Use of Direct Antigen Coating and Protein A Coating ELISA Procedures for Detection of Three Peanut Viruses

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ABSTRACT

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Direct antigen coating (DAC) and protein A coating (PAC) forms of indirect enzyme-linked immunosorbent assay (ELISA) were standardized and compared with the double-antibody sandwich (DAS) form of direct ELISA for their usefulness in the detection of three peanut viruses: peanut mottle virus (PMV), tomato spotted wilt virus (TSWV), and Indian peanut clump virus (IPCV). PMV was detectable in peanut seeds and pea tissue at a 1:10,000 dilution in buffer with the DAC and at a 1:1,000 dilution with the PAC procedures. With the DAS procedure, PMV was detectable in pea tissue at a 1:1,000 dilution and in peanut seed at a 1:100 dilution. TSWV was detectable in peanut leaves at a 1:1,000 dilution with the DAC method and at 1:100 with the PAC and DAS methods. IPCV was detectable in peanut leaves at a 1:100 dilution by the DAC, PAC, and DAS methods. Sensitivity of DAC and PAC methods was, therefore, comparable to that of the DAS procedure under the short incubation period (1–2 hr at 35 C) conditions employed in the experiments.

Several indirect ELISA procedures have been used in the detection of plant viruses. Indirect ELISA systems employing F (ab')$_2$ fragments (2) and antibodies from different animal species (15) have been used successfully. Recently, an indirect ELISA procedure employing direct antigen coating (DAC) followed by the addition of virus-specific immunoglobulins or crude antisera and detection of antigen-antibody complexes with enzyme-conjugated protein A was described by Mowat (11).

Another indirect system using an initial protein A coating (PAC) for binding and orientation of virus-specific antibodies added in the subsequent step was recently used successfully by Edwards and Cooper (7). The protein A binds to the immunoglobulins at the Fc region, leaving the F (ab')$_2$ region available for antigen binding. The bound antigen is then detected by another layer of antibodies, which are in turn detected by enzyme-conjugated protein A. The system was termed PAS ELISA, acronym for protein A sandwich.

This paper reports the use of these two systems, DAC and PAC, modified by substituting antirabbit Fc-specific (prepared in goats) conjugate for protein A conjugate. The two systems are compared with the double-antibody sandwich form of direct ELISA (DAS) for their usefulness in the detection of peanut viruses from three virus groups: peanut mottle virus (12) (PMV, potyvirus group), tomato spotted wilt virus (8) (TSWV, monotypic group), and Indian peanut clump virus (13) (IPCV, proposed furovirus group) (14).

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MATERIALS AND METHODS

Leaves of TSWV- and IPCV-infected peanut plants were field-collected. Tissue from PMV-infected pea plants (*Pisum sativum* L., cv. Bonneville) maintained in a greenhouse was collected fresh or frozen at −60 C for later use. Seeds of peanut (*Arachis hypogaea* L.) collected from field-inoculated plants were identified as infected with PMV by previously described ELISA procedures (3) and stored at 5 C.

For the DAS procedure, immunoglobulins were extracted from antisera produced in rabbits by sodium sulfate precipitation followed by dialysis as described by Rajeshwari et al (12). Immunoglobulins were conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) by the one-step glutaraldehyde method (4). Immunoglobulins extracted from rabbit Fc-specific antiserum produced in goats (Cappel Laboratories, Inc., West Chester, PA), employed in the DAC and PAC procedures, were conjugated to alkaline phosphatase in a similar fashion.

All ELISA incubation periods prior to substrate addition were 1–2 hr at 35 C. Volumes added at each step were 200 µl/well. Buffer preparation and plate-washing procedures were similar to those of Clark and Adams (4); however, 0.01 M sodium diethyldithiocarbamate (Na DIECA) (Sigma) was added to all antigen buffers (1). When antigen samples were simultaneously tested by more than one ELISA procedure, they were initially extracted at a 1:10 dilution in 0.02 M potassium phosphate buffer, pH 7.5, and subsequent dilutions were made in the appropriate antigen buffer. All coating steps were done with 0.05 M sodium carbonate buffer, pH 9.6. For the DAC method, carbonate buffer containing 0.01 M Na DIECA was used as the antigen buffer because extraction and coating were combined into one step. For the DAS and PAC methods, the antigen buffer was PBS-Tween-PVP (0.02 M potassium phosphate buffer [pH 7.3], 0.15 M NaCl, 0.003 M KCl; 0.05% Tween 20; and 2% polyvinyl pyrrolidone) containing 0.01 M Na DIECA. All antisera and enzyme-conjugated immunoglobulins were diluted in PBS-Tween-PVP-ovalbumin (4).

DAC method. Plant samples ground in a mortar and pestle were applied to the microtiter plate (Dynatech, Zug, Switzerland). Optimum dilutions of crude antisera were then added: 1:10,000 for PMV and IPCV and 1:1,000 for TSWV. This was followed by addition of rabbit Fc-specific conjugated immunoglobulins at a 1:1,500 dilution for PMV and 1:750 for TSWV and IPCV. Then *p*-nitrophenyl phosphate substrate (Sigma) was added at 0.25 mg/ml. Substrate incubation times (room temperature) were 30 min for PMV and TSWV and 2 hr for IPCV. Reactions were stopped using 50 µl of 3 M NaOH per well. Each treatment was replicated in three wells in each of three experiments. Absorbance readings (A_{410nm}) were taken with a Dynatech Micro-ELISA Minireader MR 590 calibrated to zero without an ELISA plate on the reader. Readings twice those of healthy plant control averages were considered positive.

PAC method. Protein A (Sigma, Cat. No. P-8143) diluted in carbonate buffer, pH 9.6, was added to the plate at a concentration of 1 ng/ml. Crude antisera were subsequently added at optimum dilutions (1:100,000 for PMV and IPCV, 1:1,000 for TSWV), then plant samples were added. Second antisera were added next at the same dilutions as the first antisera. This was followed by rabbit Fc-specific conjugate at a 1:750 dilution. Substrate (*p*-nitrophenyl phosphate)

incubation times (room temperature) were 90 min to 2 hr for PMV, 1 hr for TSWV, and 2 hr for IPCV. Reactions were stopped and absorbances recorded as in the DAC procedure.

In testing for PMV in pea tissue by DAC and PAC methods, dilution of antiserum and second antiserum, respectively, in 1:20 (w/v) pea tissue extract was necessary for reducing absorbance due to values of healthy plant constituents. It was also necessary to dilute antiserum for the DAC method in peanut seed extract for experiments with seeds. In preliminary TSWV testing, dilution in 1:20 peanut leaf extract was necessary as a diluent for antisera with both DAC and PAC methods. The extract was used routinely to reduce high absorbance values of healthy plant constituents. For IPCV detection, dilution of antiserum in 1:20 peanut leaf extract was necessary for reducing healthy plant absorbance values with the DAC method. In each of these cases, healthy plant extract was prepared by grinding the plant material in a mortar and pestle in PBS-Tween-PVP-ovalbumin. The extract was then filtered through cheesecloth. Antiserum was then added to the extract to obtain the desired dilution for addition to the ELISA plate. Similar dilution of antibodies in healthy plant extract reduced absorbance values of healthy plant constituents in indirect ELISA for detection of the clover phyllody MLO (5) and in direct ELISA for the detection of soybean viruses (10).

DAS method. Methods used were similar to those described earlier (4,12). Immunoglobulin concentrations were 1 μg/ml for PMV, 2 μg/ml for TSWV, and 1.25 μg/ml for IPCV. Enzyme-conjugated immunoglobulins were used at dilutions of 1:500 for PMV, 1:200 for TSWV, and 1:400 for IPCV. Substrate incubation times were 30–40 min for PMV, 15–30 min for TSWV, and 2 hr for IPCV.

RESULTS AND DISCUSSION

In preliminary testing of the PAC method, 1 ng/ml was found to be the

Table 1. Detection of peanut mottle virus (PMV) in field-grown peanut seed and greenhouse-grown pea (*Pisum sativum*) tissue by the direct antigen coating (DAC), protein A coating (PAC), and double-antibody sandwich (DAS) forms of ELISA

	A_{410nm}[a]					
	DAC[b]		PAC[b]		DAS[b]	
Sample	$\bar{x}$	Range	$\bar{x}$	Range	$\bar{x}$	Range
Healthy peanut seed						
Diluted 1:100	0.07	0.07–0.09	0.11	0.08–0.13	0.08	0.06–0.09
Diluted 1:1,000	0.07	0.07–0.08	0.10	0.08–0.12	0.07	0.06–0.08
Buffer	0.07	0.06–0.08	0.09	0.06–0.12	0.07	0.05–0.10
Infected peanut seed						
Diluted 1:100	1.72	1.49–1.94	1.40	1.15–1.67	*0.43*	*0.29–0.60*[c]
Diluted 1:1,000	1.39	1.14–1.62	*0.97*	*0.44–1.47*	0.16	0.12–0.22
Diluted 1:10,000	*0.42*	*0.37–0.46*	0.63	0.19–1.07	0.08	0.07–0.08
Healthy pea tissue						
Diluted 1:100	0.08	0.07–0.09	0.16	0.11–0.21	0.09	0.06–0.12
Diluted 1:1,000	0.08	0.07–0.08	0.13	0.08–0.16	0.08	0.06–0.10
Buffer	0.08	0.07–0.08	0.13	0.09–0.17	0.07	0.05–0.09
Infected pea tissue						
Diluted 1:100	...	1.15–2+[d]	1.25	1.07–1.45	0.69	0.62–0.78
Diluted 1:1,000	1.41	0.86–1.95	*0.58*	*0.45–0.74*	*0.32*	*0.28–0.37*
Diluted 1:10,000	*0.46*	*0.17–0.77*	0.39	0.22–0.55	0.11	0.07–0.16

[a] Absorbances of three wells for each dilution in buffer in each of three experiments. In experiments with simultaneous testing of the DAC procedure and one or both of the others, samples were initially extracted at a 1:10 (w/v) dilution rate in 0.02 M potassium phosphate buffer, pH 7.5, and subsequent dilutions made in the appropriate antigen buffer: carbonate buffer, pH 9.6, containing 0.01 M sodium diethyldithiocarbamate (Na DIECA) for DAC and PBS-Tween-PVP containing 0.01 M Na DIECA for PAC and DAS. Substrate incubation times (room temperature) were 30 min for DAC, 90 min to 2 hr for PAC, and 30–40 min for DAS.

[b] Antiserum dilutions were 1:10,000 for DAC and 1:100,000 for PAC. Rabbit Fc-specific conjugate dilutions were 1:1,500 for DAC and 1:750 for PAC. PMV-specific immunoglobulins and enzyme-conjugated immunoglobulins were used at 1 μg/ml and 1:500, respectively.

[c] Highest positive dilution shown in italics.

[d] Readings above the range of ELISA reader (absorbance of 2.0) were recorded as 2+.

Table 2. Detection of tomato spotted wilt virus (TSWV) in peanut leaf field samples using the direct antigen coating (DAC), protein A coating (PAC), and double-antibody sandwich (DAS) forms of ELISA

	A_{410nm}[a]					
	DAC[b]		PAC[b]		DAS[b]	
Sample	$\bar{x}$	Range	$\bar{x}$	Range	$\bar{x}$	Range
Healthy leaves						
Diluted 1:10	0.22	0.20–0.24	0.12	0.09–0.13	0.34	0.30–0.37
Diluted 1:100	0.19	0.17–0.21	0.10	0.07–0.12	0.28	0.26–0.30
Buffer	0.09	0.07–0.11	0.09	0.07–0.11	0.09	0.07–0.14
Infected leaves						
Diluted 1:10	1.45	1.24–1.58	0.72	0.54–0.87	1.34	1.18–1.49
Diluted 1:100	1.24	1.18–1.29	*0.36*	*0.30–0.42*[c]	*1.05*	*0.78–1.28*
Diluted 1:1,000	*1.10*	*1.07–1.12*	0.15	0.11–0.19	0.59	0.39–0.81

[a] Absorbances of three wells for each dilution in antigen buffer in each of three experiments. Antigen buffers were PBS-Tween-PVP containing 0.01 M sodium diethyldithiocarbamate (Na DIECA) for PAC and DAS and carbonate buffer, pH 9.6, containing 0.01 M Na DIECA for DAC. Substrate incubation times (room temperature) were 30 min for DAC, 1 hr for PAC, and 15–30 min for DAS.

[b] Antiserum dilution was 1:1,000 for DAC and PAC forms. Rabbit Fc-specific conjugate dilution was 1:750 for DAC and PAC forms. TSWV-specific immunoglobulins and enzyme-conjugated immunoglobulins for DAS were used at 2 μg/ml and 1:200, respectively.

[c] Highest positive dilution shown in italics.

Table 3. Detection of Indian peanut clump virus (IPCV) in peanut leaf field samples by the direct antigen coating (DAC), protein A coating (PAC), and double-antibody sandwich (DAS) forms of ELISA

	A_{410nm}[a]					
	DAC[b]		PAC[b]		DAS[b]	
Sample	$\bar{x}$	Range	$\bar{x}$	Range	$\bar{x}$	Range
Healthy leaves						
Diluted 1:100	0.12	0.09–0.13	0.14	0.13–0.15	0.09	0.07–0.11
Diluted 1:1,000	0.12	0.11–0.14	0.17	0.14–0.19	0.09	0.07–0.12
Buffer	0.08	0.07–0.09	0.19	0.19–0.20	0.09	0.07–0.10
Infected leaves						
Diluted 1:100	*0.38*	*0.32–0.46*	*0.46*	*0.41–0.47*	*0.31*	*0.28–0.32*
Diluted 1:1,000	0.27	0.21–0.38	0.27	0.24–0.30	0.13	0.11–0.15
Diluted 1:10,000	0.18	0.15–0.22	0.20	0.19–0.21	0.10	0.08–0.12

[a] Absorbances of three wells for each dilution in buffer in each of three experiments. In experiments with simultaneous testing of the DAC procedure and one or both of the others, samples were initially extracted at a 1:10 (w v) dilution rate in 0.02 M potassium phosphate buffer, pH 7.5, and subsequent dilutions made in the appropriate antigen buffer: carbonate buffer, pH 9.6, containing 0.01 M sodium diethyldithiocarbamate (Na DIECA) for DAC and PBS-Tween-PVP containing 0.01 M Na DIECA for PAC and DAS. Substrate incubation times (room temperature) were 2 hr for all forms.

[b] Antiserum dilution was 1:10,000 for DAC form and 1:100,000 for PAC form. Rabbit Fc-specific conjugate dilution was 1:750 for DAC and PAC forms. IPCV-specific immunoglobulins and enzyme-conjugated immunoglobulins were used at 1.25 μg ml and 1:400, respectively, for the DAS form.

[c] Highest positive dilution shown in italics.

optimum protein A concentration and was used thereafter for detection of all three viruses. Higher concentrations gave nonspecific reactions to healthy plant samples and buffer controls alike. Lower concentrations yielded weaker positive reactions from virus-infected samples. These results contrast with those of Edwards and Cooper (7), who found that a 1-μg ml protein A coating concentration was optimal for their PAS-ELISA system. PMV was detectable by the DAC method at a 1:10,000 (w v) dilution in buffer and at a 1:1,000 dilution with the PAC method in both infected seed and pea tissue (Table 1). With the DAS procedure, PMV was detectable at a 1:1,000 dilution of pea tissue and at a 1:100 dilution of seed extract. Because the DAC method is more convenient, it is now used in screening tests for seed transmission (3).

TSWV was detectable at 1:1,000 dilution of infected peanut leaves in buffer with the DAC and at a 1:100 dilution with the DAS and PAC methods (Table 2). Relatively high absorbance values were observed for healthy plant extracts in the DAS and DAC procedures, whereas the PAC procedure gave negligible healthy plant reaction. Therefore, the PAC procedure is preferred for routine detection of TSWV in plant extracts.

IPCV was detectable at a 1:100 dilution of infected peanut leaves in buffer with all three procedures (Table 3). It was necessary to employ 2-hr substrate incubation times to obtain absorbances from infected samples that were twice those of healthy sample means for all three ELISA forms.

Both of the indirect forms of ELISA performed as well as the routinely used DAS form for detecting the three peanut viruses under the short incubation period conditions employed in the tests. Moreover, the two indirect forms have the advantages over the DAS method of the use of crude antiserum instead of extracted immunoglobulins and a single-enzyme conjugate with all detection systems. These features make the methods preferable to the DAS form in developing countries, where limited resources and facilities make simplified procedures particularly valuable.

The DAC form is very useful for field surveys, because plant extracts can be used for coating plates without the necessity of adding either antisera or immunoglobulins. This permits greater flexibility for choosing appropriate antisera when plates are processed upon return to the laboratory. We are currently using the DAC form in surveys for several peanut viruses. With the DAC procedure developed for PMV detection, peanut stripe virus, another potyvirus infecting peanuts (6), has recently been detected in disease surveys in Thailand and the Philippines. We have also succeeded in detecting cowpea mild mottle virus, a carlavirus reported to infect peanut (9), by the DAC procedure.

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Sample	A_{410nm}[a]					
	DAC[b]		PAC[b]		DAS[b]	
	$\bar{x}$	Range	$\bar{x}$	Range	$\bar{x}$	Range
Healthy leaves						
Diluted 1:100	0.12	0.09–0.13	0.14	0.13–0.15	0.09	0.07–0.11
Diluted 1:1,000	0.12	0.11–0.14	0.17	0.14–0.19	0.09	0.07–0.12
Buffer	0.08	0.07–0.09	0.19	0.19–0.20	0.09	0.07–0.10
Infected leaves						
Diluted 1:100	*0.38*[c]	*0.32–0.46*	*0.46*	*0.41–0.47*	*0.31*	*0.28–0.32*
Diluted 1:1,000	0.27	0.21–0.38	0.27	0.24–0.30	0.13	0.11–0.15
Diluted 1:10,000	0.18	0.15–0.22	0.20	0.19–0.21	0.10	0.08–0.12

[a] Absorbances of three wells for each dilution in buffer in each of three experiments. In experiments with simultaneous testing of the DAC procedure and one or both of the others, samples were initially extracted at a 1:10 (w/v) dilution rate in 0.02 M potassium phosphate buffer, pH 7.5, and subsequent dilutions made in the appropriate antigen buffer: carbonate buffer, pH 9.6, containing 0.01 M sodium diethyldithiocarbamate (Na DIECA) for DAC and PBS-Tween-PVP containing 0.01 M Na DIECA for PAC and DAS. Substrate incubation times (room temperature) were 2 hr for all forms.

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