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Purification, serology and physico-chemical properties of a peanut mottle virus isolate from India*

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A procedure developed for the purification of peanut mottle virus (PMV) isolated from peanuts in India yielded 30–40 mg of virus/kg of plant tissue. Purified virus sedimented as a single component of 151 S. The virus coat protein migrated as a single component in each of two polyacrylamide gel concentrations and had an estimated molecular weight of 34 000 daltons. The molecular weight of the nucleic acid was $3{\cdot}10 \times 10^6$ daltons. In the precipitin ring interface test (PRIT) PMV showed a serological relationship with soybean mosaic virus (SMV). Using the double antibody sandwich form of ELISA, PMV was shown to be distantly related to adzuki bean mosaic (ABMV), amaranthus leaf mottle (ALMV), clover yellow vein (CYVV) viruses and SMV. Immunosorbent electron microscopy (ISEM) showed PMV to be closely related to ABMV, ALMV, CYVV and SMV. In PRIT, ELISA and ISEM tests no specific serological reaction was noted between PMV and antisera to groundnut eye spot, peanut green mosaic, pepper veinal mottle, potato virus Y, sugarcane mosaic and turnip mosaic viruses.

INTRODUCTION

Peanut mottle virus (PMV) has been reported to infect peanuts in India (Reddy *et al.*, 1978). On the basis of electron microscopy of virus particles and inclusions produced in infected tissue, the virus was placed in the potyvirus group (Kuhn & Bock, 1973). The adoption of a purification procedure developed for the severe mosaic strain of PMV (Sun & Hebert, 1972) to purify a mild mottle isolate resulted in a total loss of infectivity (Paguio & Kuhn, 1973). Methods for the purification of mild mottle isolates of PMV occurring in East Africa and the U.S.A. were devised by Paguio & Kuhn (1973) and Bock *et al.* (1978). However, when these procedures were used to purify the Indian PMV isolate, either considerable infectivity was lost after initial clarification or yields were less than 15 mg from 1 kg tissue. A method was developed to obtain higher yields of total and infective virus. In addition, the molecular weights of the coat protein and nucleic acid and the sedimentation coefficient of purified virus were determined.

The serological relationship of PMV with other potyviruses was investigated by agar gel diffusion, microprecipitin and precipitin tube tests (Sun & Hebert, 1972; Bock, 1973). In this study the serological relationship of PMV with 10 other potyviruses was tested by employing the precipitin ring interface test (PRIT), the double antibody sandwich form of the enzyme linked immunosorbent assay (ELISA) and immunosorbent electron microscopy (ISEM) as described by Derrick (1973).

MATERIALS AND METHODS

Virus culture and purification

The virus was maintained in peas (*Pisum sativum*, cv. Bonneville) by periodic mechanical inoculation of extracts prepared in 0·05 M phosphate buffer, pH 7·0, containing 0·02 M 2-mercaptoethanol.

Infected pea leaves exhibiting systemic mosaic symptoms were homogenized (1 g/4 ml)

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in a blender with cold 0·1 M phosphate buffer, pH 8·0, containing 0·01 M sodium diethyldithiocarbamate and 1% 2-mercaptoethanol. The sap was filtered through cheesecloth and shaken with 10% chloroform (v/v) for 3–4 min. The emulsion was broken by centrifugation at 3000 g for 15 min. Polyethylene glycol (PEG) and sodium chloride were added to the clarified extract to final concentrations of 4% and 0·2 M, respectively. The mixture was incubated at 4°C for 90 min before centrifugation at 5000 g for 10 min. The pellets were resuspended in 0·01 M borate-phosphate buffer, pH 8·3, containing 0·2 M urea (BPU). Twenty-five ml of the virus preparation was then layered on a 13 ml column of 30% sucrose prepared in BPU containing 4% PEG and 0·2 M NaCl. Following centrifugation at 24 000 rpm for 90 min in a Beckman SW 27 rotor the pellets were resuspended in BPU and subjected to rate-zonal density gradient centrifugation. The gradient columns were prepared by layering, respectively, 6, 9, 9 and 9 ml of 100, 200, 300 and 400 g/l of sucrose in BPU and then stored overnight at 4°C before use. Five ml of the virus preparation were layered on each gradient column which was centrifuged at 24 000 rpm for 2 h in a SW-27 rotor. A diffuse light-scattering zone at 6·3–7·0 cm and a single clear zone at 5·6–6·0 cm from the bottom of the tube were evident. Virus particles were detected in the lower zone.

Sedimentation coefficient

The sedimentation coefficient of the purified virus was determined in a Beckman Model E analytical ultracentrifuge using Schleiren optics with an An-D rotor. After attaining a speed of 21 000 rpm photographs were taken at 2-min intervals.

Molecular weight determination of the PMV polypeptide and PMV RNA

The molecular weight of the virus polypeptide was determined in 7·5 and 10% polyacrylamide gels using a 3% spacer gel (Reddy & Black, 1977). Purified virus pellets were solubilized in 0·0625 M Tris-HCl buffer, pH 6·8, containing 2% SDS, 1% 2-mercaptoethanol and 6 M urea and co-electrophoresed with markers at 50 V for 3–6 h. The gels were stained in 2% coomasie blue and scanned at 540 nm in a Gilford 250 recording spectrophotometer. Phosphorylase B (90 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 000) and lysozyme (14 300), obtained from Bio-Rad Laboratories, were used as molecular weight markers.

Nucleic acid was extracted from purified PMV by the method described by Kirby (1965). Nucleic acid was also obtained by treating purified virus with pronase, SDS, 2-mercaptoethanol and urea (Sreenivasulu *et al.*, 1981). The basic procedure of gel electrophoresis was that of Loening (1967). The nucleic acid was analysed in tube gels (0·6 × 8 cm) containing 2·4% acrylamide, 0·12% bis-acrylamide and 0·5% agarose. The electrophoresis buffer contained 0·02% diethyl pyrocarbonate. The nucleic acid samples and marker RNA samples were co-electrophoresed at 50 V for 3 h at room temperature. The gels were stained in 0·1% toluidine blue O and scanned at 540 nm. Tobacco mosaic virus RNA ($2{\cdot}05 \times 10^6$ daltons), brome mosaic virus RNAs (1·09, 0·99, 0·75 and $0{\cdot}28 \times 10^6$ daltons) and *Escherichia coli* ribosomal RNAs (0·56 and $1{\cdot}1 \times 10^6$ daltons) were used as molecular weight markers.

Precipitin ring interface test (PRIT)

The test was done as described by Reddy *et al.* (1969). Antisera were used at 1 : 20 dilution and titrated against serial two-fold dilutions of purified virus.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA procedure employed was similar to that described by Lister (1978). The γ-globulins were extracted with 18% sodium sulphate and adjusted to 1 mg/ml (A 280 nm = 1·4) and then conjugated with alkaline phosphatase (type VII, Sigma Chemical Co.) at an enzyme to globulin ratio of 2 : 1. Wells in polystyrene micro ELISA plates were filled with 0·2 ml of reagents. Coating globulin was used at 10 μg/ml for PMV and all other antisera employed. Extracts from healthy and infected leaves at a 1 : 10 dilution and purified PMV at 500 ng/ml were used in all tests.

Conjugated globulins were used at a concentration of 2·5 μg/ml. The assay was completed by measuring the absorbance (A 405 nm) in a Gilford 250 spectrophotometer.

Immunosorbent electron microscopy (ISEM)

The method of Derrick (1973) was adopted and the tests were conducted at the Institut für Viruskrankheiten der Pflanzen, Braunschweig, West Germany, where it was possible to include a few other potyviruses as suitable controls. Formvar-coated 200-mesh grids were exposed for 10 s to glow discharge. The grids were coated with protein A (Sigma Chemicals) by floating them on a 10 μl drop (5 μg protein A/ml) for 5 min. Coated grids were washed twice in 0·1 M phosphate buffer, pH 7·0 (PB) by floating for 15 min for each wash. Then grids were floated on a 10 μl drop of diluted globulin from antisera or normal rabbit serum for 1 h at 37°C. Five different dilutions of gamma globulins were used with each serum. The grids were washed in PB as described above. After drawing off excess PB with the edge of a filter paper, the grids were floated on a 20 μl drop of 10 μg/ml purified virus. In addition to PMV and other potyviruses, cowpea mild mottle virus (CMMV) was used to check for any non-specific trapping by any of the antisera. After incubating for 2 h at 4°C the grids were washed with 30 drops of distilled water and stained for 5 min in neutral 2% phosphotungstate. The grids were viewed under a Philips 201 C electron microscope operated at 60 kV. Particle counts were made at an instrument magnification of X40 000. Particle numbers on 12 viewing fields, chosen randomly from different grid squares, were counted on two duplicate grids. Results represent mean values from two grids.

RESULTS

Purification procedure

Maximum infectivity from systemically infected peas was obtained at 2–3 weeks following inoculation, under greenhouse conditions (22–35°C). Accordingly, the tissue was harvested 2 weeks after inoculation. Extraction in 0·01 M phosphate buffer, pH 8·0, gave the maximum infective virus in the clarified preparation compared to other buffers (Table 1). No loss in infectivity was detectable following treatment with 10% chloroform or 50% carbon tetrachloride (Table 2). However,

Table 1. Relative infectivity of peanut mottle virus extracted in different buffers

Buffer[a]	No. of lesions/half leaf at 10^{-1} dilution[b]	% relative infectivity[c]
0·5 M phosphate, pH 7·0	64	47
0·5 M phosphate, pH 8·0	122	90
0·1 M phosphate, pH 7·0	70	51
0·1 M phosphate, pH 8·0	126	93
0·05 M phosphate, pH 7·0	68	50
0·05 M phosphate, pH 8·0	130	96
0·01 M phosphate, pH 7·0	72	53
0·05 M phosphate, pH 8·0	136	100
0·1 M histidine, pH 7·0	38	28
0·1 M tris, pH 8·0	12	9
0·5 M sodium citrate, pH 8·0	75	55

[a]All buffers contained 0·01 M sodium diethyldithiocarbamate and 1% 2-mercaptoethanol.
[b]Assays were performed on *Phaseolus vulgaris* (cv. Topcrop). Each value is an average of eight replicates. Leaf tissue (110 g) was cut into small pieces; a 10 g sample was triturated in each buffer and filtered through cheesecloth.
[c]Assuming the infectivity with 0·01 M phosphate buffer, pH 8·0, as 100%.

Table 2. Relative infectivity of peanut mottle virus following treatment with organic solvents

Treatment[a]	Number of lesions/half leaf at 10^{-1} dilution[b]	% relative infectivity[c]
10% chloroform	142	100
33% chloroform	129	91
50% chloroform	120	85
8% n-butanol	26	18
8% n-butanol + chloroform (1 : 1)	42	30
50% carbon tetrachloride	139	98

[a]Extracts were prepared in 0·01 M phosphate buffer, pH 8·0, containing 0·01 M sodium diethyldithiocarbamate and 1% 2-mercaptoethanol.
[b]Assays were performed on *Phaseolus vulgaris* (cv. Topcrop). Each value is an average of eight replicates.
[c]Assuming the infectivity with 10% chloroform treatment as 100%.

treatment with carbon tetrachloride failed to remove host contamination as effectively as chloroform as judged by the zonal separation in rate zonal centrifugation. Different concentrations of NaCl and PEG were tested for precipitating the virus and the best was 0·2 M NaCl and 4% PEG. Identical infective virus recovery resulted when 0·01 M phosphate or 0·01 M borate-phosphate buffer containing 0·2 M urea or 0·001 M Cleland's reagent were tested for suspending virus pellets and for preparing sucrose gradients. The use of BPU consistently yielded virus preparations, after rate zonal centrifugation, with no detectable host contamination as judged by the electron microscopic examination of PTA-stained samples. The amount of infective virus recovered in various steps of the purification procedure is recorded in Table 3. Assuming an extinction coefficient of 3·0, 30–40 mg of virus was obtained from 1 kg of leaves.

Properties of the purified virus

The UV absorption spectrum of the purified virus had a shoulder at 290 nm, and the A 260/280 ratio was 1 : 20. The A 260/245 ratio was 1 : 26. Purified virus in BPU sedimented as a single component with a sedimentation coefficient (S°_{20W}) of 151 S.

Table 3. Relative infectivity of peanut mottle virus at different steps of virus purification

Steps of purification	No. of lesions/half leaf at 10^{-1} dilution[a]	% relative infectivity
Clarification	165	100
Chloroform treatment	165	100
Polyethylene glycol precipitation	154	93
Concentration in PEG-sucrose solution	108	66
Rate zonal gradient centrifugation	79	48

[a]Assays were performed on *Phaseolus vulgaris* (cv. Topcrop). Each value is an average of eight replicates. Dilution of each preparation was based on the original weight of leaf tissue.

Polyacrylamide gel analysis of virus polypeptide and nucleic acid

The PMV polypeptide migrated as a single component with an estimated molecular weight of 34 000 daltons in both 7·5 and 10% gels. The PMV nucleic acid extracted by Kirby's[9] method and from purified virus disrupted with SDS, mercaptoethanol and urea migrated as a single band with an estimated molecular weight of 3·10 X 10^6 daltons.

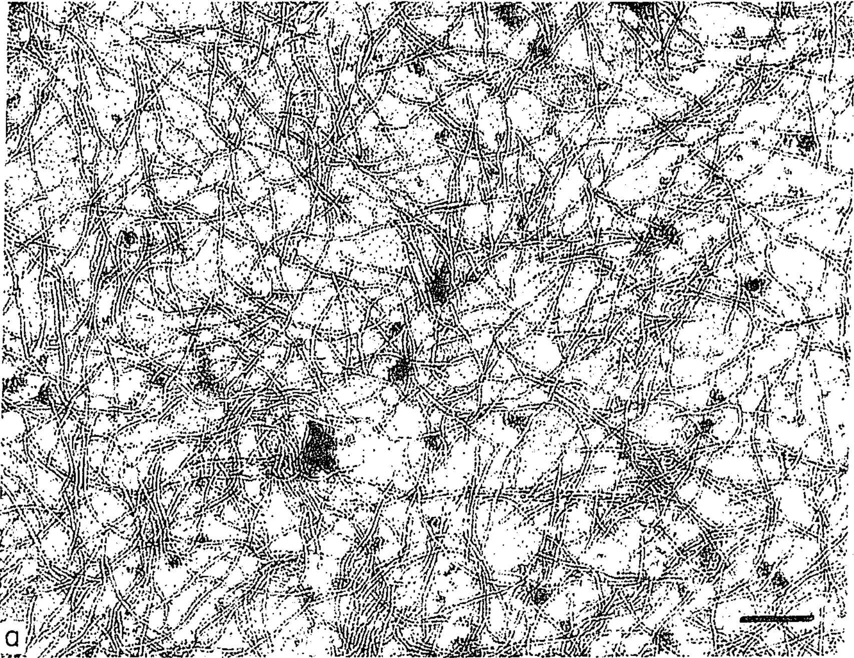
Serological relationships

In PRIT the minimum concentration of virus detected with homologous and PMV 'S' antisera was 10 μg/ml. With soybean mosaic virus (SMV) antiserum, PMV up to 40 μg/ml was detected. None of the other antisera reacted with purified PMV. In ELISA, PMV cross-reacted strongly with the PMV 'S' strain antiserum. Heterologous reactions were detectable with adzuki bean mosaic (ABMV), amaranthus leaf mottle (ALMV), clover yellow vein (CYVV) and soybean mosaic (SMV) virus antisera. Among all the antisera tested, only groundnut eye spot (GESV) gave a maximum absorption with healthy pea extracts (Table 4). In the ISEM technique the number of PMV particles trapped with the PMV 'S' strain (Fig. 1a), CYVV and SMV antisera was comparable to that with the homologous antiserum. A relatively large number of PMV particles were also trapped by ABMV and ALMV (Fig. 1b) antisera. Results with GESV (Fig. 1c), peanut green mosaic (PGMV), pepper veinal mottle (PVMV), potato virus Y (PVY), sugarcane mosaic (ScMV) and turnip mosaic (TuMV) virus antisera were comparable to that with normal serum (Table 5). Relatively large numbers of virus particles were trapped when some of the antisera were tested with related viruses (Table 5). The number of CMMV particles trapped with all antisera was comparable to that with normal rabbit serum, eliminating the possibility of non-specific trapping.

Table 4. Absorbancies (A 405) in ELISA with potyviruses in homologous and heterologous reactions

Antisera tested[a]	Healthy pea extract at 1 : 50 dilution	PMV-infected pea extract at 1 : 50 dilution	Purified PMV at 500 ng/ml
1 Adzuki bean mosaic virus (1/800)	0·001	0·142	0·210
2 Amaranthus leaf mottle virus (1/256)	0·019	0·216	0·320
3 Clover yellow vein virus	0·000	0·167	0·290
4 Groundnut eye spot virus (1/500)	0·116	0·118	0·006
5 Peanut green mosaic virus (1/500)	0·049	0·072	0·009
6 Peanut mottle virus 'Homologous' (1/600)	0·013	0·742	0·896
7 Peanut mottle virus 'S' strain (1/800)	0·016	0·783	0·809
8 Pepper veinal mottle virus (1/400)	0·026	0·032	0·010
9 Potato virus 'Y' (1/3200)	0·016	0·029	0·019
10 Soybean mosaic virus (1/800)	0·020	0·169	0·360
11 Sugarcane mosaic virus, strain 'A' (1/128)	0·016	0·020	0·016
12 Turnip mosaic virus	0·013	0·013	0·012
13 Normal rabbit serum	0·000	0·012	0·015

[a]Values given in parenthesis are homologous titres by precipitin ring test. Antisera 1, 10 and 12 were produced in Japan by Dr N. Iizuka; antiserum 2 was supplied by Dr V. Lisa (Italy); 3 by Dr A. Cockbain, 4 and 8 were supplied by Dr C. Fauquet (Ivory Coast), 5 and 6 were produced at ICRISAT; 7 was supplied by Dr T. T. Hebert (U.S.A.) and 9 and 11 were obtained from the American Type Culture Collection.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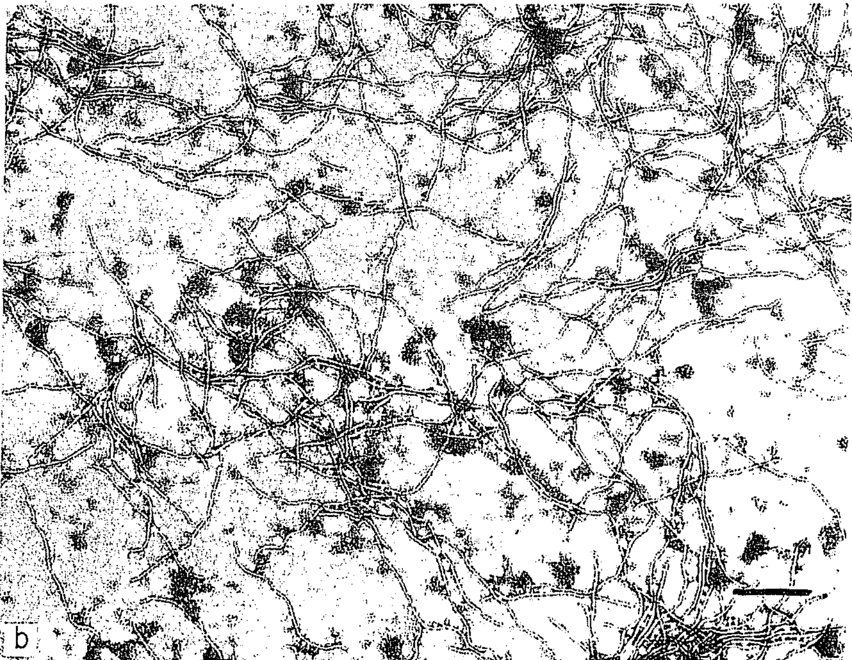
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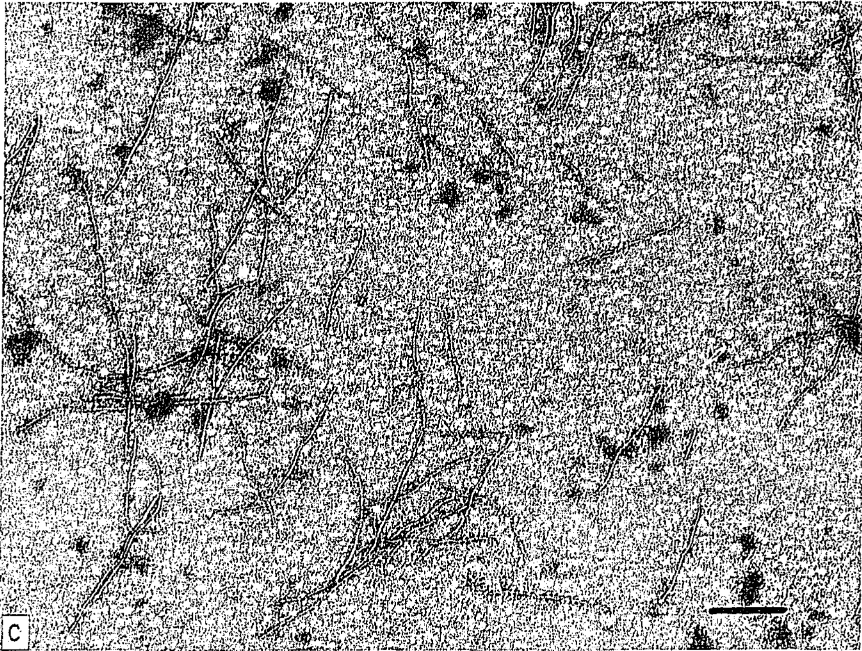

Fig. 1. Serological relationships of peanut mottle virus using immunosorbent electron microscopy. Grids were exposed to 5 μg protein A/ml, then coated with 10 μg/ml globulins followed by 10 μg/ml purified peanut mottle virus. Bar represents 400 nm. (a) Grid coated with globulins from peanut mottle virus 'S' antiserum. (b) Grid coated with globulins from adzuki bean mosaic virus antiserum. (c) Grid coated with globulins from groundnut eyespot virus antiserum; particles trapped were equivalent to negative controls.

DISCUSSION

The purification method devised yielded twice as much purified virus as the procedure described by Paguio & Kuhn (1973). The important difference between the two procedures was that in this work the virus was concentrated by using a sucrose column containing PEG and NaCl. Using 50 g of leaf tissue, the yields of infective purified virus from both procedures have been compared simultaneously in two independent experiments. PMV purified by the procedure of Paguio & Kuhn (1973) retained 22–25% of the infectivity and yielded 0·7–0·8 mg purified virus from 50 g tissue whereas our procedure gave 55% retention of infectivity and a corresponding yield of 1·9–2·2 mg. Since extracts prepared in 0·5 M sodium citrate buffer containing 1% 2-mercaptoethanol followed by treatment with either 33% or 50% chloroform (Bock *et al.*, 1978) (Table 1) and treatment of extracts with chloroform-butanol (Sun & Hebert, 1972) resulted in over 30% loss in infectivity of clarified extracts (Table 2), these methods were not compared with our PMV purification procedure.

The sedimentation coefficient of the virus particles and the molecular weight of the virus polypeptide and nucleic acid were similar to those reported for other potyviruses (Hollings & Brunt, 1981). Only freshly purified virus preparations were used for the estimation of the polypeptide molecular weight. An additional low-molecular-weight polypeptide, present in several other potyviruses, was not observed.

The precipitin tube test has been employed to detect serological relationships among potyviruses (Hollings *et al.*, 1977). However, using this test, PMV did not react with bean yellow mosaic, celery mosaic, CYVV, cowpea aphid-borne mosaic, iris mosaic, PVY, ScMV, SMV and tobacco severe etch virus antisera (Bock, 1973). In agar gel-diffusion and microprecipitin tests PMV antisera did not react with PVY, SMV or tobacco etch virus;

Table 5. Serological trapping of purified peanut mottle and other potyvirus particles on electron microscope grids sequentially coated with 5 μg protein A and antisera of potyviruses

Antisera tested[a]	Number of PMV particles trapped[b]	Other potyviruses[c]
1 Adzuki bean mosaic virus	404	–[d]
2 Amaranthus leaf mottle virus	345	–
3 Clover yellow vein virus	525	–
4 Groundnut eye spot virus	66	–
5 Peanut green mosaic virus	73	610[e]
6 Peanut mottle virus 'Homologous'	556	–
7 Peanut mottle virus 'S' strain	570	–
8 Pepper veinal mottle virus	76	–
9 Potato virus Y	65	570[f]
10 Soybean mosaic virus	490	510[g]
11 Sugarcane mosaic virus	65	–
12 Turnip mosaic virus	66	470[h]
13 Normal rabbit serum	68	67[e], 55[f], 69[g], 59[h]

[a]Source and homologous titres are given in Table 4.
[b]Formvar coated grids exposed for 10 s to a glow discharge were coated with 5 μg/ml protein A then floated on a 10 μl drop of gamma globulin at 10 μg/ml globulin concentration, which trapped the maximum number of virus particles. Details are given in the text. Counts represent mean values from 12 random viewing fields on each of two duplicate grids at a magnification of X 40 000.
[c]Purified viruses were employed at 10 μg/ml concentration.
[d]Not tested.
[e]Peanut green mosaic virus.
[f]Potato virus Y.
[g]Soybean mosaic virus.
[h]Turnip mosaic virus.

nor did purified PMV react with the antisera to these three viruses (Sun & Hebert, 1972). With the exception of the heterologous reaction detected with SMV antiserum our results with PRIT confirmed earlier reports that PMV was not serologically related to several other potyviruses. Results with ELISA and ISEM tests conclusively show serological relationships of PMV with ABMV, AMLV, CYVV and SMV. The degree of cross-reaction observed in the ISEM test was greater than that observed with the ELISA test. Although we were unable to check the ability of GESV, PVMV and ScMV antisera to trap their respective closely related viruses, it was unlikely that the sera used had lost their ability to trap virus particles. Using similar ELISA and ISEM tests, the PMV isolate from India was shown to be distantly related to colombian datura virus, Lü and Geo isolates of PVY and tobacco etch virus (S. Meyer and R. Casper, personal communication). Thus the high specificity reported for the double antibody sandwich form of ELISA (Koenig, 1978) was not detected with the PMV isolate from India. Serological relationships among potyviruses employing ELISA and ISEM tests have not been extensively reported. It would be interesting to know if, with these two tests, other potyviruses would show distant serological relationships undetectable by tube precipitin, PRIT, microprecipitin and agar gel-diffusion tests.

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