Tristeza and *Toxoptera citricida* in Cuba: Incidence and Control Strategies

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ABSTRACT. In Cuba, 130,000 ha are dedicated to citriculture and the predominant rootstock is sour orange. Citrus tristeza virus (CTV) is present at low incidence (0.1 to 3.0%) in all citrus areas surveyed, except in the Isle of Youth where the incidence was approximately 15%. Although CTV-infected trees were asymptomatic in the field, isolates that reacted to strain-differentiating monoclonal antibody MCA-13 were found. These isolates had coat protein gene sequence similar to known isolates of CTV that cause stem-pitting in grapefruit and/or decline on sour orange rootstock. Several isolates caused moderate decline reactions or stem pitting when biologically indexed in greenhouse host-range tests. The brown citrus aphid, *Toxoptera citricida* (Kirkaldy), was first detected in Guantanamo Province in March 1993, and has spread slowly and irregularly in the Eastern and Central regions. CTV incidence in the field increased after the appearance of brown citrus aphid. A summary of the multi-phased control strategies for the CTV/brown citrus aphid complex in Cuba is presented, which includes a certification program, eradication of CTV-positive plants, and efforts to reduce brown citrus aphid populations.

Tristeza is a highly destructive disease of citrus that has caused the death of millions of plants all over the world (2). It is caused by citrus tristeza closterovirus (CTV). The threat of CTV to the Caribbean Basin region has been greatly increased by the establishment of the brown citrus aphid (BrCA), *Toxoptera citricida* (Kirkaldy), in this important citrus-growing region. The most commonly used rootstock in the Caribbean Basin is sour orange which is highly susceptible to CTV-induced decline. The combined presence of BrCA, CTV, and widespread use of sour orange rootstock poses a great threat to continued citrus production in Cuba and the rest of the Caribbean Basin.

Cuba has been conducting surveys for CTV in the major citrus areas beginning in 1992. After the initial finding of the CTV in field surveys, research was begun on characterization of selected isolates and epidemiology to determine the rate of natural spread of CTV by the exotic BrCA. In this paper, we summarize the results of the CTV surveys, characterization of Cuban CTV isolates, and discuss a multi-phased control strategy being used to reduce the economic impact of the CTV/BrCA complex in Cuba.

MATERIALS AND METHODS

Surveys for CTV. Two percent of all the trees throughout Cuba were sampled. Four young stems (10 to 15 cm) from opposite sides of each tree were collected during the cool part of the year. They were grouped into composites of three plants, stored in plastic bags at 4°C for a few days until processed for ELISA. Data on variety, rootstock, planting date, field location were recorded when the samples were collected, and filed in a computerized data base.

After finding CTV in some areas in the 2% survey, follow-up surveys were conducted in CTV-infected areas. In each of these areas, leaf samples (5 to 6 per plant) were collected and handled as above but were processed as single plant samples for serology.

DAS ELISA. Samples collected from field surveys were analyzed by double antibody sandwich (DAS) ELISA in one of eight laboratories in

the different regions of Cuba. The general protocols described by Bar-Joseph et al (1) was used. Microtiter plates were coated with IgG from the broad spectrum Cuban MAb, $3C_1F_{10}$, (3, 4) using a concentration of 1 µg/ml in coating buffer for 4 hrs at 37°C. Samples were prepared by chopping young bark tissue into small pieces, adding extraction buffer at a 1:5 (w:v) ratio, and homogenizing with an Ultraturax homogenizer. After homogenization, the extracts were filtered through gauze, and aliquots placed in duplicate wells and incubated 16 hrs at 4°C. MAb $3C_1F_{10}$ IgG conjugated with alkaline phosphatase diluted at 1/3,000 in conjugate buffer was added and incubated for 3 hrs at 37°C. One mg/ml of p-nitrophenyl phosphate in substrate buffer was then added. The OD_{405} was read after 3 hrs using an ELISA plate reader. Samples which gave OD readings greater that twice that of the healthy controls were considered to be positive.

DAS-I ELISA. The protocol used for DAS-indirect (I) ELISA was similar to that described for DAS ELISA except for these details. Microtiter plates were coated with 1 µg/ml of the polyclonal antibody 1052 made against purified unfixed CTV isolate T36 (8) for 4 hrs at 37°C. Leaves were washed in tap water and the sap was extracted with a leaf roller. After dilution with five parts extraction buffer, aliquots were loaded in duplicate wells and incubated 16 hrs at 4°C. The secondary antibody was diluted appropriately in conjugate buffer (1/10,000 for ascites fluid of MAb MCA-13 (11) for detection of severe strains or 1/10,000 of ascites fluid of MAb 17G11 or ascites fluid of Cuban Mab $3C_1F_{10}$ for broad spectrum detection of CTV). Goat anti-mouse conjugate was used at a dilution of 1/30,000 in conjugate buffer for detection of the MAb.

Monitoring CTV spread in the presence of BrCA. Each of the trees in five fields in Guantanamo province were sampled for CTV: four fields had CTV-positive plants and the fifth field was CTV-negative. Each tree was sampled at 6-mo intervals for presence of CTV.

Biological characterization of CTV isolates. Some CTV isolates from Cuba were sent to the quarantine greenhouse in Beltsville, MD where they were biologically indexed on a host range of three plants each of Mexican lime, sour orange seedlings, sweet orange on sour orange rootstock, Madam vinous sweet orange, and Duncan grapefruit (6). Other isolates were indexed in Mexican lime in a greenhouse in Cuba.

Molecular characterization of the coat protein gene of Cuban CTV isolates. Reverse transcriptase polymerase chain reaction (RT-PCR) as previous described (10) was used to amplify the coat protein gene (CPG) of several Cuban CTV isolates. The amplified PCR product was cloned into PUC 118, cloned and sequenced as previously described (10). The sequence of the CP genes from Cuban isolates was compared to the sequence from other CTV isolates by the Seqaid program (12) and Clustal analyses (5, 7).

Detection and study of BrCA. A sampling methodology was developed to monitor the spread of BrCA and to estimate BrCA populations. This methodology was based on surveying 10% of the plantations every 10 days, using a 50 × 50 cm frame. Young shoots, capable of supporting aphid reproduction, were counted including those colonized by aphids and the aphids were classified by species. In each field, five trees were selected and sampled using an S-shape frame. Two collections were carried out, one on each side of the tree. When shoots were colonized with BrCA, the presence of natural enemies was also reported as the percent of shoots colonized and natural enemies as percent of colonized shoots affected.

RESULTS AND DISCUSSION

CTV has been detected in all citrus areas surveyed in Cuba. Table 1 summarizes the results of the initial survey of two percent of the trees. The highest CTV incidence was found in the Isle of Youth (14.8%) followed by Ciego de Avila (1.7%). In the rest of the areas, the percentage of trees with CTV was less than 1%. The CTV distribution in Cuba is shown in Fig. 1. Although some areas were not surveyed, it is evident that CTV is distributed throughout the country. No trees showing tristeza symptoms were found, even though sour orange is the predominate rootstock. This suggests the presence of virus strains that do not cause decline.

Survey results from the secondary survey using MAbs $3C_1F_{10}$ and MCA13 are shown in Table 1. The number of CTV-positive plants using MAb $3C_1F_{10}$ is greater than obtained during the initial 2% survey. This is probably due to the fact that a smaller number of trees were sampled as individual trees instead of composites of three plants. In addition, this survey was targeted to areas known to have CTV. In Jaguey Grande, Troncoso and the Isle of Youth areas, plants positive to MCA-13 were found. This suggests presence of CTV strains capable of producing decline in trees on sour orange or stem pitting in grapefruit or sweet orange, although this must be confirmed by biological tests. In some instances more trees were identified with the use of MCA13 than with MAb $3C_1F_{10}$ (Table 2). This may be because each monoclonal antibody recognizes a specific epitope, and some isolates may be lack the specific epitope recognized by an antibody. In the case of MCA13, the epitope for recognition coincides with presence

TABLE 1
INCIDENCE OF CITRUS TRISTEZA VIRUS (CTV) IN DIFFERENT REGIONS OF CUBA

Initial CTV survey using composite samples			Secondary survey for severe strains in areas having CTV		
Region	No. of composites[z]	CTV incidence[y]	No. trees	% CTV positive[x]	% positive for severe CTV[w]
Sandino	15,645	0.4	88	1.1	5.7
Troncoso	1,271	2.8	460	2.2	0.4
C. Tormas	2,473	0.0	—	—	—
Ceiba	10,633	0.4	241	1.3	1.3
V. Giron	62,621	0.6	873	0.3	0.3
Ciego	7,748	3.1	—	—	—
Moron	1,700	1.1	—	—	—
Sola	2,825	2.0	—	—	—
Banes	693	0.4	—	—	—
A. Libre	17,328	0.1	—	—	—
Yateras	2,064	0.6	—	—	—
Vilorio	3,517	0.1	—	—	—
Isle of Youth	8,420	4.7	186	36.6	1.1
C. de Avila	—	—	241	1.3	1.3
Guantanamo	—	—	16	12.5	0.0

[z]Expressed as the total number of trees sampled. Samples were each a composite of three trees and tested for CTV by using DAS ELISA and MAb $3C_1F_{10}$ antibody.
[y]Expressed as the percent of the total number of trees tested which were CTV positive.
[x]Expressed as the percent of trees which were CTV positive using DAS ELISA and MAb $3C_1F_{10}$ antibody. Single tree samples were used for assay.
[w]Expressed as the percent of trees which reacted against MCA-13 antibody in DASI ELISA.

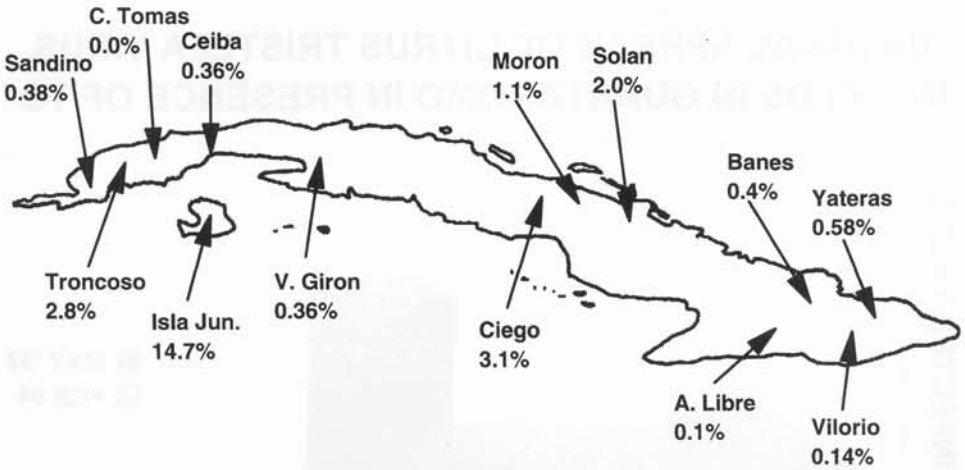


Fig. 1. Map of Cuba showing the citrus areas which were surveyed for presence of citrus tristeza virus (CTV). Following is a list of enterprises surveyed in the two percent survey, number of samples tested for CTV, and % CTV-positive: Sandino, 15,645 and 0.38%; Troncoso, 1,271 and 2.8%; C. Tomas, 2,473 and 0%; Ceiba, 10,633 and 0.36%; V. Giron, 62,633 and 0.36%; Ciego, 7,748 and 3.1%; Moron, 1,700 and 1.1%; Solan, 2,825 and 2.0%; Banes, 693 and 0.4%; A. Libre, 17,328 and 0.1%; Yateras, 2,064, 0.58%; Vilorio, 3,517 anf 0.1%; and Isla Juv. 8,420, 14.7%.

of severe biological activity in the CTV isolate (9, 11).

The study of natural CTV diffusion carried out in the five fields in Guantanamo Province infested by BrCA showed that the control field (CTV-free) remained CTV-free, while the CTV incidence in the other fields increased, becoming much higher in one field (Fig. 2). Hence, in this study, BrCA spread CTV in the location near sources of inoculum in a rapid fashion but did not vector CTV to more distant areas.

Biological tests in Cuba with some CTV isolates suggests the

TABLE 2
RESULTS OF BIOLOGICAL INDEXING OF CUBAN ISOLATES OF CITRUS TRISTEZA VIRUS ON INDICATOR PLANTS AT THE QUARANTINE GREENHOUSE, BELTSVILLE, MD.

CTV isolates		Indicator plants				
Beltsville code	Cuban code	ML[z]	SO[y]	SwO/SO[x]	Gpft[w]	MVSwO[v]
B303	HV 5/5	0.5	0	1.0	2.0	0
B305	CA 20/79	?	0	0	0	0
B310	JG 5/5	0.5	0	2.0	1.5	0
B314	JG 11/72	1.0	0	0	1.0	0

[z]ML = Mexican lime grafted on *C. macrophylla* evaluated for stunting, vein clearing and stem pitting: 0 = healthy; 1 = mild symptoms; 2 = moderate symptoms; 3 = severe symptoms.
[y]SO = Sour orange seedlings evaluated for seedling yellows (SY): 0 = healthy; 1 = mild; 2 = moderate; 3 = severe.
[x]SwO/SO =sweet orange on sour orange budlings evaluated for decline reaction: 0 = healthy; 1 = mild decline; 2 = moderate decline; 3 = tree death.
[w]Gpft = Duncan grapefruit seedling evaluated for stem pitting (SP): 0 = healthy; 1 = mild SP; 2 = moderate SP; 3 = severe SP.
[v]MVSwO = Madam Vinous sweet orange seedling evaluated for SP: 0 = healthy; 1 = mild SP; 3 = severe SP.

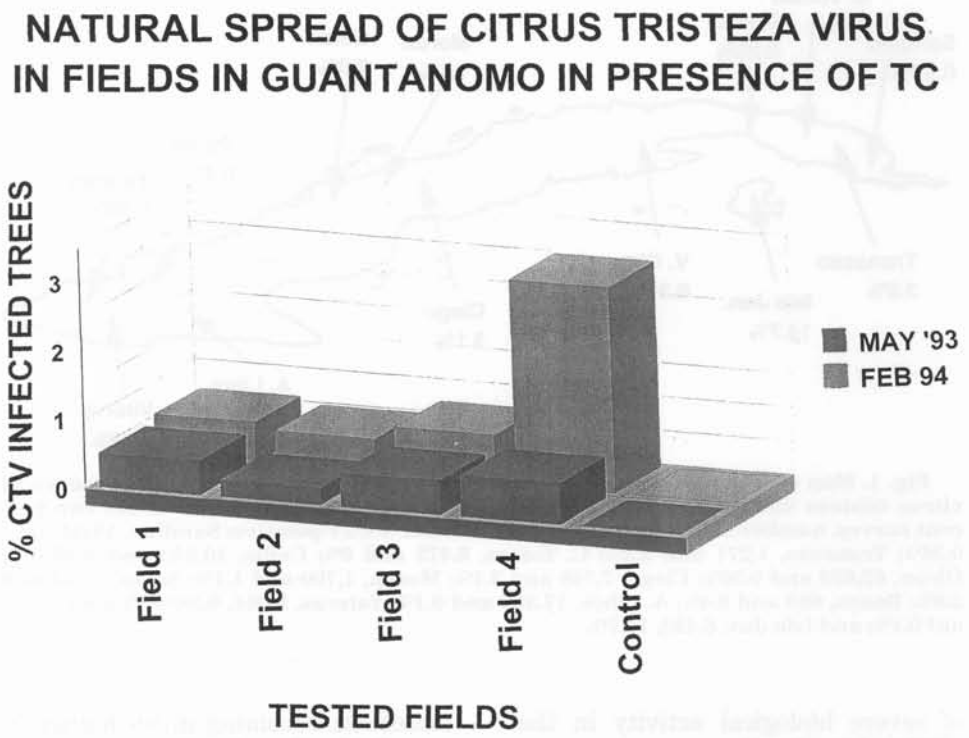


Fig. 2. Natural spread of citrus tristeza virus in fields in Guantanomo in the presence of *T. citricida*.

presence of mild strains, because only slight symptoms were observed in Mexican lime. Nevertheless, in Beltsville tests, some Cuban isolates caused mild to moderate stem-pitting in grapefruit and mild to moderate dwarfing in sweet orange grafted on sour orange (Table 2). These results suggest the presence of more severe strains, although no symptoms of CTV have been observed in the field in Cuba.

The PCR products from the CPG of the isolates B305 and B310, when sequenced, all had 669 base pairs (10). The computer analysis of the CPG sequence from several isolates shows a correlation with sequence relationships and the biological activity (10). The dendogram illustrating the clustering relationships among the deduced amino acid sequences of the coat proteins of the Cuban CTV isolates compared with several other CTV isolates constructed from the deducted amino acid sequence is shown in Fig. 3. Cuban isolate B305 is clustered with mild isolates similar to T30 from Florida and differs from T30 by only one amino acid in position 15. Cuban isolate B310 clustered with severe, stem pitting isolates B53 from Spain (illegally introduced from Japan) and B227 from India. Cuban isolate B310 differs in amino acid sequence from Florida isolate T36 in five amino acids at positions 31, 41, 100, 115 and 208.

The distribution of BrCA in Cuba as of November 1995 included the eastern two thirds of the island. The sampling methodology developed to monitor the spread of BrCA and to estimate BrCA populations was useful in developing a strategy for control. Numerous parasitoids, predators, and pathogens of BrCA have been identified in Cuba from these surveys (Table 3). Further research is underway to utilize these

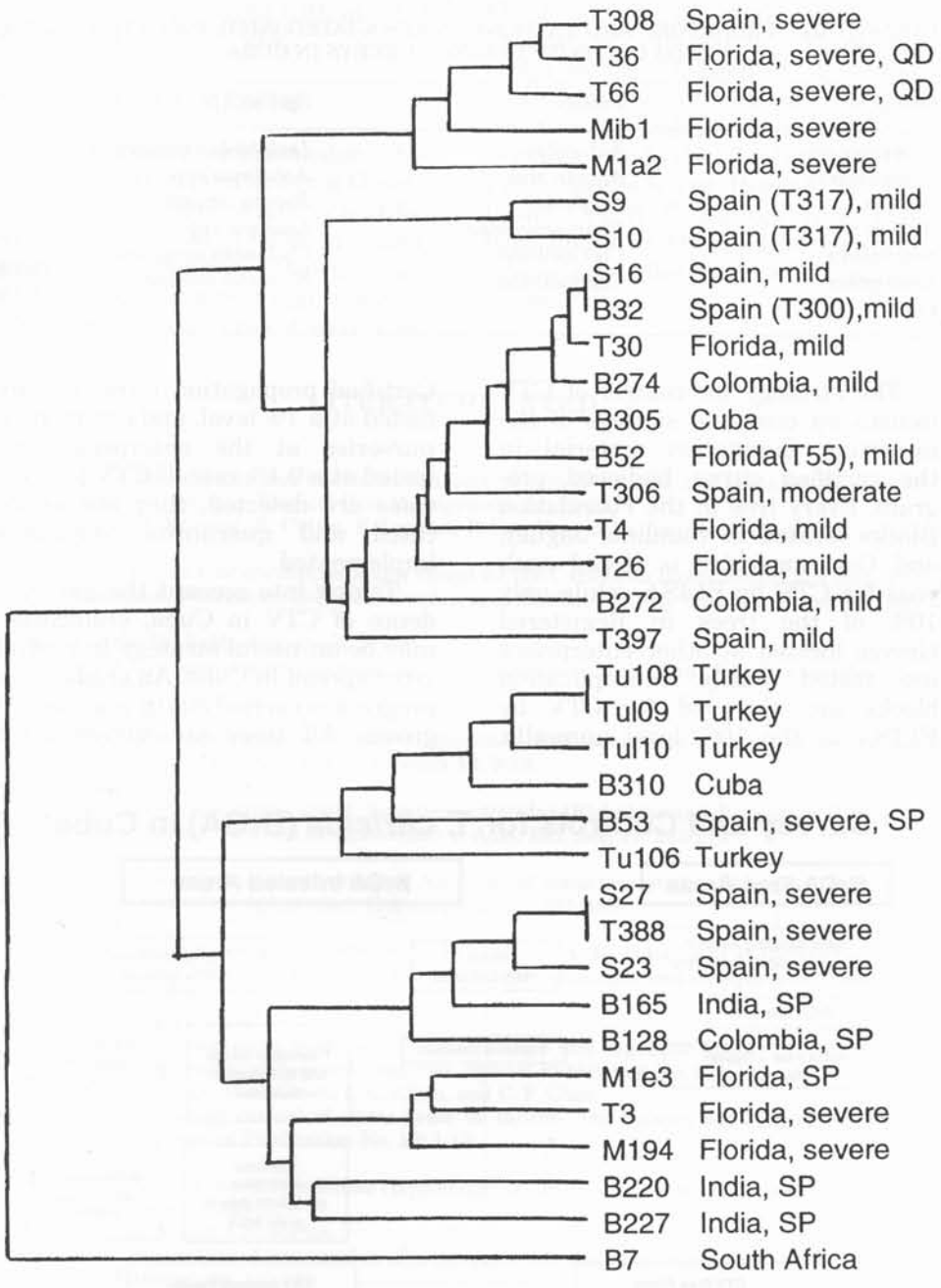


Fig. 3. Dendrogram showing the relationship of Cuban isolates of citrus tristeza virus (CTV) with other isolates of CTV. The dendrogram is developed from the amino acid sequence of the coat protein gene of CTV.

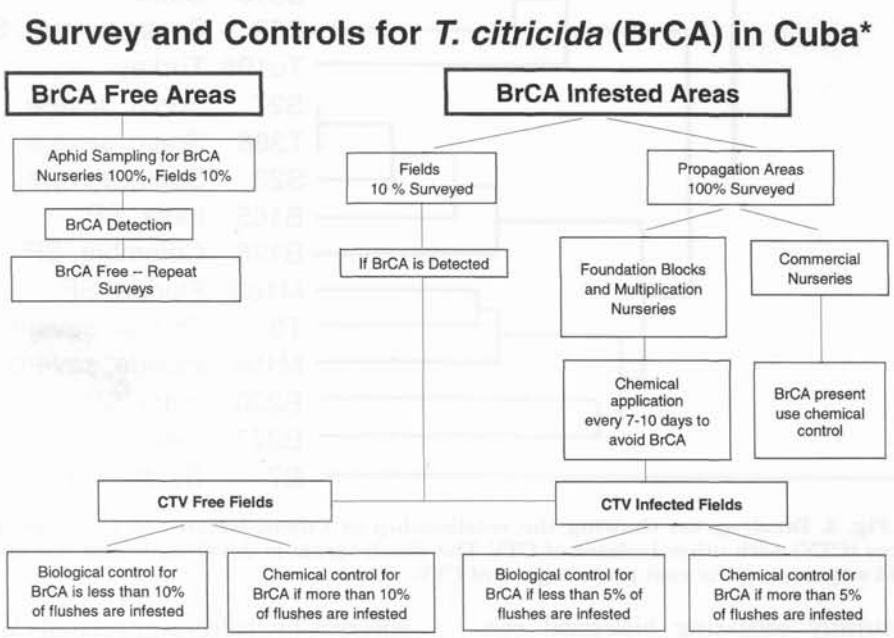
naturally occurring biological control agents to maintain low population levels of BrCA. The strategy for control of BrCA is outlined in Fig. 4. Chemical control is used in propagation areas for control of BrCA, whereas biological control and chemical control are used in field situations. The threshold where chemical control is used is lower in fields infected with CTV (5%) as compared to CTV-free fields (10%).

TABLE 3
PARASITOIDS, PREDATORS AND PATHOGENS ASSOCIATED WITH *TOXOPTERA CITRICIDA* COLONIES DURING SURVEYS IN CUBA

Group	Family	Species
Hymenoptera	Aphididae	*Lysiphlebus testaceipes*
Hymenoptera	Aphelinidae	*Aphelinus* spp.
Diptera	Syrphidae	*Baccha clavata*
Diptera	Chamaemyiidae	*Leucopis* spp.
Neuroptera	Coccinellidae	*Cycloneda sanguinea*
Neuroptera	Coccinellidae	*Scymnus roseicollis*
Fungi	—	—

The strategy for control of CTV focuses on complete surveys of the important foundation material in the certified citrus budwood program. Every tree in the Foundation Blocks located at Sandino, Jaguey, and Contramaestre is tested each year for CTV by ELISA, while only 10% of the trees in Registered Groves located at other enterprises are tested yearly. Multiplication blocks are surveyed for CTV by ELISA at the 10% level annually. Certified propagation nurseries are tested at a 1% level, and commercial nurseries at the enterprises are tested at a 0.1% rate. If CTV positive trees are detected, they are eradicated and quarantine measures implemented.

Taking into account the low incidence of CTV in Cuba, eradication may be an useful strategy to control virus spread in Cuba. An eradication program is carried out in commercial groves. All trees are surveyed for



* Traps, inspection and sampling for BrCA throughout the country each 7-10 days. All (100%) of the foundation blocks, commercial and multiplication nurseries and 10% of the citrus fields were surveyed.

Fig. 4. Survey and controls for brown citrus aphid (BrCA), *Toxoptera citricida*, in Cuba.

CTV in areas where the preliminary 2% survey indicated the presence of CTV. CTV infected trees are removed, and CTV tolerant rootstocks are now being used for new propagations. In the Isle of Youth, the CTV-infected trees could be eliminated with the sole purpose of reducing the inoculum. When CTV-infected trees are found, they are removed.

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