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Biological stability of a strain of *Cowpea severe mosaic virus* over 20 years[1]

Estabilidade biológica de uma estirpe do *Cowpea severe mosaic virus* ao longo de 20 anos

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Abstract - Cowpea (*Vigna unguiculata*) is an important crop of the traditional agriculture system in the Northeast of Brazil. It can be infected by more than 20 virus species and *Cowpea severe mosaic virus* (CPSMV) is one of the most important pathogens that naturally infect cowpea in Brazil. Several CPSMV isolates were obtained and characterized in the Plant Virus Laboratory at the Federal University of Ceará: CPSMV-CE - the first characterized isolate of the virus obtained from cowpea in the State of Ceará; CPSMV-AL - isolated from cowpea in Alagoas; CPSMV-PE - isolated from cowpea in Pernambuco; CPSMV-PR - obtained from soybean (*Glycine max*) in Paraná and CPSMV-CROT - isolated from *Crotalaria paulinea*, in Maranhão. An isolate of CPSMV with the property to infect the cv. Macaibo, a cowpea cultivar immune to most of CPSMV isolates was also biologically and serologically characterized as a new strain of the virus (CPSMV-MC). The CPSMV-MC was isolated in January 1990 and has been evaluated over 20 years by host range studies and maintenance *in vivo* by periodical mechanical inoculations in cowpea. The results of this periodical evaluation revealed that the biological integrity and the serological properties of CPSMV-MC were preserved over 20 years, indicating that the genetic preservation of a virus strain could occur over the years. Molecular studies involving part of the coat protein (CP) gene of CPSMV-MC and five other Brazilian CPSMV isolates indicated a high degree of conservation, with 92-100% nucleotide sequence identity among the isolates.

Key words - Genetic stability. Cowpea. Biological properties. Biological integrity.

Resumo - O feijão-caupi (*Vigna unguiculata*) é uma cultura do sistema tradicional do Nordeste do Brasil, que pode ser infetada por mais de 20 espécies de vírus, sendo o vírus do mosaico severo do caupi (*Cowpea severe mosaic virus,* CPSMV) um dos mais importantes patógenos que infeta naturalmente essa leguminosa no Brasil. Vários isolados do CPSMV foram obtidos e caracterizados no Laboratório de Virologia Vegetal da UFC: CPSMV-CE - o primeiro isolado do vírus obtido no Ceará; isolados obtidos de feijão-caupi: CPSMV-AL (Alagoas) and CPSMV-PE (Pernambuco); CPSMV-PR - isolado de soja (*Glycine max*) no Paraná e CPSMV-CROT - isolado de *Crotalaria paulinea* no Maranhão. Um isolado de CPSMV capaz de infetar o cv. Macaibo, cultivar de ffeijão-caupi imune a todos os demais isolados de CPSMV foi biológica e sorologicamente caracterizado e designado de CPSMV-MC. O CPSMV-MC obtido em janeiro de 1990 vem sendo avaliado através de estudos de gama de hospedeiro e mantido *in vivo* através de inoculações periódicas em feijão-caupi. Os resultados dessas avaliações revelaram que a integridade biológica e as propriedades sorológicas do CPSMV-MC foram preservadas ao longo dos anos, indicando que a preservação das propriedades genéticas de uma estirpe viral pode acontecer através dos anos. Estudos moleculares adicionais revelaram que a seqüência de nucleotídeos correspondente a parte do gene da capa protéica de cinco isolados de CPSMV, incluindo o CPSMV-MC, possui elevado grau de conservação, com 92-100% da seqüência de nucleotídeos idêntica entre os isolados.

Palavras-chave - Estabilidade genética. Feijão de corda. Características sorológicas. Integridade biológica.

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Introduction

Cowpea [*Vigna unguiculata* (L.) Walp. subsp. *unguiculata*] is an important crop of the traditional agriculture system in the semiarid regions of Northeast of Brazil. Cowpea is largely cultivated by small growers and presents great commercial cultivated areas in the North and Northeast of Brazil. Its seeds when dried posse relevant nutritional properties that are superior to those of common bean (*Phaseolus vulgaris,* L.) and have lower fat rate than those from soybean [*Glycine max* (L.) Merrill] (PHILIPS et al., 2003).

The virus diseases are still the most important limiting factors for cowpea production in several countries, including Brazil (BOOKER; UMAHARAM; MCDAVID, 2005; GHORBANI; SHAHRAEIN; ELAGINA, 2008; GONÇALVES; LIMA, 1988; HAMPTON; THOTTAPPILLY; ROSSEL, 1997; LIMA; SITTOLIN; LIMA, 2005; ORAWU et al., 2005). Cowpea can be infected with more than 20 virus species, among which the *Cowpea severe mosaic virus* (CPSMV), family *Comoviridae*, genus *Comovirus* is one of the most important that infect cowpea in Brazil (LIMA; SITTOLIN; LIMA, 2005). The CPSMV represents serious problems for this leguminous crop where it is cultivated (LIMA; SITTOLIN; LIMA, 2005). In the State of Ceará, Brazil, the first characterized isolate of CPSMV was designated as CPSMV-CE (LIMA; NELSON, 1974). Several others CPSMV isolates were obtained and characterized in the Plant Virus Laboratory at the Federal University of Ceará: CPSMV-AL - isolated from cowpea in the State of Alagoas; CPSMV-PE - isolated from cowpea in the State of Pernambuco; CPSMV-PR - obtained from soybean [*Glycine max* (L.) Merrill] in the State of Paraná and CPSMV-CROT - isolated from *Crotalaria paulinea* L. in the State of Maranhão (LIMA et al., 2005). All these CPSMV isolates and some others obtained from *Canavalia brasiliensis* Mart., *C. ensiformis* (L.) DC and *Macroptilium lathyroides* (L.) Urban had already been purified, biologically and serologically characterized and identified as strains of CPSMV (LIMA et al., 2005; LIMA; SITTOLIN; LIMA, 2005). An isolate of CPSMV with the property to infect 'Macaibo', a cowpea cultivar immune to most of the CPSMV isolates was purified, and biologically and serologically characterized as a new strain of the virus, designated as CPSMV-MC (LIMA et al., 1992). The cowpea cv. Macaibo is originated from the germ plasma collection from the Institute of Agriculture Research (IPA) in Pernambuco, and it was identified as immune to CPSMV in the Plant Virus Laboratory at the UFC, back in 1970 decade (LIMA; NELSON, 1974). Its immunity properties were confirmed to all CPSMV isolates known up to that time in Brazil (LIMA; SITTOLIN; LIMA, 2005). The CPSMV-MC isolate was obtained from a cowpea field experiment in the State of Piaui in January 1990 and it could be distinguished from the other CPSMV isolates for causing only mild symptom in the cv. Pitiuba and infecting the cv. Macaibo (LIMA et al., 1992). The CPSMV-MC was evaluated over 20 years by cowpea genotype inoculation and serology, and maintained *in vivo* at greenhouse conditions by periodical mechanical inoculations in plants of cowpea cv. Macaibo. Molecular studies were also developed involving a nucleotide sequence corresponding to part of the coat protein (CP) gene from CPSMV-MC and other five CPSMV isolates.

Material and methods

Virus isolation, maintenance and cowpea genotype evaluation

An isolate of CPSMV was obtained from leaf samples of cowpea plants growing at experimental field conditions in the State of Piaui in January 1990 (LIMA et al., 1992). Among the leaf samples tested, some were collected from a cowpea cultivar originated from genotype cross including the cv. Macaibo as an ancestral. All the samples were serologically tested against antisera specific for the viruses from the following families and genera: family *Potyviridae,* genus *Potyvirus: Blackeye cowpea mosaic virus* (BlCMV) and *Cowpea aphid-borne mosaic virus* (CABMV); family *Bromoviridae,* genus *Cucumovirus: Cucumber mosaic virus* (CMV) and family *Comoviridae,* genus *Comovirus:* CPSMV, through double immune diffusion test and indirect enzyme linked immunoabsorbent assay (ELISA). One sample that reacted only with the antiserum specific to CPSMV was used to inoculate health cowpea plants cultivars Pitiuba and Macaibo, maintained at greenhouse conditions. Based in its specific property to infect the cv. Macaibo, a cowpea cultivar immune to all others CPSMV isolates it was designated as CPSMV-MC, with MC standing for Macaibo. Both cowpea cultivars were used to maintain the CPSMV-MC over 20 years, by periodical mechanical inoculation at greenhouse conditions.

A total of 44 cowpea genotypes (TAB. 1) was evaluated against CPSMV-MC, at greenhouse conditions. Eight plants of each cowpea genotype were inoculated with the CPSMV-MC and the plants were daily inspected for symptoms. For each genotype two non inoculated healthy plants were maintained as control. After 25 days from the virus inoculations, all inoculated plants including the asymptomatic ones and those with very mild symptoms were tested by serology in double immune diffusion tests against antiserum for CPSMV-MC. Similar experiments were repeated every

five years with the following cowpea genotypes: CE-002 - Bengala, CE-003 - Vinagre-1, CE-004 - Cabecinha, CE-005 - Lisão, CE-006 - Vinagre-2, CE-007 - Das Almas, CE-008 - Ritinha, CE-010 - Isabel-1, CE-011 - Quebra Cadeira, CE-012 - Rita Joana, CE-013 - Roxão-1, CE-014 - Potomac, CE-015 - Cabecinha Roxo, CE-018 - Quarenta dias, CE-025 - Sempre Verde, CE-031 - Pitiúba, CE-046 - Milagroso, CE-066 - Carrapicho, CE-073 - Rouxinho 1, CE-524 - Macaibo, CE-596 - Setentão, CE-611 -BR1-Poty and CE-930 - Pingo de Ouro. All the symptom caused by CPSMV-MC were confirmed by serology.

According to the symptoms and the serological results, the cowpea genotype responses inoculated with the CPSMV-MC were classified as: Immune (extreme resistance) - plants without symptoms and negative for serology; Resistant - plants with mild mosaic and positive for serology; Susceptible - plants with mosaic and positive for serology and Highly Susceptible - plants with severe mosaic, other systemic symptoms, including necrosis and positive for serology.

Virus purification, protein molecular weight evaluation and antiserum production

The CPSMV-MC was purified from systemically infected cowpea cv. Macaibo leaves by a similar method used by Nascimento et al. (2010) to purify *Papaya lethal yellowing virus* from infected papaya (*Carica papaya* L.), using *n*-Butanol for plant sap clarification and virus particles precipitation with polyethylene glycol MW 6,000 (PEG), followed by a further ultra-centrifugation for virus particles precipitation and concentration. The final pellet from the high speed centrifugation was resuspended in 0.02 M Tris buffer pH 7.5, and the virus purity and concentration was evaluated in an ultraviolet spectrophotometer VARIANT DMS - 70, using wavelength range of 220 to 340 nm. The virus infectivity in the purified preparation was evaluated by mechanical inoculation in healthy cowpea cv. Macaibo.

The purified CPSMV-MC preparation was used to prepare policlonal antiserum by rabbit immunizations. A White New Zealand rabbit with approximately six months was used to be immunized with the virus. The rabbit received three injections in the foot pad with the virus preparation emulsified with incomplete Freud adjuvant. After 30 days from the last injection the rabbit was bled by the marginal vein of the ears, using xylene to dilate the veins and vaseline to avoid blood coagulation and vein obstruction.

The antiserum specificity was evaluated in double immune diffusion tests in Petri dishes with the wells opened in hexagonal arrange in the agar medium. The serological relationship among the CPSMV isolates was studied by reciprocal double immune diffusion tests with the antiserum obtained for CPSMV-MC and antisera produced for the isolates CPSMV-AL, CPSMV-CROT, CPSMV-PE and CPSMV-CE. The virus antigens were arranged in adjacent peripheral wells to possibility a spur formation between wells with serological distinct virus isolates.

Molecular characterization

The viral RNA was extracted from a purified preparation of CPSMV-MC using 10% of SDS, followed by heating at 65 ^{o}C for 10 min. The viral RNA was precipitated with 0.3 M sodium acetate and three volumes of ethanol, and the precipitate was ressupended in pure water with DEPC. A first strand of cDNA was synthesized from the viral RNA using M-MLV Reverse Transcriptase (Promega), according to the manufacturer's instructions.

Viral fragments corresponding to a portion of the CP gene were amplified by PCR using 3 µl of the cDNA, 5 µl of buffer, 5 µl of $MgCl_2$ 25 mM, 1 µl from the mixture of desoxinucleotideos (0.01 M), 1 unit of *Taq* DNA polymerase and 25 pml of each one of the universal primers for the genus *Comovirus*: F, 5'-GCA TGG TCC ACW CAG GT-3' and R, 5'-YTC RAA WCC VYT RTT KGG MCC ACA-3' (CAMARÇO et al., 2009). PCR amplification was performed with an initial heating at 94 ºC for 5 min followed by 30 cycles of the program: denaturizing (94 ºC/1 min), annealing (41 ºC/2 min) and extension (72 ºC/1 min), followed by a final extension at 72 ºC for 5 min. The amplified products were visualized in a 1% agarose gel stained with ethidium bromide under UV light.

The PCR products with an estimated size of 500-600 bp were cloned using the pGEM-T easy Vector (Promega) according to the manufacturer's instructions. Competent cells of *Escherichia coli* JM109 were used for transformation and the *E. coli* plasmid DNA was purified as described (ROTT; JELKMANN, 2001). The purified plasmid was cleaved with *Eco*R I to liberate the insert, which was sequenced in both directions using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer).

The CP gene sequences of 593 bp from CPSMV-MC and from the other CPSMV isolates were compared with each other, and the sequences were analyzed using the Blast*n* algorithm (www.ncbi.nlm.nih.gov/blast) and DNAMAN 4.0 (Lynnon Biosoft). The nucleotide sequences were aligned using the Clustal W program (www.ebi.ac.uk/clustalw), and a phylogenetic analysis was done using MEGA v. 4.0 (www.megasoftware.netI) (BASIC, 2011).

Table 1 - Symptoms and genotype response classification of cowpea (*Vigna unguiculata*) genotypes inoculated with *Cowpea* severe mosaic virus (CPSMV) strain CPSMV-MC.

Cowpea (*Vigna unguiculata*) Genotype	Symptoms*	Serology	Genotype Response
CE-002 - Bengala	mM	+	Resistant
CE-003 - Vinagre-1	ClL, mM	+	Resistant
CE-004 - Cabecinha	NeLl, mM	+	Resistant
CE-005 - Lisão	ClL, mM	+	Resistant
CE-006 - Vinagre-2	ClL, mM	+	Resistant
CE-007 - Das Almas	ClL, mM	+	Resistant
CE-008 - Ritinha	ClL, mM	+	Resistant
CE-009 - Cara suja	NeLl, ClL, M, LD	+	Highly Susceptible
CE-010 - Isabel-1	NeLl, ClL, mM	+	Susceptible
CE-011 - Quebra Cadeira	M	+	Susceptible
CE-012 - Rita Joana	(-)	-	Immune
CE-013 - Roxão-1	M, LD	+	Susceptible
CE-014 - Potomac	M, Dwf	+	Susceptible
CE-015 - Cabecinha Roxo	NeLl, M	+	Susceptible
CE-018 - Quarenta dias	ClL, M, LD	+	Susceptible
CE-025 - Sempre Verde	Dwf, sM	+	Highly Susceptible
CE-031 - Pitiúba	M	+	Susceptible
CE-046 - Milagroso	M	+	Susceptible
CE-066 - Carrapicho	ClL, M, LD	+	Highly Susceptible
CE-073 - Rouxinho 1	mM	+	Resistant
CE-077 - n- Rouxinho 2	mM	+	Resistant
CE-091 - Tvu-966	(-)	-	Immune
CE-113 - Purple Knucke Hull-55	sM	+	Highly Susceptible
CE-147 - Tvu-382	(-)	-	Immune
CE-159 - Tvu-379	(-)	-	Immune
CE-176 - AU94 – 418-07-01	M	+	Susceptible
CE-178 - MNC – 03-720-11	mM	+	Resistant
CE-293 - 1423-P1	mM	+	Resistant
CE-315 - Tvu-2331	ClL, sM, LD	+	Highly Susceptible
CE-524 - Macaibo	M	+	Susceptible
CE-596 - Setentão	mM	+	Resistant
CE-611 - BR1-Poty	mM	+	Resistant
CE-665 - [L 1325 (IPA)]	mM	+	Resistant
CE-674 - CNCX-252-9E	(-)	-	Immune
CE-790 - CNC X 251-11E	(-)	-	Immune
CE-798 - CNC X 251-76E	(-)	-	Immune
CE-681 - CNC x 284-66E	(-)	-	Immune
CE-930 - Pingo de Ouro	mM	+	Resistant
CE-933 - BRS Marataoã			
mM	+	Resistant	
CE-937 - BRS Rouxinol	mM	+	Resistant
CE-939 - Paulistinha	sM	+	Highly Susceptible
CE-940 - BRS Punjante	sM	+	Highly Susceptible
CNCX-676-51 F	(-)	-	Immune
Tvu-3961	(-)	-	Immune

***ClL** - Chlotic local lesions; **Dwf** - Dwarfism; **LD** - Leaf distortion; **Lye** - Leaf yellowing; **M** - Mosaic; **mM** - Mild mosaic; **sM** - Severe mosaic; **NeLl** - Necrotic local lesions; (-) No symptoms

Results and discussion

The appearance of CPSMV-MC as a new virus strain with the property to infect the cowpea genotype Macaibo immune to all other CPSMV isolates is an indication that new virus strains could surge by genetic mutation, rearrangement of genome components and adaptation to new cowpea cultivars or leguminous species over the years (FAUQUET et al., 2005). The genetic variability presented by plant viruses, mailing and most commonly by those with divided genome such as the case of CPSMV is evidenced by the great number of isolates biologically and genetically different from the wild type of the virus (DE JAGER, 1979; FAUQUET et al., 2005). The great range of CPSMV isolates obtained and characterized in the Plant Virus Laboratory at the Federal University of Ceará is a great evidence of that (LIMA; NELSON, 1974; LIMA et al., 2005; LIMA; SITTOLIN; LIMA, 2005).

The biological properties of CPSMV-MC confirmed over the years, differently, from the others CPSMV isolates, indicated that it infected both Pitiuba and Macaibo cowpea cultivars, causing vary mild symptoms in `Pitiuba`. Considering the property that the virus isolate infected 'Macaibo', a cowpea cultivar immune to all others CPSMV isolates characterized in Brazil, it was designated as CPSMV-MC (LIMA et al., 1992; LIMA; SITTOLIN; LIMA, 2005b). The maintenance of the genetic integrity and the biological properties of CPSMV-MC over 20 years constantly propagated *in vivo* by frequent and periodical inoculations indicate that its biological properties could be preserved over the years. The biological stability of CPSMV-MC indicates that the development of new virus strains does not imply in the extinction of those that already exist, which must be always considered in the breeding programs to avoid that the existing virus problems be forgotten in function of new strains (HAMPTON; THOTTAPPILLY; ROSSEL, 1997).

The purified virus preparation recently obtained from infected `Macaibo` showed an excellent and clear aspect, which virus purity was confirmed by the obtained ultraviolet absorption spectrum (FIG. 1) and its high infectivity in cowpea cv. Macaibo (FIG. 2). The antiserum produced against the CPSMV-MC purified preparation reacted with extracts from CPSMV infected plants without reactions with extracts from health plants and presented a titer of 1:1024 in double immune diffusion tests. The CPSMV immunogenicity was also demonstrated by Swiss mice oral immunization (FLORINDO et al., 2002).

The host range studies with 44 cowpea genotypes confirmed by several experiments over the years indicated that the biological properties of CPSMV-MC were preserved over 20 years. Besides symptom observations, the presence of the virus in the infected plants was confirmed by serology. All 44 cowpea genotypes evaluated were distributed in the following classification groups: Immune (Extreme resistance) - 10 (22.73%) cultivars; Resistant - 17 (38.63%); Susceptible - 10 (22.73%) and

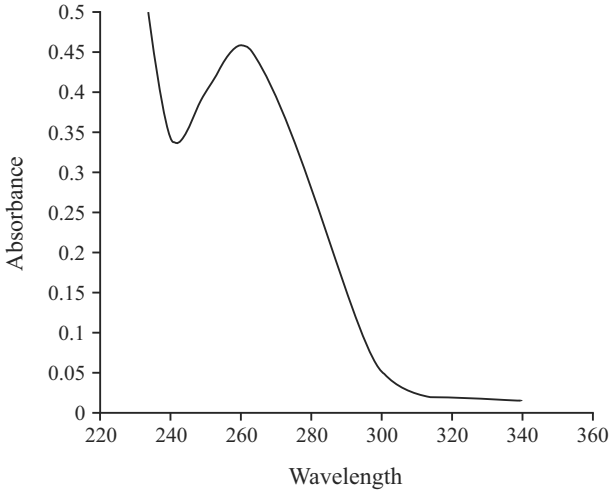

Figure 1 - Absorption spectrum of purified preparation of the *Cowpea severe mosaic virus* CPSMV-MC strain isolated from cowpea cv Macaibo in 0.05 M Tris buffer

Figure 2 - Cowpea cv. Macaibo inoculated with purified preparation of the *Cowpea severe mosaic virus* strain CPSMV-MC

Highly Susceptible - 7 (15.91%) (TAB. 1). Most of the genotypes (61.36%) showed to be immune or resistant to the virus, but 17 genotypes, corresponding to 38.64% were susceptible or highly susceptible. The cowpea cv. Macaibo was infected only by CPSMV-MC among all the others CPSMV isolates, confirming previous studies (LIMA et al., 1992; 2005b), and 27 genotypes were immune or resistant to CPSMV-MC, including 'CE-012 - Rita Joana', C'E-937 - BRS Rouxinol' and 'CE-933 - BRS Marataoã'. The immunity or extreme resistance of 'CE-933 - BRS Marataoã' to another CPSMV isolate was previously identified (FREIRE FILHO, et al., 2005), indicating that this is an adequate commercial cultivar to control CPSMV. On the other hand, the immunity of 10 cowpea genotypes to CPSMV-MC (TAB. 1) showed the viability of it being controlled by resistant commercial cultivars. On the other hand, the immunity of 12 cowpea genotypes to CPSMV-MC (TAB. 1) showed the viability of it being controlled by resistant commercial cultivars. Studies developed by Booker, Umaharam and McDavid (2005) showed that CPSMV can have a significant and important effect on cowpea yields, demonstrating that yield losses associated with CPSMV infections can vary from as little as 2% to as much as 85%, depending on the time of virus infection and the cultivar. A strain of CPSMV was found to be the most prevalent virus in all the surveyed cowpea growing areas in Uganda (ORAWU et al., 2005). According to Hampton, Thottappilly and Rossel (1997), an effective control of virus diseases in cowpea have been done by the use of resistant cultivars. Other sources of resistance to CPSMV in cowpea were also obtained by others (ASSUNÇÃO et al., 2005; LIMA et al., 2003). According to Vale and Lima (1999) the inheritance of resistance to CPSMV in cowpea cv. Macaibo is controlled by a recessive gene, but Assunção et al. (2005) found that different genes of cowpea confers resistance to CPSMV in other cultivars. The multiple sources of resistance to CPSMV-MC (TAB. 1) are going to offer a relevant genetic background turning possible and speeding up the work that have been done by the plant breeders to develop cowpea cultivars resistant to viruses and with different maturity time, type of seeds and other agronomic properties to attend the multiple market exigencies.

The results obtained with several greenhouse experiments for CPSMV-MC maintained *in vivo* by periodical inoculations in cowpea showed that its serological properties and biological integrity were preserved over the last 20 years, indicating that the genetic preservation of a virus strain could occur over the years. The symptoms and serological reactions for CPSMV-MC were the same over the years, specially for the following cowpea genotypes: 'CE-002 - Bengala', 'CE-003 - Vinagre-1', 'CE-004 - Cabecinha', 'CE-005 - Lisão', 'CE-006 - Vinagre-2', 'CE-007 - Das Almas', 'CE-008 - Ritinha', 'CE-010 - Isabel-1', 'CE-011 - Quebra Cadeira', 'CE-012 - Rita Joana', 'CE-013 - Roxão-1', 'CE-014 - Potomac', 'CE-015 - Cabecinha Roxo', 'CE-018 - Quarenta dias', 'CE-025 - Sempre Verde', 'CE-031 - Pitiúba', 'CE-046 - Milagroso', 'CE-066 - Carrapicho', 'CE-073 - Rouxinho 1', 'CE-524 - Macaibo', 'CE-596 - Setentão', 'CE-611 -BR1-Poty' and 'CE-930 - Pingo de Ouro'. The source of resistance or extreme resistance (immunity) in cowpea genotypes (TAB. 1) will also pave the way of the plant breeders to use an adequate breeding program to develop commercial CPSMV-MC resistant cultivars.

The RT-PCR results revealed the presence of a band in the agarose gel corresponding to approximately 593 bp for the CPSMV-MC RNA (FIG. 3A). The success of cloning part of the CP gene from CPSMV-MC was confirmed by the products of the digested plasmid which showed one band of approximately 593 bp for the CPSMV-MC gene and of 3015 bp for the linear plasmid DNA (FIG. 3B).

The CP gene sequences of 593 bp from CPSMV-MC and the other CPSMV isolates showed to have a high degree of conservation, with 92-100% nucleotide sequence identity among the isolates. The CPSMV-PR obtained from soybean in the State of Paraná showed 85% homology with the RNA2 from the CPSMV isolate deposited in the GenBank (SOUTO et al., 2002).

Despite the low variability of the CP gene among the isolates, there is a notable difference in their symptoms on cowpea genotypes (CAMARÇO et al., 2009), which indicates that, probably, other parts of the viral genome may be more variable, and the virus symptom determinants could be linked to these regions.

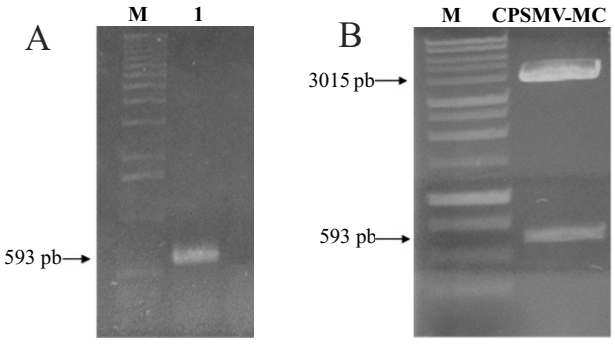

Figure 3 - Molecular studies with the *Cowpea severe mosaic virus* CPSMV-MC strain purified from cowpea. **A-** The RT-PCR results revealing a band in the agarose gel corresponding to approximately 593 bp for the purified CPSMV-MC (Line 1); Line M- DNA ladder with standards of indicated length in bp. **B-** Products from the digested plasmid which showed one band of approximately 593 bp for the CPSMV-MC gene and of 3,015 bp for the linear plasmid DNA; Line M- DNA ladder with standards of indicated length in bp

Conclusions

1. Differently from the others CPSMV isolates, the CPSMV-MC infected both Pitiuba and Macaibo cowpea cultivars, causing vary mild symptoms in `Pitiuba`;
2. Based on biological and serological experiments it was possible to conclude that the biological integrity of CPSMV-MC was preserved *in vivo* over 20 years. This is an indication that a genetic preservation of a CPSMV strain could occurs over the years, although the appearance of new strains could surge by genetic mutations, genome components reorganization, and virus adaptations to leguminous species and/or new immune cowpea cultivars over the years;
3. The development of new virus strains does not imply in the extinction of those that already exist, which must be always considered in the breeding programs to avoid that the existing virus problems be forgotten in function of new strains;
4. Molecular studies involving a nucleotide sequence of part of the CP gene from CPSMV-MC and others CPSMV isolates showed a high degree of conservation among the isolates.

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