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Elevated Background in Double Antibody Sandwich-Indirect Enzyme-Linked Immunosorbent Assay for the Detection of *Citrus tristeza virus* in Mandarin Varieties

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ABSTRACT. Healthy tissue extracts from mandarin cultivars induced a non-specific reaction in double antibody sandwich-indirect (DASI-) enzyme-linked immunosorbent assay (ELISA) to detect *Citrus tristeza virus* (CTV) at the Central California Tristeza Eradication Agency (CCTEA), Tulare, CA. This problem was seasonal, occurring from March to May in samples collected from field and nursery trees in central California. The elevated absorbance in DASI-ELISA occasionally exceeded twice the healthy control of sweet orange. Problem samples were tested by immunocapture reverse transcription polymerase chain reaction and found to be negative for CTV. Several variations in the DASI-ELISA procedure were tested and results indicated that the non-specific reaction was related to the high sensitivity of the conjugate and high binding capacity of the ELISA plates used. Two solutions to eliminate this problem were tested, (i) - use a different enzyme conjugate, and (ii) - block by incorporating extracts from uninfected mandarin tissue into the antiserum buffer. Since the elevated background in DASI-ELISA was not observed with several other CTV antisera tested, another solution was to use a different detecting CTV antiserum. The non-specific reaction in DASI-ELISA was a problem only in healthy mandarin cultivars; mandarin samples infected with CTV always gave high absorbance values and were never in question.

Index words. Serology, False-positives, DASI-ELISA, reverse transcription polymerase chain reaction.

The Central California Tristeza Eradication Agency (CCTEA) was formed in 1963 for the purpose of survey, detection and eradication of *Citrus tristeza virus* (CTV) in participating Pest Control Districts (PCDs) in California (5). These PCDs represent ~111,000 acres (about 45,000 ha) of commercial citrus within portions of Fresno, Tulare, and Kern Counties. Each year, the CCTEA surveys ~31,400 acres (12,700 ha) of citrus and processes ~375,000 samples using double antibody sandwich indirect (DASI-) enzyme-linked immunosorbent assay (ELISA) as described by Dodds (2). The trapping antiserum is a goat polyclonal made to whole CTV virions whereas the detecting antibody is a rabbit polyclonal made to CTV coat protein expressed in engineered *E. coli* (9). The benchmark for a positive ELISA is an optical density (OD) of ≥ twice that of the healthy control.

Periodically since 1999, extracts from healthy mandarin cultivars sampled in spring (March to May) have reacted nonspecifically and given elevated OD results in DASI-ELISA. In some cases, the OD exceeded twice that of the healthy control (10). This poses a serious problem in accurately diagnosing material submitted to the CCTEA by regulatory agencies that collect random samples (e.g. from nursery trees) for CTV testing. It is often difficult for an inspector to go back and take another sample from a tree if the result is questionable or just above the positive threshold. Furthermore, by the time of resampling this phenomenon may no longer be observed.

Because of the seasonal nature of this elevated OD problem, temperature, water stress, or type of tissue sampled were suspected. The non-specific reaction with mandarin samples was found regardless of

the type of tissue tested (e.g. new shoots, new leaves, older leaves and bark) except for flower buds. Moving plants from an evaporative cooled greenhouse to the hot ambient outside temperature, water deprivation or watering excess had no effect on changing the elevated OD. In addition, freezing, thawing, and leaving sample tissue exposed to ambient outside temperature for 48 h did not change the results. The CCTEA also found nonspecific DASI-ELISA reactions with samples from several plant species in the Oleaceae family including privet, lilac, olive, Modesto ash, and jasmine. None of these plants was infected by CTV.

The study presented here was conducted to help solve this problem of nonspecific reaction in DASI-ELISA with mandarin cultivars experienced by the CCTEA.

MATERIAL AND METHODS

Sample collection. Samples from commercial citrus nurseries were collected by California Department of Food and Agriculture (CDFA) and local County inspectors. Additional test samples were collected by the CCTEA from infected and healthy mandarin trees grown either in the CCTEA's greenhouse (Tulare, CA) or in the field at the Lindcove Research and Extension Center (Exeter, CA). The CCTEA conducted all sample extractions.

IC-RT-PCR. PCR was conducted using virions immunocaptured by CTV 1212, a polyclonal antibody made to whole virions from a stem pitting isolate from Australia (Dept Plant Pathology, Univ. Florida, Gainesville, FL) and reverse transcription was performed to produce cDNA (11). PCR followed using the primers for the CTV coat protein (CP) gene described by Hilf and Garnsey (6). Primers were obtained from Invitrogen Life Technologies (Carlsbad, CA). Products were analyzed by agarose gel electrophoresis and the ethidium bromide–stained product was compared with standard markers and evaluated by an image analyzer (AlphaImager 2200, Alpha InnoTech, Corp., San Leandro, CA).

DASI-ELISA. Petioles from nearly to fully expanded leaves from new flush were homogenized 1:20 (wt/vol) in grinding buffer consisting of PBST+PVP, pH 7.4 in a Kleco Tissue Homogenizer (Garcia Machines, Visalia, CA). DASI-ELISA was conducted using plates coated with a whole CTV virus polyclonal goat antibody provided by the University of California Citrus Clonal Protection Program (CCPP) (Dept. Plant Pathology, UC Riverside, CA). The detecting antiserum was a rabbit polyclonal antibody made from the p25 major capsid protein of CTV expressed in *E. coli* (9) and the DASI-ELISA performed essentially as described by Dodds (2) using alkaline phosphatase as the enzyme.

The CCTEA used NUNC™ PolySorp plates (Nalge Nunc International, Rochester, NY) and Sigma A3687 conjugate (Sigma-Aldrich Corp., St. Louis, MO) at a dilution of 1:18,000. The Agricultural Research Service (ARS), Parlier, CA used Immulon 4HBX (Thermo Labsystems, Franklin, MA) and Sigma A8025 conjugate at a dilution 1:7,000. Otherwise, all other ELISA parameters were the same for the CCTEA and ARS.

The CCTEA also conducted a cross absorption experiment mixing extracts from healthy Clementina Fina Sodea, Clementina de Nules, and W. Murcott Afourer at 10% (vol./vol.) with the detecting antiserum or the conjugated anti-rabbit IgG.

RESULTS AND DISCUSSION

Problem illustration and IC-RT-PCR. Using CCTEA test procedures, healthy Navel sweet orange had an average OD_{405} of 0.101 (Table 1); whereas 66 Minneola (low Minneola) samples averaged 0.112 (Table 1). However, four Minneola (high Minneola) samples had OD values which exceeded twice the healthy control and had to be considered as suspect for CTV-infection. The independent DASI-ELISA conducted by ARS on some of these samples resulted in no detection of elevated OD readings compared to healthy controls (Table 1). ARS correctly identified two blind samples (Blind 1 and Blind 2) as CTV-positive which were included as a check by the CCTEA (Table 1).

TABLE 1
COMPARISON OF TWO PROTOCOLS OF DOUBLE ANTIBODY SANDWICH INDIRECT (DASI-) ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTION OF *CITRUS TRISTEZA VIRUS* (CTV) IN MINNEOLA TANGELO SAMPLES

	Avg. OD_{405}	
Description	CCTEA[z]	ARS[y]
Buffer	0.085	0.168
Navel	0.101(20)[x]	Nd[w]
Low Minneola[v]	0.112 (66)	0.153 (70)
High Minneola[u]	0.222 (4)	Nd[w]
Blind[t] 1	Nd[w]	1.038
Blind 2	Nd[w]	0.267
Virus control[s]	1.116	1.432
Healthy control[r]	0.083	0.139

[z]DASI-ELISA by the Central California Tristeza Eradication Agency (CCTEA), Tulare, CA. Samples were collected from trees in commercial groves.
[y]DASI-ELISA by Agricultural Research Service Parlier, CA. Samples provided by the CCTEA from trees grown in a commercial nursery.
[x]Numbers in parenthesis are number of samples analyzed .
[w]Nd = No data
[v]Samples without elevated OD readings.
[u]Samples with elevated OD readings (> 2x healthy control).
[t]Blind samples are extracts from CTV infected plants that the CCTEA included in the 72 samples tested by ARS for quality control purposes.
[s]CTV positive controls. These included a mix of CTV-infected Madam Vinous, grapefruit, Mexican lime, and Etrog citron plants from the CCTEA greenhouse.
[r]Healthy control was a mixture of extracts from the same varieties in the virus control but CTV free.

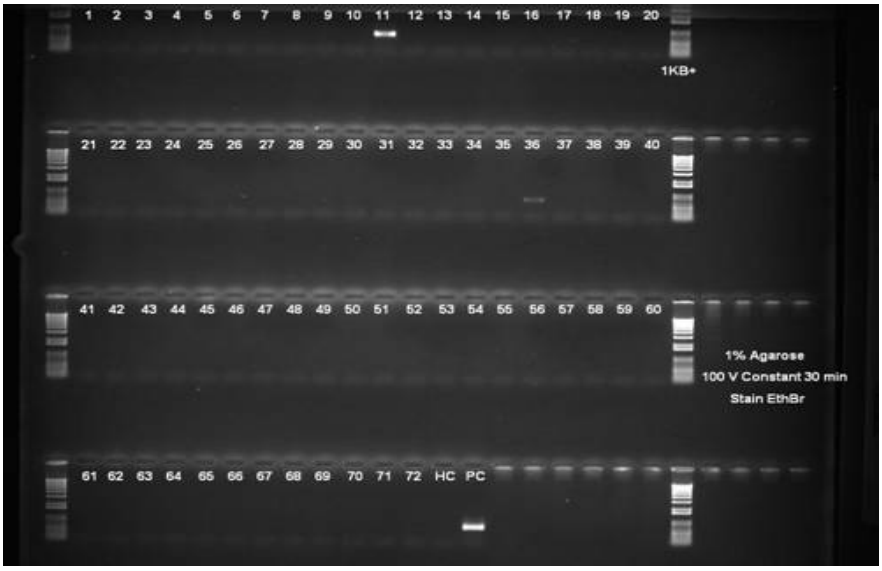

Fig. 1. Ethidium bromide stained agarose gel resulting from immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) of 72 samples, showing positive *Citrus tristeza virus* amplicons in samples 11 and 36. No other samples were found to be positive. HC = healthy control; PC = positive control. Primers targeting the CTV coat protein gene were used for the RT-PCR.

IC-RT-PCR confirmed the CTV status of all 72 samples tested (Fig. 1). Samples #11 and #36 gave an amplicon 672 bp in size, indicating presence of the CTV CP gene sequences. These samples were equivalent to Blind 1 and Blind 2, respectively, in Table 1. All remaining questionable mandarin samples were determined to be CTV negative.

In a second trial we compared the CCTEA protocols to those of the ARS. A new set of Minneola samples was used and similar results were obtained as in the first test. Of the 44 Minneola samples tested by the ARS and using CCTEA protocols, 36 were in the low Minneola group (mean OD = 0.115; range 0.098 to 0.176) and eight samples were in the high Minneola group (mean OD = 0.253; range 0.204 to 0.340). The positive virus control was 2.938 whereas the healthy control (Madam Vinous) was 0.097. Nonspecific reactions did not occur when ARS protocols were followed for the 44 Minneola samples (mean OD = 0.118; range 0.098 to 0.154; the positive virus control was 0.932 and the healthy control was 0.103). Like the previous test, all 44 samples were evaluated by IC-RT-PCR and confirmed to be CTV negative (data not shown). When Immulon 4HBX plates were used with the CCTEA reagents, the non-specific reaction was exacerbated (data not shown).

No non-specific reaction in DASI-ELISA with mandarin samples was observed (data not shown) when either a CTV ELISA kit from Agdia (Elkhart, IN) or a polyclonal antiserum made to a recombinant CTV coat protein (7) was used. Therefore, the non-specific reaction with these samples was related to the reagents used including the antiserum and was exacerbated by the binding capacity of the plates.

A separate experiment was performed altering the working dilutions of

the coating antiserum or conjugate concentrations since Dubitsky (3) has indicated a balance between the primary and secondary antibody concentrations can affect sensitivity and background of an assay. In this case, however, no difference was obtained when coating antiserum at dilutions of 1,000 or 2,500 and conjugate dilutions of 18,000 or 30,000 were used. Unfortunately, we did not test other concentrations of the detecting antiserum. Nahm and Goldblatt (8) indicated that a second antibody with a high working dilution may bind the antigen and may lead to high background binding.

TABLE 2
EFFECT OF CROSS ABSORPTION OF DETECTING ANTISERUM AND CONJUGATED ANTI-RABBIT IgG WITH HEALTHY MANDARIN VARIETIES ON THE OPTICAL DENSITY OF DOUBLE ANTIBODY SANDWICH INDIRECT (DASI-) ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTION OF *CITRUS TRISTEZA VIRUS*

Description[x]	OD_{405}		
	CCTEA[y]	Cross-absorbed detecting antiserum[z]	Cross-absorbed conjugated IgG[z]
PEP buffer	0.086	0.078	0.083
W. Murcott healthy	0.104	0.081	0.097
Clementina Fina Sodea healthy	0.112	0.083	0.114
Minneola CTV-infected	0.295	0.230	0.288
Pixie CTV-infected	0.284	0.238	0.297
Unknown 1 (high)	0.289	0.100	0.263
Unknown 2 (high)	0.203	0.087	0.192
Av. of 7 unknown (low)	0.120	0.084	0.121
Virus control	1.574	1.277	1.506
Healthy control	0.084	0.081	0.083

[x]All tests conducted by the Central California Tristeza Eradication Agency (CCTEA), Tulare CA.
[y]Standard DASI-ELISA conducted by the CCTEA including the goat anti-rabbit conjugate A3687 (Sigma-Aldrich).
[z]Using a mixture of extracts from healthy Clementina Fina Sodea, Clementina de Nules, and W. Murcott Afourer.

Problem resolution. Garnsey and Cambra (4) indicated that if the buffer control is negative but the healthy controls show a non-specific reaction, the antiserum could contain antibodies to some healthy plant antigen. This was not likely in our case since the detecting antibody was made to CTV recombinant capsid protein. However, whole virus was used as antigen for production of the goat polyclonal antibody used for trapping. Both protocols used in this study incorporated bovine serum albumin as a blocking agent in the grinding buffer. Dubitsky (3) mentions that it is critical to block any non-specific binding sites on the substrate and that insufficient

blocking can lead to high background, while overblocking may decrease sensitivity. Further, the correct type and amount of blocking agent is important. Cross absorption of the detecting antiserum with healthy extract eliminated the high background and improved the DASI-ELISA (Table 2); whereas cross absorption of the conjugate IgG did not help. Table 2 also shows that DASI-ELISA sensitivity to CTV-infected mandarin and hybrids was not affected by the addition of healthy extracts to the detecting antiserum. Hence, it seems apparent that blocking by BSA was insufficient for the mandarin samples in question. However, use of the appropriate blocking agents did solve the problem. Following ARS protocols and using the same conjugate and plate combination was found to be an alternative solution to the problem of non-specific reactivity.

The non-specific reaction in DASI-ELISA was a problem only in mandarin cultivars. Bokx et al. (1) reported that the difference between healthy and diseased potato tubers infected with potato virus S and M was not great at all sampling times, but diseased tubers could be detected reliably. Similarly, mandarin samples infected with CTV always resulted in high absorbance values in DASI-ELISA and were never in question. In any event, the secondary test utilizing IC-RT PCR with an aliquot of the extract giving questionable DASI-ELISA results was found to be valuable since resampling was then not necessary. A second DASI-ELISA test with the same extract can also serve this purpose if an alternative protocol is followed that eliminates the nonspecific reactivity.

CONCLUSIONS

An apparent seasonal problem regarding the detection of CTV by DASI-ELISA in mandarin cultivars was caused by occasional non-specific reactions. Problem samples, shown by independent tests to be virus-free, had elevated absorbance values which in some cases exceeded twice the healthy control but were significantly less than the positive control. The nonspecific reaction was associated with the high binding capacity of the ELISA plates and the high reactivity of the conjugate used by the CCTEA. The problem was also resolved by either using a less sensitive conjugate/ELISA plate combination or by cross absorption of the detecting antiserum with healthy mandarin extracts. Another solution was to switch to a different, but proven, antiserum since the problem was not encountered with several other CTV antisera tested. The CTV-status of all samples was readily confirmed by IC-RT-PCR using an aliquot of the same sample extract used for DASI-ELISA. This non-serological reaction can be used as a confirming test as it is quite independent of the DASI-ELISA system.

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