Detection of Peanut Stripe Virus in Peanut Seed by an Indirect Enzyme-Linked Immunosorbent Assay Using a Monoclonal Antibody

J. N. CULVER and J. L. SHERWOOD, Department of Plant Pathology, Oklahoma State University, Stillwater 74078-0285

ABSTRACT

Culver, J. N., and Sherwood, J. L. 1988. Detection of peanut stripe virus in peanut seed by an indirect enzyme-linked immunosorbent assay using a monoclonal antibody. Plant Disease 72:676-679.

Indirect and double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) formats, using polyclonal antibodies alone, monoclonal antibodies (MAB) alone, or the two in combination, were compared for the detection of peanut stripe virus (PStV) in peanut seed. The MAB indirect ELISA format was shown to be the most sensitive, detecting 2.5 ng/ml of virus and detecting one PStV-infected seed part diluted in 32 healthy seed parts. The MAB indirect ELISA was also used to screen seed from the PStV-infected peanut cultivars Spanco, Pronto, Tamnut 74, Argentine, and Florunner. A small portion of cotyledon from each seed was removed and tested for PStV by the MAB indirect ELISA without adversely affecting the seed's viability. The seeds were then planted and the resulting seedlings tested for PStV infection by ELISA, symptomatology, and local lesion assay on *Chenopodium amaranticolor*. The MAB indirect ELISA readily detected PStV antigen in peanut cotyledonary tissue. Seeds determined to be virus-free by the MAB indirect ELISA always produced virus-free seedlings. However, a few seeds determined to have detectable amounts of PStV antigen by the MAB indirect ELISA also produced virus-free seedlings. All five peanut cultivars were shown to transmit PStV by seed at rates ranging from 0.4 to 5.0%.

Peanut stripe virus (PStV), a potyvirus infecting peanut (*Arachis hypogaea* L.), is widespread in Southeast Asia (9). In 1982, PStV was identified in the United States (4). The movement of PStV to the United States was apparently facilitated by the importation of PStV-contaminated peanut seed (3,4). Within the United States, contaminated seed has also been responsible for the spread of PStV to most of the major peanut-producing states (3). Although PStV has been confined primarily to institutional plantings, concerns about the spread and control of this virus have led to restrictions in germ plasm exchanges and extensive screening of peanut seed for PStV (5). The screening of peanut seed for PStV has mainly been done by an enzyme-linked immunosorbent assay (ELISA), using anti-PStV polyclonal antibodies to detect PStV in small portions of peanut cotyledon seed tissue (5).

Hybridoma technology permits the production of large quantities of uniform and highly specific antibodies that lower the chance of nonspecific reactions and allow for the standardization of tests among different laboratories (7). The objective of this study was to apply hybridoma technology to the problem of detecting PStV in peanut seed. A comparison of ELISA formats using monoclonal antibodies (MAB) alone, polyclonal antibodies alone, or the two in combination, was conducted to permit the selection of a format that is timesaving and sensitive for the detection of PStV in peanut seed.

Journal Series Article No. 5262 of the Oklahoma Agricultural Experiment Station.

Accepted for publication 17 February 1988 (submitted for electronic processing).

MATERIALS AND METHODS

Virus source, virus purification, and infected seed lot production. An isolate of PStV was acquired from J. W. Demski, University of Georgia. The virus was maintained and purified as previously described (3). Dilutions of purified virus were prepared based on the assumption that a 1-mg/ml suspension of the virus had an absorbance at 260 nm of 3.0. Seed from the peanut cultivars Spanco, Tamnut 74, Florunner, Argentine, and Pronto were acquired from J. S. Kirby, Oklahoma State University. To obtain PStV-infected seed lots, seedlings in their third and fourth true leaf stages were mechanically inoculated with PStV-infected *Lupinus albus* L. leaf tissue ground in 0.01 M of phosphate buffer (PB), pH 8.0. After approximately 2 wk, 12 PStV-inoculated seedlings, from each of the five cultivars, showing symptoms of PStV infection (3) were transplanted into bushel baskets containing a 1:4 sand to soil mixture and grown to maturity in a greenhouse. Infected seed lots were obtained in this manner for both a winter and summer growing season. Seeds harvested from each cultivar were dried and stored at room temperature for 6–9 mo before being screened for PStV.

Antibody production and purification. Polyclonal antibodies and MAB to PStV were obtained as previously described (10). Polyclonal antibodies were purified by precipitation with an equal amount of water-saturated ammonium sulfate (2) and the IgG fraction obtained by ion-exchange chromatography with DEAE Trisacryl M (LKB, Gaithersburg, MD 20877, No. 250771). Part of this IgG fraction was then conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO 63178, No. P-5521) as previously described (2). Monoclonal antibodies were also purified by ammonium sulfate precipitation. A goat anti-mouse IgG alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO 63178, No. A-5781) was used to detect the MAB.

Comparison of ELISA formats for the detection of PStV mixed with cotyledonary tissue. Four ELISA formats that used polyclonal antibodies alone, MAB alone, or a combination of the two were compared for the detection of PStV in peanut cotyledonary tissue. Peanut stripe virus-infested peanut cotyledonary tissue was obtained by adding varying concentrations of PStV to healthy cotyledonary tissue ground in the appropriate buffer (1:50, w/v). Optimal conditions for antibody dilutions in each ELISA format were determined in order to minimize nonspecific reactions and the amounts of reagents used. Absorbance values at 405 nm were taken at 0.5, 1, and 2 hr using a BIO-TEK EIA plate reader (BIO-TEK Instruments Inc., Burlington, VA 05401). Samples were repeated four times for each ELISA format and the experiment was repeated twice.

Polyclonal indirect ELISA. Sample cotyledonary tissue was ground in 0.05-M sodium carbonate buffer, pH 9.6 (coating buffer), added to microtiter plates, and incubated overnight at 4 C. Plates were then rinsed three times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tween) followed by the addition of a 1/1,000 dilution of rabbit anti-PStV polyclonal IgG conjugated to alkaline phosphatase. Plates were incubated 2 hr at room temperature, rinsed three times with PBS-Tween, and the enzyme substrate (*p*-nitrophenyl phosphate 1 mg/ml) in 0.01 M of glycine buffer containing 1 mM of $MgCl_2$ and 1 mM of $ZnCl_2$, pH 10.4, was added.

Monoclonal antibody indirect ELISA.

Sample cotyledonary tissue was ground in coating buffer, added to microtiter plates, and incubated overnight at 4 C. Plates were rinsed three times with PBS-Tween, followed by the addition of a 1/500 dilution of mouse anti-PStV MAB. Plates were incubated 2 hr at room temperature, rinsed three times with PBS-Tween, and a 1/1,000 dilution of goat anti-mouse IgG conjugated to alkaline phosphatase was added. After an additional incubation at room temperature for 2 hr, plates were rinsed with PBS-Tween and the enzyme substrate was added.

Polyclonal sandwich ELISA. Rabbit anti-PStV IgG at 1 μg/ml in coating buffer was added to microtiter plates and incubated at room temperature for 2 hr. Plates were then rinsed three times with PBS-Tween, sample cotyledonary tissue ground in PBS-Tween containing 2% polyvinylpyrrolidone (PVP, mol wt 40,000) was added, and the plates were incubated overnight at 4 C. Plates were then rinsed three times with PBS-Tween and a 1/1,000 dilution of rabbit anti-PStV polyclonal IgG conjugated to alkaline phosphatase was added. Plates were incubated for 2 hr at room temperature, rinsed three times in PBS-Tween, and the enzyme substrate was added.

Monoclonal antibody sandwich ELISA. Rabbit anti-PStV IgG at 1 μg/ml in coating buffer was added to microtiter plates and incubated at room temperature for 2 hr. Plates were then rinsed three times with PBS-Tween, sample cotyledonary tissue ground in PBS-Tween containing 2% PVP was added, and the plates were incubated overnight at 4 C. Plates were rinsed three times with PBS-Tween, and a 1/500 dilution of mouse anti-PStV MAB was added. Plates were incubated 2 hr at room temperature, rinsed three times with PBS-Tween, and a 1/1,000 dilution of goat anti-mouse IgG conjugated to alkaline phosphatase was added. Plates were incubated at room temperature for 2 hr, rinsed three times with PBS-Tween, and the enzyme substrate was added.

Screening of peanut seed for PStV. The MAB indirect ELISA, as described above, was used for the detection of PStV in peanut seed. A small portion of cotyledonary tissue (0.02–0.05 g) was removed from the nongerm end of each seed, ground in 600 μl of coating buffer, and 200 μl was applied to two wells in a microtiter plate. Extract from four healthy control seeds was placed in four separate wells in each microtiter plate. Absorbance values were taken after 2 hr incubation with substrate. The mean of the two absorbance values was taken as the reading for that sample. A known positive control was run with every set of plates to ensure the reactivity of the reagents.

Grow-out tests. Seeds screened by the MAB indirect ELISA were germinated, individually planted in a greenhouse, and observed for symptoms characteristic of PStV infection (4). Seedlings from these seeds, in their third and fourth true leaf stages, were then tested for PStV infection by the MAB sandwich ELISA as described above, except the samples were foliar tissue. Leaves of individual seedlings for the ELISA were removed and the sap was expressed by roller press and diluted 1:50 (w/v) in PBS-Tween containing 2% PVP before adding to the plates. A positive response was considered to be an absorbance value at 405 nm greater than the mean of the four healthy peanut leaf tissue control wells in that plate plus four standard deviations.

Local lesion assays on *Chenopodium amaranticolor* Coste & Reyn. were performed on seedlings that gave questionable or distinctly positive ELISA readings in the ELISA on seeds. Local lesion assays were conducted by grinding seedling leaf tissue in 0.01 M PB, pH 8.0, and rubbing this inoculum onto corundum-dusted leaves of *C. amaranticolor* with small cheesecloth pads.

Sensitivity of detecting PStV in seed. Peanut seeds that were determined by MAB indirect ELISA ($A_{405nm} \geq 2.0$) to be infected with PStV were used to determine the limit of detection of PStV in cotyledonary tissue. The cotyledonary tissue of infected seed was ground in coating buffer at a 1:50 (w/v) dilution. The ground cotyledonary tissue was then diluted 1/2, 1/4, 1/16, 1/32, and 1/64 with a 1/50 dilution of healthy cotyledonary tissue in coating buffer. Dilutions of infected cotyledonary tissue were then tested by the MAB indirect ELISA.

RESULTS

Comparisons of ELISA formats for the detection of PStV in peanut seed. Results indicated that all four ELISA formats easily detected PStV in peanut cotyledonary tissue. However, the sensitivity and background levels varied between each format (Fig. 1). The polyclonal indirect ELISA was adequate for detecting PStV concentrations down to

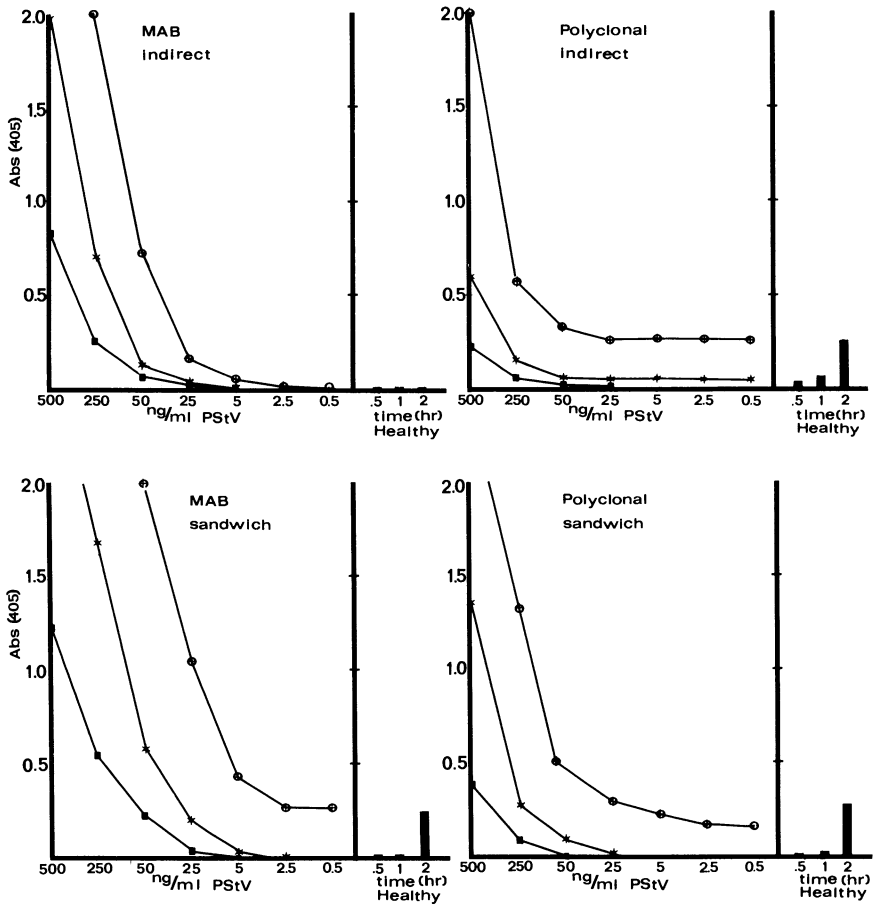

Fig. 1. Comparison of four enzyme-linked immunosorbent assay (ELISA) formats at different incubation times for detection of peanut stripe virus (PStV) in peanut cotyledonary tissue. The four formats are 1) monoclonal antibody (MAB) indirect, 2) polyclonal indirect, 3) MAB sandwich, and 4) polyclonal sandwich. Incubation times are 0.5 hr (square), 1 hr (star), and 2 hr (circle) for different amounts of PStV in cotyledonary tissue presented in the line graphs. Absorbance values for healthy controls are indicated in bar graphs to the right of each line graph. See text for details of each ELISA format.

250 ng/ml of virus after 30 min incubation with substrate. The polyclonal sandwich ELISA was five times more sensitive and able to detect PStV concentrations as low as 50 ng/ml of virus after 1 hr of incubation with substrate. The MAB sandwich ELISA was capable of detecting 5 ng/ml of PStV after a 1 hr incubation with substrate. The most sensitive ELISA format tested was the MAB indirect ELISA. Although taking twice as long to achieve the same level of intensity as both sandwich ELISA formats, the MAB indirect ELISA was capable of detecting 2.5 ng/ml of PStV after 2 hr incubation with substrate (mean absorbance for 2.5 ng/ml PStV = 0.050 ± 0.005, mean absorbance for healthy check = 0.001 ± 0.001). For this reason, the MAB indirect ELISA format was chosen to screen the PStV-infected seed lots.

Correlation of the MAB indirect seed ELISA and grow-out tests. Out of 1,544 seeds tested by ELISA, 1,184 produced seedlings. The seedlings that gave positive ELISA readings in the ELISA on seedling tissue always had symptoms typical of PStV infection. No symptoms were observed on seedlings with negative ELISA readings in the ELISA on seedling tissue. A total of 85 local lesion assays were done for seedlings germinated from seed that had tested questionably or distinctly positive in the MAB indirect ELISA of cotyledonary tissue. Local lesions were always obtained on *C. amaranticolor* from seedlings that tested positive for PStV infection by ELISA and symptomatology. Local lesions were never obtained on *C. amaranticolor* from seedlings that tested negative by ELISA. Results of grow-out tests (ELISA, symptomatology, and local lesion assay) for each seedling were then compared with the response of the seed in the MAB indirect ELISA in order to determine a positive-negative threshold for the seed assay.

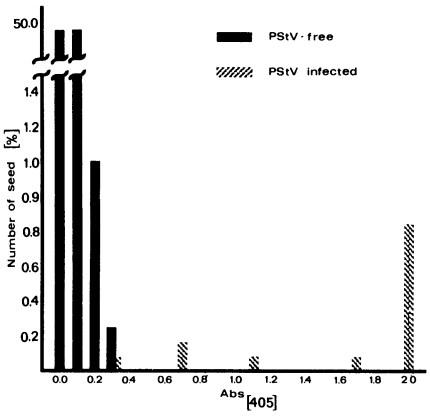

Fig. 2. Ranking of monoclonal antibody indirect enzyme-linked immunosorbent assay values (absorbance at 405 nm) of peanut seed tested for peanut stripe virus.

Determination of a positive-negative threshold for the MAB indirect ELISA was made using only absorbance values from those seeds that produced seedlings. An initial attempt to use the average of the healthy seed controls plus four standard deviations gave inconsistent results for individual microtiter plates and cultivar groupings. The problem encountered with this method was that it gave positive responses to several seeds that produced healthy seedlings in the grow-out test. The alternative method used was a visual ranking of seed cotyledon sample ELISA absorbance values similar to that described for squash mosaic virus (8). A positive-negative threshold could more readily be observed using this method (Fig. 2). When a positive-negative threshold of 0.1 OD (based on absorbance values of healthy controls) was used, 1.3% of the 1,184 seeds assayed that grew to seedlings could not be correctly assigned as negative based on the results of the ELISA on the seed alone. When a positive-negative threshold based on the ranking of absorbance values of <0.3 OD was used, only three seeds of the 1,184 seeds assayed that grew to seedlings (less than 0.3% of the seeds assayed) could not be correctly assigned as distinctly positive or negative. The three seedlings from these seeds were healthy based on ELISA, symtomatology, and local lesion assay.

Sensitivity of detecting PStV in seed. Tests for the detection of PStV in peanut seeds were performed on 10 seeds that had previously given strong positive responses ($A_{405nm} \geq 2.0$) for PStV in the MAB indirect ELISA. This group of seed was chosen because it was the largest group of seed that gave a uniform ELISA absorbance value. The mean absorbance value for the healthy control cotyledonary tissue was 0.016, while the 10 infected cotyledonary tissue samples, diluted 32 times with healthy cotyledonary tissue, gave absorbance values between 0.078 and 0.669. This indicated that the MAB indirect ELISA was capable of detecting one infected seed part diluted with 32 healthy seed parts for these seeds. Individual seed that gave initial ELISA readings lower than $A_{405nm} = 2.0$ could not be diluted to this extent and still obtain absorbance values greater than the controls.

Seed transmission of PStV. The percentage of seed transmission of PStV was determined for the five peanut cultivars tested (Table 1). All seed tested by the MAB indirect ELISA, including the seed that did not produce seedlings, was used to determine the percentage of transmission. Results indicated that PStV could be transmitted in all five cultivars. The percentage of transmission ranged from a low of 0% to a high of 5%, depending on the cultivar and the growing season.

Table 1. Frequency of seed transmission of peanut stripe virus in five susceptible cultivars of peanut grown in the greeenhouse in two different seasons

Cultivar	Number of seed tested	Number of plants tested	Percent seed[a] transmission
	Winter growing season[b]		
Argentine	92	69	3
Florunner	100	97	5
Pronto	88	76	0
Tamnut 74	100	91	3
Spanco	132	121	2
	Summer growing season[c]		
Argentine	153	133	0.5
Florunner	219	160	0.4
Pronto	220	133	0.8
Tamnut 74	220	112	0
Spanco	220	192	0

[a] Frequency of seed transmission determined by a monoclonal antibody indirect ELISA.
[b] October until March.
[c] May until September.

DISCUSSION

As previously reported (1,5), a portion of a cotyledon could be removed from a peanut seed without substantially lowering the seed's viability. Using no special modifications for germinating or planting test seed, we obtained a 76% survival rate for peanut seeds tested in this manner. The high survivability of peanut seeds in this assay makes it possible to obtain PStV-free germ plasm lines directly from tested seeds.

Of the four ELISA formats evaluated, the MAB indirect ELISA was determined to be the most sensitive for the detection of PStV in peanut cotyledonary tissue. However, the MAB sandwich ELISA also worked well for detecting PStV in peanut seeds, being only slightly less sensitive than the MAB indirect ELISA. A polyclonal sandwich ELISA, similar to the one previously reported for the detection of PStV in peanut seed (5), proved to be 20 times less sensitive than either the MAB sandwich or MAB indirect ELISA formats. The polyclonal

indirect ELISA was the least sensitive format tested. The polyclonal indirect ELISA and the MAB indirect ELISA were not strictly parallel tests. Initial comparisons between two polyclonal indirect ELISA formats using the probe IgG conjugated to alkaline phosphatase, and probe IgG followed by goat anti-rabbit conjugated to alkaline phosphatase, indicated the former of the two formats resulted in less background interference. Therefore, the better of the polyclonal indirect ELISA formats was used in comparison with the MAB indirect ELISA.

It is preferred to be able to set a definitive positive-negative threshold based on the average and standard deviation of the absorbance readings of the healthy controls. In the case of differentiating PStV-infected seed and uninfected seed it was not possible to set a definitive positive-negative threshold based solely on absorbance readings of healthy controls. An alternative method of ranking the seed cotyledon sample ELISA absorbance values was satisfactory, and a discernable difference in A_{405nm} values between seed with PStV and seed without PStV was evident. With this ranking method, only three of the 1,184 seeds assayed that grew to seedlings could not be definitively classified as negative based on ELISA results from the cotyledonary tissue assay alone. No seed that developed into PStV-infected seedlings was identified as negative for PStV by this method.

Although PStV has been found in both the cotyledon and embryo of the peanut seed (5), it is possible that on some occasions virus or PStV antigen may be present only in the cotyledon and not in the embryo, thus creating a false positive reading in the ELISA. False positive readings could also be obtained from nonviable virus particles that would not cause infection but would still produce a positive ELISA reading. Degradation of viral particles may have occurred during the 6–9 mo seed storage period before testing, thus causing a reduction in the number of infected seedlings. The lowering of the percentage of seed transmission, due to increased seed storage times, has already been reported for squash mosaic virus in cantaloupe seed (8).

The presence of false-positive readings would be undesirable for the certification of virus-free seed, because this would lead to the abandonment of clean seed lots (7,8). However, in the case of PStV, where some quarantine procedures are in effect, false-positive readings produced by the presence of nonviable viral antigen would be acceptable, because this indicates the seed originated from a virus-infected plant (7).

The MAB indirect ELISA was able to detect PStV (in some, but not all cases tested) in one infected seed portion diluted up to the equivalent of 32 healthy seed portions. Dilutions of this magnitude would enable the screening of large seed lots in a short amount of time and at less expense. Being able to screen a large number of seeds at once would also give a more accurate estimate of the extent of PStV infection in a seed lot. However, because some seed has lower amounts of virus, the amount of seed that could be tested would depend on the acceptable level of infected seed. We have not tried to address this issue in this study.

The highest observed percentage of peanut seed transmission for PStV was 5% for cv. Florunner and 3% for cv. Argentine in the winter growing season. This amount of seed transmission is substantially lower than previous reports of 11.0% for cv. Florunner and 10.0% for cv. Argentine in a winter growing season at a different geographical area (5). This difference in seed transmission indicates that environmental factors may play a large role in the transmission and retention of PStV in the peanut seed.

The testing of peanut seed is important in the control of the spread of PStV. A MAB indirect ELISA has provided a very sensitive way to detect PStV in peanut seed. The availability of a MAB to PStV eliminates some of the problems, such as limited amounts of antiserum or variability among antiserum lots, associated with the use of polyclonal antibodies (6). The incorporation of a MAB in an indirect ELISA also allows for the development of standardized tests among laboratories working with PStV. Indirect ELISA formats also require fewer steps, are simpler, faster, and more conservative of reagents.

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