Detection of Three Plant Viruses by Dot-Immunobinding Assay

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ABSTRACT

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An indirect dot-immunobinding assay (DIA) and a double antibody sandwich (DAS)-DIA were evaluated and compared with DAS-ELISA for detection of tobacco mosaic virus (TMV), tobacco ringspot virus (TbRSV), and tomato ringspot virus (TmRSV). Indirect DIA successfully detected 30 pg (30 ng/ml in a 1-μL sample) of purified TMV or TbRSV and 100 pg (100 ng/ml in a 1-μL sample) of TmRSV. DAS-DIA and ELISA detected 50 ng (10 ng/ml in a 5-ml sample) and 2.5 ng (10 ng/ml in a 250-μl sample), respectively, of all three purified viruses. Detection levels similar to those given above for indirect DIA, DAS-DIA, and ELISA were achieved when purified virus was diluted in healthy tobacco or *Chenopodium quinoa* sap. With indirect DIA, absorption of the first antibody with healthy plant sap and incubation of the membrane, on which the sample had been adsorbed, in 2% Triton X-100 was necessary to eliminate background color. Absorption of antisera was not necessary with DAS-DIA or ELISA. Both DIA procedures compared favorably with ELISA in discriminating between healthy and TmRSV-infected *C. quinoa*, geranium, dandelion, tobacco, and plantain plants.

Blotting techniques have become widely used for the specific identification of nucleic acids and proteins. These techniques have been simplified for routine detection of nucleic acids by eliminating the electrophoretic fractionation and transfer; the sample is applied directly to the nitrocellulose membrane as a spot, followed by hybridization to a labeled probe (5). This dot assay was modified to detect protein by spotting the antigen on a nitrocellulose membrane and incubating the membrane in test antibody followed by incubation in peroxidase-conjugated second antibody to the first antibody, and by development in 4-chloro-1-naphthol. The above procedure, termed dot-immunobinding assay (DIA), was used to screen the supernatants of hybridomas for monoclonal antibodies and to screen human pathological sera for multiple antibodies (4).

Immunological detection of plant pathogens (most notably viruses) has a wide range of applications including basic research, epidemiology, taxonomy, and certification of plant material. Enzyme-linked immunosorbent assay (ELISA) (3) has satisfied most of these needs because of its simplicity and high sensitivity. However, DIA has the potential for rapid, simple analysis of large numbers of samples for plant viruses at a lower cost than ELISA. This report compares double antibody sandwich (DAS)-ELISA with indirect and DAS-DIA for the detection of tobacco mosaic virus (TMV), tobacco ringspot virus (TbRSV), and tomato ringspot (TmRSV).

MATERIALS AND METHODS

Viruses and antisera. Antisera were produced in rabbits to three plant viruses: TmRSV-A, originally isolated from a rootstock sucker of a Red Delicious-MM. 106 apple tree with the apple union necrosis and decline (AUND) disease (10) and propagated for the last 3 yr in *Chenopodium quinoa* Willd.; TbRSV, originally transmitted to cucumber by *Xiphinema rivesi* from grape vineyard field soil and propagated for the last 4 yr in *C. quinoa*; and TMV of unknown origin that had been stored in frozen tobacco 5 yr. TmRSV was purified from *C. quinoa* as previously described (9). TbRSV was purified from *C. quinoa* by the same procedure, except the trituration buffer was 0.1 M potassium phosphate buffer, pH 7. TMV was purified from Samsun tobacco as previously described (7). Polyclonal antisera to these three viruses were produced in rabbits as previously described for TmRSV (9). The titers of the sera in Ouchterlony double diffusion tests were 1:256, 1:1024, and 1:2048 for TmRSV, TbRSV, and TMV, respectively. Unfractionated antisera to the viruses were used in these studies.

Plants and sample preparation. Sap extracts for DIA and ELISA analysis were obtained from five species of plants: *C. quinoa*, tobacco (*Nicotiana tabacum* L. 'Samsun'), common dandelion (*Taraxacum officinale* Weber), common plantain (*Plantago rugelii* Decne.), and geranium (*Pelargonium×hortorum* L.). The *C. quinoa* was infected with TmRSV or TbRSV by sap inoculation and likewise for the tobacco with TMV. The dandelions were infected with TmRSV via seed transmission from naturally infected parent plants (6). The TmRSV-infected plantains were transplanted from a peach orchard where the *Prunus* stem-pitting disease (PSP) (2) was prevalent. The TmRSV-infected geraniums were collected from commercial greenhouses. Infected dandelions, plantains, and geraniums showed no symptoms. Virus presence was confirmed, before comparative ELISA and DIA tests, by sap inoculation to *C. quinoa* and tobacco indicator plants followed by identification by ELISA. Healthy controls of each species were confirmed as noninfected by the same procedure.

A plant sample consisted of a single young leaf from an individual plant. Sap was prepared from each sample with a mortar and pestle or Tissumizer (Tekmar Co., Cincinnati, OH) in 5 ml of buffer. The size of each sample was approximately the same. Sample preparation for indirect DIA, DAS-DIA, ELISA, and mechanical assay was the same except for the extraction buffer (see below).

Indirect DIA. All DIA procedures were performed at room temperature. A grid consisting of 1.0 or 0.25 cm squares was drawn on a 9 × 15-cm nitrocellulose membrane sheet (Bio-Rad, Richmond, CA) with a pencil. The sheet was then cut to a size that would accommodate the number of samples in an individual test. When necessary, individual areas of the grid were labeled with a cryomarker pen (Vanguard, Neptune, NJ). Samples were triturated in TBS buffer (0.02 M Tris-Cl, 0.5 M NaCl, pH 7.5). The nitrocellulose membrane was dipped in TBS and placed on filter paper for 5 min to dry. One microliter of sample was spotted in the center of each grid square on the nitrocellulose membrane and dried for 5 min. Care was taken not to deposit any particulate material on the membrane. The membrane was then placed in a glass petri dish (plastic is more likely to adsorb protein) containing 50 ml of blocking solution (3% gelatin, 2% Triton X-100 solution in

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TBS buffer). The gelatin adsorbs to the membrane and blocks future nonspecific attachment of antibodies; the Triton solubilizes sap components, removing the green stain from the membrane. In some initial experiments, the Triton was omitted. The petri dish was gently agitated (40 oscillations per minute) for 1 hr.

The membrane was removed from the blocking solution with forceps, dipped in distilled water, and transferred to a second glass petri dish containing 50 ml of antivirus antiserum (about 1 mg/ml of total protein as determined spectrophotometrically; about a 1:40 dilution of each of the sera) and 1% gelatin in TBS. In all but some of the initial experiments, the solution also contained clarified plant sap (1 g of healthy plant leaf tissue per 10 ml of TBS buffer triturated in a Waring Blendor and clarified by centrifugation at 8,000 rpm for 20 min in a Beckman 45 Ti rotor). The source of the absorbing sap was always the same plant species as the test sample.

The membrane was removed from the first antibody solution, dipped in distilled water, and washed twice by agitation for 10 min in TBS containing 0.05% Tween 20 (TTBS). The membrane again was dipped in distilled water and transferred to a third glass petri dish containing a 1/1,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) and 1% gelatin in TBS. (The optimum conjugate dilution for each batch must be determined experimentally.) The dish was gently agitated for 1 hr.

Finally, the membrane was removed from the second antibody solution, dipped in distilled water, washed for 10 min in TTBS, washed for 10 min in TBS, and transferred to the substrate solution. The substrate solution was made during the final wash by dissolving 0.06 g of 4-chloro-1-naphthol (Bio-Rad) in 20 ml of 4 C methyl alcohol, then adding 100 ml of room-temperature TBS (cold TBS may precipitate the substrate) and 0.06 ml of 30% hydrogen peroxide. The membrane was incubated in the substrate in the dark for 10–30 min.

DAS-DIA. DAS-DIA followed the indirect DIA protocol with the following modifications. Individual dried nitrocellulose squares were spotted with 1 μl of antiserum (2 mg of protein per milliliter in TBS buffer). After drying, the squares were placed in blocking solution (3% gelatin in TBS) for 30 min. The membrane squares were then incubated in 5 ml of an antigen solution in TTBS for 1 hr. After washing, the squares were incubated in a 1/1,000 dilution of alkaline phosphatase-conjugated homologous antivirus antiserum in TBS for 1 hr. The squares were then washed and incubated in substrate solution for 10–30 min. The squares were not treated with Triton X-100; the sera were not absorbed with healthy sap. The substrate solution for alkaline phosphatase was made by adding 0.05 ml of 50 mg/ml 5-bromo-4-chloro-3-indoyl phosphate (BCIP) in N,N-dimethylformamide to 15 ml of 0.33 mg/ml nitro blue tetrazolium (NBT) in 0.1 M Tris-Cl, 0.1 M NaCl, 0.005 M $MgCl_2$, pH 9.5, buffer.

ELISA. DAS-ELISA was performed as previously described (3,8). The concentration of the coating antibody was 10 μg/ml in 0.05 M sodium carbonate buffer, pH 9.6. Samples were triturated in 0.02 M potassium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride, 0.05% Tween 20, and 2% polyvinylpyrrolidone 40,000 (PBS-Tween-PVP). The detecting (second) antibody was a 1/1,000 dilution of alkaline phosphatase- (Sigma, Type VII) conjugated antiserum in PBS-Tween-PVP. The substrate was *p*-nitro-phenyl phosphate (1 mg/ml in 10% diethanolamine, pH 9.8).

Biological assay. Sap from TmRSV-infected and uninfected plants was prepared in PBS-Tween-PVP buffer as described above and rubbed on the youngest, fully expanded, Carborundum-dusted leaves of *C. quinoa* plants. The *C. quinoa* were examined for necrotic local lesions 7 days after inoculation.

RESULTS

Initially, indirect DIA did not effectively discriminate between healthy and virus-infected plants because colored precipitates formed on dots containing healthy plant sap, as well as on those containing purified virus or virus in sap (Fig. 1A). Absorbing the antivirus antibody with healthy sap was not sufficient to eliminate the color from the healthy plant controls (Fig. 1B). Incubation of the membrane and adsorbed plant sap in 2% Triton X-100 for 1 hr removed the green stain and much of the healthy background color (Fig. 1C). Absorbing the antivirus antibody with healthy plant sap in addition to the Triton X-100 treatment eliminated the remainder of the background (Fig. 1D). Thus, with these two modifications, indirect DIA could detect TMV in tobacco and TbRSV or TmRSV in *C. quinoa*.

Two additional modifications failed to significantly reduce colored precipitate on grids spotted with healthy sap. The first of these substituted alkaline phosphate-conjugated goat anti-rabbit serum for perioxidase-conjugated serum and used the NBT-BCIP substrate. The second of these substituted mouse monoclonal anti-TmRSV IgG 2a for the rabbit polyclonal anti-TmRSV serum and used peroxidase-conjugated rabbit anti-mouse IgG as the second antibody. In both cases, it was still necessary to absorb the antivirus sera with healthy sap and incubate the spot in 2% Triton X-100 to eliminate background. Thus, nonspecific precipitation of substrate occurred with two different enzyme systems and with an

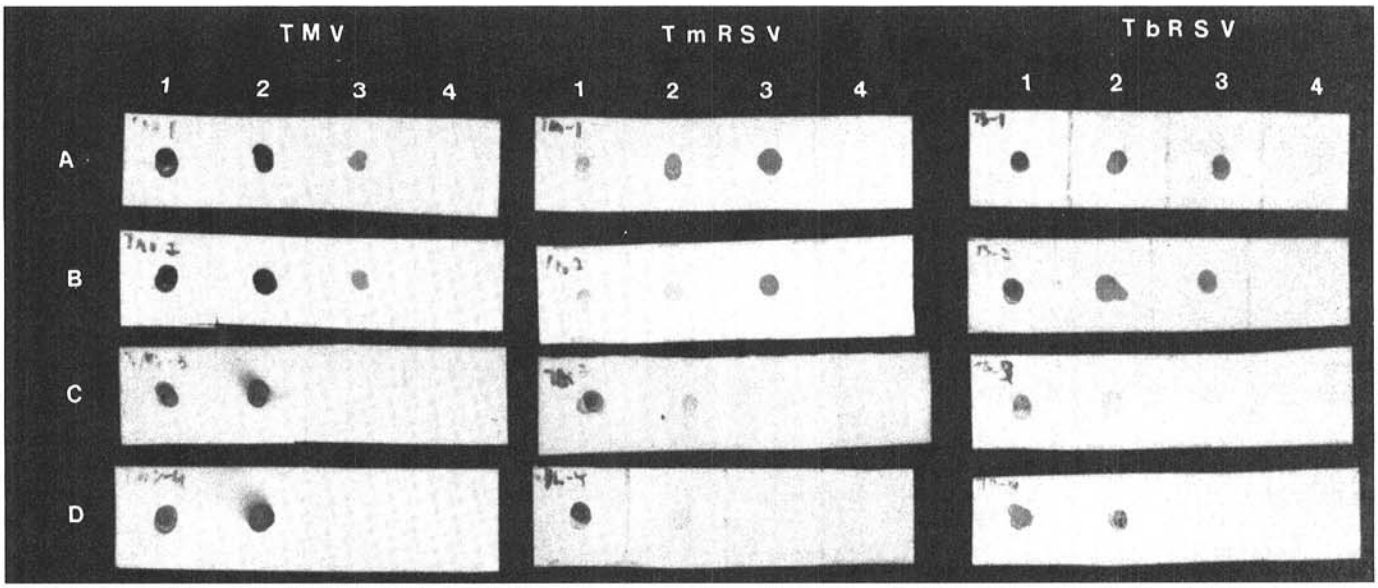

Fig. 1. Indirect DIA results for TMV, TmRSV, and TbRSV. **A,** Dots were not treated with Triton X-100, sera were not cross-absorbed. **B,** Dots were not treated with Triton X-100, sera were cross-absorbed. **C,** Dots were treated with Triton X-100, sera were not cross-absorbed. **D,** Dots were treated with Triton X-100, sera were cross-absorbed. **1,** purified virus; **2,** sap from virus-infected plant; **3,** sap from uninfected plant; and **4,** TBS buffer.

TABLE 1. Comparison of the sensitivity of ELISA, indirect DIA, and DAS-DIA for detecting three viruses

Sample[b]	Lowest concentration detected (ng/ml)[a]		
	ELISA	Indirect DIA	DAS-DIA
Purified TMV	10	30	10
Purified TbRSV	10	30	10
Purified TmRSV	10	100	10
TMV in tobacco sap	10	30	10
TbRSV in *Chenopodium quinoa* sap	10	30	10
TmRSV in *C. quinoa* sap	10	100	10

[a] The sample volumes for ELISA, indirect DIA, and DAS-DIA were 250 μl, 1 μL, and 5,000 μL, respectively. The concentrations tested were 1, 3, 10, 30, 100, 300, 1,000, 3,000, 10,000, 30,000, and 100,000 ng/ml. Detection was based on visual observation of yellow pigment (ELISA) or purple precipitate (indirect or DAS-DIA) compared with that observed with controls. Controls were buffer or healthy plant sap for purified virus diluted in buffer or purified virus diluted in healthy plant sap, respectively. The numbers are the median end points from 10 replicate experiments.

[b] Samples consisted of either purified tobacco mosaic virus (TMV), tomato ringspot virus (TmRSV), tobacco ringspot virus (TbRSV), or purified virus diluted in healthy sap (1 g of leaf tissue triturated in 5 ml of buffer).

antibody that contains no molecules produced in response to healthy sap.

The relative sensitivities of indirect DIA, DAS-DIA, and DAS-ELISA for TMV, TbRSV, and TmRSV were determined by comparing the ability of the three methods to detect various concentrations of the three viruses in buffer and plant sap (Table 1). Indirect DIA detected 30 pg (30 ng/ml) of TMV in buffer or tobacco sap, 30 pg (30 ng/ml) of TbRSV in buffer or *C. quinoa* sap, and 100 pg (100 ng/ml) of TmRSV in buffer or *C. quinoa* sap. DAS-DIA detected 50 ng (10 ng/ml) and ELISA detected 2.5 ng (10 ng/ml) of TMV, TbRSV, and TmRSV in buffer and healthy sap. Thus, detection by indirect DIA required a 3- to 10-fold higher virus concentration than by DAS-DIA or ELISA. However, indirect DIA could detect much lower amounts of virus because of the very small sample volume (1 μL compared with 250 μl for ELISA and 5,000 μL for DAS-DIA) used in the assay.

The relative effectiveness of mechanical assay, ELISA, indirect DIA, and DAS-DIA for detecting virus in *C. quinoa*, geranium, dandelion, tobacco, and plantain plants known to be infected with TmRSV was compared. Each of the four methods detected TmRSV in 100% of 50 infected *C. quinoa* plants, 15 infected geraniums, 20 infected dandelions, 10 infected tabaccos, and 10 infected plantains. None of the methods detected TmRSV in any of five uninfected control plants of each species. Thus, each of the four methods was equally effective, detecting TmRSV in 100% of each plant type.

A potential drawback to the indirect DIA is that a large volume (50 ml) of relatively concentrated (1 mg/ml) antivirus antiserum was needed. To determine if the procedure retained its sensitivity with pre-used serum, the same 50 ml of anti-TmRSV antiserum solution was used to test 100 TmRSV-infected *C. quinoa* samples per month over a 6-mo period. After the last test, the ability of the indirect DIA to detect varying concentrations of purified TmRSV was determined using the 6-mo-old antiserum solution. The procedure still detected 100 pg (100 ng/ml) of TmRSV.

DISCUSSION

DIA procedures are potentially useful research and diagnostic tools in plant pathology. They are reliable in detecting low concentrations of antigen, as has been shown for the three plant viruses. Indirect DIA should be especially useful for screening supernatants from hybridomas (4). Both indirect and DAS-DIA should be useful for routine diagnosis.

The most important criteria for evaluating a diagnostic technique are reliability, sensitivity, cost, and labor. For several years ELISA has been the method of choice for detecting plant viruses because no other technique could meet all these criteria without radioisotopes. However, both DIA procedures compare favorably with ELISA in these criteria. In the limited experiments that have been performed, DIA was reliable, always detecting virus in infected plants and never giving a false positive reading. DAS-DIA was as sensitive as ELISA. Although indirect DIA could not detect as low a virus concentration as ELISA, its sensitivity would be satisfactory for most purposes. The volume (1 μl) required by indirect DIA could be an important advantage in analyzing small samples without dilution or in screening hybridoma supernatants for reaction with an antigen in short supply.

DIA is more cost efficient than ELISA. The cost of nitrocellulose is less than that of plastic supports. Although the amount of antisera needed to test a single sample is greater with DIA than with ELISA, the antivirus antibody solution can be stored at least 6 mo and reused for at least 600 samples without appreciable loss of sensitivity. Indirect DIA also involves less labor than ELISA unless a highly automated ELISA system is used. DAS-DIA involves more labor than ELISA for large numbers of samples if nitrocellulose squares are handled individually. This drawback can be overcome by using a plastic template to confine the sample to a specific region on the nitrocellulose (1). The incubation times for DIA are also shorter than those usually recommended for ELISA, presumably because the nitrocellulose membrane has a greater affinity for protein than plastic supports.

One major advantage of ELISA is that it can be quantitated. Although spectrophotometers for reading ELISA reactions are expensive, they are widely available. DIA can also be quantitated by reflectance densitometry but these instruments are not available to many individuals involved in virus diagnosis. However, quantitation is not important in most diagnostic work where the purpose is to determine virus presence and not virus titer.

A trace of purple precipitate on blots from uninfected plants could drastically reduce the reliability and sensitivity of DIA. With indirect DIA it was necessary to completely remove the green color from the membrane with Triton X-100 and to use serum absorbed with healthy sap to eliminate this background. There were no similar background problems with DAS-DIA, and neither Triton X-100 treatment nor serum absorption was required. The reasons for nonspecific healthy reaction with indirect DIA and not with direct DAS-DIA are not clear. Similar results were obtained in a comparison between direct and indirect ELISA (9). However, with the indirect assay, a component in the healthy sap would only need to bind one antibody molecule to immobilize the enzyme, whereas, with the direct DAS assay, the sap component must bind two antibody molecules (one of which is conjugated) to immobilize the enzyme.

The source of the background in uninfected samples has not been identified. Three possibilities are sap-catalyzed reaction of the substrate, the presence of antibody to healthy sap antigens, and nonspecific interaction between healthy sap components and antibody. A monoclonal antibody to TmRSV also had to be absorbed with *C. quinoa* sap to eliminate background, indicating that at least some nonspecific interaction between a sap component and IgG is occurring.

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