# PROCEEDINGS

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# Methods For The Detection of Seed-Borne Viruses in Groundnut (*Arachis hypogaea* L.)

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## Introduction

Knowledge of transmission is essential for undertaking studies on the epidemiology of diseases caused by plant viruses. A majority of plant viruses are transmitted by biotic agents such as, insects, nematodes and fungi. Apart from these, seeds, especially in the case of legumes, pollen and plant parts used in vegetative propagation can transmit viruses. Of 36 plant virus groups described so far, one or more members in 12 groups are seed transmitted in legumes. Of several plant viruses reported to naturally infect groundnut (*Arachis hypogaea* L.), at least five viruses are known to be seed-transmitted in groundnut (Table 1).

**TABLE 1. Seed-transmitted viruses in groundnut**

| Group | Virus | References |
|---|---|---|
| Cucumovirus | Cucumber mosaic virus | Xu and Barnett (1984) |
| | Peanut stunt virus | Troutman *et al.* (1967) |
| Furovirus | Peanut clump virus | Reddy and Hobbs (1988) |
| Potyvirus | Peanut mottle virus | Kuhn (1965) |
| | Peanut stripe virus | Demski *et al.* (1984) |

Seed transmission generally occurs due to infection of gametes. Infected seeds often serve as primary source of inoculum. Additionally, seed transmission aids in the long distance spread of viruses. Thus it is essential to develop sensitive methods for virus detection in seed, especially in quarantine. In order to produce improved cultivars several developing countries are currently importing large amounts of germplasm. Unfortunately methods for virus detection in seed are largely lacking in developing countries. As a result chances for introducing seed-transmitted viruses are high. A good example is in the case of peanut stripe virus (PStV) which was known to be restricted to Southeast Asian countries prior to 1980. Within a decade the virus had spread to several countries due to constant exchange of germplasm from countries

known to PStV (D.V.R. Reddy, Personal Communication). In this paper we give methods which can be employed for the detection of viruses in groundnut seed. They can also be used to detect viruses in seed of other crop plants.

**Methods for detection**

Viruses in seeds are detected either by a direct methods (grow-out and infectivity assay) or by indirect methods (serological tests and complimentary DNA probes).

**Grow-out test** : Grow-out test is the easiest to perform and the method of choice, if seed-transmitted viruses produce external symptoms. The test should be done in containment. Seed sample taken at random is sown in pots or wooden trays in sterile soil and the seedlings are raised and plants grown up till symptoms of virus infection are apparent. Identification is based on the presence of external symptoms.

**Infectivity assay** : Diagnostic hosts are currently known (Table 2) for all the seed-transmitted viruses in groundnut. Extracts from seed or from leaves prepared in suitable buffer are mechanically inoculated on to diagnostic hosts. This test can be performed with minimum laboratory facilities. It is especially valuable for virus detection in plants exhibiting no symptoms (symptomless carriers) or typical symptoms in grow-out tests.

**TABLE 2. Local lesion hosts for infectivity assay of seed-borne groundnut viruses**

| Virus | Assay host | Reaction |
|---|---|---|
| CMV[1] | *Chenopodium amaranticolor* | Necrotic lesions |
| | *C. quinoa* | Necrotic lesions |
| | *Vigna unguiculata* | Necrotic lesions |
| PCV[1] | *C. amaranticolor* | Necrotic lesions |
| | *C. quinoa* | Necrotic lesions |
| | *Phascolus vulgaris* cv. Topcrop | Veinal necrosis |
| PMV[1] | *P. vulgaris* | Necrotic lesions |
| PSV[1] | *C. amaranticolor* | Chlorotic lesions |
| | *C. quinoa* | Chlorotic lesions |
| | *V. unguiculata* | Chlorotic lesions |
| PStV[2] | *C. amaranticolor* | Chlorotic lesions |

[1]Boswell and Gibbs (1983)
[2]Demski *et al.* (1984)

**Main disadvantages in direct methods** : They are time consuming, not very sensitive, dependent on how well the diagnostic hosts can be raised, and additionally permits virus spread if proper containment facilities are not available

**Serological tests** : Serological tests which are based on specific antigen antibody reactions offer convenient methods to diagnose the presence of viruses in seeds. Among the several serological tests that are available, those used

in the detection of seed-borne viruses are gel diffusion (Hamilton, 1965), latex bead agglutination test (Phatak, 1974), enzyme-linked immunosorbent assay (ELISA) (Bharathan *et al.*, 1984), dot immunobinding assays (DIBA) (Lange and Heide, 1986) and immunosorbent electron microscopy (ISEM) (Hamilton and Nicholas, 1978). Gel diffusion and latex agglutination tests are difficult to perform on seeds since the starch granules present in the seeds may interfere with the reaction. ISEM needs facilities for electron microscopy and additionally difficult to perform on large number of samples. DIBA and ELISA are the most preferred among the serological tests. Adoption of DIBA in developing countries is more difficult than ELISA because materials and reagents required for the test are not easily available. In case of ELISA, it is possible to obtain the necessary supplies, especially if penicillinase-based ELISA (Sudarshana and Reddy, 1989) is adopted. We give below details for performing this ELISA on groundnut seed.

**Materials required for ELISA**

(a) ELISA-plates (Dynatech, Nunc, or any other reliable brand available in India). In India only polystyrene plates are available.

(b) Antisera

(c) Micropipettes (10-100$\mu$l and 100-1000$\mu$l). Several brands (Eppendorf, Finipette, Gilson, etc.) are available. They are available in India on rupee payment. Those which provide adjustable volumes are preferable.

(d) Repeatable micropipette (100 or 200 $\mu$l volume). It is possible to dispense desired volumes of liquids repeatedly from a reservoir attached to a micropipette. Multi-channel micropipettes which can hold 4, 8, or 12 microtips, thus permitting dispensing of desired volumes simultaneously into several wells are available. These repeatable or multi-channel micropippettes are not readily available in India. Although they save considerble amount of time, a single channel pipette (described above) can be used in their places.

(e) ELISA plate reader—manual or automatic. Several ELISA plate readers are available ranging in price from $2000 to $ 15000. Some of the popular brand names are Biorad, Biotek, Dynatech, Flow Laboratories, Inter-read Immunoreader etc. However plate readers are only required for quantitative estimation of virus concentration.

**Conjugation of immunoglobulins with penicillinase**

**Materials**

(a) IgG (antirabbit IgG, Sigma Chemical Co. or antirabbit-Fc specific globulin (Cappel Laboratories) produced in goats.

(b) Phosphate buffer: (PBS) $Na_2HPO_4$, $2H_2O$ 2.88 g or $Na_2HPO_4$ 2.38 g; $KH_2PO_4$ 0.4 g, KCl 0.4 g; NaCl 16.0 g; Dist. water 2 L. pH of the solution is generally 7.4.

(c) Glutaraldehyde (25% Sigma G 5882).
(d) Penicillinase. Generally supplied as lyophilised powder from Sigma (P 0389) or from Hindusthan Antibiotics Ltd., Pimpri, India. ELISA grade.
(e) Dialysis bag (0.6 cm dia.).
(f) Micropipette.
(g) Bovine serum albumin. Highly pure chemical should be used (Sigma A 7638).

**Procedure**

(a) Take IgG, 1 mg/ml (supplied as 8 or 10 mg/ml—make an appropriate dilution in PBS) in a dialysis bag and add 2 mg of penicillinase. Solutions of IgG and penicillinase can be made at higher concentrations and mixed to get 1 mg IgG/ml and 2mg/ml penicillinase.
(b) Dialysis in PBS in a beaker for 1 h at room temperature.
(c) Transfer the dialysis bag containg IgG and enzyme into a beaker containing PBS with 0.06% glutaraldehyde (mix 1 ml of glutaraldehyde in 400 ml PBS to get 0.07% glutaraldehyde) and dialyse for 3-4h at room temperature.
(d) Replace the buffer containing glutaraldehyde with 500 ml PBS containing sodium azide (0 02%) and dialyse for 18 h at 4°C with at least three changes of buffer. For each change use 500 ml PBS containing azide.
(e) Transfer the conjugate into a new glass or plastic vial and add bovine serum albumin at 5 mg/ml concentration. Store in small aliquots (200 µl) at 40°C. Do not freeze the conjugate. Shelf life of conjugated globulin, if stored properly exceeds 1 year.

**ELISA test procedure**

**Materials**

(a) Carbonate buffer (coating buffer) : $Na_2CO_3$ 1.50 g ; $NaHCO_3$ 2.93 g ; Distilled Water 1 L. pH of buffer should be 9.6. No need to adjust the pH.
(b) Phosphate-buffered saline with tween (PBS-tween) : PBS 1 L ; Tween-20 0.5 ml.
(c) Antiserum buffer : PBS-tween 100 ml ; polyvinyl pyrollidone (40,000 MW) (Sigma PVP-40T 2.0 g ; ovalbumin (crystallized, Sigma Chemicals Grade II or Grade III) 0.2 g.
(d) Substrate buffer : Dissolve 20 mg bromothymol blue (BTB) in 50 ml of 0.1 M NaOH. Neutralize the alkali by adding conc. HCl dropwise. Make up the volume to 100 ml. Incorporate sodium penicillin-G (Potassium penicillin-G and procaine penicillin can also be used) at 0.5 mg/ml and adjust the pH to 7.2 using either HCl or NaOH (0.01 to 0.1 M). Store the mixture at 5°C. It is essential to adjust the pH to 7.2 before use.

**lelpful hints**

a) New plates should be used as supplied by manufacturers. Do not wash or rinse them prior to use.

b) Do all incubations in a humid chamber. A small rectangular plastic box with moist paper towels is adequate.

c) Take care not to contaminate the glassware intended for storing penicillin BTB solution with buffering salts because the reaction between penicillin and penicillinase is detected by changes in pH due to production of penicilloic acid.

d) Unless the antisera used are of very high quality, cross adsorption of crude antisera with healthy leaf freed extracts is recommended. This is done by grinding healthy leaves/seeds in antibody buffer to give a 1:20 dilution, then filtering through two thickness of cheese cloth. Prepare antiserum dilution in healthy plant extracts suspended in antibody buffer. Incubate at 37°C (for a minimum of 45 min.) prior to dispensing in ELISA plates. Cross-adsorption of antisera substantially reduces the non-specific reaction due to precipitation of antigens of plant origin.

**Procedure for ELISA for the detection of viruses in groundnut**

(a) Construct a wooden tray with 8 rows of 12 slots, similar to wells arranged in a microtitre ELISA plate, to accommodate 25 seeds in each slot.

(b) Slice out a portion of cotyledon, opposite to the embryo (blunt end of seed) and collect slices from 25 seeds and triturate with a mortar with 0.5 to 1.0 ml of carbonate buffer.

(c) Using a micropipette or pasteur pipette transfer about 200 µl of seed extract into each well of a microtitre ELISA plate, and place seed lot in corresponding slots in wooden tray. The position of seed sample in the wooden tray should not be disturbed till the test is completed.

(d) Fill all the wells in the plate leaving the last three empty. Utilize one well for adding extracts of seed containing the virus (positive control), one well for extracts from healthy seed (negative control) and the last one to serve as buffer control. Incubate the plate at 37°C for 1 h.

(e) Rinse the plate with a small quantity of PBS-T to remove the seed debris still remaining in the well. Wash the plate three times with PBS-T leaving PBS-T at least for 1 min in the well between washes.

(f) Dilute virus-specific antiserum raised in rabbits to the working dilution in antiserum buffer. If the test involves detection of more than one virus and precise identification of each of them is not necessary then a cocktail of antisera can be used. Dispense 200µl of diluted antisera to each well and incubate at 37°C for 1 h.

(g) Wash as in (e) using PBS-T.

(h) Dilute penicillinase labelled-antirabbit IgG antiserum buffer to working dilution (1:5000 or 1:10000), dispense 200 µl to each well and incubate at 37°C for 1 h.

(i) Wash as in (e) but using distilled water containing 0.05% tween-20.
(j) Dispense 200 µl of substrate to each well and incubate at room temperature. Read the results after 1-2 h. Presence of virus is indicated by the change in the color of the substrate from bluish green to yellow. Wells with green color indicate a lower virus titre, but nevertheless, are positive.

The lots of 25 seeds positive for virus presence can now be used either for detecting virus in individual seed or in lots of five, in order to save ELISA plates. If large quantities of seed are available it is economical to discard the lot (containing 25) which gave a positive reaction. In groundnut, the two widely distributed seed-transmitted viruses, peanut mottle virus (PMV) and PStV, have seed transmission rates often less than 3%. In the case of peanut clump virus (PCV) seed transmission often exceeds 5%. Thus for detection of PMV it is advisable to use lots containing less than 10 seed. Utilizing the ELISA procedure described above, PMV, PStV were detected in embryo, cotyledon, and seed coat. Good correlation was observed between grow-out, infectivity assay, and ELISA tests for detecting PMV (Bharathan *et al.*, 1984) and PStV (Demski and Lowell, 1935). However, in case of PCV a small proportion of seeds, though gave negative reaction in serological tests, showed virus presence in grow-out tests (A.S. Reddy, Unpublished). Utilizing extracts from 25 seed in each well of ELISA plate, it is now possible to process over 18,000 seeds in a day without destroying the viability of seed. Excluding the cost towards manpower and for producing antiserum, processing of 18,000 seeds costs about 10 US $ (Rs. 170). Utilizing these procedures it is possible to maintain virus-free germplasm, to detect viruses in quarantine and to develop cultivars with negligible or no seed transmission.

**Utilization of Complementary DNA (cDNA) probes for virus detection in seeds**

All currently known seed-transmitted viruses in groundnut possess single stranded RNA. It is possible to produce cDNA probes for these viruses. Using cDNA probes PMV and PStV were detected in groundnut seeds at lower levels than those detected using ELISA (Bijaisoradut and Kuhn, 1988). Highly perishable reverse transcriptase is required for producing cDNA probes. Additionally, expensive and short-lived $^{32}P$ is required for the production of highly sensitive cDNA probes. Recently non-radioactive cDNA probes have also been used for the detection of plant viruses in seeds (Roy *et al.*, 1988). Nevertheless they are expensive. Until more readily available and inexpensive probes are developed this method is not expected to be utilized for virus detection in large quantities of seed.

**Future research**

The presence of serologically different isolates is a major problem in quarantine. Broad-spectrum probes using monoclonal antibodies (MAb) will have

major role in the near future. Additionally, production of MAb would ensure unlimited supply of antibodies. Similarly, viral genes once successfully cloned in bacteria can be maintained permanently and can be utilised for producing desired cDNA probes. If non-radioactive probes are developed they can be transported safely and can be used in developing countries. We plan to produce group-specific MAb for various PCT isolates present in India. Recent work done by Shukla and Ward (1989) on the polypeptides of potyviruses has shown that it is possible to produce polyclonal antisera to the core region of the viral polypeptide which is highly conserved in different potyviruses. Antisera produced for core region react with several potyviruses. We are in the process of producing broadly specific antisera which can detect several potyviruses.

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