Direct Tissue Blotting for Detection of Tomato Spotted Wilt Virus in *Impatiens*

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ABSTRACT

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Direct tissue blotting for detection of tomato spotted wilt virus (TSWV) was compared with enzyme-linked immunosorbent assay (ELISA) and a dot-blot immunoassay (DBIA). TSWV was readily detected in tissue blots of infected *Nicotiana benthamiana* leaves. When biotinylated mouse monoclonal antibodies were used, DBIA was nearly eight times more sensitive than ELISA for detection of TSWV in extracts from infected *N. benthamiana* leaves. Leaf and stem samples from *Eustoma* (lisianthus) and several *Impatiens* plants showing viruslike symptoms tested positively by double-antibody sandwich ELISA and by direct tissue blottings. Asymptomatic leaves or stems from the same plants were negative in ELISA tests. Although tissue blots of asymptomatic leaves and stems were negative to the naked eye, positive reactions were observed when blots were examined at ×10–20 with a dissecting microscope.

Serological test methods widely used in plant virology include enzyme-linked immunosorbent assay (ELISA), dot-blot immunoassay (DBIA), and immune-specific electron microscopy (ISEM) (3,5, 13,16). Although ISEM is very sensitive, it is essentially a research procedure because electron microscopes are not often available for routine diagnosis.

ELISA and DBIA methods for plant virus detection depend on two assumptions. First, viral antigens or antibody molecules must attach to a solid-phase substrate where they retain antigenicity. Second, antibodies must be linked to an enzyme with both antibody- and enzyme-retaining activity. Experience has shown that both assumptions are true in assays of many plant viruses. Antibody molecules and plant virus antigens retain activity, although they attach to several different types of supports, including filter paper, nitrocellulose or nylon membranes, and plastic surfaces such as polyvinyl chloride or polystyrene (4,7,17). Antibodies have been linked to a variety of enzymes, such as β-galactosidase, urease, horseradish peroxidase, and alkaline phosphatase, yielding stable, highly reactive reagents (2,14,15).

Both ELISA and DBIA require extraction of viral antigens into a suitable buffer. Direct tissue blotting requires minimal preparation of tissue samples for detection of plant viruses and a mycoplasmalike organism (12). In this report, we compare tissue blotting with ELISA and DBIA for detection of tomato spotted wilt virus (TSWV) in infected plants.

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MATERIALS AND METHODS

Virus cultures. Isolates of TSWV were obtained from several sources (Table 1). All isolates, except the Mojave isolate from *Impatiens* cv. Mojave, were propagated in *Nicotiana benthamiana* Domin by inoculating healthy plants with an extract from leaves infected by TSWV prepared in a buffered solution containing 0.033 M KH_2PO_4, 0.067 M K_2HPO_4, and 0.01 M Na_2SO_3, pH 7.2 (1). Several *Impatiens* plants infected by TSWV and a lisianthus plant were obtained from Costa Rica. All TSWV isolates were received under a permit issued by the U.S. Department of Agriculture, Animal and Plant Health Inspection Service.

Immunological reagents. Mouse monoclonal antibodies to TSWV were produced at the Florist and Nursery Crops Laboratory in Beltsville, MD (10). IgG2a mouse monoclonal antibodies were purified from ascitic fluids by column chromatography through protein A-sepharose (8). Biotinylated monoclonal antibodies were used at 1 ng/ml antibody concentration (9). Avidin-alkaline phosphatase conjugate (Sigma Chemical Company, St. Louis, MO) was used at a 1:4,000 dilution. Rabbit polyclonal antisera and alkaline phosphatase-labeled or horseradish peroxidase-labeled rabbit antibodies to TSWV-L and a distinct isolate of TSWV (TSWV-I) (11) were obtained from Agdia, Inc. (Elkhart, IN) and were used at 1:1,000 dilutions. Alkaline phosphatase-labeled goat anti-mouse immunoglobulins and alkaline phosphatase-labeled goat anti-rabbit immunoglobulins (Kirkegaard and Perry Laboratory, Inc., Gaithersburg, MD) were reconstituted according to the manufacturer's direction and were used at 1:1,000 dilutions.

Preparation of tissue extracts. One part of plant tissue was triturated in 19 parts of the buffered inoculum solution

in a mortar with a pestle. Extracts were filtered through a layer of Miracloth (Calbiochem Corporation, San Diego, CA) before being transferred to test tubes. Further dilutions were made in a phosphate-buffered saline solution (PBS) (0.02 M K_2HPO_4, 0.15 M NaCl, pH 7.4).

Avidin-biotin mediated ELISA. Polyvinyl chloride plates were coated with 150 μl of rabbit polyclonal antibodies or mouse monoclonal antibodies (1 μg/ml in 0.1 M carbonate solution, pH 9.6) for 1–2 hr at room temperature. After one washing with PBS-Tween (PBS containing 0.05% Tween 20), plates were blocked with 1% bovine serum albumin (BSA) in PBS-Tween for 1 hr at room temperature. Various dilutions of extracts from leaf tissues infected by TSWV and healthy control leaf tissues were then incubated in triplicates at room temperature for 2 hr or 4 C overnight. After three washings for 3 min each with PBS-Tween, 150 μl of biotinylated mouse monoclonal antibodies was added to each well and incubated at room temperature for 2 hr before the next washing with PBS-Tween and incubation with avidin-alkaline phosphatase conjugate at room temperature for an additional 2 hr. After washing with PBS-Tween, substrate solution (*p*-nitrophenyl phosphate at 1 mg/ml in 10% diethanolamine, pH 9.8) was added to each well. Color intensities were measured in an MR700 Dynatech ELISA Reader (Dynatech Laboratories, Inc., Chantilly, VA).

Double-antibody sandwich (DAS) ELISA. The procedure used in DAS-ELISA was similar to that in avidin-biotin mediated ELISA except that after the addition of antigens, alkaline phosphatase-labeled rabbit anti-TSWV immunoglobulins were used to detect the virus antigens. For horseradish peroxidase-labeled rabbit anti-TSWV immunoglobulins, *o*-phenylenediamine was used as a substrate.

Sample application in DBIA. Nitrocellulose (NC) membranes, 0.45-μm pore size (Schleicher & Schuell, Inc., Keene, NH), were used in DBIA. Samples (50 μl) were applied with the aid of a minifold apparatus (Scheicher & Schuell, Inc.) under vaccuum to NC membranes that had been previously wetted. Each well was washed with 100 μl of PBS before removing the NC membrane from the apparatus.

Tissue blotting on NC membranes. Sections were cut from fresh tissues by hand with a new razor blade for each sample. Leaves were first rolled into a tight core before cutting. Tissues were held in one hand and cut with a steady motion with the other hand to obtain a single-plane cut surface. Tissue blots were made by pressing, with a firm but gentle force, the newly cut surface onto a 0.45-μm pore size NC membrane.

Detection of TSWV antigens on NC

Table 1. Sources of tomato spotted wilt virus and their propagation hosts

Isolate	Propagation host	Source
Impatiens	*Nicotiana benthamiana*	USDA[a], Beltsville, H. T. Hsu
Lettuce	*N. benthamiana*	Maui, Hawaii, H. T. Hsu
Mojave	*Impatiens* cv. Mojave	USDA, Beltsville, R. H. Lawson
Tomato-Can	*N. benthamiana*	Agriculture Canada[b], Ontario, J. A. Matteoni
Tomato-MD	*N. benthamiana*	MDA[c], Annapolis, M. Putnum

[a] U.S. Department of Agriculture, Beltsville Agricultural Research Center, Beltsville, MD.
[b] Research Station, Vineland Station.
[c] Maryland Department of Agriculture.

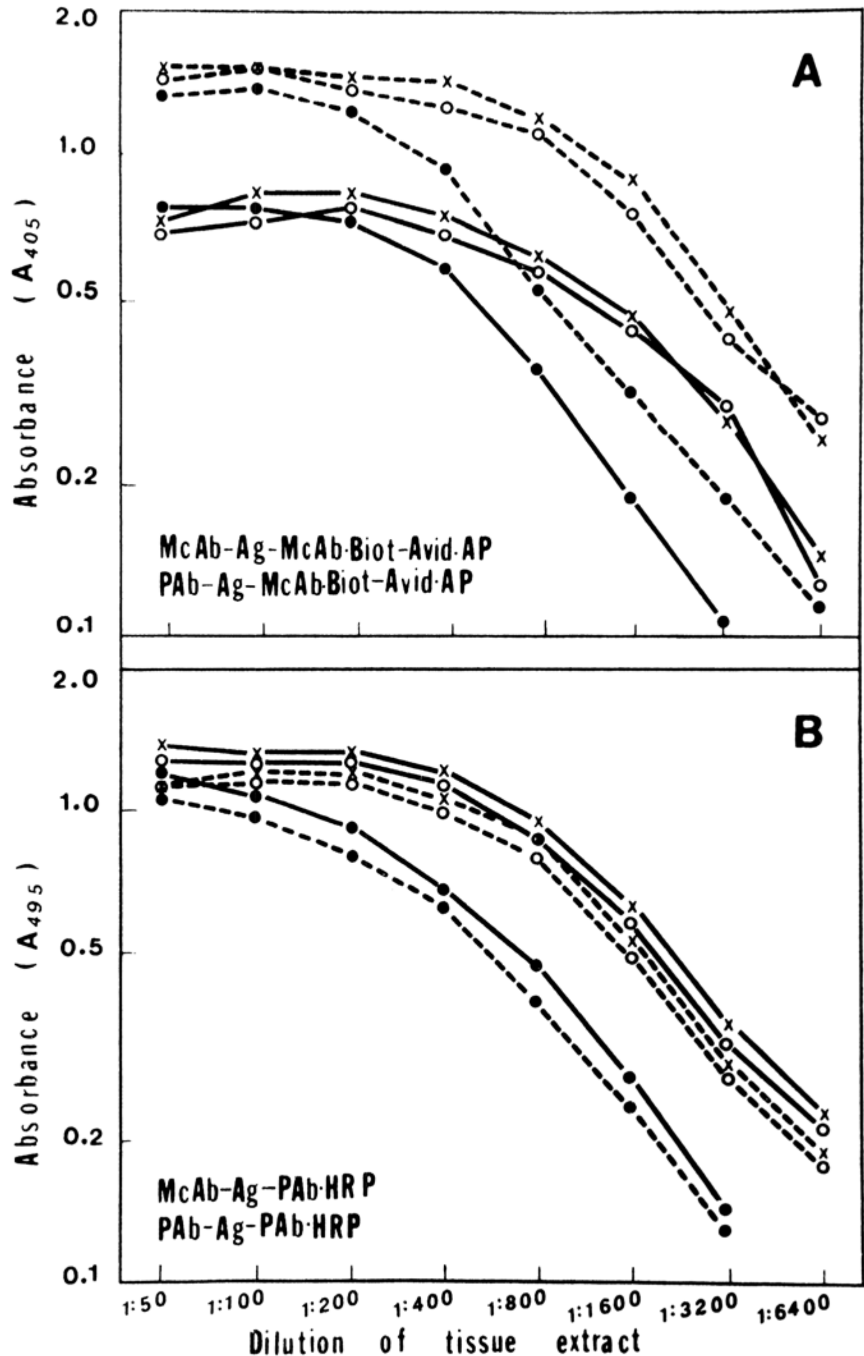

Fig. 1. ELISA detection of tomato spotted wilt virus (TSWV) antigens in extracts of infected *N. benthamiana* leaves. **(A)** Biotinylated mouse monoclonal antibodies and avidin-alkaline phosphatase conjugate or **(B)** horseradish peroxidase-labeled rabbit anti-TSWV immunoglobulins were used for detection of TSWV. X = Lettuce isolate, ○ = tomato-MD isolate, and ● = tomato-Can isolate. TSWV antigens were trapped by either mouse monoclonal antibodies (dotted lines) or rabbit polyclonal antibodies (solid lines).

membranes. Dot blots or tissue blots were first immersed in PBS containing 1% BSA for 60 min with gentle shaking at room temperature, followed by a brief rinse in PBS-Tween. For direct immunological detection, the blots were incubated for 60 min at room temperature with alkaline phosphatase-labeled TSWV-specific antibodies diluted in PBS. The blots were then washed three times by gentle shaking in PBS-Tween, 10 min each time, before incubation in an enzyme substrate solution. In indirect immunological detection, NC membranes with dot blots or tissue blots were incubated with TSWV-specific primary antibodies (rabbit polyclonal antisera or mouse monoclonal antibodies) diluted in PBS at room temperature for 60 min. After three washings in PBS-Tween, the blots were reacted with enzyme-labeled species-specific secondary antibodies (goat anti-rabbit immunoglobulins for rabbit antisera or goat anti-mouse immunoglobulins for mouse antibodies) for an additional 60 min. Before the addition of substrate, the blots were washed again three times in PBS-Tween with gentle shaking. In tests where the avidin-biotin system was employed, the blots were incubated in TSWV-specific biotinylated antibodies, followed by reaction with avidin-alkaline phosphatase conjugates.

For color development, the blots were immersed in a solution containing 14 mg of nitroblue tetrazolium and 7 mg of 5-bromo-4-chloro-3-indolyl phosphate in 40 ml of substrate buffer of 0.1 M Tris, 0.1 M NaCl, and 5 mM $MgCl_2$, pH 9.5. A positive result was indicated by the development of a purple color on the blots. A negative reaction developed no color. The reactions were rated by direct observation of the membrane and, in some cases, with the aid of a dissecting microscope.

RESULTS

Trapping antibodies. The relative efficiencies of mouse monoclonal antibodies and rabbit polyclonal antibodies for trapping TSWV antigens were compared in two different ELISA formats. The absorbance values of monoclonal antibody-coated wells were about two times those of polyclonal antibody-coated wells in indirect ELISA with the use of biotinylated-mouse monoclonal antibodies with avidin-enzyme conjugates (Fig. 1A). In direct ELISA with enzyme-labeled TSWV-specific rabbit polyclonal antibodies, the absorbance values of monoclonal antibody-coated wells were slightly lower than those of polyclonal antibody-coated wells (Fig. 1B).

TSWV detection by DBIA and ELISA. The sensitivities of DBIA and ELISA for detection of TSWV antigens in infected leaf tissues were compared by determining dilution endpoints of the same extract of infected *N. benthamiana.* In DBIA, the use of biotinylated mouse monoclonal antibodies and avidin-enzyme conjugates improved the sensitivity by about four times when compared with the use of unlabeled mouse monoclonal antibodies and enzyme-labeled goat anti-mouse immunoglobulin conjugates. When biotinylated mouse monoclonal antibodies and avidin-alkaline phosphate conjugates in mouse monoclonal antibody-coated ELISA plates were used, the sensitivity for detection of TSWV antigens in infected leaf tissue was only about one-eighth of that of DBIA with the same biotinylated antibody and avidin-enzyme conjugate preparations as detecting reagents.

TSWV detection by tissue blotting. TSWV was readily detected in tissue blots from infected leaves and stems (Fig. 2). The presence of TSWV antigens in blots of infected tissues is evidenced by the development of intense purple color (Fig. 2A,B). When primary antibodies were omitted from the reaction mixture, tissue blots from infected plants did not develop purple color (Fig. 2C,D). The healthy control leaf and stem blots did not develop purple color, but leaf blots retained the green color of chlorophyll (Fig. 2E,F). TSWV antigens were detected by a rabbit polyclonal antiserum or mouse monoclonal antibodies prepared to TSWV-L in tissue blots from *N. benthamiana* infected with lettuce, tomato-MD, or tomato-Can isolates. TSWV-I rabbit polyclonal antisera was used to detect virus antigens in tissue blots of *N. benthamiana* leaves infected with an *Impatiens* isolate of TSWV. Detection of TSWV from infected Mojave was unsuccessful because of a high concentration of colored pigments in the tissue blots.

Comparison of ELISA and tissue blottings for TSWV detection. One lisianthus plant and samples of symptomatic and asymptomatic leaves and stems from several *Impatiens* plants with suspected TSWV infections were obtained from Costa Rica and tested by both ELISA and direct tissue blottings. All samples bearing viruslike and necrotic symptoms tested positively at 1:100 dilutions by DAS-ELISA and by tissue blottings with TSWV-I rabbit polyclonal antibodies; extracts from asymptomatic leaves and stems tested negatively by DAS-ELISA, even at 1:20 dilution (Table 2). Although tissue blottings of asymptomatic leaves and stems were negative to the naked eye, careful examination of tissue blots at ×10–20 under a dissecting microscope revealed the presence of positive reactions (Table 2). All samples tested negatively by both ELISA and tissue blottings with monoclonal antibodies prepared to a lettuce isolate of TSWV.

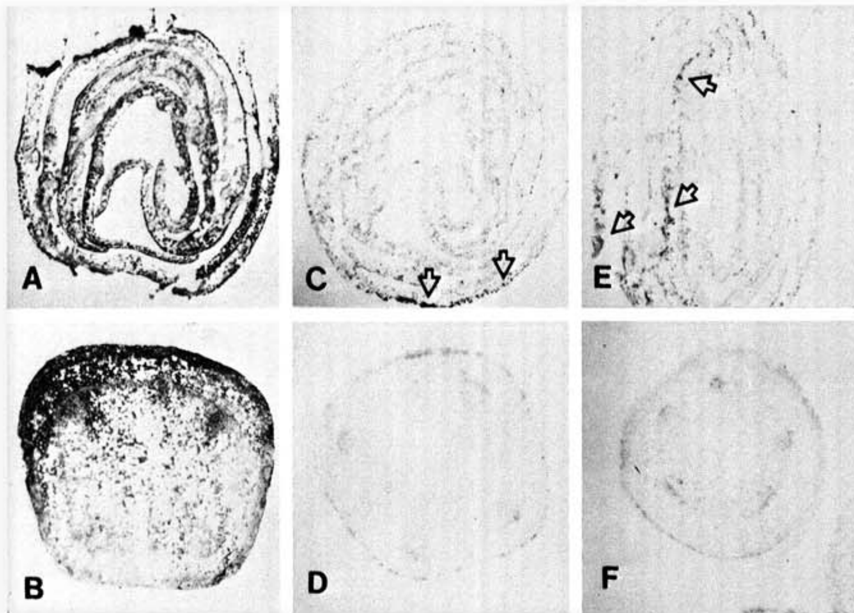

Fig. 2. Immunological detection of tomato spotted wilt virus (TSWV) in tissue blots of *Impatiens* **(A)** leaves and **(B)** stems of infected tissues where primary antibodies were used, **(C)** leaves and **(D)** stems of infected tissues where primary antibodies were not used, and **(E)** leaves and **(F)** stems of healthy control plants on nitrocellulose membranes. Blots were reacted in TSWV-specific rabbit antiserum (primary antibodies) and detected with alkaline phosphatase-labeled goat anti-rabbit immunoglobulins. TSWV was detected in infected tissues **(A and B)** but not in infected tissues **(C and D)** where primary antibodies were not used. Healthy controls **(E and F)** did not develop color reaction. Sporatic dark images (arrows) in **(C)** and **(E)** were attributable to the green color of chlorophyll.

DISCUSSION

Both ELISA and DBIA are routinely used for detection of plant viruses and diagnosis of infection. Our results show that higher dilution endpoints of TSWV antigens in extracts of infected leaf tissues were demonstrated by DBIA than by ELISA. This may be attributable to

Table 2. Detection of TSWV antigens by enzyme-linked immunosorbent assay and direct tissue blotting

Plant	Tissue/source	Blot examination[a]		ELISA[b]	
		Visual	Microscopical	1:20	1:100
67-1 (#11)	Symptomatic leaf	+	+	1.16	0.99
Impatiens	Asymptomatic leaf	−	+	0	0
	Necrotic stem	+	+	0.83	0.46
67-1 (#11d)	Symptomatic leaf	+	+	1.1	0.81
Impatiens	Asymptomatic leaf	−	+	0.02	0
	Necrotic stem	+	+	0.53	0.2
1088-3 (#22)	Symptomatic leaf	+	+	1.0	0.84
Impatiens	Asymptomatic leaf	−	+	0	0
1088-3 (#22a)	Symptomatic leaf	+	+	1.1	0.95
Impatiens	Asymptomatic leaf	−	+	0	0
WRG 163-C5 (#13)	Symptomatic leaf	−	−	0	0
Impatiens	Asymptomatic leaf	−	−	0	0
L13C491-11-1-4-5 Lisianthus	Symptomatic leaf	+	+	1.2	1.1
Positive control		+	+	1.2	...[c]
Negative control		−	−	0	0

[a] Indirect immunological method using rabbit antibodies specific to TSWV-I followed by alkaline phosphatase-labeled goat anti-rabbit immunoglobulins

[b] Double-antibody sandwich ELISA using alkaline phosphatase-labeled rabbit anti-TSWV-I immunoglobulins

[c] Not tested.

the deposition of more TSWV antigens on NC membranes than in polyvinyl chloride ELISA plates. Tests with several other viruses indicated that only about 25–37% of antigens applied to each well were actually trapped by specific antibodies coating the ELISA plates (H. T. Hsu and J. Aebig, *unpublished data*). In addition, only a limited small sample volume, usually no more than 200 μl, can be added to each well. For immunoassays of viruses on NC membranes, antigens are efficiently bound to solid support. In DBIA, TSWV antigens were detected only on the side of the membrane where the samples were applied. Because no antigens were detected on the opposite side, loss of antigens through filtrations had not occurred. It also indicates that the NC membrane had not been saturated with antigens. Complete adsorption of viral antigens in dilute sample solutions on NC membranes is possible. It was estimated that a protein-binding capacity of 80 $\mu l/cm^2$ was reported for NC membranes (6). Furthermore, a volume over 200 μl can be applied because virus antigens are collected and bound to NC matrix under vacuum.

Reactions produced by viral antigens can be observed with enzyme-labeled antibodies in tissue blots on NC membranes. In addition to TSWV, immunological analysis of plant virus antigens by direct tissue blottings has been described for several viruses in cucumovirus, luteovirus, potexvirus, and potyvirus (12). The presence of specific antigens is characterized by the development of a purple color reaction of the tissue blots on the membrane. Localization of specific antigens such as a luteovirus and a mycoplasmalike organism in specialized tissue (phloem) has also been clearly demonstrated (12). However, detection of viral antigens in infected tissue such as *Impatiens* cv. Mojave in current studies is hampered by a high concentration of colored pigments in tissue blots. Development of purple color attributable to the reaction of viral antigens is masked by the presence of anthocyanin.

Establishment of the dilution endpoint of the virus in infected tissues by direct tissue blotting on NC substrate is not possible. Positive reactions that escape examination by the naked eye can be identified under a dissecting microscope. This further increases the usefulness of tissue blotting for virus detection. Tissues that are positive by microscopic observation may contain a small portion of infected cells and an uneven distribution of the viral antigen. These tissues may give only a weak signal or may be undetected by ELISA.

Detection of TSWV antigens from infected plants by direct tissue blotting demonstrates the basic value of immunological detection of plant virus antigens with the methods described here. In addition to the advantages of specificity, sensitivity, reliability, and rapidity that ELISA and DBIA offer, the direct tissue blotting technique also provides simplicity and convenience for the assay of larger numbers of samples.

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