey (2).

Of the 49 trap plants, 5 reacted positively with the Taiwan antiserum. Two of these positive plants also reacted with MCA 13. Both of the latter plants reacted with probe I, which detects decline strains, and one plant also reacted with probe VIII, which detects mild strains. Interestingly, the other three plants had negative MCA 13 reactions, but they also reacted to probe V, which detects stem-pitting strains of CTV. All three of these plants also reacted with probe VIII, which detects mild strains of CTV.

Aphid transmission. Of 67 aphids collected from Immokalee area commercial groves in December 1998, 12 transmitted CTV (17.9%). CTV infection was detected

in receptor plants using the Taiwan antiserum. None of these samples reacted with MCA 13, and no molecular assays were done.

DISCUSSION

As has been experienced in other countries (23,24), there has been an increase in incidence of both mild and severe strains of CTV in Kendall, Ft. Lauderdale, and Delray Beach since the introduction of BrCA. In 1997, about 2 years after BrCA was first detected in the Immokalee area, there was evidence of a decline epidemic. The relatively high number of infected plants in Immokalee and the rapid progress of quick decline were probably the result of a relatively high incidence of Florida severe strains prior to the introduction of BrCA (2). Furthermore, the infection of the healthy trap plants placed in commercial groves in Immokalee indicates that the CTV strains were being spread naturally in this major citrus production area.

Mild and decline strains of CTV have been found previously throughout Florida (2,4,14). However, the incidence and spread of stem-pitting strains has not been documented. Molecular analyses of selected samples with the strain-specific probes indicate the incidence of stem-pitting strains of CTV in southeastern Florida (18). Samples reacting with probe III were found from trees in Kendall, Ft. Lauderdale, and Delray Beach. Samples reacting with probe V were found in Kendall, Ft. Lauderdale, and Immokalee. One sample collected in Ft. Lauderdale reacted with probe IV. The stem-pitting found upon biological indexing of the samples collected in 2000 from Delray Beach shows an association with reactivity against probes III, IV, or V and the presence of stem-pitting strains of CTV (Table 1). Further, the relationship between the reactivity of the probes and the biological activity (6) has been demonstrated by probe reactions with strains of known biological activity and also with field strains whose biological activity was determined in standard citrus indicator hosts (18,27). Reactions to probes III and V have been relatively rare in Florida samples prior to the sampling represented here (3,8,14,20). Our data suggest that the incidence of previously uncommon strains of CTV that may cause significant pathology in groves could be on the increase as a result of the introduction of BrCA.

The molecular data suggest that there has been an increase in strain diversity and in incidence and severity of CTV infections. The data also suggest that strains prevalent in one area may not be common in another area. For example, in Delray Beach, strains that react to probe III were found in nearly every sample subjected to molecular analysis; however, only three of eight samples from Kendall reacted to probe III. Similarly, strains reacting to

probe V were found in four of five Ft. Lauderdale samples, but in only two of eight samples from Kendall and in no samples from Delray Beach.

The Florida Citrus Budwood Registration Program currently utilizes MCA 13 to detect severe strains of CTV. Scion trees reacting positively to MCA 13 antiserum are no longer allowed to be used for budwood. Our results suggest that use of MCA 13 alone may be an unsafe practice. Although all eight samples from Kendall that were shown by the probes to contain severe decline or stem-pitting strains of CTV also reacted with MCA 13 (Table 4), the correspondence between MCA 13 and the probe reactions was poor elsewhere. In all, 11 of the 21 samples tested from Ft. Lauderdale and Delray Beach, and 3 of the 5 trap plants from Immokalee, gave either negative or borderline reactions with MCA 13; yet, the probes indicated that all 19 of these samples were infected with decline or stem-pitting strains, or both. The higher reliability of the strain-specific probes to detect severe strains probably results from the greater sensitivity of PCR and the subsequent amplification of millions of copies of the amplicons for detection by hybridization. There is concern that decline and stem-pitting strains could be present in the Florida citrus budwood scion trees and remain undetected by the current screening technology. This suggests that the scion trees should be free of all CTV.

The surveys in Delray Beach in 1996, when the severe stem-pitting strain was discovered, and the follow-up surveys in 1998 and 2000, indicated that the strain had not spread in the neighborhood, in spite of infestation by BrCA for several months prior to removal of the infected tree. The lack of spread of this isolate is further corroborated by laboratory aphid transmission studies in which no infection was found in 705 attempts with single BrCA (S. E. Halbert and K. L. Manjunath, unpublished).

The limited field infectivity assay may be the only one reported for the BrCA-CTV system. Thus, it is not possible to say whether 17.9% is high or low relative to other findings. A transmission rate of 15% is in the low-to-average range for laboratory transmission experiments with CTV (26), and other phloem-limited viruses, such as *Potato leafroll virus* (11). However, 17.9% is quite high in comparison with field infectivity data on *Barley yellow dwarf virus*, another phloem-limited virus (12). Many more field infectivity tests should be done to determine whether infectivity varies seasonally or with location.

The correlation between the various analyses performed on the sampled trees in Delray Beach is not exact. For example, in 1998, D6 reacted to probe I, which detects decline strains, but there was no reaction to probe I in 2000, and there was never any reaction to MCA 13. To make matters even

more complex, there was mild stem-pitting in grapefruit caused by this isolate in the biological indexing experiment. Sample D13 was positive for the orange stem-pitting detection ELISA, but did not react to any stem-pitting detection probes and caused no stem-pitting in the biological indexing. Finally, D36 was positive for the orange stem-pitting detection ELISA and caused mild stem-pitting in sweet orange, but it was not positive for any of the probes that detect stem-pitting. We suspect that these anomalies are due to the mixtures of CTV strains found in field isolates. It has been shown, for example, that stem-pitting strains can be recovered by aphids from Florida CTV isolates that are negative for stem-pitting by all known tests, including biological indexing (25). Much more robust analyses are needed if budwood sources are to be protected adequately.

Most of the trees sampled in the urban areas were mature trees. The incidence of infection found by Brown and Davison (2) represents an equilibrium achieved over several decades between prevalent strains of CTV and common citrus aphids, including *Aphis gossypii* Glover, *Aphis spiraeola* Patch, *Aphis craccivora* Koch, *Toxoptera aurantii* (Boyer de Fonscolombe), and a few other relatively rare species. Our results demonstrate that CTV has spread rapidly since the establishment of BrCA. They also demonstrate that previously undetected severe strains of CTV are present in these areas of Florida. Our results also suggest that some severe strains may not be detected in Florida citrus budwood sources by current ELISA technology. These results have potentially serious implications for citrus production in Florida, especially given the history of the occurrence of severe stem-pitting strains of CTV following in the years after the introduction of the BrCA in other countries (1,23). We recommend continued surveys, research, and vigilance in Florida and other citrus-producing states.

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