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Improved Serological Techniques for the Detection and Identification of Groundnut Viruses

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Serology is indispensable for the detection and identification of plant viruses. Recently, the highly sensitive enzyme-linked immunosorbent assay (ELISA) and immunosorbent electron microscopy (ISEM) techniques have been developed and may replace some of the conventional serological methods. The direct or standard double-antibody sandwich (DAS) form of ELISA. as first described by Clark and Adams (1977), has wide applications in plant virology. An indirect form of ELISA (I-ELISA) has recently been developed by Barbara and Clark (1982). The ISEM procedure developed by Derrick (1973) combines the specificity of serology with coventional electron microscopy. DAS-ELISA, I-ELISA and ISEM have been adapted for the detection and identification of several groundnut viruses. This paper will provide a description of each technique and its application for the detection and characterization of viruses occurring on groundnut in India.

MATERIALS AND METHODS

Maintenance of viruses. Cowpea mild mottle (CMMV), peanut clump (PCV), peanut mottle (PMV) and tomato spotted wilt (TSWV) viruses were all isolated from groundnut in India and maintained on the following hosts : CMMV on soybean, PCV on *Nicotiana glutinosa X N. Clevelandii* (N. hybrid), PMV on peas and TSWV on groundnut.

Antiserum production. In the standard procedure, 1.0 ml of purified virus (ca. 1 mg/ml concentration) was emulsified with an equal volume of Freund's incomplete adjuvant and injected intramuscularly into New Zealand White rabbits at weekly intervals. Serum was collected 2 weeks after the fourth injection and titred by the precipitin ring (est. The titres for antisera used in this study were CMMV 1/2560, PCV 1/1280, PMV 1/2560 and TSWV 1/256.

γ - globulin extraction and enzyme conjugation. The extraction and conjugation of γ-globulins (lgG) was similar to that described by Clark and Adams (1977) and Lister (1978). The r-globulins were pre cipitated from crude serum with 18% sodium sulphate, washed with phosphate-buffered saline (PBS) and then dialyzed against PBS to remove residual sodium sulphate. Extracted γ-globulins were adjusted to a 1 mg/ml concentration (A_{280}= 1.4) and conjugated with alkaline phosphate (type VII, Sigma Chemical Co.; at an enzyme to γ-globulin ratio 'of 2 : I.

Preparation of immunoglobulin fragments. $F(ab')_2$ fragments of IgG were prepared by pepsin digestion as described by Barbara and Clark (1982). IgG was adjusted to a 1 mg/ml concentration with 0.07 M sodium acetate buffer, pH 4.0, containing 0.05 M NaCl. Pepsin (Sigma 1 : 10,000) in distilled water, was added to a final concentration of 45 µg/mg IgG and incubated overnight at 37°G. $F(ab')_2$ fragments were separated from excess enzyme and digestion breakdown products by dialysis against three changes of PBS.

DAS-ELISA Procedure. The method employed was similar to that described by Clark and Adams (1977) and Lister (1978) (Fig.1.).

Fig. 1

DAS-ELISA Procedure

Coat wells with γ-globulins (coating antibodies)
Incubate 3 hrs at 37 C
↓
wash
Add sample extracts to the coated wells
Incubate overnight at 4 C
↓
wash
Add, γ-globulins conjugated with the enzyme alkaline phosphatase (detecting antibodies)
Incubate 3 hrs at 37 C
↓
wash
↓
Add enzyme substrate p-mtrophenyl phosphate
Incubate 30 min at room temperature
↓
add 3N NaOH
↓
yellow colour = positive reaction
Measure absorbance at 405 nm

In the first step extracted γ-globulins (coating antibodies) were adsorbed to the surface of wells in a special microtitre plate (Dynatech Laboratories). Following incubation, any excess unadsorbed coating antibodies were removed from the well by thorough washing. Next was added the test sample which was a crude leaf, insect or seed extract or purified virus. If the test sample contained viral antigens serologically related to the coating antibody, they would be bound to the well surface by the coating antibodies. After washing away any unbound material, trapped viral antigens were detected by adding alkaline phosphatase -labelled- γ- globulins (detecting antibodies). The γ-globulins used for labelling were the same as the coating antibodies. If viral antigens were bound to the surface of the well by the coating antibodies the detecting antibodies would bind to the trapped antigens. The presence of the enzyme-labelled detecting antibody can be assayed by the addition of the substrate p nitrophenyl phosphate (Sigma Chemical Co). The product of the enzyme-substrate reaction is yellow in colour and can be assessed visibly or quantitatively at A_{405} in a spectrophotometer since colour intensity is proportional to the virus concentration.

I-ELISA procedure. The I-ELISA method employed was similar to that described by Barbara and Clark (1982) (Fig. 2) The surface

Fig. 2

I - ELISA Procedure

Coat well with F (ab^1) fragments (coating antibodies)
Incubate 3 hrs at 37 C
↓
wash
↓
Add sample extracts to the coated wells
incubate overnight at 4 C
↓

wash

↓

Add crude serum or r - globuiins (detecting antibodies)
incubate 3 hrs at 37 C

↓

wash

↓

Add commercially available F - specific anti-rabbit
r-globulins conjugateed with the enzyme
alkaline phoshatase
Incubate 3 hr at 37 C

↓

wash

↓

Add enzyme substrate **p** - nitrophenyl phosphate
Incubate 30 min at room temperature

↓

add 3N NaOH

↓

yellow colour = positive reaction
Measure absorbance at 405 nm

of wells in microtitre plates was coated with $F(ab^1)_2$ fragments (coating antibodies . Test samples were added to the well after washing. If viral antigens in the test sample were related to the $F(ab^1)_2$ fragments coating the well they would be retained after washing. Next, unlabelled intact IgG(detecting antibodies) were added which would bind to trapped viral antigens. Crude serum can be used for detecting antibodies. The presence of the unlabelled detecting antibody is determined by adding enzyme-labelled anti-rabbit r-globulins produced in goats which are specific for the F_c portion of the detecting antibody The conjugated F_c - specific anti-rabbit r-globulins are commercially available (Cappel Laboratories, USA)

Immunosorbent electron microscopy procedure (ISEM). The method used is similar to that described by Derrick (1973) and Milne and Luisoni (1977) (Fig. 3),

Fig. 3

ISEM Procedure

Coat carbon-formvar grids by floating on drops of antiserum
Incubate 5 min at room temperature

↓

wash with 20 drops 0.1 M KPO_4, pH 7. 0

↓

Float coated grids on drops of sample extract
Incubate 15-30 min at room temperature

↓

wash with 40 drops distilled water

↓

Stain grids with 6 drops *2%* aqueous uranyl acetate

Formvar-coated copper grids were made hydrophilic by exposure for 10 sec to a carbon glow discharge. The grids were coated with virus-specific r-globulins by floating film-side down on drops of either crude antiserum or extracted r-globulins. After washing away excess r-globulins with drops of phosphate buffer, the coated grids were placed film-side down on drops of the test sample. If viral antigens in the test sample are serologically related to the coating antibodies, they will be bound to the surface of the grid. Any unrelated, therefore unbound, viral antigens or host plant constituents were washed from the surface of the grid with distilled water. The washed grids were negatively stained with 2% aqueous uranyl acetate and examined for the presence of trapped virus particles with the electron microscope.

RESUSTS AND DISCUSSION

Virus detection in seed using DAS - ELISA. The DAS-ELISA procedure has been adapted for rapid screening of large numbers of groundnut seed samples for the presence of PMV Since PMV. is seed-transmitted in groundnuts, it is important to identify non-seed-transmitted lines. However, this requires the assaying of thousands of seeds each season which would be difficult with conventional serological techniques or by bioassaying for virus on indicator hosts. DAS-ELISA is sensitive enough to detect virus antigen up to a l0ng/ml concentration ; therefore only a small portion of an individual seed is required for the assay. The test is reliable even when portions from five different seeds are assayed together in the same well. By testing five seeds/well, over 2500 seeds can be assayed in a single day. Every single seed which contained viral antigen as determined by ELISA also transmitted the virus in growing-on tests. Several germplasm lines have been identified which do not transmit PMV through seed.

Virus detection in insect vectors using DAS-ELISA. The DAS - ELISA test can detect the presence of TSWV in groups of 5-10 thrip insects. The test can be used in studying the epidemiology of the thrip-transmitted bud necrosis disease. The thrip population can be sampled throughout the groundnut growing season for the presence of viruliferous thrips. This facilitates scheduling of chemical control measures at the time when the proportion of viruliferous insects in the population reaches a critical level.

Determining serological relationships. DAS-ELISA, I-ELISA and ISEM tests have been employed for determining the serological relationships of CMMV, PCV and PMV. All three tests were used to establish that CMV is related to carnation latent, chrysanthemum B, dulcamara, helenium S, hop mosaic and potato virus M viruses; but unrelated to lily, symptomless and potato virus S viruses Table 1.

Table 1. The relationship of CMMV with other carlaviruses as determined by DAS-ELISA, I-ELISA and ISEM

	DAL-ELISA	I - ELISA	ISEM
Antisera tested	% Relative affinity	% Relative affinity	% Relative affinity
Carnation latent	35	58	42
Chrysanthemum 8	30	59	34
Cowpea mild mottle	100	100	100
Dulcamara	18	42	29
Helenium S	34	59	31
Hop mosaic	23	55	35
Lily symptomless	None	None	None
Potato Virus M	24	67	74
Potato virus S	None	None	None

Interestingly, these serological relationships could notbe established using the conventional microprecipitin test. DAS-ELISA and ISEM were used to show a serological relationship between PMV and several members of the potyvirus group.

PMV is related to adzuki bean mosaic (ABMV), amaranthus leaf mottle (ALMV), bean common mosaic (BCMV), clover yellow vein (CYVV), and soybean mosaic (SMV) viruses, but unrelated to groundnut eye spot, peanut green mosaic, pepper veinal mottle, potato virus y, sugarcane mosaic and turnip mosaic viruses. (Table 2.) These serological relationships could not be detected with the agar gel diffusion and microprecipitin tests.

Table 2. The relationships of PMV with other potyviruses as determined by DAS-ELISA and ISEM.

Antisera tested	DAS-ELISA % Relative affinity	ISEM % Relative affinity
Adzuki bean mosaic	19	68
Amaranthus leaf mottle	27	56
Bean common mosaic	16	17
Clover yellow vein	23	94
Groundnut eye spot	None	None
Peanut green mosaic	None	None
Peanut mottle	100	100
Pepper veinal mottle	None	None
Potato virusy	None	12
Soybean mosaic	20	86
Sugarcane mosaic	None	None
Turnip mosaic	None	None

Using I-ELISA and ISEM,no serological relationship could be detected between PCV and antisera to any of the soil-borne nematode-transmitted (tobacco rattle PRN and CAM strains and pea early browning) and *Polymyxa* - transmitted (barley yellow mosaic, beet necrotic yellow vein, rice necrosis mosaic, wheat mosaic and wheat yellow mosaic) viruses tested. Interestingly, PCV reported from West Africa is not serologically related to the PCV occurring in India.

Studying morphological [characteristics. The length distribution of PCV particles was established with the aid of ISEM. Insufficient numbers of particles are adsorbed on conventional leaf dip grids for accurate length measurements. However, by pre-coating grid with PCV antiserum over a 100-fold increase in the particle count is observed, thus facilitating an accurate assessment of the particle length distribution. The width of the PCV particle was determined to be 24 + 2 nm with modal lengths of 249 + 8 nm and 184 + 4 nm.

DAS-ELISA has proved to be a sensitive and rapid test for the detection of groundnut viruses in a large number of test samples. The test is especially amenable to screening seeds and insect vectors for the presence of viruses. As a result of successful application of DAS-ELISA for the detection of PMV in seed, several hundred seed samples can now be tested at one time for the presence of

PMV, thus facilitating the identification of non seed-transmitting genotypes and providing a useful tool for plant quarantine programs. Breeding for high yielding, non seed-transmitting cultivars would greatly reduce the spread of PMV in the field.

DAS-ELISA has also been used to detect TSWV in the thrip vector of the economically important bud necrosis disease The test can be employed to assess the proportion of viruliferous insects in field samples.

The I-ELISA test is an improvement over the conventional DAS-ELISA technique because higher dilutions of the coating and detecting antibodies can be used thus conserving antisera. Furthermore I-ELISA obviates the need for a conjugated detecting antibody,

DAS-ELISA, I-ELISA and ISEM have successfully been used to study the serological relationships of the carlaviruses, potyviruses and PCV. However, I-ELISA has been shown to detect broader serological reactions than DAS-ELISA and ISEM.

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