The Use of Enzyme-Linked Immunosorbent Assay for Detection of Citrus Tristeza Virus

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ABSTRACT

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An enzyme-linked immunosorbent assay (ELISA) test was used to identify citrus tristeza virus (CTV) in extracts from citrus tissues. Alkaline phosphatase conjugates were prepared with partially purified γ-globulin from antiserum to purified CTV. Citrus tristeza virus was detected quickly in extracts of experimentally inoculated plants kept in various indoor facilities, and in extracts of infected samples collected from the field. The ELISA procedure was equally effective for detection of most common and seedling yellows isolates of CTV from Israel and from Florida. Isolates that produced only mild symptoms on lime (*Citrus aurantifolia*) 'Mexican' indicator seedlings could be detected by ELISA. The virus was detected in various phloem-containing tissues during warm and cold seasons, but was most readily detected from fruit pedicel bark. A test procedure that incorporated composite sampling and mechanical homogenization was developed to index large numbers of field trees.

Additional key words: closterovirus.

Citrus tristeza virus (CTV), an aphid-borne closterovirus, has been one of the most important citrus diseases for more than two decades, especially in areas where extensive plantings still exist on the CTV-sensitive sour orange (*Citrus aurantium* L.) rootstock. Large-scale indexing of field trees currently is practiced in the San Joaquin Valley of California (16) and in Israel (4,15) to detect and eradicate foci of infection and suppress further natural spread of the virus. The CTV has not been transmitted mechanically to herbaceous plants, and large-scale indexing is conducted by graft inoculation of lime (*Citrus aurantifolia* [Christm.] Swingle 'Mexican') indicators, which are observed later for vein clearing and stem pitting (20). This procedure has several disadvantages: (i) it is laborious and expensive, (ii) it requires 1-6 mo until symptoms can be read, and (iii) certain mild CTV isolates do not produce clear symptoms. A method for rapid diagnosis, based on electron microscopy (EM) of partially purified CTV particles, has been developed (1) and was used until recently in Israel. The EM method enabled the rapid detection of new sources of CTV infection and, when combined with the lime test (4), it conserved indicator plants without adding much to detection time. The main limitations of the EM method are its high cost of operation and low sensitivity in diagnosing CTV in certain cultivars.

Bar-Jospeh et al (2,3) reported the purification of CTV from the leaves and bark of infected citrus seedlings, by polyethylene glycol (PEG) precipitation and density-gradient centrifugation in CsCl after fixation with formaldehyde. By use of a similar purification procedure, antisera to CTV have been produced (9,11). Antisera to unfixed purified CTV and to CTV coat protein degraded with sodium dodecyl sulfate (SDS) were used successfully in an SDS-immunodiffusion procedure to detect CTV in infected citrus (9–11). Garnsey et al (9) investigated many of the factors affecting application of serological procedures for field detection of CTV in Florida and showed that serological detection by SDS immunodiffusion was practical. However, tissues must be collected at the proper time if the samples are to have a CTV titer high enough for detection. Tsuchizaki et al (18) recently purified CTV from diseased fruits, and found that fluorescent-labeled antibodies to the Hassaku strain of CTV could be used to detect the virus within tissues of several citrus species.

Recently, the microplate enzyme-linked immunosorbent assay (ELISA) method, which is based on the double antibody sandwich technique used by Voller et al (19) and Clark and Adams (5), has been shown to be an inexpensive, reliable, and sensitive assay method for several plant viruses (6,13,17). This paper reports detection of various CTV isolates from Israel and Florida by ELISA, and application of this method for large-scale field detection of CTV.

MATERIALS AND METHODS

The experimental procedures for graft transmission of CTV and the isolates used extensively in these studies have been described previously (3). Table 1 summarizes the biological properties of five of the Israeli CTV isolates used in this study. The Florida isolates included mild quick decline, seedling yellows, and uncharacterized field isolates (9).

Greenhouse conditions in Florida essentially were as described by Garnsey et al (8). In Israel, Egyptian (=Mexican) lime seedlings were grown in a screenhouse in 5-L plastic pots that contained a mixture of sand, loam, and peat (1:2:1). For some tests, commercially grown 2-yr-old sweet orange (*C. sinensis*[L.] Osb.), grapefruit (*C. paradisi* Macfad. 'Marsh Seedless'), mandarin (*C. reticulata* Blanco), and lemon (*C. limon*[L.] Burm. f. 'Eureka') budded on sour orange rootstock were grown in 10-L plastic bags in a cool (22±2 C) plastic chamber.

Tissues were collected also from field-grown trees in Israel and Florida, and processed fresh or after being stored frozen at −20 C.

The CTV antiserum used in most tests was the antiserum prepared to formaldehyde-fixed whole virus (CTV-FW) and

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described by Gonsalves et al (11). This antiserum reacted to whole CTV in microprecipitin tests, but did not react to SDS-degraded virus in SDS-immunodiffusion tests.

The partially purified γ-globulin fraction was purified from CTV antiserum, as described by Clark and Adams (5), by use of DE 22 or DE 23 diethylaminoethyl cellulose (Whatman) for column chromatography. Conjugates of alkaline phosphatase Type VII (Sigma P-4502, Sigma Chemical Co., St. Louis, MO 63178 USA) and the purified γ-globulin were prepared by glutaraldehyde fixation (5). Tests were conducted in polystyrene microtiter plates (Dynatech M 29 ARE or Cooke 1223-24, Dynatech Laboratories, Inc., 900 Slaters Lane, Alexandria, VA 22314 USA) essentially as described by Clark and Adams (5). For large-scale field detection, IS-MRC-96 (Linbro Scientific Co., Hamden, CT 06514 USA) plates were used. Sodium azide was omitted from the washing solution in some tests, without apparent effect. In some tests, a modified procedure (Flegg and Clark, as cited in Clark et al[7]) was used, in which the enzyme-conjugated γ-globulin was added to the test sample about 30 min after the test sample was placed in the antibody-coated well, and the mixture was incubated overnight at 6 C. Several sources of substrate (*p*-nitrophenyl phosphate) were used, but the tablet form (Sigma 104-105) was most convenient.

Extracts were prepared by grinding, with a mortar and pestle, weighed amounts of tissue (0.2-0.4 g) in phosphate-buffered saline that contained 0.5 ml/L Tween-20 (PBS-Tween) and 20 g/L polyvinylpyrrolidone (Sigma) (PVP-40), or by homogenizing tissue in 5–7 ml of the same solution with an Ultra Turrax (Janke and Kunkel KG, Staufen, West Germany = SDT Tissumizer, Tekmar Co., Cincinnati, OH 45222) high-frequency dispersion homogenizer equipped with a T-18 shaft and generator. In the latter case, samples were homogenized in ice-cooled, 22-×100-mm U-bottom glass tubes. Extracts usually were transferred immediately to the test plates, but could be stored for up to 48 hr at 4 C.

Test results were scored visually as positive or negative in routine work and in large-scale surveys. Otherwise, the intensity of reaction was measured colorimetrically at 405 nm (5). Color differences equal to an OD_{405} change of 0.15-0.2 were apparent. With extracts from healthy tissues readings of 0.12 at 405 nm were usual. Samples with readings 0.15 above that of the healthy control were considered positive.

For detection of CTV by electron microscopy, fruit pedicel bark was diced on a glass slide in 0.02 M phosphate buffer, pH 7.2. A drop of the resulting extract was placed on a Formvar carbon-coated 74-μm (200-mesh) grid for 1-2 min, washed with 20-30 drops of buffer, and negatively stained with 1% uranyl acetate.

TABLE 1. Some biological properties of five citrus tristeza virus (CTV) isolates from Israel used in enzyme-linked immunosorbent assay (ELISA) tests

CTV isolate	Reaction on indicator plants[a]			Transmissibility by *Aphis gossypii* (Ref. 15)
	Lime	Sour orange	Lemon	
ST	++	−	−	low
VT	++	+	++	high
CT	++	++	−	low
HT	+++	−	−	high
AT	±	−	−	low

[a]Reaction rating symbols: − symptomless; ± very mild; + mild; ++ strong; +++ severe.

RESULTS

Initial tests were conducted with samples from Egyptian limes inoculated with three CTV isolates and samples from healthy (uninfected control) limes. Extracts of uninfected control plants, prepared in PBS-Tween + 2% PVP 40, either did not react or reacted very slightly in ELISA plates, whereas extracts of leaf or bark samples taken from plants inoculated with ST, HT, and VT tristeza isolates reacted positively (Table 2). Mechanical homogenization of infected tissue was equally as effective as grinding with a pestle and mortar. The T-18 shaft and generator unit was more effective for tough citrus tissue than the smaller T-10 unit.

Satisfactory results were obtained with the original reactant-loading procedure of Clark and Adams (5) and the modified procedure of Flegg and Clark (cited in [7]). However, we noted some apparent interference with conjugate binding in the modified procedure when extracts contained a large amount of host debris. For example, the reading for a 1/10 extract of CTV-infected bark was 0.18, whereas the reading for a 1/100 dilution from the same source was 0.86. This interference was reduced by removal of debris by low-speed centrifugation of extracts (5,000 *g* for 10 min).

Reaction with different tristeza isolates and other citrus viruses. Clear, positive ELISA reactions were obtained with all CTV isolates tested from Israel and Florida. These included, in addition to the ST, HT, and VT isolates, the AT, BT, CT, and MT isolates found in Israel. Isolate AT caused very mild symptoms in Egyptian lime indicator plants, yet gave strong, positive ELISA values (0.70–0.95 OD_{405}) when 1/10 extracts of bark tissue from Valencia sweet orange were tested. In Florida, 30 isolates (including the known mild, quick decline, and seedling yellows isolates used in SDS tests, and some uncharacterized field isolates) all gave positive results.

No reaction beyond the background level for extracts from healthy controls was obtained with extracts of young bark from greenhouse-grown citrus infected with exocortis, psorosis, xyloporosis or citrus variegation viruses (Table 3). No interference with the CTV reaction was apparent in extracts from plants doubly infected with CTV and exocortis virus or CTV and psorosis virus.

Detection of CTV in different hosts. The ELISA procedure worked well for all CTV hosts tested. We detected CTV readily in extracts from Shamouti, Valencia, Washington navel, Hamlin, Pineapple, and seedling sweet oranges, Marsh Seedless, Duncan, and Ruby Red grapefruit, Orlando and Minneola tangelos (*C. reticulata* × *C. paradisi*), Michal mandarin, Murcott "orange" (*C. reticulata* hybrid?), Etrog citron (*C. medica* L.), Mexican lime, Eureka lemon; and sour orange. Extracts from healthy trees of the different cultivars gave essentially the same low background readings. ELISA values (OD_{405}) for assays of leaf and bark of several citrus cultivars infected for 9 mo with the VT isolate and kept in a cool (22±2 C) plastic cabinet are given in Table 4.

Sensitivity, seasonal, and tissue effects. For comparison of the sensitivity of SDS immunodiffusion and ELISA for CTV

TABLE 2. Mean ELISA values (OD_{405}) of duplicate samples for extracts of leaves and bark from Egyptian limes infected with three citrus tristeza virus (CTV) isolates and from healthy (uninfected control) limes

Extract[a]	Preparation	CTV isolate			Healthy
		ST	HT	VT	
Leaves	Mortar & pestle	0.76[b,c] ± 0.06	1.05 ± 0.18	0.64 ± 0.14	0.08 ± 0.03
Bark	Mortar & pestle	0.92 ± 0.11	1.20 ± 0.07	0.78 ± 0.13	0.06 ± 0.01
Bark	Homogenizer	1.04 ± 0.08	1.18 ± 0.08	0.96 ± 0.16	0.08 ± 0.03

[a] Extracts prepared in PBS-Tween + 2% PVP-40 at tissue:buffer ratio of 1:20.
[b]Plate coated with (CTV-FW) γ-globulin, 1.5 μg/ml. Conjugate (CTV-FW) γ-globulin used at 40 μl/plate (in 12.5 ml of buffer).
[c] Mean ± standard deviation.

detection, we prepared and tested serial, twofold dilutions of comparable aliquots of extracts of bark tissue from young, greenhouse-grown sweet oranges. Extracts for SDS immunodiffusion were prepared in 0.5% SDS and those for ELISA in PBS-Tween + 2% PVP-40. The end points were at dilutions of 1/80 for SDS and 1/5120 for ELISA. In addition, we tested ELISA with extracts from Valencia orange seedlings infected with three CTV isolates and grown during July and August in a warm glasshouse (avg 27 C, max 40 C) at Bet Dagan, Israel. ELISA values of 0.72, 1.1, and 0.84 were obtained for the AT, BT, and VT isolates, respectively.

We also checked two stored samples collected from field trees in Florida during warm weather (35-37 C max) in late June. Other aliquots of these samples had not reacted or had reacted weakly in SDS-immunodiffusion tests at a 1/4 dilution. Both samples were strongly positive at 1/20 dilution by ELISA, and one was positive at a 1/100 dilution.

Bark extracts generally gave higher ELISA values than did comparable leaf extracts (Table 2). In a comparative test, fruit pedicel bark from CTV-infected, field-grown Shamouti trees gave a markedly higher ELISA value than did leaf or young shoot-bark tissue from the same source (Fig. 1). This finding confirmed previous observations made with EM on negatively stained extracts of Shamouti bark, leaf, and pedicel tissue during December 1976 and July 1977 (Bar-Joseph, *unpublished*). Particle counts per 74-μm (200-mesh) grid opening (avg of four samples, 10 grid openings for each) were 4.1 (0-12), 1.6 (0-7) and 18.2 (4-32) for bark, leaf, and pedicel, respectively. The ELISA readings for fruit pedicel bark also were higher than for albedo of the same mature Valencia fruit.

A simple device for precise identification of positive reactive wells in the ELISA plate. The microplates used in the ELISA tests are usually imprinted with numbers laterally and with letters vertically to identify each well. The necessity for cross referencing slows identification of the doublet-sample wells. A simple device was developed that was expected to permit more rapid identification. Numbers 1 to 48 were printed on the bottom of a flat-bottom microplate or on a transparent plate cover. Photocopies of the latter were made on transparent backgrounds. When the microplate under test is placed on the letter-printed base plate, the code number for each doublet is seen at the bottom of the sample well (Fig. 2). This device was used in large-scale testing for 3 mo. It also facilitated the programmed application of samples to wells that had been selected previously and enabled easy and precise visual recording of results.

Field indexing. The ELISA method was tested for detection of CTV in a grove of 50-yr-old Shamouti sweet orange trees at Miqwe Yisra'el, Israel. The high ELISA values obtained from fruit pedicel bark (Fig. 1) suggested that CTV could be detected in composite samples of this tissue from several trees, even if only one was infected. Such composite or multiple testing is advantageous for large-scale indexing of populations in which disease incidence is low.

TABLE 3. ELISA values (OD_{405}) for extract of young bark and leaf midrib tissue from glasshouse-grown *Citrus* infected with different viruses and from healthy *Citrus* tested against citrus tristeza virus (CTV) antiserum

Sample[a]	Virus	Tissue	OD_{405}
T-4	CTV T-4	Sweet orange	0.71[b] ± .06
E-9	Exocortis	Citron	0.06 ± .02
P-2	Psorosis	Sweet orange	0.06 ± .01
X-2	Xyloporosis	Sweet orange	0.04 ± .02
CVV	Infectious variegation	Citron	0.06 ± .01
Healthy	none	Citron	0.07 ± .02

[a]Tissue to buffer (PBS-Tween + 2% PVP 40) ratio of 1:20.
[b]Plate coated with 2.0 μg/ml CTV-FW γ-globulin. Conjugated CTV-FW γ-globulin used at 25 μl/plate (in 12.5 ml buffer).
[c]Mean of four replications ± standard deviation.

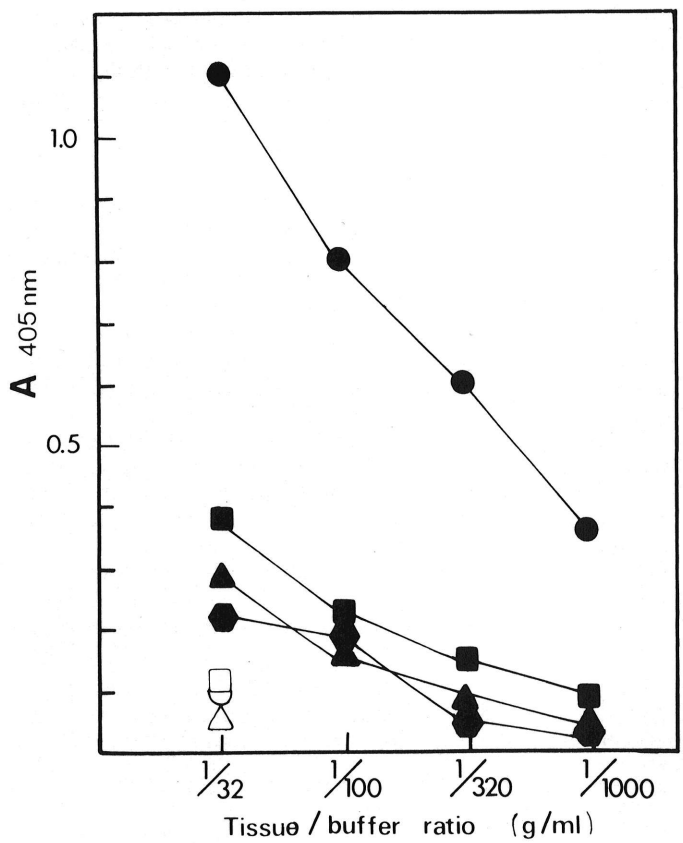

Fig. 1. Enzyme-linked immunosorbent assay (ELISA) values (OD_{405}) of extracts from Shamouti sweet orange tissues, prepared with different ratios of extracting buffer (PBS-Tween + 2% PVP 40) to tissue. Legend: pedicel = ●, young bark = ■, leaf = △, midvein = ⬢. Open symbols, healthy; solid symbols, infected.

TABLE 4. Mean ELISA values (OD_{405}) of duplicate samples for extracts of leaves and bark from *Citrus* cultivars infected for 9 mo with the VT isolate of citrus tristeza virus (CTV) and kept in a cool (22 ± 2 C) plastic cabinet, and from healthy citrus

Citrus species	Cultivar	Leaves[a]		Bark[a]	
		CTV	Healthy	CTV	Healthy
C. sinensis	Shamouti	0.85[b,c] ± 0.09	0.08 ± 0.06	1.25 ± 0.18	0.07 ± 0.03
	Valencia	0.58 ± 0.06	0.07 ± 0.01	1.15 ± 0.10	0.08 ± 0.04
	Washington	0.80 ± 0.06	0.07 ± 0.01	1.15 ± 0.20	0.08 ± 0.03
C. paradisi	Marsh Seedless	0.85 ± 0.04	0.06 ± 0.01	1.10 ± 0.15	0.07 ± 0.05
C. limon	Eureka	0.48 ± 0.03	0.04 ± 0.01	0.25 ± 0.04	0.07 ± 0.01
C. reticulata	Michal	0.72 ± 0.14	0.08 ± 0.01	0.94 ± 0.14	0.08 ± 0.07
C. aurantium	Local	0.32 ± 0.03	0.07 ± 0.01	0.60 ± 0.17	0.06 ± 0.03

[a]Extracts prepared in PBS-Tween + 2% PVP-40 at tissue:buffer ratio of 1:15.
[b]Plate-coated with (CTV-FW) γ-globulin 1.5 μg/ml. Conjugate (CTV-FW) γ-globulin used at 40 μl/plate (in 12.5 ml buffer).
[c]Mean ± standard deviation.

Three 5- to 10-cm-long twigs carrying fruit pedicels were collected from each tree to be tested for the composite sample, three halved fruit pedicels (0.25-0.35 g) from each of four or five trees were combined, and the extract was prepared in 7 ml of PBS-Tween + 2% PVP-40. The remaining pedicel halves were stored at 4 or −18 C; when a composite sample tested positive, the stored halves of the appropriate pedicels were tested separately. With this method, within 6 days we detected 33 infected but symptomless trees in a plot of 581 trees, in which a single tree had been observed to have symptoms. The infection of all trees that tested positive by ELISA was confirmed by EM of negatively stained extracts. In a separate test, 400 Shamouti trees were examined simultaneously by ELISA and by indexing on indicator plants. Sixteen trees were positive by ELISA and 15 of these gave positive vein-clearing reactions on the indicator seedling. Electron microscopy confirmed the infection of all the 16 trees found positive by ELISA.

DISCUSSION

In contrast with the detection of many other plant viruses, conventional serological techniques were introduced only recently for detection of CTV. Lack of specific antisera and the belief that CTV could be found only in low concentration in citrus hosts probably contributed to the neglect of this approach. However, continued work on purification (2,3,11) and the recent development of SDS immunodiffusion (9,10,11) and immunofluorescent antibody techniques (18) have shown that antisera to CTV can be produced and that certain phloem-rich tissues can, at times, contain serologically detectable quantities of virus.

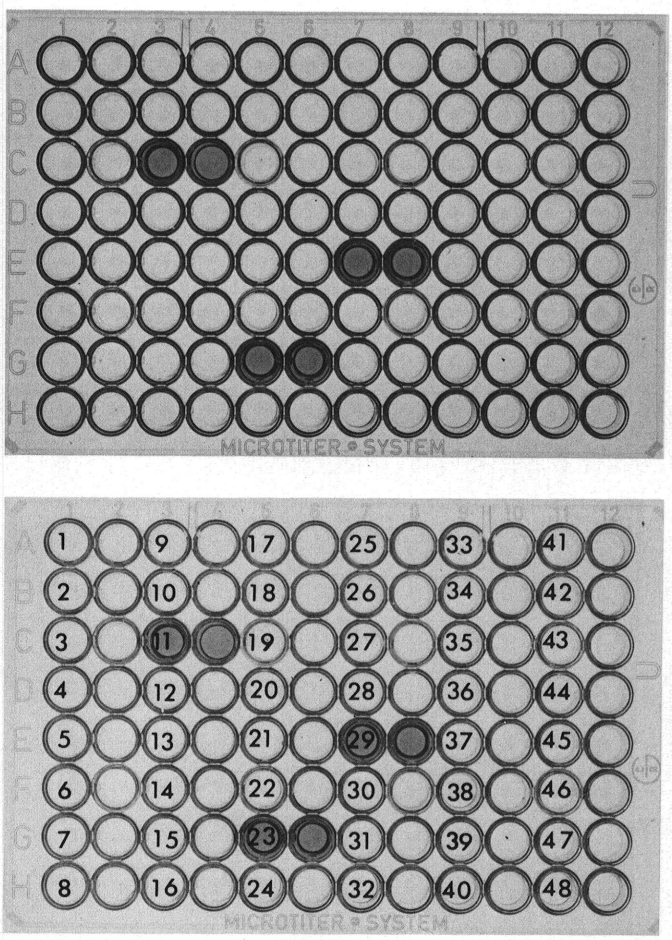

Fig. 2 Use of numbered template for rapid, accurate recording of reactions in ELISA microtiter plate. Plate containing three positive samples in doublet wells without template (upper) and with transparent coding template (lower). Sample color was enhanced for black-and-white photography.

Our results show that ELISA procedures can provide rapid, sensitive, and economical detection of CTV in crude extracts from citrus trees. Rapidity of identification is especially desirable for eradication attempts, because infected trees can now be identified and removed before additional natural spread occurs.

The ELISA procedure shares the advantages of SDS immunodiffusion (9) over previous indexing procedures. In addition, it offers greater sensitivity and more efficient use of antiserum. We calculate that several thousand samples can be indexed per milliliter of antiserum by ELISA versus about 200 by SDS immunodiffusion. The exact number varies with the efficiency of γ-globulin purification and enzyme conjugation and with the test conditions. As compared with tests involving immunofluorescence, ELISA is easier to perform, and its results can be measured quantitatively.

Although ELISA is sensitive, tissues with high CTV titer are still the best for testing, especially in composite samples. Observations made during EM (2) and immunodiffusion studies (9) are also relevant for ELISA. In addition, we have shown clearly the advantages of fruit pedicel as a test tissue. The higher content of CTV in pedicel bark, as compared with the bark of branches of the same age, might be due to a higher ratio of phloem in the pedicel bark or might indicate a more active virus synthesis there, possibly correlated with the phloem metabolic sink toward the fruit.

As was pointed out by Thresh et al (17), the main factor that limits large-scale ELISA tests is preparation of extracts, especially when the plant material is tough and difficult to grind. The use of the mechanical homogenizer resolved this problem, and both grinding and thorough between-sample washing can be done within 20-30 sec. The use of mechanical homogenization and bulk sampling has facilitated large-scale indexing of field samples for CTV by ELISA, and these procedures currently are used routinely in the CTV suppression program in Israel.

Antisera to the T-4 and ST (Bar-Joseph, *unpublished*) isolates of CTV are almost as effective for detection of various common CTV isolates as for those of the seedling yellows type (14). Mild isolates from Florida and the AT isolate, which causes a very mild, almost undetectable reaction on lime, were detected by ELISA. Koenig (13) reported recently that for several viruses, eg, the Andean potato latent virus, the specificity of ELISA was so great that conjugates prepared to one strain failed to detect other, serologically closely related strains. The avocado strain of tobacco mosaic virus (TMV) failed to react with the common type of antiserum to TMV in ELISA although it reacted positively to the common type in agar double-diffusion tests (Bar-Joseph, *unpublished*). The apparent broad-spectrum reaction of the CTV antiserum against diverse isolates of CTV is advantageous for field screening in which numerous CTV isolates may be present (12).

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