Detection of Citrus Tristeza Virus. II. Light and Electron Microscopy of Inclusions and Viral Particles*

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In a companion paper (Bar-Joseph *et al.*, 1980), two serological indexing procedures for citrus tristeza virus (CTV) are described which offer significant advantages over conventional indexing by graft inoculation of Mexican lime. In this paper, we report three microscopy procedures for detecting CTV which provide additional alternatives for very rapid CTV indexing.

Inclusions commonly form in many virus-infected plants, and can be used for diagnostic purposes (Christie and Edwardson, 1977; McWhorter, 1965). Inclusions, first reported in phloem tissues of CTV-infected citrus by Schneider (1959), can be detected easily with a simplified staining procedure devised by Christie and Edwardson (1977) for detecting plant virus inclusions in fresh plant tissues. The CTV inclusions apparently are composed largely of aggregated virus particles and were found consistently in different citrus varieties infected by mild and severe CTV isolates (Christie and Garnsey, manuscript in preparation).

Electron microscopy (EM) of negatively stained extracts has been used for indexing purposes (Bar-Joseph *et al.*, 1974) but the technique received limited application since tissue extracts had to be concentrated and few CTV particles were found in extracts from infected Valencia oranges. With new information on the relative titer of CTV in different tissues of different ages (Garnsey *et al.*, 1979; Bar-Joseph *et al.*, 1979), sampling was improved and the need for sample concentration was reduced (Gonsalves *et al.*, 1977).

A technique termed "serologically specific electron microscopy" (SSEM) has been developed for plant virus detection by Derrick (1973). With SSEM, virus particles become specifically attached to virus antibodies adsorbed to filmed electron microscope grids. The development of specific antisera to CTV (Gonsalves *et al.*, 1977, 1978) made SSEM possible for CTV.

The use of EM, SSEM and light microscopy for detecting CTV is described, and the special merits of these procedures for indexing are discussed.

METHODS AND MATERIALS

Light microscopy. Sections were cut freehand with a razor blade from fresh tissue or tissues were: mounted on brass specimen holders; frozen in Cryoform (Damon/IEC Div., Needham Hts., MA); and sectioned with a cryostat. In some cases, tissue was fixed in 3 per cent glutaraldehyde (in 0.1 M potassium phosphate buffer, pH 7.2) before sectioning. Sections were collected in water or in stain solution.

Sections were stained in 0.05 to 0.1 per cent azure A (Pfaltz and Bauer, Inc., Flushing, N.Y.) in 2-methoxyethanol (Eastman Kodak Co., Rochester, NY) and buffered with 0.2 M Na_2HPO_4 just prior to use (Christie and Edwardson, 1977). Stained sections were washed sequentially in 95% ethanol and 2-methoxy ethyl acetate (Eastman Kodak Co.) and mounted in Euparal (GBI [Labs] Ltd., Manchester, England). Staining and washing periods varied

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slightly according to section thickness and stain concentration but each step generally required several minutes.

Electron microscopy. Several methods were used to prepare negatively stained extracts for EM examination. In Florida, tissue was diced in several small drops of 1 to 2 per cent phosphotungstic acid (PTA) (Matheson Coleman & Bell PX1055) dissolved in 0.1 M potassium phosphate buffer and adjusted to pH 7.0 to 7.2. Bovine serum albumin, 0.05 per cent, (Sigma Chemical Co. A-4503) was added as a spreader. Small aliquots of stain solution were freeze dried and reconstituted as needed. The light green extract was placed on formvar-coated carbon-stabilized grids (75 x 300 or 200 mesh) for several minutes. The grids were washed several times with fresh stain, and the excess was wicked off with filter paper. For further detail see Gonsalves *et al.* (1977). Grids were scanned at an instrument magnification of 5,000 to 10,000 X (Phillips 201) through a 7 X binocular.

In Israel, extracts were prepared by dicing tissue in 0.02 M phosphate buffer, pH 7.2. The extract was placed on a grid as above, and the grid was rinsed with buffer after several minutes. The rinsed grid was then stained with 1 per cent uranyl acetate, and the excess was removed.

Serologically specific electron microscopy. Grids were prepared as described by Derrick and Brlansky (1976). The CTV antiserum used was antiserum to unfixed whole virus of the T-4 isolate (Gonsalves *et al.*, 1977). Extracts were prepared by grinding approximately 200 mg of young bark tissue with 1.8 ml of extraction buffer (0.05 M Tris-HC1, pH 7.2 containing 0.15 M NaC1 and 0.4 M sucrose). Electron microscope grids with carbon-Parlodion films were placed for 30 minutes on drops of serum diluted 1:500 with Tris buffer (0.05 M Tris-HC1, pH 7.2). The grids were then washed with Tris buffer and placed on drops of plant extract for 1 to 4 hours. Following the reaction period, the grids were washed with extraction buffer and distilled water, dipped for 30 seconds in 5×10^{-4} M uranyl acetate (in 95 per cent ethanol), and rinsed for 10 seconds in 95 per cent ethanol.

RESULTS

Light microscopy. Inclusions were found in young phloem tissues of all CTV-infected hosts examined. Inclusions appeared similar for all strains of CTV. In some cases, quantitative differences in inclusions between strains were noted but, since inclusions are not regularly distributed in a single plant or tissue, accurate quantitative comparisons are difficult.

Inclusions were observed in single, widely scattered cells and in clusters of cells. These clusters were more common in young than in mature tissue. Inclusions sometimes were observed in parenchyma cells immediately outside the phloem fiber. Although inclusions were somewhat sporadically distributed, examination of 5-10 sections 1-1.5 cm long from several tissue pieces usually was sufficient for reliable diagnosis. The magenta CTV inclusions were usually prominent, and accurate diagnosis could be made by a rapid scan of the sections at 100 X magnification (fig. 1). Inclusions were found in all phloem-containing tissues examined, including feeder roots, bark, flowers, fruit pedicel and "button" areas, and leaf petioles. Fruit pedicel bark and petioles of young, expanded leaves were the most reliable sources tested. Leaf petioles were generally selected for most routine tests because they were convenient to sample, were present on all plants, and were from phloem tissue of approximately known age. Accumulations of inclusions were often observed near the abscission zone between the petiole and the base of the leaf blade, and long sections of petioles were usually cut to include that area (fig. 1).

Occasionally, a few stained bodies were observed in cells of healthy tissue or tissue infected with other viruses, especially exocortis, which could be confused with CTV inclusions at low magnification. The paracrystalline

Fig. 1. Long section of leaf petiole near base of leaf blade. Darkly stained CTV inclusions can be seen singly and in several larger clusters. Magnification approximately 100X.

Fig. 2. Paracrystals in highly magnified (~ 1000X) CTV inclusion.

(fig. 2) or banded structures typical of CTV inclusions were not observed in plants not infected with CTV when observed at higher magnification (> 800 X). Large clusters of inclusions were also seen only in CTV-infected tissue.

To test inclusion detection as a CTV-indexing procedure, leaf samples were collected several times during the growing season from field-grown orange and grapefruit trees infected with several isolates of CTV (table 1). Pieces of two or three petioles were mounted on a single holder. Sections were cut, stained, and mounted unsorted on coded glass slides for examination. Normally, 8 to 12 sections were examined from each sample. All CTV infections were readily detected in spring-flush tissues collected in April and May (table 1), and all but two were detected in similar spring-flush tissue collected in August after 3 months of hot weather (daily maximum often >34°C). Inclusions were found readily in young, summer-flush tissue collected in August. Inclusions were found consistently in sections of fruit pedicel bark collected in May and August.

Slides prepared as described are semipermanent and have been re-examined successfully 6-9 months after preparation. Inclusions were similar in fresh and glutaraldehyde-fixed tissue. Young tissue sectioned more easily when fixed, but normal cell components stained more intensely and small inclusions were less obvious on rapid examination.

Electron microscopy of negatively stained extracts. In Florida, we have detected CTV particles readily in extracts prepared from bark of young growth flushes. Particles were detected in tissues from greenhouse- and field-grown plants of all major varieties including Valencia, navel, Parson Brown, and Pineapple sweet oranges; Temple orange; Marsh and Duncan grapefruits; Mexican lime; sour orange; and Eureka lemon. Particles of CTV were detected from plants infected with more than 20 isolates ranging from mild to very severe. Some isolates were checked only in a limited number of hosts. Bark from new shoots at points where leaves were nearly fully expanded, but still soft, yielded the greatest number of particles. Spring-flush tissues gave better results than summer-flush tissues. Numerous needlelike crystals, probably hesperidin, were present in extracts of some field-grown tissue prepared in PTA, and heavy concentrations of these crystals hampered rapid scanning of some grids. These crystals could be removed from extracts by low-speed centrifugation before staining.

Extracts from fruit pedicel bark in Israel have given good results for EM indexing, without extract concentration. Although ELISA (enzyme-linked immunosorbent assay) is now used for most CTV indexing in Israel (Bar-Joseph *et al.*, 1980), the EM procedure is still used to verify CTV infections in new locations located by ELISA tests.

Examination of preparations by EM remains an important tool for research purposes, such as virus purification in which treatment effects on the particle need to be observed (fig. 3).

Serologically specific electron microscopy. The amount of electron microscope time required per grid for CTV identification using SSEM was minimal. Due to the large number of virus particles and particle fragments and the good contrast provided by the uranyl acetate stain (fig. 4), a positive identification of CTV-infected trees could invariably be made immediately upon the first observation of the grid in the microscope. Virus particles were not found on grids prepared using normal serum and extracts from infected tissue (fig. 5) or on grids with extracts from plants free of CTV. In mixtures of plant tissue from known infected and healthy trees, CTV particles could be detected consistently in pooled samples representing one infected plant in 10 or even one in 100. Virus particle fragments were observed infrequently in samples representing one infected plant in 1,000. We were able to show by SSEM that CTV is present in commercial citrus

TABLE 1
DETECTION OF CITRUS TRISTEZA VIRUS (CTV) INCLUSIONS BY LIGHT MICROSCOPY OF SECTIONS FROM PETIOLES OF FIELD TREES, STAINED WITH AZURE A.

Host	CTV isolate*	No. trees	Sampling Date†			Lime test‡
			Apr. 1	May 20	Aug. 22	
Valencia	T-3	2	+	+	+	+
Valencia	T-4	2	+	+	+	+
Valencia	T-26	2	+	+	+	+
Valencia	field	1	+	+	+	+
Valencia	field	1	++	nt	nt	+
Valencia	none	3	-	-	-	-
Marsh	field	1	++	+	+	+
Marsh	none	1	-	-	-	-
Duncan	T-3	2	+	+	+	+
Duncan	T-4	2	++	+	+(-)	+
Duncan	T-26	2	+	+	+(-)	+
Duncan	none	1	-	-	-	-

*T-3 is a severe seedling yellows (SY) isolate; T-4 causes strong symptoms in Mexican lime, but mild symptoms in sweet orange on sour orange rootstock, and does not cause SY; T-26 causes mild or negligible symptoms in all hosts. Field isolates were uncharacterized.

†All samples from comparable shoots of the spring flush which were approximately 2, 9 and 21 weeks old, respectively; ++ = unusually strong symptoms; NT = not tested; +(-) = one tree positive, other negative.

‡Test trees indexed on Mexican lime seedlings.

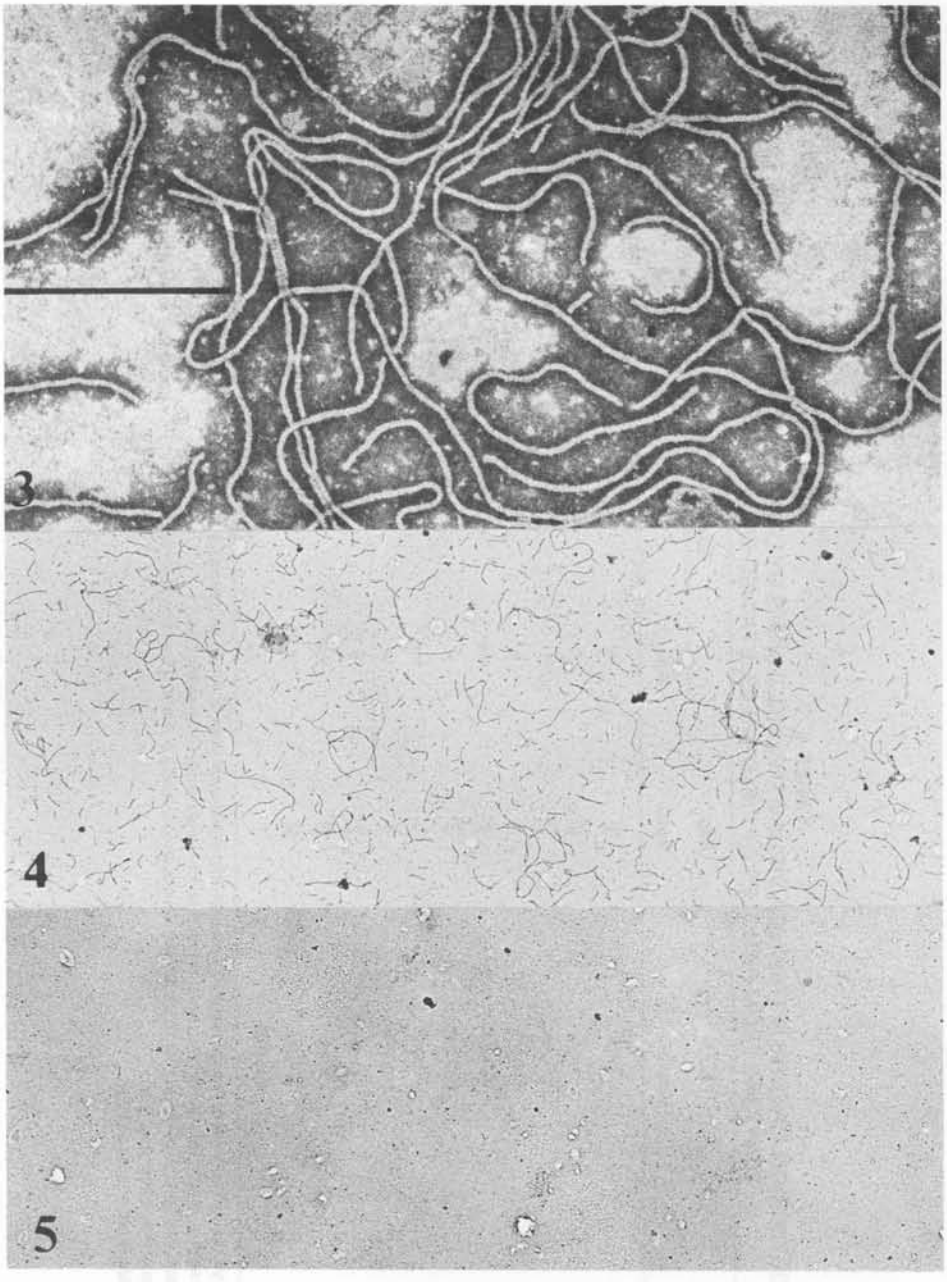

Fig. 3. Electron micrograph of partially purified CTV stained with potassium phosphotungstate. Note flexuous nature of particles and presence of broken fragments. Bar = 0.5μ.

Fig. 4. Serologically specific electron microscopic (SSEM) assay of an extract from young CTV-infected bark tissue. Numerous CTV particles and fragments are adsorbed to grid membrane coated with dilute CTV antiserum.

Fig. 5. SSEM assay of same extract used for Fig. 4 on grid membrane coated with dilute normal serum.

plantings in Louisiana, although most trees are on tolerant rootstocks.

DISCUSSION

The microscopy techniques described have several significant advantages over other procedures. Perhaps, most importantly, CTV infection can be detected within minutes, whereas even the relatively rapid serology procedures described in the companion paper normally require at least 24 hours. These microscopy procedures also provide physical information about the CTV particles and/or their location within the host. This may not be critical for practical, routine indexing but is important for many research applications.

The detection of stained CTV inclusions by light microscopy requires no antiserum and only simple laboratory equipment. Slides can even be prepared from freehand sections and examined in the field with a portable microscope. Similar inclusions were observed earlier by Schneider (1959) and were interpreted as viral inclusions; however, the ultrastructure of the inclusions was not known at that time, and inclusions have not been utilized for routine diagnosis of CTV.

The azure A staining method for CTV inclusion detection is simpler for routine purposes than the fluorescent antibody technique described by Tsuchizaki *et al.* (1978), although the latter would be sensitive to unaggregated CTV or small aggregates not visible by light microscopy.

We could not discriminate between strains as suggested by Sasaki *et al.* (1978).

The conventional EM negative staining procedure requires access to an electron microscope, but sample preparation is simple and rapid. The SSEM procedure requires access to an electron microscope and a source of antiserum, although only very small amounts of dilute antiserum are required (Derrick, 1973). The SSEM procedure takes slightly longer than the EM procedure, but it provides greater sensitivity.

We believe that CTV infections can be reliably detected by the microscopy techniques described here. As with other procedures, a certain degree of skill and experience is required. Some care in selection of samples may also be required for easy detection and optimum accuracy.

It may also be more difficult to prepare and examine large numbers of specimens by these procedures than to conduct the serological indexing procedures described in the companion paper.

No single procedure can be ideal for all applications, and it is important to realize that several options are available for CTV detection. These should be selected on specific needs and availability of appropriate materials, equipment, and skills. Two or more procedures can be combined to confirm results and provide additional information. Highly reliable diagnosis of CTV infection within 24 hours is now fully practical, even without elaborate test facilities.

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