# First report of *Cowpea mild mottle virus* in chía (*Salvia hispanica*)

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**Abstract**

Chía (*Salvia hispanica*), an herbaceous plant of the *Lamiaceae* family, has displayed a growing popularity in the lasts years due to its high concentrations of the health-beneficial Omega-3 fatty acid. The aim of this study was to characterize the viruses affecting this cultivation. A total of forty chía plants showing leaf deformation, dwarfism or chlorosis were collected from production fields located in Argentina northeast. The samples were screened for the presence of *Alfalfa mosaic virus* (AMV), *Cowpea mild mottle virus* (CPMMV), *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV) and *Tospovirus* group (I, II and III), *Potyvirus* and *Begomovirus* genus. Three plants were positive for CPMMV. When CPMMV positive samples were analyzed by electron microscopy, presence of feather-like inclusions formed by presumed virions were observed. Moreover, ORF2 to ORF6 from the CPMMV viral genome was amplified in by RT-PCR. Viral coat-protein (CP – ORF5) was analyzed against viral CPMMV isolates annotated in GenBank. The nucleotides identity was between 75.6% and 99.0%, above given International Committee on Taxonomy of Viruses (ICTV) criteria for differentiation of Carlavirus species. The phylogenetic analysis revealed that the isolates found in chía plants grouped with other isolates from Brazil, Ghana and USA, but separate from those from India. In the present work we report, for the first time, the presence of *Cowpea mild mottle virus* in chía.

**Keywords:** Carlavirus, CPMMV, *Lamiaceae*,……

Chía (*Salvia hispanica*) is an herbaceous plant of the *Lamiaceae* family. It is grown commercially for its seeds, which are rich in the α-linolenic acid (omega-3). (Ayerza and Wayne, 2006).. Chía grow well in tropical and subtropical regions. This crop requires abundant sun and is frost intolerant. In Argentina, chía is cultivated in the northwest region between February and July (Zavalía et al., 2011).

Despite viral symptoms have been observed in chía plants since 2012, there is scarce information about the viruses capable of infecting this crop (Celli et al., 2014). In 2014, we first reported two bipartite begomoviruses affecting chia plants: the *Tomato yellow spot virus* (ToYSV) previously detected in bean, *Leonurus sibiricus*, tomato (*Solanum lycopersicum)*, *Euphorbia heterophylla*, *Crotalaria* sp., *Leucas martinicensis*, in Brazil and Argentina; and the *Sida mosaic Bolivia virus* 2 (SiMBoV2), previously found in weed *Sida micrantha* in Bolivia.

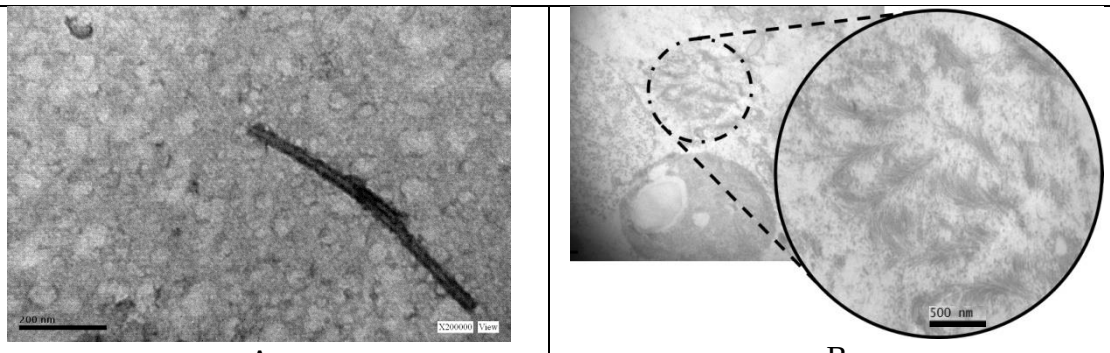
In May-June 2014, 40 plants showing leaf deformation, dawrfism or chlorosis (all symptoms reported in virus-infected plants) were collected in the northwest region of Argentina (Salta and Jujuy province). The samples were screened for the presence of *Alfalfa mosaic virus* (AMV), *Cowpea mild mottle virus* (CPMMV), *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV) and *Tospovirus* group (I, II and III) by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and

*Potyvirus* genus by plate-trapped antigen (PTA), using commercial antisera (BIOREBA SRL Latin America), according to the manufacturer's instructions.

Three out of 40 plants reacted with the antisera against CPMMV, all of them from north of Salta (22°12'S 63°26'W). Additionally, the plants positive for CPMMV were also tested for Begomovirus by PCR, using the begomovirus universal primers PAL1v1978/PAR1c496 (Rojas et al., 1993). No amplification was observed in the samples; therefore, they were considered negative for the infection with Begomovirus.

Figure 1. Chía plant (Sample MA01) showing stem and leaf deformation, dwarfism, chlorosis and leaf necrosis typical of viral infection.

A positive plant (sample MA01, Fig. 1) were analyzed by immunosorbent electron microscopy plus decoration (ISEM-D) according to Milne and Luisoni (1977) with the CPMMV antiserum (BIOREBA SRL Latin America) and by ultra-thin sections of leaf that was fixed and embedded in plastic for routine morphology, examined in a JEOL 1200 (Jeol, Tokyo, Japan) transmission electron microscope. The ISEM-D showed the presence of flexuous and filamentous decorated particles (Fig. 2A) and the ultra-thin sections revealed the presence of feather-like inclusions formed by presumed virions (Fig. 2B) described for CPMMV (Brunt *et al*., 1983).




A B

Figure 2. Electron micrograph of chía leaf (*Salvia hispanica* L.) sample MA01 infected by *Cowpea mild mottle virus*: A. immunosorbent electron microscopy plus decoration (ISEM-D) with the CPMMV antiserum; B. ultra-thin sections.

Total nucleic acids were extracted from leaf sample MA01 using CTAB method (Hoisington *et al*., 1994) and tested by reverse transcription-polymerase chain reaction

(RT-PCR) using specific primers ORF2 F/ORF2 R, ORF3 F/ORF3 R and ORF4 F/ORF6 R (Zanardo *et al*., 2014). Three fragments of expected size (938, 552 and 1676 base pairs) were amplified corresponding to ORF 2 to the 3' terminus of CPMMV genome. The RT-PCR amplicons were purified with the Qiagen PCR purification kit (Qiagen, Inc. Valencia, CA, USA), and then directly sequenced in both directions.

Nucleotide (nt) sequences were assembled using the SeqMan program (DNASTAR, Inc., Madison, WI, USA), and manual adjustments were done when necessary. The assembly of the three genomic fragments retrieved a contig with (2462 nt), which was deposited in GenBank (Accession No. KP402890). The BLAST analysis revealed 99% nt identity (coverage 100%) when compared to an isolate of CPMMV from USA (Accession No. KC774020).

According to the species demarcation criteria of the International Committee on Taxonomy of Viruses (ICTV) (Adams et al., 2012), carlavirus isolates of different species should have less than 72% shared nt identity, or less than 80% aa identity, in their coat protein (CP) or polymerase genes to be considered as different species.

The nt identity of the gene encoding the CP was calculated using MegAlign program (DNASTAR, Inc., Madison, WI, USA). We observed that the nt identity, among Argentinean isolates and 29 other sequences published in GenBank (2015.01.08), ranged from 75.6% (JX524198, an Indian isolate) to 99.0% (KC774029, an isolate from USA), which is above given ICTV criteria for differentiation of Carlavirus species (Adams et al., 2012).

A phylogenetic analysis of the CP sequences was performed with Mega 5.2 software (Tamura et al., 2011) using the Neighbor-Joining, Tamura model with Invariant sites (I). The bootstrap consensus tree was inferred from 2,000 replicates. The analysis displayed two defined clusters; one includes the Argentinean sequence studied in this work among others from Brazil, Ghana, Taiwan and USA, whereas the other group includes only the three isolates from India (Figure 3). Any cluster division related to the host was observed. The existence of a cluster with Indian viral isolates is in Yadav et al. (2013), who observed that the Indian isolate found in soybean clustered together with two other Indian isolates reported in peanut (Naidu et el., 1998).

Zanardo et al. (2014) reported a relationship between the severity of many Brazilian CPMMV isolates found in soybean and the genomic regions of the virus. They observed that the ORF5 (CP) grouped the severe isolates of the virus. In contrast, we did not observed severe Brazilian isolates from soybean grouping together (Figure 3), which may be explained by the higher number of sequences used, the different hosts and the geographical regions included in our analysis.

CPMMV is a member of genus *Carlavirus* and was reported for infecting plants of *Fabaceae* family (*Arachis hypogaea, Arachis repens, Canavalia ensiformis, Desmodium tortuosum, Glycine max, Phaseolus vulgaris, Vigna subterranean, Vigna unguiculata*), and Solanacea (*Lycopersicon esculentum*) (ICTVdB Management, 2006; Muniyappa and Reddy, 1983) but, to our knowledge, this is the first report of CPMMV infection chía, a member of *Lamiaceae* family.

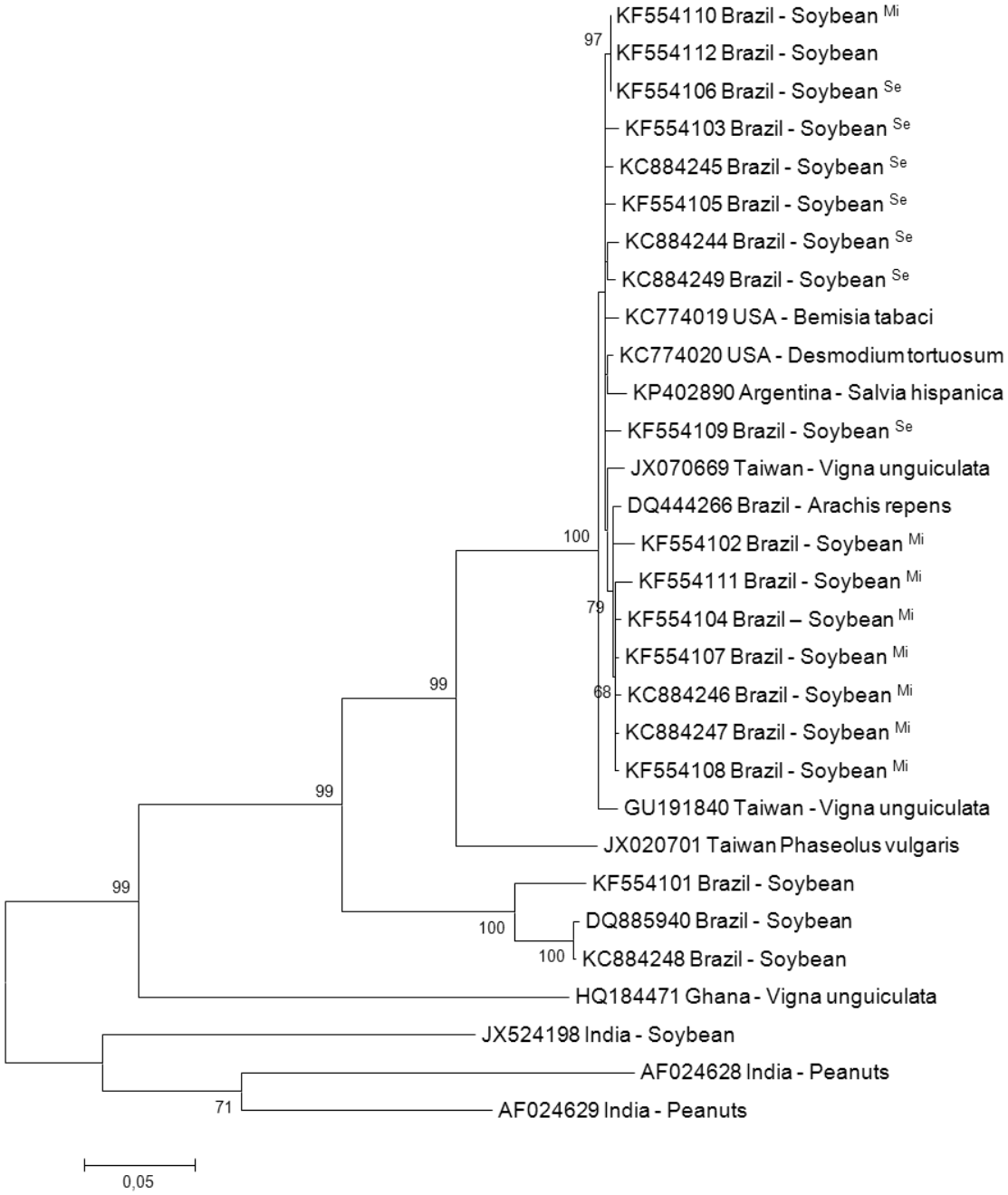



Figure 3. Consensus phylogenetic tree constructed using the software Mega 5.10 (Tamura et al., 2011), Neighbor-Joining, Tamura model with Invariant sites (I) and 2,000 replicates based on the alignment of nucleotide sequences encoding the coat protein of Cowpea mild mottle virus (CPMMV). "Se" and "Mi" are an isolates causing severe or mild symptoms in soybean plants described by Zanardo et al. (2014).

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