

Immunological Detection of Plant Viruses and a Mycoplasma-like Organism by Direct Tissue Blotting on Nitrocellulose Membranes

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ABSTRACT

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A technique of tissue blotting on nitrocellulose membranes was described for detection of plant viruses and a mycoplasma-like organism (MLO) in infected plants. Tissue blots were made by pressing, with a firm and gentle force, the freshly cut tissue surface on nitrocellulose membranes. Antigens in tissue blots on nitrocellulose membranes were detected by enzyme-labeled immunological probes. In indirect immunological methods, the blots were reacted with antigen-specific primary antibodies and detected with enzyme-labeled species-specific secondary antibodies. Alternatively, the blots were reacted with antigen-specific biotinylated primary antibodies and detected with avidin-enzyme conjugates. In direct immunological methods, the blots were reacted and detected with enzyme-

labeled antigen-specific antibodies. The tissue blotting technique was applied to detect viruses in cucumovirus, luteovirus, potyvirus, potyvirus, and tomato spotted wilt virus groups and an MLO. Passionfruit woodiness, papaya ringspot, sweet potato feathery mottle, bean yellow mosaic (BYMV), bamboo mosaic (BoMV), cymbidium mosaic, cucumber mosaic, and tomato spotted wilt viruses were detected in blots of infected leaf tissue cross sections. BYMV and BoMV were also detected in tissues of newly emerged shoots of gladiolus and bamboo, respectively. In addition, BYMV was detected in tissue blots made from dormant corms of gladiolus. In barley yellow dwarf virus and tomato big bud-MLO infected leaves, antigens were localized in phloem cells of the vascular system.

Additional keywords: antigen localization, disease indexing method, immunohistochemical detection, serological diagnosis.

Rapid and accurate detection of the causal agents of diseases is essential for plant health certification. Immunological procedures are commonly utilized in pathogen detection. The specificity, rapidity, and reliability of various immunological methods have been reviewed (17). Improved detection procedures are to suit specific applications in diagnosis of plant diseases caused by various pathogens.

Immunological detection of plant viruses requires either preparation of tissue specimen sections (15) or extraction of virus antigens from infected tissues (2,4). Sample preparation for tissue sectioning or tissue extraction is often cumbersome and time consuming. Methods that reduce the time required for sample preparation are desirable for routine indexing large numbers of samples and in studies of plant virus epidemiology. In this report we describe a simple and rapid nitrocellulose membrane system of plant tissue blotting for detection of plant virus or mycoplasma-like organism (MLO) infections. Tissue printing on nitrocellulose paper has been utilized to study the development of soybean seed coats (1).

MATERIALS AND METHODS

Pathogens and host plants. Nine different viruses representing five different taxonomic plant virus groups and an MLO were

used in this study. Passionfruit woodiness potyvirus (PWV) (14); papaya ringspot potyvirus (PRV, a gift from S. D. Yeh, National Chung-Hsing University, Taichung); bamboo mosaic potyvirus (BoMV, Y. H. Hsu, *unpublished*); cymbidium mosaic potyvirus (CyMV) (13); and RPV isolate of barley yellow dwarf luteovirus (BYDV, a gift from S. M. Gray, USDA-ARS, Ithaca) were from naturally infected hosts. Tomato spotted wilt virus (TSWV) was isolated from *Impatiens* and periwinkle (*Catharanthus roseus* (L.) G. Don) in Beltsville, and a lettuce isolate was collected from Maui, Hawaii, and maintained in *Nicotiana benthamiana* Domin. Bean yellow mosaic potyvirus (BYMV) was from naturally infected gladiolus (a gift from K. Kamo, USDA-ARS, Beltsville). Cucumber mosaic cucumovirus (CMV) (11) was maintained in *Nicotiana tabacum* L. 'Van-Hicks'. An isolate of sweet potato feathery mottle potyvirus (SPFMV) was graft transmitted from *Ipomoea batatas* (L.) Lam. and maintained in *Ipomoea setosa* Ker. An MLO-inducing big bud disease of tomato (TBB-MLO) was maintained in periwinkle (*C. roseus*) and was a gift from I. M. Lee (USDA-ARS, Beltsville).

Virus-specific antisera and monoclonal antibodies. Polyclonal antisera to BoMV, CMV, purified PWV capsid proteins, and PWV cylindrical inclusion proteins (PWV-CIP) were previously described (11,14). Rabbit antiserum to PRV was a gift from S. D. Yeh (19). They were used at 1:1,000 dilutions. Rabbit antiserum prepared to an *Impatiens* isolate of TSWV (TSWV-I) and alkaline phosphatase-labeled anti-TSWV-I rabbit polyclonal antibodies was obtained from Agdia, Inc. (Elkhart, IN) and used at a 1:1,000 dilution. Ascitic fluids containing monoclonal antibodies to BYDV-RPV (RPV-1) (6), tomato big bud (TBB)-MLO (10B3G11)

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(9), SPFMV (7H8, H. T. Hsu, unpublished) or CyMV (3C8H10, H. T. Hsu, unpublished) were used at 1:5,000 dilutions. Cultured fluids containing mouse monoclonal antibodies PTY1, 14, 30, and 36 (12) were used at 1:10 dilutions for detection of BYMV. Alkaline phosphatase-labeled goat anti-rabbit immunoglobulin antibodies (Sigma Chemical Company, St. Louis, MO; Promega Corp., Madison, WI; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) or alkaline phosphatase-labeled goat anti-mouse immunoglobulin antibodies (Kirkegaard and Perry Laboratories, Inc.) were used at manufacturer's suggested dilutions.

Antibody purification and biotinylation. IgG2a mouse monoclonal antibodies were produced by a hybridoma cell line (8C4) derived from a mouse immunized with TSWV-L (10). Immunoglobulins were purified from ascitic fluids of BALB/c mice by column chromatography through protein A-sepharose (5). For biotinylation, 100 μ l biotin solution (1 mg/ml *N*-hydroxy-succinimidobiotin (Sigma Chemical Company) in dimethyl sulfoxide) were added dropwise, while slowly stirring, to 1 ml of purified immunoglobulin solution (1 mg/ml in 0.2 M borate buffer, pH 8.5). After 10 min of continuous stirring, the solution was left at room temperature for 4 hr before dialysis against two changes of phosphate-buffered saline solution (PBS, 0.02 M K_2HPO_4 , 0.15 M NaCl, pH 7.4) at 4 C overnight. The biotinylated antibodies were stored in 0.1 ml of aliquots at -20 C. The biotinylated 8C4 TSWV monoclonal antibodies were diluted to 1 ng/ml antibody concentration for use. Avidin-alkaline phosphatase conjugate (Sigma Chemical Company) was used at a 1:4,000 dilution according to the manufacturer's instruction.

Tissue preparation and tissue blotting. Sections were cut from fresh tissues by hand using 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 obtained by pressing, with a firm but gentle force, the newly cut surface onto 0.45 μ m pore size nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA; Schleicher & Schuell, Inc., Keene, NH). In tissues such as papaya leaves containing a high concentration of latex, the cut surface was first drained on tissue paper to remove excess exudate before blotting onto membranes.

Immunological detection of antigens on tissue blots. Tissue blots were first immersed in PBS containing 2% bovine serum albumin (BSA) for 60 min at room temperature. Indirect immunological procedures were used for detection of PWV, PRV, SPFMV, BYMV, BoMV, CyMV, CMV, BYDV, TSWV-L, and TBB-MLO. After blocking with 2% BSA, the nitrocellulose membranes were then incubated with antigen-specific primary antibodies diluted in PBS at room temperature for 2 hr or 4 C overnight. Following three successive washings in a PBS-Tween solution (PBS containing 0.05% Tween 20), 10 to 15 min each time, the membrane was incubated with alkaline phosphatase-labeled secondary antibodies (goat anti-rabbit immunoglobulins for rabbit polyclonal antisera or goat anti-mouse immunoglobulins for mouse monoclonal antibodies) for 2 hr at room temperature. A modified indirect immunological procedure using biotinylated monoclonal antibodies was also employed to detect TSWV-L antigens. The alkaline phosphatase-avidin conjugates were used in place of enzyme-labeled secondary antibodies.

A direct immunological procedure was used for detection of TSWV-I antigens. Tissue blots were first blocked with 2% BSA in PBS and then reacted with antigen-specific alkaline phosphatase-labeled antibodies.

Before incubation in the substrate solution for color development, the nitrocellulose membranes were again washed three times, 10 to 15 min each, in PBS-Tween and were then immersed in a substrate 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, 5 mM $MgCl_2$, pH 9.5. A positive result was indicated by the development of a purple color on the tissue blot. A negative reaction developed no color on the blot. The results were photographed using a dissecting scope with an optical magnification of 20 \times .

RESULTS

Potyvirus. Potyvirus (PWV, PRV, SPFMV, or BYMV) antigens were readily detected immunologically in infected leaf tissue blots on nitrocellulose membranes. PWV antigens were detected both in leaf and vine (stem) tissue blots of infected passionfruit (Fig. 1A and B). Using rabbit anti-PWV-CIP immunoglobulins, cylindrical inclusion antigens were detected in the tissue blots of infected leaves (Fig. 1C), but not in tissue blots of infected vines. Similar procedures revealed the presence of PRV or SPFMV antigens in tissue blots of leaves but not petioles of PRV-infected papaya or SPFMV-infected sweet potato or *I. setosa* (data not shown). BYMV antigens were detected by PTY 1 or a mixture of PTY 14, 30, and 36 in tissue blots made from dormant corms or newly emerged shoots of infected gladiolus (data not shown). No viral antigens were detected in blots of healthy control tissues (Fig. 1D and E).

Potexvirus. BoMV antigens could be easily detected immunologically in tissue blots of infected leaves (Fig. 2A), petioles, and newly emerged shoots and sheath blades (Fig. 2B), but not from healthy controls (Fig. 2C). High concentrations of BoMV antigens were distributed throughout cross sections of the leaf tissues of infected bamboo. In the newly emerged shoots, BoMV antigens localized in the older, outer shoot sheath. CyMV antigens in cross sections of infected *Cattleya* leaves were observed in tissue blots (Fig. 2D). CyMV antigens were detected in all five consecutive blots of a single cut surface of an infected *Cattleya* leaf (data not shown). Healthy tissue blots did not show presence of antigens (Fig. 2E).

Cucumovirus. CMV antigens were detected in both tissue blots of leaves and petioles (data not shown). Distribution of CMV antigens was observed in tissue blots of cross sections of infected leaves; less CMV antigen accumulated in mesophyll compared to epidermis. In petioles, CMV antigens were deposited mostly in epidermal layers of the petiole. No CMV was detected in both healthy leaves and petioles.

Luteovirus. Detection of BYDV-RPV antigens in tissue blots of a cut leaf section was not consistent. BYDV-RPV antigens were not detected in blots from every cross section. This is probably due to uneven distribution of BYDV antigens in phloem tissues of infected leaves. Antigen was localized primarily in the phloem of infected leaves (Fig. 3A). Tissue blots of leaves from healthy barley did not show antigen-specific labels (Fig. 3B).

Tomato spotted wilt virus. TSWV was readily detected in tissue blots from infected leaves (Fig. 4). Both the periwinkle isolate and lettuce isolate of TSWV could be identified from tissue blots of infected periwinkle or *N. benthamiana*, respectively, by biotinylated monoclonal antibodies prepared to TSWV-L (Fig. 4A). The TSWV-I isolate could be detected in tissue blots from infected *N. benthamiana* by either the indirect immunological method using TSWV-I rabbit polyclonal antibodies and enzyme-labeled goat anti-rabbit immunoglobulin antibodies or the direct method using TSWV-I specific enzyme-labeled rabbit antibodies (Fig. 4B). Monoclonal antibodies prepared for TSWV-L failed to detect TSWV-I, and the TSWV-I rabbit polyclonal antiserum did not detect TSWV-L or periwinkle isolates (data not shown). Detection of TSWV antigens from tissue blots of stem cuttings of infected plants were, however, not successful. Leaf blots of healthy plants did not show antigen-specific stainings (Fig. 4C).

Mycoplasma-like organism. MLO antigens were detected in tissue blots of infected leaves. Examination of immunologically stained tissue blots of periwinkle leaves revealed that TBB-MLO antigens were restricted only to midrib phloem cells and secondary veins of the leaves of TBB-MLO infected plants (Fig. 5A). Blots from healthy leaves gave a negative reaction (Fig. 5B).

DISCUSSION

Our studies show that the technique of tissue blotting on nitrocellulose membranes can be readily applied in detection of plant viruses including cucumovirus, luteovirus, potexvirus, potyvirus, and tomato spotted wilt virus groups, and an MLO. The

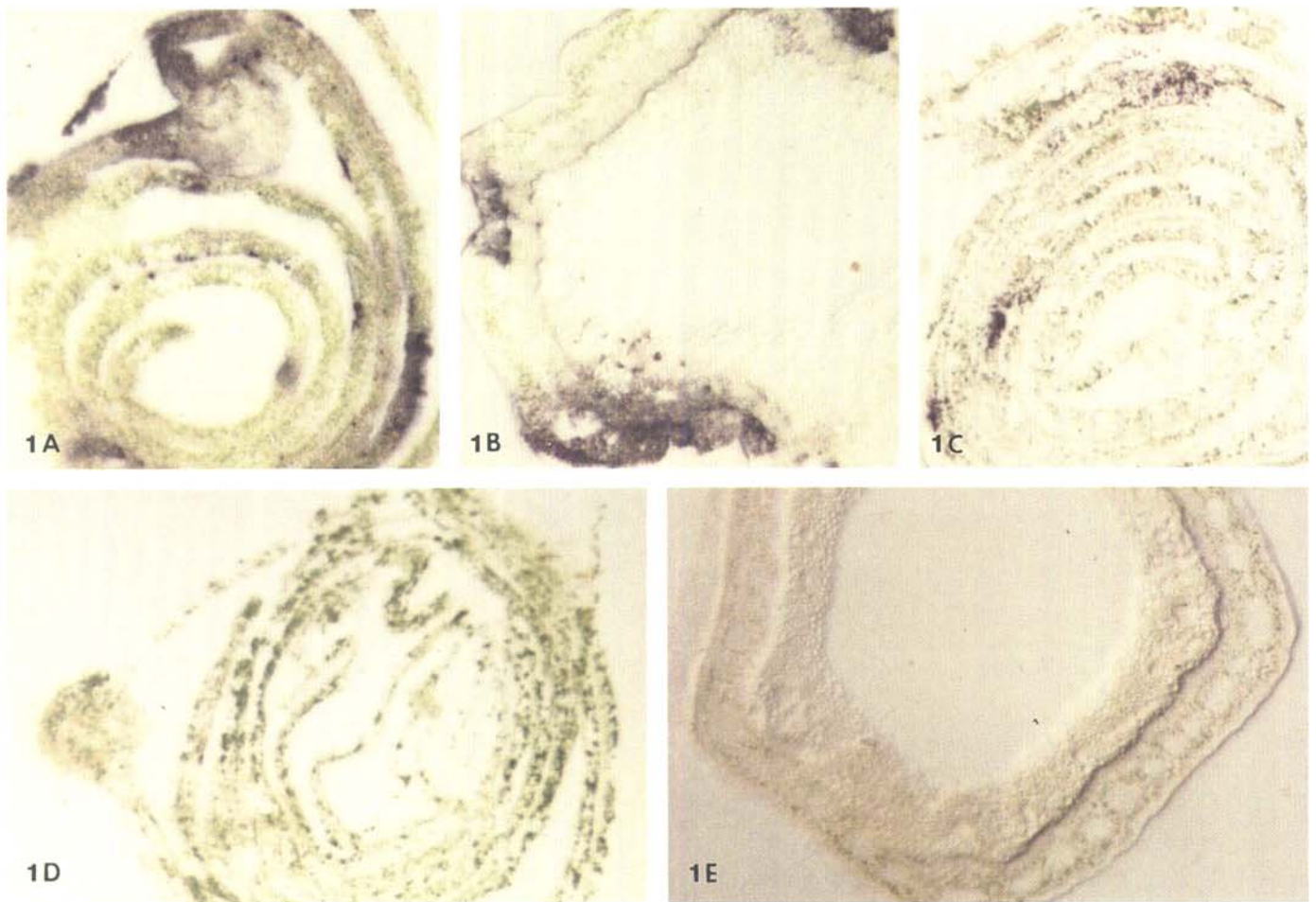


Fig. 1. Immunochromatogram detection of passionfruit woodiness potyvirus (PWV) in infected tissue blots on nitrocellulose membranes. The blots were reacted with PWV coat protein (PWV-CP) or cylindrical inclusion protein (PWV-CIP) rabbit polyclonal antibodies and detected with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin antibodies. **A**, PWV-CP antigens in blot of an infected passionfruit leaf; **B**, PWV-CP antigens in blot of an infected passionfruit vine; **C**, PWV-CIP antigens in blot of an infected passionfruit leaf; **D**, healthy passionfruit leaf; and **E**, healthy passionfruit vine.

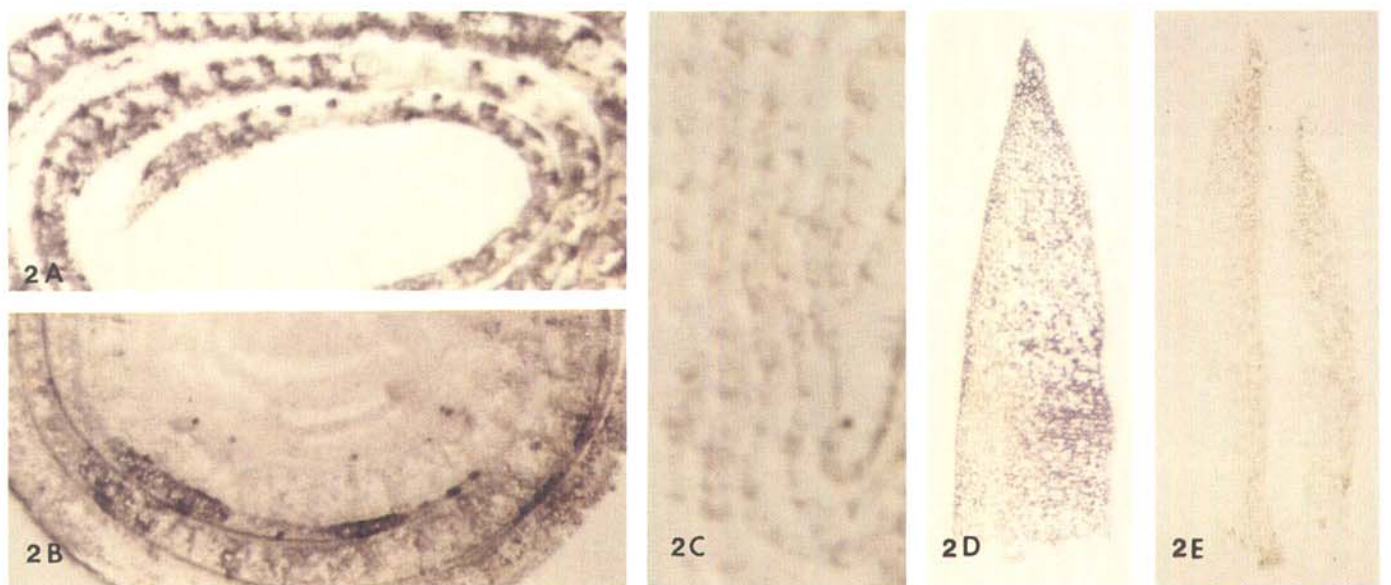


Fig. 2. Immunochromatogram detection of potexviruses in infected tissue blots on nitrocellulose membranes. **A-C**, The blots were reacted with bamboo mosaic virus (BoMV) rabbit polyclonal antibodies and detected with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin antibodies. **A**, BoMV antigens in blot of an infected bamboo leaf; **B**, BoMV antigens in blot of a newly emerged shoot of an infected bamboo; and **C**, a healthy bamboo leaf. **D-E**, The blots were reacted with cymbidium mosaic virus (CyMV) mouse monoclonal antibodies and detected with alkaline phosphatase-labeled goat anti-mouse immunoglobulin antibodies. **D**, CyMV antigens in blot of an infected *Cattleya* orchid leaf; and **E**, healthy orchid leaf.

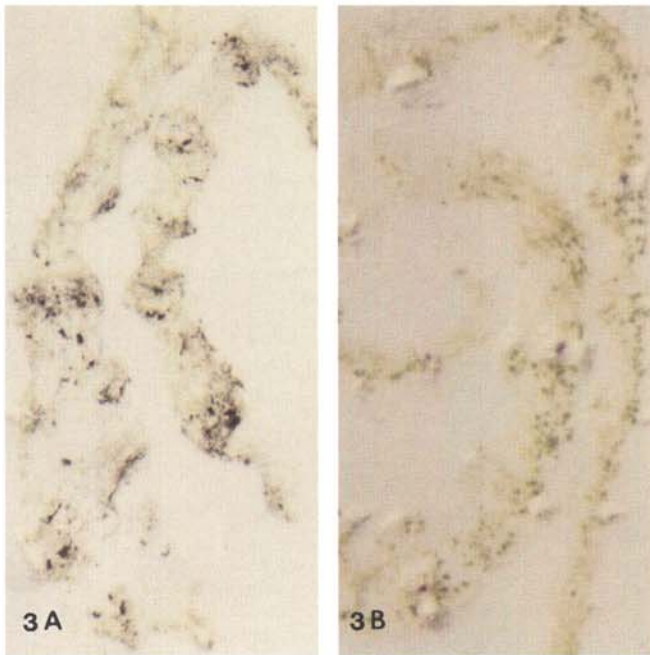


Fig. 3. Immunochemical detection of the RPV isolate of barley yellow dwarf luteovirus (BYDV) in tissue blots of infected barley on nitrocellulose membranes. The blots were reacted with mouse monoclonal antibodies and detected with alkaline phosphatase-labeled goat anti-mouse immunoglobulin antibodies. **A**, BYDV-RPV antigens in a leaf of infected barley; and **B**, healthy barley leaf.

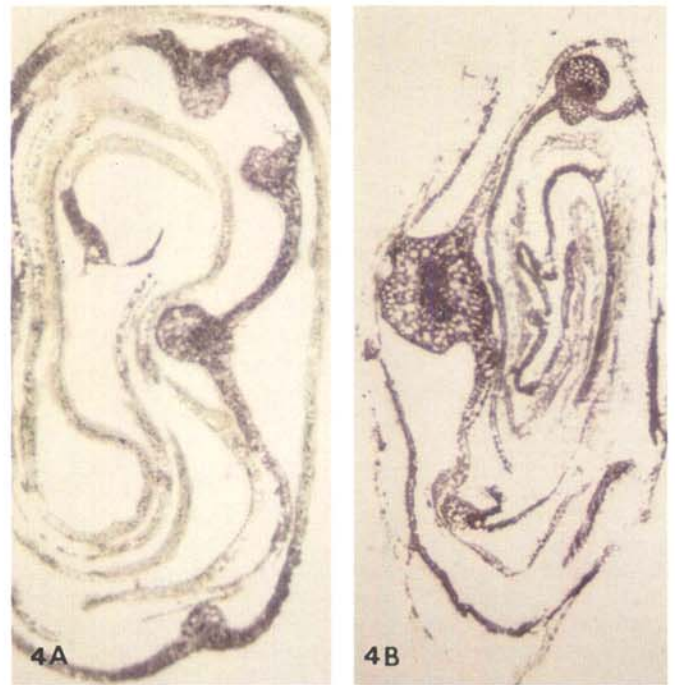
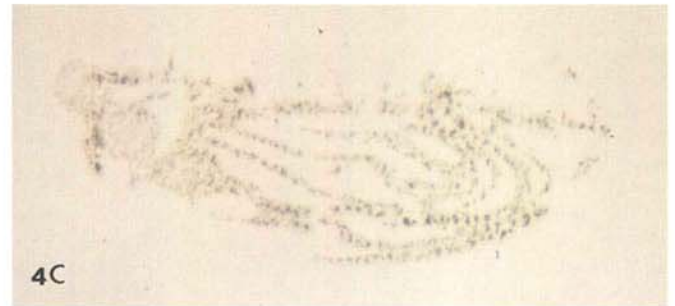


Fig. 4. Immunochemical detection of tomato spotted wilt virus (TSWV) in tissue blots of leaves of infected *Nicotiana benthamiana* on nitrocellulose membranes. **A**, The blot was reacted with biotinylated mouse monoclonal antibodies (prepared for the lettuce isolate of TSWV) and detected with avidin-alkaline phosphatase conjugates; **B**, the blot was reacted with TSWV-I rabbit polyclonal antibodies and detected with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin antibodies; and **C**, healthy *N. benthamiana* leaf.



technique can be modified to suit individual requirements. For example, the results of a 2-hr incubation were just as good as those of an overnight incubation of primary antibodies. Overnight incubation also can be used for secondary (enzyme-labeled) species-specific antibodies. This will provide a stage that the entire procedure can be carried on to the next day without interference of results (*unpublished data*). The entire process of the indirect immunological method using secondary labeled antibodies can be easily completed during a working day. Where enzyme-labeled primary antibodies are available, the direct method of immunological detection of antigens in tissue blots on nitrocellulose membranes can be completed within 3 hr. An overnight incubation of primary antibodies or secondary (enzyme-labeled) species-specific antibodies in the indirect immunological method in current studies provided convenience to investigators. It allows investigators to complete an assay in a manageable manner without interference of results.

Various variations of tissue blotting with different reagents may be a matter of choice by individual investigators. In indirect methods, species-specific enzyme-labeled antibodies (such as goat anti-mouse or goat anti-rabbit immunoglobulins) or enzyme-labeled protein A are commonly employed. In procedures where biotinylated primary antibodies are used, the avidin-enzyme conjugate is a logical choice. In direct methods, antigen-specific antibodies are, however, tagged with appropriate enzymes.

The technique of tissue blotting for antigen detection is similar to that used in immunofluorescence microscopy to visually localize specific antigens in tissue sections or cultured cell monolayers (3,7,15). A single positively stained cell justifies the conclusion of infection. In the tissue blotting technique, the specific antigens are immunologically localized with enzyme-labeled antibodies on

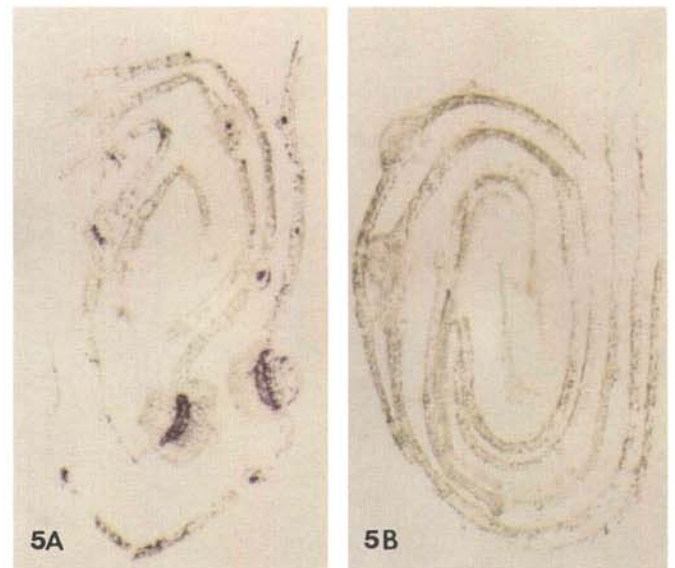


Fig. 5. Immunochemical detection of a mycoplasma-like organism (MLO) in tissue blots of infected leaves on nitrocellulose membranes. The blots were reacted with mouse monoclonal antibodies (prepared to the MLO-inducing tomato big bud disease) and detected with alkaline phosphatase-labeled goat anti-mouse immunoglobulin antibodies. **A**, Tomato big bud MLO antigens in leaf blot of an infected periwinkle; and **B**, healthy periwinkle leaves.

nitrocellulose membranes. Precise deposition of antigens in individual cells is sometimes difficult to analyze. Nevertheless, localization of specific antigens in specific tissues can be clearly demonstrated. The technique provides means of studying translocation of viral antigens.

Although similar in principles to dot-blot immunoassays of antigens on various membrane supports (16), the tissue blotting technique does not require mechanical disruption of tissues for the extraction of antigen or require different buffers for each antigen. The application of samples occurs simply by blotting the cut tissue surface on a supporting membrane rather than by adding a certain amount of antigen extract to the membrane with the aid of a commonly used dot-blot apparatus. In addition, pathogens that are irregularly distributed with only a few cells highly affected will show up clearly in tissue blots but would give only a weak signal or even go undetected if tested as extracts (4).

Immunological detection of viral or MLO antigens on nitrocellulose membranes is very sensitive (9). Although it was not determined in current studies, we expect that the sensitivity of immunological detection of viral antigens in tissue blots should be similar or equal to that of dot-blot immunoassays. In addition, this method maintains the specificity of antigen-antibody reactivities. TSWV was not readily detected by enzyme-linked immunosorbent assay (ELISA) in asymptomatic leaf tissues of infected plants (8). The presence of TSWV antigens was, however, detected in tissue blots of asymptomatic leaves of infected *Impatiens* plants (H. T. Hsu and R. L. Lawson, *unpublished data*). The failure to detect viral antigens in petiole blots of some potyvirus (e.g., PRV and SPFMV) infected leaves could be due to an extremely low concentration or uneven distribution of the antigens in the tissue. Detection of bean common mosaic virus in bean seeds by putting cotyledons on nitrocellulose papers was reported. Details of methods were, however, not described (18).

One of the advantages of the tissue blotting technique for detection of plant pathogens is that the tissue blot nitrocellulose membranes can be prepared in any laboratory, greenhouse, or even in fields at any locations by persons with just a few instructions. Furthermore, the tissue blot membranes can be stored and/or transported and processed 2-3 wk after the samples are applied. The technique also offers the advantage that a large number of samples can be processed in a short time. This new procedure should be useful in clinical diagnostic laboratories and in large-scale disease indexing and germ plasm certification programs.

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