









A survey of RNA genome viruses in Lima bean crops of Northeastern Brazil

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ABSTRACT: Lima bean plants showing mosaic and leaf distortion symptoms, similar to those induced by viruses, were observed in several production areas in the states of Ceará and Piauí, Northeastern Brazil. The aim of this study was to identify RNA genome viruses that infect lima bean. Field research was conducted from 2017 to 2018. Fifty-five symptomatic samples were randomly collected from seven properties in five municipalities and tested by RT-PCR and DNA sequencing with specific or universal primers for two viruses and two virus groups. Four virus species were identified: cucumber mosaic virus (CMV), cowpea mild mottle virus (CPMMV), cowpea aphid-borne mosaic virus (CABMV) and cowpea severe mosaic virus (CPSMV), which had infection levels of 21.8, 52.7, 47.2, and 1.8%, respectively, as well as double and triple infections. The CMV isolates belonged to subgroup IA. The CPMMV isolates had high nucleotide identity with CPMMV isolates from Brazil, USA and Mexico. The CABMV isolates showed moderate nucleotide identity with Brazilian isolates. Only one sample was infected with CPSMV. This is the first record of CPMMV and CPSMV naturally infecting lima bean. Approaches to virus control are discussed.

Key words: carlavirus, comovirus, cucumovirus, *Phaseolus lunatus*, potyvirus, virus identification.

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INTRODUCTION

Lima bean (*Phaseolus lunatus* L.) is an annual legume crop of great importance for the food and nutritional security of rural communities in the Northeast region of Brazil due to its good adaptation to the regional climate and soil conditions and its high content of protein (26%), making it an easily accessible protein source (Barreto Neto et al. 2015). This region is responsible for more than 94% (11.193 tonnes) of Brazilian lima bean production, with the states of Ceará, Paraíba, Pernambuco, and Piauí being the main producers (IBGE 2018). However, due to the low levels of technology and other factors, the yield obtained in the northeastern region with this crop is around 300 kilograms/hectare.

Viruses have efficient forms of dissemination, are difficult to control, and are considered a major phytosanitary problem. They induce significant yield losses, limiting the production of many economically important crops such as beans (Lima et al. 2005). In Brazil, at least nine viruses that infect beans, such as cowpea [*Vigna unguiculata* (L.) Walp.] and common bean (*Phaseolus vulgaris* L.), have been reported. Those with the most predominance in the field are cucumber mosaic virus (CMV) (Eiras et al. 2004), cowpea aphid-borne mosaic virus (CABMV) (Nicolini et al. 2012), cowpea severe mosaic virus

(CPSMV) (Abreu et al. 2012) and cowpea mild mottle virus (CPMMV) (Lamas et al. 2017). There are, however, few reports of viruses infecting lima bean (Carvalho et al. 2015), possibly because lima bean is mainly cultivated by small farmers and has less economic importance than cowpea and common bean. The only comprehensive survey of lima bean viruses in Brazil was carried out for begomoviruses, and two species were identified as causal agents of golden mosaic disease (Ramos Sobrinho et al. 2014).

To fill this gap of knowledge regarding viral diseases of the lima bean, a survey was carried out in cultivated areas in the states of Piauí and Ceará, Brazil, to identify viruses infecting this crop and assess their potential economic importance.

MATERIAL AND METHODS

Collection of plant material

Lima bean samples with mosaic and leaf distortion symptoms were collected during the growing season (May to June) of 2017 and 2018 in the states of Piauí and Ceará, Brazil. In Piauí, a total of 43 samples were collected in Várzea Grande do Piauí (n = 9), Tanque do Piauí (n = 9), Barra D'Alcântara (n = 12), in the experimental area of the Center for Agricultural Sciences of Universidade Federal do Piauí, Teresina (n = 13). In the state of Ceará, 12 samples were collected in the city of Tianguá. Collected leaf samples were placed in plastic bags and the site registered by global positioning system (GPS) coordinates. Back in the laboratory, samples were preserved at -80 °C.

RNA extraction

Total RNA from leaf tissue was extracted with TRIzol reagent (Life Technologies) following the manufacturer's recommendations. The pellets were washed with 75% ethanol, air-dried for 20 min and resuspended in 50 µL nuclease-free ultrapure distilled water and stored at -80 °C.

Diagnostic test

Some viruses are prevalent in bean fields in Northeastern Brazil, including CMV, CABMV, CPMMV and CPSMV (Lima et al. 2005; Lamas et al. 2017). Therefore, this work aimed to determine if the same viruses were infecting lima bean in this region. Samples were tested by reverse transcription-polymerase chain reaction (RT-PCR) with CMV- and CPMMV-specific primers and universal primers for potyviruses and comoviruses (Table 1). For the synthesis of complementary DNA (cDNA) and PCR, the enzymes GoScript Reverse Transcriptase (Promega, Madison, WI, USA) and GoTaq Hot Start polymerase (Promega, Madison, USA) were used, respectively, following the manufacturer's recommendations. The PCR

Table 1. Oligonucleotides used in the RT-PCR reactions to detect cucumber mosaic virus (CMV), cowpea mild mottle virus (CPMMV), potyviruses and comoviruses in lima bean.

Oligonucleotide	Orientation	Sequence (5' - 3')	Reference
CMVF	Forward	TGGTCGTCCTCACTATTAACCAC	Kim et al. (2014)
CMVR	Reverse	TACTGATAAACCAGTACCGGTGA	
CIFor	Forward	GGIVVIGTIGGIWSIGGIAARTCIAC	Ha et al. (2008)
CIRev	Reverse	ACICCRTTYTCDATDATRTTIGTIGC	
ComovirusF	Forward	GCATGGTCCACWCAGGT	Brioso et al. (1996)
ComovirusR	Reverse	YTCRAAWCCVYTRTTKGGMCCACA	
CPMMV-4000F	Forward	AACTTGGCCTTAGTGAACCTCTACA	Lamas et al. (2017)
CPMMV-4500R	Reverse	ATTAGCTCTGTGCCTGGGGT	

products were analyzed on 1% agarose gels and stained with ethidium bromide. Amplified fragments of all potyviruses and comoviruses, as well as some CMV and CPMMV samples, were purified and sequenced in both directions at Macrogen Inc. (Seoul, South Korea).

Phylogenetic analysis

Phylogenetic trees based on the nucleotide sequences were generated by Bayesian inference using MrBayes version v. 3.2 (Ronquist et al. 2012) with nucleotide substitution model (SYM+I+G for CMV), (HKY+I for CPMMV) and (GTR+G for CABMV) selected by MrModeltest 2.3 (Posada and Buckley 2004) in the Akaike information criterion (AIC). The analyses were carried out for 10,000,000 generations and sampling was done at every 1,000 steps to produce the distribution tree. The first 2,500 trees were discarded as burn in. Tree was visualized using Figtree (Rambaut 2009).

Mechanical transmission experiments

After RT-PCR assays, samples infected singly by one of the tested viruses were used for mechanical transmission assays to observe the symptoms each virus induced in lima bean, cowpea, and common bean. Extracts of infected leaves were prepared in 0.01 mol·L⁻¹ phosphate buffer (pH 7.5), and rubbed onto the first pair of expanded leaves of three weeks old lima bean plants, previously dusted with carborundum (particle size 0.037 mm). Twelve lima bean plants (six of 'Branquinha' and six of 'Boca de Moça') were inoculated, in addition to two plants of each of the cowpea genotypes line TE 97-309G-3, 'Pampo' and 'Imponente', and the common bean 'Carioca'. The plants were grown in 2.8 L pots on a mixture of soil, charred rice husk and vegetable substrate (1:1:1 ratio) and kept in cages protected with antiaphid mesh during all experiments. Inoculated plants were periodically observed, registering the onset and the type of symptoms. Virus infection was confirmed by RT-PCR.

RESULTS AND DISCUSSION

RT-PCR for virus detection

Amplicons were successfully obtained with the set of primers to detect CMV, CPMMV, poty- and comovirus in the sampled plants. Sequence analysis of the amplified fragment using primers for poty- and comovirus allowed for the identification of the detected potyvirus as CABMV, and the comovirus, as CPSMV. Among RT-PCR detected viruses, CABMV and CPMMV were the most prevalent. Of 55 samples, 27 (49.0%) were positive for CABMV and 29 (52.7%), for CPMMV (Table 2). CPMMV and CABMV were found in all sampled localities, except Teresina. CMV was found in 12 samples (21.8%) in three municipalities, while CPSMV was found only in one sample (1.8%) from Teresina.

Table 2. Detection of lima bean (*Phaseolus lunatus*)-infecting viruses in five municipalities of the states of Ceará and Piauí, Brazil.

City	No. of samples	Number and percentage of infected samples (%)							CMV+ CPMMV+ CABMV
		CABMV	CMV	CPMMV	CPSMV	CABMV+ CMV	CABMV+ CPMMV	CMV+ CPMMV	
Barra D'Alcântara (PI)	12	9 (75.0)	1 (8.3)	5 (41.6)	0 (0.0)	1 (8.3)	2 (16.6)	0 (0.0)	0 (0.0)
Tanque (PI)	9	4 (44.4)	0 (0.0)	8 (88.8)	0 (0.0)	0 (0.0)	3 (33.3)	0 (0.0)	0 (0.0)
Teresina (PI)	13	0 (0.0)	9 (69.2)	9 (69.2)	1 (7.6)	0 (0.0)	0 (0.0)	7 (53.8)	0 (0.0)
Tianguá (CE)	12	10 (83.3)	0 (0.0)	2 (16.6)	0 (0.0)	0 (0.0)	1 (8.3)	0 (0.0)	0 (0.0)
Várzea Grande (PI)	9	4 (44.4)	2 (22.2)	5 (55.5)	0 (0.0)	1 (11.1)	1 (11.1)	2 (22.2)	1 (11.1)
Total	55	27 (49.0)	12 (21.8)	29 (52.7)	1 (1.8)	2 (3.6)	7 (12.7)	9 (16.3)	1 (1.8)

The high incidence of CABMV, CPMMV and CMV is most likely associated with the high occurrence of insect vectors of these viruses in lima bean plantations, which are often cultivated concurrently and in the same period as cowpea. However, transmission through lima bean infected seeds cannot be ruled out. The low incidence of CPSMV in lima bean observed in this study is probably due to the fact that lima bean is not a preferred host of its insect vectors (chrysomelid beetles).

Of the 55 evaluated samples, 16 (29.0%) were infected with more than one virus (Table 2). Double infections of CABMV and CMV (3.6%), CABMV and CPMMV (12.7%), and CMV and CPMMV (16.3%), were more frequent, while triple infection of CABMV, CMV and CPMMV was recorded in 1.8% of the plants. Mixed infections have biological, epidemiological and economic implications, as there may be synergistic relationships, leading to amplification of disease symptoms, changes in systemic movement of viruses, and increased or decreased concentration of viruses in the plant (Taiwo et al. 2007).

When inoculated simultaneously with CMV, CABMV and CPSMV, susceptible cowpea plants showed more severe symptoms, while more resistant plants showed milder symptoms (Oliveira et al. 2012).

Sequence identity and phylogenetic analysis

Virus-derived DNA fragments of 320, 500, 700 and 536 bp specific for CMV, CPMMV, potyviruses and comoviruses, respectively, were successfully amplified (data not shown), confirming the occurrence of these viruses in lima bean in

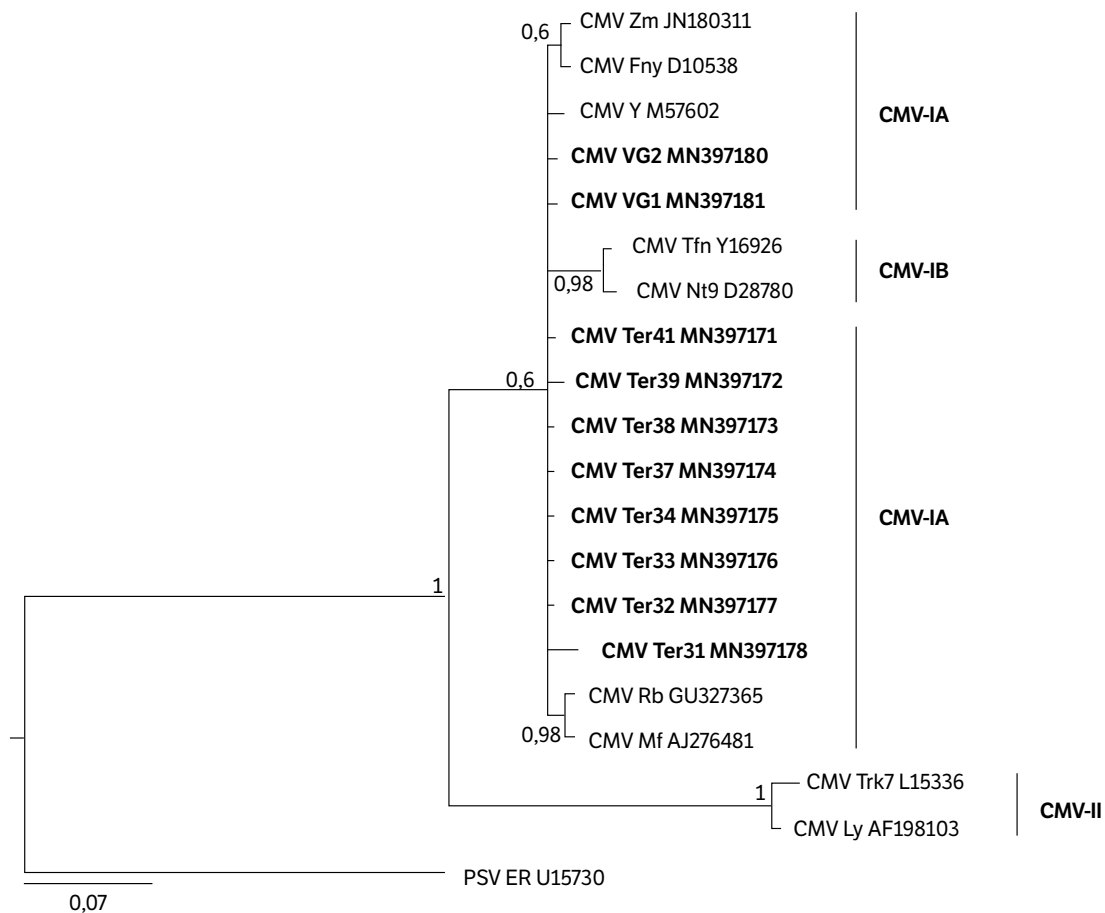


Figure 1. Bayesian phylogenetic tree based on the ORF 3b nucleotide sequence from cucumber mosaic virus (CMV) isolates obtained in this study (bold) and isolates recovered from the GenBank, with their respective accession numbers. Peanut stunt virus (PSV) was used as the outgroup.

Northeastern Brazil. Pairwise sequence comparisons for the capsid protein (CP) gene of 10 CMV isolates from this study showed 96–100% nucleotide (nt) and 100% amino acids (aa) identities, demonstrating a high conservation of the CP gene. Pairwise sequence comparisons with isolates from GenBank revealed identity values of 94–99% for nt and 96–100% for aa with CMV subgroup I isolates, and 80–81% for nt, and 84–85% for aa with CMV subgroup II isolates. In the phylogenetic analysis, our isolates grouped with the CMV subgroup IA (Bayesian posterior probability = 0,6) (Fig. 1). These results reinforce those of Eiras et al. (2004) that in Brazil, there is a prevalence of CMV subgroup I.

Five CPMMV isolates were sequenced and showed 97–100% nt identity amongst themselves and 97–99% nt and 100% aa identities with other CPMMV isolates from Brazil, USA and Mexico (Fig. 2). This virus is considered reemergent and has also been reported in high incidence in common bean (80–100%) and in a wide range of uncultivated hosts in Northeastern Brazil (Lamas et al. 2017). The high incidence of CPMMV in lima bean crops as well as in other cultivated Fabaceae such as common bean and soybean (Zanardo et al. 2014; Lamas et al. 2017) reinforces the importance of managing this pathogen.

Pairwise sequence comparisons for the cylindrical inclusion (CI) gene of 27 CABMV isolates revealed 81–100% nt and 92–100% aa identity. Pairwise sequence comparisons with isolates from GenBank showed the highest identity of 90–95%

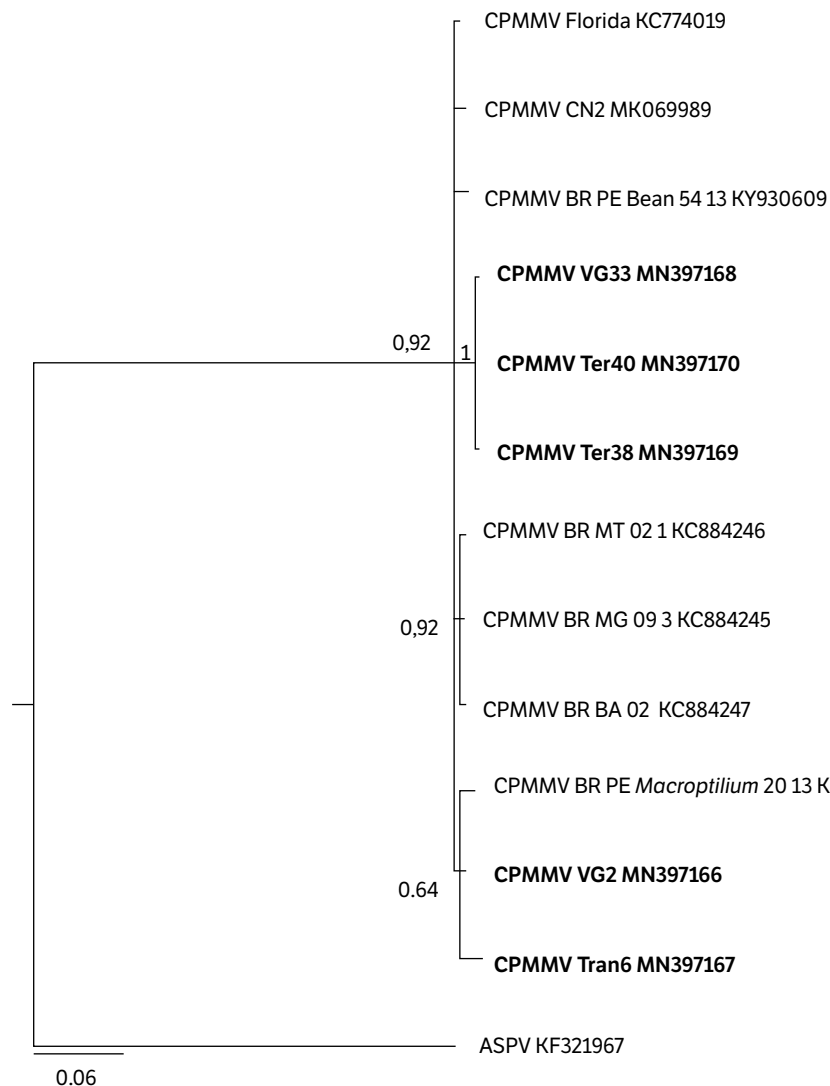


Figure 2. Bayesian phylogenetic tree based on the RdRp nucleotide sequence from cowpea mild mottle virus (CPMMV) isolates obtained in this study (bold) and isolates recovered from the GenBank, with their respective accession numbers. Apple stem pitting virus (ASPV) was used as an outgroup.

nt and 96–97% aa with CABMV MG-Avr isolate from passion fruit (HQ880243) (Barros et al. 2011; Rodrigues et al. 2015). Based on comparisons of partial genome sequences and according to the International Committee on Taxonomy of Viruses (ICTV), species demarcation criterion for the genus *Potyvirus* (< 74% sequence identity for CI coding region) (Adams et al. 2005; ICTV 2019), isolates studied in this work were confirmed as CABMV, the only potyvirus infecting lima bean plants identified in this study.

Phylogenetic analysis of 16 samples revealed the formation of two groups: the first group contained Brazilian (12 of isolates used in this study, MG-Avr and BR1), Indian (RR3) and African (Z) isolates, and the second contained four isolates used in this study and others Brazilian isolates Alvinlândia-SP, Fernão-SP and SJBV-PR, both groups with high support (Fig. 3). There was no correlation between geographic origin or host, since the MG-Avr, Alvinlândia-SP, Fernão-SP and SJBV-PR isolates were obtained from passion fruit, the BR1 isolate from peanut, and the RR3 isolate from cowpea.

Moderate sequence conservation and lack of correlation between geography, host origin, and phylogeny has been demonstrated for CABMV isolates obtained from different regions of Brazil. CABMV is the prevalent species in cowpea

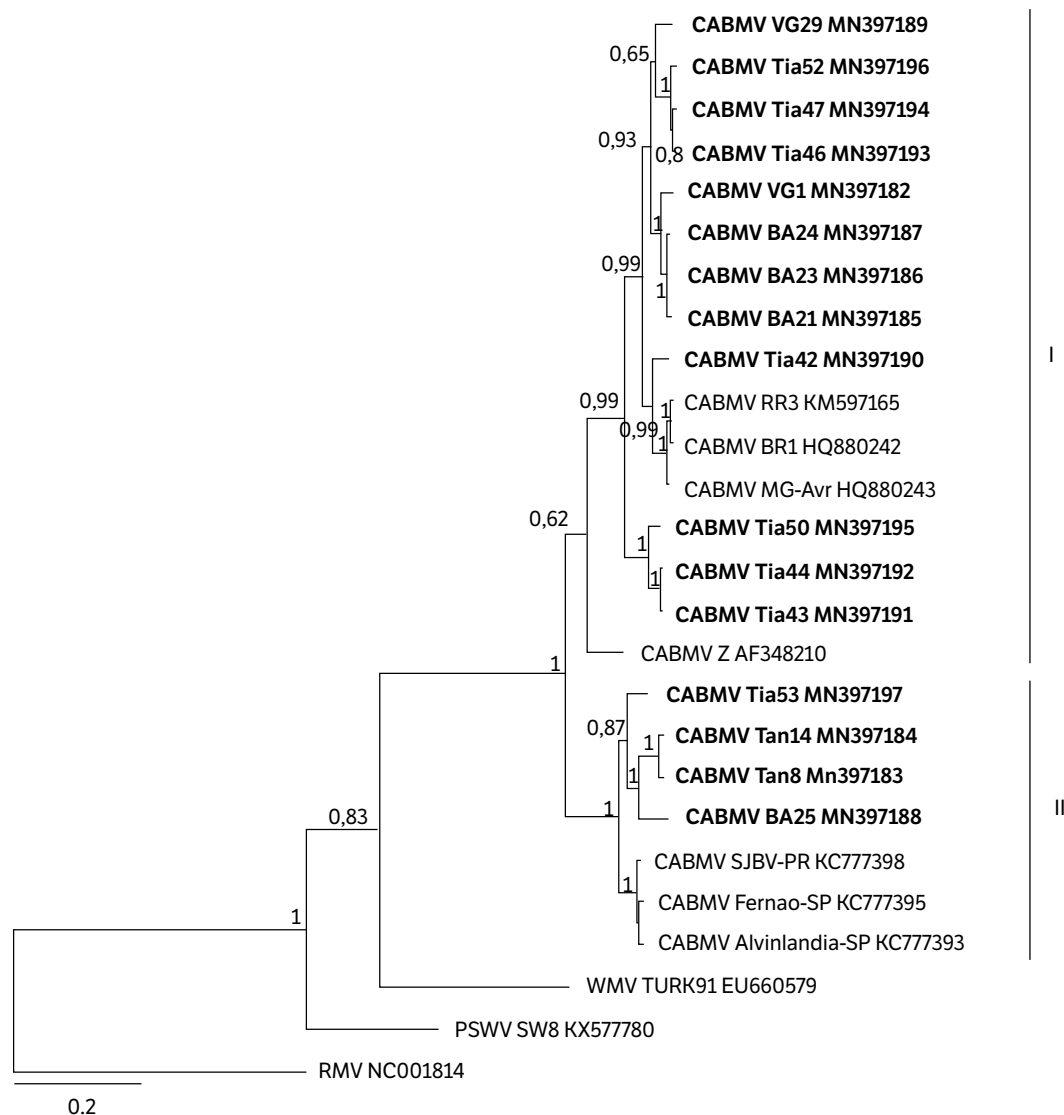


Figure 3. Bayesian phylogenetic tree based on the CI gene of cowpea aphid-borne mosaic virus (CABMV) isolates obtained in this study (bold) and isolates recovered from the GenBank, with their respective accession numbers. Ryegrass mosaic virus (RMV) was used as the outgroup.

(Freitas et al. 2012) and passion fruit (Rodrigues et al. 2015) in Brazil. Both botanical species are normally cultivated in Northeastern Brazil and therefore potential sources of lima bean virus and vice versa.

Only one sample (MN729608) was diagnosed as infected by a comovirus. The partial sequence of the CP gene presented higher identity, 100% nt and 100% aa, with the isolate CPSMV PI (HM450148) from cowpea sampled in Brazil. These data confirming CPSMV as the only comovirus infecting lima bean in this study.

Symptoms characterization

The four viruses caused symptoms in all lima bean plants following mechanical inoculation with extract of infected material (Fig. 4). CMV induced systemic symptoms of mild mosaic 20 days after inoculation (dai). At 40 dai, mosaic was observed in young leaves and in some expanded leaves (Fig. 4a). No other CMV symptoms were observed in lima beans. CABMV caused mild mosaic symptoms 8 dai; at 35 dai, the leaves had intense blistering and limb distortion and showed necrotic lesions (Fig. 4b); at 60 days after inoculation, plants were underdeveloped. CPMMV induced systemic mild mosaic symptoms in young leaves at 8 dai; at 20 dai a yellow mosaic was observed in young and fully expanded leaves and blistering was observed in young leaves (Fig. 4c). Chlorotic local lesions were observed 4 days after mechanical inoculation with CPSMV; at 35 dai, severe blistering and distortion of leaves (Fig. 4d), death of terminal shoots, leaf necrosis and premature leaf fall, and underdevelopment were observed. Infection by the inoculated virus was confirmed in all inoculated and symptomatic plants by RT-PCR. The symptoms observed in lima bean plants resemble those presented by cowpea infected with CMV, CABMV and CPSMV (Oliveira et al. 2012), including underdevelopment of the plant, implying that these viruses are real problems for the production of lima beans in Brazil. Future studies of synergistic effects between these viruses in lima beans may contribute to reveal the effects on production.

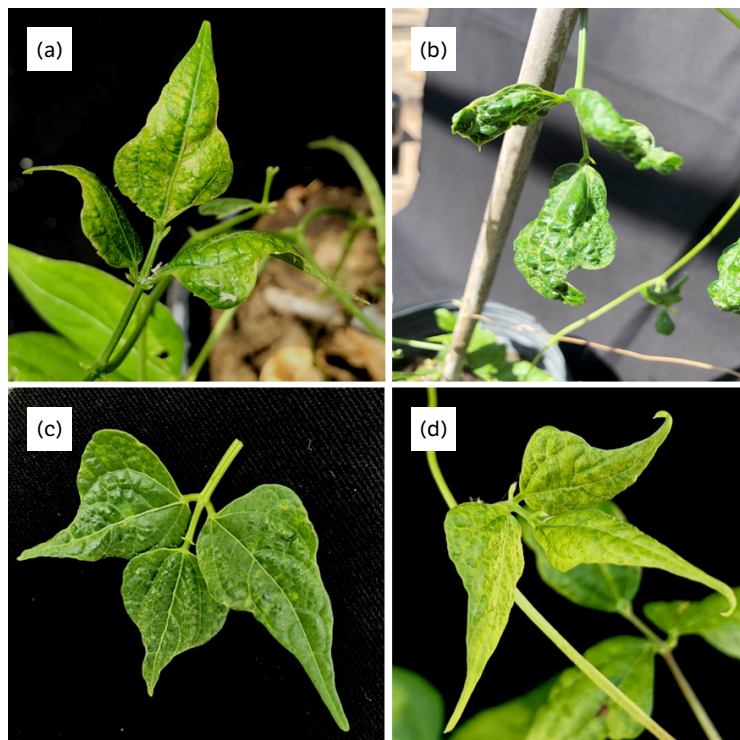


Figure 4. Symptoms observed in lima bean plants ‘Branquinha’ inoculated with: (a) cucumber mosaic virus (CMV) – mild mosaic; (b) cowpea aphid-borne mosaic virus (CABMV) – severe mosaic and leaf distortion; (c) cowpea mild mottle virus (CPMMV) – mild mosaic and crinkled leaves; (d) cowpea severe mosaic virus (CPSMV) – severe mosaic and severe leaf distortion.

CONCLUSION

The results of this study revealed four viruses infecting lima beans in Brazil. However, the presence of other viral species not covered by this study cannot be ruled out. This is the first time that CPMMV and CPSMV have been recorded naturally infecting lima beans anywhere in the world. In addition, the frequent incidence of CMV and CABMV infecting lima beans was confirmed. However, it will be necessary to expand the sampling area to obtain a more representative distribution and prevalence of these viruses. In this context, the use of RT-PCR was useful in achieving the detection of a limited number of known viruses. However, the use of more modern identification techniques such as high throughput sequencing could identify other viruses that were not the target of this research.

Lack of knowledge about viruses as a phytosanitary problem amongst farmers contributes to the high incidence of the disease. Consequently, there are no attempts to control viruses and their vectors. These results will be the basis for future studies of lima bean viruses, mainly for the genetic management of these viruses, in view of the lack of knowledge of resistant varieties, in addition to the execution of an extension program that will aim to teach producers about viruses. To our knowledge, this is the first study to identify and characterize lima bean RNA viruses in Brazil.

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