Detection of Citrus Tristeza Virus. I. Enzyme-Linked Immunosorbent Assay (ELISA) and SDS-Immunodiffusion Methods*

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Indexing procedures are an essential component of control programs for citrus tristeza virus (CTV). Thousands of trees must be indexed annually for CTV infection to maintain the eradication programs in Israel and California (Raccah *et al.*, 1976; Roistacher, 1976). In areas where tristeza is already widespread, indexing is important to research on varietal resistance, cross protection, vector relationships and therapy procedures.

The development of the lime test for CTV (Wallace and Drake, 1951) was a major advance in detection, and this procedure has been a mainstay in CTV indexing for many years. However, the lime test requires extensive plant material, greenhouse facilities, skilled personnel and considerable time (4-6 weeks minimum). It does not provide quantitative measurement or allow detection of CTV from *in vitro* sources. Mild isolates may be difficult to detect.

Detection of CTV infection by electron microscopic examination of negatively stained extracts has been used (Bar-Joseph *et al.*, 1974). This procedure provided rapid results, but limited sensitivity, somewhat laborious sample preparation, and the requirement for electron microscopy limited application.

Recently, several serological procedures have been used for CTV detection (Tsuchizaki *et al.*, 1978; Garnsey *et al.*, 1979; Bar-Joseph *et al.*, 1978, 1979*a*). Several of these procedures are well adapted for large-scale field use and, at the same time, provide essential assay tools for experimental studies.

In this paper we report application of the ELISA (enzyme-linked immunosorbent assay) and the SDS-immunodiffusion procedures to practical and experimental indexing problems and compare the advantages and limitations of each procedure. In a companion paper (Garnsey *et al.*, 1980), CTV indexing by light and electron microscopy of CTV particles and inclusions is discussed.

METHODS AND MATERIALS

ELISA. The ELISA assay method used was basically the double antibody-sandwich method (Clark and Adams, 1977) outlined in Appendix I. Several types of polystyrene microtiter plates were used, including Cooke 1223-24, Dynatech M 29 ARE and M 129 A (Dynatech Laboratories, Inc., Alexandria, VA), and Linbro IS-MRC96 (Linbro Scientific Co., Hamden, CT). Alkaline phosphatase Type VII was obtained from Sigma Chemical Co., St.

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Louis, MO. The γ - globulin was purified, and conjugates between purified γ - globulin and alkaline phosphatase were prepared essentially as described by Clark and Adams (1977). A beaded, preswollen DEAE-cellulose (Pharmacia, Uppsala) was used in place of Whatman DEAE 22 or 23 cellulose to purify some γ - globulin preparations. This reduced preparation of the cellulose for column chromatography. Other specific procedural details have been recently described by Bar-Joseph *et al.* (1979*a*).

Appropriate standards were included in test plates to compensate for quantitative deviations in color change due to

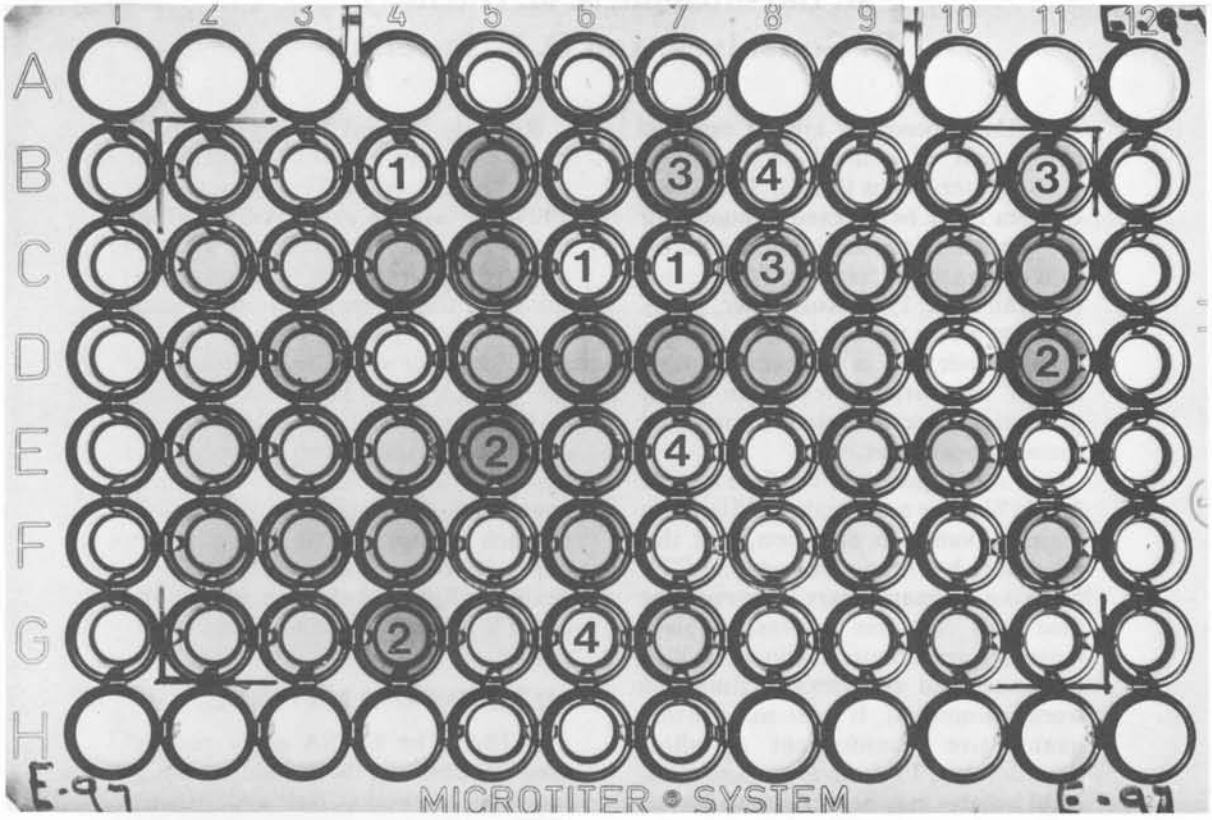

Fig. 1. Example of an ELISA (enzyme-linked immunosorbent assay) microtiter plate used for detection of citrus tristeza virus (CTV). Wells treated sequentially with γ - globulin purified from CTV antiserum, sample extracts, γ - globulin conjugated to alkaline phosphatase and the enzyme substrate *p*-nitrophenyl phosphate (Appendix 1). Amount of color change (yellow) in substrate is proportional to amount of CTV present. In this plate, test samples were loaded in a randomized pattern with three replications. Wells coded 1 contained extract from a healthy plant; wells coded 2, 3, and 4 contained extracts from a CTV-infected plant at 1/10, 1/100 and 1/500 dilutions, respectively. Although the difference is not obvious in the photograph, wells 1 and 4 differed in color. OD_{405} values were 0.07, 1.92, 1.03, and 0.33, respectively. Uncoded wells contained extracts from healthy and CTV-infected field trees. Border wells were not used.

reactant concentration, incubation conditions and plate binding capacity.

In addition to the antiserum to the formaldehyde-fixed, whole virus of the T-4 isolate of CTV used in previous ELISA studies (Bar-Joseph *et al.*, 1979*a*), we used antiserum to unfixed, whole T-4 CTV (Gonsalves *et al.*, 1978) antiserum to SDS-degraded T-4 CTV coat protein (Garnsey *et al.*, 1979), antiserum to the severe T-3 isolate prepared to a combination of unfixed-whole virus and SDS-degraded coat protein (Purcifull, Garnsey, and Gonsalves, unpublished) and antiserum to partially purified VT-CTV (Bar-Joseph, unpublished).

Microtiter plates (fig. 1) and enzyme conjugates used for large-scale indexing tests in Israel were often reused per procedures described by Bar-Joseph *et al.* (1979*b* and *c*).

SDS-immunodiffusion. The SDS-immunodiffusion methods used were as described by Garnsey *et al.* (1979) and follow the general procedures of Purcifull and Batchelor (1977). Tests were conducted in 15- x 100-mm disposable, plastic petri dishes filled with 12 ml of media containing 0.8 per cent Noble agar (Difco), 0.5 per cent SDS (Sigma), and 1.0 per cent NaN_3. Wells were usually 7 mm in diameter and spaced 5 mm apart (edge to edge) in a pattern of six peripheral wells surrounding a center well (fig. 2). Three patterns were cut per plate.

Antiserum to SDS-degraded coat protein of the T-4 isolate and antiserum to the T-3 isolate as described above were used.

Tissue sample storage and preparation. Samples were stored at 4°C for testing within a few days. Longer storage was done at -20 or -55°C, or tissue was dried under vacuum for 1-2 days and then stored at -20°C. In some cases, measured amounts of tissue were frozen in extraction buffer and stored directly in the test tubes used for sample homogenization.

Some extracts were tested both by SDS immunodiffusion and by ELISA. In this case, extracts were prepared at a 1:10 or 1:20 dilution (w:v) in extraction buffer (Appendix I). An aliquot was removed for the ELISA test, and NaCl and polyethylene glycol (PEG) 6000 were added to make final concentrations of 0.5 M and 6 per cent (w/v), respectively. Following a 1-hour incubation, the extract was centrifuged at 20,000 X g for 15 minutes and the pellet resuspended in 0.5 per cent SDS (1 ml per 0.25 g of original tissue weight).

Results of ELISA and SDS tests were scored visually on coded data sheets and photographed when necessary. We photographed SDS-immunodiffusion plates with Polaroid Type 665 black and white film under dark field illumination (Garnsey *et al.*, 1979). The ELISA plates were photographed on Kodachrome 64 film with diffused backlight supplied by Jewel Vita-Lite fluorescent tubes (5500° Kelvin).

RESULTS

Test procedures and conditions. Specific procedures and materials have been defined for ELISA (Bar-Joseph *et al.*, 1979*a*) and SDS-immunodiffusion (Garnsey *et al.*, 1979) tests which will yield consistent, reliable results. Some procedures and materials are critical, but most can be varied considerably with acceptable results.

For SDS-immunodiffusion, the critical factors are a proper choice of antiserum, an appropriate concentration of SDS, and proper ionic strength in the test media. Antiserum to a formaldehyde-fixed whole CTV reacted very weakly or not at all in our SDS-immunodiffusion tests, while antiserum to unfixed whole virus and antiserum to SDS-degraded coat protein reacted well. Best results were usually obtained when SDS was present both in the sample extract and in the agar media, but aqueous extracts gave satisfactory results. An SDS concentration near 0.5 per cent was desirable. Diluted antisera or purified γ - globulin fractions failed to react in SDS-immunodiffusion tests unless appropriate protein levels were maintained in the γ - globulin-containing solution (Purcifull and Batchelor,

1977). Normal serum, or a 5 per cent solution of bovine serum albumin, was used as a diluent to maintain protein levels when antisera were diluted and to resuspend fractionated γ - globulins. A pattern punch (Auto-gel, Grafar Corp., Detroit) to cut wells and an adjustable pipet (Rainin Instruments, Brighton, MA) to load samples greatly facilitated preparation of test plates. The pipet is also desirable for ELISA work.

The most critical factors for ELISA are a specific antiserum for preparation of the enzyme conjugate, a substrate (plate) with binding affinity for the coating γ - globulin, and appropriate concentrations of coating γ - globulin, conjugate and substrate. We have used γ - globulin purified from antisera to fixed whole virus and unfixed whole virus for coating and conjugate preparation. The γ - globulin from antiserum to SDS-degraded coat protein (SDS-CP) was used successfully in combination with γ - globulin from whole virus antiserum for the alternate step (see Appendix 1). However a higher-than-normal, nonspecific background reading of undetermined cause was observed when the γ - globulin from antiserum to SDS-CP was used for both the coating and conjugate steps. This reaction was not due to antibodies to healthy sap since it occurred with extraction buffer alone. Although our serum to unfixed whole CTV reacts weakly to healthy citrus antigens, the conjugate prepared to it could be used at a dilution (1/800) that eliminated significant reaction with healthy extracts. The specificity of coating antibodies is less critical than the specificity of the conjugate antibodies and we have used γ - globulins from antiserum to partially purified CTV for coating. Different batches of microtiter plates can vary in binding affinity and should be checked against a standard. This is especially important for plates that are to be reused. Use of standard samples is essential for accurate quantitative comparison of results from different tests.

Tissue selection. Selection of sample tissue was important for serological indexing, especially for the SDS-immunodiffusion test. Best results were obtained from samples rich in recently produced phloem tissue, such as bark from flushes of new growth. Positive results were obtained from bark of mature limbs, from young feeder roots, from leaf midribs and from various fruit tissue. Bark of the fruit pedicel and the fruit button were especially good sources and provided good results during the summer when young vegetative tissues were not available. Samples for survey purposes can be collected even at the packinghouse (Bar-Joseph *et al.*, 1978). The CTV titer peaks in new growth as leaves near full expansion, then often drops rapidly during warm weather.

Sample storage. Tissue may be collected during optimum stages of growth and stored frozen or dried for extended periods. Tissue may be air dried, or dried under vacuum and stored at -20° to -60°C.

Extracts prepared for testing by ELISA or SDS-immunodiffusion, were frozen and stored at -20°C and retested successfully. This was often convenient for comparative studies and for rechecking results. Fresh extracts were stored up to 24 hours at 4°C before testing with good results.

Efficiency and sensitivity. In the conventional SDS-immunodiffusion system described (Garnsey *et al.*, 1979), 120 tests can be done with 1 ml of undiluted antiserum (6 well pattern, 50 μl capacity). Use of smaller wells and/or diluted antiserum could increase efficiency severalfold, but 1,000 tests/ml of antiserum would probably be a practical limit. Antisera have a lower effective titer by SDS immunodiffusion than by other tests and dilutions beyond one-fourth will normally decrease sensitivity.

Normally 5 to 7 mg of "purified" γ - globulin were obtained from 1 ml of antiserum by our purification procedures. Approximately 7,000 ELISA tests are possible with 6 mg of γ - globulin (assuming a well volume of 0.2 ml, 2 μg/ml concentration of γ - globulin for coating, and a 1/400 dilution of enzyme-

γ - globulin conjugate). With especially active γ - globulin and conjugate preparations, greater dilutions are possible. Samples from several trees can be composited for testing when infection rates are low, which further extends the number of trees indexed per given volume of antiserum. When a positive reaction is obtained, all trees of the composite are rechecked individually. Conjugates can be reused up to four times where infection rates are low (<5 per cent) and still retain more than 60 per cent of their original activity (Bar-Joseph *et al.*, 1979*c*).

Microtiter plates have been successfully reused as many as six times by treating the plates for 60 minutes with 0.2 M glycine-HCl, pH 2.2, between uses (Bar-Joseph *et al.*, 1979*b*). This treatment dissociates the antigen-antibody bonds, but does not remove the coating antibody. Repeated use saves plates and time.

The SDS-immunodiffusion test is apparently sensitive to virus concentrations of 1-2 μg/ml (Garnsey *et al.*, 1979), a level adequate to detect CTV in field samples collected under favorable conditions. Extracts of young shoot bark usually react at least moderately at 1/8 dilution (tissue/buffer), and reactions are often visible at a 1/64 dilution. Samples with a low titer can be readily concentrated by a single cycle of PEG precipitation (Garnsey *et al.*, 1979).

In comparative tests with the same sample, ELISA has proven 10- to 50-fold more sensitive than SDS-immunodiffusion when both tests are scored visually. Even greater sensitivity can be achieved for ELISA by spectrophotometric analysis of the reactions, but the time, precision, and replication required to detect significant subvisual differences between samples made analysis of large numbers of samples impractical.

The ELISA test was sensitive enough that we consistently detected infections of mild and severe CTV isolates in bark extracts (10% tissue w/v) of mature spring flush of Valencia sweet orange collected in August after an extended period of hot weather ($\overline{m}$ daily maximum >33°C). Many of these samples could not be diagnosed by SDS-immunodiffusion.

Reaction to different isolates. The antisera to the Florida T-4 isolate of CTV have reacted to all CTV isolates tested so far from Florida, Israel, Texas, and Hawaii, in ELISA and SDS tests (Bar-Joseph *et al.*, 1979*a*; Garnsey *et al.*, 1979, and unpublished). These tests have included a very broad range of biological types from very mild to severe seedling yellows forms (fig. 2). We also obtained a positive reaction by ELISA to over 95 per cent of 107 isolates tested from California, which again included a broad spectrum of isolates, some of foreign origin.

Only limited tests have been performed with the antiserum to the T-3 isolate of CTV, but it reacted strongly to 12 CTV isolates ranging from seedling yellows isolates to extremely mild isolates.

Strong serological reactions have been obtained with most very mild CTV isolates which are difficult to detect by the lime test.

Host effects. Both ELISA and SDS immunodiffusion have been used successfully on most major citrus cultivars, including sweet oranges, sour orange, Eureka lemon, Mexican lime, Etrog citron, tangelos, mandarins and grapefruit. Sporadic failures have occurred with CTV-infected grapefruit, a problem apparently inherent to grapefruit and not serology related (Garnsey *et al.*, 1979).

Use of serology for field indexing. The SDS-immunodiffusion procedure was used successfully for field indexing in Florida by Garnsey *et al.* (1979), and subsequently by personnel of the Florida Department of Agriculture and Consumer Services (C. O. Youtsey, personal communication).

Over 82,000 samples have been indexed by ELISA during the past year in Israel, and ELISA has largely supplanted the lime test for routine purposes. Five new centers of CTV infection have been located in Israel, and symptomless trees on sour orange rootstock have been discovered. Experimental evaluation of

ELISA for use in the California tristeza eradication project is underway (D. Cordas, personal communication).

Research applications. We use serological indexing procedures routinely to measure titer in tissues used for purification; to check efficiency of purification procedures; to analyze fractions from density gradients and electrophoresis gels for presence of virus or virus protein; to study distribution and titer of CTV within trees; to measure rates of CTV synthesis; to index experimentally inoculated, symptomless hosts for presence of CTV; and to verify CTV content in inoculum sources and propagation sources before grafting.

DISCUSSION

Serological indexing offers some obvious benefits to CTV eradication programs. Large numbers of samples can be processed without extensive greenhouse facilities or the need to grow and maintain large numbers of indicator plants. It is flexible and adaptable to rapid changes in requirements. Results are obtained rapidly, and infected trees can be removed before secondary spread occurs. Material and labor costs per test are easily competitive with the lime test and processing can be semimechanized to handle large numbers of samples. Serological indexing is highly accurate when properly done and can detect a very wide range of CTV isolates. The failure to detect several isolates from California may be due to low titer in these specific isolate-host combinations tested, and not lack of serological reactivity between the isolates and our T-4 antiserum. Further tests are in progress. As with any procedure, including the lime test, experience is helpful in evaluating weak reactions and appropriate controls are essential.

Rapid quantitative measurement of CTV from *in vivo* and *in vitro* sources by serological indexing is advantageous to research programs. New areas of research are feasible because rapid results for several hundred samples per day can be obtained with modest labor and equipment.

Selection of an indexing procedure should be based on specific needs. The SDS-immunodiffusion procedure is simple, very rapid, and requires minimal equipment, sample preparation and operations. It, however, lacks the sensitivity of ELISA and is not as economical in use of antiserum. The latter point is quite important for large-scale applications, but less so for limited field or research use. Purified SDS-degraded coat protein for use as an inject antigen is more easily obtained than highly purified whole virus (Garnsey *et al.*, 1979). Nonspecific reactions sometimes occur in SDS-immunodiffusion systems (Gonsalves *et al.*, 1978; Purcifull and Batchelor, 1977), and these can complicate interpretation of results unless proper controls are included.

The ELISA procedure is more sensitive, requires less antiserum, and is readily adapted for large-scale use. However, it requires more sophisticated laboratory facilities, and involves more steps. ELISA is not as well suited for rapid processing of a few samples, although single test cells are available or samples can be stored until enough are available to justify a test. Purification of γ - globulin and preparation of enzyme conjugates are time consuming and expensive for only a few tests. The ELISA procedure will become more practical for limited applications if prepared materials (γ - globulin-coated plates, stock solution concentrates, and pretitered conjugate) can be made available, ready for use. False positive readings can occur if the plates are faulty, or if errors are made in procedure.

Serological differences between CTV isolates may exist, but our experience suggests that most CTV isolates are closely related serologically even though they cause very different host reactions. In contrast to the lime test, or indexing on other indicators, our serological techniques do not provide information on biological properties.

In conrast to the microscopy techniques discussed in the companion paper (Garnsey *et al.*, 1980), neither serology procedure provides information on the physical condition of the virus particle or its distribution, aggregation, etc.

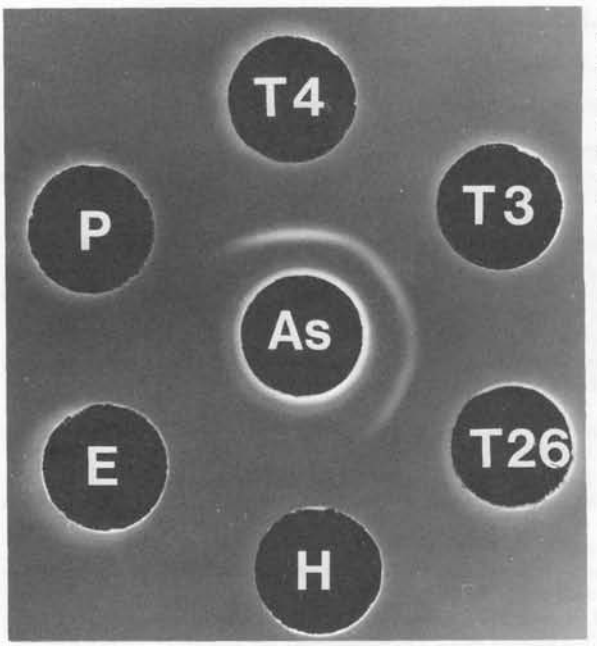

Fig. 2. Reaction of antiserum (As) to SDS-degraded CTV coat protein to extracts from sweet lime infected with a severe isolate of CTV (T-3), *C. excelsa* infected with a moderate isolate (T-4), and Duncan grapefruit infected with a mild isolate (T-26), healthy sweet orange (H), Etrog citron infected with exocortis (E), and sweet orange infected with psorosis (P). Immunodiffusion test conducted in media prepared with 0.8 per cent Noble agar, 0.5 per cent SDS (sodium dodecyl sulfate), and 1 per cent sodium azide. Extracts prepared in 0.5 per cent SDS (1 g tissue to 4 ml). Plate photographed 36 hours after loading.

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APPENDIX I. TYPICAL ELISA PROCEDURE FOR CTV.

1. **Coat Plate —** Add 0.2 ml purified γ - globulin (2 μg/ml in coating buffer[a]) per well. Incubate 2-6 hours at 37°C and wash[b].
2. **Add test samples —** Add 0.2 ml of test sample (prepared at 1:10 or 1:20 dilution in extraction buffer[c]) per well. Incubate overnight at 4-6°C, wash.
3. **Add enzyme-γ** - globulin conjugate — Add 0.2 ml per well of 1/400 dilution prepared in extraction buffer which contains 0.2 per cent ovalbumin. Incubate 3-6 hours at 30°C, wash.
4. **Add substrate —** Add 0.25 to 0.30 ml of substrate per well (0.6 mg/ml *p*-nitrophenylphosphate in substrate buffer). [d]Incubate 30 to 60 minutes at room temperature.
5. **Stop —** Add 50μl 3M NaOH per well to stop reaction.
6. **Read —** Observe visually or measure absorbance at 405 nm.

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[a]Coating buffer = 0.05 M sodium carbonate, pH 9.6 + 0.2 per cent NaN_3.

[b]Wash step = 3 washes with PBST (see c).

[c]Extraction buffer = PBST (phosphate-buffered saline with 0.5 per cent Tween 20) + 2 per cent polyvinyl pyrrolidone.

[d]Substrate buffer — 10 per cent diethanolamine adjusted to pH 9.8 with HCl.