

Serological Grouping of Cowpea Severe Mosaic Virus Isolates from Central Brazil

M. T. Lin, J. R. N. Anjos, and G. P. Rios

Associate professor and graduate student, respectively, Department of Plant Biology, University of Brasilia, 70910 Brasilia, DF, Brazil; and plant pathologist, National Research Center for Rice and Bean (CNPAP), EMBRAPA, 74000 Goiânia, Goiás, Brazil.

We thank J. P. Fulton and H. A. Scott, University of Arkansas, for providing antisera to legume comoviruses and to J. M. de Sousa for technical help.

The equipment used in this study was acquired with funds from Programa de Nutrição, FUB/FINEP/BID.

Accepted for publication 17 September 1980.

ABSTRACT

Lin, M. T., Anjos, J. R. N., and Rios, G. P. 1981. Serological grouping of cowpea severe mosaic virus isolates from central Brazil. *Phytopathology* 71:435-438.

Fourteen single-lesion isolates of cowpea severe mosaic virus obtained from central Brazil were separated by immunodiffusion in agar gel into two serologically distinct groups, designated as serotype I (13 isolates) and serotype II (one isolate), respectively. Isolates of serotype I infected soybean (*Glycine max*) cultivar IAC-2 which was immune to serotype II. This soybean cultivar is suggested as a possible diagnostic plant for this virus. Based on reactions in cowpea (*Vigna unguiculata*) cultivar IPEAN VII,

bean (*Phaseolus vulgaris*) cultivar Rico 23, and *Chenopodium amaranticolor*, the isolates in serotype I were further classified into three groups. *C. amaranticolor*, which was previously regarded as only a local lesion host for cowpea severe mosaic virus, was shown to be systemically infected by some isolates of serotype I. Serotype I also was shown to be serologically more related than serotype II to two other legume comoviruses, cowpea mosaic virus and bean pod mottle viruses.

Additional key words: antiserum production, cross-absorption test

Cowpea mosaic is the virus disease most commonly found attacking cowpea (*Vigna unguiculata* (L.) Walp.) in Brazil. It has been found in the states of São Paulo (4,5), Ceará (10), Piauí (6,11), Amazonas (9,11), Pernambuco (11,15), Goiás (11), and the Federal District (11).

A great deal of confusion has existed in the nomenclature of the viruses causing cowpea mosaic. According to the original CMI/AAB Description of cowpea mosaic virus (18), all isolates of this virus can be separated into two subgroups, severe and yellow, by serology and host reactions. Swaans and van Kammen (16) made a more thorough study of host range, symptomatology, serology, and the ratio of the virus components of the two subgroups and concluded that they should be considered as two different viruses. However, to avoid taxonomic complications and for practical reasons, these authors maintained the two subgroups as different strains of one virus (16). Later, based on serological differences, Fulton and Scott (8) proposed the serogrouping concept for legume comoviruses and considered the severe and yellow subgroups as distinct viruses which were designated the CPMV-Arkansas and CPMV-Sb serogroups, respectively. The recent CMI/AAB Descriptions of Plant Viruses also considered the severe and yellow subgroups as distinct viruses and officially designated them as cowpea severe mosaic virus (CPSMV) (3,7) and cowpea mosaic virus (CPMV) (3,19), respectively. To avoid the

confusion in terminology, the acronyms, CPSMV and CPMV, are used in this paper.

It is generally accepted that the beetle-transmitted, isometric virus that causes mosaic in cowpea in the western hemisphere is CPSMV (7,8). By using antisera to CPSMV and CPMV furnished by Fulton and Scott (8), Lin (11) found that all 44 samples of mosaic-affected cowpea from various parts of Brazil were infected with CPSMV only.

Although it has been noted that CPSMV isolates from different countries in the western hemisphere often form fine spurs with one another when tested by immunodiffusion (8), no work has been done on the serological grouping of these isolates or the isolates from a certain geographical location. We report here the separation of 14 CPSMV isolates obtained from central Brazil into two serologically distinct groups and propose the use of serotypes I and II to designate these groups.

MATERIALS AND METHODS

Sample collection and virus isolation. Cowpea samples with symptoms of severe mosaic and stunting were collected from the state of Goiás and the Federal District in central Brazil in 1979. Crude sap prepared from leaves of each sample was tested on agar gel plates against antisera to the type members of five legume comoviruses (CPSMV, CPMV, quailpea mosaic virus (QPMV), bean pod mottle virus (BPMV), and bean rugose mosaic virus (BRMV [3]) kindly supplied by Fulton and Scott (8). The antisera

were used at 1:20 dilution as recommended (8). Samples that gave a positive reaction (ie, formation of a sharp, well-defined, curved band) with the CPSMV antiserum were mechanically inoculated to *Chenopodium amaranticolor* Coste & Reyn. and *C. quinoa* Willd. plants maintained inside a greenhouse. Extracts from single well-isolated local lesions were manually inoculated to cowpea cultivar Serido which developed systemic vein-clearing in 7–10 days and mosaic 2 wk later. These single lesion isolates were used in this study.

Host reactions. To test the reactions of plant species to CPSMV isolates, at least five seedlings of each species or cultivar were mechanically inoculated with each isolate; the seedlings were kept inside a greenhouse for observation. Temperature inside the greenhouse varied from 21 to 31 C during this period. All seedlings were grown in aluminium pots because previous experiments showed that cultivar Serido seedlings grown in clay pots were not always 100% infected when mechanically inoculated with some of these isolates. Extracts from symptomless plants were tested against CPSMV antiserum in agar gel plates or assayed on *C. amaranticolor* to check for latent infection.

Virus purification. Systemically infected leaves of cowpea cultivar Serido were triturated in two volumes of 0.1 M borate buffer, pH 7.6, containing 0.01 M Na-EDTA and 0.1% Na₂SO₃ in a blender. After clarification of the extract with *n*-butanol, the virus was concentrated by precipitation with 6% polyethylene glycol (MW 6,000) and 0.01 M NaCl. Further purification was made by two cycles of differential centrifugation. The final preparation was used as immunogen for antiserum production. A double-beam spectrophotometer Model UV-200S (Shimadzu Seisakusho Ltd., Kyoto, Japan) was used to measure the ultraviolet (UV) spectra and concentrations of the virus preparations. An extinction coefficient of $E_{260\text{nm}}^{0.1\%} = 8.0$ (18) was used to estimate the concentration of the unfractionated virus preparations.

Antiserum production. Rabbits were injected intramuscularly with 1.5–4.0 mg of whole virus emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI 48232) at weekly intervals for 3 wk. Bleedings were made weekly by heart puncture beginning on the day of the last injection. The antisera were preserved by mixing with an equal volume of glycerol and kept in a freezer.

Serological tests. Immunodiffusion tests using 0.75% Noble agar, 0.85% NaCl, and 0.02% sodium azide were utilized to determine serological relationships. The tests were performed in 9-cm-diameter Formvar-treated glass petri dishes containing 16 ml of agar gel. The test pattern consisted of six peripheral wells 5 mm in diameter equally spaced around a central well of the same size with 4 mm between the closest edges of the central and peripheral wells. Serological relationships were determined by placing antigens (crude sap or purified virus) in the peripheral wells and the antiserum in the central well. To determine titers, serum dilutions in saline were placed in the peripheral wells and respective homologous or heterologous antigens at 1 mg/ml in the center well. Titers were expressed as reciprocals of the last antiserum dilution which reacted with antigen. The agar gel plates were observed for reaction and photographed after 15–17 hr of incubation in a moist chamber. Serum was cross-absorbed by mixing it with two volumes of purified heterologous antigen in a serological tube, incubating the mixture in a waterbath at 37–38 C for 6 hr and then at 4 C for 2 hr; precipitates were removed by centrifugation.

RESULTS

Reactions of plant species and cultivar to CPSMV isolates. A total of 14 single-lesion isolates were obtained. Symptoms produced by the isolates were identical in cowpea cultivars Serido and Pitiuba, bean (*Phaseolus vulgaris* L.) cultivar Manteiga, and *Chenopodium quinoa*. However, variations in the reactions of four other plant species to these isolates were noted and, based on these variations, the 14 isolates were classified into four groups (Table 1). Group 2 (isolate G-12) was differentiated from the others by being unable to infect soybean (*Glycine max* (L.) Merr.) cultivar IAC-2, while group 4 (isolate G-32) was unique in not being able to induce necrotic local lesions in cowpea cultivar IPEAN VII. Group 3 (isolates G-BE, G-7, G-22, G-25, G-28, and F-F1) was able to cause necrotic local lesions and systemic flecking and puckering symptoms in *Chenopodium amaranticolor*, while the others could only induce necrotic local lesions in this plant. Group 1 (isolates G-3, G-5, G-8, G-9, F-C18, and F-F18) resembled group 2 in host reactions in cowpea cultivar IPEAN VII, bean cultivar Rico 23, and *C. amaranticolor*, but could be differentiated from it by being able to cause local and systemic infections in soybean cultivar IAC-2.

Selection of CPSMV isolates for antiserum production. All isolates except G-12 formed one fused precipitin line when tested against CPSMV antiserum in immunodiffusion. A distinct spur was formed by extension of this line beyond the junction with the line formed between G-12 and CPSMV antiserum. The 14 isolates were separated into two apparently serologically distinct groups, with G-12 in one group and the rest in another. Isolate G-12 and three other isolates (G-BE, G-3, and G-32) which represented three different host reaction groups (Table 1) were selected for further study by purification and antiserum production.

Virus purification and antiserum production. Virus preparations of the four isolates showed UV spectra typical of nucleoprotein with maximum and minimum absorptions at 258–260 nm and 240–242 nm, respectively, and $A_{260/280} = 1.58$. The yields of virus were between 184 and 257 mg/kg of leaf tissue. All four isolates were highly immunogenic. Antisera taken 3 wk after the first injection reached a reciprocal titer of 256 or 512 to their homologous antigens and a reciprocal titer of 4 to crude sap of healthy cowpea leaves. The titers of the sera taken in three subsequent bleedings were two times higher. No attempt was made to remove the antibodies to host proteins in the sera.

Serological tests. Antisera with reciprocal homologous titers of 512 or 1,024 were diluted to 1:16 for use in immunodiffusion tests. Based on spur formation, the four isolates were separated into two serotypes, with G-BE, G-3, and G-32 in serotype I and G-12 in serotype II (Fig. 1). No serological difference was detected among the three isolates in serotype I. When the other 10 isolates (ie, G-5, G-7, G-8, G-9, G-22, G-25, G-28, F-C18, F-F1, and F-F18) were compared with G-BE and G-12 on agar gel plates against the antisera to these two isolates, the 10 isolates were serologically identical to G-BE. Therefore, 13 of the 14 CPSMV isolates studied belong to serotype I. In cross-absorption tests between G-BE and G-12, 5 mg of G-BE was sufficient to absorb all of the detectable common antibodies from 1 ml of the G-12 antiserum with a homologous titer of 512, whereas 10 mg, but not 5 mg, of G-12 was required to remove the common antibodies from 1 ml of the G-BE antiserum with a homologous titer of 1,024 (Table 2). The other 12

TABLE 1. Reactions of four plant species to four groups comprising 14 isolates of cowpea severe mosaic virus obtained from central Brazil

Isolate ^a	<i>Glycine max</i> 'IAC-2'	<i>Vigna unguiculata</i> 'IPEAN VII'	<i>Chenopodium</i> <i>amaranticolor</i>	<i>Phaseolus vulgaris</i> 'Rico 23'
Group 1	LLc/VC ^b	LLn/VC,Mo	LLn/-	LLn/-
Group 2	-/-	LLn/VC,Mo	LLn/-	LLn/-
Group 3	LLc/VC	LLn/VC,Mo	LLn/FI,P	-/-
Group 4	LLc/VC	-/VC,Mo	LLn/-	-/-

^a Isolates in each group are: Group 1 = G-3, G-5, G-8, G-9, F-C18, and F-F18; Group 2 = G-12; Group 3 = G-BE, G-7, G-22, G-25, G-28, and F-F1; Group 4 = G-32. Isolates with symbol G were obtained from Goias state while those with F were from the Federal District.

^b Numerator and denominator indicate local and systemic symptoms, respectively. Symbols for the symptoms are: - = no symptoms and no virus recovered; LLc = chlorotic local lesions; LLn = necrotic local lesions; VC = vein-clearing; FI = flecking; P = puckering; and Mo = mosaic.

TABLE 2. Comparison of titers of absorbed and unabsorbed antisera to two cowpea severe mosaic virus isolates, G-BE (serotype I) and G-12 (serotype II), reacted with their homologous and heterologous antigens^a

Antiserum	Absorbed with	Reacted with	
		G-BE ^b	G-12 ^b
G-BE	None	1,024	256
G-BE	G-12 (5 mg virus/ml serum)	512	16
G-BE	G-12 (10 mg virus/ml serum)	256	0
G-12	None	256	512
G-12	G-BE (5 mg virus/ml serum)	0	256
G-12	G-BE (10 mg virus/ml serum)	0	256

^a Antiserum titers were determined by immunodiffusion in agar gel and were expressed as reciprocals of the last antiserum dilution that reacted with the antigen.

^b Freshly purified virus preparations at 1 mg/ml.

isolates in serotype I reacted with the G-BE antiserum previously absorbed with 10 mg of G-12, forming one sharp precipitin line on agar gel plates, but did not react with the absorbed G-12 antiserum.

Relatedness of serotypes I and II of CPSMV to other legume comoviruses. Purified preparations of G-BE and G-12 at 1 mg/ml were tested against undiluted antisera to CPMV, BPMV, QPMV, and BRMV on agar gel plates. One straight, diffused line, instead of a sharp, well-defined, curved line, was formed between either one of the CPSMV isolates and any one of these antisera. Spur formation was observed when G-BE and G-12 were tested side by side against either the CPMV or the BPMV antiserum, but not when they were reacted against the antisera to BRMV and QPMV. The spur was formed by extension of the line formed between G-BE and the CPMV or BPMV antiserum beyond the junction with the line formed between G-12 and the antiserum. These results indicate that serotype I (isolate G-BE) is more closely related to CPMV and BPMV than is serotype II (G-12). Since titers of these antisera were not known and their homologous antigens were not available for comparison, it was impossible to determine the real serological relationships of serotypes I and II to these comoviruses.

DISCUSSION

The results show that 14 CPSMV isolates obtained from central Brazil could be separated into two serologically distinct groups by immunodiffusion in agar gel. Among the 14 isolates, 13 were assigned to serotype I and only one to serotype II.

Besides serological distinction, the isolates of serotypes I and II studied here differed in abilities to infect soybean cultivar IAC-2. This soybean cultivar was very susceptible to isolates of serotype I, but was immune to serotype II. The diagnostic value of soybean cultivar IAC-2 in distinguishing these two serotypes can only be known after more isolates of serotype II are available and tested. Recently, an individual *Vigna sesquipedalis* Fruwirth plant in Goiás was found to be doubly infected with isolates of both serotypes (13) and when cultivar IAC-2 plants were inoculated with sap prepared from this plant, only virus of serotype I was recovered from the soybean plants. Soybean cultivar IAC-2 seemed to be more susceptible to serotype I isolates than either cowpea or *C. amaranticolor*, both of which have been recommended as diagnostic plants for CPSMV (7,18). Similar correspondence was observed with an isolate of serotype I obtained from naturally infected *Calopogonium mucunoides* Desvaux plants in Goiás (12). Recently, a CPSMV isolate was found infecting soybeans in the fields in Illinois (14) and an isolate of serotype I was obtained from soybean plants showing bud blight symptoms in Brasília (1). The potential threat of CPSMV isolates to soybean production have been demonstrated (1,17).

Although isolates in serotype I were serologically indistinguishable by the immunodiffusion method, variations among these isolates in host reactions were noted. Based on reactions in cowpea cultivar IPEAN VII, bean cultivar Rico 23, and *C. amaranticolor*, the 13 isolates of this serotype were separated into three groups (Table 1). Of particular interest was group 3 which was able to induce local lesions and systemic flecking and puckering symptoms

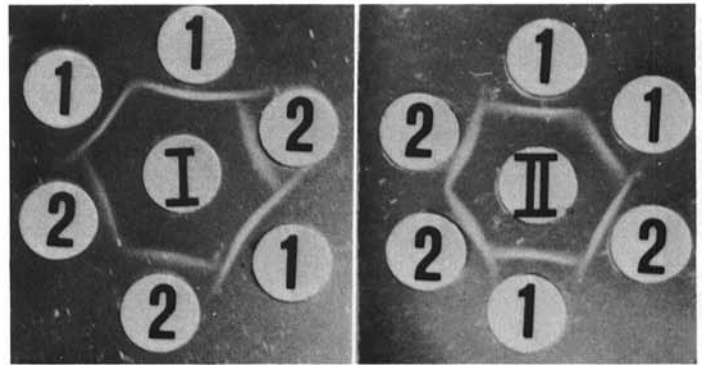


Fig. 1. Immunodiffusion in agar gel tests with serotypes I and II of cowpea severe mosaic virus showing formation of spur. I = antiserum to serotype I; II = antiserum to serotype II; 1 = antigen of serotype I; 2 = antigen of serotype II. The antisera were at 1:16 dilution and the antigens were purified virus preparations at 1 mg/ml.

in *C. amaranticolor*. A Brazilian CPSMV isolate was shown to be able to cause both local and systemic symptoms in this host (11). It has been concluded that isolates of CPSMV are able to induce only local lesions in *C. amaranticolor* (7,16,18), while those of CPMV are capable of causing both local and systemic infections in this plant (16,18,19). Apparently, reactions in *C. amaranticolor* are not reliable in distinguishing isolates of CPSMV from those of CPMV.

In Brazil, *Ceratomyza arcuata* Oliv. was reported as the only known beetle vector of CPSMV by Costa et al (6), and this finding was confirmed by Batista (2). It is not known whether the isolate used for the transmission tests by Costa et al (6) belongs to serotype I or II, because it is no longer available. However, the isolate used by Batista was found to be of serotype I (M. T. Lin, unpublished). Whether *C. arcuata* Oliv. also can transmit isolates of serotype II remains to be determined.

The CPSMV isolates from El Salvador, Puerto Rico, Costa Rica, and Venezuela are reported to spur with the Arkansas isolate and with each other in immunodiffusion (8). It seems that isolates from different geographical locations demonstrate serological differences. A comparative study with isolates from different countries in the western hemisphere will provide a better picture on the extent of serological variations within this virus. The designation of serotypes I and II for the CPSMV isolates from central Brazil serves as a starting base for this type of study and the availability of antisera to these serotypes certainly will facilitate the work. It is possible that isolates with serological properties differing from serotypes I and II will be identified in the future. In this case, the new serotypes should be named sequentially as serotypes III, IV, and so on.

LITERATURE CITED

1. Anjos, J. R. N., and Lin, M. T. 1980. Queima do broto em soja causada por um membro do serotipo I do vírus do mosaico do caupi—serogrupo Arkansas. (Abstr.) Fitopatol. Bras. 5:(In press).
2. Batista, M. F. 1979. Identificação de vectores e epidemiologia de dois vírus transmitidos por besouros Coleoptera-Chrysomelidae. M. S. thesis. University of Brasília, Brasília, DF, Brazil. 59 pp.
3. Bruening, G. 1978. Comovirus group. No. 199 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew, Surrey, England.
4. Caner, J., Silberschmidt, K., and Flores, E. 1969. Ocorrência do vírus do mosaico da *Vigna* no Estado de São Paulo. O Biológico 35:13-16.
5. Costa, A. S., Oliveira, A. R., Kitajima, E. W., and Matsuoka, S. 1969. Ocorrência do mosaico do feijão macassar em São Paulo. Rev. Soc. Bras. Fitopatol. 3:56-57.
6. Costa, C. L., Lin, M. T., Kitajima, E. W., Santos, A. A., Mesquita, R. C. M., and Freire, F. R. F. 1978. *Ceratomyza arcuata* (Oliv.), um crismelídeo vector do mosaico da *Vigna* no Brasil. (Abstr.) Fitopatol. Bras. 3:81.
7. de Jager, C. P. 1979. Cowpea severe mosaic virus. No. 209 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew Surrey, England.
8. Fulton, J. P., and Scott, H. A. 1979. A serogrouping concept for legume

- comoviruses. *Phytopathology* 69:305-306.
9. Kitajima, E. W., Noda, H., Lin, M. T., and Costa, C. L. 1979. Um mosaico em feijão-de-asa (*Psophocarpus tetragonolobus*) causado por um isolado do subgrupo severo do vírus do mosaico da *Vigna*. *Fitopatol. Bras.* 4:519-524.
 10. Lima, J. A. A., and Nelson, M. R. 1977. Etiology and epidemiology of mosaic of cowpea in Ceará, Brazil. *Plant Dis. Rep.* 61:864-867.
 11. Lin, M. T. 1979. Purification and serology of legume and corn viruses in Brazil. *Fitopatol. Bras.* 4:203-213.
 12. Lin, M. T., Anjos, J. R. N., and Rios, G. P. 1980. Ocorrência natural do serotipo I do "cowpea mosaic virus-Arkansas serogroup" em *Centrosema pubescens* e *Calopogonium mucunoides* no Brasil Central. (Abstr.) *Fitopatol. Bras.* 5:(In press).
 13. Lin, M. T., and Rios, G. P. 1980. Infecção natural e simultânea de *Vigna sesquipedalis* por serotipos I e II do "cowpea mosaic virus-Arkansas serogroup." (Abstr.) *Fitopatol. Bras.* 5:(In press).
 14. McLaughlin, M. R., Thongmeekom, P., Goodman, R. M., Milbrath, G. M., Ries, S. M., and Royse, D. J. 1978. Isolation and beetle transmission of cowpea mosaic virus (severe subgroup) from *Desmodium canescens* and soybeans in Illinois. *Plant Dis. Rep.* 62:1069-1073.
 15. Paguio, O. R. 1979. Incidência e algumas propriedades de um membro do subgrupo severo do "cowpea mosaic virus" em Pernambuco. (Abstr.) *Fitopatol. Bras.* 4:133.
 16. Swaans, H., and van Kammen, A. 1973. Reconsideration of the distinction between the severe and yellow strains of cowpea mosaic virus. *Neth. J. Plant Pathol.* 79:257-265.
 17. Thongmeekom, P., Paschal, E. H., and Goodman, R. M. 1978. Yield reductions in soybeans infected with cowpea mosaic virus. *Phytopathology* 68:1549-1551.
 18. van Kammen, A. 1971. Cowpea mosaic virus. No. 47 in: *Descriptions of Plant Viruses*. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew Surrey, England.
 19. van Kammen, A., and de Jager, C. P. 1978. Cowpea mosaic virus. No. 197 (No. 47 revised) in: *Descriptions of Plant Viruses*. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kew, Surrey, England.