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RT-PCR and CP gene based molecular characterization of a cucumber mosaic cucumovirus from Aligarh, U.P., India

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ABSTRACT

A virus disease of garden sage (*Salvia splendens* Ker-Gawl.) was observed and characterized showing symptoms of severe mosaic, mottling and distortion of leaves being remain shortened and growth retarded. The virus was transmitted to the healthy plants of *Salvia* spp. as well as many other hosts by mechanical inoculation, *Myzus persicae* Sulzer and *Aphis gossypii* Glover transmit the virus in non-persistent manner. Purified sample in EM showed spherical particles c.28 nm in diameter. DAC- ELISA [1] was performed with crude sap, 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 recorded four-times more than the value of negative controls with values that ranged between 0.289 ± 0.005 and 0.325 ± 0.003. RT-PCR was performed using total RNA as templates and CMV Coat Protein (CP) gene specific reverse and forward primers, gel was electrophoresed on 1% agarose, an amplification of expected size 650 bp fragment was obtained only in the infected sample which proved that the present virus is a strain of CMV, the type member of the genus cucumovirus belonging to the family Bromoviridae.

Keywords: Salvia; Mosaic; Non-persistent; DAC-ELISA; RT-PCR; Cucumovirus

1. INTRODUCTION

Salvia is a large genus of aromatic and ornamental herbs and shrubs distributed in temperate and tropical regions. About 24 species are known to occur in India. Among them *Salvia splendens* Ker-Gawl; *S. officinalis* L., *S. farinacea* Benth. and *S. patens* are widely grown in Indian gardens. The whole plant or different parts of the plant are used in medicine. The oil extracted from *Salvia* spp. *i.e*. sage oil is used as antibacterial, antiviral, antispasmodic and also violent epileptiform convulsant.

During the survey of viral disease on ornamental plants in literature it was found that the virus reported on *Salvia sclarea* was CMV [2] from Italy. Symptoms were also observed during disease evaluation on *Salvia uliginosa i.e*. Bog Salvia [3]. An isolate of Potato virus-Y tuber necrotic strain was also reported on *Salvia sclarea* [4] and *Datura Stramonium* Linn. as a natural host.

Cucumber Mosaic Virus (CMV) of family Bromoviridae is one of the most important widespread viruses in the world infecting the largest number (approximately 1000) of plant species [5]. The genome of the virus consists of positive-sense single stranded three RNAs (RNA 1, RNA 2 and RNA 3) and a sub-genomic RNA (RNA 4) which is encoded by the 3'-half of RNA 3 [6] and which is involved in encapsidation [7].

A number of CMV strains reported from all over the world have been placed in two subgroups I and II on the basis of serology [8-10], nucleic acid hybridization [11], gene sequences [12,13] and RFLP [14,15]. CMV subgroup I has recently been subdivided into IA and IB on the basis of gene sequence available for CMV strains and phylogenetic analysis [16-18]. Further asian strains of CMV have been placed in subgroup IB [16].

2. MATERIALS AND METHODS

Transmission Studies

Mechanical transmission: Naturally infected leaves were used for preparing inoculum with 0.1 M phosphate buffer pH 7.0. Pure culture was maintained by obtaining

single lesion from *Chenopodium amaranticolor* Coste & Reyn and inoculating it on to *Nicotiana glutinosa* L. The virus was further multiplied on the hosts such as *N. tabacum* cv. Xanthi and *N. tabacum* cv. Harrison spl. For hosts range studies different hosts belonging to different families were grown in an insect proof glass house and screened against the virus obtained from pure culture.

Transmission by aphids: Healthy colony of aphids namely *Myzus persicae* Sulzer and *A. gossypii* Glover were raised in cages and the healthy nymphs of these aphids were used for transmission. The aphids were given a pre-acquisition starvation of 2 h, acquisition access 2 minutes, inoculation access 2 h using at least 10 aphids/plant and 10 recipient plants for each aphid species.

Purification: The present virus was purified [19] with slight alteration in pH of buffer and molarity. About 200 grams of infected leaves of *N. tabacum* cv. Samsun inoculated 10 days earlier were harvested and homogenized in 400 ml of phosphate buffer pH 7.0 of the same molarity containing 0.1% of thioglycolic acid and 0.1% sodium sulphite. Clarification of the extract and virus precipitation with 6% PEG was done [20]. The purified virus obtained from method given above was diluted with the phosphate buffer having the same molarity. After making dilution UV spectra of the virus characteristic of nucleoprotein was obtained with DU-6 spectrophotometer.

Electron microscopy: The electron microscopic studies were carried out by using clarified virus concentrate prepared [21]. Antisera of different plant viruses viz. Potato Leaf Roll Virus (PLRV), Cowpea mosaic virus (CPMV), Soybean stunt strain (CMV-SS), Pea strain (CMV-P) and MMV strain were used in IEM studies. All the antisera were diluted 200 folds with 0.88% NaCl solution before use. Trapping, decoration and mounting of trapped virions was done according to Garg and Khurana [22,23].

Serology: The identification of the CMV subgroup specific antibodies along with alkaline phosphatase-linked antibodies was used for ELISA [1].

Total RNA extraction and RT-PCR: The total RNA isolated from infected leaves of *Salvia splendens* and *Nicotina glutinosa* by using RNAqueous (Ambion, USA). Viral RNA was isolated by disrupting the particles by 1% SDS followed by extraction with Phenol-chloroform method [24]. Reverse Transcription Polymerase Chain Reaction (RT-PCR) was performed to amplify the CP gene of present isolate with some flanking regions using total RNA (approximately 5 µg) and viral RNA (approximately 2 - 3 µg) both as template and CP gene specific forward and reverse primers [25]. First strand cDNA synthesis was performed using total RNA, 50 pM reverse primers and 200 µ Moloney Murine Leukemia virus reverse transcriptase enzyme in 20 µL reaction mixture, incubated at 42˚C for 90 minutes. PCR was carried out in 50 µL reaction using 5 µL cDNA as a template, 3 µL *Taq* DNA polymerase, 25 pM forward and reverse primers each, 10mM dNTPs of each in GeneAmp PCR 9700 system thermal cycler (Applied Biosystem, USA). The procedure is as follows: initial denaturation at 94˚C for 3 minutes 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 5 minutes.

3. RESULTS AND DISCUSSION

Transmission Studies

Mechanical inoculation made it possible to infect several hosts including *Amaranthus gracilis*, *A. retroflexus*, *Antirrhinum majus*, *Apium graveolens*, *Arachis hypogea*, *Beta vulgaris*, *Brassica campestris*, *B.juncea*, *Capsicum annuum*, *Cucumis sativus*, *C. melo*, *Cucurbita pepo*, *Citrullus vulgaris*, *Physalis peruviana*, *Datura metel*, *Daucus carota*, *Dianthus barbatus*, *Gomphrena globosa*, *Lycopersicon esculentum*, *Pisum sativum*, *Raphamus sativus*, *Solanum melongena*, *S. nigrum* and *Spinacea oleracea* showed symptoms or were latently infected (**Table 1**).

In aphid transmission tests *Myzus persicae* and *Aphis gossypii* transmitted the virus to 8/10, 5/10 plants respectively. The symptoms produced were identical to those induced by sap inoculation but *Bemisia tabaci* was failed to transmit the disease.

Mechanical inoculation from naturally infected *Salvia* plants transmitted the virus to *C. amranticolor* and *C. album* produced chlorotic local lesions (**Figure 1**) followed by systemic infection (**Figure 2**) while on *C. quinova* and *C. murale* the virus produced necrotic lesions (**Figure 3**). Symptoms on other species were systemic, mild to severe mosaic, and leaf deformation except *C. pepo* which produced only mild yellowing on the emerging leaves (**Figure 4**).

N. glutinosa and *C.amaranticolor* produced systemic symptoms of mosaic mottling and downward curling (**Figures 5(a)** and **(b)**) while *Brassica oleracea* showed upward curling of the leaves (**Figure 5(c)**). Systemic symptoms appeared on *N. rustica* in the form of green mosaic and at advance stage of infection plants showed deformation of leaves, reduced leaf lamina and growth retardation. Inoculated C*apsicum annuum* leaves showed necrotic lesions after 3 - 5 days of inoculation (**Figure 6**) *Cucumis melo* and *Citrullus vulgaris* produced discrete local lesions when the virus is inoculated on cotyledons which on later stage produced mosaic on young emerging leaves (**Figure 7**)

The following species which failed to develop symptoms after inoculation and virus was not recovered on

Table 1. Host range of different virus isolates.

Family/Hosts	MM1	MM2	MM3	MM4	MM5
Amaranthaceae	LL	LL	LL	-	-
Amarnthus gracilis Desf.	LL	LL	-	-	-
A. retroflexus L.	LL, M	LL	-	-	-
Chenopodiaceae					
C. amaranticolor Coste & Reyn.	LL	LL	LL	LL	LL
C. murale L.	LL	DLL	DLL	-	-
C. quinova Wild.	LL	LL	LL	LL	LL
C. album L.	LL	LL	-	-	-
Spinacea oleracea L.	MM	LL	-	LL	-
Beta vulgaris L.	BL,M	BL,M	-	-	-
Asteraceae					
Dianthus barbatus L.	MM	MM	MM	-	-
Cucurbitaceae					
Cucumis sativus L.	LL	LL	LL	LL	-
C. melo L.	LL	CS	CS	-	-
Cucurbita pepo L.	CS	CS	CS	-	-
Citrullus vulgaris Schrad.	LL,M	LL,M	VC	VC	-
Leguminoceae					
Vigna radiate (Linn.) Wilczec.	LL	LL,M	LL,M	-	-
Arachis hypogea L.	LC	LC,M	LC,M	-	-
Pisum sativum L.	LL,M	VC	VC	-	-
Solanceae					
Datura metel L.	CLL,M	-	LD	LD	-
D. stramonium L.	CLL,MM	LD	ST	ST	ST
N. glutinosa L.	LL,MM	CL,M	CL	-	-
N. tabacum cv. Xanthi	LL,M,VC	LL,VC	-	-	-
N. tabacum cv. Harrison spl.	LL,M	LL,M,LD	LL,M,LD	-	-
Physalis peruviana L.	VC,LD	VC,LD	-	-	-
Solanum nigrum L.	MO,LP	Mo,BL	-	-	-
S. melongena L.	VC,MM	Mo,M	MOM	-	-
Capsicum annuum L.	CLL,M	CLL,M	-	-	-
Lycopersicon esculentum L.	M,Mo	M,LD	M,LD	-	-
Brassicaceae					
Raphanus sativus L.	VC, VB	VC,LP	Mo	-	-
Brassica campestris L.	VC,M	M,Mo	-	-	-

Abbreviations: LL = Local Lesion, CL = Leaf Curling, CLL = Chlorotic Local Lesion, M = Mosaic, Mo = Mottling, MM = Mild Mosaic, VC = Vein Clearing, VB = Vein Banding, LD = Leaf Deformation, BL = Blister Like, LP = Line Pattern, CS = Chlorotic spots, DLL = Discrete Local Lesions.

Figure 1. Chlorotic local lesions.

Figure 2. Coalesces on *C. amranticolor.*

Figure 3. Necrotic lesions on *C. quinova.*

Figure 4. Mild yellowing on emerging leaf.

(a)

(b)

(c)

Figure 5. (a) Mottling on *N. glutinosa*; (b) Down curling of *Chenopodium* leaf; (c) Upward curling of *B. oleracea.*

Figure 6. Necrotic lesions on on *C. annuum.*

Figure 7. Mosaic on *C. pepo* leaf.

back inoculation include *Ageratum maxicanum*, *Chrysanthemum indicum*, *Cosmos bipinnata*, *Dahlia pinnata*, *Benincasa cerifera*, *Curcurbita maxima*, *Lagenaria siceraria*, *Trichosanthus anguina*, *Alium cepa*, *Althea rosea*, *Lathyrus odoratus*, *Dolicos lablab*, *Phlox* and *Carolina*.

Particle Morphology: The Purified preparation of the virus obtained from the infected leaves of *Nicotiana glutinosa* L. showed isometric virus particles of size c. 28 nm that is typical of CMV (**Figure 8**). Trapping, decoration and counting of the trapped virions was done according to Garg and Khurana [22] (**Figure 9)**.

ELISA: A high contrast of colour (yellowish green) showing decrease in intensity with increase in dilutions developed by the CMV infected plants. The mean absorbance values at 405 nm.

PCR: The result revealed the amplification of ~650 bp size band from the Salvia isolate, but not from asymptomatic when subjected to electrophoresis. The strong evidence was obtained when the infected samples was subjected to southern hybridization using α^{32}P labeled DNA probe prepared from a CMV CP clone (EU140547). However, no such hybridization signal were found with healthy samples. The amplified product was gel purified (Au-Prep Sigma gel extraction kit) and cloned in pGEM-T easy vector system (Promega, USA). Three positive clones were sequenced and the data were evaluated for a consensus sequence and submitted to GeneBank database (Accession number EU 600215).

Basic Local Alignment Tool (BLAST) analysis of the CP gene of the *Salvia* isolate revealed the maximum (97%) nucleotide (nt) identity with CMV isolate of sub group IB (EF 178298 and DQ 910858), compared with medium identity (89% - 88%) with isolate of CMV sub group IA (D 01538 and D 12499) and minimum identity (70% - 67%) with sub group II (M 21464 and L 15336) CMV sequences (**Table 2**). Multiple nucleotide and de-

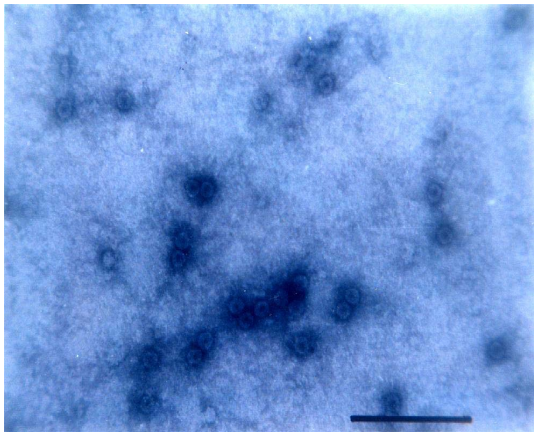

Figure 8. Isometric particle size of c. 28 nm.

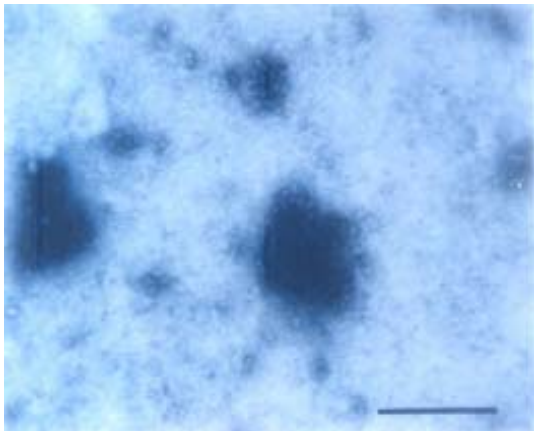

Figure 9. Trapped isometric particle of CMV.

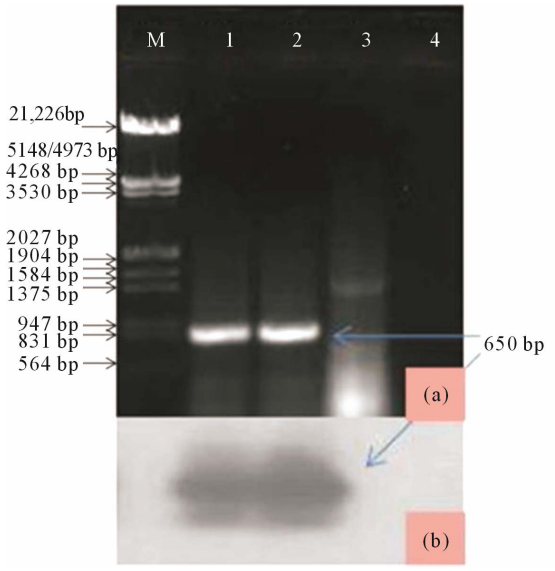

Figure 10. (a) Lane M: Lamda DNA digested with *Eco*RI and *Hind*III as DNA marker; Lane 1: CMV positive control; Lanes 2 and 3: Salvia infected and apparently healthy samples; Lane 4: RT-PCR without template (b) Southern hybridization of the same gel using specific probe.

duced amino acid (aa) alignment with the available sequence were performed using CLUSTAL-W program v1.82 [25] and aligned files were bootstrapped 100 times generating a neighbour-joining a phylogenetic tree using tree explorer. The CMV isolate also showed close phylogenetic relation ship with an Indian strain of CMV of sub group IB, where as more distant relation ships were observed for subgroup I and II, when analysed by Mega 4 [26]. These biological, serological and molecular studies confirmed the isolate under study is CMV member of sub group IB.

4. DISCUSSION

Based on DAC-ELISA, RT-PCR and phylogenetic analysis of CP gene results indicate that the virus concerned was an isolate of CMV that was serologically related to CMV-K8 and CMV- SS but not with the other antisera. Two common species e.g. *M. persicae* and *A. gossypii* are able to transmit the disease in non-persistent manner. Although CMV previously has been reported to infect the genus Salvia naturally, however, such reports are rare. Therefore, the use of host plants and symptomatology to define strain is not practical. The symptom expression can vary according to temperature, age of indicator plants and source of inoculums. CMV concentration can vary between plants and between leaves of the same plants [28] and a single amino acid in coat protein can also alter the development and diversity of symptoms [29,30]. Several lines of evidence show that the virus concentration in *C. murale*, *D. metel* and also in experimentally infected *C. quinova* is low. The virus detected in the crude sap of these plants by biophysical means, serology and by external morphology.

The virus produced local lesions on *C. amaranticolor* and *C. quinova*, Earlier workers [2,27] also reported that their isolate from Salvia is a strain of CMV. However, the Aligarh isolate is different from the isolate reported by these earlier workers in their biophysical properties. The isolate of CMV reported by [3] from *A. majus*, *S. splendens* and *S. uliginosa* was compared with CMV of Aligarh isolate from *S. officinalis* but found to be different from CMV of above workers in their host reactions and also some variations in their bio-physical properties. However, the serological studies based on immuno-electron microscopy further revealed that the virus isolated

Table 2. Percentage nucleotide (nt) and amino acid (aa) identity of CMV *Salvia* isolate (accession No. EU 600215) with the selected CMV strains of subgroup IA, IB and II with other member of cucumovirus.

Accession	Strain/Abbreviations	Natural host	Subgroup	Location	% identity obtained by multiple alignment	
					nt	Aa
EF178298	CMV-Ban	*Musa paradisiaca* L.	IB	India	97	96
DQ510858	CMV-Ban-L	*Musa paradisiaca* L.	IB	India	97	96
EU310928	CMV-Cath	*Catharanthus roseus* G. Don.	IB	India	95	95
EF608461	CMV-Pep	*Capsicum annuum* L.	IB	Thailand	95	96
AY560556	CMV-Pep-T	*Capsicum annuum* L.	IB	Thailand	95	96
AM158321	CMV-Ban-M	*Musa paradisiaca* L.	IB	India	95	96
EU429567	CMV-BT	-	IB	China	93	94
DQ640743	CMV-Ban	*Musa paradisiaca* L.	IB	India	94	95
AY560555	CMV-Pep-T	*Capsicum annuum* L.	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-Trk-7	-	II	Hungary	67	80
EF153735	TAV	*Chrysanthemum morifolium* L.	OG	India	39	40
NC_002040	ER-PSV	*Vigna unguiculata* (Linn.) Walp.	OG	China	39	44

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.

from *Salvia* at Aligarh has sero-affinity with CMV-SS and CMV-K8 but not with CMV-P and CMV-M, which shows that the isolate from *Salvia* is a strain of Cucumber mosaic virus of cucumo virus group.

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