Two New Serotypes of Cowpea Severe Mosaic Virus

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We thank G. Pio-Ribeiro, Departamento de Agronomia, Universidade Federal Rural de Pernambuco, Recife, Pernambuco, and F. P. Cupertino, Departamento de Biologia Vegetal, Universidade de Brasília, 70910 Brasília, DF, Brazil, for supplying the V-1 and bean isolates, respectively, of cowpea severe mosaic virus.

Part of the work was performed at Iowa State University, where the senior author was on leave supported in part by the Brazilian National Research Council for Scientific and Technologic Development (CNPq) and the World Food Institute, Iowa State University (Project 0052). Journal Paper J-11029 of the Iowa Agriculture and Home Economics Experiment Station, Project 2428.

Accepted for publication 30 September 1983.

ABSTRACT

Lin, M. T., Hill, J. H., Kitajima, E. W., and Costa, C. L. 1984. Two new serotypes of cowpea severe mosaic virus. Phytopathology 74:581-585.

Two cowpea severe mosaic virus isolates, V-1 and one from bean, were identified as new serotypes III and IV, respectively. Serotype III differed from the other serotypes by infecting *Nicotiana tabacum* 'TNN.' Serotype IV was unable to infect *Chenopodium amaranticolor*, a known diagnostic host for this virus. Two beetle species, *Cerotoma arcuata* and *Diabrotica speciosa*, transmitted serotype IV from beans to beans. Cytological alterations induced in plants infected by these four serotypes were similar, except the fibrous inclusions appeared to be induced less frequently by serotypes III and IV than by the other two. Two major proteins with apparent molecular weights of 40,300–41,800 daltons (d) and 20,100–21,400 d were detected in the middle components of the four serotypes. A minor protein of about 22,400–23,300 d was detected in serotypes I, III, and IV, but not in serotype II. Serological analysis showed that cross reactivity among the four serotypes was due to the common antigenic determinant A. Serotypes I, III, and IV share an additional common determinant designated as B. Serotypes I and IV also have the common determinant designated C. Each serotype also has a specific antigenic determinant D, E, F, and G for serotypes I, II, III, and IV, respectively, which contribute to their serological distinction.

Additional key words: antigenic determinants, beetle transmission.

Cowpea severe mosaic virus (CPSMV), a member of the comovirus group, commonly infects cowpea (*Vigna unguiculata* (L.) Walp.) in many countries of the Americas (6). Isolates of CPSMV from different geographical locations are often related, but not serologically identical (7). Recently, Lin et al (11) began a systematic study of the serological relationship among CPSMV isolates and separated 14 isolates from central Brazil into two serotypes, designated I and II. These authors proposed that additional serotypes should be numbered sequentially.

We now report the discovery and distinctive characteristics of two new serotypes, which have been designated III and IV. Part of this work has been reported previously (14).

MATERIALS AND METHODS

Source of virus isolates. Brazilian isolates G-BE and G-12, designated serotypes I and II, respectively, were described previously (11). Serotype III (V-1 isolate), originally isolated in Venezuela, was supplied by G. Pio-Ribeiro, Universidade Federal Rural de Pernambuco, who received it from J. P. Fulton, University of Arkansas. Serotype IV was isolated from naturally infected bean (*Phaseolus vulgaris* L.) in central Brazil (5) and provided by F. P. Cupertino, Universidade de Brasília. Serotypes I, II, and III were maintained in and purified from *Vigna unguiculata* (L.) Walp. 'Seridó' or 'Blackeye Pea,' while serotype IV was propagated in and purified from the bean cultivars Rico Pardo or Rico 23. Procedures for virus assay, host reactions, virus purification, and antiserum production were described previously (11,12).

Beetle transmission. *Cerotoma arcuata* Oliv. and *Diabrotica speciosa* Thom. were collected from a field at the University of Brasilia. They were freed from possible contamination with CPSMV or any other viruses by consecutive transfer to two sets of healthy plants of bean cultivar Jalo and cowpea cultivar Seridó at 4-day intervals. The nonviruliferous beetles, in groups of five, were used in 48-hr acquisition access feedings on plants of cultivar Jalo bean infected with serotype IV, followed by inoculation feedings of 48 hr on healthy cultivar Jalo bean plants. The inoculated plants were indexed for CPSMV 2–3 wk later by serology.

Cytology. Infected leaf samples from plants of cultivar Seridó cowpea or cultivar Rico 23 bean (for serotype IV) were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.05 M phosphate buffer, pH 7.2, for 1–2 hr and postfixed in 1% OsO_4 in 0.15 M phosphate buffer, pH 7.2, for 1 hr. After dehydration in acetone, tissues were embedded in Spurr's resin sectioned, stained with uranyl acetate and lead citrate, and examined in a JEOL JEM 100C electron microscope.

Molecular weight estimation of coat protein. Electrophoresis in 10% polyacrylamide gels containing sodium dodecyl sulfate (9) was used to compare and estimate molecular weights of the coat proteins of the four serotypes. Only the middle component, isolated by density-gradient centrifugation, was used in comparisons because it is easier to obtain in sufficient purity for this purpose. Centrifugation of the virus preparation, in linear 10–40% sucrose density-gradients prepared in 0.01 M potassium phosphate buffer, pH 7.6, was at 27,000 rpm for 3.5 hr in a Beckman SW 27 rotor. Gradients were fractionated with an ISCO density-gradient fractionator. To avoid cross contamination, the leading half of the middle component was collected, pelleted by high-speed centrifugation, and separated again in a density gradient.

Serology. Serological comparison by immunodiffusion tests employed a test pattern consisting of six wells around a central well (11) and a pattern consisting of rows of wells 5 mm in diameter arranged alternately with similar wells in adjacent rows. Spacing between two adjacent wells was 5 mm. In intragel absorption tests,

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the absorbing antigen was removed from the antiserum well 24 hr after loading.

RESULTS AND DISCUSSION

Host reaction. Serotypes I, II, and III induced necrotic local lesions in *Chenopodium amaranticolor* Coste & Reyn. and necrotic local lesions and severe mosaic in cowpea cultivar Seridó. However, serotype III could be differentiated from the other two serotypes by inducing chlorotic local lesions in *Nicotiana tabacum* L. 'TNN.' Serotype IV did not infect any of these plants. *C. amaranticolor* is regarded as a useful diagnostic host for CPSMV (6). CPSMV isolates obtained from legumes other than cowpea failed to infect, as previously described (12) and shown above, lessens the value of *C. amaranticolor* as in a diagnostic host for CPSMV. Serotype IV seems to be adapted to bean because it infects most of the bean cultivars, but very few of the cowpea cultivars tested (5).

Beetle transmission. None of the 38 bean and 30 cowpea plants used to feed the *C. arcuata* or *D. speciosa* that were collected directly from the field became infected with CPSMV or any other viruses. These results indicate that the beetles were free of CPSMV when collected. Both species fed well on cultivar Jalo bean, but *D. speciosa* did not feed on cultivar Seridó cowpea. *C. arcuata* and *D. speciosa* transmitted serotype IV from beans to beans at a rate of 50% (nine of 18 plants inoculated) and 43% (three of seven plants inoculated), respectively. *C. arcuata* and *D. speciosa* have previously been reported as vectors for a cowpea isolate (3) and a bean isolate (4) of CPSMV, respectively.

Cytological comparison. Examination of thin sections of cowpea or bean plant tissue, experimentally infected with each one of the four serotypes, revealed basically similar features (Fig. 1). Putative CPSMV particles were commonly found in the vacuole; they probably also occur in the cytoplasm, but cannot be differentiated from the ribosomes. Most parenchyma cells from infected leaves exhibited one or more cytoplasmic areas rich in vesicles with aggregates of mitochondria occasionally observed (Fig. 1C). Fingerlike cell wall outgrowths, more conspicuous in the vascular region, were commonly found. In some sections, tubules containing viruslike particles appeared embedded in the cell wall outgrowths or between the cell wall and plasmalemma (Fig. 1D). These features correspond to those previously described in CPSMV-infected cells (10) and were common to all four serotypes. However, there appeared to be a difference in the frequency of occurrence of a fibrous cytoplasmic inclusion (Fig. 1A and B) of proteinaceous nature (2), which was more frequent in serotypes I and II than in serotypes III and IV. These observations were later confirmed in a more thorough comparison of nine different isolates of CPSMV, including the four serotypes studied here (E. W. Kitajima, *unpublished*).

Purification and gel electrophoresis. Cultivar Blackeye Pea cowpea was a better host for purifying of serotypes I, II, and III than was cultivar Seridó. Between 0.75 and 0.5 g of purified CPSMV per kilogram of leaf tissue was obtained from Blackeye Pea, while the yield from Seridó never exceeded 0.5 g/kg. Serotype IV was purified from bean cultivar Rico Pardo or Rico 23 with a yield of ~0.5 g/kg of leaf tissue.

Purified preparations of serotypes III and IV had typical nucleoprõtein UV spectra with a maximum:minimum and $A_{260/280}$ similar to those reported for serotypes I and II (11). Sucrose density

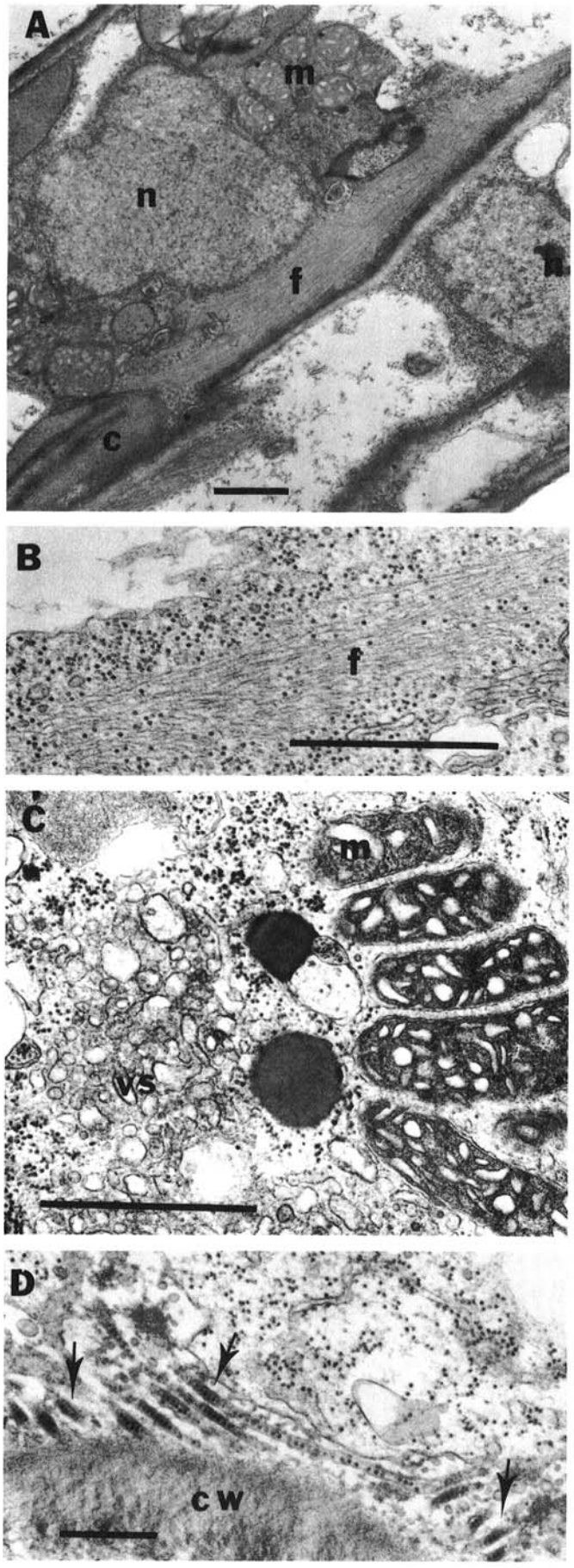

Fig. 1. Electron micrographs of thin sections of cowpea (**A and C**) and bean (**B and D**) leaf tissues infected with different serotypes of cowpea severe mosaic virus. **A,** Low magnification view of palisade parenchyma cells infected with serotype II. Large fibrous inclusion (f) is noticeable in the cytoplasm. **B,** Detail of the fibrous inclusion (f) in the cytoplasm of a spongy parenchyma cell infected with serotype IV. **C,** Aggregate of mitochondria (m) and vesicles (vs) in a spongy parenchyma cell infected with serotype I. **D,** Tubular structures containing viruslike particles (arrows) associated with the cell wall (cw) of a spongy parenchyma cell infected with serotype IV. c = Chloroplast; n = nucleus; bar = 1 μm.

gradient centrifugation showed the presence of top (T), middle (M), and bottom (B) components in all four serotypes, as previously reported (6), with very little contamination of host material near the meniscus (Fig. 2).

Gel electrophoresis showed two major proteins, bands 1 and 3, in the M components of all four serotypes and of a bean pod mottle virus (BPMV) isolate (13). In addition, a minor protein, band 2, was observed in serotypes I, III, IV, and BPMV, but not in serotype II. Absorption profiles of the gels showed the minor band as a shoulder of the band 3 protein peak and the absence of this shoulder in serotype II (Fig. 3). Molecular weights of corresponding proteins did not differ substantially among the four serotypes (Table 1). Co-electrophoresis of serotype I and BPMV in the same gel showed apparent co-migration of the three proteins, suggesting that protein molecular weights in these two viruses were similar, if not identical. The estimated molecular weights of the two major proteins in four serotypes were between 40,300 daltons (d) and 41,800 d for band 1 and between 20,100 d and 21,400 d for band 3. These values are close to the values reported for comoviruses (15). The presence of a minor protein band in CPSMV (8,16), BPMV (13) and other comoviruses (eg, see [1] and [15]) has been reported previously and is assumed to be the result of limited proteolysis of the smaller protein (band 2) (1). If interpreted in this manner, the absence of the minor protein in serotype II (Fig. 3) would suggest the unique property of complete proteolysis of the smaller protein in this serotype. As will be shown later, serotype II is serologically very distant from the other three serotypes.

Antiserum production and serological comparison. Serotypes III and IV were highly immunogenic as reported previously for serotypes I and II (11). Antisera to serotypes III and IV had homologous titers of 1,024. Antisera to serotype I (titer 1,024) and serotype II (titer 512) were prepared previously (11).

Titration of the four antisera indicated that serotypes I, III, and IV were more closely related to each other than to serotype II

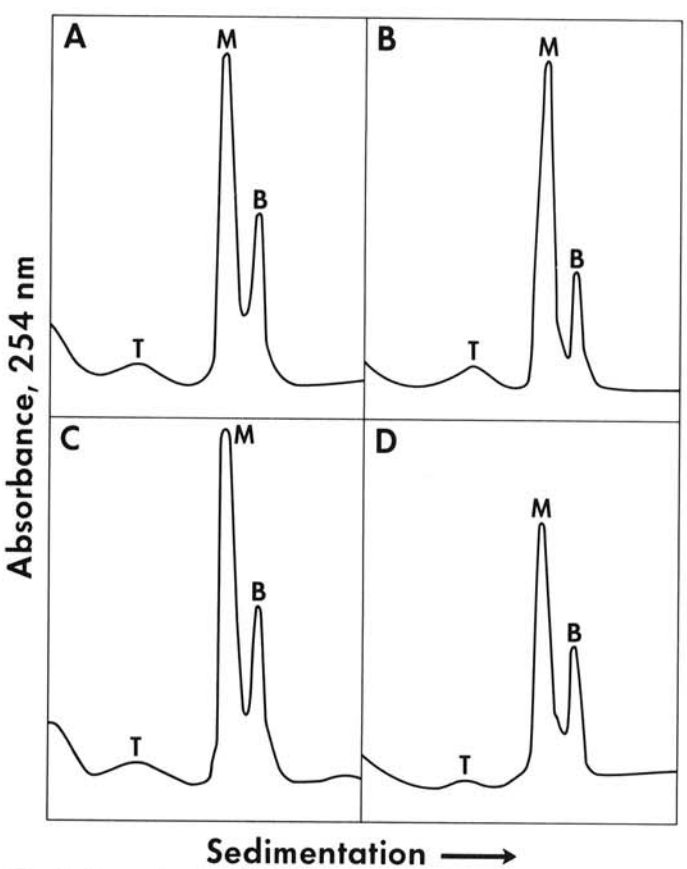

Fig. 2. Absorption profiles of cowpea severe mosaic virus in 10–40% sucrose density gradients showing the presence of top (T), middle (M), and bottom (B) components in **A,** serotypes I; **B,** II; **C,** III; and **D,** IV. Centrifugation was for 3.5 hr at 27,000 rpm in a Beckman SW 27 rotor.

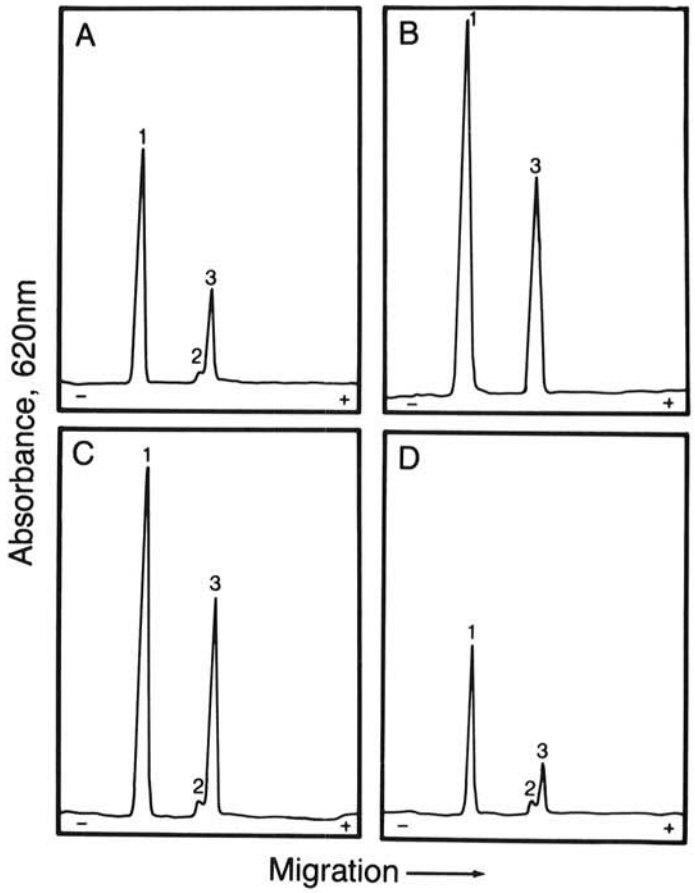

Fig. 3. Absorption profile of cowpea severe mosaic virus coat protein after electrophoresis in 10% polyacrylamide gels containing SDS. **A, B, C, and D** = serotypes I, II, III, and IV, respectively. Electrophoresis was at 8 mA per gel until a bromophenol blue tracking dye reached the bottom of the gel. Note the absence of band 2 protein in B.

TABLE 1. Molecular weight estimates of the proteins in the middle components of the four serotypes of cowpea severe mosaic virus determined by electrophoresis in 10% polyacrylamide gels containing sodium dodecyl sulfate

Serotype	Species of protein		
	Band 1	Band 2	Band 3
I	40,900 ± 200[a]	22,400 ± 400	20,400 ± 100
II	40,300 ± 700		21,400 ± 100
III	40,500 ± 500	23,000 ± 200	20,100 ± 100
IV	41,800 ± 600	23,300 ± 100	21,100 ± 100

[a] Mean and standard deviation of four (serotype III) and five (serotypes I, II, and IV) determinations. Molecular weight standards used were bovine serum albumin (mol wt = 68,000 daltons), myoglobin (mol wt = 17,200 daltons), and cytochrome C (mol wt = 11,700 daltons).

TABLE 2. Comparison of homologous and heterologous titers of antisera to four cowpea severe mosaic virus serotypes

Antiserum	Antigen in crude sap[a]			
	I	II	III	IV
I	1,024[b]	256	512	512
II	128	512	128	128
III	512	256	1,024	512
IV	1,024	512	512	1,024

[a] Serotypes I, II, and III were tested in the cowpea cultivar Seridó, and serotype IV was tested in the bean cultivar Rico 23.

[b] Titers, determined by immunodiffusion in agar gel, are expressed as reciprocals of the last antiserum dilution that reacted with the antigen.

(Table 2). Serological relationships were further evaluated by intragel absorption and evidence of spur formation in immunodiffusion tests.

The four serotypes spurred with each other when tested against serotype I antiserum (Fig. 4A). Spurs were more evident when serotype II was used as the absorbing antigen in intragel absorption tests (Fig. 4B). In this test, complete absorption was indicated by the lack of a precipitin line between serotype II and the absorbed antiserum. The appearance of a strong precipitin ring surrounding the antiserum well (Fig. 4B) demonstrates the existence of a common antigenic determinant in all four serotypes. To facilitate the interpretation, this common determinant was arbitrarily designated as A. The absorbed antiserum still reacted with serotypes I, III, and IV, forming sharp precipitin lines that spurred with each other (Fig. 4B). By the pattern of spur formation, it is evident that serotype I possesses at least three additional antigenic determinants, arbitrarily designated as B, C, and D. Of these, B is common to serotype III and IV, and C is common to serotype IV.

When serotype II antiserum was used, patterns of identity were observed among serotypes I, III, and IV, which were related to, but not identical with, serotype II (Fig. 4C). After intragel absorption with any one of the heterologous antigens, serotype II antiserum reacted only with its homologous antigen (Fig. 4D). This confirms the existence of a specific determinant in serotype II, which we now designate as E. In this test, determinant A was again demonstrated by formation of a ring surrounding the antiserum well.

When serotype III antiserum was used, the pattern of spur formation allowed the detection of at least three antigenic determinants in this serotype (Fig. 4E). Determinant A was again detected and confirmed by the intragel absorption test (Fig. 4F). Determinant B, which was identified as common to serotypes I, III, and IV by serotype I antiserum, also was detected by serotype III antiserum (Fig. 4F). The spur formation between serotype III and serotype I or IV in the intragel absorption test suggests the existence of a specific determinant in serotype III. This determinant is named F.

Serological differences among the four serotypes were further demonstrated by testing against serotype IV antiserum (Fig. 4G). The formation of a strong band surrounding the central well (Fig. 4H) in intragel absorption tests with serotype II as the absorbing antigen is again attributed to the common antigenic determinant A. The presence of determinant B in serotypes I, III, and IV and of determinant C in serotypes I and IV also was confirmed by spur formation in the intragel absorption test with serotype IV antiserum (Fig. 4H). In addition, serotype IV has a specific determinant, which is now designated as G.

Results of the serological analysis are summarized in Table 3. Serotype I is serologically most closely related to serotype IV based on their possession of three common antigenic determinants (A, B, and C). Serotype II is most distantly related to the other serotypes because it shares only determinant A in common. Serotype III is more closely related to serotypes I and IV than to serotype II because it shares two common determinants (A and B) with those two serotypes. In addition to the common determinants, each serotype has an additional specific determinant that allows serological distinction among the serotypes. Interpretation of serological analyses is greatly facilitated by assigning letters arbitrarily to designate independent antigenic determinants. This also permits rapid identification of antigenic determinants in a new serotype.

Only two to four antigenic determinants in each serotype were identified in this study. These serotypes may possess other determinants that were not detected owing to the limited sensitivity of the technique employed. The use of monoclonal antibodies may resolve this issue.

TABLE 3. Antigenic determinants in four serotypes of cowpea severe mosaic virus identified by homologous and heterologous reactions in immunodiffusion tests[a]

Antiserum	Antigen			
	I	II	III	IV
I	*A,B,C,D*	A	A,B	A,B,C
II	A	*A,E*	A	A
III	A,B	A	*A,B,F*	A,B
IV	A,B,C	A	A,B	*A,B,C,G*

[a]The antigenic determinants identified in the homologous reactions (italicized) are designated as the determinants for that particular serotype.

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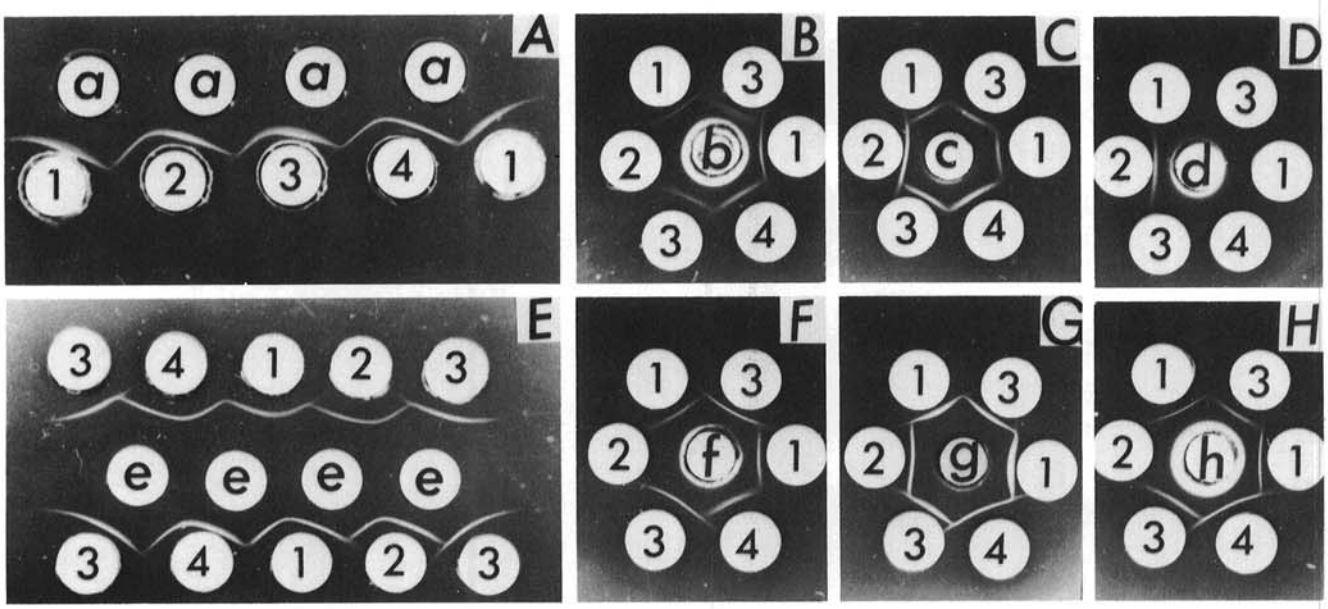

Fig. 4. Serological comparison of cowpea severe mosaic virus serotypes by immunodiffusion in agar gel. Figure elements with capital letters A–H correspond to central well designations in lowercase letters a–h. Wells 1, 2, 3, and 4 = antigens of serotypes I, II, III, and IV, respectively; a, c, e, and g = antisera to serotypes I, II, III, and IV, respectively; b, f, and h = antisera to serotypes I, III, and IV, respectively, but previously intragel-absorbed with antigen of serotype II; d = serotype II antiserum previously intragel-absorbed with antigen of serotype I.

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