Application of Citrus Tristeza Virus Antisera in Labeled Antibody, Immuno-Electron Microscopical, and Sodium Dodecyl Sulfate-Immunodiffusion Tests[1,2]

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ABSTRACT. Citrus tristeza virus (CTV) antisera were prepared to undegraded, unfixed virus (CTV-W), undegraded formaldehyde fixed virus (CTV-F), and sodium dodecyl sulfate (SDS) degraded virus coat protein (CTV-S). These antisera were compared for their ability to detect CTV using *in situ* immunofluorescence (ISIF), double sandwich enzyme-linked immunosorbent assay (ELISA), serological specific electron microscopy (SSEM), and SDS immunodiffusion (SDS-ID). Gamma globulins (IgG) were separated from whole serum by sodium sulfate precipitation or on a protein A affinity column for use in ISIF and ELISA. Unfractionated sera were used for SDS-ID and SSEM. Citrus tristeza virus could be detected in infected tissue by ISIF and SSEM with IgG prepared to CTV-W or CTV-F but not with CTV-S. In SDS-ID tests, CTV was detected with antisera to CTV-S or CTV-W but not with antiserum to CTV-F. Using ELISA, CTV was detected in buffered extracts with all three sera; however, nonspecific reactions interfered when IgG to CTV-S was used for both coating and conjugate steps. The nonspecific reaction was reduced when CTV-S was used as coating and CTV-W was used as the conjugate antiserum or when CTV-S IgG was isolated by a protein A affinity column. Sodium dodecyl sulfate degraded virus antigen was detected by ELISA when coating IgG to CTV-S and conjugate IgG to CTV-W was used. Presence of at least two serological determinants for CTV was indicated.
Index words. in situ immunofluorescence, serologically specific electron microscopy, enzyme-linked immunosorbent assay.

Many serological methods are available for the detection and identification of plant viruses in plant tissues and extracts. Serological methods currently used for detection of citrus tristeza virus (CTV) include immunoflorescence (13), enzyme-linked immunosorbent assay (ELISA) (1), serologically specific electron microscopy (SSEM) (7) and sodium dodecyl sulfate immunodiffusion (SDS-ID) (8). Tsuchizaki *et al.* (13) used an immunofluorescent microscopic technique for the detection of CTV in infected citrus tissues using fluorescein isothiocyanate (FITC). An *in situ* immunofluorescent technique using tetramethylrhodamine isothiocyanate (TRITC) for the detection of xylem-limited bacteria in plant tissues was recently reported by Brlansky *et al.* (3). Antisera have been prepared in rabbits to undegraded, unfixed CTV (9), undegraded formaldehyde fixed CTV (10), and to sodium dodecyl sulfate degraded coat protein (8). Results reported have indicated that these sera are not equally suitable for all diagnostic procedures (1, 6, 10). In this paper we compare these antisera for detecting CTV by different serological tests.

MATERIALS AND METHODS

Virus isolates. The CTV isolates T-4 and T-3 from Florida were used throughout this study. The T-3 isolate produces a seedling yellows (SY) reaction whereas T-4 does not (8).

Antisera. All antisera used in this study were prepared to the T-4 isolate of CTV. Antisera to undegraded, unfixed virus (CTV-W) and to undegraded formaldehyde fixed virus (CTV-F) were those prepared by Gonsalves *et al.* (9)

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[2]Use of a company or product name by the U.S. Department of Agriculture does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

and (10), respectively. Antiserum to SDS-degraded virus coat protein (CTV-S) was that prepared by Garnsey *et al.* (8). Gamma globulins (IgG) separated from the whole serum by the sodium sulfate procedure of Kekwick (11) or by the protein A-Sepharose affinity chromatography procedure of Miller and Stone (12) were used for *in situ* immunofluorescence (ISIF) and ELISA. The concentration of IgG was estimated spectrophotometrically using E 0.1%/280 nm = 1.4. In some tests IgG fractionated by $(NH_4)_2SO_4$) precipitation and cellulose chromatography (1, 4) was used.

Conjugation of antisera. Antisera for ISIF were conjugated with either (FITC) (Miles Laboratories, Elkhart, IN 46515) or with TRITC (Research Organics, Inc., Cleveland, OH 44125). The IgG were conjugated with FITC by the method of Blakeslee and Baines (2) and with TRITC as described by Brlansky *et al.* (3).

The IgG for double sandwich ELISA were conjugated with alkaline phosphatase Type VII (Sigma Chemical Co., St. Louis, MO 63178) as previously described (1, 4).

In situ **immunofluorescence.** Both FITC- and TRITC-labeled IgG were used in this study with a modification of the technique of Brlansky *et al.* (3). Sections of bark and petiole material of virus-infected and healthy citrus were cut using a Harris Model WRC cryostat (Harris Manufacturing, Inc., North Billerica, MA 01862). Longitudinal and transverse sections 30 to 40 μm thick were immersed in labeled IgG to CTV. Labeled normal serum IgG and labeled IgG specific to Pierce's disease bacteria were used as controls. Sections were incubated at room temperature for 1 hour or at 37 C for 30 minutes, washed in phosphate-buffered saline for 10 to 20 minutes, and mounted on microscope slides in Aqua Mount.® The slides were observed with a Leitz Dialux® fluorescence microscope (E. Leitz, Inc., Rockleigh, NH 07647) equipped with a 50-V mercury lamp and viewed in the 560- to 590-nm range for TRITC (Leitz filter block N2) and in the 380- to 420-nm range (Leitz filter block H2) for FITC. Photographs were taken with Kodak Ektachrome EC 400 color film® and/or Kodak Technical Pan 2415® black and white film.

Serologically specific electron microscopy. The procedure for SSEM was that previously described for plant viruses by Derrick and Brlansky (5) and specifically for CTV by Garnsey *et al.* (7). Unfractionated antisera were used throughout at dilutions of 1:500 in 0.05 M Tris buffer, pH 8.0. Parlodion-coated copper grids were commonly used; although freshly prepared formvar-coated grids were also useful. In the latter case, grids were coated with 0.5% formvar in ethylene dichloride. After the IgG-sensitized grids were exposed to test extracts and rinsed, the grids were positively stained with a 5% solution of uranyl acetate in 50% ethanol for 1 minute and washed in 95% ethanol for 30 seconds. The grids were viewed with a Philips 201 transmission electron microscope.

ELISA. The double sandwich ELISA procedure developed for CTV by Bar-Joseph *et al.* (1) was used. Immulon II Microtiter® plates (Dynatech Laboratories, Inc., Alexandria, VA 22314) were coated with 1 μg IgG per ml for 4 hours at 37 C. Antigen preparations were prepared as previously described (1) or by grinding either healthy or CTV-infected tissue at a ratio of 1 g fresh weight per 24 ml of 0.05 M Tris pH 8.0. Antigen preparations were then additionally diluted 1 to 50 (w/v) with either Tris buffer or Tris buffer

containing SDS so that the final SDS concentration was 0.1%. Antigen preparations were incubated overnight at 4 C in the microtiter plates. Conjugates were diluted 1 to 1,000 (about 1 μg IgG per ml) in a phosphate-buffered saline solution (PBS) containing 0.5% (v/v) Tween 20, 2% (w/v) polyvinylpyrrolidone (40,000 molecular weight, Sigma) and 0.02% (w/v) bovine serum albumin (fraction V powder, Sigma) and incubated in the plates for 24 hours at 4 C. After rinsing, substrate (*p*-nitrophenylphosphate, 1.0 mg/ml in 10% triethanolamine, pH 9.8) was added. After 30 minutes at room temperature, the $A_{405\ nm}$ was read on each well using a Bio-Tek EIA Reader®, Model EL 307 (Bio-Tek Instruments, Inc., Burlington, VT 05401). Some samples were diluted with water and read in a spectrophotometer.

SDS immunodiffusion. The SDS-immunodiffusion method used was that described by Garnsey *et al.* (8).

RESULTS

Citrus tristeza virus was detected in infected citrus bark and petiole tissue sections by ISIF using IgG prepared to CTV-W or CTV-F and conjugated to FITC or TRITC (table 1). Less interference from autofluorescing tissues was found using a wavelength of 560-590 nm. Fluorescence was not observed when infected tissue sections were incubated in labeled IgG to CTV-S, labeled normal serum IgG, or in labeled IgG to the Pierce's disease bacterium. Sections of healthy tissue also did not fluoresce after exposure to any of the labeled CTV IgGs.

Similar results were obtained when these same antisera were tested using SSEM. Thread-like CTV particles were consistently found on grids that were coated with CTV-W or CTV-F and incubated on CTV-infected tissue extracts or on purified preparations of CTV. The average number of virions detected at 7,000 X on three 8.25 x 10.0 cm electron micrographs was 108 for grids coated with CTV-W antiserum and 43 for grids coated with CTV-F antiserum. Occasional CTV virions were seen on grids coated with CTV-S and exposed to CTV extracts, but similar numbers were seen on uncoated grids exposed by the quick dip method. No virions were found on CTV-W or CTV-F coated grids ex-

TABLE 1
SEROLOGICAL DETECTION OF CITRUS TRISTEZA VIRUS USING VARIOUS METHODS AND ANTISERA

Method of detection*	Antisera prepared against:†		
	CTV-W	CTV-F	CTV-S
ISIF	+‡	+	—
SDS-ID	+	—	+
SSEM	+	+	—
ELISA (antigen in Tris buffer)	+	+	+
ELISA (antigen in 0.1% SDS)	—	—	+

*ISIF = *in situ* immunofluorescence; SDS-ID = sodium dodecyl immunodiffusion; SSEM = serologically specific electron microscopy; ELISA = enzyme-linked immunosorbent assay.
†CTV-W = antisera prepared to undegraded unfixed CTV, isolate T-4; CTV-F = antisera prepared to undegraded formaldehyde-fixed CTV, T-4; CTV-S = antisera prepared to sodium dodecyl sulfate degraded CTV, T-4 coat protein.
‡† + = CTV detected; — = CTV not detected.

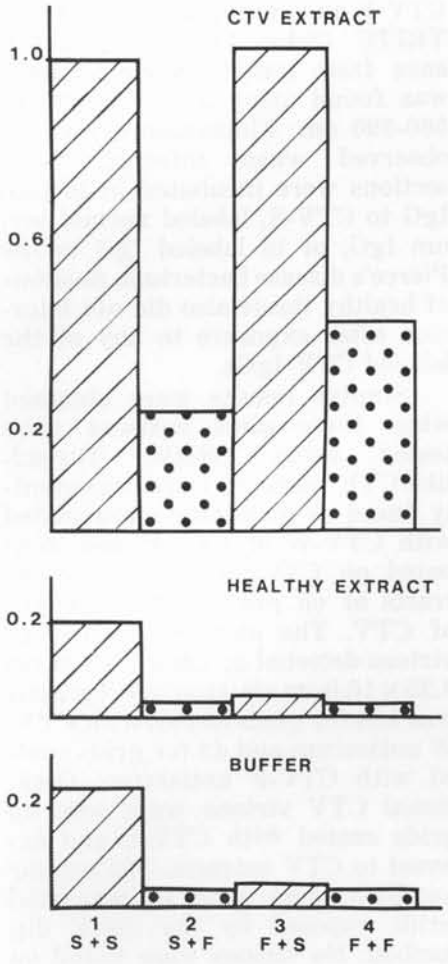

Fig. 1. Detection of citrus tristeza virus with different combinations of immunoglobulins prepared to sodium dodecyl sulfate (SDS)-degraded coat protein (CTV-S) and undegraded, formaldehyde-fixed virus (CTV-F) for coating and conjugate steps in double sandwich enzyme - linked - immunoabsorbent assay tests. In columns 1 and 2, IgG to CTV-S was the coating antibody and in columns 3 and 4, IgG to CTV-F was the coating antibody. Conjugates to both IgGs were tested with each coating as indicated. IgG concentration for coating was 2 μg/ml and conjugates were used at 1/400 dilution for all combinations. OD_{405} values are for 1/4 dilutions of reacted, stopped substrate.

posed to extracts of healthy tissue.

In SDS immunodiffusion tests, CTV could be detected in crude extracts or in purified preparations with antisera to CTV-S or CTV-W but not with antiserum to CTV-F (table 1).

When extracts were prepared in Tris buffer, CTV could be detected by ELISA with all 3 CTV antisera. However, when the extracts were prepared in 0.1% SDS, CTV could only be detected by the antiserum specific for CTV-S and not by the antisera to CTV-W or CTV-F.

When the IgG fraction for CTV-S antisera was prepared by ammonium sulfate fractionation as described by Clark and Adams (4) and used for the coating IgG and conjugate steps, there was a high, nonspecific background reaction even in wells exposed only to buffer (fig. 1). For example, using IgG coating and conjugates as prepared by Clark and Adams (4), a healthy citron leaf homogenized 1/50 (w/v) in 0.05 M Tris buffer pH 8.0 yielded an average A_{405} value of 0.308 using CTV-S coating and conjugate, whereas an average A_{405} value of 0.031 for the same sample was obtained with CTV-W IgG coating and conjugate. The nonspecific reaction was consistently lower when CTV-S IgG isolated on a protein A affinity column was used (table 2). No nonspecific reaction was observed when CTV-S IgG was used only for the coating or the conjugate step and CTV-F or CTV-W IgG for the other (fig. 1). The SDS-degraded virus and whole virus in Tris buffer were both detected when plates were coated with IgG to CTV-S and trapped antigen was recognized with conjugate for CTV-W. In a test of CTV extracts prepared in phosphate-buffered saline (1), with different combinations of CTV-S and CTV-F IgG's for coating and conjugate steps, CTV-S and CTV-F coatings were equally good for CTV-S conjugate, but CTV-F coating was superior to CTV-S coating when combined with CTV-F conjugate (fig. 1).

TABLE 2
COMPARISON OF THE ABILITY OF ANTISERA PREPARED AGAINST UNDEGRADED, UNFIXED CTV (CTV-W), UNDEGRADED FORMALDEHYDE-FIXED CTV (CTV-F) AND SDS-DEGRADED CTV COAT PROTEIN (CTV-S) TO DETECT CTV USING DOUBLE SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Tissue	Buffer	A_{405} nm		
		CTV-W*	CTV-F*	CTV-S*
Healthy Mexican lime	Tris	0.032†	0.006	0.084
Healthy Mexican lime	Tris + 0.1% SDS	0.024	0.078	0.108
T4-infected Mexican lime	Tris	1.462	1.604	1.605
T4-infected Mexican lime	Tris + 0.1% SDS	0.035	0.049	0.982
Healthy *Citrus excelsa*	Tris	0.034	0.008	0.085
Healthy *C. excelsa*	Tris + 0.1% SDS	0.008	0.053	0.086
T3-infected *C. excelsa*	Tris	0.892	1.033	1.167
T3-infected *C. excelsa*	Tris + 0.1% SDS	0.018	0.067	0.448
None	Tris	0.009	0.042	0.096
None	Tris + 0.1% SDS	0.019	0.055	0.081

*The IgG used for coating was isolated by using protein A affinity chromatography (11).
†Mean of six replicates.

DISCUSSION

The simultaneous comparison of four serological procedures using CTV antisera prepared by three different methods provides new information on the complexity of the serological reactions involving CTV. The ability to detect CTV by different serological techniques was confirmed, but the success of a particular technique was dependent on the method used to prepare the immunogens for production of anti-CTV IgG. The ability of CTV-F antiserum to detect whole, undegraded CTV and its inability to detect SDS-degraded virion coat protein confirmed an earlier report (9). The failure of CTV-S to trap CTV particles in SSEM was also more thoroughly documented. The failure of CTV-S-labeled IgG to function in ISIF procedures reinforced the indication from SSEM that IgG to CTV-S do not react well to the surface antigen sites of intact CTV. The failure of CTV-S IgG to recognize whole CTV in both SSEM and ISIF suggests that failure in ISIF is not because affinity is altered when IgG is labeled, since the IgG was not labeled for SSEM. Negative SSEM results probably can not be explained by lack of CTV-S antibody adsorption to grids since IgG adsorption to a substrate was not required for ISIF. Since CTV-S antiserum works well in the relatively insensitive SDS-immunodiffusion procedure, lack of reaction in SSEM and ISIF should not be due to insufficient antibody titer.

Results from SDS-immunodiffusion, SSEM and ISIF would all suggest that two antigenic determinants exist for CTV. On this basis, CTV-S IgG when used as a coating antibody for ELISA should not trap intact CTV antigen and, conversely, CTV-F antibody should not trap subunit antigen. However, actual ELISA results show that heterologous combinations of CTV-S and CTV-F for coating and conjugate steps do work with only a minor difference between homologous and heterologous systems. This suggests that the antigen-antibody reaction may involve more than two simple determinants or that affinity requirements differ between SSEM, ISIF and ELISA for trapping of intact CTV antigen by CTV-S antibody.

It is apparent that, the serological detection of CTV antigen is

complex and results can be affected by choice of antiserum. At this point, CTV-W appears to be the only IgG source satisfactory for nearly all serological procedures. Presence of antibodies to multiple determinants in this antiserum may contribute to its outstanding performance in ELISA.

Accurate quantitation of CTV antigen by ELISA or radio immunoassay (RIA) will require knowledge of the relative titers of the different antibody species present and the form of the test antigen. Although CTV is relatively stable, sap extracts presumably contain both subunit proteins and intact virus. Since CTV is easily sheared during extraction, particles with exposed cryptic determinants could also be present in tissue extracts.

Reduction of the nonspecific reaction with CTV-S following purification of the IgG by protein-A affinity chromatography suggests that the nonspecific response observed is associated with a serum fraction other than IgG.

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