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Natural infection of periwinkle (*Catharanthus roseus*) with *Cucumber mosaic virus*, subgroup IB

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Abstract. This paper reports the characterisation of a naturally occurring *Cucumber mosaic virus* (CMV) infection, causing mosaic, leaf distortion and stunting of periwinkle (*Catharanthus roseus*). The virus isolate showed wide host range, biophysical properties similar to CMV, isometric particles of 28 nm, capsid protein of 26 kDa, serological reaction with CMV-S and non-persistent transmission by aphids. The CP gene of the virus was amplified using reverse transcriptase–polymerase chain reaction (RT-PCR) with CP-specific primers, cloned and sequenced (657 bp). Sequence analysis of the PCR product with other CMV isolates revealed the closest identity with *Rauvolfia serpentina* isolate of CMV (98%) and the phylogram revealed that CMV naturally infecting periwinkle belongs to subgroup IB.

Catharanthus roseus, commonly known as periwinkle (Fig. 1), is a tropical perennial herb of the family Apocyanaceae. It is cosmopolitan in distribution and cultivated in Madagascar, India, Israel and USA (Stearn 1975). The species contains a large variety of alkaloids (Svoboda and Blake 1975) and some of them are of importance mainly for their anti-cancer (vincristine and vinblastine) medicinal values.

Cucumber mosaic virus (CMV, genus: *Cucumovirus*, family: Bromoviridae) is one of the most widespread plant viruses in the world with an extensive host range infecting ~1000 species including cereals, fruits, vegetables, ornamentals, medicinal and aromatic plants (Roossinck 1999). More than 75 species of aphids can transmit CMV in a non-persistent manner (Palukaitis *et al*. 1992). CMV was first reported in *Cucumis sativus* from the USA (Prince 1934). In India, the occurrence of CMV has been reported from many hosts such as Egyptian henbane (Samad *et al*. 2000), gladiolus (Raj *et al*. 2002), *Lycopersicum esculentum* (Sudhakar *et al*. 2006), geranium (Verma *et al*. 2006), banana (Aglave *et al*. 2007), *Rauvolfia serpentina* and *Jatropha curcas* (Raj *et al*. 2007, 2008). However, only limited reports are available on biological and molecular characterisation of these isolates and their exact identification remained unaddressed. In this article, we report a natural occurrence of CMV on *C. roseus* and its biological and molecular properties.

Fig. 1. Healthy plants of periwinkle (*C. roseus*).

In early summer 2003 (March–May), several plants of *C. roseus* exhibited bright chlorotic spots on the upper leaves, gradually developing to a green mosaic with leathery, irregular and deformed leaves and an overall stunted growth in comparison to healthy plants (Fig. 2). Flowers exhibited symptoms of faded petals and were malformed. Incidence in commercial fields was from 70 to 80%, with highest incidence in poorly drained areas of the field. Flower production in commercial fields was reduced by ~30%. In advanced stages of the disease, premature death and drying of infected plants was observed.

Several diseases caused by bacteria, fungi, nematodes, phytoplasmas and viruses are reported on periwinkle from different parts of the world. A review of literature revealed that periwinkle has been reported as a susceptible host of many distinct viruses such as *Pepper ringspot virus*, *Narcissus mosaic virus*, *Poplar mosaic virus*, *Cacao yellow mosaic virus*, *Tobacco streak virus*, *Alfalfa mosaic virus*, *Tobacco ringspot virus* (Plant viruses online, http://image.fs.uidaho.edu/vide/famly011.htm#Catharanthus%20roseus, verified 27 March 2008) *Zantedeschia mild mosaic virus* (ZaMMV, Huang and Chang 2005), *Carnation mottle virus* (Singh *et al*. 2005), *Potato yellow vein virus* (PYVV, Salazar *et al*. 2000), *Tomato spotted wilt virus* (TSWV, Chatzivassiliou *et al*. 2000). Recently *R. serpentina*, another member of the Apocyanaceae is recorded as a natural host of CMV (Raj *et al*. 2007). However,

 10.1071/DN08013 1833-928X/08/010030

Fig. 2. Naturally infected *C. roseus* twig showing mosaic, leaf and flower deformation.

natural occurrence of CMV on *C. roseus* has not been previously recorded.

Infected plant tissues were homogenised in 0.1 M phosphate buffer (pH 7.2) containing 0.02% thioglycollic acid and sodium sulfite, squeezed through muslin cloth and mechanically applied onto leaves using carborundum (600 mesh) as an abrasive. Seedlings treated with buffer only served as negative controls for inoculation. Both inoculated and control plants were kept under observation in an insect-proof glasshouse (Noordam 1973). The virus isolate was efficiently sap transmitted from naturally infected periwinkle to healthy periwinkle and a variety of plant species mainly from Chenopodiaceae, Cucurbitaceae and Solanaceae. Our studies used a single lesion from periwinkle obtained by passage through *C. amaranticolor* and further propagated in *Nicotiana benthamiana.* Periwinkle plants inoculated mechanically reacted with symptoms similar to those observed on naturally infected plants. The mechanically inoculated plant species that showed local and/or systemic symptoms included; *Gomphrena globosa*, *N. benthamiana*, *N. glutinosa*, *N. occidentalis*, *N. rustica*, *N. tabacum* cv.DR-1 (Harrison Special), *N. tabacum* Samsun NN, *N. tabacum* White Burley, *N. xanthi*, *Cucumis melo*, *C. sativus*, *Pisum sativum*, *Capsicum annuum*, *Solanum lycopersicum*, *Lycopersicum esculentum*, *Petunia hybrida*, *Chenopodium album*, *C. amaranticolor*, *C. quinoa*, *Cucurbita pepo*, *Datura stramonium*, *D. inoxia*, *Glycine max*, *Solanum melongena*, *Vigna mungo* and *Solanum nigrum*. However, the following plants failed to develop either local or systemic symptoms; *Arachis hypogaea*, *Brassica campestris*, *Dahlia hybrida*, *Chrysanthemum cinerariifolium*, *Dianthus barbatus*, *Helianthus annuus*, *Luffa cylindrical*, *Mirabilis jalapa*, *Tagetes erecta*, *Zinnia elegans*, *Mentha arvensis*, *M. spicata*, *M. citrata*, *Withania somnifera* and *Physalis floridana.* Selected hosts of the periwinkle CMV isolate are compared with the host range studies of others CMV isolates (Table 1) from vanilla (Madhubala *et al*. 2005), Egyptian and black henbane (Zaim and Khan1988; Samad *et al*. 2000), *R. serpentina* (Zaim and Khan 1988), banana (Aglave *et al*. 2007) and lily (Terami *et al*. 2004).

Groups of five aphids (*Myzus persicae*) were transferred to glass tubes and starved for 2 h. They were allowed to feed for 15 min on infected periwinkle or *N. benthamiana* leaves and transferred to a healthy periwinkle or *N. benthamiana* plant for a 24 h inoculation/feeding period. *Myzus persicae* transmitted the virus in a non-persistent manner to healthy periwinkle (5/10 plants) and *N. benthamiana* (7/10 plants). Symptoms developed after 15–20 days of feeding. For soil transmission, periwinkle plants bearing viral symptoms along with soil collected from infested fields were planted individually in pots. Two weeks later, three healthy periwinkle seedlings at the five to six leaf stage were planted next to the symptom bearing diseased plants. Control arrangements were set up using apparently healthy periwinkle in autoclaved soil. Plants were grown in a glasshouse and observed daily for symptom development over 6 weeks. Neither disease symptoms nor virus particles were detected in the seedlings transplanted adjacent to infected plants.

Serial 10-fold dilutions of crude sap in 0.1 M phosphate buffer (pH 7.0) were prepared from infected *N. benthamiana* for determination of bio-physical studies, dilution end point (DEP), thermal inactivation point (TIP) and longevity *in vitro* (LIV) of the virus (Noordam 1973). Infectivity was assayed on *C. amaranticolor.* Virus retained infectivity in crude sap up to a

Table 1. Comparison of selected hosts with different isolates of CMV

B, blister; CLL, chlorotic local lesion; LD, leaf distortion; LI, latent infection; LS, leaf shedding; M, mosaic; NLL, necrotic local lesion; NS, no symptoms; SN, systemic necrosis; SS, systemic symptoms; StS, streaks on stem; VC, vein clearing; –, not tested

Host tested	Isolates of CMV					
	Periwinkle	Vanilla	Henbane	Rauvolfia	Banana	Lily
C amaranticolor	CLL	CLL, NLL	NLL	NLL	CLL	CLL, NLL
C. annuum	M	M, LC	NS	–	M	–
C. sativus	M	VC	M	SS	CLL, M, VC	M
D. stramonium	M, B	–	CLL, M LS, LD	CLL	–	NS
G. globosa	NLL, LD	–	–	NLL	–	SN
G. max	M, VC	NS	–	–	CLL, M	LI
L. esculentum	M, LD	StS, LD	M, LD	SS	M, LD	M
N. glutinosa	M	M, B	NLL, M	–	M, LD	M
Z. elegans	NS	–	–	SS	–	M

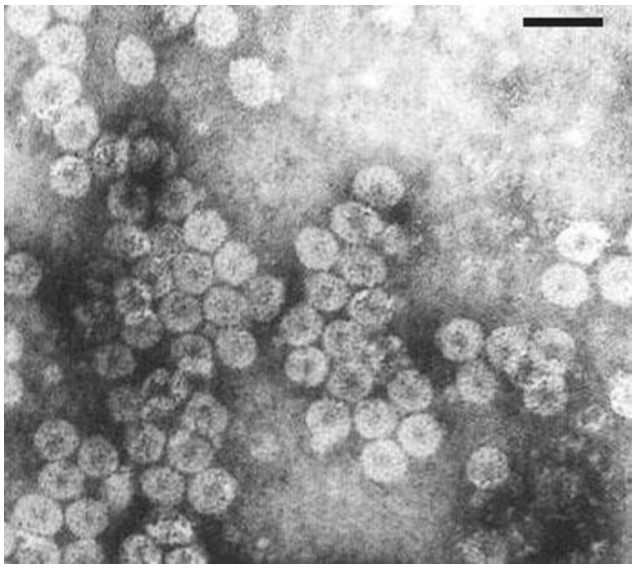

Fig. 3. Electron micrograph of a purified virus preparation stained in 2% uranyl acetate showing CMV particles (bar = 55 nm).

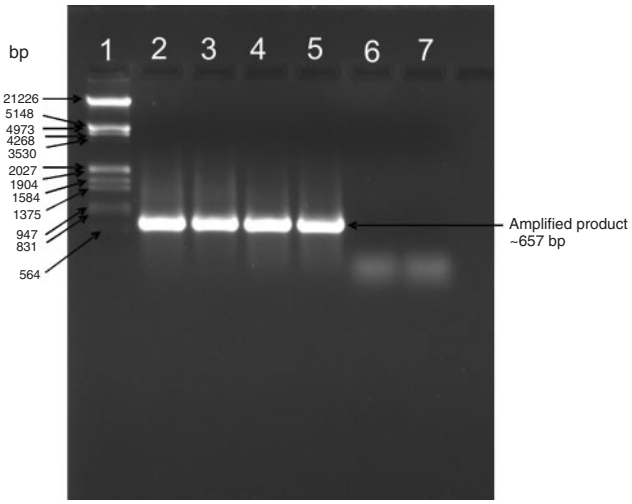

Fig. 4. RT-PCR amplification of CMV coat protein gene (~657 bp). Lane 1, λ DNA/*Hind*III/*Eco* R1 marker; lanes 2–5, amplified DNA fragment from infected samples; lanes 6–7, healthy samples.

Table 2. Comparison of coat protein gene sequence identity

The sequence of the India C18 isolate (EU310928) is compared at the nucleotide (nt) and amino acid (aa) level using the BLAST and DiAlign tools with isolates of *Cucumber mosaic virus* (CMV) reported from India and elsewhere

Accession	Strain	Natural host	Location	Subgroup	%Identity	
					nt	aa
EU310928	CMV-C18	*Catharanthus roseus*	Lucknow, India	Ib	100	100
X89652	CMV-PM	*Physalis minima*	Lucknow, India	Ib	98	98
EF593024	CMV-DI	*Datura innoxia*	Lucknow, India	Ib	98	96
AF281864	CMV-DI	*Datura innoxia*	Lucknow, India	Ib	98	96
EF593023	CMV-AT	*Amaranthus tricolour*	Lucknow, India	Ib	98	96
AF198622	CMV-AS	*Amaranthus* sp.	Lucknow, India	Ib	98	96
EF153733	CMV-CM	*Chrysanthemum mor.*	Lucknow, India	Ib	97	95
EF593026	CMV-JC	*Jatropha curcus*	Lucknow, India	Ib	98	98
EF153739	CMV-JC	*Jatropha curcus*	Lucknow, India	Ib	98	98
EF593025	CMV-RS	*Rauvolfia serpentina*	Lucknow, India	Ib	98	99
DQ914877	CMV-RS	*Rauvolfia serpentina*	Lucknow, India	Ib	98	99
AF350450	CMV-HM	*Hyoscyamus muticus*	Lucknow, India	Ib	98	98
AJ810260	CMV-Ch	*Capsicum annum*	India	Ib	96	97
AY754359	CMV-VP	*Vanilla planifolia*	Kerala, India	Ib	95	98
AY545924	CMV-PN	*Piper nigrum*	Kerala, India	Ib	95	99
AY861397	CMV-WP	*Vanilla tahitensis*	New Zealand	Ib	94	97
EF153737	CMV-LG	*Lemon grass*	Lucknow, India	Ib	95	96
AY125575	CMV-ban	*Musa paradisiacal*	Kerala, India	Ib	95	96
AY690620	CMV-PB	*Piper betle*	Kerala, India	Ib	95	97
AY690621	CMV-PL	*Piper longum*	Kerala, India	Ib	95	97
D43800	CMV-Pepo	–	Japan	Ia	90	94
L36251	CMV-Kor	–	Korea	Ia	89	91
AJ006988	CMV-Pl	–	China	Ia	91	95
U43888	CMV-MA	*Musa acuminata*	Israel	Ia	92	95
D10538	CMV-Fny	–	NY, USA	Ia	92	95
AJ585086	CMV-Li	*Lilium longifolium*	HP, India	II	75	79
D00463	CMV-Wl	–	USA	II	76	81
L15336	CMV-Trk7	–	Hungary	II	76	80
NC_002040	PSV	*Vigna unguiculata*	USA	Out-group	46	46

dilution of 10^{-3} TIP at 65°C and *LIV* at 4°C for 7 days. Fresh *N. benthamiana* leaves showing distinct symptoms were harvested after 2 weeks of inoculation and used as a source for virus purification as described previously (Samad *et al*. 2000). The purified virus suspension revealed a typical UV absorbance spectrum (Gibbs and Harrison 1976) of nucleoprotein showing a *A*260/*A*280 ratio of 1.7. Purified virus preparations stained with 2% uranyl acetate revealed the presence of typical isometric particles of ~28 nm diameter under Philips EM420 transmission electron microscope (Fig. 3). The periwinkle virus isolate reacted positively with antiserum of CMV (CMV; PVAS 242a, American Type Culture Collection, USA) in a gel double diffusion test (Raj *et al*. 2007). Electrophoretic analysis of SDS-dissociated virus revealed the presence of a single polypeptide with a relative molecular weight of ~26 kDa (Sambrook and Russell 2001). Leaf dip preparations from infected periwinkle and *N. benthamiana* plants contained spherical particles similar to those in purified preparation. Virion particles with similar profiles were frequently observed in ultrathin sections of infected tissues from leaves and petioles.

Total RNA was extracted using an RNeasy Mini kit (QIAGEN) following the manufacturer's instructions. The isolated RNA was used for cDNA synthesis using reverse transcriptase for polymerase chain reaction (PCR) amplification. The cDNA synthesis was carried out using Avian Myeloblastosis Virus Reverse Transcriptase

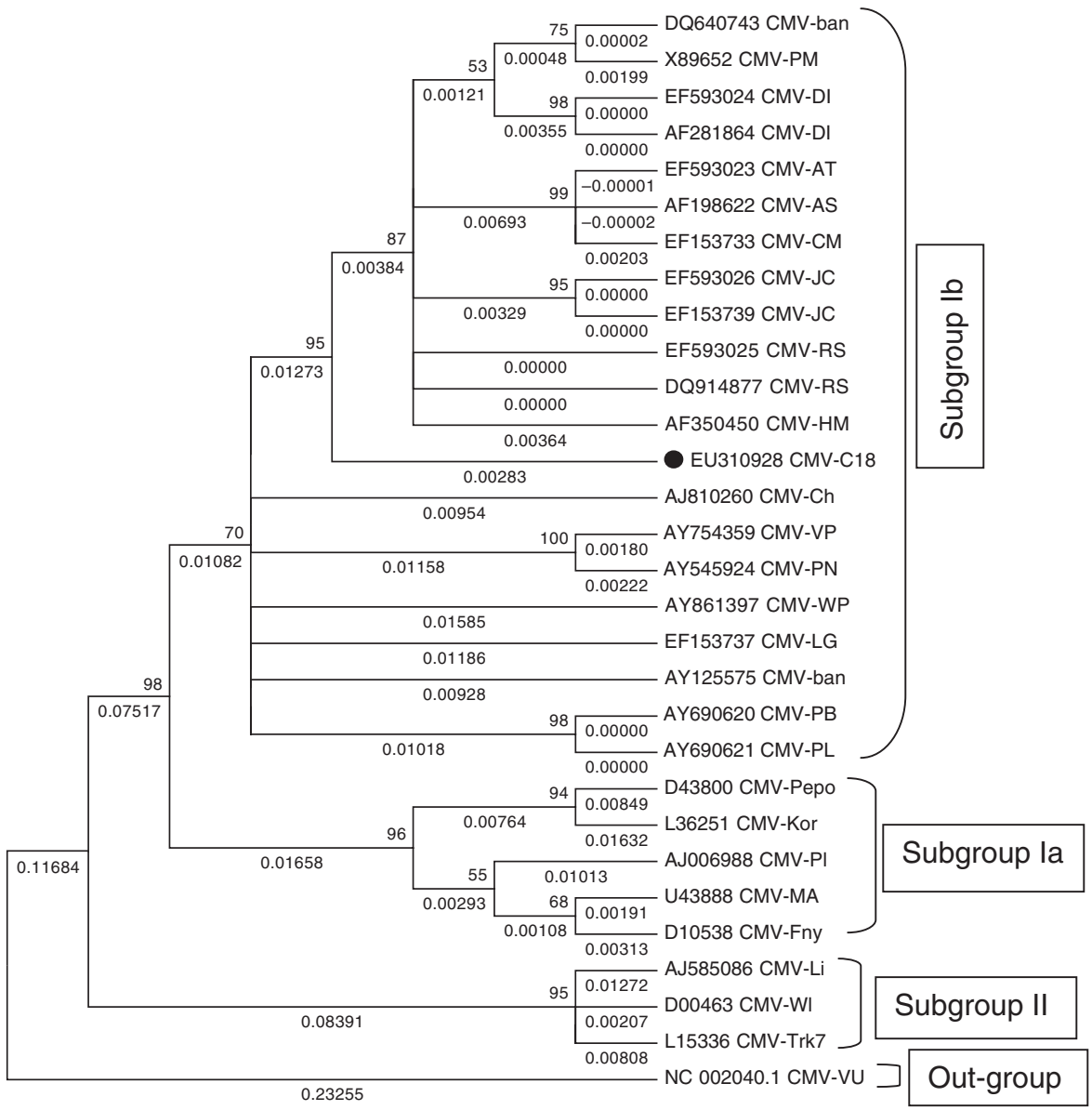

Fig. 5. Phylogenetic relationship of the virus isolated from the CIMAP-India C18 isolate (EU310928) with strains of *Cucumber mosaic virus* (CMV) subgroup I (Ia and Ib) and II based on the nucleotide sequence alignment using Mega4.0 neighbour-joining tree method. The tree was rooted on peanut stunt virus (PSV; Nc_002040) as an out-group. The virus from the present study is highlighted with a black dot.

AMVRTase (10U) at 42°C for 90 min, in a total volume of 20 µL containing RNase inhibitor (25U), 1 mM each dNTP and a downstream primer (5′GCAT GGTACCTCAAACTGGGAGC AC-3′). The PCR (25 µL) was performed using 2 µL of cDNA and primers specific to CMV coat protein (CP, Srivastava *et al*. 2004) (F: 5′-GCATTCTAGA TGGACAAATCTGAATC-3′ and R: 5′GCATGGTACCTCA AACTGGGAGCAC-3′) in an automated thermal cycler (Perkin Elmer-Gene Amp PCR system 2400). The PCR cycles were, one initial cycle of denaturation at 94°C for 5 min followed by denaturation at 94°C (1 min), annealing at 52°C (1 min) and extension at 72°C for 1.30 min. The final extension at 72°C was maintained for 5 min. The PCR amplification product was separated by electrophoresis in 1.2% agarose gel in 1X TAE and stained with ethidium bromide revealing an amplified DNA fragment of the expected size (~650 bp) from virus infected samples only but not from healthy samples (Fig. 4). DNA marker λ/*Eco*RI+*Hind*III double digests were used as size markers.

The amplified PCR product was cloned into pGEM-T Easy vector kit (Promega, USA), recombinant clones were identified by restriction digestion using the restriction endonuclease enzyme *Eco*R1 and selected clones were sequenced using XL 3130 Genetic Analyser (ABS, USA). The sequenced region was submitted to GenBank (Accession number EU310928) and contained a single open reading frame, which comprised 657 bases of nucleotides potentially coding for 218 amino acids. The new sequence was compared using bioinformatics tools DiAlign (Genomatrix) MEGA 4.0 with CP gene sequences of all available CMV isolates from India as well as representative isolates from other parts of the world belonging to both the subgroups (I and II) (Table 2). Nucleotide and deduced amino acid sequence of the CP gene of CMV infecting periwinkle showed closest identity (98%) with the subgroup IB CMV isolates from *Rauvolfia serpentina* (EF 593025) and with GenBank accession numbers. DQ640743 and EF593026 (Fig. 5). On the basis of these biological and molecular studies it is concluded that the virus naturally infecting periwinkle is a subgroup IB CMV. The high sequence identities observed between Indian isolates of CMV compared with isolates from other localities indicate that periwinkle isolate of CMV originated locally.

Acknowledgements

The authors are thankful to Dr S.P.S. Khanuja (Director, CIMAP) for encouragement and providing necessary facilities. Thanks also to Dr AK Gupta, National Gene Bank of Medicinal and Aromatic Plants, CIMAP, Lucknow for providing the germplasm of periwinkle (*C. roseus*) for this study.

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Manuscript received 30 January 2008, accepted 19 March 2008