



Biological, serological and molecular comparison between isolates of *Cowpea severe mosaic virus*

Rosa Felicia E. Araújo Camarço¹, Aline K. Queiroz do Nascimento¹, Eduardo C. de Andrade² & José Albersio A. Lima¹

¹Laboratório de Virologia Vegetal, Departamento de Fitotecnia, Universidade Federal Ceará, 60360 842, Fortaleza, CE, Brazil; ²Embrapa Mandioca e Fruticultura, 44380 000, Cruz das Almas, BA, Brazil

Author for correspondence: José Albersio A. Lima, e-mail: albersio@ufc.br

ABSTRACT

Cowpea, *Vigna unguiculata*, can be affected by several diseases, and those caused by viruses are considered of great importance. *Cowpea severe mosaic virus* (CPSMV), which belongs to the family *Comoviridae*, genus *Comovirus*, stands out for its severity and degree of incidence. Its genetic variability can give rise to different strains around the world, including in Brazil. The objective of the present research was to establish a biological, serological and molecular comparison between five CPSMV isolates obtained from naturally infected plants in different States of the Northeast of Brazil. Isolates were obtained from cowpea: CPSMV-AL (State of Alagoas), CPSMV-CE (Ceará); CPSMV-MC (Piauí), CPSMV-PE (Pernambuco) and an isolate obtained from *Crotalaria paulinea*, in the State of Maranhão - CPSMV-CROT. A host range study evidenced biological differences among the virus isolates, especially in cowpea genotypes. The isolates were showed to be serologically and molecularly related by polymerase chain reaction – PCR, using degenerated primers which amplified two conserved regions in the coat protein and in the replicase genes. Cloning and sequence of CPSMV-PE made possible its comparison with other CPSMV isolates and other virus species from the genus *Comovirus*.

Keywords: *Vigna unguiculata*, *Comovirus*, CPSMV, molecular differentiation.

RESUMO

Comparação biológica, sorológica e molecular de isolados de *Cowpea severe mosaic virus*

O feijão caupi, *Vigna unguiculata*, pode ser afetado por várias moléstias, entre as quais, destacam-se as viroses, cuja incidência e severidade variam, dependendo do hospedeiro, do vetor e da fonte do inóculo no campo. O vírus do mosaico severo do caupi (*Cowpea severe mosaic virus*, CPSMV) é o único vírus pertencente ao gênero *Comovirus* já identificado no Brasil infetando o feijão caupi. A presente pesquisa teve por objetivo estudar e comparar as características biológicas, sorológicas e moleculares de cinco isolados do CPSMV originários de diferentes estados do Nordeste: Isolados de feijão caupi em Alagoas (CPSMV-AL), no Ceará (CPSMV-CE); no Piauí (CPSMV-MC), em Pernambuco (CPSMV-PE) e o isolado obtido de *Crotalaria paulinea* no Maranhão (CPSMV-CROT). Os estudos de gama de hospedeiro evidenciaram diferenças biológicas entre os isolados, sobretudo em genótipos de caupi. As análises sorológicas em dupla difusão em agar confirmaram o forte relacionamento entre os isolados, e os estudos moleculares pela reação em cadeia da polimerase - PCR, utilizando oligonucleotídeos degenerados que amplificam seqüências conservadas dos genes do capsídeo e da replicase, não permitiram nenhuma diferenciação entre os isolados. A clonagem e o sequenciamento do CPSMV-PE possibilitaram sua comparação com outros isolados de CPSMV e outras espécies de vírus do gênero *Comovirus*.

Palavras-chave: *Vigna unguiculata*, *Comovirus*, CPSMV, Diferenciação molecular.

Cowpea, *Vigna unguiculata* (L.) Walp. susp. *unguiculata* is an important legume crop, with great capacity to supply the population with protein and carbohydrates in the Northeast of Brazil. The most important viruses that infect cowpea in Brazil belong to the following families and genera: family *Comoviridae*, genus *Comovirus*; family *Potyviridae*, genus *Potyvirus*; family *Bromoviridae*,

genus *Cucumovirus*, and family *Geminiviridae*, genus *Begomovirus* (Lima et al., 2005a). *Cowpea severe mosaic virus* (CPSMV), which belongs to the family *Comoviridae* and genus *Comovirus*, has stood out for its severity and degree of incidence. Several CPSMV isolates have been biologically and serologically characterized in the State of Ceará and most of them have been isolated from naturally infected cowpea (Lima et al., 2005b). The first CPSMV isolate obtained in the State of Ceará was designated CPSMV-CE (Lima & Nelson, 1974; 1977). Another isolate obtained from cowpea cv. Macaibo was designated CPSMV-MC (MC for Macaibo), considering the fact that cv. Macaibo

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was immune to all the CPSMV isolates obtained in Brazil up to that period (Lima et al., 1998). Other virus isolates obtained from *Canavalia brasiliensis*, *C. ensiformis*, *Crotalaria paulinea* and *Macroptilium lathyroides* were also biologically and serologically characterized and identified as strains of CPSMV (Lima & Souza, 1980; Lima et al., 2005a, b).

The present paper has the objective of developing comparative studies involving biological, serological and molecular characteristics of five CPSMV isolates obtained in the Northeast of Brazil. Isolates were obtained from cowpea: CPSMV-AL (State of Alagoas), CPSMV-CE (Ceará); CPSMV-MC (Piauí), CPSMV-PE (Pernambuco) and one isolate was obtained from *C. paulinea*, in the State of Maranhão - CPSMV-CROT. Each isolate was maintained in a separate anti-aphid screen cage with temperature varying from 26° to 32°C. All isolates were mechanically inoculated in the plant species from the families Chenopodiaceae (*Chenopodium amaranticolor*, *C. murale* and *C. quinoa*; Leguminosae (*Cassia occidentalis*, *Clitoria ternatea*, *Leucaena leucocephala*, *Phaseolus vulgaris*) and 20 genotypes of cowpea, and Solanaceae (*Nicotiana benthamiana* and *N. tabacum*)

The isolates CPSMV-PE, CPSMV-CROT and CPSMV-AL were purified based on the method used by Lima & Nelson (1974) with some modifications. The purified preparations were used to prepare polyclonal antisera by rabbit immunizations, and the specificity of each antiserum was evaluated in double immune diffusion tests. Antisera for the isolates CPSMV-CE and CPSMV-MC were previously produced by Lima & Nelson (1974) and Lima et al. (1998). The serological relationship among the virus isolates was studied by reciprocal double immune diffusion tests with the respective antisera and antigens.

For molecular characterization, total RNA was extracted from infected leaf samples of each virus isolate using the protocol developed by Rott & Jelkmann (2001), and used for synthesis of correspondent complementary DNA (cDNA). Two PCR methods were used to amplify genomic fragments: PCR 1 and nested PCR 2. The first method followed the standard technique using degenerated primers corresponding to a conserved genome sequence specific for the coat protein gene from the genus *Comovirus*: 5'-YTCRAAWCCVYTRTTKGGMCCACA-3' - R and 5'-GCATGGTCCACWCAGGT-3' - F. The amplification was performed according to the program: initial heating at 94°C for 5 min and 25 cycles involving denaturation (94°C/1 min), annealing (41°C/2 min) and extension (72°C/3 min), followed by a final extension at 72°C for 7 min. The amplified products were visualized in a 1% agarose gel treated with ethidium bromide, under ultra violet light. In the nested PCR-2 method the first step of reverse transcription was performed according to Maliogka et al. (2004) to obtain the cDNA for each virus isolate. PCR with the obtained cDNAs was carried out using a pair of degenerated primers corresponding to a nucleotide sequence from the replicase

gene specific for the genus *Comovirus*. The primers COM F1 (5'-ACIWSIGARGGITWYCC-3') and COM R2 (5'-AVRTRTRTCRTCICCRTA-3') were selected to initiate the PCR and the reactions were performed according to the program described above. The primers COM NeF3 (5'-TACGWSGGARGGGT WYCC-3'), COM NeR4 (5'-ARRTRTRTCRTC GCCRTAIAAC-3') and COM NeR5 (5'-AVRTRTRTCRTC GCCRTAIGT-3') were used to continue the nested PCR according to the following sequence of cycles: PCR a - Initial heating at 94°C for 2 min; five cycles of segmented steps: 95°C for 30 s; 40°C for 10 s; 37°C for 5 s and 72°C for 30 s, followed by 35 segmented cycles: 95°C for 30 s; 42°C for 30 s and 72°C for 25 s, followed by a final extension at 72°C for 2 min. For nested PCR b a denaturation step at 94°C for 2 min; three cycles of segmented steps: 95°C for 30 s, 48°C for 30 s and 72°C for 20 s, followed by 35 segmented cycles with the following steps: 95°C for 30 s; 50°C for 30 s; 72°C for 25 s and a final extension at 72°C for 2 min. The amplified products were visualized in 1.5% agarose gel (w/v) treated with ethidium bromide under ultra violet light (Maliogka et al., 2004).

The amplified PCR-1 products with estimated size of 593 bp from all CPSMV isolates were cloned using the pGEM-T® Vector System I (Promega, Madison, WI) according to the manufacturer's instructions. Competent cells of *Escherichia coli* XL1 were used for transformation. *E. coli* plasmid DNA was purified as described (Rott & Jelkmann, 2001) and was digested with *Eco* RI to liberate the insert. Inserts were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer), according to the manufacturer's instructions.

The nucleotide sequence corresponding to the coat protein gene from CPSMV-PE was compared with the nucleotide sequences of other virus species from the genus *Comovirus*. Sequences were analyzed using the programs DNAMAN 4.0 and Blastn (www.ncbi.nlm.nih.gov/blast). The sequences were further aligned using the program Clustal W (www.ebi.ac.uk/clustalw) and the phylogenetic tree was construed using the MEGA v. 4.0 program.

Although CPSMV presents a large biological variability with a wide host range in the leguminous family (Lima et al., 2005a), neither isolate studied infected *C. occidentalis*, *C. ternatea*, *Leucaena* sp. and *P. vulgaris*. All the isolates caused necrotic local lesions in *C. amaranticolor*, *C. murale* and *C. quinoa* and systemically infected most of the cowpea genotypes tested (Table 1). The symptoms in cowpea genotypes varied from mild mosaic to severe mosaic and leaf distortion, according to the isolate and the genotype involved, confirming that CPSMV-CE was the most severe isolate and CPSMV-MC was the mildest. The study permitted the identification of those genotypes with higher and lower degree of resistance and susceptibility to each isolate (Table 1). For example: 'CE-13' was not infected by CPSMV-CE; 'CE-440' was highly susceptible to CPSMV-AL, CPSMV-CE, CPSMV-CROT and CPSMV-PE, but showed only mild mosaic when infected with

TABLE 1 - Symptoms and serology of plant species inoculated with five isolates of *Cowpea severe mosaic virus* (CPSMV): CPSMV-AL; CPSMV-CE; CPSMV-CROT; CPSMV-MC e CPSMV-PE

Family/Species/Genotype	Symptoms and Serological Results				
	CPSMV-AL	CPSMV-CE	CPSMV-CROT	CPSMV-MC	CPSMV-PE
Chenopodiaceae					
<i>Chenopodium amaranticolor</i>	NeLl	NeLl	NeLl	NeLl	NeLl
<i>Chenopodium murale</i>	NeLl	NeLl	NeLl	NeLl	NeLl
<i>Chenopodium quinoa</i>	NeLl	NeLl	NeLl	NeLl	NeLl
Leguminosae					
<i>Cassia occidentalis</i>	nS/ (-)	nS/ (-)	nS/ (-)	nS/ (-)	nS/ (-)
<i>Clitoria ternatea</i>	nS/ (-)	nS/ (-)	nS/ (-)	nS/ (-)	nS/ (-)
<i>Leucaena</i> sp.	nS/ (-)	nS/ (-)	nS/ (-)	nS/ (-)	nS/ (-)
<i>Phaseolus vulgaris</i>	nS/ (-)	nS/ (-)	nS/ (-)	nS/ (-)	nS/ (-)
<i>Vigna unguiculata</i>					
CE 08	sM;Ld/(+)	mM/(+)	mM/(+)	mM/(+)	mM/(+)
CE 13	mM/(+)	nS/(+)	mM/(+)	mM/(+)	mM/(+)
CE 36	mM/(+)	sM/(+)	mM/(+)	mM/(+)	sM/(+)
CE 45	sM;Ld/(+)	sM;Ld/(+)	sM;Ld/(+)	mM/(+)	M;Ld/(+)
CE 46	sM;Ld/(+)	sM;Ld/(+)	sM;Ld/(+)	mM/(+)	M;Ld/(+)
CE 49	sM;Ld/(+)	sM;Ld/(+)	sM;Ld/(+)	mM/(+)	M;Ld/(+)
CE 85	sM;Ld/(+)	sM;Ld/(+)	sM;Ld/(+)	mM/(+)	sM;Ld/(+)
CE 97	M/(+)	sM;Ld/(+)	sM;Ld/(+)	mM/(+)	sM;Ld/(+)
CE 144	sM;Ld/(+)	sM;Ld/(+)	sM;Ld/(+)	mM/(+)	M;Ld/(+)
CE 315	M/(+)	sM/(+)	M/(+)	mM/(+)	M/(+)
CE 440	sM;Ld/(+)	sM;Ld/(+)	sM;Ld/(+)	mM/(+)	sM;Ld;Pdt/(+)
CE 877	nS/(-)	nS/(-)	nS/(-)	nS/(-)	nS/(-)
CE 923	sM/(+)	sM/(+)	mM/(+)	mM/(+)	sM/(+)
CE 924	mM/(+)	sM/(+)	M/(+)	mM/(+)	mM/(+)
CE 929	M/(+)	mM/(+)	mM/(+)	nS/(-)	mM/(+)
Epace 10	M/(+)	M;Ld/(+)	M;Ld/(+)	mM/(+)	mM/(+)
Pitiuba	mM/(+)	sM;Ld/(+)	M;Ld/(+)	mM/(+)	M;Ld/(+)
Roxinho	M;Ld/(+)	sM;Ld/(+)	M;Ld/(+)	mM/(+)	M;Ld/(+)
Setentão	M/(+)	M;Ld/(+)	M;Ld/(+)	mM/(+)	M;Ld/(+)
Macaibo	nS/(-)	nS/ (-)	nS/ (-)	M;Ld/(+)	nS/ (-)
Solanaceae					
<i>Nicotiana benthamiana</i>	nS/ (-)	nS/ (-)	nS/ (-)	nS/ (-)	nS/ (-)
<i>Nicotiana tabacum</i>	nS/ (-)	nS/ (-)	nS/(-)	nS/ (-)	nS/ (-)

*Ld – leaf distortion / NeLl – necrotic local lesions / mM – mild mosaic / M – mosaic / Pdt – plant death / sM – severe mosaic / nS – no symptoms / (+) – positive in serology / (-) – negative in serology.

CPSMV-MC; ‘CE-923’ showed severe mosaic upon infection by CPSMV-AL, CPSMV-CE and CPSMV-PE, but only mild mosaic upon infection by CPSMV-CROT

and CPSMV-MC; ‘CE-929’ was not infected by CPSMV-MC. The genotypes ‘CE-45’, ‘CE-46’, ‘CE-49’, ‘CE-85’ and ‘CE-144’ were susceptible to all isolates and, on the

other hand, the genotype 'CE-877' showed resistance to all virus isolates (Table 1). The genotype CE-659 was showed to be resistant to CPSMV-CE (the most severe isolate) and to CPSMV-MC (the least severe isolate). Based on the symptoms, the virus isolate severity decreased in the following order: CPSMV-CE, CPSMV-CROT, CPSMV-PE, CPSMV-AL and CPSMV-MC. The immunity of cv. Macaibo to CPSMV-AL, CPSMV-CE, CPSMV-CROT and CPSMV-PE, but not to CPSMV-MC, confirmed its importance for CPSMV-MC differentiation. These results represent the first biological criteria for differentiation of CPSMV isolates.

The purified preparations of CPSMV-AL, CPSMV-CROT and CPSMV-PE showed ultra violet spectra typical of polyhedral virus particles and the final virus concentrations (33 mg of virus/Kg of infected tissue) were compatible for CPSMV (Lima & Nelson, 1974; Lima et al., 1998; Lima et al., 2005a). The absence of spur formation between the different virus isolates in double immune diffusion is an indication of their serological identity. Similar results had been already obtained for some CPSMV isolates, but Lima et al. (1998) also found serological differences between CPSMV-MC and another CPSMV isolate obtained from soybean (*Glycine max*) in Paraná State (Bertacini et al., 1994).

The amplified products by PCR 1 and PCR 2 did not show any difference among the CPSMV isolates (Figure 1). The results involving the replicase gene, considered a conserved protein for ssRNA viruses (Dovas & Kátis, 2003a, b), demonstrate potential for detection of unknown viruses from the family *Comoviridae*. The possible molecular difference among the CPSMV isolates could, probably, be detected when the whole polyprotein was sequenced and analyzed (Dovas & Katis, 2003a; Maliogka et al., 2004). Amplified DNA fragments with estimated size of 593 bp from the CPSMV isolates were cloned and the fragments from the CPSMV-PE isolate showed a small difference in size (Figure 1C), indicating a possible molecular difference in this virus isolate. The sequence of the CPSMV-PE genome fragment (Figure 2) was compared with virus sequences deposited in the GenBank, and the comparative analysis revealed a similarity of 93% with the sequence of a CPSMV isolate obtained in the State of Paraná, CPSMV-PR (Bertacini et al., 1994). The results are consistent with previous data obtained for other CPSMV isolates and are in accordance with ICTV rules (Fauquet et al., 2005), confirming that CPSMV-PE and CPSMV-PR are closely related. Lima et al. (1998) also found serological differences between CPSMV-MC and CPSMV-PR, and in this study CPSMV-PE was showed to be serologically identical to CPSMV-MC. The molecular comparison between CPSMV-PE and *Cowpea mosaic virus* (CPMV), a different but related virus species from the genus *Comovirus* (Swaans & Kammen, 1973), showed a close relationship between them (Figure 2). The occurrence of CPMV has not yet been reported in Brazil (Lima & Nelson, 1974, 1977; Lin et al.,

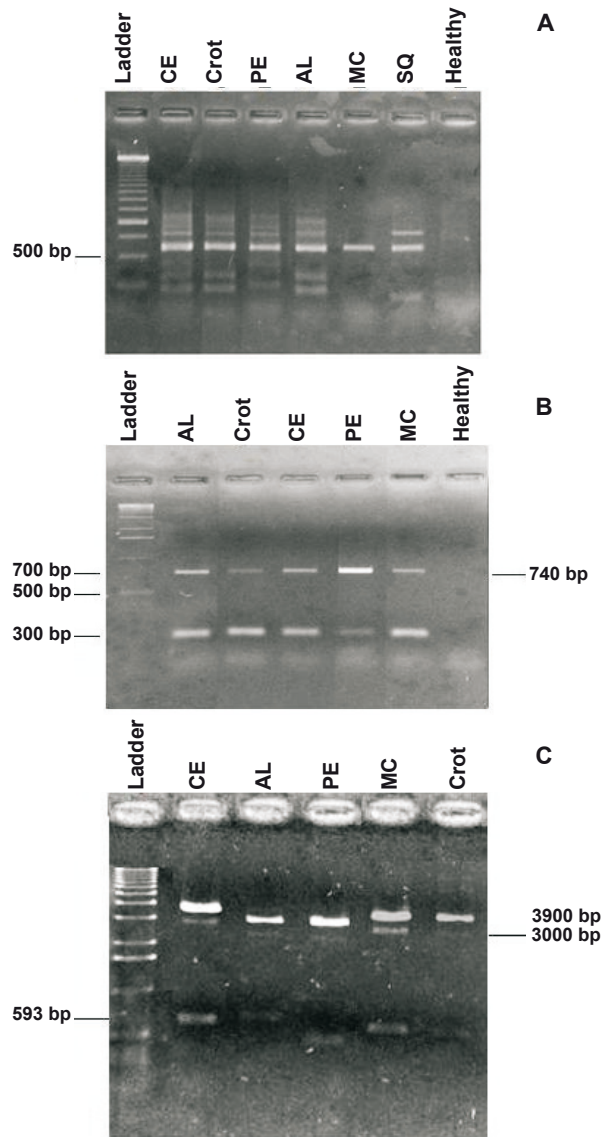


FIGURE 1 - Amplified genomic fragments of *Cowpea severe mosaic virus* isolates: CPSMV-AL, CPSMV-CE, CPSMV-MC, CPSMV-PE and CPSMV-CROT in 1% agarose gel. **A.** Genomic fragments from CPSMV and *Squash mosaic virus* (SqMV) isolates amplified by polymerase chain reaction (PCR-1) using degenerated primers for the coat protein gene; **B.** Genomic fragments from CPSMV isolates amplified by PCR (nested PCR-2) using degenerated primers for the replicase gene and **C.** Genomic fragments, of the coat protein gene, from CPSMV clones upon digestion with *Eco* RI. The first lanes were loaded with a 1kb DNA ladder.

1981; Lima et al., 2005a, 2005b). A low sequence similarity (74%) was observed between CPSMV-PE and an isolate of CPSMV obtained in Central America designated CPSMV-VU (Kammen & Jager, 1978; Jager, 1979), revealing a

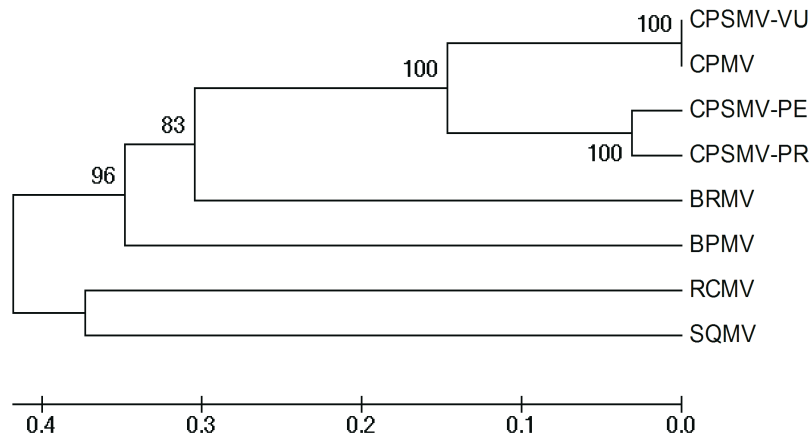


FIGURE 2 - Phylogenetic tree based on the capsid protein gene (nucleotide sequences) showing the relationship between CPSMV-PE (GQ229416) and other viruses from the genus *Comovirus* using the UPGMA method. The numbers of each branch represent bootstrap values (1,000 replications). BPMV (AY744933) - *Bean pod mottle virus*; BRMV (AF263548) - *Bean rugose mosaic virus*; CPSMV-Cowpea severe mosaic virus: CPSMV-PR (AF263549) isolate from Paraná – Brazil and CPSMV-VU (M83309) obtained in Central America; CPMV (X00729) - *Cowpea mosaic virus*; RCMV (M14913) - *Red clover mottle virus* and SQMV (AB054689) - *Squash mosaic virus*. Horizontal distances are proportional to the genetic distances between the isolates.

significant genetic difference that could be attributed to geographic localization. In the phylogenetic tree (Figure 2), isolates CPSMV-PE and CPSMV-PR clustered together, confirming their close relationship. Furthermore, isolate CPSMV-VU clustered with CPMV, indicating that its identification as CPSMV is incorrect.

The close serological relationship observed among the CPSMV isolates could explain the strong similarity in the obtained PCR products for the coat protein gene. Although closely related serologically and molecularly, the CPSMV isolates showed biological differences when inoculated in cowpea genotypes, which need to be considered in breeding programs to produce resistant varieties. The appearance of a CPSMV strain with the ability to infect a cowpea genotype (Macaibo) immune to all other virus isolates is evidence that new strains can be developed by genetic mutation, rearrangement of genome components and adaptation to new cowpea cultivars or leguminous species over the years.

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