Comparison of Detection Methods for Citrus Tristeza Virus in Field Trees During Months of Nonoptimal Titer

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ABSTRACT

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Enzyme-linked immunosorbent assay (ELISA) can reliably detect citrus tristeza virus (CTV) in samples collected during approximately 6 months of a typical year. Two reverse transcriptase polymerase chain reaction (RT-PCR) methods (total nucleic acid extract and immunocapture based) were evaluated and compared to ELISA in order to develop a more sensitive assay for CTV. From May 1994 to October 1995, 6 sweet orange trees infected with CTV from each of 2 geographic areas (Riverside and the San Joaquin Valley) were tested monthly by each method. In the months of August (San Joaquin Valley samples) and September (Riverside and San Joaquin Valley samples) several of the trees had a significant loss of virus titer such that CTV was not reliably detected by ELISA. By contrast, the 2 PCR methods gave definitive positive results for CTV in samples collected during these months. Different tissue types were analyzed by each of the above assays. Petioles and midribs, both phloem-rich tissues, were each satisfactory for ELISA, while distal leaf tips did not always produce a positive result. All tissue types were equally efficient in producing a positive result in both PCR-based assays. The results of this study provide a basis for CTV testing by PCR in months when virus titer drops to a level generally unacceptable for using ELISA.

Citrus tristeza closterovirus (CTV) has a positive sense, single-stranded RNA genome of approximately 20 kb in size, encapsidated in a flexuous rod 2,000 nm long composed of coat protein subunits of 25 kDa (2,12,26). CTV causes some of the most economically important diseases of citrus worldwide, including stem pitting, which is often associated with reduced tree and fruit size and quick decline leading to tree death when sour orange is used as a rootstock (4). CTV is a phloem-limited virus transmitted semi-persistently by aphid feeding and by grafting infected budwood (3).

A testing program run by the Central California Tristeza Eradication Agency (CCTEA, Tulare, CA) uses the enzyme-linked immunosorbent assay (ELISA) to identify trees infected with CTV (7). Samples are recollected from trees initially identified as CTV-positive, and the diagnosis is confirmed by a second ELISA test before the tree is removed. At least two problems are encountered with the current testing method. The first occurs when ELISA values are not at least 2.5 times the value of the healthy control plant (the threshold used for scoring a positive sam-

ple at the CCTEA), but are obviously higher than a normal healthy control sample. The tree must be resampled and additional ELISA tests must then be done in the following weeks or months to confirm whether or not the tree is infected with CTV. The second is that in a typical year, ELISA testing is only reliable during certain months, when virus titer is high (8). Therefore, field collections are limited to these times. A more sensitive test for CTV in field-collected samples would alleviate these two problems and could increase the effectiveness of the CTV eradication project in California.

Sensitive molecular techniques such as reverse transcriptase polymerase chain reactions (RT-PCR), both total nucleic acid- and immunocapture-based, may be able to extend the time of testing throughout the year, resolve the interpretation of ambiguous ELISA results, and allow pooling of samples. We present a comparison of ELISA, RT-PCR and immunocapture RT-PCR (IC-PCR) assays for testing field samples during each month of the year, and a determination of the optimal tissue type for each assay. Data on pooling samples are presented elsewhere (17).

MATERIALS AND METHODS

Tissue collection. All plants used to analyze titer variability were varieties of sweet orange, *Citrus sinensis* L. (Os.), and were determined to be CTV-positive when initially tested by ELISA. Three different surveys were conducted. First, leaves were collected weekly from ap-

proximately 20 field trees from commercial groves in the San Joaquin Valley, California, and were tested by ELISA from March 1993 to February 1995. The resulting absorbance values were averaged to produce a single data point for the week. For the second survey, six trees from three different locations on the University of California/Riverside campus were sampled each month from May 1994 to October 1995. Six to eight twigs 8 to 12 inches long with leaves were harvested from positions around the entire perimeter of each tree. Leaves were removed and pooled for each individual tree and subsamples were removed for analysis by ELISA, RT-PCR, and IC-PCR. Finally, in the summer of 1995 (July to October), leaf samples from six infected trees from commercial orange groves in the San Joaquin Valley (three each from Fresno and Bakersfield, CA) were analyzed by ELISA, RT-PCR, and IC-PCR.

ELISA. Standard double antibody sandwich, indirect ELISA was carried out using: goat anti-CTV IgG (1 µg/ml) as the coating antibody; antigen sample (1/10); rabbit anti-CTV IgG (2 µg/ml) (19); commercially obtained goat anti-rabbit alkaline phosphatase conjugate (0.05 µg/ml, Sigma Chemical Co., St. Louis); and *p*-nitrophenyl phosphate (1 mg/ml, Sigma) as the substrate (6,9). Antigen samples were prepared by grinding 0.5 g petiole tissue in 5 ml phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20, 2% polyvinylpyrrolidone (MW 40,000) and 0.2% bovine serum albumin using either a Tekmar Tissuemizer (Cincinnati, OH) or a Kleco Pulverizer (Garcia Machine, Visalia, CA). The absorbance at 405 nm was measured after 90 min of substrate development at room temperature using an Emax plate reader and SoftMax software (Molecular Devices Corp., Menlo Park, CA).

Total nucleic acid based RT-PCR. Three hundred milligrams of petiole tissue were ground in liquid nitrogen, extracted with 400 µl 0.1 M Tris-Cl, pH 8.0, 2 mM EDTA, and 2% sodium dodecyl sulfate (TES), and 450 µl phenol:chloroform:isoamyl alcohol (25:24:1), heated for 5 min at 70°C, and centrifuged for 5 min at full speed in a microcentrifuge (21). The aqueous phase (total nucleic acid, TNA) was recovered and 100 µl was further purified through a 1 cc Sephadex G-50-80 column (16). A 353 bp fragment at the carboxy terminal half of the coat protein gene of CTV was amplified by adding 20 µl of the

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column-purified TNA which had been heat denatured for 5 min at 65°C, to 80 µl of a single tube RT-PCR reaction mix consisting of 0.4 mM dNTPs, 200 ng each of primer 64 (5'-TGACATTAGTAACGACGATCATCAGCCC-3', anti-sense) and primer 65 (5'-ATGACGACGCCACGGGTA-

TAACGTACACTC-3', sense) (14), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100 (1X Thermophilic Buffer, Promega Corp., Madison, WI), 2.5 mM MgCl₂, 10 mM dithiothreitol, 10 U RNasin (Promega), 15 U AMV reverse transcriptase (Promega), and 2.5 U *Taq* DNA po-

lymerase (Promega). The mixture was incubated at 42°C for 45 min to allow for cDNA production, then the product was denatured at 95°C for 2 min before performing 40 amplification cycles consisting of 95 C/1'; 50 C/1'; 72 C/2', followed by a final 5 min at 72 C using an EriComp twin-block Easy Cyclor (EriComp, Inc., San Diego, CA).

Immunocapture RT-PCR. ELISA was performed as above and, after recording the absorbance values, the substrate liquid was washed out of each well of the microtiter plate using PBS buffer (without Tween, PVP, or BSA). One hundred microliters of the RT-PCR mix above were then added to each well to be assayed (20,29). The plate was placed at 37°C for 1 h, then the sample liquid was transferred to a microfuge tube, placed in the thermocycler, and incubated an additional 15 min at 42°C, denatured for 2 min at 95°C, and cycled 40 times as above.

Detection of PCR products. Five microliters of each PCR product were added to 100 µl of denaturing solution (1.25 ml TE-buffer, 2.5 ml 20X SSC, and 1.0 ml 37% formaldehyde), heated at 55°C for 20 min, and cooled on ice (14). Samples were blotted to a nitrocellulose membrane (MSI, Westborough, MA) using a slot blot apparatus, then baked for 30 min at 80°C under vacuum. The oligonucleotide probe CTV-P3 (5'-CTGATTTAGAATGTGCTGTGTACA-3', genomic sense, which binds near the 3' end of the coat protein ORF) (14) was end-labeled using ³²P-ATP and T4 polynucleotide kinase (16). Membranes were prehybridized, hybridized, and washed as previously described (14). Blots were exposed to Fuji RX film (Tokyo) for 18 to 20 h at -80°C and developed using a Konica QX-70 automatic processor.

Optimum leaf tissue sample. In August 1995, 3 of the San Joaquin Valley trees were selected for further testing in order to determine if some parts of leaf samples were preferable to others for ELISA, RT-PCR, and IC-PCR. Eight leaves were stacked and sectioned as follows: the terminal 6 to 7 mm of petiole (small scale petiole); the remainder of petiole (8 to 10 mm) and the first 4 to 5 mm of midrib and laminar tissue (large scale petiole); a 1 cm square from the middle of the leaf stack including the midrib surrounded by laminar tissue (midrib); and 8 to 10 mm of the distal tips of the leaves (leaf tips). TNA was extracted as above and used either directly with no further purification, after column purification, or after column purification and sixfold concentration by ethanol precipitation in an RT-PCR reaction and blotted as above. Identical samples were also prepared and analyzed by ELISA followed by IC-PCR.

RESULTS

Monthly changes in ELISA-based CTV titer in the San Joaquin Valley.

Variation in CTV titre from 1993-95

Fig. 1. Variation in citrus tristeza closterovirus (CTV) titer during a typical season, 1993 to 1994, and during 1994 to 1995. The three plots represent: —◆— average positive threshold of 0.33, ($2.5 \times$ healthy absorbance value), average obtained from weekly enzyme-linked immunosorbent assays conducted at the Central California Tristeza Eradication Agency during 1993 to 1995; —■— plot of weekly average absorbance value for 20 positive trees in the San Joaquin Valley from March 1993 to February 1994; and ---▲--- plot of weekly average absorbance value for 20 positive trees in the San Joaquin Valley from March 1994 to February 1995.

Table 1. Enzyme-linked immunosorbent assay (ELISA) absorbance values for three individual sweet orange trees having values that fell below the cutoff threshold during at least 1 month of the year, and the average of these and an additional three trees tested in Riverside, California, during 1994 and 1995. Underlines denote values that fell below the cutoff threshold of $2.5 \times$ the healthy control absorbance value

Month	Sample no.			Average (6) $\pm$ SD
	14-7	15-8	2-12	
1994				
May	1.59	1.75	1.63	1.76 ± 0.17
June	1.15	1.19	1.11	1.17 ± 0.04
July	0.76	0.79	0.79	0.82 ± 0.05
August	0.33	0.62	0.66	0.59 ± 0.13
September	<u>0.22</u>	<u>0.25</u>	<u>0.21</u>	0.31 ± 0.08
October	0.65	0.44	0.44	0.60 ± 0.18
November	0.88	1.02	0.67	0.82 ± 0.14
December	0.71	0.98	0.73	0.77 ± 0.15
1995				
January	1.13	1.28	0.73	1.09 ± 0.19
February	1.52	1.59	1.54	1.53 ± 0.06
March	1.26	1.27	1.22	1.27 ± 0.03
April	1.61	2.07	1.70	1.84 ± 0.17
May	1.78	1.78	1.73	1.78 ± 0.06
June	1.11	1.54	1.40	1.43 ± 0.15
July	1.32	1.34	1.30	1.34 ± 0.07
August	0.62	0.89	0.89	0.80 ± 0.09
September	<u>0.17</u>	0.52	0.48	0.41 ± 0.12
October	0.29	0.56	0.40	0.51 ± 0.21
Controls				
Buffer	0.11			
Healthy	0.11	$2.5 \times = 0.28$		
Positive	1.05			

ELISA absorbance values dropped to or below the threshold level used at the CCTEA for a positive sample (2.5 times the absorbance value for the healthy control), from August to November 1993 and again in January of 1994 (Fig. 1). This is the general pattern observed for the past several years of testing by the CCTEA. During the study period from March 1994 to February 1995 this pattern did not recur. September 1994 was the only month in which the average absorbance value of 20 trees dropped below the threshold level (Fig. 1). Although titer was reduced in the other summer months and slightly reduced in the winter, absorbance values did not drop markedly.

Monthly comparison of ELISA, RT-PCR, and IC-PCR. Samples were collected for 18 months between May 1994 and October 1995 from the Riverside location, and for 4 months from the San Joaquin Valley site, July to October 1995.

Each of the 3 assays was completed for individual trees each month (as opposed to pooled data for 20 trees above). IC-PCR was begun in July 1994 and completed for every month thereafter. The same trend of virus titer variation was observed at the Riverside collection sites for 1994 and 1995. ELISA detected CTV in samples from all 6 Riverside trees every month except September 1994, when the values for 3 trees fell below the cutoff point of 2.5 times the healthy control (Table 1). In September 1995 only 1 of the same 3 trees fell below the cutoff value. ELISA values peaked in April and May, then steadily decreased each month until September each year, when they reached their lowest point. There was an $83 \pm 3.8\%$ SD reduction in the ELISA absorbance values from May to September in 1994 and a $78 \pm 6.0\%$ SD reduction between April and September 1995. Virus titer recovered significantly in October, leveled off in

November and December, and then began to gradually increase through April 1995.

The absorbance values for the six San Joaquin Valley samples were lower for each respective month than those of the Riverside samples, but the same trend was followed. However, two of the six samples fell below the cutoff value in August 1995 and all six samples were below the cutoff in September 1995 (Table 2).

In contrast to the ELISA data, results from individual trees were very consistent from month to month for both of the PCR methods. Therefore, only PCR slot blot data from one tree are presented. Both PCR methods detected CTV easily in all 6 Riverside trees, all months of the year (Fig. 2, panels A and B). In September, when ELISA did not reliably detect CTV, PCR gave strong positive results, and even detected CTV in the Riverside (Fig. 2, panels A and B, lanes 3 and 15) and San Joaquin Valley (Fig. 2, panels C and D, lanes 14 and 15) trees that were scored negative by ELISA. IC-PCR was more variable than TNA RT-PCR on a month to month basis, but still gave positive results for every month (Fig. 2, panel B). Aliquots from several PCR reactions were also analyzed by agarose gel electrophoresis and all gave the expected size (353 bp) fragment (data not shown). In this study, slot blotting was consistently more sensitive than gel electrophoresis and was used as the routine method of choice.

Optimum tissue for RT-PCR. In order to compare tissue types as good sources of viral RNA for PCR, four tissue types, small and large scale petioles, midribs, and leaf tips were compared. Crude aqueous phase TNA could not be amplified successfully regardless of tissue type or CTV isolate (Fig. 3, lane 1, panels A to D). However, when the aqueous phase was column purified, large amounts of the expected 353 bp fragment were made (Fig. 3, lane 2, panels A to D). When column purified TNA was concentrated sixfold by ethanol precipitation, no increase in signal strength was obtained (Fig. 3, lane 3, panels A to D). All tissue types gave identical results when analyzed by IC-PCR (Fig. 3, lane 4, panels A to D). When these same tissues were tested by ELISA, petioles and midribs gave similar results while absorbance

Fig. 2. Detection of citrus tristeza closterovirus (CTV) in sweet orange trees on a monthly basis. Autoradiograph of slot-blotted reverse transcriptase polymerase chain reaction (RT-PCR) (A and C) and IC-PCR (B and D) products from CTV-infected trees from Riverside (tree 14-7, A and B) and the San Joaquin Valley (tree 3-22, C and D), hybridized with a ^{32}P -labeled probe. Lane numbers are supplemented by control types (H = healthy, + = positive) and month beginning with September 1994 (S, lane 3). The box encloses those months in which enzyme-linked immunosorbent assay values were below the cutoff threshold of 2.5 times the healthy control. Blotting results were consistent for each month, therefore results are only shown from the tree that underwent the greatest amount of suppression from each location.

Table 2. Enzyme-linked immunosorbent assay (ELISA) absorbance values for six sweet orange trees from the San Joaquin Valley in California during 1995. Underlines denote values that fell below the cutoff threshold of $2.5 \times$ the healthy control absorbance value

Month	Sample no.						Average (6) $\pm$ SD
	1-27	3-22	5-16	47-28	49-29	57-24	
July	0.93	0.96	0.67	0.68	0.86	0.91	0.83 ± 0.12
August	0.54	<u>0.18</u>	0.30	0.44	<u>0.21</u>	0.37	0.34 ± 0.13
September	<u>0.16</u>	<u>0.20</u>	<u>0.16</u>	<u>0.24</u>	<u>0.26</u>	<u>0.22</u>	<u>0.21 ± 0.04</u>
October	0.71	0.92	1.02	1.12	1.06	0.57	0.90 ± 0.19
Controls							
Buffer	0.11						
Healthy	0.11	$2.5 \times = 0.28$					
Positive	1.24						

values for leaf tips were approximately 45% lower, including one sample which was below the cutoff value (Table 3). Only slight differences were seen in the amounts of amplification products from each tissue type for both RT-PCR and IC-PCR, indicating that all are equally acceptable for use in PCR based assays.

DISCUSSION

Two types of RT-PCR were compared to ELISA for each month of the year. Not unexpectedly, PCR of either type was more sensitive than ELISA. However, ELISA was more sensitive in this study than anticipated during the summer and in the winter, especially for the Riverside samples. Previous data (8), and the initial ELISA data presented here, showed that during the summer, ELISA and dsRNA analysis were not sensitive enough to use as a reliable assay. The 1994 to 1995 growing season was not typical regarding the monthly change of virus titer based on ELISA testing. There was only one month in which ELISA did not yield positive results, instead of the usual five or six months. This was demonstrated independently in two distinct geographic areas so that we have a high degree of confidence in these data. A casual look at weather records was not able to explain the lack of virus suppression in the summer and winter months in 1994 and 1995. Improve-

ments to the CTV ELISA system that increased its sensitivity, and the use of test trees with well-established infections, may help account for these results. Caution in using ELISA to detect CTV in arbitrarily selected field trees during the summer is still recommended. Since they may harbor lower titer infections than the test trees surveyed here, virus suppression would more readily occur in the summer and ELISA could miss low-level infections of CTV. While the use of 2.5 times the healthy control as the cutoff level for a positive sample is considered conservative, we maintain its use in this study because it is the method in use at the CCTEA, and for the following reasons: 1) since only two replications are used per sample at the CCTEA due to the large number of samples tested each year (approximately 500,000), it is not feasible to use a standard deviation based cut-off value that would require 6 to 8 healthy control replications per plate and 3 or 4 replications per experimental sample; and 2) due to legal issues that arise in diagnosing a tree as positive and its subsequent removal, a more conservative approach is desired at this time.

PCR reliably detected CTV in field samples when ELISA could not, specifically for those trees that would have been scored as negative by ELISA. Other researchers have also reported success in using PCR to detect a variety of viruses in field crops including plum pox potyvirus (13,29), luteo and potyviruses in potato (27,5), bean yellow mosaic potyvirus in gladiolus bulbs (28), a variety of woody host viruses including CTV (25), cucumber mosaic virus in banana (11), and also mycoplasma-like organisms in pear trees (10). However, to date, there have been no reports of PCR applied to an actual field situation which took into account the effect of weather and temperature on detection.

Several different leaf tissues were tested by RT-PCR to determine the optimal sample type. ELISA for detection of phloem-limited viruses has commonly been carried out on phloem-rich samples such as petioles (1,22). Using RT-PCR, there were no detectable differences between the results for any of the four tissue types tested. Unexpectedly, the TNA extracts from distal

leaf tips produced results similar to those from petioles. Leaf tips are also preferable to petioles due to their ease of grinding, and because leaf tips are available after the petioles are removed for ELISA, so additional collecting is not necessary. Regardless of tissue type, total nucleic acid extracts required column purification for efficient RT-PCR amplification. This has also been observed by other researchers for the RT-PCR amplification of viral RNA from dasheen mosaic potyvirus (21) and bean yellow mosaic potyvirus (28).

IC-PCR also readily detected CTV in all tissue types. The ELISA absorbance values for the leaf tips were approximately 45% lower than those for petioles and midribs, while IC-PCR gave similar results regardless of the tissue type. IC-PCR has several benefits over TNA based RT-PCR including savings in time and effort (no organic extraction or column purification, etc.) and ambiguous ELISA results can be easily and quickly resolved immediately after the ELISA has been completed. Two major problems exist, however, in using IC-PCR to the exclusion of TNA-based RT-PCR. First, due to the number of samples being processed daily, complete cleaning of equipment between samples is not guaranteed, which does not affect an ELISA result, but could have a great effect on the number of false positives obtained when using IC-PCR. Second, the CCTEA usually does two freeze/thaw cycles prior to grinding for ELISA, due to handling and storage requirements. This treatment greatly decreases the amount of amplification product obtained from IC-PCR, often to almost non-detectable levels (D. Mathews, unpublished results). Recent tests have shown that acceptable IC-PCR data can be obtained after one freeze/thaw cycle, but optimum amplification occurs when tissue is processed fresh within several days of harvest and storage at 4°C. We propose that the freeze/thaw cycles disrupt plant cells and virions such that when virions are bound to the coating antibody, the viral ssRNA is no longer available or suitable for reverse transcription and PCR amplification.

Less than 0.4% (70,000 to 80,000 trees) of the citrus trees grown in the San Joaquin

Fig. 3. Determination of optimum tissue type for detection of citrus tristeza closterovirus (CTV) by reverse transcriptase polymerase chain reaction (RT-PCR). Autoradiography is of slot-blotted RT-PCR products hybridized with a ³²P-labeled probe. Tissue types assayed: A = large-scale petioles; B = small-scale petioles; C = midribs; and D = distal leaf tips. Source of nucleic acid for PCR reaction: lane 1 = crude aqueous phase; lane 2 = column purified aqueous phase; lane 3 = column purified aqueous phase concentrated sixfold by ethanol precipitation; and lane 4 = virions trapped on an enzyme-linked immunosorbent assay plate, after substrate development (IC-PCR).

Table 3. Enzyme-linked immunosorbent assay (ELISA) absorbance values for various leaf tissue types from citrus tristeza closterovirus (CTV)-infected trees. Samples were collected during August 1995 from the San Joaquin Valley in California. Underlines denote values that fell below the cutoff threshold of 2.5 × the healthy control absorbance value

Tissue type	Sample no.		
	1-27	47-28	57-24
Petioles (large)	0.54	0.44	0.37
Petioles (small)	0.53	0.46	0.37
Midribs	0.57	0.50	0.40
Leaf tips	0.32	0.30	<u>0.19</u>
Controls			
Buffer	0.11		
Healthy	0.12	2.5 × = 0.29	
Positive	1.32		

Valley in California (Kern, Tulare, and Fresno counties) are believed to be infected with CTV (7). This low incidence is the product of a combination of efforts including a clean stock program (the UC Citrus Clonal Protection Program, 18), quarantine of imported citrus material by the County Agricultural Commissioner Offices, monitoring of registered nursery trees by the California Department of Agriculture/Nursery Services Division, and an active suppression program by the CCTEA. The CCTEA in Tulare, California is an ongoing grower-funded program. An average of 500,000 trees are tested for CTV by ELISA each year, but sample collection can only be done in those months when the virus titer is high. Based on the PCR results obtained in this study, some testing could continue throughout the year, notably confirmation testing that may have to wait until the next year if ELISA were used.

The primary aphid vector of CTV in California, *Aphis gossypii* Glover, is generally not very efficient at transmitting strains of CTV (24). However, *Toxoptera citricidus* (Kirkaldy) the brown citrus aphid, is very efficient at transmitting most known strains of CTV (15,30). Recently, this vector has established itself in Florida as well as in Central America and Cuba. Countries where *T. citricidus* and CTV have been present together for decades have experienced major losses of citrus on the order of millions of trees worldwide (23). It is therefore advisable for California agencies to identify and remove as many CTV-infected trees as possible in the next few years so that when the new vector arrives in California the incidence and distribution of CTV will be lower than at present.

Our findings show that the use of RT-PCR based techniques will further this goal by permitting early detection of infections and allowing for the testing of a greater number of trees throughout the year. The uninterrupted management of this aphid-transmitted virus is an important objective for California. The ability to pool multiple field samples into a single PCR test will also increase the efficiency of testing (17).

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