Serological Methods for Detection of Pea Seed-Borne Mosaic Virus in Leaves and Seeds of Pisum sativum

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ABSTRACT

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Immunodiffusion in gels containing 0.5% sodium dodecyl sulfate, enzyme-linked immunosorbent assay (ELISA), and serologically specific electron microscopy (SSEM) were evaluated for the detection of pea seed-borne mosaic virus (PSbMV) in pea tissues. The virus was detected readily by immunodiffusion of leaf homogenates from single infected plants but not in homogenates from composite samples containing less than 25% PSbMV-infected leaves. In contrast, detection of PSbMV in composite samples of leaves containing 5-10% PSbMV-infected leaves was obtained using ELISA or SSEM. Each of these latter methods also detected the virus in homogenates of seed from a seedlot containing 25% infected seed; levels of 1% seed infection were detected consistently by SSEM. The application of these methods for the detection of PSbMV in breeding programs is discussed.

Pea seed-borne mosaic virus (PSbMV) is the causal virus of a potentially serious disease in pea. The virus, which has been reported to occur in several countries (5, 8), was thought to have been eradicated from North America but the recent detection of it in Canadian pea breeding lines (6) suggested that breeding lines may be the major source of the virus. Evidence also is accumulating that about 23% of the lines in the P.I. collection, a major germ plasm pool for pea breeders, are infected (R. O. Hampton, *personal communication*). These observations underscore the need for virus-free plants in any program of pea improvement.

At present, indexing programs for the presence of PSbMV in pea involve visual inspection of individual plants for characteristic symptoms followed by infectivity assays on Perfection-type pea cultivars and *Chenopodium amaranticolor,* a local lesion host. Because non-Perfection-type cultivars often do not show typical symptoms, a screening program based primarily on symptoms will not be satisfactory in detecting infected plants.

The purpose of this report is to evaluate three serological methods, immunodiffusion in gels, enzyme-linked immunosorbent assay (ELISA), and serologically specific electron microscopy (SSEM) for the rapid detection of PSbMV in pea seedlings and in homogenates of infected seeds.

MATERIALS AND METHODS

Virus source.—An isolate of PSbMV was obtained from a breeding line of pea furnished by A. Slinkard, Crop Science Department, University of Saskatchewan, Saskatoon. A single local lesion isolate was obtained from this isolate by inoculation to *Chenopodium amaranticolor*. The resulting culture was propagated in *Pisum sativum* '447' in which it caused symptoms typical of the disease. To provide virus for purification, it was manually inoculated to pea 447 at the two-node stage; inoculated plants were maintained at 23 C with a photoperiod of 16 hr in a greenhouse. Plants showing typical symptoms were harvested at 21 days postinoculation and chilled overnight at 3 C prior to processing.

Virus purification.—Virus was extracted and purified by a modification of the method of Huttinga (7). Chilled tissue was homogenized in an ice-cold mixture of 0.1 M Tris/HCl, pH 9.0, containing 0.2% 2-mercaptoethanol (150 ml), carbon tetrachloride (40 ml), and chloroform (40 ml) using a Waring Blendor. The resulting slurry was stirred for 15 min and then was centrifuged at 10,000 *g* for 20 min. Virus was pelleted from the supernatant fluid by centrifugation for 1.5 hr at 26,500 *g* (No. 30 rotor, 18,000 rpm Spinco L-2 ultracentrifuge) and the resulting pellets were resuspended in 0.1 M Tris/HCl, pH 8.0, to make a 10-fold concentrate. After clarification by centrifugation at 10,000 *g*, the supernatant was made 0.1% with Igepon T-73 and centrifuged for 5 hr at 25,000 rpm through a sucrose cushion (5.0 ml virus suspension floated on 6.0 ml of 0.1 M Tris/HCl, 0.1% Igepon T-73, pH 8.0, containing 45 mg sucrose/ml) in the SW27 rotor. Mineral oil was floated on top of the virus preparation to prevent tube collapse. The pellets, invariably glass-clear, were resuspended in 0.1 M Tris/HCl, pH 8.0, to which was added a few crystals of 1,1,1-trichloro-2-methyl-2 propanol (chlorbutanol) to prevent microbial growth. Virus was stored at 3 C until further use. Virus yields were approximately 5.0 mg/100 g tissue based on ultraviolet absorption measurements.

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Antiserum production.—Purified virus (1.2-1.8 mg/rabbit/injection) was injected intramuscularly into the hind legs of several rabbits in a series of injections at 2-week intervals followed by a booster dose at 10 mo. Bleedings were done from the ear at 3, 4, 5, 15, 16, 43, 44, and 46 wk after the first injection. The serum fraction was made 50% (v/v) with glycerin and stored at −20 C until use. The serum dilution endpoint was determined by the microprecipitin procedure using purified virus at 0.1 mg/ml in 0.01 M potassium phosphate, 0.14 M NaCl, pH 7.2.

Immunodiffusion.—A modification of the immunodiffusion procedure of Purcifull et al. (9) was developed. Agar gels (0.8% special Noble agar) containing 0.5% sodium dodecyl sulfate (SDS), 1% sodium azide, and distilled water were cast in petri dishes and six-way test patterns with wells 0.65 cm in diameter were cut on a center-to-center spacing of 1.1 cm. Crude sap from pea tissue, diluted with an equal volume of 1.0% SDS, was deposited in the peripheral wells; nondiluted antiserum (titer 1:2,048) was deposited in the central well. Plates were incubated for 12-15 hr at room temperature and then observed for the presence of precipitin lines.

Serologically specific electron microscopy (SSEM).—The procedure of Derrick and Brlansky (3) was adapted for use in detecting PSbMV in sap from infected plants or in homogenates of seeds. Antiserum was diluted 10^{-2} in 0.05 M Tris/HCl, pH 7.2 (Tris buffer), and support grids coated with a film (1% solution of Parlodion in amyl acetate, carbon-fronted) were floated on the diluted serum for 30 min. Nonadsorbed serum proteins were removed by rinsing the grids in a stream of Tris buffer, and they were then floated for one hr on homogenates of leaf tissue or of mature seeds previously soaked overnight in water. Homogenates were prepared by homogenizing the tissue or seeds in 10 times its fresh weight of Tris buffer containing 0.4 M sucrose and 0.15 M NaCl. Then the grids were rinsed thoroughly in a stream of distilled water, floated on 1% uranyl acetate in 50% ethanol for 10 min, washed in 50% ethanol, and then dried. They were examined with a Philips EM 300 electron microscope.

Enzyme-linked immunosorbent assay (ELISA).—The ELISA procedure (2, 11) was used with purified virus (0.5 - 50 μg/ml) and with homogentates from leaves and from seeds softened by soaking in water overnight. All antigens were diluted or extracted in a phosphate-buffered saline (PBS) solution (0.012 M KH_2PO_4, 0.008 M $Na_2HPO_4 \cdot 12 H_2O$, 0.137 M NaCl, 0.003 M KCl, pH 7.4) containing 0.02% sodium azide, 2% polyvinyl pyrrolidone, and 0.2% egg albumin. The leaf and seed homogenates (1 g fr. wt. tissue + 10 ml buffer) are considered to be 10^{-1} dilutions of the concentration of virus in the original tissue; these homogenates were further diluted in some experiments. The γ-globulin (1.4 mg) was conjugated with 2.8 mg of alkaline phosphatase (E.C. 3.1.3.1, Sigma type VII) using 0.06% glutaraldehyde as the coupling agent. The serological test was done in polystyrene microhemagglutination plates (Cooke M29AR) and results were assessed by visual scanning or by photometric scanning at 405 nm.

RESULTS

Characterization of the antiserum.—Antisera produced by the three rabbits used in this study were comparable in dilution endpoint (1:2,048) in microprecipitin tests with purified virus (0.1 mg/ml). There was no detectable reaction with preparations from noninfected plants processed in the same manner as the immunizing antigen. None of the antisera reacted with purified virus or with crude sap from infected plants in the conventional immunodiffusion test (i.e., immunodiffusion in gels without added detergents or other virus-degrading agents).

Immunodiffusion.—Several modifications of the conventional double-diffusion procedure were tried before adopting the SDS-agar gel system. The incorporation of sodium dibutylnaphthalene sulfonate (Leonil SA) (4) or sodium-N-methyl-N-oleoyl taurate (Igepon T-73) was unsatisfactory, largely because of copious nonspecific precipitates in the region of the gel proximal to the antiserum. However, a prominent single precipitin band, specific for purified virus or for sap from PSbMV-infected pea, was obtained with agar gels containing 0.5% SDS (Fig. 1-b). No precipitin bands were observed with normal serum or in gels which did not contain SDS (Fig. 1-a). Good precipitin bands were obtained only with serum collected after an antibody production period of 4 mo and an antibody dilution endpoint (microprecipitin test) in excess of 1:1,024. Good precipitin bands were obtained with such an antiserum diluted only to 1:2 with PBS but when normal serum was used as a diluent this mixture consistently extended the dilution endpoint to 1:4 and occasionally to 1:8. Precipitin bands were obtained consistently when a mixture of one infected pea seedling and three healthy seedlings were used as the test antigen. However, further dilutions of virus using mixtures of one infected plant and seven healthy plants give inconsistent results. Fusion of precipitin bands was obtained between four isolates of PSbMV from Canada and two from the U.S. with no indication of spur formation. Excellent correspondence (100%) between the presence of the PSbMV-specific precipitin band and the results of infectivity tests on *Chenopodium amaranticolor* was obtained in several trials using manually inoculated seedlings and seedlings infected via seed transmission as sources of the virus.

Serologically specific electron microscopy (SSEM).—With this method consistent detection of PSbMV virions in homogenates of leaves and moistened seeds was obtained (Fig. 2). An antiserum dilution of 10^{-2} was selected for routine analyses of homogenates from either tissue. Dilutions to 10^{-3} and 10^{-4} could be used, but with less reliable results. Virions trapped on the grid were too numerous to count when leaf homogenates from single plants were tested. However, the sensitivity of the method could be determined by examining homogenates from physical mixtures of infected and healthy pea tissue constructed to correspond to levels of 10, 5, 1, and 0% infection. The numbers of virions observed in 5-min scans of such homogenates were, respectively, 204, 78, 32, and 0.

Virions could also be detected in homogenates of seeds from a seedlot known to contain 25% PSbMV-infected seed (by SDS gel immunodiffusion assay of seedlings). To further test the sensitivity of this technique, a series of infected seedlots with levels of 5, 2, 1, and 0% PSbMV-

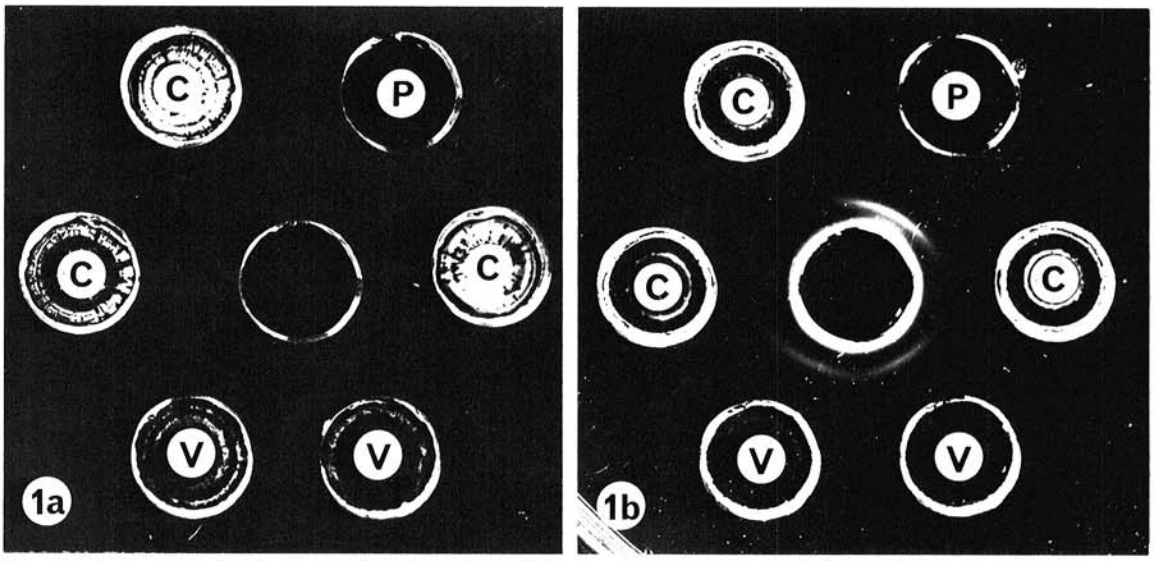

Fig. 1-(a, b). Immunodiffusion method for detecting pea seed-borne mosaic virus PSbMV) in plant extracts. **a)** Agar gel without SDS. **b)** Agar gel containing 0.5% SDS. Wells marked "P" contained purified PSbMV (0.5 mg/ml). Wells marked "C" and "V" contained extracts from healthy and PSbMV-infected pea, respectively. Center wells contained nondiluted antiserum to PSbMV (titer 1:2,048).

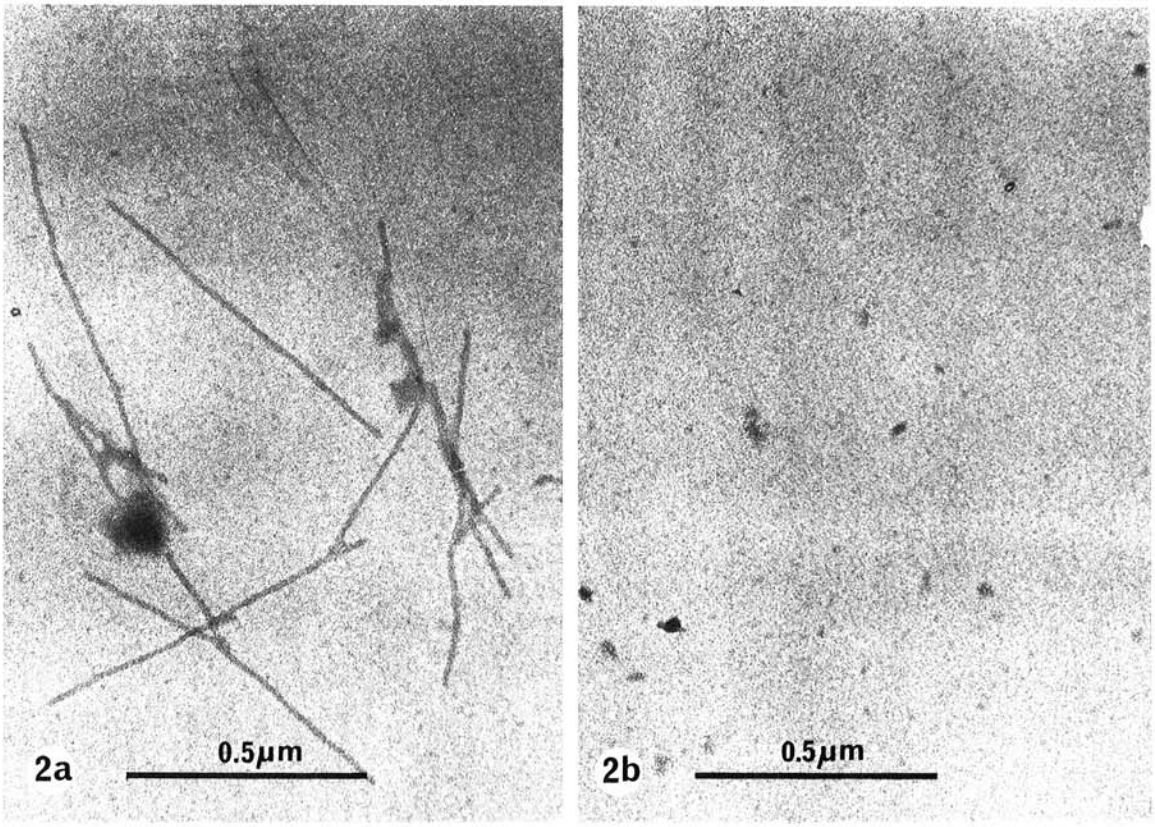

Fig. 2-(a, b). Detection of pea seed-borne mosaic virus in homogenates of infected pea seed by serologically specific electron microscopy (SSEM). **a)** Electron micrograph of grid upon which homogenate from infected seed was deposited. **b)** Electron micrograph of grid upon which homogenate from virus-free seed was deposited. Electron microscope grids were coated with PSbMV antiserum (titer 1:2,048) diluted 10^{-2}.

infected seed was constructed by mixing 25 seeds from the infected seedlot with seeds from a virus-free seedlot. The numbers of virions recorded in 2-min scans of the grids were 65, 35, 25, and 0, corresponding to 5, 2, 1, and 0% infection, respectively. In a separate trial done 1-mo later with the same seedlot, 84, 27, and 8 virions were observed in 5-min scans of grids corresponding to 25, 5, and 1% PSbMV-infected seed, respectively. In a final test of the method, six seeds taken at random from the infected seedlot were cut into corresponding halves. One half of each seed was mixed with 50-200 seeds from virus-free plants and the mixtures were processed for SSEM. Only one mixture (one half-seed + 100 seeds) contained virions detected by SSEM and the corresponding half seed was also the only one containing virions when the remaining halves were assayed by SSEM.

Enzyme-linked immunosorbent assay (ELISA).—Preliminary experiments established that a 10^{-2} dilution (6.7 μg/ml) of the purified γ-globulin was optimal for coating the microtiter plates. Purified virus in concentrations as low as 0.5 μg/ml was easily detected with enzyme-conjugated antibody diluted to 1:500 or 1:1,000, but less readily detectable at antibody diluted to 1:2,500. There were no nonspecific reactions in the controls which consisted of purified preparations of tobacco mosaic virus at the same concentrations as PSbMV, and PBS-Tween containing 2% polyvinyl pyrrolidone and 0.2% egg albumin. The results of assays for PSbMV in homogenates of leaves and of seeds (25% infection, determined by immunodiffusion tests of seedlings) are given in Table 1, Experiment 1. The virus could be detected in leaves of infected plants when homogenates of these leaves were diluted to 10^{-2} but not always when the dilution was 10^{-3}. Virus also could be detected in the crude homogenates of artificial mixtures of infected and healthy leaves containing 5 and 10% infected leaves and in the crude homogenate from a seedlot containing 25% infected seed. In a second experiment, artificial mixtures of seed from the infected seedlot and healthy seed which contained 25, 5, 1, and 0% infected seed were assessed for PSbMV. The virus could be detected in the mixture containing 25% infected seed but not in the mixtures containing 5% and 1% infected seed or in the healthy seed control (Table 1, Expt. 2). However, when comparable mixtures were prepared for SSEM, 83, 27, 8, and 0 virions were detected in 5-min scans of grids corresponding to mixtures containing 25, 5, 1, and 0% infected seed, respectively.

TABLE 1. Detection of pea seedborne mosaic virus (PSbMV) in homogenates of leaves and seeds of pea by enzyme-linked immunosorbent assay (ELISA)

Expt. no.	Sample	Dilution ($\log_{10}$)	Assessment procedure: A_{405}	Assessment procedure: Visual
1	PSbMV-infected leaves (100%)	−1	0.57	+
		−2	0.27	+
		−3	0.14[a]	−
	PSbMV-infected leaves (10%)[b]	−1	0.26	+
		−2	0.12	−
	PSbMV-infected leaves (5%)[b]	−1	0.26	+
		−2	0.14	−
	Healthy leaves	−1	0.14	−
		−2	0.12	−
	PSbMV-infected seed (25%)	−1	0.53	+
		−2	0.19	−
	Healthy seeds	−1	0.11	−
	PSbMV (50 μg/ml)		2.84	+
2	PSbMV-infected seed (25%)	−1	0.84	+
		−1.3	0.41	+
	PSbMV-infected seed (5%)[b]	−1	0.19	−
		−1.3	0.04	−
	PSbMV-infected seed (1%)[b]	−1	0.06	−
		−1.3	0.01	−
	Healthy seed	−1	0.13	−
		−1.3	0.01	−
	PSbMV (40 μg/ml)		2.55	+

[a]Values of A_{405} less than 0.2 were considered negative.
[b]Mixtures (w/w) of diseased and healthy tissues or seeds adjusted to contain the indicated percentage of diseased tissue.

DISCUSSION

The objective of this investigation was to provide a serological procedure for the rapid detection of PSbMV-infected plants. Such a method would have to be relatively simple in its implementation and unequivocal in its interpretation. Three procedures, immunodiffusion in SDS-agar gels, ELISA, and SSEM were evaluated with these two criteria in mind.

The use of the SDS-agar gel immunodiffusion system resulted in consistent detection of the virus in infected seedlings and in older plants, in contrast to the negative results obtained earlier by Stevenson and Hagedorn (10) using a similar system. It would appear to be the routine procedure of choice for determining the status of individual parental plants in a breeding program. However it is not suitable for screening large populations of plants using composite samples because the method is not sensitive enough to detect low levels of PSbMV infection. At best, a composite sample containing tissue from one infected and three healthy plants represents the upper limit in the reliability of the immunodiffusion procedure. When applied on a single-plant basis, the procedure is adequate, providing unequivocal results within 12-15 hr.

For detecting PSbMV in larger populations by bulk sampling, both ELISA and SSEM have potential applications. Plant populations containing 5% infected plants can be detected readily by both methods (Table 1); thus, they are suitable for sampling populations of progeny lines or other bulk samples in which the incidence of virus infection might be low. There was no advantage to scanning the ELISA samples at 405 nm over visual scanning by eye although Clark and Adams (2) were able to increase the sensitivity of detection 10-fold for some viruses by photometric scanning.

Of particular interest is the detection of PSbMV by ELISA and SSEM in homogenates of seeds. There always has been a need for a rapid procedure to detect virus infection in seeds; both ELISA and SSEM appear to be useful procedures. Detection of PSbMV at a level of 1% seed infection has been consistent with SSEM and is similar to that reported for tobacco ringspot, barley stripe mosaic, and lettuce mosaic viruses in seeds (1). A direct comparison of ELISA and SSEM for detecting PSbMV in a graded series of seed infection demonstrated that SSEM could detect PSbMV in seedlots containing 1-5% infected seed, whereas negative results were obtained with ELISA at these levels (Table 1, Experiment 2), suggesting that SSEM is superior to ELISA for detecting very low levels of infected seed. The apparent superiority of SSEM for detecting the virus in seed homogenates may be due to the relatively high A_{405} (0.1-0.15) of healthy seed homogenates. Such a background would interfere with detecting very low levels of infected seed by ELISA, but its effect may not be apparent in assays by SSEM where the criterion of infection is the visualization of typical virions on the antibody-coated grid. The presence of antibodies to normal pea proteins may explain the background effect because such antibodies would be bound to the plastic surface and would compete for enzyme-labeled antibody in proportion to their concentration. The use of antiserum cross-absorbed with normal pea proteins might alleviate this difficulty.

Both ELISA and SSEM are uniquely advantageous in the screening of seedlots before they are introduced into a germ plasm pool, thus minimizing the chance for PSbMV infection in the pool. They also should be useful for plant quarantine and other agencies in screening seedlots for the presence of seed-borne viruses.

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