SEROLOGICAL DETECTION OF PEANUT STRIPE VIRUS

By

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PREFACE

The focus of this study was to develop monoclonal and polyclonal antibodies to peanut stripe virus (PStV). These antibodies were then characterized and compared for the ability to detect PStV by various serological methods in both peanut leaf and seed tissues. The serological methods used were enzyme-linked immunosorbent assay, dot-immunobinding assay, and Western blotting.

Methods for the detection of PStV were then applied to the problem of finding resistance to PStV in peanut germ plasm lines and the development of a peanut seed assay for the detection of PStV. For both applications the serological detection methods developed proved invaluable in the discovery of resistance and the development of a seed detection assay. The findings of this study should provide a basis for the standardization of detection assays and the control of PStV.

I wish to express my sincere appreciation to all the people who have assisted me throughout my Master's program here at Oklahoma State University. Special gratitude is extended to my major adviser, Dr. John L. Sherwood, for his guidance, encouragement, and friendship. I also extend my appreciation to the other members of my

committee, Dr. Mark R. Sanborn and Dr. Hassan A. Melouk, for their helpful suggestions and professional expertise.

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CHAPTER I

INTRODUCTION

In 1982 a new virus infecting peanut appeared in the United States with the potential to endanger commercial peanut production. The virus was identified as peanut stripe virus (PStV) and had apparently been transported to the United States in infected peanut seed from the Peoples Republic of China in 1979 (1,2). The infected seed was processed through the USDA Plant Germplasm Quarantine Center and the Plant Introduction Office at Beltsville, Maryland and then sent to the Southern Regional Plant Introduction Station in Experiment, Georgia for grow-out tests and inspections (1). However, PStV remained undetected for three years before being isolated and characterized as a new virus in 1982 (1). The delay in recognizing PStV as a new virus was attributed to its mistaken identification as peanut mottle virus which is endemic throughout the United States (1). Between 1979 and 1986 PStV has spread to most of the major peanut producing states. These states include Georgia, North Carolina, Florida, Virgina, Texas, and Oklahoma (1,2).

PStV is classified as a member of the potyvirus group and characterized by a filamentous rod shaped particle of

752 nm in length with a 33.5 kilodalton coat protein and a single strand of RNA of 3,100 kilodaltons (2). Physical properties of the virus include a longevity *in vitro* of 3 days at 20 C, a temperature inactivation point between 60-65 C, and a dilution end point between 10^{-3} and 10^{-4} (2). PStV produces local lesions on *Chenopodium amaranticolor*, is aphid transmitted by *Aphis craccivora* in a non-persistant manner, and is seed transmitted in peanuts (2,3). The host range of PStV includes several common weeds eg. *Desmodium* (beggarweed) and *Indigofera* (indigo), soybeans, and other legume crops (2,4). PStV has been reported to be serologically related to the potyviruses blackeye cowpea mosaic virus, soybean mosaic virus, and clover yellow vein virus (2).

Yield reductions in peanut due to PStV infection are still uncertain. However, greenhouse studies have indicated that losses could be as high as 20% for some peanut cultivars (2). It has also been estimated that for every one percent of the peanut crop infected with PStV an average loss of 425,000 dollars could occur (1).

The spread of PStV has been facilitated by the passage of infected peanut seed from one researcher to another (1,3). Studies have also shown that peanut plants infected with PStV have occured at the same location for four consecutive growing seasons while not being found in fields near that location (4). This suggests that PStV is persistent in the environment but that once established does

not spread rapidly. However, several characteristics of PStV could significantly enhance this spread. These characteristics include a high rate of seed transmission, transmission by an aphid vector, and several common weed hosts (1,6).

PStV is reportedly wide spread throughout much of Southeast Asia (7). To stop the movement of PStV into and around the United States the USDA-ARS has suggested that all Arachis germ plasm exchange be channeled through the Plant Germplasm Quarantine Center, Beltsville, Maryland, shipments abroad be given phytosanitary certifications, and all materials being brought into the United States be given import permits (8). Quarantine restrictions have also been placed on peanut seed from China, Thailand, and the Philippines (8).

Once PStV has been identified in a field or seed lot the current control measures have been to destroy the infected plants or seeds (1,6). Such measures have lead to destruction of seed production fields, delays in the release of new peanut cultivars, and the loss of research data (6,9). This type of control relies heavily on the rapid detection of the virus and is difficult to do using conventional methods such as host ranges and local lesion assays.

Currently the detection of PStV is done mainly through serological methods which utilize polyclonal serum (1,3). It has been the purpose of the following research to

improve this detection through the development of monoclonal antibodies to PStV and to employ these antibodies for detecting PStV in peanut plant and seed tissues. The aim being to eliminate many of the problems associated with polyclonal serum by using monoclonal technology to aquire large quantities of highly specific antibodies that can provide more sensitive and standardized detection assays (5).

This thesis includes three manuscripts, each representing an independent chapter written in a format that will facilitate their submission for publication to a national scientific journal. The approval of this format is in accordance with the Graduate College policy of accepting a thesis in manuscript form.

Chapter II, is entitled "Production and Characterization of Monoclonal Antibodies and Polyclonal Serum for the Detection of Peanut Stripe Virus by Enzyme-Linked Immunosorbent Assay, Dot-Immunobinding Assay and Western Blotting". This chapter compares monoclonal and polyclonal antibodies in enzyme-linked immunosorbent assay (ELISA), dot-immunobinding assay, and Western blot for the detection of PStV in both peanut leaf and seed tissue and will be submitted to the disease detection section of "Phytopathology". Chapter III, entitled "Resistance to Peanut Stripe Virus in Arachis Germ Plasm", provides the first report of resistance to PStV in any peanut germ plasm line or cultivar and has been accepted with revision in

the resistance section of "Plant Disease". Chapter IV, entitled "Detection of Peanut Stripe Virus in Peanut Seed by a Monoclonal Antibody Based Non-Sandwich Enzyme-Linked Immunosorbent Assay", reports on the use of a monoclonal antibody in an ELISA to detect PStV in peanut seed and will also be submitted to "Plant Disease".

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CHAPTER II

Production and Characterization of Monoclonal Antibodies and Polyclonal Serum for the Detection of Peanut Stripe Virus by Enzyme-Linked Immunosorbent Assay, Dot-Immunobinding Assay, and Western Blotting

ABSTRACT

Monoclonal antibodies (MCA) to peanut stripe virus (PStV) were obtained by fusing spleen cells from mice immunized with PStV to a mouse myeloma cell line. MCAs against PStV, produced by hybridoma cell lines 3AB5 and 7C14, were of the IgG_{2a} subclass and bound to different antigenic sites of PStV. MCAs from 3AB5 and 7C14 were compared to rabbit anti-PStV polyclonal serum for the detection of PStV in peanut leaf and seed tissues by enzyme-linked immunosorbent assay (ELISA), dot-immunobinding assay, and Western blotting. ELISA was also used to determine the reactivity of both MCA and polyclonal serum to other plant viruses. In ELISA, MCAs from both 3AB5 and 7C14 detected PStV with lower background levels in both peanut leaf and seed tissues than did the polyclonal serum. In Dot-immunobinding assays, MCA from 7C14 was superior to polyclonal serum for detecting PStV in either infected peanut leaf or seed tissue. MCA from 3AB5 was unable to detect PStV in leaf tissue by dot-immunobinding assay but

was superior to polyclonal serum for detecting PStV in peanut seed tissue. MCA from 7C14 and polyclonal serum were equally satisfactory for detecting PStV by Western blot. MCA from 3AB5 was unable to detect PStV in infected peanut leaf or seed tissue by Western blot. In ELISAs against several other plant viruses, polyclonal serum and MCAs from 3AB5 and 7C14 reacted strongest to PStV and slightly to several other plant viruses. Both MCAs and polyclonal serum reacted with peanut mild mottle virus.

INTRODUCTION

Peanut stripe virus (PStV) was introduced into the United States in 1979 in infected peanut seed from the Peoples Republic of China (2,3). PStV has since spread to most of the major peanut producing states but has been contained primarily to research plots and fields (2,3,5). The introduction and spread of PStV has resulted in reduced germ plasm exchange, destruction of field plots and breeder seed, delays in the release of new peanut cultivars, and the loss of research data (13,18). Because control efforts to eliminate PStV have been dependent on the detection of the virus in peanut seed and leaf tissues, the need has arisen for reliable and quick methods of PStV detection (2,4). This is difficult if not impossible to obtain using traditional detection methods such as local lesion assays and host ranges.

Currently, detection of PStV is by serological methods

using anti-PStV polyclonal serum (2,4,14). However, the use of polyclonal serum holds several problems which may interfere with the reliability of test results. These problems include difficulties in obtaining large quantites of antiserum, possible non-specific reactions to other antigens, and the variability of different polyclonal serums to the same antigen (9,11,15). Such problems can generally be circumvented by the use of monoclonal antibody technology for the production of highly specific antibodies to be used for the serological detection of plant viruses (7,9).

The purpose of this study was to produce and characterize monoclonal antibodies (MCA) and polyclonal serum to PStV. The serological reactivity of both MCA and polyclonal serum was then compared by enzyme-linked immunosorbant assay (ELISA), dot-immunobinding assay, and Western blotting for the detection of PStV in peanut leaf and seed tissues. MCA and polyclonal serum were also compared for serological reactivity to other plant viruses.

MATRIALS AND METHODS

PStV source and purification. An isolate of PStV, obtained from J. W. Demski, Department of Plant Pathology, University of Gerogia, Experiment, GA, was maintained in Lupinus albus L. by mechanical inoculation of infected leaf tissue ground in 0.01 M phospate buffer (p.b.), pH 8.0 and applied to leaf surfaces of the peanut cultivar Argentine,

dusted with 400 mesh corundum, using small cheesecloth pads.

PStV purifications were done at 4 C by homogenizing freshly cut infected L. albus leaf tissue in 0.1 M p.b., pH 8.0 with 0.001 M dithioerythritol (1 g tissue : 2.5 mls buffer). The homogenate was filtered through 2 layers of cheesecloth and clarified by shaking vigorously for 5 min with chloroform (10% v/v) followed by centrifugation at 10,000 rpm for 10 min in a Sorvall GSA rotor. The supernatant was then removed and the virus precipitated by the addition of 4% (w/v) polyethlene glycol (mol. wt. 8,000) and 1.5% (w/v) potassium chloride. This was then stirred for 30 min, allowed to stand for 2 hrs, and the resulting precipitant collected by centrifugation as above. The pellet was then resuspended overnight in approximately 1/10 its original volume in 0.1 M p.b., pH 8.0. A final clarification was preformed by shaking for 1 min with chloroform (10% v/v) followed by centrifugtion for 10 min at 10,000 rpm in a Sorvall SS-34 rotor. The supernatant was then layered onto 10-40% sucrose gradients made up in 0.1 M p.b., pH 8.0. The viral fraction was separated by ultracentrifugation at 24,500 rpm for 2:20 min in a Beckman SW-25.1 rotor and collected by an ISCO density gradient fractionator and UV analyzer (Lincoln, NE 68504). The remaining sucrose was removed by centrfugation at 29,500 rpm for 2 hrs in a Beckman number 30 rotor. The resulting viral pellet was then resuspended in 0.01 M p.b., pH 8.0 and the virus concentration determined based on an absorbance of 3.0

at 260 nm being equivalent to 1 mg/ml (17).

Production and purification of polyclonal serum to PStV. A New Zealand white rabbit was injected intramuscularly twice weekly with 1 mg/ml purified PStV emulsified in an equal volume of Freund's complete adjuvant until a antiserum titer of 1024, as determinined by microprecipitin test against purified PStV, was obtained. The antiserum was then purified by precipitation in an equal amount of water saturated ammonium sulfate (1) and the IgG fraction obtained by ion-exchange chromatography with DEAE Trisacryl M (LKB, Gaithersburg, Maryland 20877, No. 250771). The IgG fraction was then conjugated to alkaline phosphtase (Sigma Chemical Co., St. Louis, MO 63178 No. P-5521) as previously discribed (1).

Monoclonal antibody production and characterization. Mouse myeloma cell line p3x63Ag8.653 (653) was obtained from E. L. Halk (Agrigenetics Corporation, Madison, WI 53716) and maintained in RPMI 1640 medium (K. C. Biological, Inc., Lenexa, KS 66215) supplemented with 10% horse serum (Hyclone Laboratories, Logan, UT 84321), 1.0 g/L $NaCO_3$, 0.06 g/L penicillin-G, 0.10 g/L streptomycin sulfate, and 0.30 g/L L-glutamine (Sigma Chemical Co., St. Louis, MO 63178). Cells were grown in a humidified incubator at 37 C with 5% CO_2. Cells were also maintained in SP medium (40% RPMI 1640, 40% RPMI 1640 conditioned by 48 hrs growth of myeloma cells, 20% horse serum, and 0.05 mg/ml gentamicin) for 1 wk prior to fusion to insure log phase growth at the time of fusion.

Balb/c mice were injected weekly for three weeks with 250-500 ug of purified PStV emulsified in an equal volume of Freund's complete adjuvant. Four days prior to the fusion an injection of 250 ug/ml purified virus in distilled water was made.

The procedure used for cell fusion was adapted from a method used by Kohler and Milstein (12). Spleens of the immunized mice were removed aseptically and washed sequentially three times with a balanced salt solution of 8.0 g/L NaCl, 0.40 g/L KCL, 0.35 g/L $NaCO_3$ and 0.002% phenol red. Washed spleens were then macerated through a 0.1 mm^2 nylon mesh into approximatly 40 mls of serum-free RPMI 1640 medium. Spleen cells were then counted with a hemocytometer and an equal volume of serum-free RPMI 1640 medium, containing 2 to 3 times as many 653 cells, was mixed with the spleen cells and centrifuged for 5 min at 400 g. Cells were then resuspended in serum-free RPMI 1640 medium containing 35% PEG (mol. wt. 1000) and centrfuged at 400 g for 5 min followed by 30 sec at 2000 g. The undisturbed pellet was incubated at 37 C for 6 min and the PEG removed by two consecutive centrifugations at 500 g for 5 min with each pellet being resuspended in 40 mls of SP medium. Resultant cells were incubated overnight in a 75 cm^2 tissue culture flask. The next day, cells were centrfuged at 400 g for 5 min and resuspended in HAT medium (SP medium supplemented with 10^{-4} M hypoxanthine, 4 x 10^{-7} M aminopterin, and 1.6 x 10^{-5} M thymidine) and distributed

into 24-well tissue culture plates. Cells were kept in HAT medium for 1 wk and then placed in HT medium (HAT medium w/o aminopterin) for an additional wk followed by regular feedings in SP medium.

Two weeks post fusion, hybridomas were screened for antibody production to PStV by ELISA. All ELISA incubation periods were for 2 hrs at room temperature unless otherwise stated. Microtiter plates were also washed three times between each step with phosphate buffered saline containing 0.05% Tween-20 (PBS-Tween). ELISA was preformed by coating microtiter plates with 1 ug/ml of the IgG fraction of the rabbit anti-PStV polyclonal serum. PStV infected or healthy L. albus leaf tissue, triturated in phosphate buffered saline (PBS) w/ 2% polyvinyl prrolidone (PVP, mol. wt. 40,000), was then added to the plates and incubated overnight at 4 C. Supernatant from each hybridoma cell line was added to both infected and healthy control ELISA wells followed by the addition of an anti-mouse alkaline phosphatase cojugate (Sigma Chemical Co., St. Louis, MO 63178, No. A-5781). Enzyme substrate was then added and absorbance at 405 nm compared for infected and healthy leaf tissue by way of a BIO-TEK EIA plate reader (Bio-Tek Instruments, Inc., Burlington, VT 05401).

Hybridoma cells positive in ELISA for infected tissue and negative for healthy tissue were then cloned using a soft agar method. This was done by adding 50 ul of a hybridoma cell suspension to a 10 cm petri dish containing

10 ml of SP medium. A volume of a 5% Difco agar solution was then added to obtain a final agar concentration of approximatly 0.3%. Petri dishes were then incubated for two weeks and single colonies removed and expanded in 24-well tissue culture plates. Cell lines were then retested for antibody production to PStV, followed by a second cloning and expansion.

Isotyping of antibodies, produced by cloned hybridoma cell lines, was done using a kit for the identification of the subclass specific antisera; IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA, IgM, kappa light chain, and lamba light chain. (Zymed Laboratories, Inc., San Francisco, CA 94080).

A double antibody binding ELISA was used to compare the antigen binding sites of different MCAs to PStV (8). The ELISA incubation times, reagents, and plate washings were the same as before unless otherwise stated. In the double antibody binding ELISA, microtiter plates were coated with anti-PStV polyclonal serum followed by the addition of a 1/100 dilution of PStV infected leaf tissue. A saturating amount of a MCA was then added and incubated at room temperature for 2 hrs. After which a second saturating amount of a different MCA was added, incubated for 2 hrs at room temperature, and followed by the addition of anti-mouse conjugate and enzyme substrate. The design of this ELISA was such that the response of two MCAs could be measured (absorbance at 405 nm) separately or in competition with each other for a PStV antigenic binding site.

Dot-immunobinding assay and Western blotting. Samples for dot-immunobinding assays were prepared by trituration of healthy or infected peanut leaf and cotyledon seed tissues in Tris-buffered saline (TBS). Ten ul of each triturated sample were spotted on strips of nitrocellulose paper. Remaining binding sites on the nitrocelulose strips were then blocked by incubation in TBS containing 5% Carnation non-fat dry milk (Los Angeles, CA 90036) for 30 min at room temperature. Nitrocellulose strips were washed 3 times in TBS followed by incubation with either polyclonal serum or monoclonal antibodies at 1/200 dilutions in TBS for 1 hr at room temperature. Strips were then washed 3 times with TBS and incubated for 1 hr at room temperature in anti-rabbit or anti-mouse IgG alkaline phosphatase (Sigma Chemical Co., St. Louis, MO 63178, No. A-8025 & A-5781). Strips were washed a final 3 times with TBS and enzyme substrate applied (5 mg nitro blue tetrazolium in 15 ml of 0.01 M Tris-buffer, pH 9.5, with 0.1 M NaCl and 5 mM $MgCl_2$, with 2.5 mg of 5-bromo-4-chloro-3-indoyl-phosphate in 50 ul of N,N-dimethyl-formamide). This substrate produces a purple precipitate indicating a positive reaction.

Western blot samples were prepared by mixing peanut leaf and seed tissues, triturated in TBS, with an equal volume of sample buffer (62.5 mM Tris-HCl buffer, pH 6.8, containing 10% SDS, 5% 2-mercaptoethanol, and 0.002% Bromophenol blue) and heating for 5 min at 95 C. Infected peanut seed samples were either naturally or artificially

obtained by spiking healthy cotyledon seed tissue with 50 ug/ml of purified PStV.

Sample proteins were then separated by electrophoresis on a 1.5 mm 12% polyacrylamide separating gel (pH 8.8) with a 5% polyacrylamide stacking gel (pH 6.8) in Mighty Small I or SE 600 vertical slab gel apperatus (Hoefer Scientific, San Fransisco, CA 94107). Electrophoresis buffer contained 25 mM Tris, 192 mM glycine, and 3.46 mM SDS, pH 8.3. Samples were electrophoresed at 40 mA for the Mighty Small I gels and 25 mA for the larger SE 600 gels until the bromophenol blue dye font reached the bottom of the gel.

Separated proteins were then transblotted from the polyacrylamide gel to nitrocelulose paper using a TE 42 Transphor unit (Hoefer Scientific, San Fransisco, CA 94107) at 100 V for 1 hr. Transfer buffer contained 25 mM Tris, 129 mM glycine, and 20% (v/v) methanol, pH 8.3. Once transblotting was complete the nitrocellulose paper recieved the same treatment as described in the dot-immunobinding assay. However, protien-A-peroxidase (PAP), 1ug/ml, (Sigma Chemical Co., St. Louis, MO 63178, No. P-8651) was also used in place of anti-rabbit and anti-mouse alkaline phosphatase conjugate. The substrate for PAP was one part 4-chloro-1-napthol (3mg/ml in methanol), five parts TBS, and 0.018 parts of a 3% hydrogen peroxide. A positive reaction gave a purple precipitant.

ELISA procedures. Polyclonal serum and MCA were compared in sandwich, non-sandwich, direct, and indirect

ELISA formats. A sandwich ELISA was done by coating microtiter plates with either a 1/1000 dilution of anti-PStV polyclonal IgG or a 1/500 dilution of anti-PStV MCA in 0.05 M sodium carbonate buffer, pH 9.6, (coating buffer) and incubating for 2 hrs at room temperature. Plates were then washed 3 times with PBS-Tween and a 1/50 dilution of PStV infected or healthy peanut leaf and cotyledon seed tissues, triturated in PBS-Tween containing 2% PVP, added and incubated overnight at 4 C. Infected seed was artificially obtained by spiking triturated healthy seed tissue with 50 ug/ml of purified PStV. In non-sandwich ELISA, microtiter plates were first coated with a 1/50 dilution of either triturated PStV infected and healthy peanut leaf tissue or titurated cotyledon seed tissue, with and without 50 ug/ml of purified PStV, in coating buffer and incubated overnight at 4 C.

Both sandwich and non-sandwich ELISA plates were then washed 3 times with PBS-Tween followed by the addition of either anti-PStV polyclonal serum and anti-PStV MCA for indirect ELISA formats or anti-PStV rabbit IgG, conjugated to alkaline phosphatase, for direct ELISA formats. Plates were incubated again for 2 hrs at room temperature followed by another 3 washes in PBS-Tween at which time plates with indirect ELISA formats were filled with a 1/1000 dilution of anti-mouse or anti-rabbit IgG alkaline phosphatase, while plates with direct ELISA formats were filled with enzyme substrate (p-nitrophenylphosphate 1 mg/ml in 0.1 M glycine

buffer with 1mM of $MgCl_2$ and $ZnCl_2$, pH 10.4). After an additional 2 hrs incubation at room temperature indirect ELISA plates were washed 3 times in PBS-Tween and substrate added as in the direct ELISA format. Absorbance values at 405 nm were then taken at 30 and 60 min using a BIO-TEK EIA plate reader.

ELISA with other plant viruses. The serological reactivity of MCA and polyclonal serum to several plant viruses was compared by both sandwich and non-sandwich ELISAs (Table 1). Steps for the two ELISA formats were identical to those previously described except that viral samples were obtained from infected host leaf tissue at dilutions of 10^{-1} to 10^{-3} and sandwich ELISA plates were coated first with antiserum specific to each respective virus.

RESULTS

MCA characterization. Out of 19 hybridoma cell lines found to be producing antibodies to PStV two (3AB5 and 7C14) were selected for comparison to the anti-PStV polyclonal serum for the detection of PStV by ELISA, dot-immunobinding assay, and Western blot. Both 3AB5 and 7C14 lines were shown to produce antibodies of the IgG_{2a} subclass. For the purpose of discussion the antibodies produced by these two cell lines will be refered to as MCA 3AB5 and MCA 7C14. Absorbance values from the double antibody binding ELISA were used to determine the additivity

index of MCA 3AB5 against MCA 7C14. The additivity index gives a percentage of the response (absorbance at 405 nm) of a MCA alone and in competition with a second MCA for an antigen binding site. The closer this percentage is to 100 the less the competition is between the two MCAs for the same antigen binding site. The additivity index for MCA 3AB5 against MCA 7C14 was greater than 63 percent. This indicated that MCAs from the two cell lines bound to different antigenic sites of PStV. Attempts to conjugate MCAs from cell lines 3AB5 and 7C14 to alkaline phosphatase were unsuccesful as indicated by a total a loss of serological activity of the antibodies after conjugation.

Dot-immunobinding assay and Western blotting for the detection of PStV. Polyclonal serum, MCA 3AB5, and MCA 7C14 were compared for the detection of PStV in a dot-immunobinding assay with purified PStV, PStV infected peanut leaf, and PStV infected cotyledon seed tissue (Fig. 1). Infected leaf tissue was detectable at a 1/1000 dilution only with MCA 7C14. Infected cotyledon seed tissue could be detected at a 1/1000 dilution with both MCA 3AB5 and MCA 7C14 with MCA 3AB5 producing the lowest background response in the healthy seed tissue control. MCA 3AB5, MCA 7C14, and polyclonal serum all detected purified PStV at a 1/1000 dilution of a 50 ug/ml viral suspension representing approximatly 0.5 ng of PStV placed on the nitrocellulose paper. However, MCA 3AB5 responded less intensely than did MCA 7C14 or polyclonal serum for the detection of purified

PStV. Polyclonal serum proved to be of little use in detecting PStV infected leaf or seed tissue in dot-immunobinding assays due to high background responses in the healthy controls.

Approximately 1.5 ug of purified PStV could be detected by Western blot with MCA 3AB5, MCA 7C14, and polyclonal serum, however, polyclonal serum produced the strongest response. MCA 7C14 and polyclonal serum could also detected PStV in infected leaf and cotyledon seed tissues, again with the polyclonal serum producing the strongest response. Polyclonal serum also reacted slightly to proteins in the healthy leaf and cotyledon seed tissues (Fig. 2). MCA 3AB5 could not detect PStV in either infected leaf or cotyledon seed tissues using the Western blot (Fig. 3). There was no detectable differance between reactions for either naturally or artificially obtained infected cotyledon seed tissue.

ELISA formats for the detection of PStV. Sandwich, non-sandwich, direct, and indirect ELISA formats with various combinations of polyclonal serum, MCA 3AB5, and MCA 7C14 were used for the detection of PStV in peanut leaf and cotyledon seed tissues. In sandwich ELISA, plates were first coated with an antigen specific antibody followed by the addition of sample and then probe antibody. In non-sandwich ELISA, plates were coated first with antigen and then probe antibody. Sandwich and non-sandwich ELISA were either direct, using an enzyme-labled probe antibody, or indirect, using a second enzyme-labled antibody specific for

the probe antibody.

The eight different ELISA formats used to detect PStV varied greatly for response time and background levels (Table 2). The formats with the quickest response and the lowest background levels for detecting PStV in peanut leaf tissue were a direct sandwich ELISA using only the polyclonal serum and an indirect sandwich ELISA in which polyclonal serum was coated to the plate and MCA 3AB5 used as a probe for PStV. MCA 7C14 in an indirect non-sandwhich ELISA also worked well for detecting PStV in peanut leaf tissue, although taking four times as long to reach an equvialent level of response. PStV was not readily detected in infected peanut leaf by conjugated polyclonal serum in a direct non-sandwich ELISA, MCA 3AB5 in an indirect non-sandwhich ELISA, and ELISA formats which coated first with either MCA 3AB5 or MCA 7C14.

MCA 7C14 in an indirect non-sandwhich ELISA or polyclonal serum in a direct sandwich or non-sandwhich ELISA gave the most satisfactory results for the detection of PStV in spiked cotyledon seed tissue (Table 2). All other formats resulted in either slow response times or high background levels.

Reaction of MCA and polyclonal serum to other plant viruses. Polyclonal serum, MCA 3AB5, and MCA 7C14 were tested for their serological reaction to 16 plant viruses by both sandwhich and non-sandwich ELISA (Table 3). For all three antibodies the strongest response was obtained

from the PStV infected plant tissue. In a sandwich ELISA the IgG fraction of the polyclonal serum, conjugated to alkaline phosphatase, also reacted slightly with tomato spotted wilt virus (TmSWV), an isolate of peanut mottle virus (PMV-OK), and peanut mild mottle virus (PMMV). Also in the sandwich ELISA, MCA 7C14 reacted slightly to watermelon mosaic virus 1 (WMV-1) and potato virus Y (PVY), while MCA 3AB5 reacted slightly to only PMMV. Results of polyclonal serum in the non-sandwich ELISA formats proved to be unuseable because of extremely high background responses. However, in the non-sandwich ELISA, MCA 7C14 again reacted slightly to WMV-1 and PVY as well as slightly to blackeye cowpea mosaic virus and PMMV, while MCA 3AB5, as in the sandwich ELISA, reacted only slightly to PMMV.

DISCUSSION

The use of MCA in a dot-immunobinding assay provided the quickest and most convenient way for detecting PStV in large numbers of samples. On average this assay takes only three hours to complete and is very conservative of reagents (10). The use of polyclonal serum in this assay was unsuccessful for detecting PStV in leaf and seed tissue due to extremely high background responses of healthy tissue controls. This problem of using polyclonal serum in dot-immunobinding assays has previously been reported (16).

Western blotting using MCA or polyclonal serum also provided a quick method for detecting PStV, between 7-8 hrs

to complete, although being much more labor intense than either the dot-immunobinding assay or ELISA. Previous reports have indicated that Western blot sensitivity for detecting PStV is simalar to that of ELISA (17). Western blotting also has the added feature of separating samples by molecular weight. This isolates proteins that are normally mixed together and allows easier access for antigen antibody binding. With the interfering proteins separated, the problem of background responses to non-specified proteins can be eliminated. This would explain the success of the polyclonal serum in Western blots and its failure in dot-immunobinding assays. Molecular weight determinations made from Western blotting can also be useful in detecting mixed infections of viruses (17).

Although taking two days to complete, ELISA provided an easy and effectve way to assay large numbers of samples. Of the eight ELISA formats tested only one, a direct sandwich ELISA using polyclonal serum, worked well for both peanut seed and leaf tissue. However, ELISA formats using MCA 3AB5, in an indirect sandwich ELISA with leaf tissue, or MCA 7C14, in an indirect non-sandwich ELISA with seed tissue, worked equally well and gave lower background responses then that of the polyclonal serum.

Both sandwich and non-sandwich ELISAs were used for testing the reaction of MCA 3AB5, MCA 7C14, and polyclonal serum to other plant viruses. Comparisons of the two ELISA formats showed differences among the response of an antibody

to the same virus. Such differences have been associated with the use of a capture antibody which can enhance or retard the binding of a second antibody (6). However, some responses were the same for both ELISA formats. These included responses of MCA 7C14 to WMV-1 and PVY, MCA 3AB5 to PMMV, and polyclonal serum to PMMV. Since MCA 3AB5 and MCA 7C14 were shown to bind to different antigenic sites of PStV, the responses of both MCAs and the polyclonal serum to PMMV would indicate a close serolgical relationship to PStV. This is of intrest since both PStV and PMMV originated in China (19). Of the antibodies tested, MCA 3AB5 was the most specific for PStV reacting only slightly to PMMV.

Detection of PStV in peanut leaf and seed tissue has been accomplished by ELISA, dot-immunobinding, and Western blotting. In all three methods MCAs provided detection of PStV which was equal or superior to that of the polyclonal serum. The incorporation of MCAs into these methods will bring the advantages of more reliable and standardized tests for the detection of PStV.

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Table 1. Viruses tested by sandwich and non-sandwich ELISA for serological reaction with polyclonal serum and monoclonal antibodies to PStV.

Virus	Group	Leaf tissue tested
Peanut stripe (PStV)	Potyvirus	Lupinus albus L. cv. Tiftwhite
Peanut mottle isolate OK (PMV)	Potyvirus	Pisum sativum L. cv. Little Marvel
Watermelon mosaic one (WMV-1)	Potyvirus	Cucrbita pepo L. cv. Lemon drop
Bean yellow mosaic (BYMV)	Potyvirus	P. sativum L. cv. Littel Marvel
Soybean mosaic (SMV)	Potyvirus	Glycine max L. cv. Forrest V
Blackeye cowpea masaic (BICMV)	Potyvirus	Vigna sinensis (Torner & Savi)
Tobacco etch (TEV)	Potyvirus	Nicotiana tabacum L. cv. Xanthi-nc
Peanut mild mottle (PMMV)	Potyvirus	Lupinus albus cv. Tiftwhite
Potato virus Y (PVY)	Potyvirus	N. tabacum cv. Xanthi-nc
Wheat streak mosaic (WSMV)	Potyvirus	Triticum aestivum L. cv. Bluejacket
Brome mosaic (BMV)	Bromovirus	Hordeum vulgare L. cv. Post
Tobacco mosaic (TMV)	Tobamovirus	N. sylvestris (Speg. & Comes)
Wheat soilborne mosaic (WSBMV)	WSBMV group	T. aestivum L. cv. Vona
Cucumber mosaic (CMV)	Comovirus	N. tobacum L. cv. Xanthi-nc
Tomato spotted wilt (TmSWV)	TmSWV group	Datura stramonium L.
Tobacco Streak (TSV)	Ilarvirus	N. tabacum L. cv. Xanthi-nc

Table 2. Comparison of sandwich and non-sandwich ELISA with various direct and indirect combinations of polyclonal serum and monoclonal antibodies for the detection of PStV in leaf and cotyledon seed tissues.

Coating antibody	none[a]		R[b]		none		R		none		R		3AB5		7C14	
Probe antibody	--		--		3AB5[d]		3AB5		7C14[d]		7C14		RS[c]		RS	
Conjugate	R-E[e]		R-E		M-E[f]		M-E		M-E		M-E		AR-E[g]		AR-E	
Time for reaction (min)	30	60	30	60	30	60	30	60	30	60	30	60	30	60	30	60
PStV infected leaf tissue	.12[h]	.20	2.0	2.0	.03	.05	1.4	2.0	.32	.52	.23	.38	1.5	2.0	.95	1.6
Healthy leaf tissue	.09	.13	.23	.39	.02	.00	.08	.11	.00	.00	.08	.09	.39	.67	.53	.90
PStV spiked seed tissue	1.2	1.9	1.7	2.0	.06	.19	.76	1.3	2.0	2.0	.85	1.4	.16	.26	.32	.53
Healthy seed tissue	.02	.47	.01	.02	.08	.00	.13	.22	.00	.00	.13	.22	.17	.26	.18	.30

[a]Sample coated directly to microtiter plate.
[b]IgG fraction of rabbit polyclonal serum.
[c]$AmSO_4$ purified rabbit polyclonal serum.
[d]$AmSO_4$ purified mouse monoclonal antibody.
[e]IgG fraction of rabbit polyclonal serum conjugated to alkaline phosphatase.
[f]Goat anti-mouse conjugated to alkaline phosphatase.
[g]Goat anti-rabbit conjugated to alkaline phosphatase.
[h]Average absorbance at 405 nm for four readings.

Table 3. Reaction of monoclonal antibodies and polyclonal serum to different plant viruses in sandwich and non-sandwich ELISA.

	Sandwich[a]			Non-sandwich[b]	
Virus[c]	7C14	3AB5	Poly	7C14	3AB5
PStV	+++[d]	+++	+++	+++	+++
PMV	−	−	++	−	−
WMV-1	+	−	−	++	−
BYMV	−	−	−	−	−
SMV	−	−	−	−	−
BICMV	−	−	−	++	−
TEV	−	−	−	−	−
PMMV	−	+	++	++	+
PVY	++	−	−	+	−
WSMV	−	−	−	−	−
BMV	−	−	−	−	−
TMV	−	−	−	−	−
WSBMV	−	−	−	−	−
CMV	−	−	−	−	−
TmSWV	−	−	+	−	−
TSV	−	−	−	−	−

[a]ELISA plates coated first with polyclonal antiserum specific to each respective virus.
[b]ELISA plates coated directly with virus samples.
[c]Virus acronyms and hosts are as in Table 2.
[d]+++, A_{405} = 0.6 to 2.0; ++, A_{405} = 0.1 to 0.6; +, A_{405} = 0.03 to 0.1; −, A_{405} < 0.03. All values read approximatly 30 min after adding substrate.

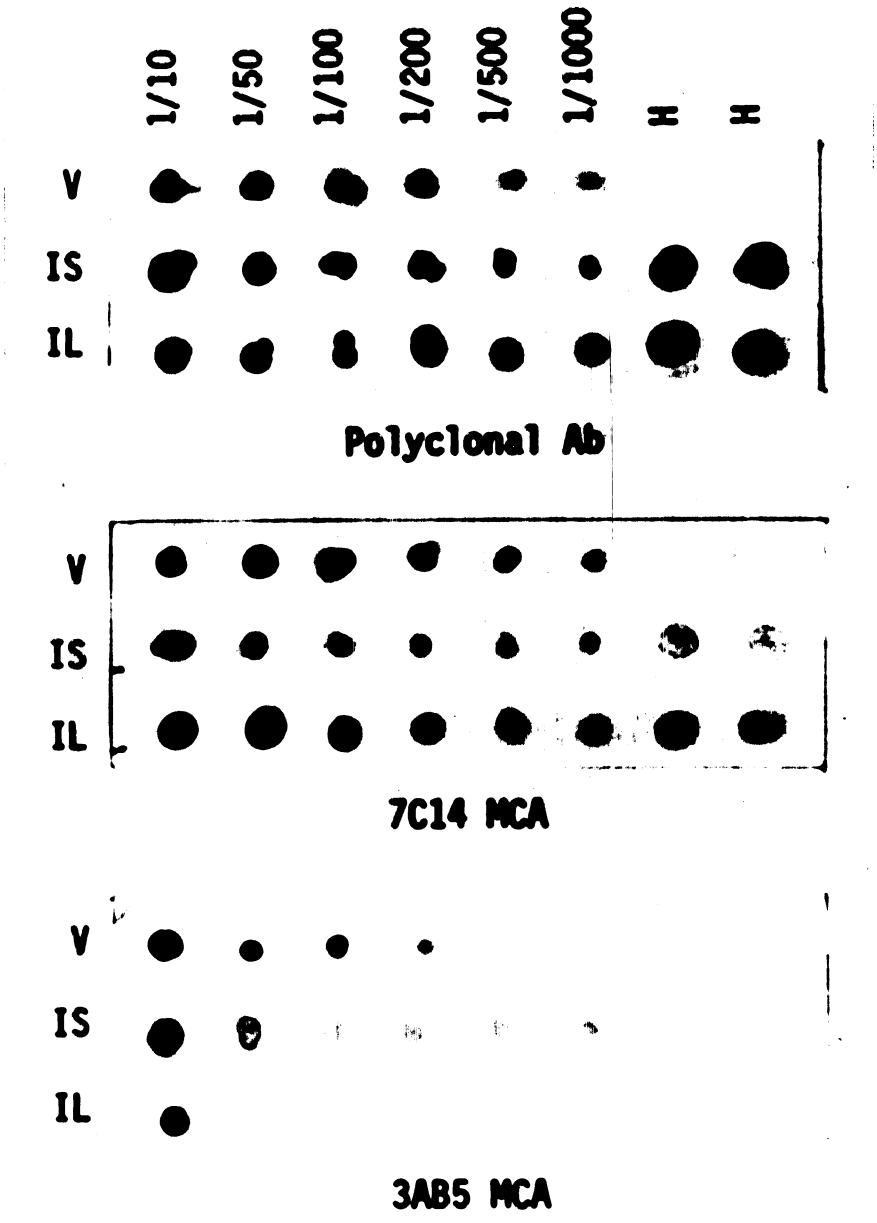

Fig. 1. Dot-immunobinding assays with polyclonal serum and monoclonal antibodies 7C14 and 3AB5. Dilutions were from 1/10 to 1/1000 of purified PStV (V), PStV infected peanut cotyledon seed tissue (IS), and PStV infected peanut leaf tissue (IL). Dilutions of healthy controls (H) for each sample were 1/50.

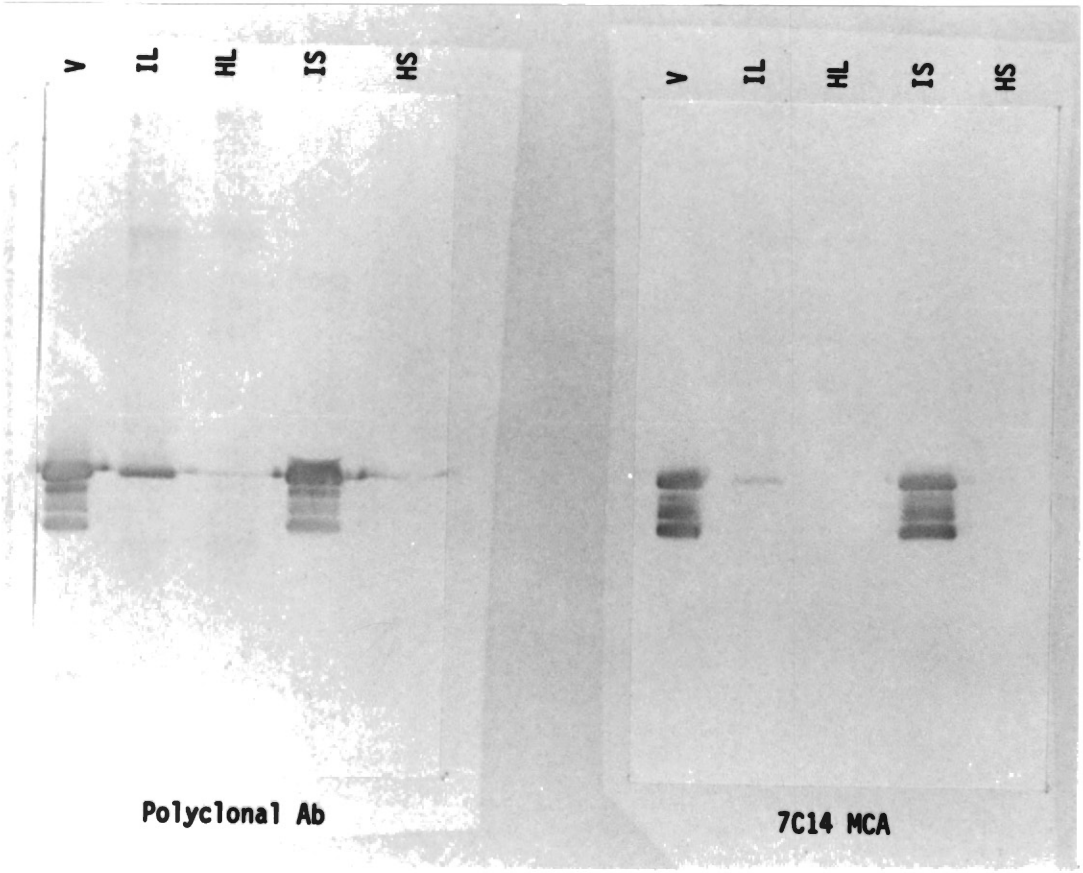

Fig. 2. Western blots using polyclonal serum and monclonal antibody 7C14. Lanes were loaded with purified PStV (V), PStV infected peanut leaf tissue (IL), healthy peanut leaf tissue (HL), healthy peanut cotyledon seed tissue (HS), and artifically infected peanut cotyledon seed tissue (IS).

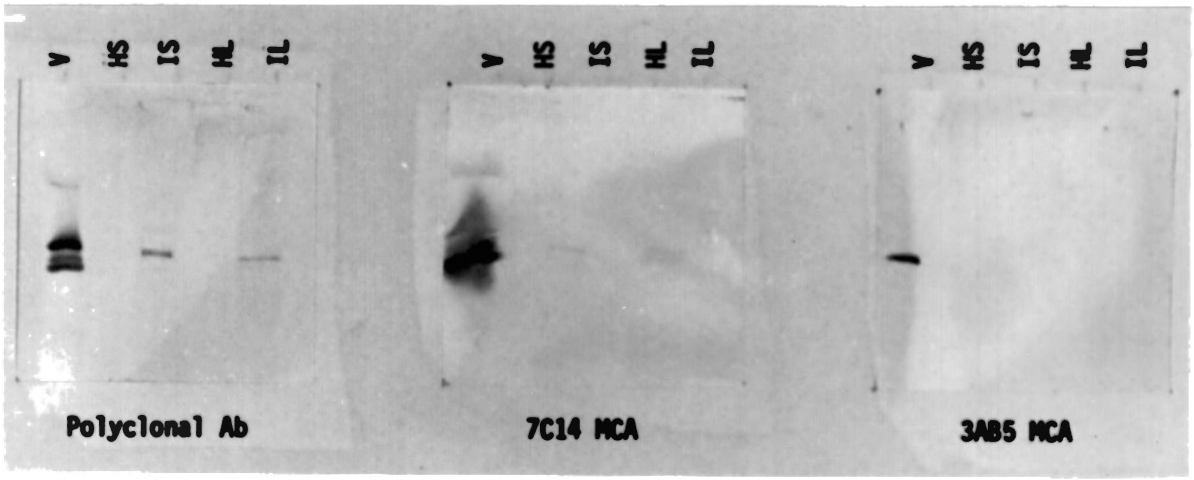

Fig. 3. Western blots using polyclonal serum and monoclonal antibodies 7C14 and 3AB5. Lanes were loaded with purified PStV (V), PStV infected peanut leaf tissue (IL), healthy peanut leaf tissue (HL), PStV infected cotyledon seed tissue (IS), and healthy cotyledon seed tissue (HS).

CHAPTER III

Resistance to Peanut Stripe Virus in *Arachis* Germ Plasm

ABSTRACT

Peanut accessions of the Arachis section; *A. diogoi* (PI 468141 and PI 468142), *A. helodes* (PI 468144), *Arachis sp.* (PI 468345 and PI 468169), and of the Rhizomatosae section (PI 468174, PI 468363, and PI 468366), were evaluated for resistance to peanut stripe virus (PStV). These entries and a susceptible cultivar Argentine were mechanically inoculated with PStV. Three to 4 weeks after inoculation, both inoculated and subsequently formed leaves from each entry were tested for PStV infection. Symptomatology, local lesion assay on *Chenopodium amaranticolor*, enzyme-linked immunosorbent assay, and electron microscopy were used to evaluate susceptibility or resistance. This inoculation and testing sequence was repeated three times for each entry. All entries except for PI 468169 and the susceptible cultivar Argentine were negative for virus infection. To our knowledge, this is the first report of resistance to PStV in *Arachis*.

INTRODUCTION

Peanut stripe virus (PStV) was first detected in the United States in Georgia in 1982 (2). It had apparently been introduced through germ plasm lines from the Peoples Republic of China (1). Since then PStV has also been detected in Florida, North Carolina, Texas, Virgina, and Oklahoma (1,2).

Reports indicate that PStV infections have been confined to research plots (1). However, there is a potential for the spead of PStV to areas of peanut cultivation. The spread of PStV is facilitated by high rates of transmission in seed, transmission by aphid vectors, and a host range that includes several common weeds which may serve as reservoirs for the virus (1,2). The potential for economic losses in peanut due to PStV infection is uknown, although previous studies have indicated a yeild loss in peanuts of up to 20% (1).

To date, there has been no report of resistance to PStV in any cultivar or germ plasm line of peanut. However, entries in the taxonomic sections of Arachis and Rhizomatosae have been shown to be resistant to peanut mottle virus (PMV) (5). Resistance to PStV in the section Arachis would be important because of its cross-compatibility with cultivated peanuts. On the basis of resistance to to PMV, germ plasm lines of the Arachis and Rhizomatosae sections were selected for testing resistance

to PStV. Selected entries of the Arachis and Rhizomatosae sections showing resistance to PStV are reported in this paper.

MATERIALS AND METHODS

Selected accesions were propagated vegetatively by removing stem sections with 4-5 fully expanded leaves. Ten to 15 stem sections from each entry were rooted in Hoagland's solution in 1 x 14 cm test tubes placed in clear polyethlene chambers on a greenhouse bench (4). Once rooted, the stem cuttings were moved into 11 cm pots containing a 1 : 4 sand to soil mixture.

PStV was obtained from J. W. Dempski, University of Gerogia, Georgia Experiment Station, Experiment, GA and maintained in both Lupinus albus, L. and peanut cv. Argentine. Inoculum was prepared by grinding infected leaf tissue in 0.01 M phosphate buffer, pH 8. New growth on each of the test plants was dusted with 400 mesh corundum and inoculum applied to the leaf surfaces with small cheesecloth pads.

Two to three weeks after inoculation symptoms were recorded. Both inoculated and subsequently formed leaves from each entry were removed and tested for PStV infection by enzyme-linked immunosorbent assay (ELISA) and local lesion assay. This inoculation and testing sequence was repeated three times for each test plant. During the final test, leaves were also removed for examination by electron

microscopy.

Polyclonal and monoclonal antibodies to PStV were prepared as previously described (7). Microtiter plates for ELISA were coated with a 1/1000 dilution of anti-PStV polyclonal antibody in 0.05 M sodium carbonate buffer, pH 9.6, for 2 hr at room temperature. Plates were then washed three times with phosphate buffered saline containing 0.05% Tween-20 (PBS-Tween) and a 1/10 and a 1/100 dilution of test peanut tissue ground in PBS-Tween with 2% polyvinyl pyrrolidone (PVP, mol. wt. 40,000) was added. After an overnight incubation at 4 C plates were washed three times with PBS-Tween and a 1/100 dilution of mouse anti-PStV monoclonal antibody in PBS-Tween was added and incubated for 2 hr at room temperature. Plates were again washed three times in PBS-Tween and a 1/1000 dilution of anti-mouse IgG alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO 63178, No. A-5781) in PBS-Tween was added and incubated for 2 hr at room temperature. Plates were washed a final three times in PBS-Tween, followed by the addition of alkaline phosphatase substrate (p-nitrophenylphosphate 1 mg/ml) in 0.1 M glycine buffer with 1 mM of $MgCl_2$ and $ZnCl_2$, pH 10.4, and incubated for approximately 20 min. Absorbance at 405 nm was recorded by use of a BIO-TEK EIA plate reader (BIO-TEK Instruments, Inc., Burlington, VT 05401). Tissue from the healthy peanut cv. Tamnut was used as a negative control and tissue from the PStV infected peanut cv. Argentine as a positive control. The minimum threshold

positive value was taken as three times the mean absorbance (A_{405}) for two wells of the healthy control on that plate.

Local Lesion assays were performed by using foliar tissue from each test entry, ground in PBS-Tween with 2% PVP. The ground tissue was inoculated as described above onto several leaves of Chenopodium amaranticolor Coste & Reyn. (2). One to two weeks after inoculation plants were examined for local lesion development.

Electron microscopy was done by leaf dip assay (3). Two ul of a saline solution were placed on Formvar-coated electron microscopy grids. A leaf from a test plant was cut perpendicular to the mid-vien and the cut edge allowed to make contact with saline on the grid for 2 min. The grid was negatively stained with 1% uranyl acetate for two minutes and wicked dry with filter paper. Ten to fifteen grid openings in each grid were examined to determine the presence or absence of PStV.

RESULTS AND DISCUSSION

The germ plasm entries selected were wild peanut lines from the USDA-ARS germ plasm collection at Stillwater, OK, that were maintained through vegetative propagation. Attempts to propagate several test plants from each entry varied in success due to the difficulty in rooting and maintaining wild peanuts. This left some entries with low numbers of test plants. However, plants with the same PI number are genetically similar and any further vegatative

propagations from these PI numbers should reflect the results of this study. Thus, we have included entries with low test plant numbers in our results.

ELISA and local lesion assay provided the clearest evidence for the resistance to PStV for each of the three inoculating and testing sequences preformed on each germ plasm entry (Table 1). Absorbance readings for positive reactions were between 0.03 and 2.00 optical density units. All reactions obtained from the healthy cv. Tamnut control and for the negative reactions ranged from 0.00 to 0.06 optical density units. Results of local lesion assays compared directly to those of the ELISA. In electron microscopy, positive samples had rod-shaped particles of the same length and diameter as particles found in the PStV infected cv. Argentine control. Negative samples showed no such particles. Results of electron microscopy matched the results obtained from ELISA and local lesion assay. Symptomatology of inoculated germ plasm lines could not be used to determine resistance or susceptibility due to a lack of clearly defined symptoms in susceptible test plants.

Of the eight entries tested for resistance to mechanical inoculation with PStV, seven were consistently shown to be free of virus by ELISA, local lesion assay, and electron microscopy. The seven entries were PI 468141, PI 468142, PI 468144, and PI 468345 of the Arachis section and PI 468174, PI 468363, and PI 468366 of the Rhizomatosae section (Table 2). PI 468169 and the control cv. Argentine

always produced a positive reaction for the presence of a PStV infection.

Among the germ plasm lines shown to be resistanct to PStV, five have been previously reported to be resistant to PMV. These five are PI 468141, PI 468142, PI 468174, PI 468363, and PI 468366 (5). In addition PI 468141 and PI 468142 have previously been shown to be resistant to early leaf spot caused by Cercospora arachidicola Hori (4). The discovery of resistance to PStV in germ plasm lines and the cross-compatibility of these lines with cultivated peanuts should provide a means for moving PStV resistance into cultivated peanuts. This is important because PStV is found not only in the United States and the Peoples Republic of China but also throughout Southeast Asia where peanut is an important agricultural commodity (6). Hence, the movement of PStV resistance into cultivated peanut would provide a basis for the future control of this disease.

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Table 1. Reaction of selected germ plasm accessions and one susceptible cultivar to inoculation with peanut stripe virus.

Entry	Number of plants tested	Number of inoculation	Reaction of tests for PStV[a]			
			ELISA[b]	Local-lesion[c] assays	Electron[d] microscopy	Symptoms[e]
Resistance						
PI 468141	2	3	–	–	–	–
PI 468142	10	3	–	–	–	–
PI 468174	2	3	–	–	–	–
PI 468363	3	3	–	–	–	–
PI 468366	6	3	–	–	–	–
PI 468144	1	3	–	–	–	–
PI 468345	10	3	–	–	–	–
Susceptible						
PI 468169	5	3	+	+	+	–
Argentine	5	3	+	+	+	+

[a]Tests conducted 3-4 wks after each of the three inoculations, except for electron microscopy (– = negative, + = positive for PStV).
[b]Reaction of an indirect double antibody sandwich ELISA.
[c]Assay done by mechanical inoculation on Chenopodium amaranticolor Coste & Reyn.
[d]Uranyl acetate negative-stained preparations of leaflets conducted 4 wks after third inoculation.
[e]Presence of mosaic and striping pattern typical of PStV infection.

Table 2. Indexing of *Arachis* germ plasm tested for resistance to peanut stripe virus.[a]

Entry	Taxonomic Section	Origin, Collectors[b]	Resistance to PStV
PI 468141 (*Arachis diogoi* Hoehne)	Arachis	Brazil, GK 30001	Yes
PI 468142 (*A. diogoi*)	Arachis	Brazil, GK 30005	Yes
PI 468174 (*Arachis sp.*)	Rhizomatosae	Brazil, GKPSc 30131	Yes
PI 468363 (*Arachis sp.*)	Rhizomatosae	Paraguay, GKPSc 30116	Yes
PI 468366 (*Arachis sp.*)	Rhizomatosae	Paraguay, GKPSc 30119	Yes
PI 468144 (*Arachis helodes*)	Arachis	Brazil, GK 30029	Yes
PI 468345 (*Arachis sp.*)	Arachis	Bolivia, GKSSc 30102	Yes
PI 468169 (*Arachis sp.*)	Arachis	Brazil, GK 30037	No

[a]As determined by independent conformation in ELISA, local lesion assay, and electron microscopy (See Table 1).
[b]Collector's initials: G = W. C. Gregory, K = A. Krupovickas, P = J. Pietratrelli, Sc = A. Schinini, S = Simpson.

CHAPTER IV

Detection of Peanut Stripe Virus in Peanut Seed by a Monoclonal Antibody Based Non-Sandwich Enzyme-Linked Immunosorbent Assay

ABSTRACT

An assay for the detection of peanut stripe virus (PStV) in peanut seed was developed using a monoclonal antibody in a non-sandwich enzyme-linked immunosorbent assay (ELISA). This ELISA was then used to screen seed from the PStV infected peanut cultivars Spanco, Pronto, Tamnut, Argentine, and Florunner. A small portion of cotyledon from each seed was removed and tested for PStV by ELISA. The seeds were then planted and the resulting seedlings tested for PStV infection by ELISA, symptomatology, and local lesion assay on Chenopodium amaranticolor. Results indicated that ELISA readily detected PStV antigen in peanut cotyledon tissue. Seeds determined to be virus-free by ELISA always produced virus-free seedlings. However, several seeds determined to have detectable amounts of PStV antigen by ELISA also produced virus-free seedlings. All peanut cultivars transmitted PStV by seed at rates ranging from 0.4% to 5.0%. The ELISA was determined to have a sensitivity of five ng/ml virus and could detect one part

infected seed diluted in 32 parts healthy seed.

INTRODUCTION

Peanut stripe virus (PStV) was apparently brought to the United States in infected peanut (Arachi hypogaea L.) seed from the Peoples Republic of China (2,3). Although being a wide spread disease throughout most of Southeast Asia (11), in the United States PStV has been contained primarily to institutional plantings (2,4). Little is known about the epidemiology of PStV. However, seed transmission has played a major role in the movement of this disease in the United States (2,4). To date, the control of PStV has been dependent on the screening of peanut seed for the presence of the virus (2,4). Seed assays for the detection of PStV and peanut mottle virus in peanut seed have previously been reported (1,4). These assays utilize polyclonal serum in a sandwich enzyme-linked immunosorbent assay (ELISA) to detect virus in small portions of peanut seed without decreasing the viability of the seed (1,4).

The purpose of this study was to apply the advatages of monoclonal antibody (MCA) technology to an ELISA for the detection of PStV in peanut seed. MCA technology can provide large quantites of uniform and highly specific antibodies that lower the chance of non-specific reactions and allow for standardized screening among different laboratories (7). A MCA based non-sandwich ELISA was developed for the detection of PStV in peanut seed. Unlike

the previously used ELISA methods, this assay was unique because it utilized only one MCA to detect PStV antigen bound directly to microtiter plates. Thereby providing a very simple and direct way for the detection of PStV in peanut seed.

MATERIALS AND METHODS

Virus source and infected seed lot production. An isolate of PStV was aquired from J. W. Demski, University of Georgia, Gerorgia Experiment Station, Experiment, GA 30212 and maintained as previously described (3). Seed from the peanut cultivars Spanco, Tamnut, Florunner, Argentine, and Pronto were aquired from J. S. Kirby, Oklahoma Sate University, Stillwater, OK 74078. To obtain PStV infected seed lots, seedlings in their 3rd and 4th true leaf stages, were mechanical inoculated with PStV infected Lupinus albus L. leaf tissue ground in 0.01 M phosphate buffer (p.b.), pH 8.0. After approximatly 2 wks, twelve PStV inoculated seedlings from each cultivar 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 dryed and stored at room temperature for 6 to 9 months before being tested.

Seed ELISA procedure. A non-sandwich ELISA using a monoclonal probe antibody was developed for the detection of

PStV in peanut seed. A MCA specific to PStV was produced as previously described (12). A small portion of cotyledon tissue (0.02 - 0.05 g) was removed from the non-germ end of each seed, ground in 600 ul of 0.05M sodium carbonate buffer, pH 9.6 (coating buffer), and applied to microtiter plates which were then incubated overnight at 4 C. The next day plates were rinsed three times with phosphate buffered saline containing 0.05% Tween-20 (PBS-Tween) and a 1/500 dilution of mouse anti-PStV MCA in PBS-Tween applied and incubated for 2 hrs at room temperature. Plates were again rinsed three times with PBS-Tween and a 1/1000 dilution of goat anti-mouse IgG alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO 63178, No. A-5781), in PBS-Tween, added. After 2 hrs of incubation at room temperature plates were rinsed a final time with PBS-Tween followed by the addition of alkaline phosphatase substrate (p-nitrophenylphosphate 1 mg/ml) in 0.01 M glycine buffer containing 1 mM of $MgCl_2$ and $ZnCl_2$, pH 10.4. After an additional 2 hrs of incubation at room temperature absorbance at 405 nm was read using a BIO-TEK EIA plate reader (BIO-TEK Instruments, Inc., Burlington, VT 05401).

Growout tests. Seeds screened by the MCA based non-sandwich ELISA were individually planted in a greenhouse. Seedlings from these seeds, in their 3rd and 4th true leaf stages, were then tested for PStV infection by ELISA, symptomatology, and local lesion assay on Chenopodium amaranticolor Coste and Reyn.

A sandwich ELISA using a polyclonal capture antibody and a MCA probe was used to test the seedling leaf tissue for the presence of PStV infection. Polyclonal serum was obtained as previously described (12). Microtiter plates were first coated with a 1/1000 dilution of anti-PStV polyclonal serum in coating buffer. Plates were then incubated for 2 hrs at room temperature, rinsed three times with PBS-Tween, and a 1/50 dilution of leaf sap, obtained from a roller press, in PBS-Tween containg 2% polyvinyl pyrrolidone (PVP, mol. wt. 40,000) added and incubated overnight at 4 C. The remaning steps of this procedure are identical to those of the MCA based non-sandwich ELISA. A positive response was considered to be an absorbance value (405 nm) greater than the mean of the healthy peanut leaf tissue controls plus four standard deviations.

Individual seedlings in their 4th and 5th true leaf stage were observed for symptoms characteristic of PStV infection. Local lesion assays were preformed only on seedlings that gave questionable or distinctly positive ELISA readings in the seed assay. Local lesion assays were done by grinding seedlings leaf tissue in 0.01 M p.b. pH 8.0, and rubbing this inoculum onto corundum dusted leaves of C. amaranticolor with small chessecloth pads.

Detection of PStV in seed lots. PStV infected peanut seeds, determined by MCA non-sandwich ELISA, with absorbance values of 2.0 optical density units (O.D.) were ground in a 1/50 dilution of coating buffer. The ground

cotyledon tissue was then diluted 1/2, 1/4, 1/16, 1/32, and 1/64 with a 1/50 dilution of healthy cotyledon tissue in coating buffer. Dilutions of infected cotyledon tissue were then tested by the MCA based non-sandwich ELISA format.

Comparisons of ELISA formats. ELISA formats using MCA and polyclonal serum were compared to the MCA non-sandwich ELISA for their ability to detect PStV in peanut cotyledon seed tissue. The ELISA formats consisted of a non-sandwich ELISA utilizing an anti-PStV polyclonal serum, conjugated to alkaline phosphatase, and two sandwich ELISA formats that utilized an anti-PStV polyclonal capture antibody followed by either an anti-PStV polyclonal conjugate or anti-PStV MCA. The procedures for these three formats were simialar to the previously described non-sandwich ELISA for testing seed and sandwich ELISA for testing seedlings. For comparing ELISA formats, infected cotyledon seed tissue was artificialy obtained by adding varing concentrations of purified PStV to 1/50 dilutions of healthy cotlyedon tissue ground in the appropriate buffer. Upon the addition of enzyme substrate to the microtiter plates absorbance values at 405 nm were taken at 0.5, 1, and 2 hrs using a BIO-TEK EIA plate reader.

RESULTS

Optimization of seed ELISA. The optimal conditions for the seed assay were established in order to lower the background response and minimize the use of reagents.

Dilutions of 1/10, 1/50, and 1/100 of ground cotyledon tissue in coating buffer and dilutions of 1/100, 1/500, and 1/1000 of anti-PStV MCA in PBS-Tween were compared in the MCA based non-sandwich ELISA. Results of ELISA using 1/50 dilutions of ground cotyledon tissue and 1/500 dilutions of anti-PStV MCA gave the most satisfactory responses. Variations of coating buffer (used to grind seed) and PBS-Tween (used to dilute MCA) with and without 2% PVP and 0.2% ovalbumin were also tested for their effect on the detection of PStV in the non-sandwich ELISA. Results indicated that the variations made in either coating or PBS-Tween buffers had no significant effect on the detection of PStV in peanut seed by the MCA based non-sandwich ELISA.

For the seed assay, microtiter plate arangements were such that individual seed samples were tested twice and the mean of the two absorbance values taken as the reading for that sample. Four healthy seed controls, each duplicated, were run per plate and a known positive control run with every set of plates to insure the reactivity of the reagents.

Correlation of seed ELISA and grow-out tests. Out of 1,544 seeds tested for PStV only 1,184 seeds produced seedlings. Seedlings were individually tested for PStV infection by ELISA and symptomatology. Seedlings which gave positive ELISA readings always produced symptoms typical of PStV infection. While no symptoms occured on seedlings with negative ELISA readings. A total of 85 local lesion assays

were done on seedlings whose seed had tested questionably or distinctly positive in ELISA. Local lesions were always obtained on Chenopodium amaranticolor from seedlings that tested positive for PStV infection by ELISA and symptomatology and never from seedlings that tested negative. Results of grow-out tests (ELISA, symptomatology, and local lesion assay) for each seedling were then compared to the response of the seed in the MCA based non-sandwich ELISA in order to determine a positive-negative threshold for the seed assay.

Determination of a positive-negative threshold for the MCA based non-sandwich ELISA was made using only absorbance values from those seeds that produced seedlings. An intial attempt to use the average of the healthy seed controls plus four standard deviations gave inconsistant results for individual plates and cultivar groupings. The problem encountered with this method was that it consistantly gave positive responses to several seeds that tested healthy in the grow-out tests. The alternative method used was a visual ranking of seed sample absorbance values simialar to that described for squash mosaic virus (10). A positive negative threshold could easily be observed using this method since only 1.5% of all healthy seedlings gave seed ELISA values greater than 0.1 O.D. in the seed assay while 93.3% of all infected seedlings gave seed ELISA values greater than 0.6 O.D. Ranking of seed ELISA values easily showed a three to ten fold increase in absorbance values

between the highest negative value and the lowest positive value. This gap between negative and positive values was difficult to see on a plate to plate basis. However, when values from one cultivar and growing season were ranked together this positive-negative gap became very apparent. This gap was also visible when all seed values were ranked together (Fig. 1). Only one positive seed, out of the 1,184 tested could not be ranked in this fashion.

PStV detection in seed lots. Tests for the detection of PStV in peanut seed lots were done using 10 seeds that had previously given strong positive responses for PStV in the MCA based non-sandwich ELISA. Portions from the cotyledons of these infected seeds were diluted with healthy seed cotyledon tissue. The diluted infected seed tissue was then tested by the MCA non-sandwich ELISA. Each dilution of infected seed was duplicated in microtiter plates and the mean absorbance value determined. The average absorbance value for the healthy control cotyledon tissue was 0.016, while the ten infected cotyledon tissues, diluted 32 times with healthy cotyledon tissue, gave absorbance values between 0.078 and 0.669. This indicated that the MCA non-sandwich ELISA was capable of detecting one infected seed part in 32 healthy seed parts.

Seed transmission rates of PStV. Peanut seed transmission rates were determined for the five peanut cultivars tested (Table 1). All seeds tested by the MCA non-sandwich ELISA, including those that did not produce

seedlings, were used to determine transmission rates. Results indicated that PStV could be transmitted in all five cultivars. Transmission rates ranged from a low of 0.4% to a high of 5.0% depending on the cultivar and the growing season.

Comparisons of ELISA formats. The MCA based non-sandwich ELISA was compared to three other ELISA formats for the detection of PStV in peanut seed. Experiments were repeated twice with samples being assayed 4 times in the same plate. Positive results were considered to be any absorbance value (405 nm) twice the mean of the healthy seed tissue controls.

Results indicated that all ELISA formats easily detected PStV in peanut cotyledon tissue. However, sensitivity and background levels varied between the ELISA formats (Fig. 2). Non-sandwich ELISA, utilizing only polyclonal serum, was adequate for detecting PStV concentrations down to 250 ng/ml virus after 30 min incubation with substrate. A sandwich ELISA, utilizing polyclonal capture and probe antibodies, was five times more sensitive and able to detect PStV concetrations as low as 50 ng/ml virus after 1 hr of incubation. The MCA sandwich ELISA, utilizing a polyclonal capture antibody, was capable of detecting 5 ng/ml of PStV after a 1 hr incubation. The most sensitive ELISA format tested was the MCA based non-sandwich ELISA. Although taking twice as long to achieve the same level of intensity as the sandwich ELISA formats,

the MCA non-sandwich ELISA was was capable of detecting 0.5 ng/ml of PStV.

DISCUSSION

As previously reported (1,4), a portion of peanut cotyledon could be removed from the seed without substantially lowering the seed's viability. Using no special modifications for germinating or planting test seed, a 76% survival rate was obtained for peanut seeds tested in this manner. The high survivablity of peanut seeds in this assay makes it possible to obtain PStV-free germ plasm lines directly from tested seeds.

A MCA based non-sandwich ELISA readily detected PStV in peanut seed. This ELISA gave no false negative readings, in that all peanut seeds testing virus-free gave rise to healthy seedlings. However, a problem was encountered with false positive readings, in that some peanut seeds testing positive for PStV gave rise to healthy seedlings. The false positive readings accounted for approximatly 1.6% of the healthy seeds and consisting of sample values that were higher then 0.1 absorbance values. This problem was substantially reduced by a visual ranking of absorbance values for seed samples. By ranking samples, a visable gap between positive and negative seeds could be observed and used as the positive-negative threshold for the assay.

The false positive readings obtained from ELISA were most likely due to PStV antigen present in the cotyledon

tissue but not causing infection in the seedling. Although PStV has been found in both the cotyledon and embryo of the peanut seed (4), it is possible that on some occasions virus 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 unassembled viral coat proteins or degraded virus particles. Such non-viable viral antigen could not cause infection but still give a positive ELISA reading. Degradation of viral particles may have occured during seed storage prior to testing and caused a reduction in the number of infected seedlings. The lowering of seed transmission rates by increasing storage time of seed has already been reported for squash mosaic virus in cantelope seed (10). Finally, the high sensitivity of the indirect ELISA may lead to detection of low concentrations of viral antigen that do not always lead to infection in the seedling.

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 (9,10). However, in the case of PStV, where quarantine procedures are in affect, false positives readings produced by the presence of non-viable viral antigen would be acceptable, since this indicates the seed originated from a viral infected plant (9).

The MCA non-sandwich ELISA was able to detect PStV in one infected seed portion diluted with the equvalent 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.

The higest observed rates of peanut seed transmission for PStV were 5.0% for cv. Florunner and 3.3% for cv. Argentine in the winter growing season. These rates were substantially lower then previous reports of 11.0% for cv. Florunner and 18.0% for cv. Argentine in a winter growing season (4). This differance in seed transmission may be accounted for by the number of seeds containing PStV antigen, as indicated by the false positive ELISA readings, that did not produce infected seedlings.

The MCA based non-sandwich ELISA was determined to be the most sensitive ELISA format for the detection of PStV in peanut cotyledon tissue. However, the MCA based sandwich ELISA, used commonly in the detection of other plant viruses (6,8), also worked well for detecting PStV in peanut seed being only slightly less sensitive than the MCA non-sandwich ELISA. A sandwich ELISA format similar to the ones previouly reported for the detection of PStV and PMV in peanut seed, using only polyclonal serum, proved to be ten times less sensitive than either the MCA based sandwich or non-sandwich ELISA formats. A non-sandwich ELISA using only polyclonal serum was the least sensitive format tested.

The testing of peanut seed is important if the spread

of PStV is to be halted. A MCA based non-sandwich ELISA has provided the most sensitive way to detect PStV in peanut seed. Besides sensitivity, the MCA non-sandwich ELISA has the advantage of not being dependent on polyclonal serum. Thus, some of the problems, such as limited amounts of antiserum or variablity among antiserum lots, associated with the use of polyclonal serum can be avoided (5,7). The incorporation of a MCA in this assay also allows for the development of standardized tests among laboratories working with PStV. Non-sandwich ELISA formats also require fewer steps, making them simpler, faster, and more conservative of reagents. And by using a second commercially available antibody conjugate it was possible to elimminate the loss of serological activity that can occur during conjugation (5).

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Table 1. Seed transmission of peanut stripe virus in five susceptible cultivars of peanut over two growing seasons under greenhouse conditions.

Cultivar	Number of seed tested	Number of plants tested	Percent seed[a] transmission
Winter season			
Argentine	92	69	3.28%
Florunner	100	97	5.04%
Pronto	88	76	0.00%
Tamnut	100	91	2.52%
Spanco	132	121	2.10%
Summer season			
Argentine	153	133	0.46%
Florunner	219	160	0.42%
Pronto	220	133	0.84%
Tamnut	220	112	0.00%
Spanco	220	192	0.00%

[a]Percent of seed transmission determined by a monoclonal antibody based non-Sandwich ELISA.

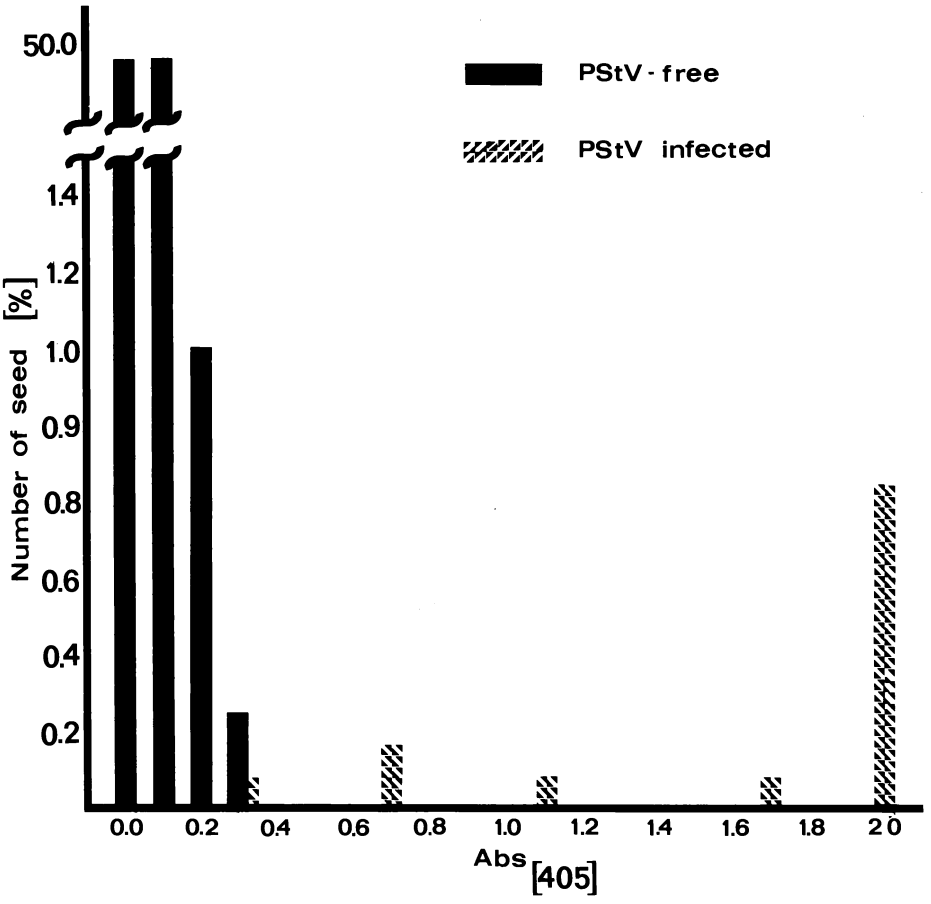

Fig. 1. Ranking of 1,184 peanut seeds tested by a monoclonal antibody based non-sandwich enzyme-linked immunosorbent assay for the presence of peanut stripe virus (PStV).

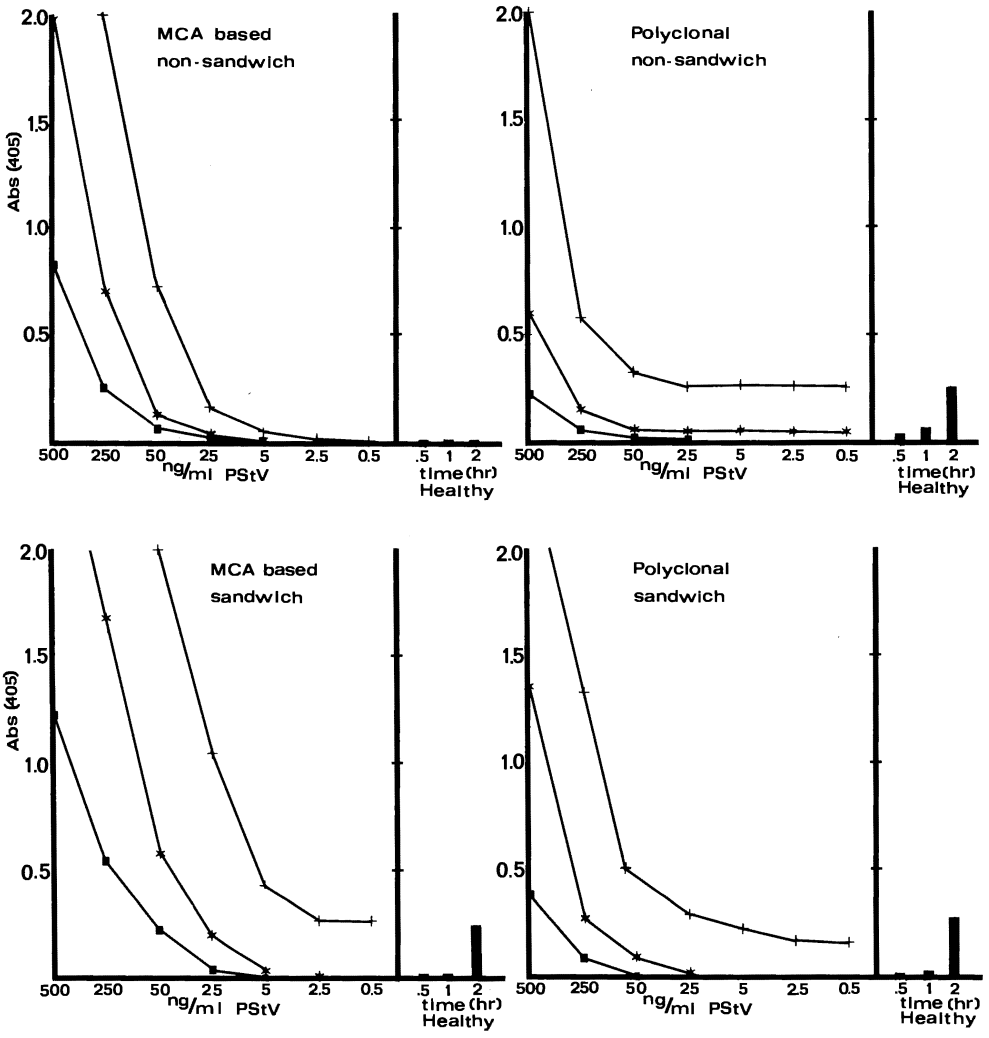

Fig. 2. Comparison of four different enzyme-linked immunosorbent assay formats for the detection of peanut stripe virus in peanut cotyledon seed tissue. Readings were taken at 30 min ( ), 1 hr (*), and 2 hrs (+) incubation with substrate. Histogram represents reaction of healthy controls for each ELISA format.

VITA 2

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