Direct Tissue Blot Immunoassay (DTBIA) for Detection of Citrus Tristeza Virus (CTV)*

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ABSTRACT. A direct tissue blot immunoassay (DTBIA) procedure was tested for detection of citrus tristeza virus (CTV). Freshly cut stem, petiole or fruit pedicel tissue was carefully pressed to nitrocellulose membranes. The membranes were blocked by incubation in dilute bovine serum albumin and then incubated with unlabeled or biotinylated monoclonal or polyclonal antibodies. Antigen-bound biotinylated antibodies were detected by exposure to a streptavidin-alkaline phosphatase conjugate (APC) and antigen-bound unlabeled antibodies were detected by a goat anti-mouse or goat anti-rabbit IgG-APC. The substrate was NBT-BCIP. Localized areas of the tissue imprints of CTV-infected plants stained intensely and were easily recognized under 10X magnification. Location of CTV in phloem tissues was determined easily without sectioning or other cytological techniques. No comparable staining was observed in imprints of healthy tissue. Assays of 858 healthy and CTV-infected trees in Florida and 560 trees in Spain by ELISA and by DTBIA indicated similar rates of CTV infection. Strain differentiation was accomplished by making duplicate impressions on different test sheets and processing one with the strain-selective monoclonal CTV-MCA13 and the other with polyclonal antibodies, or a mixture of monoclonal antibodies which react to all isolates. DTBIA is rapid, requires little sample preparation, and tissue blots could be stored at room temperature at least 30 days prior to assay. Blotted membranes can be sent safely to another location for testing. DTBIA has been adapted for commercial diagnostic purposes.

Index words. CTV-MCA13, 3DF1, and 3CA5 monoclonal antibodies, biotinylated antibody, streptavidin, ELISA, immunoblotting.

The use of enzyme-labeled antibodies in serological assays has provided diagnostic probes with a high level of sensitivity, stability, low cost, and safety (7, 12). ELISA is the most commonly used diagnostic procedure for plant viruses which combines use of an enzyme-labeled antibody and binding of the antigen or antibody to a solid phase (the ELISA plate). A number of variations of ELISA have been developed for CTV, and sensitivity has been enhanced through use of secondary antibodies and biotin-streptavidin linkages (8). Immunoblot procedures are a form of ELISA where one of the reactants (usually the antigen) is bound to a membrane, such as nitrocellulose, which has protein binding properties, and is detected directly or indirectly with a labeled probe. An immunoblotting procedure for CTV was recently described by Rocha-Peña et al. (20, 21). Immunoblotting procedures are rapid, require only minimal equipment, and can have good sensitivity, but background color and lack of quantitative measurement of results can be a problem in some applications (12).

Lin et al. (15) recently described a variation of the immunoblot technique where the tissue sample is blotted directly to the membrane. They obtained good results with several virus and mycoplasmalike pathogens, including two which are phloem-limited. Application of this technique to tomato spotted wilt virus has also been reported (14). The direct tissue blotting assay (DTBIA), also described as an immunoprinting ELISA, requires no sample preparation or extraction and provides information on distribution and localization of the pathogen in host tissues. We felt that DTBIA should also work well with CTV because it is phloem-limited, the tissue area to observe for a virus-specific reaction is well defined and previous cytological studies have indicated that large amounts of virus are present in some cells of the phloem of CTV-infected plants (3, 9).

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Polyclonal antisera have been prepared to several CTV isolates and work well for general detection of CTV (1, 2, 8). Monoclonal antibodies have also been developed. Some are specific to well conserved epitopes and react to most isolates (22, 23). A strain-selective monoclonal, CTV-MCA13, has also been described (18). The large variety of serological detection methods which have been developed for CTV since the advent of high quality, virus-specific antibodies was recently reviewed (19).

This paper reports development and evaluation of DTBIA for CTV, which is sensitive, reliable, requires minimal equipment and sample preparation, and is adaptable for large scale testing. An abstract has been previously published (17).

METHODS AND MATERIALS

Tissue blotting technique. Tissue blots were prepared essentially as described by Lin et al.(15). Blots were made from stem pieces, leaf petioles, fruit pedicel, vascular cores, bark cut from larger stems, and roots. Vascular cores of fruit and bark samples were trimmed to an appropriate size for blotting. A smooth fresh cut was made with a razor blade and the cut surface was pressed gently and evenly to the membrane. In some cases, especially with succulent tissue, two blots were made sequentially from the same cut. Both ends of stem pieces were frequently blotted to increase testing of each sample. To compare different antibodies, or different treatments, blots were made from the same tissue piece on separate membranes. A fresh cut was made between each blot and only a thin slice of tissue was removed so that the blots would be as comparable as possible. Disposable gloves or tweezers were used when handling the membranes and in the process of blotting.

Blotted membranes were allowed to dry for 10 - 30 minutes. In most cases blots were processed within several hours, but in some cases blotted membranes were stored for longer periods, and a comparison was made of temperature, duration and desiccation effects on DTBIA.

Membranes and membrane processing. Bio-Rad Trans-Blot nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA 94547) were used for most studies. The 15-cm^2 membranes were cut to an appropriate size for the number of samples to be blotted. The membranes were usually premarked with an indexed grid of suitable size so the position of individual samples on a membrane could be recorded. Other membranes tested included Bio Blot nitrocellulose (Costar, Cambridge, MA 02140), Millipore 0.45-μm filter membranes (Millipore Corp., Bedford, MA 01730), Photogene nylon membrane (GIBCO BRL, Gaithersburg, MD 20877), and Zeta Probe membranes (Bio-Rad Laboratories, Hercules, CA 94547).

Blocking. After the membrane was imprinted with the tissue samples and dried, it was usually placed in a solution of 1% BSA in PBS and incubated for 1 hr at 25 C, or overnight at 4-6 C to block any remaining protein binding sites. Other blocking agents were used in specific tests as described below.

Incubation. Membranes were incubated in plastic dishes on a bench top shaker, in resealable plastic bags attached to a slowly rotating wheel, or in a Robbins Model 310 Hybridization Incubator (Robbins Scientific Corp., Sunnyvale, CA 94086). Incubation times were normally 1 to 2 hr at room temperature for the virus-specific antibody or secondary antibodies, and 1 hr for streptavidin conjugates.

Washing. Membranes were washed three times between steps in PBS-Tween (7) for 5 min under gentle agitation.

Immunological methods and antibody sources. *Immunoblots.* Four basic procedures were used and are diagramed in Fig. 1. The first was a direct method where the blotted membranes were exposed to CTV-specific antibodies conjugated to alkaline phosphatase (1, 7). The second procedure was an indirect method where the blot-

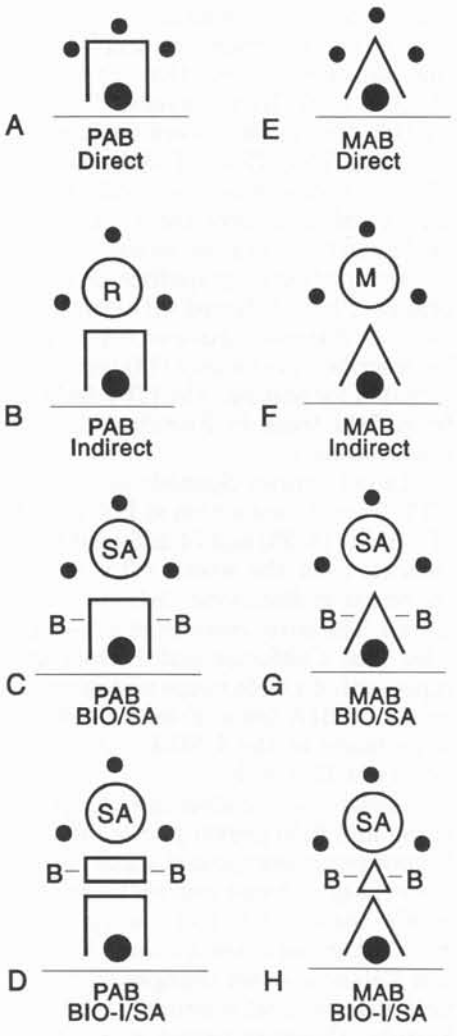

Fig. 1. Diagram of four protocols for direct tissue blot assays for citrus tristeza virus (CTV) used in this study are illustrated for both polyclonal (PAB) and monoclonal antibodies (MAB). A and E show the direct procedure where the virus-specific antibody conjugated with alkaline phosphatase (AP) ⊓ or △, is used directly to detect the antigen —. B and F show the indirect procedure where an unlabeled CTV-specific antibody is reacted to the antigen and then detected by an AP-conjugated secondary antibody, Ⓡ, Ⓜ. C and G show a biotin-streptavidin procedure (BIO/SA) where the antigen is reacted with a biotinylated CTV-specific antibody △, ⊓ and then detected by streptavidin conjugated to AP, Ⓢⓐ. D and H show an indirect biotin-streptavidin (BIO-I/SA) procedure where the antigen is reacted to unlabeled CTV-specific antibody, △, ⊓ which is in turn exposed to a biotinylated secondary antibody (goat anti-mouse or goat anti-rabbit) △, ▭ and then to the streptavidin-AP conjugate.

ted membrane was exposed first to unlabeled CTV-specific antibodies and then to commercially prepared alkaline phosphatase-labeled secondary antibodies (goat anti-rabbit for polyclonals and goat anti-mouse for monoclonals). In the third method, the blotted membranes were incubated with biotinylated CTV-specific antibodies (13) and then with a commercially prepared streptavidin-alkaline phosphatase conjugate. In the fourth variation, the blotted membranes were incubated sequentially with unlabeled CTV-specific antibodies, a commercially prepared biotinylated secondary antibody, and a commercially prepared streptavidin-alkaline phosphatase conjugate. The source of commercial alkaline phosphatase and biotinylated antibodies was Boehringer Mannheim Biochemicals, Indianapolis, IN 46250.

The CTV polyclonal antibody (PAB) 1052 to the Florida isolate T36 (18) was used for most tests. Several other polyclonals were used in limited tests. The 873, 894 and 879 PABs are to the Florida CTV isolate T4 as described previously (2). The 1051 and 1053 PABs are to the Florida CTV isolates T30 and T26, respectively, and have also been described (20). The 908 PAB was prepared to whole unfixed virus of the Florida CTV isolate T3 and has been used successfully for ELISA (Garnsey, unpublished).

Several different monoclonal antibodies (MABs) were used. The 3DF1 and 3CA5 MABs (23) are reactive to most isolates of CTV, and are specific to two separate and widely conserved epitopes on the CTV coat protein (11). A mixture of 3DF1 and 3CA5 was used in some cases to ensure detection of all isolates (5). The CTV-MCA13 is a MAB which reacts with severe sources of CTV, but does not react to mild isolates from Florida and some other countries (18). The 3E10 MAB is a broadly reactive MAB from Taiwan (22).

In most cases, purified IgG was used as a source of polyclonal antibody. Ascites and purified IgG were used as sources for MABs. Dilutions were made in PBS or in PBS which contained

1% BSA (8). Concentrations of IgG varied with the different sources and applications but, in general, dilutions for unlabeled CTV-specific antibodies ranged from 1/5,000 to 1/50,000 when made from ascites or from 1 mg/ml stock solutions of purified IgG. Commercially labeled secondary antibodies and streptavidin conjugates were used at the manufacturer's recommended dilution.

ELISA. Double antibody sandwich (DAS) and double antibody sandwich indirect (DAS-I) procedures (4, 8) were used in different studies. The 1052 PAB was used for coating and conjugate in DAS and as the coating antibody for DAS-I. Several monoclonals, including 3DF1, 3CA5, a mixture of 3DF1 and 3CA5, and CTV-MCA13 were used as intermediate antibodies. The labeled secondary antibody was as described above.

Substrates. In most tests, the substrate was a freshly prepared mixture of NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) (12). Stock solutions were made in N, N' dimethylformamide (DMF) at 75 and 50 mg/ml respectively. The substrate mixture was 0.33 mg/ml NBT and 0.175 mg/ml BCIP in substrate buffer (0.1 M Tris-HCl, 0.1 M NaCl, 5 mM MgCl, pH 9.5). In some tests substrate was prepared from Sigma Fast BCIP/NBT tablets (Sigma Chemical Co., St. Louis, MO) or from Vector Stain (Vector Laboratories, Inc., Burlingame, CA 94010). Incubation time in the substrate solution varied 5 to 20 min. The reaction was stopped by washing the membranes in distilled water or in 0.001 M EDTA prepared in 0.01 M Tris-HCl, pH 7.5.

Observation of blots. The processed membranes were placed in water in a petri dish or in a plastic bag with a small quantity of water and examined under a dissecting microscope at a 10 to 25X magnification. Dried membranes were stored in envelopes in the dark for future reference.

Virus isolates and tissue sources. A large number of CTV isolates were tested. The Florida isolate T36 (18, 20) was used in many routine tests to define optimum parameters and for testing differential reaction of CTV-MCA13 in DTBIA. Several different Florida mild isolates were also tested, including T30, T55-1 (T55a) and T69. These isolates cause very mild symptoms on Mexican lime and do not cause decline in trees grafted on sour orange or stem pitting in grapefruit or sweet orange. Plants infected with citrus tatter leaf virus and citrus exocortis viroid as described previously (18) were also included for testing. The CTV isolates from field trees in Florida were not characterized.

Twenty-three Spanish isolates of CTV from the collection at I.V.I.A. at Moncada (16, 23) and 74 different CTV isolates from the exotic CTV isolate collection at Beltsville, MD (10) were tested. The latter came from nine countries plus California and Hawaii and represented a wide range of strain severity. DTBIA tests of exotic isolates were made at the USDA quarantine facility at Beltsville.

Tissues were collected from glasshouse and field grown plants. Madam Vinous sweet orange and Mexican lime were the glasshouse sources most commonly tested, but blots were made from other varieties as well. Hamlin and Valencia sweet oranges were the field sources most commonly tested in Florida. Varieties tested in Spain included Washington Navel, Clementines and Nova. Where possible, tissue sources were stem or petiole tissue from a new or recent flush of growth. In field tests in Spain, blots were made of a composite sample which consisted of three twigs from each of five trees (6). A cut was made across a bundle of 15 twigs and the ends were blotted simultaneously to the membrane. Tissues were stored at 4-6 C if blots could not be done at the time of collection.

RESULTS

Initial tests were made by making blots of CTV-infected tissue and healthy citrus stem tissue on nitrocellulose membranes with procedures similar to

those described by Lin et al. (15). These blots were tested by the MAB-indirect and the MAB-BIO/SA methods (Fig. 1) with 3DF1 MAB as the CTV-specific antibody. Under 10X magnification, the outline of the stem imprint was clearly visible and intense areas of deep purple staining were present in the imprint area which corresponded to the phloem of CTV-infected stems (Fig. 2 C-D). These intensely stained areas were not present in blots of comparable healthy tissue (Fig. 2 B). When appropriate antibody concentrations and incubation times were used, the uninfected tissue imprint was pink, and the remaining membrane was white or a faint pink. The pink background was easily distinguished from the intensely stained areas in the phloem of CTV-infected tissue. Best results were obtained when the tissue was pressed to the membrane just firmly enough to leave a faint green image of the tissue without a strong imprint in the membrane. Nonspecific background increased when imprints were made too forcefully onto the paper.

Generally, a number of intensely stained areas were present and these sometimes coalesced to form a ring of staining corresponding to the phloem region. In most cases, positive blots were instantly and easily identified, even when only one or two small areas of intense staining were present. As with ELISA, inclusion of known healthy and infected controls with each sheet was essential to confirm that the reactant concentrations and test procedure were appropriate and to determine the normal background color to be expected. A set of standard controls for a series of blots was generated by blotting a single membrane repeatedly with CTV-infected and healthy tissues freshly cut for each impression. Portions of this membrane with paired CTV-infected and healthy tissue imprints were included with a series of test sheets as a reference standard.

Comparison of procedures. The MAB-indirect, MAB-BIO/SA, and the MAB-BIO-I/SA methods were similar and gave better signal to background ratio and a more sensitive assay than the direct method. The MAB-BIO/SA requires an additional step, but has the advantage that no preparation or labeling of the CTV-specific antibody is required. It has been used extensively for commercial applications during the past year with excellent results. The considerations which affect choice of method for DTBIA are essentially similar to those indicated for ELISA (8).

Membranes. All sources of nitrocellulose membranes tested gave acceptable results. Bio Blot nitrocellulose tore less than the other membranes tested. Differences were noted between different lots of membrane from the same source. Photogene nylon and Zeta Probe membranes also worked. The Zeta Probe, generally used for binding nucleic acids, showed a marked overall color development, but the CTV-specific stained areas could be clearly differentiated. Nitrocellulose membranes were white immediately after incubation in substrate, but frequently developed a general pink cast with time, especially if exposed to light. This color development varied from test to test and did not interfere with readings. Membranes stored in the dark could be read for up to 12 months.

Blocking agents. Blocking with 0.5 or 1% BSA gave satisfactory results and was used-routinely. Tests with Blotto (5% non-fat dry milk with 0.02% NaN_3 in PBS), Blotto plus 0.2% Tween, and 1% milk did not show marked differences in a MAB BIO-I/SA, and, in fact, the control without blocking ingredients produced a usable blot. Ovalbumin was unsuitable as a blocking agent.

Incubation schedules. A typical incubation schedule for DTBIA is indicated in Fig. 3. Considerable flexibility was found in incubation times and conditions as previously indicated (15). The blocking steps or one of the antibody incubations can be done overnight at 4-6 C rather than at room temperature. Two-hour incubations were used initially for the various antibody incubation steps, but later, shorter periods

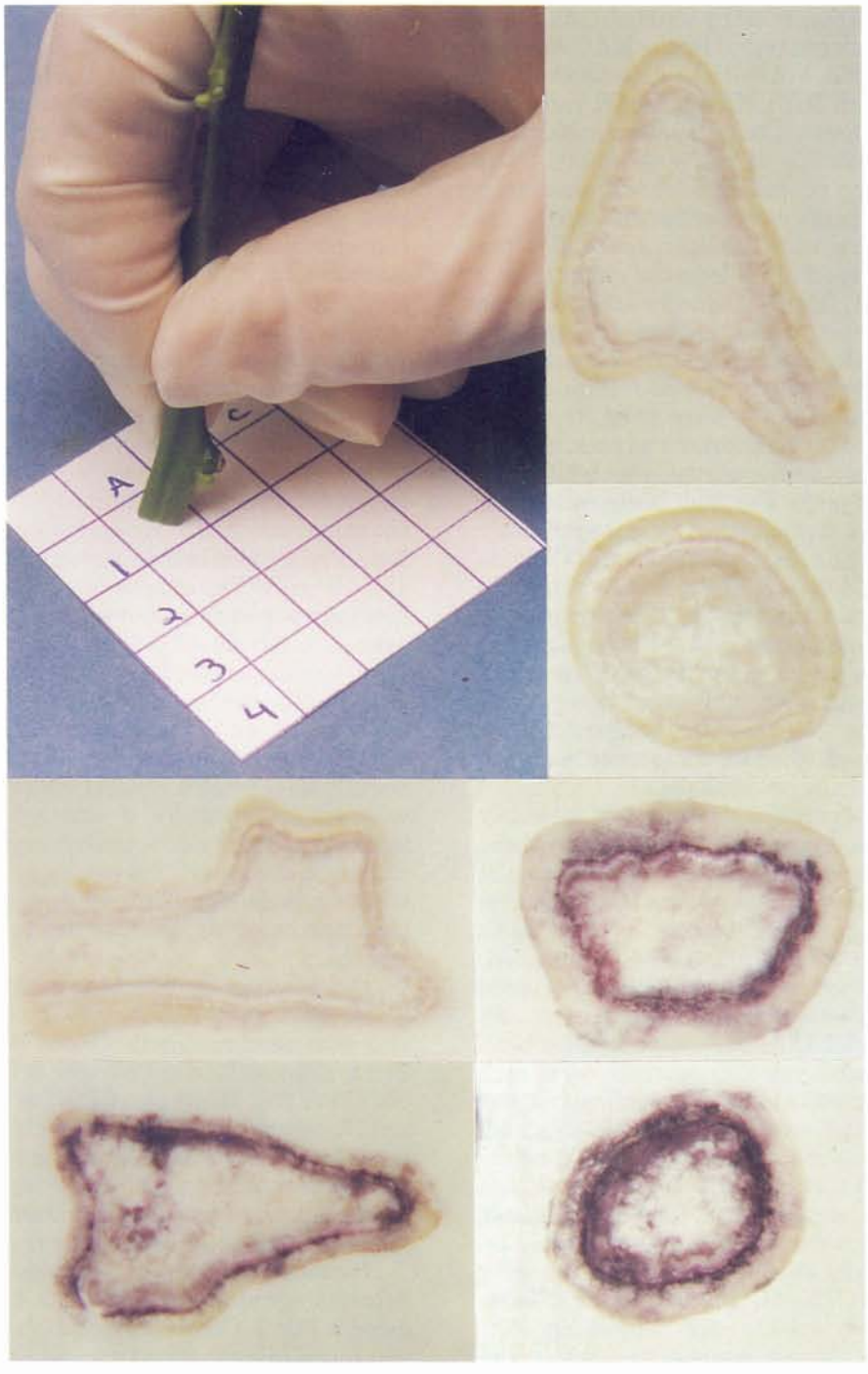

Fig. 2. Direct tissue blot immunoassay for citrus tristeza virus (CTV). A) Freshly cut surface of stem is blotted on a nitrocellulos membrane and exposed to CTV-specific antibodies which are directly or indirectly labeled with alkaline phosphatase and detected by exposure to a NBT-BCIP substrate (see Fig. 1 B). B) Sections of healthy citrus stem, C) sections of stem infected with mild isolate T30, and D) section of stem infected with the decline isolate T36. In B, C and D the upper section was tested against the strain-selective monoclonal antibody MCA13 (18) and the lower section against the 3DF1 monoclonal which reacts to mild and severe isolates (23). Sections are shown at approximately 15X magnification.

DIRECT TISSUE BLOT IMMUNOASSAY FOR CTV

1. BLOT THE FRESHLY CUT SUFRACE OF TISSUE ON MEMBRANE*

2. BLOCK MEMBRANE WITH 1% BOVINE SERUM ALBUMIN (BSA)

Wash Membrane 3x **

3. INCUBATE MEMBRANE 1-2 HR WITH ANTIGEN-SPECIFIC, BIOTIN-CONJUGATED ANTIBODY***

Wash Membrane 3x

4. INCUBATE MEMBRANE 1 HR WITH STREPTAVIDIN-ALKALINE PHOSPHATASE CONJUGATE***

Wash Membrane 3x

5. INCUBATE MEMBRANE WITH NBT-BCIP SUBSTRATE 5-20 MIN.

6. STOP REACTION BY WASHING IN 0.001M EDTA

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* Use nitrocellulose or other membrane suitable for protein blotting. Grids can be marked on paper with pencil or blue ball point pen.

** Use PBS-Tween for washing and agitate gently for 5 min.

*** For indirect assay use unlabeled virus-specific antibody in step 3 and secondary antibodies conjugated to alkaline phosphatase in step 4 (goat) anti-mouse for monoclonals and goat anti-rabbit for polyclonals).

Fig. 3. Outline of basic direct tissue blot immunoassay (DTBIA) for citrus tristeza virus (CTV) with BIO/SA method (Fig. 1 C, G).

were used and background color decreased. Incubations were done in glass cylinders of a hybridization oven, in flat plastic containers placed on a bench top shaker, and in sealed plastic bags attached to a slowly rotating wheel oriented at a 45-degree angle. Results were comparable, but changing solutions was easier with the bag or dish system, and the bag system required the least antibody solution.

Incubation time in the substrate was critical. Over incubation increased background color and did not increase the specific signal. Color development usually began within 5 min after addition of the substrate and the reaction was stopped 5-10 min later, or as soon as any color appeared in the membrane away from the imprint areas. The most convenient procedure was to observe the imprint of a known positive control and to stop the reaction when the desired reaction appeared. A strong background color soon after addition of the substrate indicated that concentration of the antibodies or enzyme conjugate was too high. As a general rule, we found that a concentration approximately one-half that used for ELISA

was optimum. Initial tests with several 10-fold dilutions around the anticipated optimum should be made and the greatest dilution which permits full color development should be selected.

Comparison of different polyclonal and monoclonal antibody sources. Several different polyclonal antisera and monoclonal antibodies were tested. Results of a comparative test of seven PABs in a PAB BIO-I/SA protocol are shown in Table 1. Antisera to five different isolates worked, and antisera to fixed whole virus, unfixed whole virus and to SDS-degraded coat protein (2) of a single isolate also worked. A nonspecific background reaction was observed with PAB 894 as observed previously in ELISA (2). It did not prevent detection of the CTV-specific reaction. Correspondingly, four different MABs (3DF1, 3CA5, CTV-MCA13, and 3E10) also all worked well in a MAB BIO-I/SA protocol.

The specificity of CTV-MCA13 for certain CTV isolates observed in ELISA (17) was also true for DTBIA. Isolates inducing decline and stunting in Florida which reacted to CTV-MCA13 in ELISA also gave a strong reaction in DTBIA. Isolates which did not cause decline and stunting did not react in ELISA or DTBIA using CTV-MCA13, but did react strongly to 3DF1 MAB and the 1052 PAB. Differentiation of isolates could be done by blotting each sample to two separate membranes and processing these with CTV-MCA13 and with a broadly reactive antibody (Fig. 2). Results for a comparative assay of 13 different isolates of CTV by ELISA and DTBIA using the broadly reactive 3DF1 MAB and the severe-strain-selective CTV-MCA13 MAB are shown in Table 2.

Isolate and host effects. DTBIA detected the wide variety of CTV isolates tested in Florida and Spain, and detected all 74 sources tested from the international CTV collection at Beltsville. Direct tissue blots were done successfully with numerous citrus hosts including Hamlin, Valencia, and navel sweet oranges, Marsh and Red Blush grapefruit, Mexican lime, alemow, *Citrus hystrix*, pummelo, and rough lemon. There was no evidence for host-associated nonspecific reactions with any of the varieties tested. As expected, negative tests were obtained with hosts that are immune to CTV such as trifoliate orange or Carrizo citrange. Blots of tissue infected with tatter leaf virus or citrus exocortis viroid were negative.

Tissue source. CTV infection was detected by DTBIA from different infected tissues, including stems and leaf petioles of different ages, fruit pedicel, the vascular core of mature fruit, bark

TABLE 1
REACTION OF DIFFERENT POLYCLONAL ANTIBODIES (PAB) TO CITRUS TRISTEZA VIRUS (CTV) IN DIRECT TISSUE BLOT IMMUNOASSAYS (DTBIA)

Antibody	Isolate	Inject antigen[y]	Reaction in DTBIA[z]				
			Healthy	T-30	T-55-1	T-68	BKGD[z]
873	T4	Whole F	0/2	2/2	2/2	2/2	low
879	T4	Whole UF	0/2	2/2	2/2	2/2	low
894	T4	Coat P	0/2	2/2	2/2	2/2	mod.
908	T3	Whole UF	0/2	2/2	2/2	2/2	low
1051	T30	Whole UF	0/2	2/2	2/2	2/2	low
1052	T36	Whole UF	0/2	2/2	2/2	2/2	low
1053	T26	Whole UF	0/2	2/2	2/2	2/2	low

[z]Number of imprints positive over number tested. Stem imprints were made on nitrocellulose membranes, and processed with PAB-B10-I/SA procedure (Fig. 1). Concentration of PAB was 1 μg/ml, the biotinylated goat anti-rabbit was used at 1/5000 and the streptavidin-alkaline phosphatase conjugate was used at 1/4000.
[y]Whole F = formalin-fixed purified virus, Whole UF = untreated whole virus, and Coat P. = denatured coat protein from purified virus.
[z] BKGD = Background color reaction in tissue.

TABLE 2
COMPARISON OF ELISA AND DIRECT TISSUE BLOT IMMUNOASSAY (DTBIA) FOR DIFFERENTIAL DETECTION OF MILD AND SEVERE ISOLATES OF CITRUS TRISTEZA VIRUS IN FLORIDA

	3DF1 Antibody		CTV-MCA13 Antibody		
Isolate	ELISA[z]	DTBIA[y]	ELISA	DTBIA	Bioassay[x]
T-30	+	+	–	–	M
T-36	+	+	+	+	S
T-55-1	+	+	–	–	M
T-66	+	+	+	+	S
FS-506	+	+	+	+	S
FS-537	+	+	–	–	M
FS-539	+	+	+	+	S
FS-542	+	+	–	–	M
FS-546	+	+	+	+	S
FS-549	+	+	+	+	ND
FS-550	+	+	+	+	S
FS-556	+	+	–	–	M
FS-557	+	+	–	–	M
Healthy	–	–	–	–	0

[z]ELISA was done by DAS-I method with PAB 1052 used as coating antibody.
[y]DTBIA was done by BIO-SA procedure in Fig. 1.
[x]M = no symptoms in infected sweet orange grafted on sour orange; S = stunting and/or decline effects in infected sweet orange grafted on sour orange; ND = not determined and 0 = no reaction.

patches cut from the trunk of large trees, and roots. In general, the best reactions were obtained from young flush tissue or from twigs directly below a young flush with good cambial activity. Good reactions were also obtained with bark from older limbs and main stem (trunk). The stained areas in trunk bark were often scattered and small, but were very distinct. Stem pieces 3-7 mm in diameter and leaf petioles were the easiest to blot and were used in most tests.

To test location effects within a plant, a chronically infected 2-yr-old navel orange was sampled at multiple sites. Stem pieces from at least four distinct growth flushes were tested. All 17 sites tested were positive. The strongest reactions were obtained in new flush tissue. The oldest stem pieces gave weaker but clearly positive reactions. In several experiments large numbers of twigs or leaves were taken from a single infected tree and all tested positive. In tests to compare membranes and other variables, a large number of blots from a single stem were made. A thin slice was removed between blots so that, in effect, multiple sites were tested along the stem. All 48 blots made from individual stems infected with each of four different isolates were positive.

Storage of blotted membranes prior to assay. To test storage effects on the blot assay, blots were made of healthy and T36-infected sweet orange. Each sample set consisted of two blots each of healthy tissue and three sources of T36-infected tissue which varied in reaction intensity. These were stored at 4 and 30 C at room humidity and over a desiccant. Assays were completed at 1, 15, 7 and 30 days after the initial blots were made. The assay system was Biotin/SA with MCA13. Membranes stored at 30 C gave a stronger reaction than those stored at 4 C. Membranes stored under normal room humidity were also slightly better than those stored over a desiccant. There were no obvious differences between the 1-day and the 15- or 30-day storage periods for the same treatment combination. Blots stored for 6 months in other tests have given good results.

Comparison of DTBIA and ELISA for field assays. In a large scale comparison of ELISA and DTBIA, shoots of new flush growth were collected from 858 vigorous 3-year-old Hamlin

and Valencia orange trees in a field planting near Clewiston, FL. These trees were part of an epidemiology experiment to study natural spread of CTV into a virus-free planting. The two previous annual surveys indicated a low, but increasing incidence of CTV. Comparative assays were made from each shoot collected. An 8-10 cm stem section was selected and each end was freshly cut and blotted to nitrocellulose. An extract from a 0.5 g sample of diced bark from the remaining stem piece was prepared and tested by DAS ELISA (1). Identical results were obtained with 852 trees by each method; 51 trees were infected, and 801 were virus-free. A discrepancy occurred with six trees. Re-assay from the original trees showed that four of the six trees had originally been misdiagnosed by ELISA and two had been misdiagnosed by DTBIA. In Spain, 560 trees were tested as five tree composites and the composite samples with infected trees were identified equally well by DTBIA and ELISA.

Comparison of sensitivity of DTBIA, ELISA, and immunoblotting. A limited test was made of tissue of different ages from sweet orange infected with mild and severe isolates of CTV. Blots were made from the different sources and extracts were made and tested by DAS-I ELISA and by immunoblotting at 1/50 and 1/500 dilutions. MAB and secondary antibody concentrations were the same for DAS-I and immunoblotting. Immunoblotting failed to detect infection at a 1/500 dilution of some extracts which were detected by ELISA. Even weak sources whose extracts were positive by ELISA only at a 1/50 dilution were detected by DTBIA.

DISCUSSION

DTBIA is a reliable and sensitive procedure for detection of CTV. Sensitivity, assay times, and costs compare favorably with other previously described procedures for serological detection of CTV. The assay makes efficient use of virus-specific antibodies, and by using an indirect or the BIO-I/SA method the assay can be done without any labeling or conjugation of antibodies. DTBIA has several advantages over conventional immunoblot procedures. It requires no preparation or extraction of the sample, eliminating the need for homogenizers, or for tubes and containers to store extracts prior to testing. It provides precise delivery of the sample to the membrane without need for manifolds or other loading devices. It can be easily tailored to varying numbers of samples by cutting the membrane to an appropriate size.

DTBIA provides direct information about distribution of the virus within the host. Even samples which give weak positive reactions by ELISA or by regular immunoblots usually give clear results with DTBIA, since only one infected cell group is needed to give a clear signal.

In general, procedures where the antigen is trapped to the solid phase are less sensitive for detection of viruses in plant extracts than procedures where the antigen is trapped by an antibody bound to the solid phase. In both ELISA and conventional immunoblots there is competitive binding of host proteins and antigens in the extract to the solid phase and when the virus titer is low there may be insufficient binding of the pathogen-specific antigen. In DTBIA there is direct binding of the virus from infected cells on the cut surface of the tissue without dilution by proteins from noninfected cells in other locations. Thus, strong signals are formed in localized areas which are easily detected. If the sample is ground and the extract is tested by ELISA or immunoblotting, the advantage of localization is lost and a weak signal is obtained.

DTBIA provides a very convenient method to ship a sample for testing from one location to another. No live tissue is present and possible introduction of other pests or pathogens is eliminated. The sample is stable on the membrane, refrigeration or protection of the sample is not required, and shipping costs are minimized. DTBIA is extremely convenient for field survey

work in remote sites. All an investigator needs to carry are several sheets of nitrocellulose membrane, a few razor blades and disposable gloves.

Because of the intense reaction in localized areas where CTV is concentrated in the phloem of infected plants, cross-reaction to host antigens by antibodies to host proteins in the serum is less of a problem than for ELISA or conventional immunoblot assays. The reaction to host proteins is more uniform and the background does not interfere with observation of the intense CTV-specific reaction sites in the blot. Several of the polyclonal antisera used successfully for DTBIA in this test give high background readings in ELISA.

The major disadvantage of DTBIA is that it is not convenient to precisely quantitate results. In many applications this is not important, but for those situations where quantitation is needed, ELISA is a preferable assay. DTBIA is also less convenient than ELISA or conventional immunoblot assays when multiple tests of a single sample by different antibodies are needed. For example, panel assays against several different monoclonals are easy to perform from a single extract in ELISA, but require preparation of separate sheets for each MAB in DTBIA.

Since only the plane of the cut surface is probed, DTBIA would be less likely to detect a poorly distributed pathogen than a procedure where a larger amount of tissue is tested. In our experience, this was not a problem with CTV and can be overcome by making multiple blots of the same sample.

We found that it takes more time to precisely log in sample information and to record results with DTBIA than it did with a computer-assisted ELISA system. Nitrocellulose membranes are also more fragile to handle than ELISA plates. Use of a commercial kit with premarked membranes and data sheets for sampling (Nokomis Corp., Altamonte Springs, FL) reduced blotting time and provided protection to the membranes.

In common with other assays, some experience is helpful to accurately read blots, especially where the reaction is weak. It is essential that appropriate healthy and infected controls be included in each membrane for reference. Some preliminary testing with known healthy and CTV-infected tissue should be done to define optimum dilutions and incubation periods for the antibodies and reagents to be used.

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