Development of Stable, Uniform Antigen Controls for Use in ELISA for *Citrus tristeza virus*

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ABSTRACT. Enzyme-linked immunosorbent assays (ELISA) are used extensively for detection of *Citrus tristeza virus* (CTV). The positive and negative controls used in ELISA vary greatly from laboratory to laboratory. Even in the same laboratory, there often is a lack of consistency over a period of time. We report a simple, easy method to prepare standardized positive and negative controls which are relatively stable over a long period of time, even at room temperature. The use of such standards may permit better direct comparison among laboratories when comparing different ELISA systems for detection of CTV.

Index words. Citrus tristeza virus, ELISA, serology.

Citrus tristeza virus (CTV), a closterovirus, is a major graft-transmissible pathogen that limits citrus productivity worldwide (2, 3, 15). CTV virions are flexuous rods of about 2000 × 11 nm and contain a non-segmented, positive-sense, single-stranded RNA genome (15). Virions are encapsidated by a major and minor coat protein (11). The major detrimental symptoms caused by CTV are decline of trees on sour orange rootstock and/or stem pitting of scions regardless of the rootstock, causing trees to die or to be debilitated with decreased fruit yields, respectively. CTV is transmitted through infected propagation material and vectored by several aphid species with *Toxoptera citricida*, commonly called the brown citrus aphid, being the most efficient. Long-distance spread is mainly through movement of infected plant material and has been responsible for the world-wide distribution of this destructive virus (15). Control measures for CTV include quarantine, certification programs, eradication in some areas, as well as the use of tolerant rootstocks and mild-strain cross protection (3, 15).

The application of the enzyme-linked immunosorbent assay (ELISA) for detection of plant viruses in 1977 was a technological landmark for detection, diagnosis and assay of plant viruses (8). ELISA was widely accepted because of the advantages the procedure offered over previously used immunoprecipitation tests: less antibody was required, increased detection sensitivity, and removal of possible inhibitory products in plant extracts by rinsing the sample after the virus was bound to the trapping antibody (8, 9). ELISA is presently the most commonly used serological method for the detection of plant viruses. One of the first successful applications of ELISA for detection of plant viruses was for the detection of CTV by Bar-Joseph et al. (1). Presently ELISA is used widely for eradication and other surveys, for the production of CTV-tested budwood in certification and clean stock programs, and as a research tool.

In all immunological tests, it is essential to include positive and negative reference samples (controls) to validate the assay and to indicate the range of test sensitivity (8, 13). The use of stabilized reference standards is widely established in the medical field for quality control of diagnostic tests. This quality

control has two major components: external quality assurance (EQA) and internal quality control (IQC). In EQA, the test performance is compared among laboratories for the purpose of harmonization and accreditation of these laboratories. It is usually part of an extensive quality control program that can include standards for testing and personnel (6, 10, 14). In Florida, citrus budwood may be cut only from registered trees which have been tested for freedom from severe strains of CTV by qualified labs using the monoclonal antibody MCA-13 (22). As an example of EQA in plant pathology, the Florida Bureau of Citrus Budwood Registration (FBCBR) requires laboratories testing budwood for freedom from severe strains of CTV to qualify by successfully testing and determining the CTV status of a panel of budsticks or other tissue provided by FBCBR.

For IQC, control materials are run repeatedly (usually 20 times), to serve as a reference for variation and standardization within a laboratory (4). The use of IQC samples increases a laboratory's confidence in the validity of the test performed and allows for the detection of batch to batch variation in commercial diagnostic kits (4).

An important criterion for antigen standards is the availability of sufficient quantities of stable, homogeneous samples which behave like the candidate samples (4, 6, 9). These sample "standards" should be as similar as possible to the material to be tested, be stable for at least the period of time needed to achieve the goal of the quality control, and be safe (non-infectious) (10). There are several factors which make creating antigen standards for CTV difficult, such as isolate diversity, the difficulty in obtaining purified virus as a reference for routine standardization purposes, and titer fluctuations of the virus in fresh extracts associated with host, isolate and tissue source and maturity of the tissue.

In our literature research we also found large variations in optical density (OD) values reported among publications (12, 21, 23, 26). In an extensive literature review, Sutula et al. (24) found large variations in ELISA results amongst 81 publications on plant viruses, and a large number of these papers did not mention the method of threshold calculation.

Antigen standards can be stabilized by several methods: filter sterilization, lyophilization, or the addition of chemicals. With lyophilization, reconstitution can be a problem, and lyophilization equipment is expensive (6). The use of chemicals offers several advantages including low cost, simple use, low matrix effect and the lack of need for a sterile environment (5, 6). Ethylene glycol has been shown to be a stabilizing agent for antigens in blood serum (5).

There is a need for standardization of diagnostic tests at the international level. Van Regenmortel (25) indicated the need for reference control antigens to evaluate antisera, to validate the detection of plant viruses in new locations, and he pointed out the problems associated with the movement of infectious material for use as positive controls for diagnostic tests. International movement of plant material is increasingly difficult; as quarantine restrictions are important to prevention the spread of virus diseases (17). Thus, validation of diagnostic test results has become more important. In the European Union, CTV is on the list of important organisms harmful to plants (7), and the standardization of diagnostic tests is part of the Standards, Measuring and Testing section of the European Union's Research program.

In this paper, we report an easy method for the development of stable, uniform antigen controls for use in ELISA for detection of CTV.

MATERIAL AND METHODS

General protocol for Preparation of antigen standards. Fresh bark and midrib tissue from CTV-infected and healthy plants was collected and chopped into 1-2 mm pieces. Tissue was ground in carbonate buffer (0.02 M sodium carbonate, 3 mM sodium azide, pH 9.6) at a ratio of 100 mg/ml, using a dispersion homogenizer (Tekmar, Cincinnati, OH), and incubated overnight at 4°C. The extract was then filtered through four layers of cheesecloth, and the filtrate mixed 1:1 (v/v) with glycerol. These preparations are referred to as standards, and they were stored at -20°C unless stated otherwise.

ELISA procedure. The ELISA procedure was similar to the one described by Nikolaeva et al. (19) so that the orange stem pitting ELISA could be performed from the same homogenized samples. Wells of Costar high binding (Corning, Acton, MA) or Immulon 2-HB (Dynex Technologies, Chantilly, VA) microtiter plates were rinsed with deionized water to remove possible polystyrene fragments (18) and drained by tapping on clean tissue paper. They were then coated with 100 µl aliquots of polyclonal coating antiserum (CREC 1052, CREC 1051 or CREC 31) diluted 1:1,000 in 0.02 M sodium carbonate buffer (pH 9.6) and incubated for 2-3 h at room temperature or for 16-24 h at 4°C. Plates were rinsed three times with deionized water and filled with PBS-T buffer (1.5 mM KH_2PO_4, 0.1 mM Na_2HPO_4, 2.7 mM KCl, and 137 mM NaCl, pH 7.4, containing 0.1% Tween-20). Plates were then incubated for at least 5 min filled with PBST. This wash procedure was then repeated two more times, and plates were then rinsed with deionized water immediately before the next step.

Plates were then loaded with 100 µl aliquots of the standards in wells containing 100 µl of carbonate buffer containing 0.2% Tween-20. The sample and carbonate buffer was mixed in the wells, then incubated for 16-24 h at 4°C, and washed as previously described.

The detecting antibody was the polyclonal antiserum G-604, produced in goat against the bacterially expressed coat protein gene of a severe isolate of CTV from India (B227) (16). A volume of 100 µl of G-604 at a 1:40,000 dilution in PBS-T buffer containing 0.2% bovine serum albumin (BSA) and 2% polyvinylpyrrolidone (PVP-40) was added to the wells, and incubated for 1-3 h at 37°C (or, alternatively, for 16-20 h at 4°C). The plates were washed as previously described. Plates were then incubated for 3 h at 37°C (or for 16-20 h at 4°C) with 100 µl aliquots of rabbit anti-goat IgG conjugated with alkaline phosphatase (Sigma A-4187) diluted 1:30,000 in PBS-T containing 0.2% BSA and 2% PVP. The plates were washed as previously described, and 200 µl aliquots of 1.0 mg/ml of p-nitrophenyl phosphate (Sigma-Aldrich N2.200-2) in 0.02 M carbonate buffer, pH 9.6, containing 0.02% sodium azide were added. The reaction was incubated at room temperature, or alternatively at 4°C overnight (18-20 h), and the OD measured at 415 nm using a microplate reader (BioRad 550, BioRad, Hercules, CA). The OD values were calculated as the measured OD minus the average OD-value for substrate incubated in uncoated wells.

Testing of standard stability – IQC. Standards prepared from tissue infected with CTV isolates T26, T30, or T36 and a healthy control were initially stored at -20°C. After 2 months, an aliquot of each standard was removed from -20°C and kept at room temperature. The remaining standards were kept at -20°C and were used as positive and negative controls in the routine ELISA tests performed in our laboratory over nine months. Comparisons of the standards stored at room

temperature and at -20°C were made at nine and 28 mo after preparation.

External quality assurance—EQA. To test the performance of the standards after shipping to laboratories in other locations, the standards were aliquoted into 0.5 ml microcentrifuge tubes, placed in small zip-seal bags in padded envelopes, and sent to 10 laboratories around the world via airmail. Two sets of standards were sent to each laboratory. One set was used by the participating laboratory and the second set was returned to our laboratory for reference testing. This enabled us to evaluate the effect of mailing on the standards as compared to constant storage at -20°C, and their usefulness to other laboratories. Each participating laboratory tested the standards using their own ELISA procedure and antibodies.

Additive and buffer effects. To compare the effect of buffers and additives, extracts of T49 (a mix of isolates T26, T30 and T55), FL169, T3800 and a healthy control were prepared in carbonate and PBS buffers alone and with the additives glycerol and Tween-20 added singly and in combination. The extracts were then stored at -20°C and tested after 14 mo. Each sample was tested using two wells in each of five microtiter plates. ANOVA and Duncan multiple range tests at 95% confidence were performed on the averages of the OD values of each sample in each plate for comparison of buffers within each standard, and of standards within each buffer.

To evaluate the effect of increasing glycerol concentration on the ELISA reaction, T49-infected tissue extracts were prepared as previously described. Dilutions were made and mixed with glycerol to create tissue extracts of 12.5, 25, 50 and 100 mg/ml in carbonate buffer with glycerol concentrations of 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, and 25%. This concentration series was tested in four microtiter plates.

RESULTS

Effect of storage—ICQ. The prepared ELISA standards remained reactive for the entire 28 months of the experiment, even when stored at room temperature (Table 1). Standards stored at room temperature gave higher OD values in ELISA than those stored at -20°C. Standards stored at -20°C were used as controls in 33 assays

TABLE 1
REACTIVITY IN ELISA OF EXTRACTS FROM HEALTHY AND *CITRUS TRISTEZA VIRUS* INFECTED CITRUS PLANTS AFTER 9 AND 28 MO STORAGE AT ROOM TEMPERATURE (RT) AND -20°C

	Optical density$_{405\,nm}$ (OD)							
		9 mo		28 mo	Average OD		C.V. (%)[1]	
Sample	0 mo	RT	-20°C	RT	RT	-20°C	RT	-20°C
Buffer	0.05	0.01	0.01	0.16	0.06	0.04	101	52
Healthy	0.04	0.01	0.01	0.12	0.04	0.03	96	59
T26	0.30	0.59	0.24	1.51	0.55	1.09	83	110
T30	0.29	0.43	0.29	1.80	0.59	0.86	97	96
T36	0.58	1.89	1.49	3.50[2]	2.08	2.35	66	50
T36a	0.56	2.92	2.32	3.20	1.96	1.92	64	51

[1]Coefficients of variation (C.V.) for all tests performed on these standard extracts during the course of the experiment: six tests for extracts stored at room temperature, 33 tests for extracts stored at -20°C. Standard extracts stored at -20°C were used in the ELISA tests routinely performed in our laboratory.
[2]This OD reached a value greater than maximum 3.50 reading of the microplate reader.

that were performed by various personnel in our laboratory with minor variations in the methodology, yet the results were consistent over a nine month period. The standards stored at room temperature were tested five times during the first 9 mo, and were tested one final time after 28 mo, at which time they were still reactive. Coefficients of variation (C.V.) for each standard in this experiment were 64-101% for standards kept at room temperature, and 50-110% for standards kept at -20°C.

EQA experiments. Differences in OD value were small between aliquots of each standard that were returned to our laboratory after mailing to other laboratories and then compared to the same set of standards kept at -20°C in our laboratory (Table 2). The C.V. values for each returned standard tested ranged from 7 to 9%. Much higher variation was found when comparing OD values of the standards tested in the ten participating laboratories, with C.V. values ranging from 67 to 122%, although all 10 laboratories could correctly identify the healthy and CTV-infected extracts.

Buffers and additives. The use of glycerol significantly increased the stability of the positive samples (Table 3). When carbonate buffer was used for extraction of standards from severe CTV isolates, no difference in reactivity was observed after 14 months of storage at -20°C. The use of carbonate buffer seems to increase the reactivity of extracts with severe isolates as compared to reactivity of the same isolates when extracted in PBS when no glycerol was used. Combinations of either buffer with Tween-20 but without glycerol rendered some of the extracts from infected plant material non-reactive (Table 3).

The use of glycerol did affect the ELISA-reaction directly: for every one percent of glycerol added to the final extract, the reactivity of the positive samples decreased between 1.1 and 1.9% (Fig. 1). Tissue dilution

TABLE 2
REACTIVITY IN ELISA OF TISSUE EXTRACT STANDARDS RETURNED BY THE PARTICIPATING LABORATORIES TO EVALUATE THE EFFECTS OF SHIPPING AND STORAGE ON ANTIGEN

	Optical density $_{405\ nm}$ of indicated extract				
Laboratory	Healthy	T30	T49	T68	FL169
1	0.07	1.07	1.61	2.77	3.03
2	0.08	1.19	1.49	2.71	2.66
3	0.09	0.91	1.35	2.70	2.96
4	0.08	1.08	1.51	2.61	2.79
5	0.08	1.21	1.69	3.07	3.06
6	0.08	1.11	1.59	3.12	3.29
7	0.09	1.06	1.61	3.06	3.14
8	0.08	1.14	1.58	2.85	2.76
9	0.09	1.07	1.42	2.57	2.62
10	0.08	1.26	1.79	3.09	3.03
Control[1]	0.07	1.26	1.70	2.62	2.80
Average[2]	0.08	1.12	1.58	2.83	2.92
Std. dev.[3]	0.005	0.104	0.128	0.214	0.212
C.V. (%)[4]	7	9	8	8	7

[1]Standards of the same source stored at -20°C were used as controls.
[2]Average reading from all 10 laboratories.
[3]Standard deviation.
[4]Coefficient of variation expressed as a percent.

TABLE 3
REACTIVITY IN ELISA OF EXTRACTS PREPARED WITH COMBINATIONS OF PBS OR CARBONATE BUFFERS WITH/WITHOUT TWEEN-20 AND/OR GLYCEROL AND AFTER STORAGE AT -20°C FOR 14 MONTHS.

	Optical density $_{405\ nm}$ of indicated extract[1]			
	Buffer only	+ Tween-20	+ Glycerol	+ Tween-20 & Glycerol
Carbonate buffer[2]				
Healthy	0.03 cE[3]	0.02 eD	0.05 bE	0.05 bE
T55/30/26	0.16 eC	0.18 eC	0.82 aC	0.73 bC
T49	0.10 eD	0.07 efD	0.67 bD	0.62 cD
FL169	0.68 dB	0.60 eB	1.40 bA	1.53 aA
T3800	0.88 dA	0.95 cA	1.37 bB	1.45 aB
PBS buffer[2]				
Healthy	0.03 eE	0.02 eC	0.06 aD	0.04 cE
T55/30/26	0.03 fD	0.03 fC	0.66 cC	0.44 dD
T49	0.04 fC	0.03 fC	0.71 aC	0.55 dC
FL169	0.04 fB	0.04 fB	1.20 cB	1.15 cA
T3800	0.10 eA	0.08 eA	1.38 bA	1.37 bA

[1]Values are average ODs of two wells in each of five plates.
[2]Carbonate Buffer is 0.02 M sodium carbonate, 3 mM sodium azide, pH 9.6, and PBS Buffer is 1.5 mM KH_2PO_4, 0.1 mM Na_2HPO_4, 2.7 mM KCl and 137 mM NaCl, pH 7.4.
[3]ANOVA and Duncan multiple range test at 95% confidence were employed, where each plate was considered a block, and the average OD of the two wells was an observation. Lower case letters indicate significant differences between buffers within each sample; upper case letters indicate significant differences between samples within each buffer.

affected reactivity more than glycerol content. Using the recommended protocol for preparing standards where the final glycerol concentration is 25% and the tissue concentration is 25 mg/ml, the loss of reactivity due to tissue dilution is estimated at 62%, while the loss of reactivity due to the presence of glycerol is estimated at 47%. The loss of reactivity due to the presence of glycerol could be reduced to 26% by an increase of tissue concentration to extraction buffer to a ratio of 100 mg/ml.

DISCUSSION

An important aspect for establishing quality control programs for ELISA, either internal or external, is the availability of standards that react similarly to the test samples and have comparable antigen levels. Ideally standards should be easily prepared in large quantities and should remain stable for long periods (4, 6, 9). Reactivity of standards prepared with carbonate buffer, glycerol (50%) and sodium azide (200 ppm) was maintained even after storage at room temperature for 28 mo. High C.V. values between readings of each standard amongst tests in the IQC experiment in our laboratory were attributed to large variations in the assay procedures. Incubation periods and time of OD readings after adding substrate varied greatly, and a more standardized procedure reduced this variation in later IQC tests where C.V. values ranged from 34-44% over 36 assays (data not shown). This could be standardized further by reading the entire plate when one standard reaches a pre-determined OD value, for example when the mild CTV-infected control reaches 0.5 OD. When ELISA samples were compared using a standardized protocol, the C.V. values in our laboratory ranged between 7-9% (Table 2). ELISA performed using standardized incubation times and temperatures should yield C.V. values of 10% or less (4, 28).

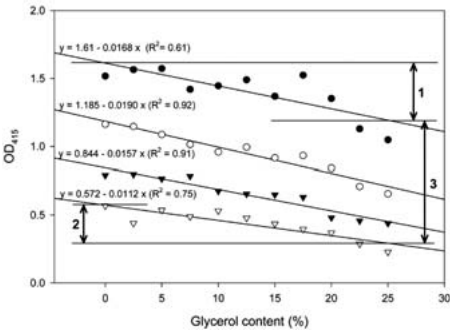

Fig. 1. Effect of glycerol concentration on reactivity of stored extracts of citrus tristeza virus infected citrus. Optical density values for extracts of T49 using a series of final glycerol concentrations in the microtiter well (0-25%) at four tissue concentrations: _ = 0.1 g/ml, _ = 0.05 g/ml, ▼ = 0.025 g/ml, and ▽ = 0.0125 g/ml. Linear regression lines with their formulas and R^2-values for reactivity against glycerol concentration for each tissue concentration level are given. Arrows 1 and 2 indicate the loss in reactivity due to the glycerol at the original and final concentrations of tissue in the extract and ELISA well. Arrow 3 indicates the loss in reactivity due to tissue dilution.

Reproducibility of OD values was inconsistent between plate brands and even batches of plates of the same brand (8), and even now C.V. values within plates were found to range between 19 and 31% when the same extract was tested in all wells of each plate (unpublished data). When we want a precise result from ELISA, we read blank plates and select plates having uniform O.D. values so that the background is as uniform as possible.

In the EQA experiment we found C.V. values to be within the 10% range when we compared OD values between standards returned by the participating laboratories. The standards remained very stable during the mailing process, irrespective of the distance and destination.

Although glycerol has some adverse effect on sample reactivity in the assays, this effect was less significant than that of the four-fold extract dilution caused by mixing the extract with glycerol to prepare the standard with the subsequent dilution with carbonate buffer at the time of the assay. This dilution could be lessened by increasing the tissue concentration in the primary extract, and/or by using carbonate buffer already mixed 1:1 with glycerol in the extraction. However, dilute samples are often desirable as standards for assays where low virus titer in the test samples is expected and assay sensitivity needs to be calibrated.

Glycerol was essential for the preservation of ELISA reactivity during long-term storage using either carbonate buffer or PBS. Carbonate buffer alone seemed to have some stabilizing effect, which we believe is caused by its higher pH. Both carbonate buffer and glycerol appear to increase ELISA reactivity (Table 3) and especially for severe strains over time (Table 1). Nikolaeva et al. (20) also observed this trend in extracts from severe stem pitting strains of CTV and utilized this in the development of the orange stem pitting ELISA assay (19). Adding Tween-20 to the standards had little effect on the stability of the extracts. We preferred to minimize the amount of additives used to stabilize extracts in order to maintain the composition of the standard as similar as possible to the extracts of the test samples. Tween-20 can be used in the extraction buffer to prevent non-specific binding. In contrast, omitting Tween-20 from the extraction buffer did not affect the reactivity with G-604 antibodies.

Ideally, samples tested by ELISA should be tested in a manner so that statistically significant differences between OD values can be estimated (27). In our experiments, statistically significant differences were found between treatments because we used four or five replications. A lot of repetitions are not practical in many situations, especially where quantitative comparisons are needed, and replications

are usually limited to two wells in one microtiter plate in routine testing. Therefore, differences between positive and negative controls should be as large as possible. Commonly OD values which are two-times or greater than those of the negative controls are used as the threshold for a positive reaction (9). In our experiments, when using a randomized block design with five repetitions for each treatment we found significant differences between values for positive and healthy samples which were below the two-times cutoff. The two-times negative control cutoff rule is also not valid when OD values for healthy controls are very low. For example, in our test of standards stored at room temperature and at -20°C after 9 mo (Table 1) the healthy controls produced OD-values of 0.006 and 0.001, respectively. From experience, cutoff values of 0.012 and 0.002 are not practical given in-plate variations, variation in loading samples to the same volume, etc. We purposefully avoided the calculation of cutoff points due to the large variation of methods that can be used for this purpose (24), and instead concentrated on demonstrating the variation that exists in OD values obtained by different laboratories.

Although lyophilization of standards may be a superior method for the long term preservation of reactivity in EQA and IQC standards, many laboratories, especially in developing countries, do not have access to expensive lyophilization equipment. This makes the production of local IQC controls and their comparison with possible lyophilized external controls difficult. In addition, errors in production and reconstitution of lyophilized samples can be a problem.

The standard extracts developed here were easily prepared and remained reactive for 28 mo at room temperature and during mailing to the participating laboratories. The large variation in OD-values found in the IQC experiments in our own laboratory could be minimized in the future by standardized test and reading protocols.

Since the original report by Clark et al. (8), plant virologists worldwide have applied the ELISA procedure for a variety of diagnostic and research purposes. The absence of standardized protocols and reagents for ELISA in published papers is notable. In general, each laboratory uses its own antibodies, reagents, and test parameters. This results in difficulties in interpretation of test results, and hinders comparison of results among laboratories and even between tests within the same laboratory. Easily produced, stable CTV extracts can help coordinate international efforts to better standardize ELISA tests for CTV in different laboratories worldwide.

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