

Immunochemical and Microscopical Techniques for Detecting Blackeye Cowpea Mosaic and Soybean Mosaic Viruses in Hypocotyls of Germinated Seeds

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ABSTRACT

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Double immunodiffusion tests were used to detect blackeye cowpea mosaic virus (BICMV) in hypocotyls of infected, 4- to 5-day-old cowpea (*Vigna unguiculata*) seedlings and soybean mosaic virus (SoyMV) in soybean (*Glycine max*) hypocotyls. Antigens consisted either of hypocotyl extracts prepared in sodium dodecyl sulfate (SDS) or of hypocotyl disks embedded in agar media containing SDS. The percentages of infected seed in five lots of cowpea seed and one lot of soybean seed as determined by the

hypocotyl disk immunodiffusion test were similar to the percentages of infection determined for these same lots by detecting symptoms in seedlings. Serologically specific electron microscopy also was used to detect both viruses in infected hypocotyls. Cytoplasmic (cylindrical) inclusions were detected in BICMV-infected cowpea hypocotyls and SoyMV-infected soybean hypocotyls by light microscopy, electron microscopy, and serology.

Additional key words: seed pathology, potyvirus, pinwheels, legume virus.

Techniques for detecting seed-borne viruses have been grouped (23) into dry examination (visual inspection of dry seeds), biological tests (growing-on and infectivity tests), biochemical tests

(colorimetric, histochemical, and serological tests), and biophysical tests (electron microscopy). According to Phatak (23), the highly specific serological tests are the best among those available to assay seed lots for the presence of virus-infected seeds. Several types of serological tests have been used for detecting virus-infected seeds (2,3,11,12,14-16,18,19,23,25,27).

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Two potyviruses, blackeye cowpea mosaic virus (BICMV) and soybean mosaic virus (SoyMV) are commonly seedborne in cowpea (29) and soybean (23), respectively. This report describes immunochemical and microscopical tests for detecting BICMV and SoyMV in the hypocotyls of 4- to 5-day-old seedlings of cowpea and soybean, respectively.

MATERIALS AND METHODS

Sources of viruses, antisera, and seed. A Florida isolate of BICMV was used for preparation of antisera to virus and inclusion proteins (14,17). These sera had titers of 1/32 and 1/4, respectively, in double-immunodiffusion tests. Seeds of cowpea (*Vigna unguiculata* [L.] Walp. 'Knuckle Purple Hull' and 'Early Ramshorn') harvested from BICMV-infected plants were obtained either from cowpea fields near Gainesville, FL or from Mr. Gilvan Pio Ribeiro, University of Georgia, Athens. As controls, BICMV-free seed produced in Texas was obtained from a local commercial source.

Antisera to American Type Culture Collection isolate PV-94 of SoyMV and its inclusion proteins were produced with antigens purified from *Nicotiana benthamiana* Domin. (Hiebert and Purcifull, unpublished). Soybean (*Glycine max* [L.] Merr. 'Jupiter') seed from plants naturally infected with SoyMV were used for serological testing.

Seed germination and preparation of antigens for immunodiffusion. Seeds were surface-sterilized in 0.5% sodium hypochlorite for 10 min, rinsed thoroughly with sterile deionized water, and placed in moistened paper towels to germinate. The towels were rolled up, placed upright in 30-ml beakers and the seeds were allowed to germinate for 3-5 days at 25-27 C in an incubator. Hypocotyls were excised with razor blades and used for testing. Disks, 1-2 mm thick, were cut from individual hypocotyls with a razor blade; the blade was rinsed with 95% ethanol and deionized water after cutting each hypocotyl. The hypocotyl disks were tested individually in double or single immunodiffusion tests by embedding them directly into the agar. Hypocotyl extracts also were used as test antigens. Single hypocotyls, or groups of five or 10 hypocotyls were ground in water or in 1.5% sodium dodecyl sulfate (SDS) (1:1, w/v) with a mortar and pestle.

Immunodiffusion tests. BICMV in cowpea was the test system used for investigating several immunodiffusion techniques. Individual hypocotyls or groups of hypocotyls from germinated seeds were routinely tested against antisera by double diffusion tests in media containing 0.8% Noble agar, 0.5% SDS, and 1.0% NaN_3 , prepared either in water (24) or in 0.05 M Tris-HCl, pH 7.2. Up to 12 hypocotyl disks were embedded in the agar 4-5 mm from each antiserum well. Undiluted antiserum for BICMV was used routinely in these tests, but antiserum dilutions of 1/2, 1/4, and 1/8 in either normal serum or in 0.05 M Tris-HCl, pH 7.2, also were tested against hypocotyl disks and extracts of hypocotyl tissue. As controls, hypocotyls from infected and uninfected seeds were tested against BICMV antiserum and normal serum. Hypocotyl extracts were added to antigen wells that were distributed in a hexagonal arrangement around a central serum well (all wells 7 mm in diameter, and the serum well was 4 to 5 mm from each antigen well). All plates were incubated in a moist chamber at 24 C for 24 to 48 hr. The sensitivity of double immunodiffusion for detection of BICMV in extracts of bulked hypocotyls of germinated Knuckle Purple Hull seeds was tested by mixing 1.0 g of infected hypocotyl tissue with different amounts of uninfected hypocotyls.

Double immunodiffusion tests with either hypocotyl disks or extracts were used also for SoyMV.

Disks and extracts of BICMV-infected hypocotyls also were tested by single radial immunodiffusion in medium containing 0.8% Noble Agar, 0.5% SDS, 1.0% NaN_3 , and 15% BICMV antiserum in 0.05 M Tris-HCl, pH 7.2. Each 90 x 15-mm plastic petri dish contained 15 ml of medium. Hypocotyl disks were embedded edgewise in the solidified agar with the aid of forceps, and the plates were incubated in a moist chamber at 24 C for 24-72 hr. Extracts from groups of five hypocotyls were placed into wells (3 mm diameter) punched into the agar medium in a row

arrangement. The wells were spaced 3-4 mm apart and as many as 230 germinated seeds could be tested in each dish. Precipitin rings around the wells charged with extracts from infected tissue could be detected between 1 and 24 hr later.

Serologically specific electron microscopy. Single or bulked hypocotyls also were checked by serologically specific electron

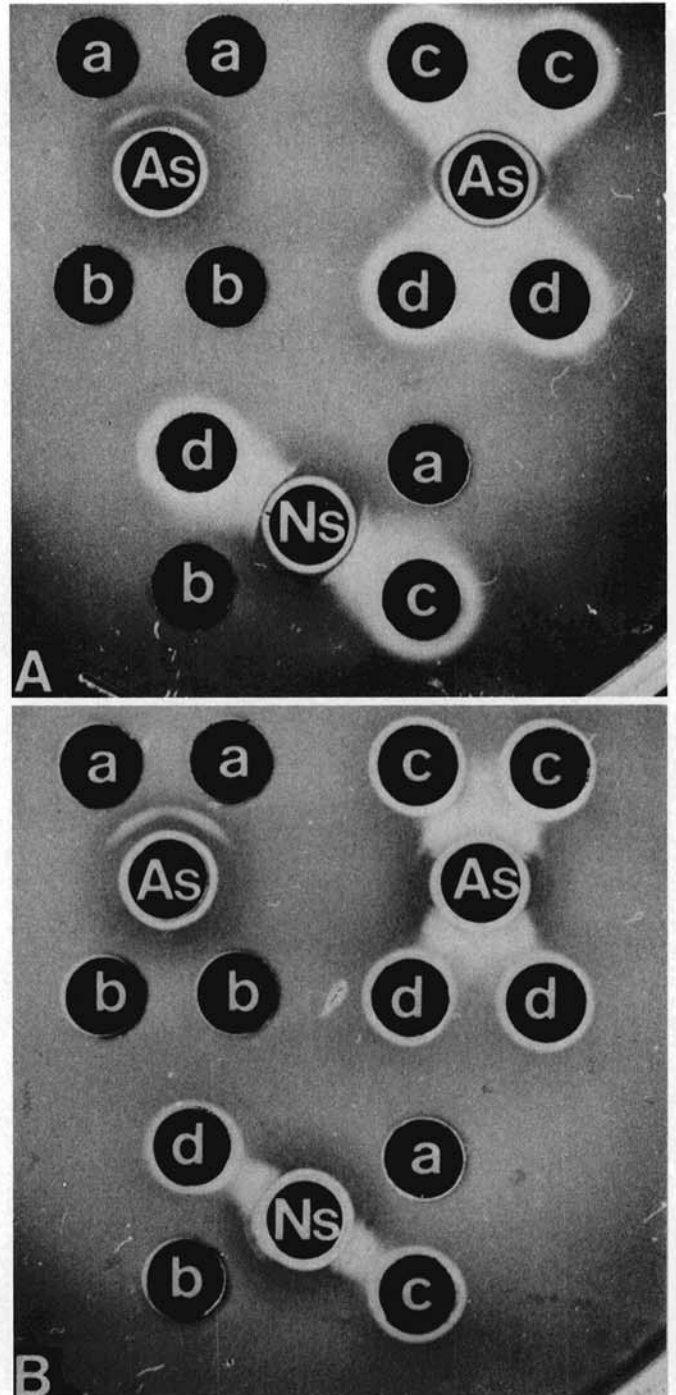


Fig. 1. Double immunodiffusion tests with extracts from different portions of blackeye cowpea mosaic virus (BICMV)-infected and healthy, 4- to 5-day-old cowpea seedlings. **A**, Medium containing 0.8% Noble agar, 1.0% NaN_3 , and 0.5% sodium dodecyl sulfate prepared in deionized water. **B**, Medium with the same ingredients, but prepared in 0.05 M Tris-HCl, pH 7.2. Center wells were charged with: (As) BICMV antiserum, and (Ns) normal serum. The peripheral wells were filled with extracts from: hypocotyls from infected (a) and healthy (b) seedlings; cotyledons and primary leaves of infected (c) and healthy (d) seedlings. Note the nonspecific precipitates formed with extracts (c and d) from a mixture of cotyledons and primary leaves.

microscopic (SSEM) techniques. Extracts were prepared in 0.05 M Tris buffer, pH 7.2, containing 0.15 M NaCl and 0.4 M sucrose, and examined by SSEM for filamentous particles as described by Derrick and Brlansky (7).

The sensitivity of SSEM was determined by diluting BICMV-infected Knuckle Purple Hull hypocotyl tissue with uninfected tissues up to a dilution of 1/50 (w/w). The different mixtures of infected and uninfected hypocotyl tissues were ground in the extracting solution (1:1, w/v) with mortars and pestles, and small drops from these extracts were placed on the antiserum-sensitized grids.

Light and electron microscopy of inclusions induced by BICMV and SoyMV in hypocotyls of germinated seeds. Germinated seeds of cowpea Knuckle Purple Hull infected with BICMV and germinated seeds of soybean Midwest infected with SoyMV were identified previously by double immunodiffusion tests using antiserum for BICMV and SoyMV, respectively.

Examinations of cytoplasmic inclusions were made in epidermal strips from BICMV- and SoyMV-infected hypocotyls of germinated seeds, after staining with a combination of calcoamine orange and "luxol" brilliant green (4).

Small pieces of virus-infected and uninfected cowpea or soybean hypocotyls were prepared for ultrathin sectioning as described elsewhere (17). Sections were cut with a diamond knife and stained

with potassium permanganate, uranyl acetate, and lead citrate. All specimens were examined with a Philips Model 200 electron microscope.

RESULTS

Immunodiffusion tests. BICMV antigens prepared from hypocotyls of germinated seeds proved to be very satisfactory for indexing seeds in immunodiffusion tests. Neither disks nor extracts from hypocotyl tissues showed any interfering nonspecific reactions. On the other hand, extracts obtained from whole seedlings, or individual seedling parts, viz. roots, cotyledons, and primary leaves, showed several types of precipitation patterns when tested against any serum in double diffusion tests (Fig. 1). Although the specific precipitates still were masked, the nonspecific precipitates were reduced somewhat when the agar medium was prepared in 0.05 M Tris-HCl, pH 7.2 (Fig. 1B).

The BICMV was detected in hypocotyl extracts from bulked seedlings and in small disks of individual hypocotyls of cowpea Knuckle Purple Hull and Early Ramshorn by double immunodiffusion tests. Virus-specific precipitin lines were observed with extracts of mixtures of BICMV-infected (one part) and uninfected (up to 29 parts) cowpea Knuckle Purple Hull hypocotyls.

The percentage of viral infection in a seed lot was determined by testing individual hypocotyl disks in double immunodiffusion. Virus-specific precipitin lines formed between BICMV-infected hypocotyl disks and antiserum wells, whereas no reaction was observed with uninfected hypocotyls (Fig. 2, left). Undiluted antiserum and antiserum diluted 1/2 and 1/4 with normal serum all reacted perceptibly with BICMV antigens. The percentages of BICMV-infected seeds in four different lots of cowpea Knuckle Purple Hull and one Early Ramshorn seed lot were estimated by this simplified double immunodiffusion test (Table 1). More than 100 seedlings tested by this technique also were checked randomly by SSEM for the presence of BICMV particles; in all cases, the results matched perfectly. The incidence of infection obtained by the hypocotyl disk-immunodiffusion method was similar to that obtained by the growing-on test (Table 1), which involves estimation of infected plants based on symptoms observed in greenhouse-grown seedlings (23).

The presence of BICMV in cowpea hypocotyl extracts also was detected by single radial immunodiffusion tests in agar medium impregnated with BICMV antiserum (Fig. 2, right). Precipitin rings were observed around the wells charged with extracts from groups of five individual hypocotyl disks in which at least one was infected with BICMV, but not around those wells charged with extracts of uninfected hypocotyls. The virus-specific reactions appeared approximately 1 hr after the hypocotyl extracts had been added to the wells and became very evident 3-4 hr later.

When small disks of individual hypocotyls were directly

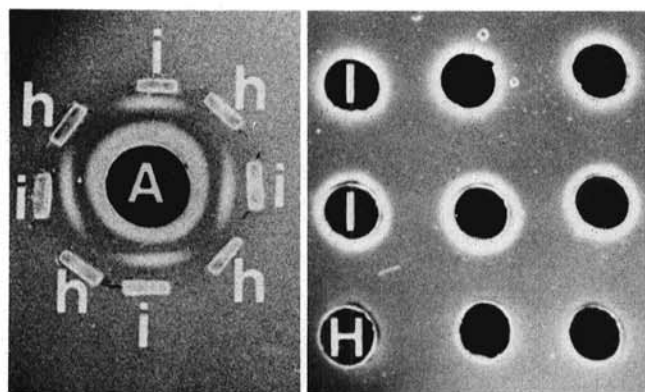


Fig. 2. Immunodiffusion tests for detection of blackeye cowpea mosaic virus (BICMV)-infected hypocotyls. Left: Double diffusion tests with hypocotyl disks embedded edgewise in agar medium; hypocotyls from infected plants (i) and healthy plants (h); A = antiserum to BICMV. Right: Single radial diffusion tests with medium containing 0.8% Noble agar, 1.0% Na₂S₂O₃, 0.5% SDS, and 15% BICMV antiserum. The six wells in the upper two rows (I) were filled with extracts from BICMV-infected cowpea hypocotyls and the bottom three wells (H) were filled with extracts from healthy hypocotyls.

TABLE 1. Comparison of double immunodiffusion tests with hypocotyl disks and growing-on tests for detection of virus-infected seeds

Seed source	Virus tested	Seed assay methods			
		Serology		Growing-on test ^a	
		Infected seed (no. infected/no. tested)	Infection (%)	Infected seed (no. infected/no. tested)	Infection (%)
Cowpea (<i>Vigna unguiculata</i> [L.] Walp.)					
Cultivar Knuckle Purple Hull					
Lot A	BICMV ^b	72/323	22.3	33/160	20.6
Lot B	BICMV	12/160	7.5	13/173	7.5
Lot C	BICMV	5/115	4.3	8/145	5.5
Lot D	BICMV	17/160	10.6	13/141	9.2
Cultivar Early Ramshorn	BICMV	5/100	5.0	34/545	6.2
Soybean (<i>Glycine max</i> [L.] Merr.)					
Cultivar Jupiter	SoyMV ^c	4/176	2.3	2/89	2.2

^aResults of growing-on tests based on appearance of symptoms in seedlings in greenhouse tests.

^bBlackeye cowpea mosaic virus.

^cSoybean mosaic virus.

embedded into the agar medium containing BICMV antiserum, the virus-specific reactions appeared more slowly. Specific reactions (viz, opalescent precipitates around infected hypocotyl disks) were most easily recognized with a binocular microscope. The precipitates, usually located at the ends of the hypocotyl disks, appeared in 24 hr, but were more evident 48–72 hr after the test was initiated. Usually, a nonspecific precipitate also started to appear throughout the agar medium after 72 hr.

Double immunodiffusion tests also successfully detected SoyMV in infected, germinated seeds. Either extracts from five bulked hypocotyls or small disks of individual hypocotyls gave positive reactions (Fig. 3). The results were confirmed by SSEM. The percentage of SoyMV-infected soybean (cv. Jupiter) in one seed lot was estimated by double immunodiffusion testing with hypocotyl disks (Table 1).

Detection of BICMV and SoyMV by SSEM. The SSEM technique (3,7) was adapted successfully in identifying BICMV-infected (Fig. 4) and SoyMV-infected seedlings. Virus particles were observed in extracts from mixtures of healthy hypocotyl tissue (49 parts) and infected hypocotyl tissue (one part).

The SSEM technique also was used for detecting SoyMV in infected hypocotyls.

Serological and microscopical detection of virus-induced inclusions in hypocotyls. Cytoplasmic inclusions induced by BICMV in cowpea and by SoyMV in soybean were detected by serology and microscopy in hypocotyls of 4- to 5-day-old seedlings. In double immunodiffusion tests, specific precipitin lines were observed with the inclusion antisera and extracts of virus-infected hypocotyls, but not with extracts from healthy hypocotyls (Fig. 5).

Light microscopic observations of epidermal strips from hypocotyls of virus-infected cowpea and soybean seedlings readily revealed the presence of cytoplasmic inclusions (Figs. 6A, 6B). Pinwheels with scrolls were observed in ultrathin sections of hypocotyl tissues infected either with BICMV (Fig. 6C) or SoyMV (Fig. 6D).

DISCUSSION

Hypocotyl tissues from young seedlings served as reliable sources of antigens for immunochemical assays. The percentages of virus infection determined for several seed lots by the hypocotyl disk-double immunodiffusion method were similar to the percentages of infection determined for these same lots by growing-on tests. The double-immunodiffusion technique detected BICMV in seed extracts diluted 1/30, whereas the more sensitive SSEM technique detected BICMV at an extract dilution of 1/50. The SSEM techniques were used to detect pea seed-borne mosaic virus

and lettuce mosaic virus in seed extracts diluted 1/100 (12) and 1/1,000 (7), respectively.

Extracts from cowpea seedling roots, primary leaves, cotyledons, or whole seedlings gave nonspecific precipitates which seriously interfered with interpretation of immunodiffusion tests. These nonspecific reactions were not observed when hypocotyl-extracts or pieces were used as antigen sources. Others have encountered problems with nonspecific reactions in serological tests with legume seeds. Cockbain et al (6) reported that embryos of *Vicia faba* L. seeds caused the agar to become clouded, thereby obscuring the virus-specific precipitin lines. Lister (18) reported similar problems concerning the interferences of nonspecific precipitates with the detection of tobacco ringspot virus immunoprecipitates in double diffusion tests. Phatak (23) assumed

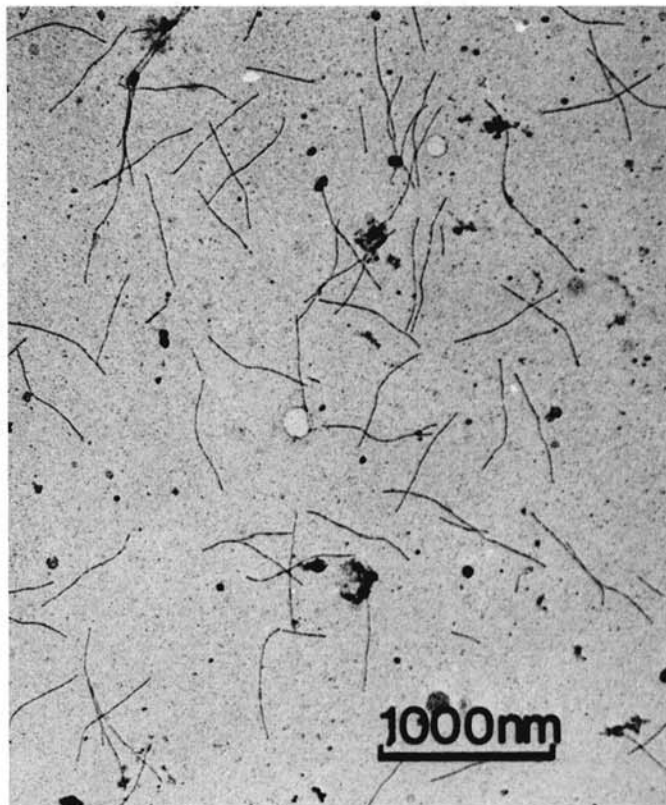


Fig. 4. Blackeye cowpea mosaic virus particles visualized by serologically specific electron microscopy (SSEM) of extracts from infected cowpea hypocotyls.

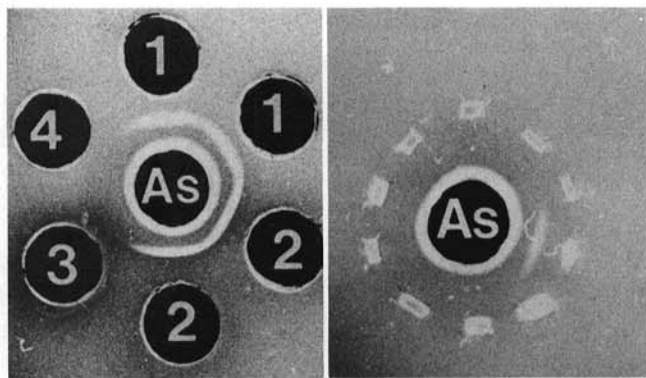


Fig. 3. Double immunodiffusion tests with soybean mosaic virus (SoyMV). Left: Tests with extracts from: (well 1) SoyMV-infected soybean hypocotyls; (well 2) SoyMV-infected *Nicotiana benthamiana* leaves; (well 3) healthy *N. benthamiana* leaves; and (well 4) healthy soybean hypocotyls. Center well (As) contains SoyMV antiserum. Right: Hypocotyl disks embedded edgewise in agar with SoyMV antiserum in center well. Note positive results with a single virus-infected hypocotyl disk; all other hypocotyls were from noninfected seedlings.

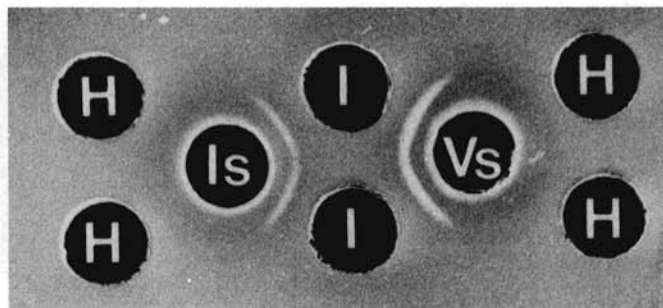


Fig. 5. Double immunodiffusion tests with hypocotyl extracts from 4- to 5-day-old seedlings of soybean using antisera for SoyMV and SoyMV cytoplasmic inclusions (SoyMV-I). The center wells were charged with: (Is) SoyMV-I antiserum and (Vs) SoyMV antiserum. The peripheral wells were charged with SDS-treated extracts from: (I) SoyMV-infected hypocotyls and (H) healthy soybean hypocotyls.

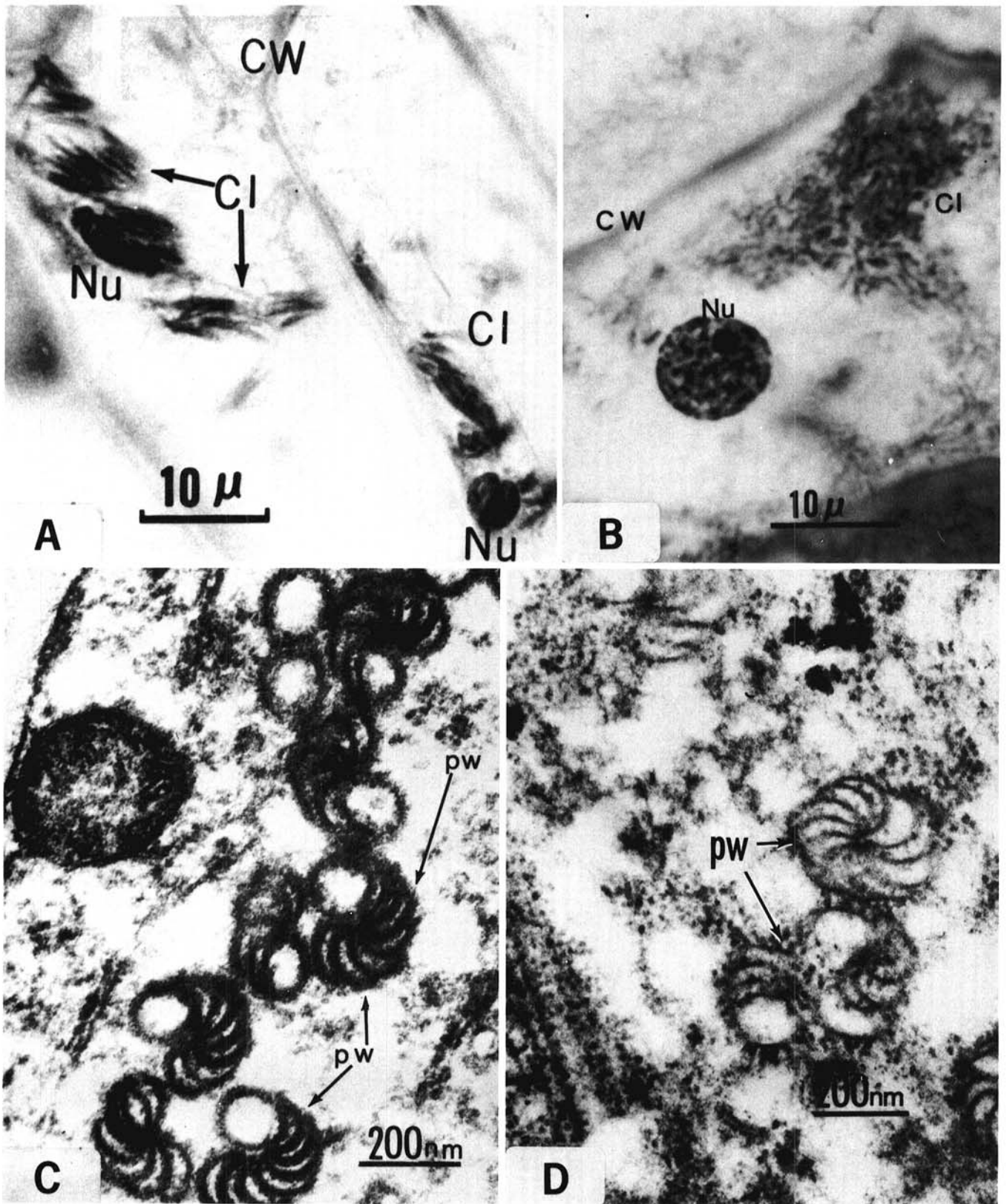


Fig. 6. Light and electron microscopy of inclusions in hypocotyl tissue. Photomicrographs of cytoplasmic inclusions induced by BICMV in epidermal strips of cowpea hypocotyl (A) and induced by SoyMV in epidermal cells of hypocotyls from 4- to 5-day-old soybean seedlings (B). The hypocotyl epidermal strips were stained with a combination of calcoamine orange and "luxol" brilliant green. CI = cytoplasmic inclusions, CW = plant cell wall, Nu = nucleus. Electron micrographs of ultrathin sections of cells from hypocotyls of 4- to 5-day-old cowpea seedlings infected with BICMV (C) and hypocotyl cells of 4- to 5-day-old SoyMV-infected soybean seedlings (D). Note pinwheel inclusions (pw).

that the high content of lectin (hemagglutinin) in soybean seeds was responsible for the unsatisfactory results of the passive hemagglutination test for SoyMV in seed extracts. Lectins have been reported to occur in high concentrations in certain legume seeds (9,22), and have been reported to bind to specific mono- and polysaccharides, to serum globulin, and to virus coat protein (10,20). There is no evidence, however, that the nonspecific reactions observed with cowpea seedling roots or leaves are due to lectins. No hemagglutinating activity was detected in extracts from ungerminated seeds of Knuckle Purple Hull or California Blackeye cowpea (S. Pueppke, *personal communication*).

Cytoplasmic inclusions induced by BICMV in cowpea hypocotyls and by SoyMV in soybean hypocotyls were detected by serology, light microscopy, and electron microscopy ([16] and Figs. 5 and 6). The cytoplasmic inclusions induced by potyviruses are widespread in systemically infected plants (1,4,8,21,26,28), but the occurrence of these inclusions in germinated seeds has been documented only recently (13,16). Light microscopy (4) was a useful method for confirming the presence of virus in particular seedlings and for selecting suitable material for cytological examination at the electron microscope level.

The results reported here and by others (12,18,23) indicate that several options exist concerning serological methods for detection of potyviruses in legume seedlings or seeds. The choice of method depends primarily on the investigator's objectives and experiences, on the sensitivity required, and on available facilities. The ELISA (2,5,12,18) and SSEM (3,7,12) tests are among the most sensitive procedures available, but they also are more complex and/or require more expensive, sophisticated instrumentation than do gel diffusion tests. The reliability and simplicity of the hypocotyl-disk-double immunodiffusion test make it suitable for commercial certification programs. For example, a trained person can test approximately 50-60 hypocotyl disks per hour by this technique. Slack and Shepherd (27) detected barley stripe mosaic virus by embedding barley leaves directly in agar plates containing antibody to the virus. They pointed out that this simplified approach of using tissue pieces as test antigens makes tissue grinding, preparation of antigen wells, and chemical treatment of the antigen prior to incorporation into the agar matrix unnecessary. Similar advantages accrue as a result of using hypocotyl disks as antigens. The hypocotyl disk immunodiffusion test in the SDS system, therefore, appears to be a feasible alternative for detecting certain seed-borne potyviruses.

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