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Natural occurrence of *Cucumber mosaic virus* associated with mottling and mosaic disease on *Salvia splendens*, a new record from India

S. Ali[A], *A. A. Khan*[A,B], *B. Afreen*[A], *R. Sharma*[A], *T. Jahan*[A] *and Q. A. Naqvi*[A]

[A]Plant Virology Laboratory, Aligarh Muslim University, Aligarh-202 002, India.
[B]Corresponding author. Email: akil_nbri@yahoo.com

Abstract. Natural occurrence of *Cucumber mosaic virus* (CMV) subgroup IB associated with mottling and mosaic symptoms on *Salvia splendens* was identified on the basis of serological and molecular tests for the first time in India.

Salvia (*Salvia splendens* Sellow ex Schult., family Lamiaceae) is an important annual ornamental plant grown worldwide. During November 2007 to March 2008, severe mottling and mosaic was observed on the leaves of *S. splendens* plants growing in the garden of Aligarh Muslim University, Aligarh, India, with a high disease incidence (~48.7%). Symptoms on the infected *S. splendens* suggest a virus as the causal pathogen.

For mechanical transmission of the virus, newly emerged leaf tissue of naturally infected *S. splendens* was macerated in 0.1 M phosphate buffer, pH 7.0 (1 : 1, w : v) and inoculated on several test species following the protocol of Noordam (1973). Necrotic lesions were obtained on *Chenopodium amaranticolor* and *C. album* 7–10 days post-inoculations (dpi), while systemic mosaic was observed on *Nicotiana benthamiana*, *N. tabacum* cv. White Burley and *Cucumis sativus* 15 dpi. To identify the virus at the serological level, direct antigen coated-enzyme linked immunosorbent assay (DAC-ELISA; Verma *et al*. 2005) was performed with crude sap obtained from infected leaf tissue, *Cucumber mosaic virus* (CMV) specific polyclonal antiserum (PVAS 242a, ATCC, USA) and alkaline phosphatase-linked secondary antibodies (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH or DSMZ, Germany). The mean absorbance at 405 nm for negative and positive controls were 0.061 ± 0.008 and 0.349 ± 0.003, respectively, while infected samples were more than four-times the value of negative controls with values that ranged between 0.289 ± 0.005 and 0.325 ± 0.003. Biological and serological identification of the virus suggests the association of CMV with the disease.

To characterise the virus at the molecular level, total RNA was isolated using RNAqueous Kit (Ambion, USA) from naturally infected and apparently healthy *S. splendens* leaf tissue from plants in the same location. Reverse transcription-polymerase chain reaction (RT–PCR) was performed using total RNA as templates and CMV coat protein (CP) gene specific reverse and forward primers (Srivastava *et al*. 2004). First strand cDNA synthesis was performed using total RNA, 50 pM reverse primers and 200 U *Moloney Murine Leukemia Virus* reverse transcriptase enzyme in a 20 µL reaction mixture, incubated at 42°C for 90 min. PCR was carried out in a 50 µL reaction using 5 µL cDNA as a template, 3U *Taq* DNA polymerase, 25 pM forward and reverse primers each, 10 mM dNTPs of each in a GeneAmp PCR 9700 system thermal cycler (Applied Biosystem, USA). The PCR conditions were as follows: initial denaturation

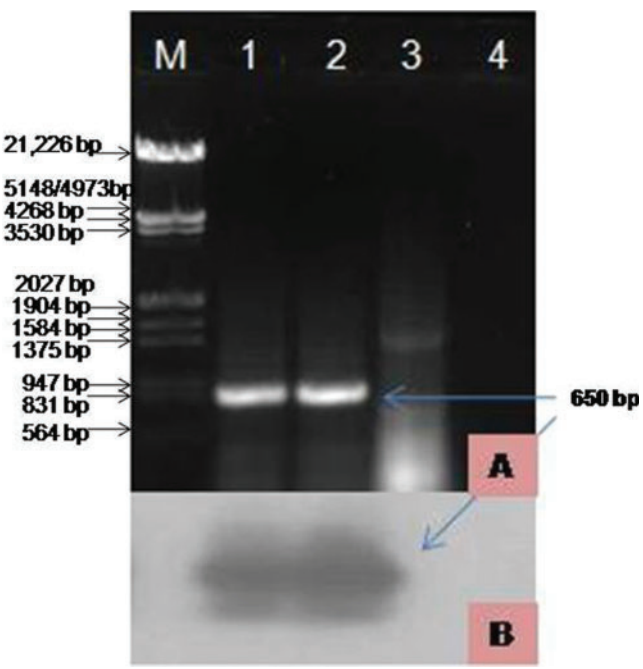

Fig. 1. (*A*) RT-PCR amplification in CMV-infected *S. splendens* with CMV-CP specific primers. Lane M: Lambda DNA digested with *Eco*RI and *Hind*III as DNA marker; lane 1: CMV positive control; lanes 2, 3: *S. splendens* infected and apparently healthy plant samples, respectively; and lane 4: RT-PCR without template. (*B*) Southern hybridisation of same gel using a CMV-CP specific probe.

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Table 1. Percentage nucleotide (nt) and amino acid (aa) identity of the CMV *Salvia* virus isolate under study (accession EU600215) with the selected CMV strains of Subgroup IA, IB and II along with other members of genus *Cucumovirus*

CMV, *Cucumber mosaic virus*; TAV, *Tomato aspermy virus*; PSV, *Peanut stunt virus*; IA, Subgroup IA; IB, Subgroup IB; II, Subgroup II; OG, out group; –, sequence/ information not provided

Accession	Strain/Abbreviation	Natural host	Subgroup	Location	% identity obtained by multiple alignment	
					nt	aa
EF178298	CMV-Ban	*Musa paradisiaca*	IB	India	97	96
DQ910858	CMV-Ban-L	*Musa paradisiaca*	IB	India	97	96
EU310928	CMV-Cath	*Catharanthus roseus*	IB	India	95	95
EF608461	CMV-Pep	*Capsicum annuum*	IB	Thailand	95	96
AY560556	CMV-Pep-T	*Capsicum annuum*	IB	Thailand	95	96
AM158321	CMV-Ban-M	*Musa paradisiaca*	IB	India	95	96
EU429567	CMV-BT	–	IB	China	93	94
DQ640743	CMV-Ban	*Musa paradisiaca*	IB	India	94	95
AY560555	CMV-Pep-T	*Capsicum annuum*	IB	Thailand	94	94
D10538	CMV- Fny	–	IA	USA (NY)	89	93
D12499	CMV-Y	–	IA	Japan	88	92
M21464	CMV-Q	–	II	Australia	70	80
L15336	CMV-Trk7	–	II	Hungary	67	80
EF153735	TAV	*Chrysanthemum morifolium*	OG	India	39	40
NC_002040	ER-PSV	*Vigna unguiculata*	OG	China	39	44

for 3 min at 94°C followed by 30 cycles of 94°C for 15 s, 54°C for 30 s and 72°C for 30 s with a final extension at 72°C for five minutes. RT–PCR resulted in the amplification of ~650 bp expected size band from infected *S. splendens*, but not from asymptomatic, when assessed by gel electrophoresis (Fig. 1*A*). To further confirm the identity of the PCR amplicons, southern hybridisation was performed using α^{32}P labelled DNA probe prepared from a CMV CP clone (accession EU140547) (Fig. 1*B*). The strong positive signals were obtained from infected samples, however, no such hybridisation signals were found from healthy samples. The amplified product was gel purified (Au-Prep Sigma gel extraction kit) and cloned in pGEM-T easy vector system (Promega, USA). The three positive clones were sequenced (Genei Pvt. Ltd, Bangalore, India) and the data were evaluated for a consensus sequence and submitted to the GenBank database (Accession EU600215).

Basic Local Alignment Search Tool (BLAST) analysis of the CP gene of CMV *S. splendens* isolate revealed the maximum (97%) nucleotide identities with CMV isolates of subgroup IB (accessions EF178298 and DQ910858), compared with medium identities (89–88%) with isolates of CMV subgroup IA (accessions D10538 and D12499) or minimal identities (70–67%) with subgroup II (accessions M21464 and L15336) (Table 1) CMV sequences. Multiple nucleotide and deduced amino acid alignments with the available sequence were carried out using CLUSTAL-W program v1.82 (Thompson *et al*. 1994) and the aligned files were bootstrapped 100 times generating a neighbour-joining phylogenetic tree using Tree-Explorer. The CMV isolate under study also showed close phylogenetic relationships with Indian strains of CMV of subgroup IB, whereas more distant relationships were observed for subgroup IA and II, when analysed by MEGA 4 (Tamura *et al*. 2007). These cumulative studies at the biological, serological and molecular level confirm the Salvia isolate of CMV to be member of subgroup IB (Fig. 2).

CMV was first recorded on *S. splendens* from Venezuela (Debrot *et al*. 1974). Afterwards, CMV was also detected in China on the same species by Jian *et al*. (2000). To the best of our knowledge, this is the first record of CMV on *S. splendens* in India.

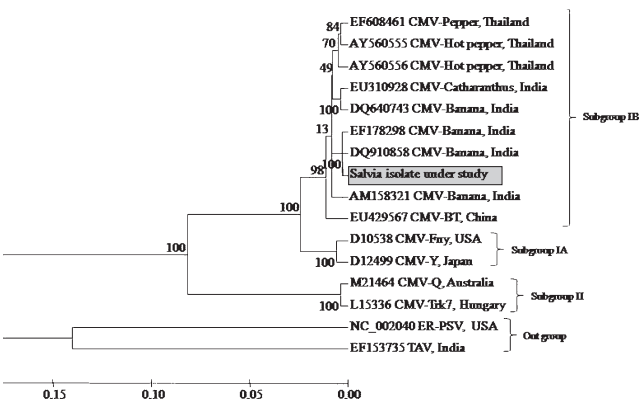

Fig. 2. Phylogenetic analysis (by MEGA 4 tool) at the amino acid level of the salvia virus isolate with the established *Cucumber mosaic virus* strains of subgroup 1A, IB and II of *Cucumoviruses*. The evolutionary history was inferred using the Neighbour-joining methods. The percentage of replicate trees in which the associated isolates clustered together in the bootstrap test (100 replicates) is shown next to the branches and distances were computed using Poisson Correction methods. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). *Tomato aspermy virus* (TAV) and *Peanut stunt virus* (PSV) are used as out group sequences for reference.

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