Rapid Dissemination of Mild Isolates of Citrus Tristeza Virus Following Introduction of *Toxoptera citricida* in the Dominican Republic

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ABSTRACT. Rapid increase in the incidence of citrus tristeza virus (CTV) infection occurred in test plots established in large commercial 4- to 6-yr-old sweet orange plantings at three locations in the Dominican Republic. Two of the 20 × 20 tree plots were Valencia sweet orange on Carrizo citrange rootstock and the third was Valencia on sour orange rootstock. In each location, some trees infected with mild isolates had apparently been established via propagation of infected budwood. Samples were collected from all trees at ca. 6 mo. intervals between September 1992 and May 1995, and these were tested for CTV by DAS-I ELISA. Polyclonal antibodies and a mixture of two broad-spectrum monoclonal antibodies (MABs) were used for general detection of CTV infection, and the selective MAB, MCA13, was used to detect decline and stem pitting isolates. Initially CTV incidence in the plots was 1, 8, and 50%, respectively. Within 32 months the CTV incidence had risen to 93, 48, and 93%. In a similar plot of Marsh grapefruit on Swingle citrumelo rootstock, infection incidence increased from 0% to only 2% during the same period. In a second grapefruit block surveyed over a shorter period, no infection was detected. Over 700 additional trees in numerous locations were also sampled. During the portion of this study reported, more than 1,420 CTV-infected trees were detected, but none tested positively with MCA13, and no decline was observed in CTV-infected trees on sour orange. MCA13-positive decline isolates were detected in June 1996 at one location approximately 10 km from one plot location. Currently, rapid spread of CTV isolates has not affected citrus production in the Dominican Republic, however, spread of severe isolates into commercial plantings from existing dooryard citrus, or from CTV-infected introductions, remains a serious threat.

Key words. ELISA, MCA13, epidemiology, virus detection, grapefruit, sweet orange, virus transmission.

Rapid spread of the brown citrus aphid, *Toxoptera citricida* (Kirkaldy) into Central America, citrus-producing Caribbean islands, and, more recently, Florida has created concern about increased losses from severe strains of citrus tristeza virus (CTV), and has prompted efforts to obtain more accurate information on distribution of the brown citrus aphid and on incidence of CTV throughout the Caribbean basin (9, 12, 15, 16).

Previous observations of CTV epidemics in Argentina, Brazil, Peru, and Venezuela indicated that CTV was spread rapidly by the brown citrus aphid, and that once it was introduced into a new area, severe isolates of CTV became more prevalent (12). However, these observations were made after epidemics of CTV were well established, and were based on observation of symptoms which do not accurately indicate the time of infection or infections by mild isolates. Only limited experimental data are available on the rate and pattern of spread of CTV by the brown citrus aphid during the early stages of an epidemic (10). Some data have recently been obtained on rates and patterns of CTV spread with the melon aphid, *Aphis gossypii* Glover, as the principal vector (7).

ELISA (enzyme-linked immunosorbent assay) provides a convenient tool to monitor the distribution and spread of CTV, even when trees do not show symptoms (6, 10). A rapid presumptive determination of strain severity can also be done by

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ELISA using the monoclonal antibody MCA13 (11). MCA13 reacts to most decline and stem pitting isolates of CTV, but does not react to mild isolates in Florida which do not cause decline or stem pitting (5, 11).

Preliminary surveys in the Dominican Republic made in 1990 and 1992 indicated that CTV was present, but was not yet widespread in recently established, large-scale commercial plantings (1). These plantings, which totaled approximately 12,000 ha, were propagated primarily from bud sources that originated from virus-free budwood imported from California or from budwood obtained from Florida of undetermined CTV status (1). Most of these trees were propagated on tristeza-tolerant rootstocks, but some plantings were made on sour orange, and most older dooryard and small commercial plantings are on sour orange. The large new commercial plantings were developed at several different areas on the island. Initial tests of dooryard trees and commercial plantings indicated the presence of both mild and MCA13-positive isolates, but most infected trees found in commercial plantings did not react to MCA13, and were presumed similar to mild isolates found in Florida. Establishment of the brown citrus aphid in the Dominican Republic was confirmed in 1992, and it was soon found in all areas (1). This situation suggested that valuable information about movement of CTV could be obtained by monitoring spread of CTV in citrus plantings in the Dominican Republic. Accordingly, survey plots were established in several different areas to monitor the rate of increase and the pattern of movement of CTV. A corresponding study was also established in Costa Rica where two serologically distinguishable isolates were present (8).

This paper describes the surveys conducted and documents the dissemination of mild isolates of CTV in citrus by the brown citrus aphid in the Dominican Republic.

METHODS AND MATERIALS

Plots. Four plots were established in September 1992. Each plot consisted of 400 trees in a 20 × 20 tree configuration. Plot DR1 was established in a planting of Valencia sweet orange on Carrizo citrange rootstock planted in February 1990 as part of a commercial sweet orange planting of approximately 1,500 ha near Villa Altagracia (Fig. 1a). Plot DR2 was established in a planting of Valencia sweet orange on Carrizo citrange within a commercial planting of approximately 500 ha of sweet orange, grapefruit and Persian lime near Bayaguana (Fig. 1b). The trees were approximately 5 yr old when the study began. This planting is about 40 km east of DR1 (Fig. 1a). Plots DR3 and DR4 were located in a large commercial planting of approximately 2,400 ha near Hato Mayor of which approximately 6% are grapefruit. This area is about 120 km northeast of Santo Domingo and about 50 km to the east of DR2 (Fig. 1a). DR3 was established in a planting of Marsh grapefruit on Swingle citrumelo planted in 1988. DR4 was established in a planting of Valencia sweet orange on sour orange rootstock planted in 1987. DR4 was approximately 2 km to the east of DR3 and the intervening area is essentially a solid planting of citrus.

Subsequently, two additional plots were established. DR5 consisted of 320 trees (16 × 20) of Marsh grapefruit on Carrizo citrange within a grapefruit planting approximately 150 m west of DR2. These trees were planted in 1987. DR6 consisted of 400 trees of sweet orange (mostly navels) on sour orange rootstock in a commercial planting of approximately 200 ha of sweet orange near La Cumbre. This planting is about 20 km north of DR1, and is geographically separated from it

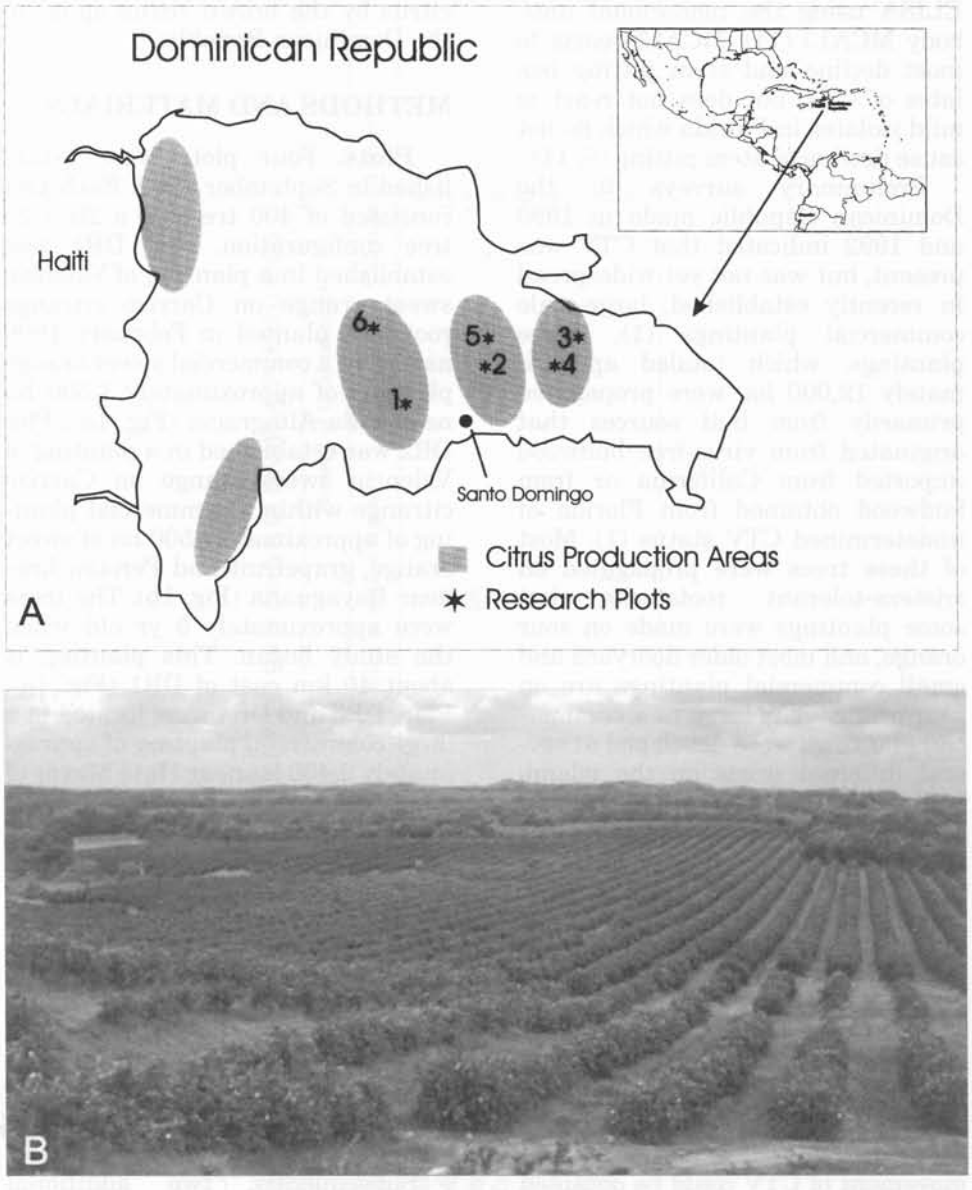

Fig. 1. A) Location of test plots established in different citrus growing regions in the Dominican Republic to monitor spread of citrus tristeza virus, B) Commercial planting which was site for test plots DR2 (sweet orange in mid-picture) and DR5 (grapefruit in far background). Picture taken May 1994.

by a low mountain. These trees were about 5 yr old when testing began.

Over 700 additional samples were collected from trees near the test plots, and from dooryard and small commercial plantings in a variety of other locations to further measure distribution of CTV in the Dominican Republic. These were collected and processed in the same manner as the plot samples.

Collection and processing of tissue samples for ELISA. The plots were sampled on approximately 6-mo intervals and observed for brown citrus aphid populations. Samples for ELISA were petioles from four leaves collected from indi-

vidual flushes of new growth around the tree. The peduncle from two or three small fruit was collected if no new flush was present. The tissue collected from each tree was placed in small white 4.5 × 7.5 cm paper bags (Amcraft) pre-marked with plot, row, and tree designation (Fig. 2a). These bags were stapled in groups of five, and placed in a ziplock plastic bag over blue indicator Silica Gel (Davison Chemicals, Inc., Baltimore, MD 21203) (Fig. 2b). The initial 2.5 g of silica gel used per sample was changed, if necessary, to complete the drying process.

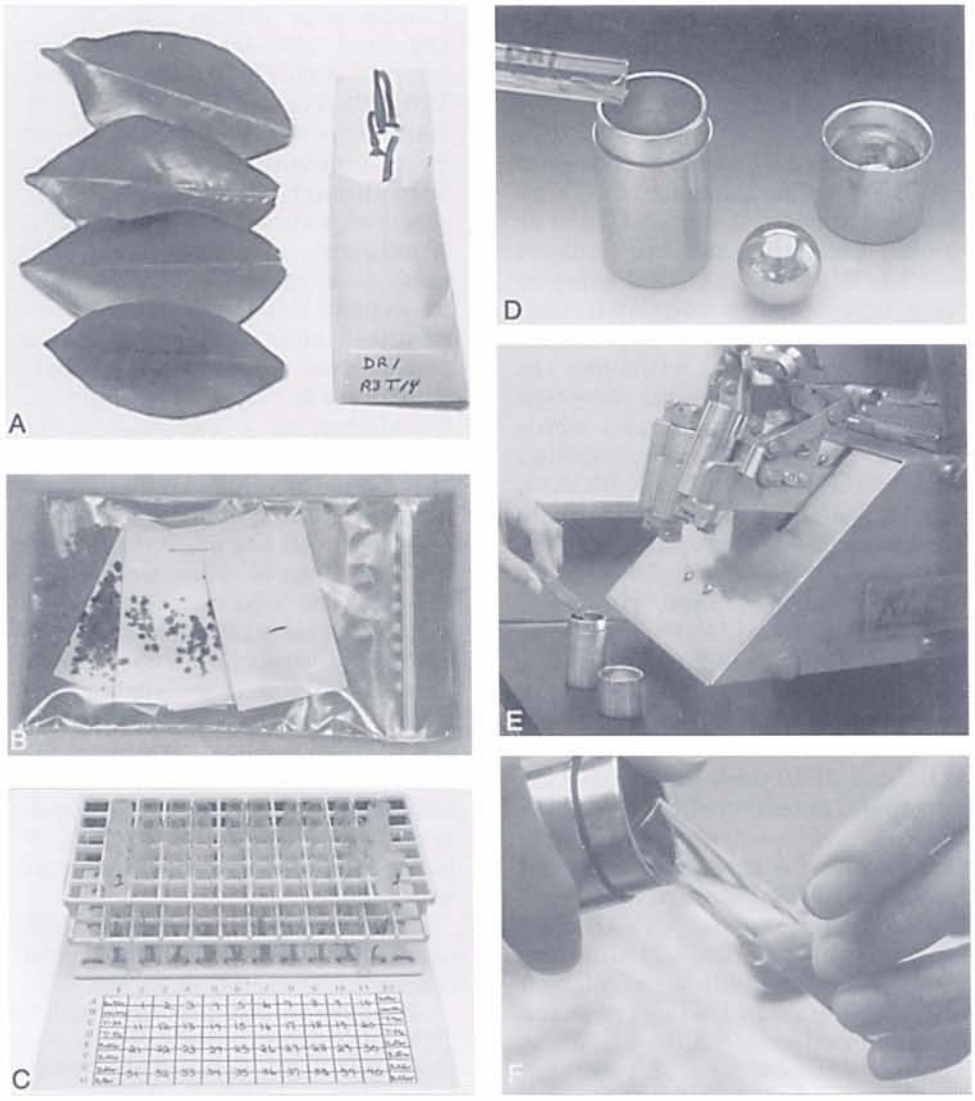

Fig. 2. Collection and processing of samples for determination of citrus tristeza virus infection by ELISA. A) Petioles removed from four leaves collected from young growth flushes at the tree periphery are separated from the leaf blade and placed in coded paper envelopes. B) Envelopes placed in plastic bags containing a self indicating silica gel desiccant for drying, shipment, and storage. C) Samples organized in tube rack according to ELISA plate loading pattern. D) Dry samples placed in canister of Kleco tissue pulverizer with PBST extraction buffer. E) Kleco pulverizer and F) Extract returned to sample tube ready for ELISA.

Hydrated silica gel was reactivated by heating at 200°C for 1 hr.

Dried samples were stored at 4°C. Most samples were extracted by use of a Kleco tissue pulverizer (Kinetic Laboratory Equipment Co., Visalia, CA 93292) (Fig. 2d and 2e). The dry samples were organized for processing (Fig. 1c) and placed in the stainless steel sample canister with 5 ml PBST (Fig. 2d). A stainless steel ball was added and the canister was oscillated vigorously for 30 sec to thoroughly disrupt the tissue (Fig. 2f).

Serological assays. Samples were assayed for presence of CTV by double antibody sandwich (DAS) or double antibody sandwich indirect (DAS-I) ELISA (3, 11). The samples were tested with a polyclonal antibody in DAS ELISA or with a mixture of monoclonal antibodies in DAS-I ELISA for general detection of all isolates. Those samples which reacted positively were tested against the selective MAB, MCA13, to test for those that would likely induce decline of stem pitting (11).

The coating antibody for all tests was IgG purified from polyclonal antisera to Florida isolates T36 (1052) or T3 (908) and was used at 1 g/ml. For DAS tests, the detecting antibody was 1052 IgG conjugated to alkaline phosphatase. The MABs 11B1 and 3E10 used in combination for general detection of CTV by DAS-I are broad spectrum MABs prepared against a Taiwanese isolate of CTV (13). In combination, they react to all known isolates of CTV (S. Garnsey, unpublished data). MABs were used either as ascites or purified IgG, and diluted in PBST to near the maximum effective dilution (highest dilution without loss of reaction). Alkaline phosphatase-labeled goat antimouse IgG (Boehringer Mannheim, Indianapolis, IN 46250) was used as directed to detect antigen-bound CTV-specific MABs.

Costar High Binding EIA plates (Costar, Cambridge, MA 02140) were used in most tests. Tissue samples were extracted at a ratio of approximately 10 parts buffer to 1 part fresh tissue and were stored at 4%C until transferred to the ELISA plates. Each sample was tested in two wells, and each plate included 40 samples plus internal controls of PBST, extracts of healthy citrus, extracts from citrus infected with Florida mild isolate T30 (MCA13-negative), and extracts from citrus infected with Florida decline isolate T36 (MCA13-positive). The control extracts were rehydrated, freeze-dried preparations and were uniform through all tests. Plates were observed visually following addition of substrate and when satisfactory color development was observed in the control wells, (normally 20 to 30 minutes after adding substrate) plates were read using a Biotek EL310 plate reader. The data was transferred to a computer spread sheet for further processing. The absorbance readings for the positive controls were usually between 1.2 and 1.5 at 405 nm while readings for healthy extracts were usually 0.05 or less. The readings for the T30 extract tested against MCA13 were similar to those for healthy tissue. Test samples were considered positive if the absorbance value exceeded the value of the healthy control plus 0.1. The differences between infected and healthy samples were usually very clear, and the average absorbance for CTV-infected field samples was > 1.0.

Some samples were also tested using a direct tissue blot assay procedure as previously described (5).

Bioassays. Samples from several CTV-infected trees within the survey plots were collected and sent to the USDA, ARS, Beltsville Agricultural Research Center, Beltsville, MD, for biocharacterization and comparison with isolates from other countries. These isolates were established in a quarantine greenhouse by graft-inoculation to healthy Madam

TABLE 1
INCREASE IN TRISTEZA VIRUS INFECTION WITHIN SURVEY PLOTS ESTABLISHED IN COMMERCIAL CITRUS PLANTINGS IN THE DOMINICAN REPUBLIC AS DETECTED BY ELISA

Date	DR1[a]	DR2	DR3	DR4	DR5	DR6
Sept. 1992	8.3[y]	49.9	0.0	1.3	ns	ns
May 1993	16.2	65.7	0.0	16.5	ns	ns
Dec. 1993	19.5	79.6	0.0	30.8	ns	ns
May 1994	27.8	86.0	0.2	49.0	0.0	ns
Dec. 1994	37.4	90.6	0.4	70.0	0.0	51
May 1995	52.0	93.8	2.1	87.3	0.0	75
trees[x]	396	371	388	400	320	398

[a]Plots DR1, DR2, DR4 and DR6 were in plantings of sweet orange. Plots DR3 and DR5 were in grapefruit plantings.
[y]CTV infection was determined by DAS-I ELISA by testing samples of young petioles as described in the text. ns = not sampled.
[x]Total number of trees in each plot. Plots were rectangular with 20 rows of 20 trees, except for DR5 which consisted of 16 rows of 20 trees.

Vinous sweet orange seedlings and were then tested on a replicated standard set of five indicators as previously described (4).

RESULTS

Initial survey. Results of the initial survey in September 1992 indicated that infection levels in plots DR1 and DR4 were low, and that no infection was present in DR3 (Table 1). In contrast, about 50% of the trees in DR2 were infected. None of the infected trees reacted to MCA13, and surveys of trees on sour orange at different locations did not reveal any decline that could be associated with CTV. Colonies of brown citrus aphid were observed on at least some trees in all plots. The level of aphid infestation was proportional to the amount of new flush present.

Increase of CTV infection in sweet orange. A rapid increase in infection was measured in the three sweet orange plots in subsequent surveys (Fig. 3, Table 1). The rapid increase of infection in DR2 is not surprising given the high initial infection rate in 1992. However, rates also increased rapidly in DR1 and DR4 where initial infection levels were lower. The increase in DR1 was slower than in DR4, but trees in DR1 were less vigorous and had less new flush than those in other locations, apparently because of damage from root weevils and less favorable soil conditions. None of the infected trees in these sweet orange plots tested positively against MCA13, and trees in DR4, which are on sour orange rootstock, have remained free of decline. A young planting of vigorous trees of sweet orange on sour orange rootstock, located within one km of DR2, was examined in 1992. Nine of 20 trees tested by ELISA were infected, but all tested negatively against MCA13, and the planting has remained free of decline. Testing was also expanded to a large sweet orange planting on sour orange north of DR1 (Fig. 1a). About 10% of the 41 trees sampled in May 1994 were infected. DR6 was established in December 1994, and 203 of 400 trees were CTV-infected, in the initial test. The number of infected trees increased to 298 by May 1995. None of these trees tested positively against MCA13 and none have yet shown decline symptoms.

Limited sampling was done during May 1994 in areas adjacent to the DR1 and DR4 plot sites to determine if the incidence of infection

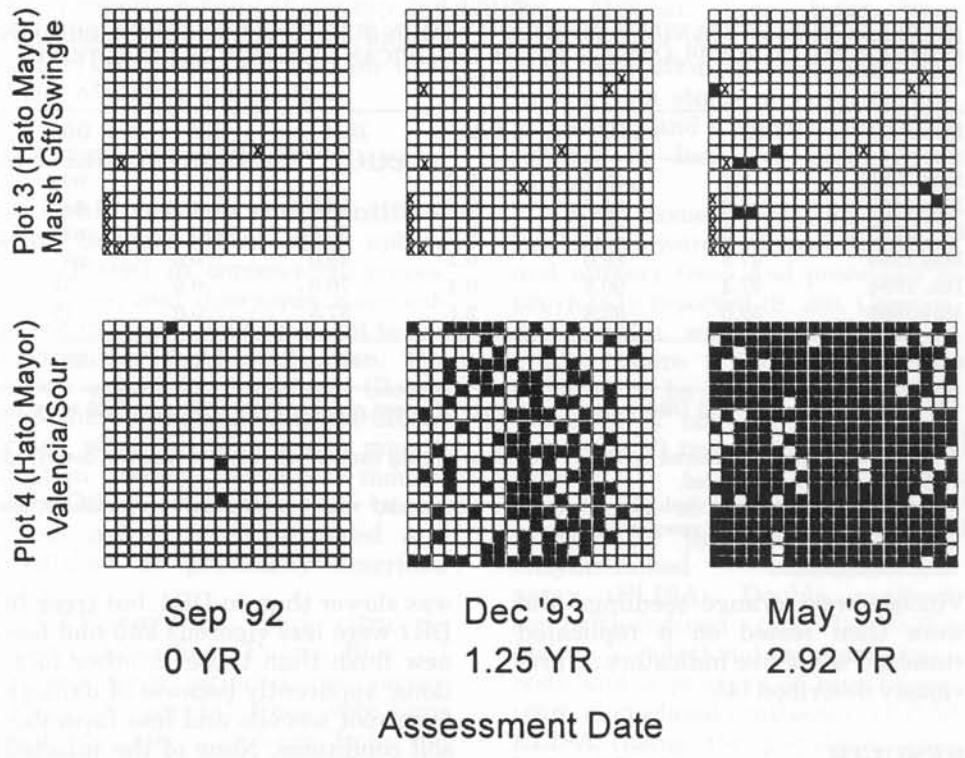

Fig. 3. Relative progression of CTV infection in sweet orange plot DR4 and grapefruit plot DR3. Each square indicates a single tree space. Shaded spaces were trees found infected with CTV by DAS-I ELISA as described in the text. Spaces marked by an (x) are missing trees.

within the plots was consistent with that in the immediate area. We collected samples along diagonal lines extending out from all four borders of the plots and also from blocks of 5 to 20 trees at various distances from the plots. The mean infection levels among trees bordering DR1 was 36% in contrast to the 28% incidence within the plot. Infection levels in trees bordering DR4 averaged 64% compared to 70% within the plot in December 1994.

Increase of infection in grapefruit. No CTV infection was observed in the grapefruit plot DR3 until May 1994 when one infected tree was detected. Eight infected trees (2%) were detected by May of 1995. This slow increase contrasted sharply to the rapid increase in the sweet orange plot DR4 approximately 2 km to the east (Table 1). Spot surveys in grapefruit next to DR3 also showed no infection. There was an apparent gradient in infection levels in sweet orange between DR4 and DR3. All 13 samples collected 0.5 km west of DR4 were infected; 17 of 20 samples collected about 1 km west of DR4 were positive, and 23 of 89 samples collected at two locations about 2 km west of DR4 (nearest to DR3) were infected. Infestations of brown citrus aphid were found in DR3 when new flush was present. Aphid colonies were even observed on the peduncles of young fruit when no new growth was present.

To further investigate the apparent low infection rate in grapefruit, DR5 was established in a block of Marsh grapefruit immediately adjacent to the sweet orange planting where DR2 was located. In contrast to DR3, infection levels in sweet

TABLE 2
SEROLOGICAL REACTIVITY OF CTV-INFECTED TREES DETECTED IN SURVEYS OF CITRUS PLANTINGS IN THE DOMINICAN REPUBLIC

Sample source[z]	CTV positive[y]	MCA13 positive[x]
DR1	206	0
DR2	347	0
DR3	6	0
DR4	349	0
DR5	0	0
DR6	298	0
Misc.	218	0
Total	1,426	0

[z]Samples were from plots established in commercial citrus plantings as described in Table 1. Misc. source includes all other samples which were collected from other locations in commercial and domestic plantings.
[y]Infection determined by DAS-I ELISA using a mixture of two monoclonal antibodies (MAB) each specific to conserved epitopes of the citrus tristeza virus as described in the text. For samples from DR1 through DR6, multiple assays of the same tree were made as indicated in Table 1.
[x]Extracts from all samples which tested positively against the MAB combination were re-tested by DAS-I ELISA against the strain-specific MAB MCA13 (8). Extracts from T30- and T36-infected plants were included in all assays to verify conditions and reactivity of antibodies used.

orange in the immediate vicinity of DR5 were high. The infection level in DR2 was nearly 50% in 1992, and has increased to 94% in 1995 (Table 1). No infections were detected in the three assays made of the 320-tree DR5 grapefruit plot.

Other surveys. Samples were collected from 726 trees outside the test plots at different locations during the six sampling trips made since September 1992. Of these, 218 were infected, but none assayed positively when tested by MCA13. The few trees on sour orange which were found in poor condition failed to index positively by ELISA. No CTV symptoms have been observed in dooryard trees of Mexican lime. Stem pitting was found in some *C. macrophylla* trees in early surveys near Hato Mayor, and several plants were found which tested positively by MCA13 (R. F. Lee, personal communication). These trees apparently were removed prior to the current surveys, but one isolate found by R. F. Lee, which tested positively on MCA13, has been established at Beltsville and its MCA13 reactivity confirmed.

During a continued survey made June 1996, some declining 12-yr-old trees of Valencia orange on sour orange rootstock, and several stunted trees of Valencia on *C. macrophylla* rootstock were discovered in a commercial planting near Sabana Grande which is approximately 10 km west of DR3. The declining trees tested positively with MCA13 and showed budunion symptoms typical of CTV. Further surveys in this area are in progress. No evidence of decline or infection by MCA13 isolates was found in other areas.

Biocharacterization. Tests of several CTV isolates collected from the survey plots were conducted at Beltsville. Symptoms in Mexican lime were mild and there was no seedling yellows or significant stem pitting in either grapefruit or sweet orange. A slight stunting was noted in several plants of sweet orange grafted on sour orange when inoculated with one Dominican Republic isolate, but this reaction has not yet been confirmed in further tests, and it is not certain that it was a CTV effect. The MCA13-positive isolate

collected earlier was also tested. It produced somewhat stronger symptoms on Mexican lime, a weak decline, and grapefruit stem pitting reactions.

DISCUSSION

Sequential sampling of test plots in commercial citrus plantings over 32 months indicated that CTV is spreading rapidly in three major citrus growing areas of the Dominican Republic. The original source of infection in each area was apparently established by planting trees which had been propagated from bud sources unknowingly infected with CTV. The similarity and the mildness of the isolates detected in all locations suggests a common origin, quite possibly CTV-infected budwood from Florida. Many trees in the newer plantings were produced from budwood source trees originally propagated from virus-free budwood obtained from California. It is probable that some budwood source trees became infected prior to their use for nursery propagations.

We assume that the rapid secondary dissemination of CTV observed is associated primarily with the brown citrus aphid since it was abundant in the plots and is a highly efficient vector. However, some spread by melon aphid or spirea aphid (*Aphis spiraecola* Patch) is also possible. While the rate of spread in all sweet orange plots was relatively high, differences between plots were noted. The plot with the lowest infection rate was the least vigorous and had less new flush leaves than other plots.

The rate of spread was low in the two grapefruit plots. The low infection rate in DR3 could possibly be attributed to low inoculum potential in the immediate area of the plot, but this was not the case in DR5. The low incidence of infection in grapefruit suggests that either the isolates present in sweet orange infect grapefruit less readily, or that there is some unknown vector-host relationship involved. The low rate of infection was not due to lack of colonization by the brown citrus aphid since these were found on grapefruit when new flush was present, and on the stems of young fruit when no new flush was present. Slower rates of ingress of CTV into grapefruit have been observed previously in other countries (12). Further tests will be made to see if infections increase in DR3 now that CTV is present in grapefruit donor hosts.

Although the test plots are limited in size we believe that the data obtained reflects the general situation in the Dominican Republic. High rates of spread in sweet orange were found in four different areas under separate management. This high rate of spread is also consistent with data from Costa Rica, Puerto Rico (8, 15), and Reunion (10). Surveys of trees in additional locations and sampling around the test plots also indicated that the infection rates within the test plots were representative.

Large scale surveys are always subject to errors in sample collection, sample processing, and assay technique. However, the collecting and processing methods used, and care in selecting young tissue for sampling, minimized errors. The strong ELISA reaction of infected field samples indicated that our collection, drying, and extraction protocols were appropriate. Once trees tested positively they generally remained positive in each subsequent sampling period. In a few cases, a tree tested positive, was negative in the next assay, and then positive in subsequent assays. This was attributed to irregular distribution of CTV in the plant in the early stages of infection. It is unlikely that any isolates were present which would not react with the mixture of the MABs 11B1 and 3E10 since this combination detects all isolates in

our extensive world-wide collection (Garnsey et al., unpublished).

The lack of reaction with MCA13 indicates that the isolates detected in the current surveys are not decline or stem pitting isolates. While some CTV decline isolates have been found in Spain, Florida, and California which do not react to MCA13 (2, C. Powell and K. Riley, personal communications, and S. Garnsey, unpublished), most decline and stem pitting isolates do react to MCA13. We believe that the experimental conditions were appropriate since the positive controls reacted strongly, and MCA13 reactive samples were detected in Costa Rica, Florida, and Puerto Rico with MCA13 (8, 15, S. Garnsey, unpublished data). Direct tissue blot assay results were also consistent with those obtained by ELISA.

The lack of decline symptoms in infected sweet orange trees on sour orange in the field also supports the serological evidence that these were not decline-inducing isolates. However, many of the CTV-infected trees on sour orange rootstocks currently under observation, have only recently become infected and these could conceivably develop decline symptoms at a later date.

The failure to find more MCA13-positive isolates was somewhat surprising since some were reported in the preliminary testing done in 1992, prior to establishment of the current surveys. Re-examination of the original 1992 ELISA data revealed that the absorbance values for some of the samples reported as MCA13-positive were near the threshold level and could have been misread. However, at least four samples gave clearly positive results. Failure to find MCA13-positive trees in the present surveys, except recently at one location, suggests that those isolates have not yet become widespread.

At this point, introduction of the brown citrus aphid has resulted in rapid spread of existing mild isolates of CTV, but has not seriously impacted citrus production in the Dominican Republic. This is in contrast to the experience in most other countries and several factors have probably contributed to this situation. Most older plantings in the Dominican Republic were established on sour orange, and there would have been a selection pressure against using budwood sources infected with decline-inducing isolates of CTV. Recent plantings were made with cultivars introduced as virus-free budwood from California or from existing budwood source trees on sour orange. Thus, the primary component in the reservoir of CTV isolates available to spread into commercial plantings following the introduction of the brown citrus aphid was a mild isolate. More severe isolates already exist in the Dominican Republic and will likely be spread into commercial plantings by the brown citrus aphid. In addition, there is always the possibility that budwood infected with a severe CTV isolate will be introduced and propagated and then be subsequently spread by aphid vectors.

A certification program is urgently needed to prevent future propagation and accidental distribution of trees infected by severe isolates. Presence of exocortis, cachexia, bark scaling psorosis, and blight (14) observed in different locations is an additional reason to develop and maintain pathogen-free budwood sources.

It is uncertain that the mild isolates now being rapidly spread will protect trees against subsequent challenge by more severe isolates in the future. However, our data indicates that where the brown citrus aphid is present, a rapid spread of a desirable protecting isolate of CTV could be achieved in citrus still relatively free of CTV by infecting trees at strategic locations and letting the aphid further disseminate the virus.

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