Occurrence of *Cucumber mosaic virus* on vanilla (*Vanilla planifolia* Andrews) in India

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Cucumber mosaic virus (CMV) causing mosaic, leaf distortion and stunting of vanilla (*Vanilla planifolia* Andrews) in India was characterized on the basis of biological and coat protein (CP) nucleotide sequence properties. In mechanical inoculation tests, the virus was found to infect members of Chenopodiaceae, Cucurbitaceae, Fabaceae and Solanaceae. *Nicotiana benthamiana* was found to be a suitable host for the propagation of CMV. The virus was purified from inoculated *N. benthamiana* plants and negatively stained purified preparations contained isometric particles of about 28 nm in diameter. The molecular weight of the viral coat protein subunits was found to be 25·0 kDa. Polyclonal antiserum was produced in New Zealand white rabbit, immunoglobulin G (IgG) was purified and conjugated with alkaline phosphatase enzyme. Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) method was standardized for the detection of CMV infection in vanilla plants. CP gene of the virus was amplified using reverse transcriptase-polymerase chain reaction (RT-PCR), cloned and sequenced. Sequenced region contained a single open reading frame of 657 nucleotides potentially coding for 218 amino acids. Sequence analyses with other CMV isolates revealed the greatest identity with black pepper isolate of CMV (99%) and the phylogram clearly showed that CMV infecting vanilla belongs to subgroup IB. This is the first report of occurrence of CMV on *V. planifolia* from India.

[Madhubala R, Bhadramurthy V, Bhat A I, Hareesh P S, Retheesh S T and Bhai R S 2005 Occurrence of *Cucumber mosaic virus* on vanilla (*Vanilla planifolia* Andrews) in India; *J. Biosci*. **30** 339–350]

1. Introduction

Cucumber mosaic virus (CMV, genus: *Cucumovirus*, family: Bromoviridae) is one of the most widespread plant viruses in the world with extensive host range infecting about 1000 species including cereals, fruits, vegetables and ornamentals (Roossinck 1999). CMV is readily transmitted in a non-persistent manner by more than 75 species of aphids (Palukaitis *et al* 1992). Weed hosts function as a reservoir for the virus and serve as sources of inoculum for the development of disease epidemics. Transmission through planting materials is also significant in some crop and weed hosts (Hsu *et al* 2000). CMV is a multicomponent virus with a single stranded positive sense RNA. RNAs 1 and 2 are associated with viral genome replication while RNA 3 encodes for movement protein and coat protein. Numerous strains of CMV have been classified into two major subgroups (subgroups I and II) on the basis of serological properties and nucleotide sequence homology (Palukaitis *et al* 1992). The subgroup I has been further divided into two groups (IA and IB) by phylogenetic analysis (Roossinck *et al* 1999).

Keywords. Coat protein gene sequence; *Cucumber mosaic virus*; host range; sequence analyses; serological detection; *Vanilla planifolia*

Abbreviations used: CMV, *Cucumber mosaic virus*; CP, coat protein; DAC-ELISA, direct antigen coated-enzyme linked immunosorbent assay; DAS, double antibody sandwich; EBIA, electroblot immuno assay; IgG, immunoglobulin G; RT-PCR, reverse transcriptase-polymerase chain reaction.

In India occurrence of CMV has been reported from many hosts. However, only limited reports are available on biological and molecular characteristics of these isolates. Vanilla (*Vanilla planifolia* Andrews) is an expensive spice cultivated for its highly priced beans. In India, large-scale extensive cultivation of vanilla started only recently. Viral diseases have become serious due to the recent intensive cultivation and occurrence of viral diseases such as mosaic and leaf curl were observed only recently in India (Bhai *et al* 2003; Sudharshan *et al* 2003). Recent survey of vanilla plantations of Kerala and Karnataka showed an average incidence of mosaic disease ranging from 0–5% (Bhat *et al* 2004b). Many of the affected vines are at their pre-bearing period and incidence of the disease is expected to increase as the crop is propagated vegetatively. Based on electron microscopy of leaf dip preparations, occurrence of three kinds of flexuous particles resembling *Potexvirus*, *Potyvirus* and *Closterovirus* and an isometric particle have been reported (Bhai *et al* 2003; Sudharshan *et al* 2003; Bhat *et al* 2004b). But exact identification of the causal viruses remained unaddressed. In this article, we report the occurrence of CMV on the basis of biological and coat protein (CP) gene sequence-based properties, a new record on *V. planifolia* in India.

2. Materials and methods

2.1 *Virus isolate*

Sixty-five vanilla plantations of Karnataka and Kerala states were surveyed and the plants showing viral like symptoms such as mosaic, mottling, chlorotic streaks parallel to venation, necrosis, leaf distortion and stunting were collected and maintained through vegetative propagation in an insect proof glasshouse.

The isolates were subjected to direct antigen coated enzyme linked immunosorbent assay (DAC-ELISA) using antisera to different viruses representing genus *Potyvirus*

Table 1. Reaction of different plant species to CMV infecting vanilla.

Family/plant species	Symptoms	Days required for symptom expression
Chenopodiaceae		
Chenopodium amaranticolor L.	CLL, NLL	3–7
Cucurbitaceae		
Benincasa hispada (Thunb.) cogn.	Mo	7–10
Cucumis sativus L.	VC	7–10
Cucurbita pepo L.	SL	–
Lagenaria siceraria (Mol.)	Mo	15–20
Trichosanthes anguina L.	CS	3–7
Fabaceae		
Cajanus cajan (L.) Millsp.	SL	–
Cicer arietinum Linn.	SL	–
Glycine max (L.) Merr.	SL	–
Vigna mungo (L.) Hepper	VN	5–10
Vigna radiata (L.) Wilczek	SL	–
Vigna unguiculata (L.) Walp	SL	–
Malvaceae		
Abelmoschus esculentus L.	SL	–
Poaceae		
Zea mays L.	SL	–
Solanaceae		
Capsicum annuum L.	M, LC	5–7
Lycopersicon esculentum L.	D, St	5–7
Nicotiana benthamiana W.	B, M, Mo, LC	7–10
Nicotiana glutinosa L.	B, M, Mo, LC	7–10
Nicotiana tabacum L.	B, M, Mo, LC	7–10

B, blisters on leaves; CLL, chlorotic local lesion; CS, chlorotic spot; D, deformation of top leaves; LC, curling of leaves; M, mosaic; Mo, mottling; NLL, necrotic local lesion; SL, symptomless; St, streaks on stem; VC, vein clearing; VN, veinal necrosis.

and CMV. A few of them which reacted positive to CMV antiserum, were initially mechanically inoculated onto *Chenopodium amaranticolor*. Local lesions produced on the leaf of *C. amaranticolor* were used as a source of single lesion isolate. Single lesion isolate obtained against a Calicut (Kerala state) isolate of vanilla was used in all the experiments. Mechanical inoculation was carried out by extracting sap in chilled 0·1 M phosphate buffer (pH 7·2) containing 0·1% 2-mercaptoethanol in a mortar kept in an ice tray. The extracted sap was rubbed on the leaves of test plants dusted with celite, which were then washed off with tap water after 2–3 min.

2.2 *Host range*

The following plant species belonging to 6 different families grown in pots under insect proof condition were mechanically inoculated: *Abelmoschus esculentus*, *Benincasa hispida*, *Cajanus cajan*, *Capsicum annuum*, *Cicer arietinum*, *Cucumis sativus*, *Cucurbita pepo*, *Glycine max*, *N. benthamiana*, *N. tabacum*, *N. glutinosa*, *Lagenaria siceraria*, *Lycopersicon esculentum*, *Trichosanthes anguina*, *Vigna mungo*, *V. radiata*, *V. unguiculata*, and *Zea mays* (table 1). A minimum of five plants of each species were inoculated and kept under observation for three weeks. Symptoms were recorded and checked for the presence of virus by back inoculation onto *C. amaranticolor*.

2.3 *Virus purification*

Virus was propagated on *N. benthamiana* plants by mechanical inoculation. Leaves of *N. benthamiana* harvested two weeks after inoculation was used for purification. Virus particles were purified using a slightly modified protocol of Lot *et al* (1972) as described in Bhat *et al* (2004a). The virus yield was determined by considering A_{260} 5·0 = 1 mg/ml (Sarma *et al* 2001).

2.4 *Electron microscopy*

Electron microscopy of the purified virus preparations negatively stained with 2% uranyl acetate (pH 4·5) were examined under JEOL-100-CF-II transmission electron microscope at the Unit of Virology, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi.

2.5 *Sodium dodecyl sulfate polyacrylamide gel electrophoresis*

SDS PAGE was carried out using 12% resolving gel and 5% stacking gel as described by Laemmli (1970). The purified virus preparation mixed with an equal volume of sample buffer heated for 3 min in boiling water was used for loading on the gel. The marker protein (Genei, Bangalore) was used as size standard.

2.6 *Antiserum production*

Polyclonal antiserum against the virus was produced in a New Zealand white rabbit by injecting purified virus preparation (0·5 mg of virus with Freund's incomplete adjuvant, 1 : 1) intramuscularly six times at 10 days interval. The animal was bled 15 days after the last injection and the antiserum was collected.

Immunoglobulin G (IgG) was purified from the crude polyclonal antiserum by affinity column chromatography using IgG purification kit (Genei, Bangalore) and quantified by taking absorbance values at 280 nm (1·4 absorbance = 1 mg/ml of IgG). One mg of this IgG was used for enzyme conjugate preparation. One step glutaraldehyde method described by Avrameas (1969) was followed for the preparation of IgG-alkaline phosphatase conjugate.

2.7 *Electro blot immuno assay*

Electro blot immuno assay (EBIA) was performed after SDS PAGE in nitrocellulose membranes at 20 V for 2 h as described in O'Donell *et al* (1982). Homologous antiserum against CMV was used at 1 : 1000 and antirabbit IgG labelled with alkaline phosphatase was used at 1 : 20,000 (Sigma Chemical Co., St: Louis, USA). Prestained marker protein (Bio-Rad, Richmond, CA, USA) was used as size standard.

2.8 *Double antibody sandwich ELISA*

Double antibody sandwich (DAS) ELISA was done on polystyrene plates using the protocol described by Clark *et al* (1986). Wells were initially coated with CMV IgG at 1 µg per ml of coating buffer. Antigen was prepared by grinding leaf tissues in 5 vol of PBS-T containing 2% polyvinyl pyrrolidone (PVP) and 0·2% BSA followed by centrifugation at 8000 rpm for 1 min. Supernatant obtained was used to load onto ELISA plates. CMV specific alkaline phosphatase conjugate was used at 1 : 2000 dilution. One hour after the addition of substrate (*p*-nitrophenyl phosphate, Genei, Bangalore), the ELISA reactions were read at 405 nm by using an ELISA reader (µQuant, Bio Tek Instruments Inc., USA).

2.9 *RNA isolation and reverse transcriptase-polymerase chain reaction*

For reverse transcriptase-polymerase chain reaction (RT-PCR), RNA was extracted using Nucleospin RNA Plant

kit (Macherey-Nagel, Duren, Germany). RT-PCR were performed in the same tube without any buffer changes in between as described by Pappu *et al* (1993). The primers designed for the CP gene sequences of CMV (based on multiple sequence alignments of CP sequences available in GenBank) were used to prime the amplification. Genome sense primer 5′ ATGGACAAATCTGAATCAAC 3′ was derived from the beginning of the first 20 bases of the coding region while antisense primer, 5′ TCAAACTGGGAGCACCC 3′ represented last 17 bases of the coding region of the CP gene. The PCR reaction (100 µl) contained 200 ng each of the primers 20 U Ribonuclease inhibitor (Genei, Bangalore), 10 U AMV reverse transcriptase (Finnzymes OY, Finland), 2·5 U *Taq* polymerase (Genei, Bangalore), 1 × PCR buffer (Genei, Bangalore), 10 mM dithiothreitol (Genei, Bangalore) and 10 µM each of the dNTPs (Finnzymes OY, Finland). PCR mix (27 µl) containing the above components was added to the tubes containing the template RNA (73 µl) resulting in a final reaction volume of 100 µl. Amplification was performed in an automated thermal cycler (Eppendorf Master Cycler Gradient) and the programme consisted of one cycle at 42°C for 45 min for cDNA synthesis followed by 40 cycle reaction profile involving 30 s of denaturation at 94°C, 1 min of annealing at 50°C and 1 min of extension at 72°C and a single cycle of final extension at 72°C for 10 min. The reaction products (20 µl) were analysed on 1% agarose gel along with 500 bp DNA ladder (Genei, Bangalore). The DNA bands were visualized and photographed using a UV transilluminator and a gel documentation apparatus (Alpha Innotech Corporation, CA, USA).

2.10 *Cloning, sequencing and sequence analyses*

The PCR product was purified using Strata Prep PCR purification kit (Stratagene, LaJolla, CA, USA) followed

Table 2. Source of CMV coat protein gene sequence used for comparisons.

CMV subgroup	Country	Designation	GenBank Accession No.
Subgroup IA	China	P1	AJ006988
	Japan	Ban	U43888
	Japan	D8	AB004781
	Japan	Leg	D16405
	Japan	N	D28486
	Japan	Pepo	D43800
	Korea	Kor	L36251
Subgroup 1B	China	ChCu	X65017
	India	SD	AB008777
	India	IN-Pn	AY545924
	India	IN-Pb	AY690620
	India	IN-Pl	AY690621
	India	IN-Am	AF198622
	India	IN-Ba	AY125575
	India	IN-Di	AF281864
	India	IN-Hb	AF350450
	India	IN-PhyM	X89652
	Korea	C7-2	D42079
	Korea	ABI	L36525
	Taiwan	M48	D49496
	Taiwan	NT9	D28780
Subgroup II	India	In-Li	AJ585086
	Australia	Q	M21464
	Hungary	Irk	L15336
	Japan	M2	AB006813
	Scotland	Kin	Z12818
	South Africa	S	AF063610
	United States	Ls	AF127976
	United States	Wl	D00463
	India	VP	AY754359 (This study)

by polishing of PCR product using P*fu* DNA polymerase (Stratagene, LaJolla, CA, USA) and dNTP mix. The resultant product was then cloned into pPCR Script Amp SK(+) cloning vector using pPCR Script Amp SK(+) cloning vector kit (Stratagene, LaJolla, CA, USA) and competent *Escherichia coli* strain DH5*a* were transformed by following standard molecular biology procedures (Sambrook and Russel 2001). Recombinant clones were identified by PCR as well as restriction endonuclease digestion and the selected clones were sequenced at the automated DNA sequencing facility available at the Avestha GenGraine Technologies Pvt Ltd., Bangalore.

Sequence data were compiled using Seqaid Version 3·6 (Rhoads and Roufa 1985). Multiple sequence alignments were made using Clustal X (1·81). Percent sequence identities were determined using Bio-Edit program version 5·0·9. Sequence phylogram was constructed by Neighborhood Joining Bootstrap method (Bootstrap analysis with 1000 replicates) in Clustal X (1·81) and rooted trees were generated using TREEVIEW software (Win 32) Version 1·6·6 (Page 1996). The CP nucleotide and amino acid sequences of other CMV isolates used for comparison (table 2) were obtained from GenBank (Benson *et al* 1999). The BLAST programme (Altschul *et al* 1997) was used to identify related sequences available from the GenBank database.

Figure 1. CMV infected *V. planifolia* leaf showing mosaic and leaf deformation under natural conditions.

3. Results

3.1 *Symptomatology and host range*

The characteristic disease symptoms associated with CMV infection in vanilla included mosaic and leaf distortion. The leaves become small and leathery with deformation (figure 1). In some cases, the internodal length was reduced leading to stunting of the plant. The virus was efficiently sap transmitted to *C. amaranticolor*. The inoculated leaves produced chlorotic local lesions within 3–5 days of inoculation, which later turned to necrotic lesions (figure 2). Back inoculated *V. planifolia* produced original symptoms. The virus was also transmitted to plant members belonging to Cucurbitaceae, Fabaceae and Solanaceae (table 1, figure 2). Back inoculation to *C. amaranticolor* from test plants showed that only susceptible hosts reproduced the symptoms on *C. amaranticolor*.

3.2 *Virus purification*

The virus was purified from *N. benthamiana* as it was a good propagating host. In sucrose gradient centrifugation, the gradient fraction from 3·25 to 3·50 cm from the top of the centrifuge tube was ascertained to have the virus particles. Yield of virus obtained per 100 g of tissue varied from 1–3 mg depending on the season and harvest time after inoculation. Electron microscopy of negatively stained purified preparations revealed the presence of typical isometric particles of about 28 nm diameter (figure 3). The purified preparation when run on SDS-PAGE produced a major band corresponding to 25 kDa (figure 4a). SDS-PAGE of purified preparation also revealed some break down of coat protein. The SDS-PAGE separated viral preparations reacted with its homologous antiserum in EBIA and reacted positively with the expected band (figure 4b). EBIA tests clearly confirmed the degradation of coat protein subunits in the purified preparations.

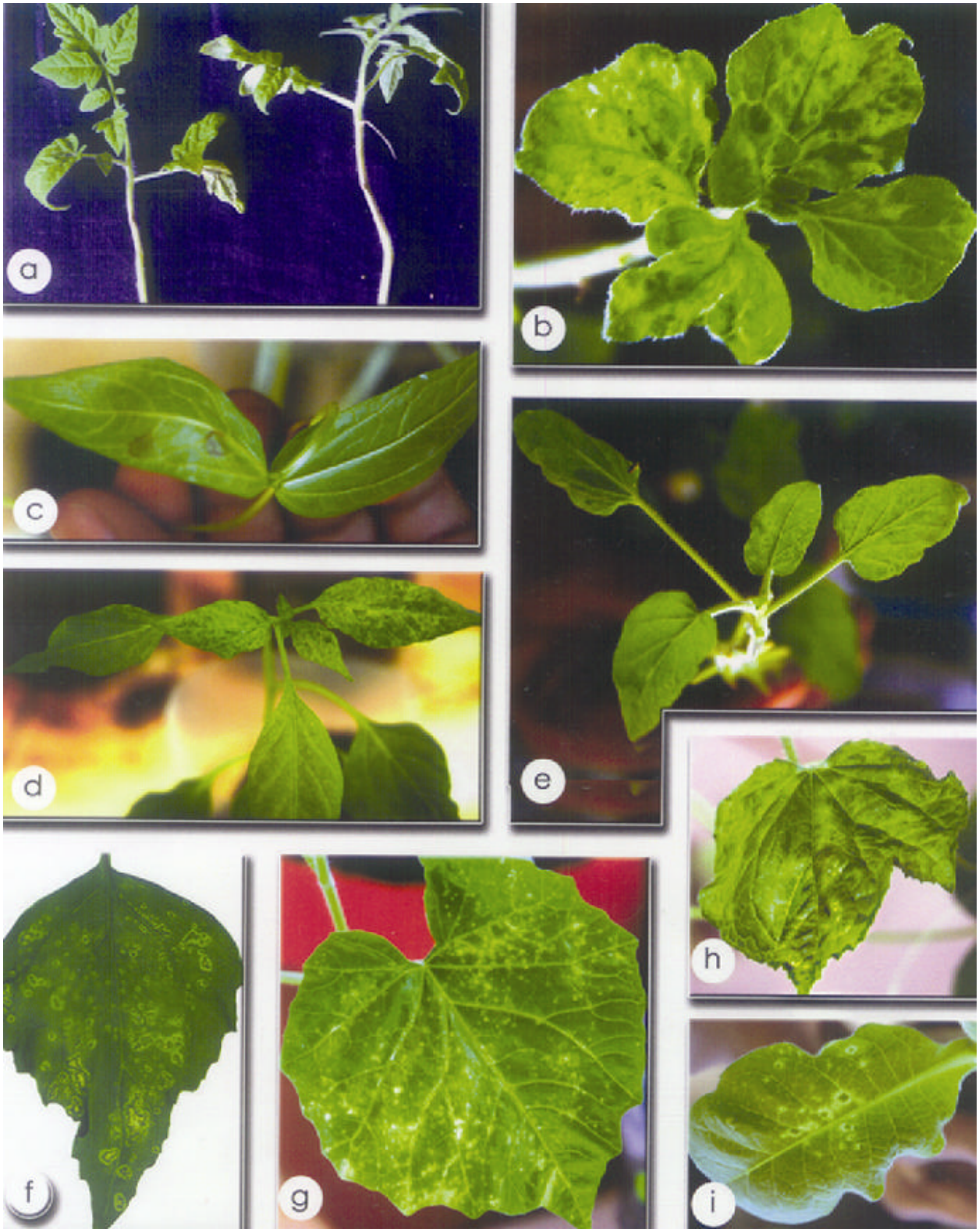

Figure 2. Plants exhibiting different kinds of symptoms upon mechanical inoculation with vanilla isolate of CMV under green house condition. (**a**) *Lycopersicon esculentum*; (**b**) *Nicotiana glutinosa*; (**c**) *Vigna mungo*; (**d**) *Capsicum annuum*; (**e**) *N. benthamiana*; (**f**) *Chenopodium amaranticolor*; (**g**) *Trichosanthes anguina*; (**h**) *Cucumis sativus*; (**i**) *N. tabacum*.

3.3 *Serological detection*

The virus was immunogenic and produced antiserum with an titre of 1 : 8000 in ELISA. In order to confirm specificity of the CMV antiserum produced, a DAC-ELISA test was performed using *Cymbidium mosaic virus* and a *Potyvirus* along with CMV infecting vanilla. The results showed positive reaction with only CMV infected samples thus indicating specificity of the antiserum produced. However antiserum cross-reacted with CMV isolates from other hosts. In order to standardize DAS-ELISA for detection of CMV, various concentrations of coating antibody (IgG) and enzyme conjugate were tried and, IgG at 1 μg/ml of coating buffer and CMV IgG-alkaline phosphatase conjugate at 1 : 2000 dilutions successfully detected the presence of CMV in the infected samples. DAS-ELISA method thus standardized was able to detect CMV infection in the vanilla plant samples. Out of 66 plants representing different locations tested, 19 showed positive reaction to CMV antiserum (table 3). Plants showing symptoms such as mosaic, mottling, leaf deformation as well as symptomless plants reacted positive to CMV antiserum.

3.4 *Coat protein gene amplification, sequencing and sequence analyses*

RT-PCR was successful in amplifying the CMV CP gene and a product of expected size (ca. 650 bp) was observed in infected sample. The resultant product was cloned and sequenced. The sequenced region contained a single open reading frame, which comprised 657 bases of nucleotides potentially coding for 218 amino acids. It was compared with CP gene sequences of all available CMV isolates from India as well as a few representative isolates from other parts of the world belonging to both the subgroups (I and II) (table 2). CP gene sequence of CMV infecting *V. tahitensis* and *V. fragrans* could not be used in the comparison due to their non-availability in the public domain (GenBank,

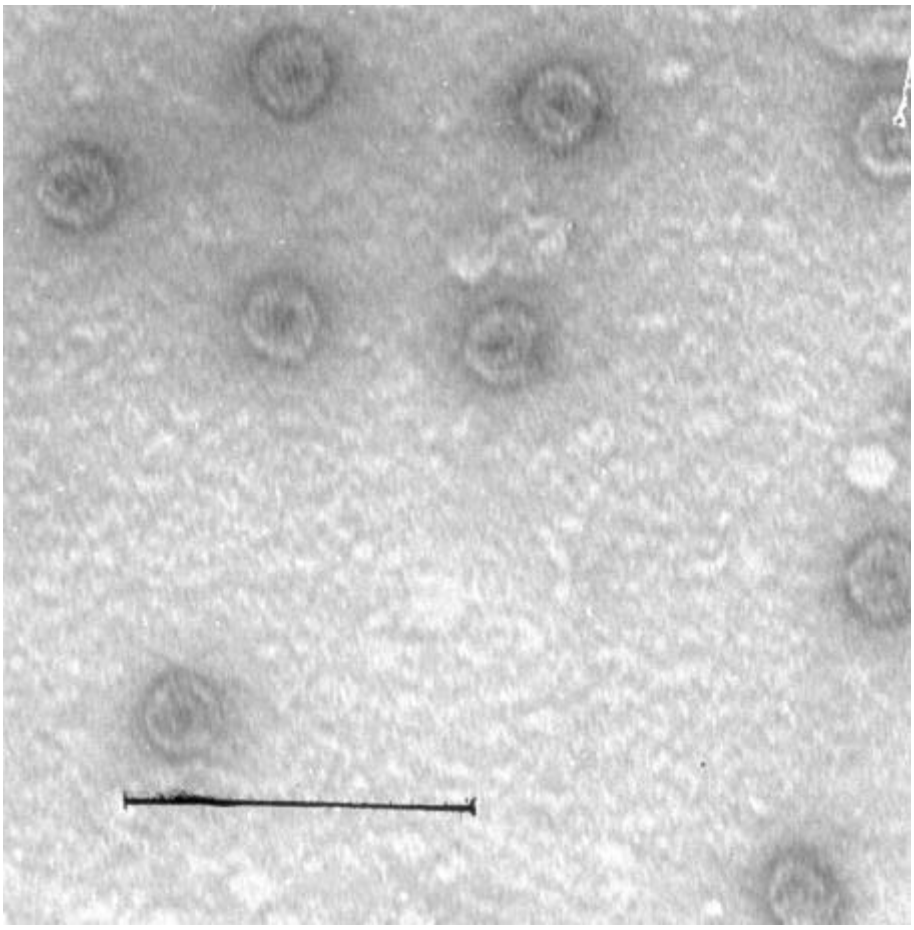

Figure 3. Electron micrograph of purified virus preparations showing isometric particles. Bar represents 100 nm.

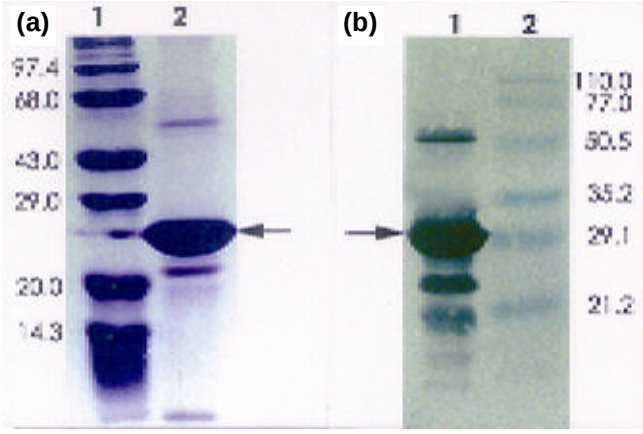

Figure 4. (**a**) SDS-PAGE analysis of coat protein of purified virus. Lane 1, marker protein and numbers on left indicate MW of marker proteins; lane 2, purified virus from vanilla. (**b**) EBIA analysis of purified preparations. Lane 1, purified virus from vanilla; lane 2, Bio-Rad pre-stained proteins and numbers on right indicate MW of marker proteins. Blotted virus protein was probed with homologous antiserum of CMV.

EMBL). Nucleotide and deduced amino acid sequence of CP gene of CMV infecting vanilla (VP) showed greatest identity with CMV isolate from black pepper (99%) (IN-Pn). Both at nucleotide and amino acid level, VP showed an identity of 89–92% and 92–95% with Subgroup IA isolates of CMV while it was 91–99% and 94–99% with Subgroup IB isolates respectively. An identity of 75–76% and 78–82% were observed with subgroup II isolates both at nucleotide and amino acid level respectively.

Multiple sequence alignment based on deduced CP amino acid sequences of subgroup I isolates showed that VP and IN-Pn differed only at one position (figure 5). Analysis of CP identified one amino acid position (G209) as unique to VP. Within subgroup IB isolates, Indian isolates were more close to each other than to subgroup IB isolates from other regions (figure 5). One amino acid position (T31) was found to be unique to all Indian isolates. Similarly one amino acid position (Y99) was unique among subgroup IA isolates.

Table 3. Detection of CMV by DAS-ELISA in vanilla plants representing different locations of Karnataka and Kerala states.

Isolates	Visual symptoms	A_{405} value* 5
Kerala state		
Calicut		
Sample 1	LD	0·36
Sample 2	NS, LD, M	0·44
Sample 3	M	0·40
Sample 4	NS	0·36
Sample 5	SL	0·35
Vandiperiyar		
Sample 1	NS, LD, S	0·40
Sample 2	M, CS, LD, NS	0·46
Sample 3	M	0·57
Sample 4	NS	0·37
Sample 5	M, NS, S	1·00
Kannur		
Sample 1	NS, CS, LD, S	0·33
Sample 2	S	0·47
Sample 3	S	0·37
Karnataka state		
Dakshina Kannada		
Sample 1	NS	0·48
Sample 2	NS	0·30
Sample 3	CS	1·00
Sample 4	NS, CS	1·23
Saklespur		
Sample 1	SL	0·91
Sample 2	SL	0·38
Healthy vanilla	SL	0·05

CS, chlorotic streaks; LD, leaf deformation; M, mosaic; NS, necrotic spot; S, stunting of plant; SL, symptomless.
*Average of three replications taken 1 h after addition of substrate.

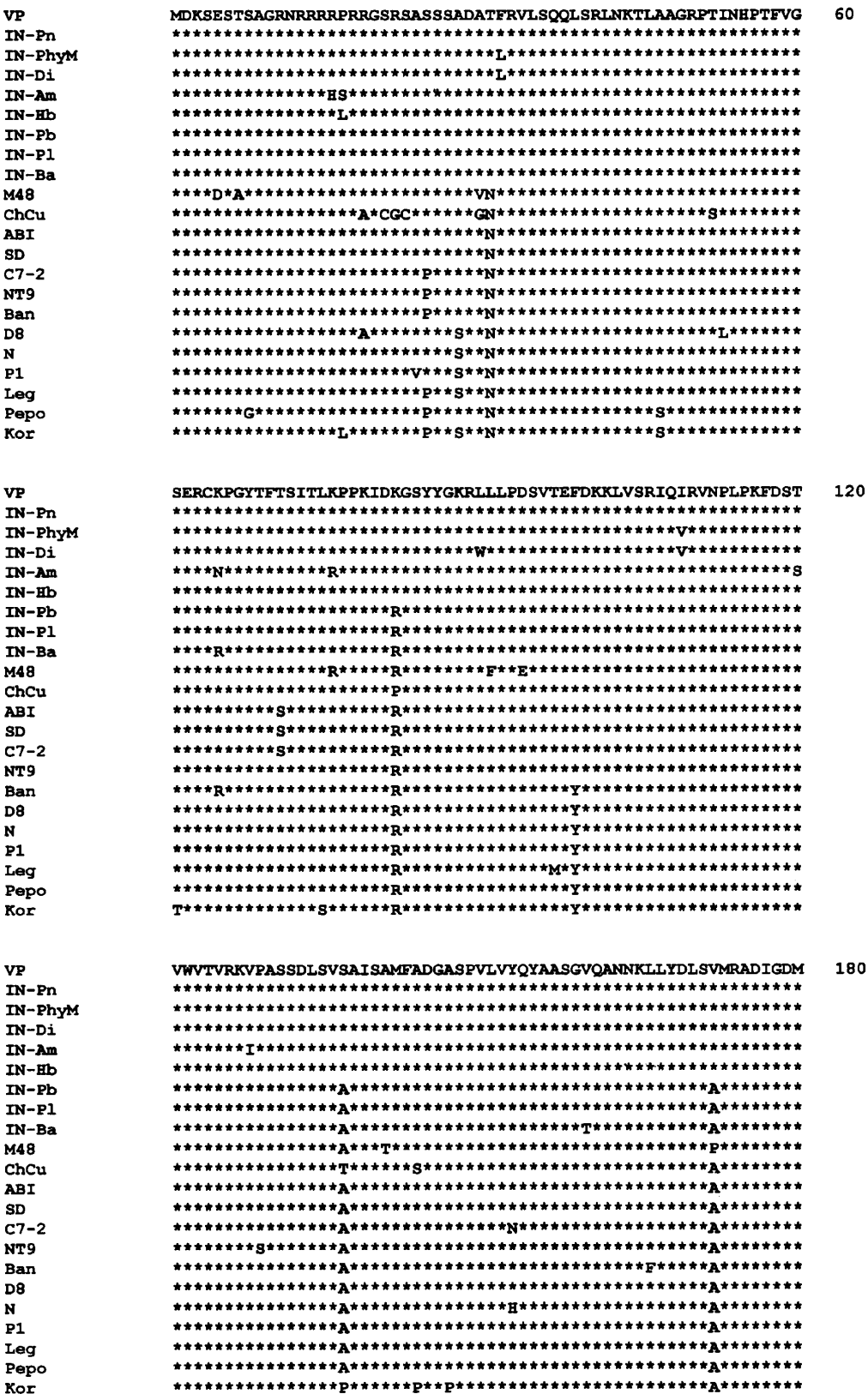

Figure 5. For caption see page No. 348.

Phylogram illustrating phylogenetic relationship among CMV isolates generated based on CP amino acid sequences showed that VP was most closely related to the members of subgroup I. In contrast, CMV isolates belonging to subgroup II formed a different cluster, well separated from subgroup I isolates (figure 6). Within subgroup I, VP showed close phylogenetic relationship with subgroup IB isolates than IA isolates as the latter formed a separate cluster. However, within subgroup IB, two major clusters were obtained. One cluster consisted of Indian isolates that aligned together as one group and the other consisted isolates from other Asian countries. The only exception was three Indian isolates (IN-Pb, IN-Pl and IN-Ba) and two other isolates (ChCu and M48) which formed two separate subclusters (figure 6). Among all the isolates, VP showed maximum evolutionary relationship with IN-Pn.

4. Discussion

The results presented show the occurrence and identification of CMV on vanilla on the basis of biological and CP sequence similarities. The virus was identified as a member of subgroup IB. As vanilla is clonally propagated through stem cuttings, DAS-ELISA method standardized in the present study could be used to detect CMV infection in vanilla as well as other hosts. This would help in identifying and certifying planting material to check spread of the virus. In a few CMV infected vanilla plants, occurrence of flexuous virus particles was also observed indicating mixed infections. Characterization of flexuous virus is under progress. This is also the first record of CMV on *V. planifolia*. Recently, CMV belonging to subgroup I was reported on *V. fragrans* and *V. tahitensis* from French Polynesia and Reunion Island (Farreyrol *et al* 2001).

CMV has a wide host range infecting over 1000 species, virus has been adapting successfully to new hosts and environments (Roossinck 2002). High sequence identities observed with all Indian isolates of CMV (Srivastava and Raj 2004) clearly indicate that vanilla isolate of CMV originated locally. Commercial scale of vanilla cultivation has started in India only recently. It is mainly grown as mixed crop with arecanut, coconut, black pepper and banana. Among these, occurrence of CMV on banana and black pepper in India is already known (source: GenBank Acc. No. AY125575; Sarma *et al* 2001). High sequence identities and evolutionary ties in the CP gene amino acid observed with CMV isolate from black pepper and vanilla (99%) indicates the probability that vanilla isolate of CMV originated from black pepper isolate. Variation in symptoms

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VP          RKYAVLVYSKDDALETDELVLHVDIEHQGIPTSGVLPV   218
IN-Pn       ********************************R*********
IN-PhyM     ********************************R*********
IN-Di       ******M*************F*******R*********
IN-Am       ******M**********************R*********
IN-Hb       **********************I*****R*********
IN-Pb       ********************************R*********
IN-Pl       ********************************R*********
IN-Ba       ********************************R*********
M48         ***************S*************R*********
ChCu        ************V***************R*********
ABI         **IT*****************************R*********
SD          ********************************R*********
C7-2        ********************************R*********
NT9         ********************************R*********
Ban         ********************************R*********
D8          ********************************R*********
N           ************************V***R*********
P1          ************************V***R*********
Leg         ************************V***R*********
Pepo        ************************V***R****R****
Kor         ********A*****A*********V***R*********
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Figure 5. Multiple sequence alignment of coat protein amino acid sequences of CMV infecting vanilla (VP) with other CMV isolates of subgroup I using Clustal X. Asterisk (*) indicates identity at a given position. Sequences for comparisons were obtained from GenBank and designation given to each of the isolates and their GenBank accession numbers are given in table 2.

of CMV infected vanilla observed in this study indicates the possibility of occurrence of different strains at different regions. This kind of symptoms variation of CMV infected *V. fragrans* and *V. tahitensis* was reported by Farreyrol *et al* (2001). Hence a number of additional CMV isolates from different locations and hosts will have to be examined to establish correlation between molecular typing and symptom types.

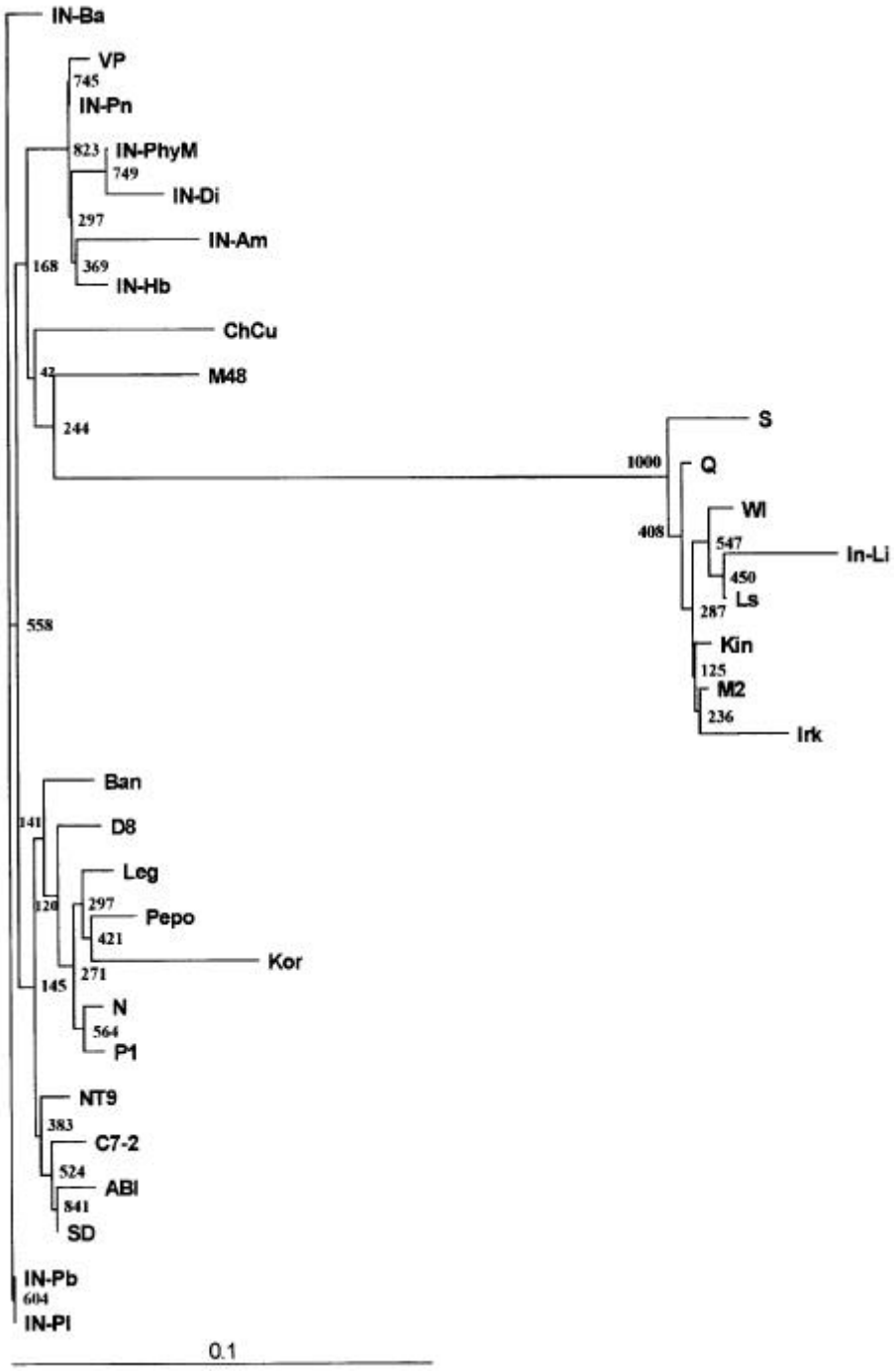

Figure 6. Phylogram, drawn by Neighborhood Joining Bootstrap method in Clustal X (1·81), illustrating phylogenetic relationships based on the multiple alignments of the coat protein amino acid sequences of 29 distinct isolates of CMV and vanilla isolate of CMV (VP). Sequences for comparisons were obtained from GenBank and designation given to each of the isolates and their GenBank accession numbers are given in table 2.

Acknowledgements

We thank Scientist-In-Charge, Virology Unit, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi for electron microscopy work.

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MS received 12 November 2004; accepted 1 March 2005

*e*Publication: 30 April 2005

Corresponding editor: SHAHID JAMEEL

Note added in proof: The occurrence of CMV from *V. fragrans* from French Polynesia and Reunion has been reported by Farreyrol *et al* (2001). *V. fragrans* and *V. planifolia* are two names for the same species.