

Techniques

Enzyme-Linked Immunosorbent Assay for Plant Viruses in Intact Leaf Tissue Disks

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Contribution 1197, Department of Plant Pathology, The Pennsylvania Agricultural Experiment Station. Authorized for publication 8 September 1980 as Journal Series Paper 6022.

This work was supported in part by funds from The Pennsylvania Flower Growers Association.

Accepted for publication 23 July 1980.

ABSTRACT

Romaine, C. P., Newhart, S. R., and Anzola, D. 1981. Enzyme-linked immunosorbent assay for plant viruses in intact leaf tissue disks. *Phytopathology* 71:308-312.

Tobacco ringspot virus (TRSV) and maize dwarf mosaic virus (MDMV) were detected by enzyme-linked immunosorbent assay (ELISA) when leaf tissue disks were substituted for leaf extracts as the test sample. The procedure entailed incubating 6-mm-diameter disks of leaf tissue, sampled with a paper punch, in microtiter plate wells containing 200 μ l of phosphate-buffered saline with Tween-20 and polyvinyl pyrrolidone. When a single virus-infected leaf disk was assayed, the absorbance value ($A_{405\text{ nm}}$) obtained was poorly reproducible, ranging from 0 to 50% of that observed by testing

the leaf extract. However, a more comparable test reliability was obtained by increasing the 2-hr substrate reaction time to 24 hr and by incubating several leaf disks in each plate well. By using this protocol, MDMV in corn and TRSV in geranium were detected from 1 wk up to at least 2 mo postinoculation with a reliability comparable to the conventional test using a leaf extract. The leaf disk sampling technique, when used to complement the ELISA test, should prove valuable for the rapid and sensitive qualitative evaluation of virus infection when dealing with large numbers of plants.

Enzyme-linked immunosorbent assay (ELISA) was first introduced in plant virology by Voller et al (14) and Clark and Adams (4) and has been widely used for the quantitative and qualitative measurement of a variety of plant viruses in both crude

plant extracts and in purified preparations (2,3,5,7-10). The economy, versatility, and high sensitivity of ELISA promises to make it practical for large scale virus disease surveys and epidemiological studies. However, the major constraint in analyzing numerous samples by ELISA, is the time needed to prepare the leaf extract test sample by grinding the plant tissue in buffer with a mortar and pestle. In the analysis of a large plant

0031-949X/81/03030805/\$03.00/0

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population, the sampling procedure would be the rate-limiting step that would dictate the size and scope of the experiment.

In trying to develop a more expeditious method of preparing the test sample, we have found that it is possible to use ELISA on intact leaf tissue disks. The feasibility of using pieces of tissue in ELISA was noted by Marco and Cohen (9) and was presented by us in a preliminary report (11). We describe here a protocol for ELISA of intact leaf disks for detecting maize dwarf mosaic (MDMV) and tobacco ringspot virus (TRSV) with a reliability comparable to the more conventional test employing leaf extract.

MATERIALS AND METHODS

Sources and maintenance of viruses. Strain A of MDMV (kindly provided by C. W. Boothroyd, Cornell University, Ithaca, NY 14853) and a geranium isolate of TRSV were used throughout this study. TRSV was maintained in frozen cucumber leaf tissue at -20°C and MDMV in *Sorghum bicolor* L. 'Sart' in a greenhouse at $25 \pm 1^{\circ}\text{C}$.

Mechanical inoculation and maintenance of plants. Inoculum for virus transfer was prepared by grinding infected leaves in two to three volumes of cold 50 mM phosphate buffer, pH 7.0. Usually 3- to 4-wk-old plants of corn (*Zea mays* L. 'SX-60') for MDMV and florist's geranium (*Pelargonium \times hortorum* Bailey) 'Nittany Lion Red' for TRSV were inoculated by dusting Carborundum on three leaves and applying the inoculum with the pestle. Healthy control plants were mock-inoculated with buffer alone. Following inoculation, corn plants were maintained in the greenhouse. Geraniums were transferred to a growth chamber at 21°C with fluorescent and incandescent lamps at 1.2 klux for a 16-hr photoperiod. Unless specified otherwise, plants were infected for at least 4 wk before being used in the experiments.

Antisera. Antiserum to MDMV strain A was kindly provided by D. T. Gordon, Ohio Agricultural Research and Development Center, Wooster 44691. Antibodies to TRSV were prepared by injecting a New Zealand white rabbit with purified virus. A purified virus preparation was emulsified with an equal volume of Freund's complete adjuvant and injected intradermally at a series of sites along the back. Two more series of injections were administered at 1-wk intervals (1 mg virus total). The rabbit was bled by cardiac puncture 2 wk after the final injection.

Preparation of γ -globulins for ELISA. The procedure for partial purification and conjugation of the γ -globulins was essentially as described by Clark and Adams (4). The γ -globulins were twice precipitated with ammonium sulphate at 50% saturation. MDMV

γ -globulins were used without further purification whereas TRSV γ -globulins were chromatographed on DEAE-cellulose (Whatman DE-22). The partially purified γ -globulins were conjugated with twice their weight of alkaline phosphatase (4) in the presence of glutaraldehyde (1).

Procedures for ELISA. The protocol for ELISA was as outlined by Voller et al (14) and Clark and Adams (4). Wells of a polystyrene microtiter plate (#14-245-3, Fisher Scientific Co., Pittsburgh, PA 15328) were incubated with partially purified nonconjugated γ -globulins (1 $\mu\text{g}/\text{ml}$, 200 $\mu\text{l}/\text{well}$) in 0.05 M sodium carbonate containing 0.02% sodium azide, pH 9.6 for 3 hr at 37°C . Test samples consisted of either leaf extracts or intact leaf tissue disks. Extracts were prepared by homogenizing one gram of leaf tissue with a mortar and pestle in 10 or 20 volumes of PBS-Tween-PVP (0.02 M phosphate, 0.15 M NaCl, 0.02% sodium azide, pH 7.4 containing 0.05% Tween-20 and 2% polyvinyl pyrrolidone [40,000 molecular weight]). The homogenate was filtered through a layer of Miracloth™ (#475855, Calbiochem, San Diego, CA 92112) and 200- μl aliquot of the filtrate was incubated in a plate well. Alternatively, a 6-mm-diameter disk of tissue was removed from the leaf with a paper punch (McGill, M. P. Co., Marengo, IL 60152) and transferred to the plate well containing 200 μl (~1:25, w/v) of PBS-Tween-PVP with a needle probe. After the test samples had been added, plates were incubated without shaking for 18–20 hr at 6°C but in later experiments they were gently agitated at approximately 100 oscillations per minute on a wrist-action shaker (Model 75, Burrell Corp., Pittsburgh, PA 15230). This enhanced the test results obtained with leaf disk test samples, but had no effect on leaf extract analyses. TRSV enzyme-conjugated γ -globulins were used at 1,000-fold dilution in PBS-Tween-PVP with 0.02% ovalbumin. MDMV-conjugated γ -globulins were diluted 200-fold before use in the same buffer also containing 10 $\mu\text{g}/\text{ml}$ of preimmune γ -globulins. The latter was included to reduce color

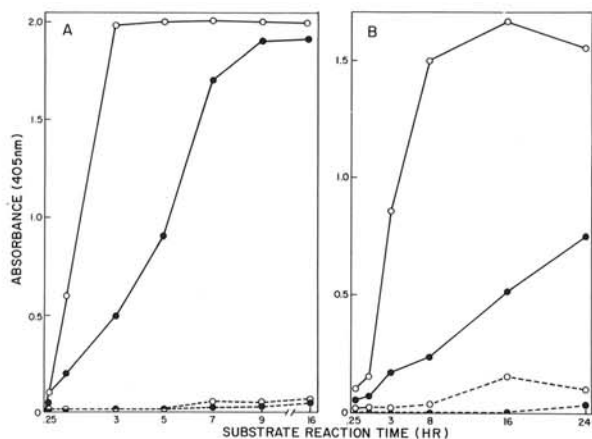


Fig. 1. Relationship between test absorbance ($A_{405\text{nm}}$) in the enzyme-linked immunosorbent assay (ELISA) and the substrate reaction time using either a leaf extract or a leaf tissue disk as the test sample for **A**, maize dwarf mosaic virus in corn and **B**, tobacco ringspot virus in geranium. A single leaf disk was incubated in the plate well with 200 μl of PBS-Tween-PVP. The enzyme-substrate reaction was terminated at the time intervals indicated by the addition of 50 μl of 3N NaOH. Healthy plant extract (○—○); healthy plant tissue disk (●—●); diseased plant extract (○—○); diseased plant tissue disk (●—●).

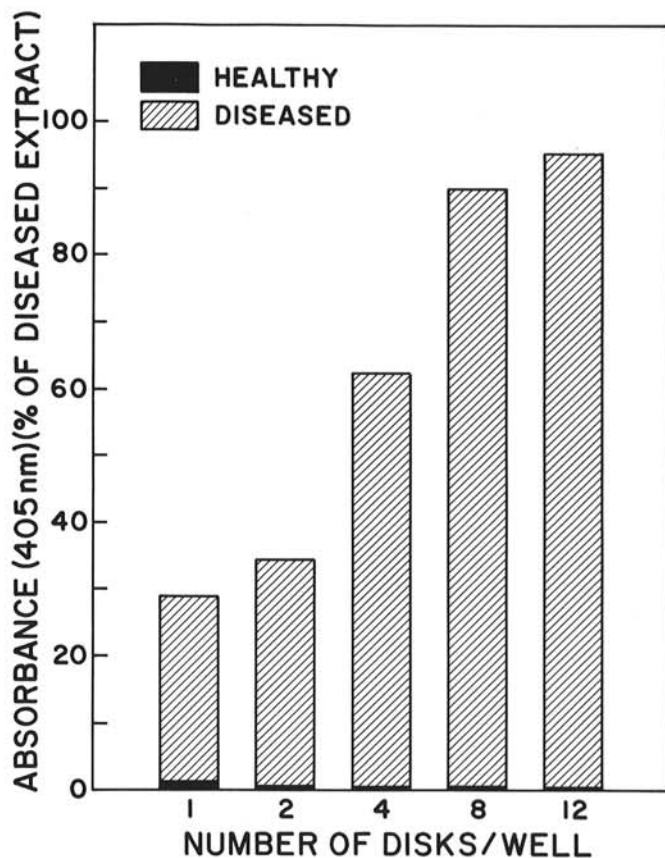


Fig. 2. Relationship between test absorbance ($A_{405\text{nm}}$) in the enzyme-linked immunosorbent assay (ELISA) and the number of leaf tissue disks from tobacco ringspot virus-infected geranium incubated in the plate well. The leaf disks were incubated in the well with 200 μl of PBS-Tween-PVP. The enzyme-substrate reaction time was 1 hr. Data are expressed as percentage of the $A_{405\text{nm}}$ obtained with an extract prepared from the infected leaf.

development due to nonspecific reactions. Conjugated γ -globulins (200 μ l) were incubated in the plate well for 3 hr at 37 C. Unless stated otherwise, a 200 μ l aliquot of substrate (*p*-nitrophenolphosphate, 1 mg/ml) in 10% diethanolamine containing 0.02% sodium azide, pH 9.6, was incubated in each well for 1–2 hr at room temperature. To assess the antibody-antigen reaction, 50 μ l of 3 N NaOH was added to each well and the yellow nitrophenolate end product was quantified by colorimetry ($A_{405\text{ nm}}$). A buffer control, consisting of PBS-Tween-PVP substituted for the test sample, was included in every experiment. Net absorbance values were calculated by subtracting the mean buffer control absorbance value from the mean apparent absorbance values for each treatment. Experiments were designed so that there was a minimum of two observations per treatment per plate from duplicate plates. All experiments were repeated at least twice. Statistical analysis was performed according to Duncan's multiple range test (12).

RESULTS

Initially, we found that replacing a leaf extract with a leaf tissue disk as the ELISA sample resulted in unreliable detection of MDMV and TRSV. Tissue disks sampled from the same leaf frequently yielded absorbance values that ranged from 0–50% of that obtained with an extract prepared from the same leaf (*unpublished*). It became apparent that the reliability of ELISA, with a single tissue disk sample, was dependent on the concentration of the virus in the leaf. Although TRSV could be reliably detected in plants at 2 wk postinoculation, when the concentration of infectious virus in the leaf was near its maximum, it could not be easily detected in the latent stage of infection when the virus titer had declined.

Effect of the substrate reaction time. Results from preliminary experiments indicated that modification of the ELISA test protocol was necessary if a tissue sample was to be used in the test without compromising sensitivity. The length of the substrate reaction on the sensitivity of the test and on nonspecific reactions was therefore studied. Absorbance values ($A_{450\text{ nm}}$) obtained with single leaf tissue disk samples from TRSV-infected, MDMV-infected, and healthy leaves were compared to those of the corresponding leaf extracts at various time intervals after initiating the substrate reaction. In all treatments, absorbance increased with substrate reaction time; however, the rate of increase and the final color intensity varied with the treatments (Fig. 1 A,B). The most rapid rate of color development occurred when leaf extracts from infected plants were used as test samples. The color intensity of the yellow *p*-nitrophenolate end product increased approximately linearly, becoming maximal at 3 and 8 hr in the MDMV and TRSV assays, respectively. At these time intervals, the corresponding leaf disk samples had yielded only 25–30% of the

maximal absorbances. However, with longer incubation (16–24 hr), $A_{405\text{ nm}}$ values of 50% or more of the maxima were obtained. In contrast, color development due to nonspecific reactions associated with healthy test samples, whether extracts or disks, were negligible at all time intervals up to 24 hr. Although differences between positive ELISA reactions in tests of infected-leaf tissue disks and healthy controls were statistically significant ($P = 0.05$) at all reaction intervals tested, a prolonged substrate reaction was desirable because infected/healthy absorbance ratios were markedly increased. Frequently, tests that were scored as negative or questionably positive by visual inspection after 1–2 hr of substrate reaction, were decisively positive upon further incubation.

Effect of the number of leaf disks per plate well. As a possible alternative or a step complementary to increasing the substrate reaction time, we examined the influence of the number of leaf disks incubated in the plate well on the sensitivity of the test. The absorbance values obtained by varying the number of TRSV-infected or healthy leaf tissue disks in the sample well were compared at a substrate reaction time of 1 hr. The data are expressed as a percentage of the absorbance obtained with an extract prepared from corresponding leaves. A statistically significant ($P = 0.05$) positive reaction resulted when even a single leaf disk from a TRSV-infected leaf was incubated in the well (Fig. 2). A successive increase in the number of disks to 12 (the maximum number accommodated) was positively correlated with an increase in absorbance up to 95% of the value obtained with the extract sample. This was not accompanied by an increase in nonspecific reactions obtained with healthy controls. A significant positive reaction ($P = 0.05$) did not result even when 12 healthy leaf disks were incubated in the plate well.

Assessing the reliability of ELISA using leaf disks. The sensitivity of the ELISA test using leaf disks was evaluated experimentally in two ways. In the first test, each of 20 geranium plants, either healthy or latently infected with TRSV (5 wk postinoculation) were indexed by using two test protocols: a single leaf disk as the test sample and a 24-hr substrate reaction, and a leaf extract as the test sample and a 1.5-hr substrate reaction. Either test protocol proved entirely reliable for detecting TRSV in the latent stage of infection (Table 1). The absorbance values obtained when infected leaf tissue disks were used averaged 58% of those obtained with the corresponding extracts, but were always significantly positive, $P = 0.05$. The minimum positive absorbance value was approximately 3.5-fold higher than the maximum absorbance obtained with healthy controls. Interestingly, the paper punch was not sterilized between sampling plants in these experiments but this did not pose a contamination problem even when a healthy plant (plant no. 11) was preceded in the sampling sequence by three infected plants.

TABLE 1. Comparative enzyme-linked immunosorbent assay (ELISA) values ($A_{405\text{ nm}}$) obtained by the leaf extract and the leaf tissue disk sampling methods for testing known healthy or tobacco ringspot virus (TRSV)-infected geraniums

Plant no. ^a	Status ^b	Absorbance (405 nm) Sampling method		Plant no.	Status	Absorbance (405 nm) Sampling method	
		Extract ^c	Disk ^d			Extract	Disk
1	Healthy	0.07	0.00	11	Healthy	0.00	0.03
2	Diseased	1.33	0.90	12	Diseased	0.85	0.17
3	Healthy	0.05	0.05	13	Healthy	0.00	0.01
4	Diseased	1.00	0.59	14	Healthy	0.00	0.00
5	Diseased	1.90	1.56	15	Diseased	1.87	1.82
6	Healthy	0.10	0.00	16	Diseased	1.31	0.34
7	Healthy	0.10	0.04	17	Healthy	0.01	0.04
8	Diseased	1.79	0.88	18	Diseased	0.97	0.37
9	Diseased	1.55	0.66	19	Healthy	0.02	0.05
10	Diseased	1.96	1.79	20	Healthy	0.07	0.05

^aThe plants were indexed consecutively in the order of appearance in the table. The paper hole-punch used to remove the leaf disks was not sterilized between plant samples.

^bHealthy plants were mock-inoculated with buffer. Diseased plants were latently-infected with TRSV 5 wk postinoculation.

^cPrepared by homogenizing leaf tissue in 20 volumes of PBS-Tween-PVP and followed by filtering through one layer of Miracloth. An enzyme-substrate reaction time of 1.5 hr was used.

^dOne leaf tissue disk was incubated in the plate well with 200 μ l of PBS-Tween-PVP. An enzyme substrate reaction of 24 hr was used.

TABLE 2. Comparison of leaf extract and leaf tissue disk sampling methods used with enzyme-linked immunosorbent assay (ELISA) for the detection of maize dwarf mosaic virus (MDMV) in corn and tobacco ringspot virus (TRSV) in geranium

Postinoculation time (wk)	Absorbance (405 nm)							
	MDMV				TRSV			
	Diseased		Healthy ^a		Diseased		Healthy	
	Extract ^b	Disk ^c	Extract	Disk	Extract ^d	Disk	Extract	Disk
1	1.42	2.08	0.00	0.02	0.15	0.19	0.01	0.02
2	1.42	2.07	0.05	0.04	2.10	1.95	0.01	0.00
4	1.51	2.06	0.06	0.00	1.21	1.95	0.00	0.00
8	1.77	2.08	0.02	0.03	0.37	1.94	0.01	0.03

^a Healthy plants mock-inoculated with buffer.

^b Prepared by homogenizing leaf tissue in 10 volumes of PBS-Tween-PVP and followed by filtering through one layer of Miracloth. A substrate reaction time of 1.5 hr was used.

^c Four tissue disks were sampled from one leaf per plant and incubated together in a plate well with 200 μ l of PBS-Tween-PVP. A substrate reaction time of 24 hr was used.

^d As in footnote b except that 20 volumes of buffer per weight of tissue was used during extraction.

In a second type of experiment, we investigated the reliability of the test for detecting MDMV and TRSV in plants at various times after infection. The plants were indexed by using the same protocols as outlined in the first test, except that four leaf disks were incubated in the plate well. Using either protocol, positive reactions ($P = 0.05$) were obtained with samples collected 1–8 wk postinoculation (Table 2). Absorbance values were usually higher using the leaf disk protocol, especially in the TRSV assays, presumably because of the longer reaction time (24 hr) with leaf disks. Measurements made under our experimental conditions, revealed that a decrease in the concentration of infectious virus along with a remission of foliar symptoms occurred at 3 wk postinoculation. This was reflected by a decrease in the amount of TRSV antigen detected by the standard protocol, from an absorbance value of 2.10 at 2 wk, to 1.21 at 4 wk, and 0.37 at 8 wk. However, test results obtained by using the tissue disk protocol were virtually unaffected by the decrease in virus titer; the absorbance values remained maximal at approximately 1.95 irrespective of the stage of infection.

DISCUSSION

We have shown that by minor modification of the standard ELISA protocol it is possible to substitute intact leaf tissue disks for leaf homogenates for virus analysis without reducing the sensitivity of the test. The adoption of the procedure does not seem contingent upon virus morphology since we have found it to work equally well with a long flexuous rod-shaped particle (MDMV, 750 nm long) and a small spherical particle (TRSV, 28 nm in diameter). Admittedly, it is difficult to accept the use of a whole tissue sample without anticipating a compromise in the reliability of the test. However, in principle, it is no more than an extension of the "leaf-dip method" that is used for rapid virus detection by electron microscopy (6). Unquestionably, comparably less virus is released from the injured cells at the periphery of the leaf disk than is contained in a tissue homogenate. It follows that less virus is available for "capture" by the antibodies bound to the plate well surface, thereby reducing the sensitivity of the test. Perhaps the most obvious countermeasure would be to increase the concentration of the enzyme-conjugated γ -globulins, although we have chosen to manipulate other test parameters. For instance, simply increasing the number of leaf disks incubated in the well and providing gentle agitation during the incubation step probably enhanced test sensitivity by increasing the concentration of virus in suspension in the well. Similarly, prolonging the substrate incubation step most likely compensated for the deficiency in virus by allowing more time for the catalytic conversion of the substrate to the colored end product.

The usefulness of the tissue disk ELISA procedure as a quantitative test is questionable since prolonged enzyme-substrate reactions frequently led to saturating enzyme kinetics marked by the development of a maximal absorbance (Fig. 1A). It is conceivable, however, that the test could be quantitative by

reducing the substrate reaction time providing that the experimental error introduced in the test by the disk sampling method per se was not a restriction. Although the leaf disk ELISA protocol requires an additional overnight incubation, it more than compensates for the time that would be required to manually prepare large numbers of leaf extracts. Nonspecific reactions that might result from a prolonged substrate incubation would certainly be a consideration in adopting this technique but this was not a concern with the two ELISA systems we have examined. Further, in our experience, the overnight substrate incubation step was often more of a convenience than a necessity since visually detectable color intensities developed after just a few hours in the majority of the cases.

In summary, the leaf tissue disk sampling method, when used with ELISA, should provide an extremely rapid and sensitive assay for the qualitative evaluation of virus infection when testing large numbers of plants. We are particularly interested in adopting this technique for virus indexing commercially produced geraniums for certification in Pennsylvania where an estimated 2,400 plants are screened annually (13). In addition to facilitating routine virus indexing programs, the sampling technique also should prove valuable in virus disease surveys and epidemiological studies. One obvious advantage is that precoated plates could be taken to the field where the plants could be sampled in situ.

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