A Comparison of an Enzyme Linked Immunosorbent Assay (ELISA) and Western Blotting for Detection of Peanut Mottle Virus and Peanut Stripe Virus[1]

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ABSTRACT

Western blotting was used to detect infections of peanut cv. Tamnut 74 with peanut mottle virus (PMV) and/or peanut stripe virus (PStV). Leaf samples were ground in electrophoresis sample buffer and heated for 5 min at 95 C prior to electrophoresis in 12% polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose sheets at 100V for 45 min. Western blots were performed by first blocking unbound sites on the nitrocellulose with 5% non-fat dry milk in Tris-buffered saline (TBS), pH 7.4 for 30 min, followed by incubation in a 1/200 dilution of PMV and/or PStV antiserum in TBS (the latter antiserum provided by J. W. Demski, U. of GA) for 45 min. This was followed by incubation in protein-A-peroxidase (2 μg/mL in TBS) for 45 min, followed by 4-chloro-1-napthol plus hydrogen peroxide in TBS. As little as 25 ng of either purified PMV or PStV was detected. This was similar to the limits of detection fo the double sandwich enzyme linked immunosorbent assay (ELISA). Because of the difference in migration of the coat proteins of PMV and PStV, both viruses may be detected in plants infected with PMV and PStV. This assay can be performed in approximately 6 h when mini-gels are used for the initial electrophoretic seperation and does not require the antiserum to be fractionated or bound to an enzyme as is the case with ELISA.

Key Words: Groundnut, PMV, PStV, potyvirus, immunoblotting

Peanut mottle virus (PMV) and peanut stripe virus (PStV) are two potyviruses capable of infecting a number of legume species (5,6,7). PMV has long been endemic, but PStV was introduced only recently into the United States (4). Peanut infected with either virus can result in significant economic loss.

Investigations of serological detection of PStV have been limited. The enzyme linked immunosorbent assay (ELISA) has been used to detect PStV and PMV (1,4). However, because of the modifications of antiserum required to run the ELISA, e.g. fractionation of the IgG and conjugation of IgG to an enzyme such as alkaline phosphatase, the double sandwich ELISA may not be suited for detection when only a small amount of antiserum is available or when only a few samples need to be assayed.

Western blotting, the serological identification of electrophoretically separated proteins tranferred to a solid support, has been used for the detection of plant viruses (2,12,16). In this technique viral and plant proteins in crude sap can be electrophoretically separated, transferred to nitrocellulose and the viral proteins probed with unmodified antiserum. The antigen-antibody complex formed can be detected by protein-A-peroxidase which, in the presence of 4-chloro-1-naphthol and hydrogen peroxide, forms a purple precipitate. The single coat proteins of PMV and PStV separate from each other during electrophoresis because they differ in molecular weight (5,14). Thus, both viruses may be detected when the proteins transferred to nitrocellulose are probed with a mixture of the two antisera to the two viruses. This paper presents the results of comparing Western blotting to the double sandwich ELISA for sensitivity in detecting PMV and PStV, and the use of Western blotting to detect infections of peanut cv. Tamnut 74 with PMV and/or PStV. A preliminary report of this work has been published (15).

Materials and Methods

Virus source, purification, and antiserum production. The PMV isolate used has been described previously (13,14). The isolate of PStV used and antiserum to PStV was obtained from J. Demski, University of Georgia. PMV was purified from *Pisum sativum* L. Little Marvel as described previously (14). Pea tissue was ground in freshly prepared 0.01 M potassium phosphate buffer, pH 8.0, with 0.001 M dithioerythritol (2 mL/g), filtered through cheesecloth, and clarified with chloroform followed by centrifugation. Virus was precipitated by the addition of polyethylene glycol (PEG) and potassium chloride (KCl). The pellet was resuspended overnight in 0.01 M borate-phosphate buffer, pH 8.3, with 0.2 M urea. Resuspended pellets were collected, clarified as above and virus pelleted with PEG and KCl. The pellet was resuspended overnight in the borate-phosphate buffer. Resuspended pellets were centrifuged for 5 min at 3,000 *g* and four mL of the supernatant was placed on 10-40% sucrose gradients in the grinding buffer. The single virus zone per tube from density gradient centrifugation was pelleted by centrifugation and resuspended in 0.01 M potassium phosphate buffer, pH 8.0. Resuspended pellets were collected and centrifuged for 5 min at 3,000 g and the supernatant collected. Virus concentration was calculated based on an absorbance of 3.0 at 260 nm being equivalent to 1 mg/mL.

PStV was purified from *Lupinus albus* as above except only freshly harvested material was used. Virus concentration was calculated based on an absorbance of 3.0 at 260 nm being equivalent to 1 mg/mL.

For PMV antiserum production, a rabbit was injected intramuscularly twice weekly with 1 mg of virus with Fruend's complete adjuvant for 5 weeks. Antiserum with a microprecipitin titer against purified PMV of 1024 was used for ELISA tests.

Enzyme linked immunosorbent assay (ELISA). The double sandwich ELISA procedure used was similar to that of Clark and Adams (3). The immunoglobulin G (IgG) fraction of PMV antisera was isolated on DEAE-Trisacryl-M and of PStV by three ammonium sulphate precipitations. ELISA plates were coated with 1 μg/mL IgG in 0.05 M carbonate buffer, pH 9.6, for 2 h at room temperature. Plates were rinsed 3 times with phosphate buffered saline containing 0.05% Tween (PBS-Tween), and samples diluted in PBS-Tween containing 2% polyvinyl pyrrolidone (PVP) were then added. After incubation at 4C overnight, plates were rinsed 3 times with PBS-Tween, and alkaline phosphatase (Sigma Chemical Co. No. P5521, St. Louis, MO 63178) labeled anti-PMV or anti-PStV IgG was added at a 1:200 dilution in PBS-Tween containing 2% PVP and 0.2% ovalbumin. After incubation for 5 h at room temperature, plates were rinsed as above, the p-nitrophenyl phosphate in diethanolamine substrate buffer was added, and plates were incubated 20 min before reading in a BIO-TEK EIA plate reader (BIO-TEK Instrument, Inc., Burlington, VT 05401).

Western blotting. Samples were prepared for Western blotting by grinding in sample buffer (62.5 mM Tris-HCl buffer, pH 6.8, containing 10% SDS, 5% 2-mercaptoethanol, and 0.002% Bromophenol blue; 3 mL/g) and heating at 95 C for 5 min. The samples were subjected to electrophoresis in a 12% polyacrylamide running gel (pH 8.8) with a 5% stacking gel (pH 6.9). Electrophoresis buffer contained 25 mM

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Tris, 192 mM glycine and 3.46 mM SDS, pH 8.3. Electrophoresis was conducted in 1.5 mm slab gels at 20 mA until the tracking dye reached the bottom of the slab (9).

Proteins were transferred to nitrocellulose with a Hoefer Scientific Transphor Unit (San Francisco, CA 94107) at 100 V for 45 min in a 25 mM Tris, 129 mM glycine, 20% (vol/vol) methanol, pH 8.3, buffer (18). After transfer, unbound sites were blocked for 30 min at room temperature in a solution of 5% Carnation non-fat dry milk (Los Angeles, CA 90036) in Tris buffered saline (TBS). The nitrocellulose sheet was rinsed 3 times in TBS and placed in a 1/200 dilution of antiserum in TBS. After incubation for 45 min at room temperature the nitrocellulose sheet was rinsed once in TBS, once in TBS containing 0.05% Tween 20, and twice in TBS. The nitrocellulose sheet was then placed in protein-A-peroxidase in TBS (2 μg/mL) for 45-60 min, then rinsed 3 times in TBS, and transferred to substrate buffer (5 parts TBS + 1 part of a 3 mg/mL solution of 4-chloro-1-napthol in methanol + .018 parts of hydorgen peroxide) for about 5 min. In the experiment where all the proteins transferred to nitrocellulose were visualized, the nitrocellulose sheet was stained (50% methanol, 10% acetic acid and 0.125% coomassie blue) for 5 min and destained in 50% methanol with 10% acetic acid.

Results and Discussion

Optimization of assays. Conditions for optimizing ELISA and Western blotting for detection of both PMV and PStV were examined in order to minimize non-specific reactions. Concentrations of 10, 1.0, and 0.1 μg/mL of IgG in coating buffer; dilutions of 1/10 and 1/100 of samples; and dilutions of 1/200, 1/800, 1/3200 of alkaline phosphatase conjugated IgG were examined with ELISA. A concentration of 1.0 μg/mL IgG in coating buffer, samples diluted 1/100 and alkaline phosphatase conjugated IgG at a dilution of 1/200 were the most satisfactory. A 4 ng/mL suspension of purified virus was detected and the response as measured by absorbance at 405 nm was linear as the virus concentration was increased to 500 μg/mL.

In the case of Western blotting, gels were loaded with 20μL aliquots of a 100 ng/mL suspension of purified virus for electrophoresis and subsequent detection by Western blotting. Nitrocellulose blots were incubated for various times (15, 30, 45, 60, 90 min) in different dilutions (1:100, 200, 400, 800) of antiserum and different dilutions (0.5, 1, 2, 4 μg/mL) and times (15, 30, 45, 60, 90 min) in protein-A-peroxidase. Satisfactory results were obtained with a 1/200 dilution of antiserum for an incubation of 30-60 min, and with protein-A-peroxidase at a concentration of 2 μg/mL for 15 min. Times longer than these in either antiserum or protein-A-peroxidase tended to increase the background. Under the incubation conditions outlined, a 20 μL sample of a 25 ng/mL suspension of purified PMV (Fig. 1a) or PStV (Fig. 1b) was detected. When Western blotting was used for detection of PMV or PStV in infected peanut tissue only a single band was found to react to the antiserum although numerous proteins were transferred to the nitrocellulose as indicated by coomassie blue staining (Fig. 2a).

Detection of PMV and PStV in single or mixed infections. ELISA and Western blotting were compared for detection of PMV and PStV alone or in combination in inoculated peanut cv. Tamnut 74. Two weeks after planting, seedlings were dusted with 225-μm corundum (Semitech, Inc., Dallas, TX 75220) and rubbed lightly with a small four-ply gauze pad saturated with inoculum (0.5 mg/mL PMV, PStV, or PMV and PStV). Samples were taken for assays 4 weeks after inoculation. Each experiment consisted of 12 plants inoculated with virus (4 with PMV, 4 with PStV, and 4 with both) and healthy controls. Experiments were repeated 3 times.

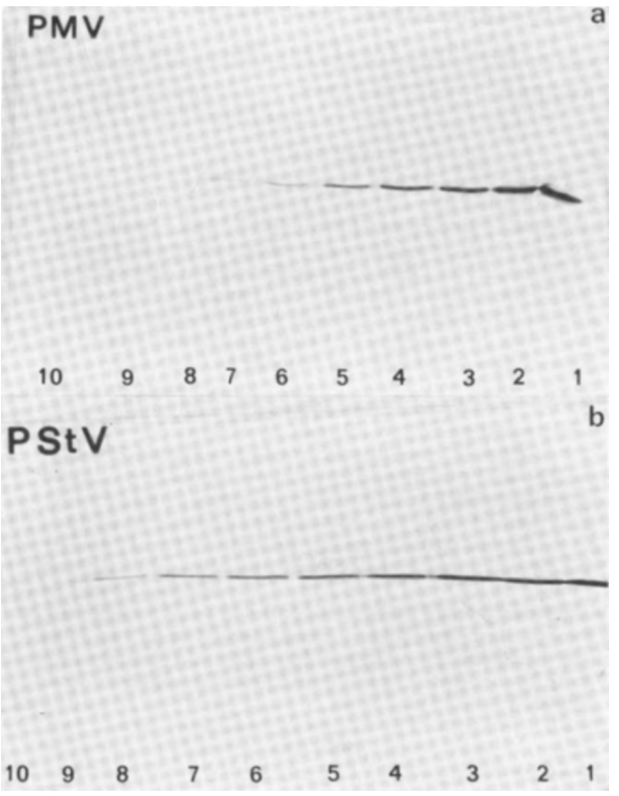

Fig. 1. Western blot of purified peanut mottle virus (PMV) and peanut stripe virus PStV). 1a. Lanes were loaded with 20 μL of purified PMV at 1000, 500, 250, 200, 100, 50, 25, 10, 5, and 1 ng/mL (lanes 1 to 10). lb. Lanes were loaded with 20 μL of purified PStV at 1500, 1000, 500, 400, 200, 100, 50, 25, 10, 5 ng/mL (lanes 1 to 10).

With ELISA samples from PMV, PStV, or PMV and PStV infected plants were differentiated easily. In all cases infected plants had 2-fold or higher increases in ELISA readings compared to control plants. In the case of PMV infected plants ELISA readings ranged from 0.500 to 2.000 with an average of 1.432 compared to 0.000 to 0.220 with an average of 0.122 for controls. With PStV infected plants ELISA readings ranged from 0.620 to 2.000 with an average of 1.566 compared to 0.100 to 0.230 with an average 0.167 for controls. With plants infected with both viruses the range of reactions for each virus was similar. The reaction of PStV antiserum with PMV infected plants and PMV antiserum with PStV infected plants did not differ from the reaction of each antiserum with healthy control tissue.

Western blotting enabled detection of single or mixed infections of the viruses because of the difference in migration of the coat proteins of the two viruses as well as the lack of serological cross reactivity of the viruses. When samples from PMV, PStV, and both PMV and PStV infected peanut were run in adjacent lanes and probed with either PMV antiserum or PStV antiserum only the coat protein bands reacting to the respective

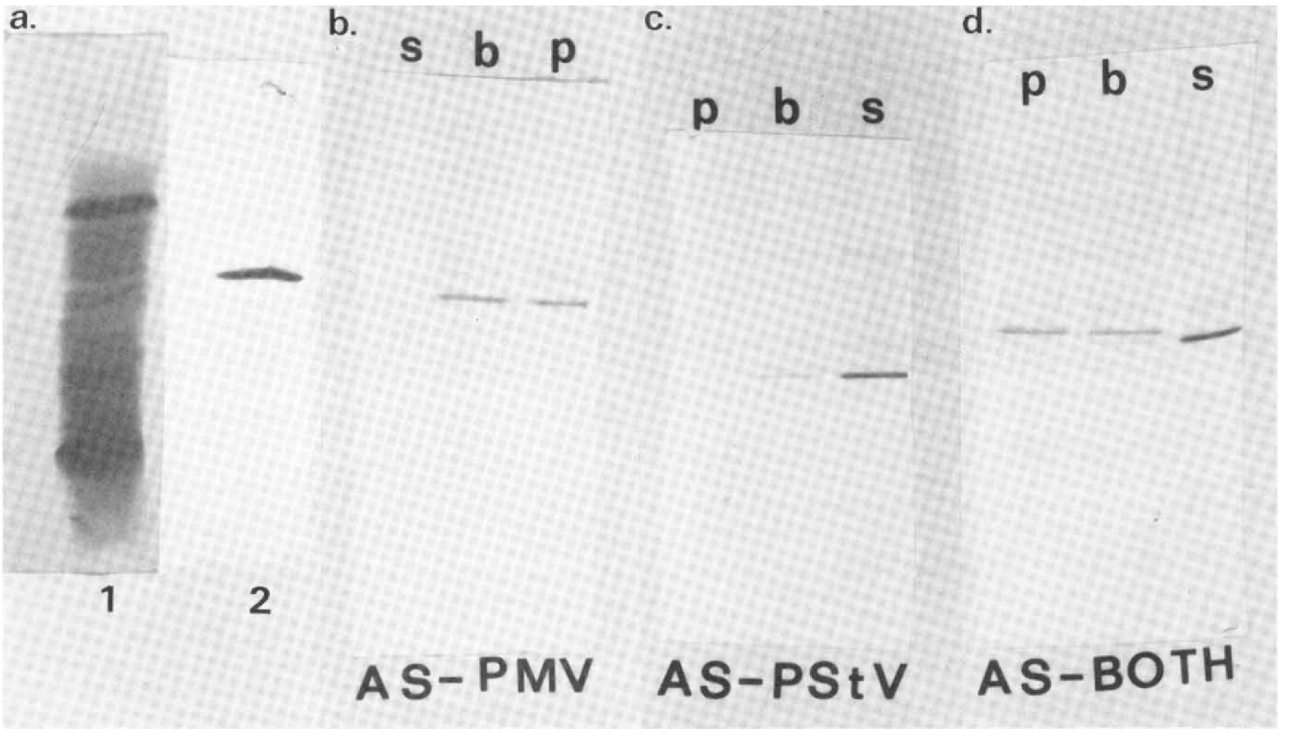

Fig. 2. Western blot of peanut mottle virus (PMV, p), peanut stripe virus (PStV, s) or PMV and PStV (b) infected peanut cultivar 'Tamnut 74'. 2a. Coomassie blue stain of proteins from PMV infected plant transferred to nitrocellulose by electroblotting (lane 1) and Western blot of same sample showing single coat protein of PMV (lane 2). 2b. Western blot of p, s, and b infected peanut probed with PMV antiserum (AS-PMV). 2c. Western blot of p, s, and b infected peanut probed with PStV antiserum (AS-PStV). 2d. Western blot of p, s, and b infected peanut probed with a mixture of PMV and PStV antiserum (AS-BOTH).

antiserum became apparent (Fig. 2b, 2c). A mixed infection could be detected in a single run by mixing antiserum to PMV and PStV and using this to probe the nitrocellulose blot. When this was done the band corresponding to the PMV coat protein was evident in PMV infected plants, the band corresponding to the PStV coat protein was evident in PStV infected plants, and bands corresponding to both viruses was evident in plants infected with both PMV and PStV (Fig. 2d).

Conclusions

Although both ELISA and Western blotting worked well for the detection of infections with PMV and/or PStV, there are situations where one assay may be preferable over the other. ELISA is the simplest to run when a large number of samples require screening. ELISA may not be suitable when only a limited number of samples are to be run because of the modifications of antiserum that are required (e.g. fractionation of the IgG component and conjugation of IgG to alkaline phosphatase), when antiserum may be in short supply, or when quantification is not required. A number of different formats of indirect ELISA for detection of plant viruses which are quicker to run than the double sandwich ELISA have been reported (8,10,11). I have observed in trying to employ an indirect ELISA for PMV and PStV, as Lommel, et al. (10) have reported with carnation mottle virus and carnation ringspot virus, that interference and high backgrounds occur when using these assays in crude plant extracts (unpublished). The use of nitrocellulose as a support medium in a dot-blot assay with rabbit serum has also been unsuccessful (17).

The Western blot technique is very conservative in antiserum requirement. A volume of diluted antiserum can be stored at 4C and used a number of times without loss of efficiency of the assay. Antiserum to PMV with microprecipitin titers of 256 and 512 worked equally as well as antiserum with a titer of 1024. It is generally recommended that antiserum have a titer of 512 or greater for ELISA. The other advantage of the Western blot technique is the time in which it can be completed. Western blotting can be completed in 6-7 h when the initial electrophoretic separation is conducted in minigels. This is far less than the total time required for ELISA, although the actual amount of "hands-on" time per sample is higher when Western blotting is used. The sensitivity of Western blotting is similar to ELISA. Western blotting is also relatively inexpensive per sample if a laboratory has the equipment required for electrophoresis and electrophoretic transfer of proteins to nitrocellulose. Although protein-A-peroxidase is one of the cheaper reagents commercially available for detection of antigen-antibody reactions, it may have a disadvantage when the plant tissue being probed has a high peroxidase concentration. Peroxidase activity can be maintained through the electrophoretic separation and transfer process and react with the peroxidase substrate resulting in faint background bands. Hence, it is best to run a known positive in the gel to know where the coat protein being probed migrates. This problem can be circumvented by

using the slightly more costly alkaline phosphatase linked anti-rabbit conjugate to probe the blot after reaction with rabbit serum. Using either protein-A-peroxidase or alkaline phosphatase linked anti-rabbit conjugate, Western blotting is an inexpensive method for sensitive detection of PMV and PStV when a few samples need to be run. The initial cost of ELISA can be high, but if numerous samples are to be examined this may be the assay of choice.

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