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Characterization of *Cucumber mosaic virus* infecting snake gourd and bottle gourd in India

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ABSTRACT

Snake gourd and bottle gourd are the common cucurbitaceous vegetables consumed in India that are prone to viral infections under field conditions. During our field survey in Southern India in 2012-14, the symptoms *viz.*, stunted growth, mosaic mottling, puckering and chlorosis in these crops were observed. Incidences of virus-like symptoms ranging between 63 and 88% were observed in three different locations. Based on the mechanical inoculations on their respective host, plants produced symptoms similar to those under field conditions. Samples tested with DAS-ELISA using CMV polyclonal antiserum were found positive for CMV. The occurrence of CMV was further confirmed by amplification of coat protein gene of CMV using RT-PCR assays. The sequence analysis of coat protein gene revealed highest nucleotide identity of > 92% with CMV subgroup IB isolates. Coding regions from CMV RNA 1, RNA 2 and RNA 3 genomic fragments were cloned and sequenced. Phylogenetic analysis of RNA1, RNA 2 and RNA 3 from CMV infecting snake gourd and bottle gourd showed close relatedness with Italian isolate that infect *Capsicum* rather than Asian isolates of CMV from India, Malaysia, China and Japan. The characterization of CMV from snake gourd and bottle gourd pertaining to biological, serological and molecular attributes are presented.

1. Introduction

Cucurbits are important vegetable crops belonging to the family Cucurbitaceae grown for domestic consumption and worldwide commercial trade. Out of 118 genera and 825 species of *Cucurbitaceae*, 36 genera and 100 species are found in India [1]. Around 5.6% of the total vegetable produced in India is contributed by cucurbitaceous crops [2]. Snake gourd also known as Chinese cucumber (*Trichosanthes cucumerina* L.) and bottle gourd (*Lagenaria siceraria* L) are important cucurbitaceous vegetables grown and consumed in Southern India, Bangladesh, and Nepal. Several bacterial, fungal and viral diseases in these species cause severe yield and economic losses to the farmers. Cucurbitaceous crops in India were reported to be infected by viruses belonging to the genera *Begomovirus, Cucumovirus*, *Potyvirus, Tobamovirus, Tospovirus,* etc. [3–7].

Cucumber mosaic virus (CMV), belongs to genus *Cucumovirus* in the family *Bromoviridae*. It has broader host range and infects more than 1200 plant species across 100 plant families with an average yield penalties ranging 10–20% [8]. CMV is widespread in Europe, Asia, Australia, Africa and North America. CMV has tripartite single-stranded positive sense RNA genome (RNAs 1, 2 and 3). Transmission of CMV mainly occurs through aphids in a non-persistent manner. Additionally, CMV can be transmitted by mechanical means [9] and through seeds in many cucurbit species *viz.*, pumpkin, cucumber, wild cucumber, etc. [10]. Based on the serological properties, CMV is categorized into subgroups I and II. Subgroup I was divided into IA and IB based on the phylogenetic analysis of coat protein gene and 5′ untranslated region (5′ UTR) [11]. CMV subgroups IA and II are present worldwide, while subgroup IB is originated from Asia [12]. Our survey on mosaic diseases of cucurbitaceous crops in Tamil Nadu confirmed the natural occurrence of CMV on snake gourd and bottle gourd. Here we report biological and molecular characterization of CMV isolates infecting snake gourd and bottle gourd in India.

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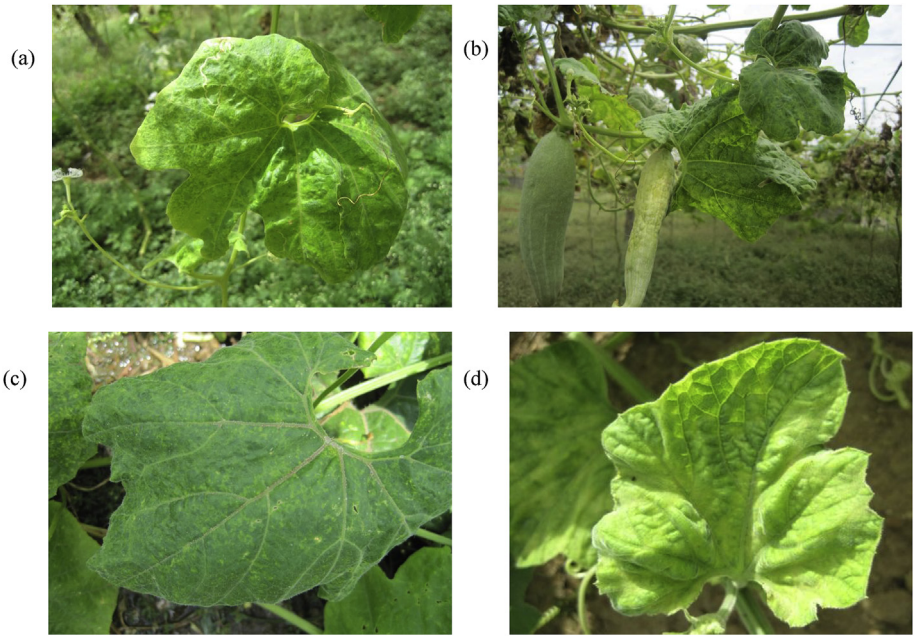

Fig. 1. Symptoms of CMV infected snake gourds plants showing mosaic mottling (a) puckering (b) and chlorotic spots (c) and chlorosis and mosaic mottling on bottle gourd leaf (d).

2. Materials and methods

2.1. Collection and maintenance of virus inoculum

Extensive surveys were conducted in the cucurbits cultivating areas of 12 districts in Tamil Nadu State, India, to study the occurrence of mosaic disease in cucurbitaceous crops during the growing season of 2012–2014. Naturally infected snake gourd and bottle gourd plants exhibiting virus like symptoms were collected from surveyed areas. Preliminary screening for the presence of CMV in these samples was done by Double Antibody Sandwich enzyme linked immunosorbent assay (DAS-ELISA) using polyclonal antibody against CMV that was obtained from DSMZ, Germany. Among them, CMV infected snake gourd (TN TNAU SG1 and TN TNV SG1) and bottle gourd (TN NGK BoG1) samples were mechanically inoculated on the snake gourd and bottle gourd plants, respectively. Virus isolates were maintained in insect-proof cages separately at the green houses of Department of Plant of Pathology, Tamil Nadu Agricultural University, Coimbatore (India) for further studies.

2.2. Host range study

CMV positive isolate (TN-TNAU-SG1) maintained on the snake gourd plants were mechanically sap inoculated onto 16 different hosts *viz.*, *Vigna unguiculata* L., *Nicotiana glutinosa* L., *Nicotiana plumbaginifolia* Viv., *Chenopodium amaranticolor* L., *Trianthema portulacastrum* L., *Datura stramonium* L., *Solanum lycopersicum* L., *Luffa aegyptiaca* Mill., *Lagenaria siceraria* L., *Cucurbita moschata* L., *Cucumis sativus* L., *Cucumis anguria* L., *Trichosanthes cucumerina* L., *Benincasa hispida* Thunb., *Luffa acutangula* L. and *Citrullus lanatus* Thunb. L. using 0.1 M sodium phosphate buffer (pH 7.0), 0.1% β-mercaptoethanol under greenhouse conditions to study the viral host range. The plants were maintained at 22–25 °C and observed for symptom development 30 dpi to record the local and systemic infection. Virus transmission was confirmed by RT-PCR using coat protein (CP) based primer pair, GK CMV F/R (Supplementary Table 1).

2.3. Cloning and sequencing of the genome

Total RNA was extracted from infected 100 mg leaf tissue of snake gourd and bottle gourd samples using Trizol reagent (Sigma Aldrich, USA). The integrity and quality of the total RNA was checked on 1% agarose gel. For the amplification of different ORFs 1a, 2a, 2 b, 3a and 3 b specific primers were designed by multiple alignments of different isolates of CMV nucleotide sequences available in NCBI GenBank using clustalW (Supplementary Table 1). First strand cDNA synthesis was carried out using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) as per manufacturer's instructions. Reactions were performed at 42 °C for 60 min followed by incubation at 70 °C for 5 min. Amplifications were carried out using Master mix (Bangalore Genei, Bengaluru, India) in a thermocycler (Eppendorf, Germany) with initial denaturation of 2 min at 94 °C followed by 35 cycles of denaturation at 94 °C for 30 s, annealing and extension temperature being specific for particular amplicon and finally an extension time of 10 min at 72 °C (Supplementary Table 1). PCR products were ligated into pGEMT-easy vector (Promega, Madison, USA) according to the manufacturer's instructions and then transformed into *E. coli* DH5α. Recombinant colonies were screened by restriction digestion. For each amplicon, at least three clones were sequenced at Excelris Pvt. Ltd., Ahemedabad, India. Sequences were assembled using CLUSTAL W2 and assembled sequences of RNA 1 (ORF 1a), RNA 2 (2a and 2 b) and RNA 3 (3a and 3 b) were deposited in the NCBI GenBank.

2.4. Phylogenetic analysis

Nucleotide sequences of CMV isolates belonging to subgroup IA, IB and II reported from different parts of the world were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov) and analyzed using MEGA 6.0 version. Phylogenetic relationships between these isolates were inferred from the nucleotide sequence alignment by Maximum likelihood method (1000 bootstrap replicates). To check the potential recombination events within CMV isolates, we used RDP4 program with default settings with all the CMV sequences available in GenBank database. Sequence identity percentage was calculated using Bioedit sequence alignment editor version 7.0.9 [13–15].

3. Results and discussion

Cucumber mosaic virus was reported on several crops including cucumber, gherkin, tomato, chilli, brinjal, potato, banana, pepper, vanilla, gerbera, etc from India. Despite very little informations were available on the infection of CMV on cucurbits at the genome level, snake gourd and bottle gourd fields were surveyed during 2012-14 for identification of viruses associated under natural field conditions in Tamil Nadu. The virus disease symptoms *viz*., mottling, leaf puckering, chlorotic spots and stunted plant growth (Fig. 1) on snake gourd and chlorosis with mosaic mottling were observed on bottle gourd. Among the 61 snake gourd and 46 bottle gourd samples collected, 14 snake gourd samples from Coimbatore and Tirunelveli and 4 bottle gourd symptomatic samples from Nagercoil tested positive for the presence of CMV using DAS-ELISA. For further studies, two snake gourd isolates collected from TNAU orchards, Coimbatore (TN TNAU SG1) and Tirunelveli (TN TNV SG1) and one bottle gourd isolate (TN NGKBoG1) from Nagercoil were used.

Mechanical sap transmission of snake gourd isolate (TN TNAU SG1) resulted in local lesions (chlorotic and necrotic) between two to ten days on the host plants *viz., Vigna unguiculata, Nicotiana glutinosa, Chenopodium amaranticolor, Trianthema portulacastrum* and *Datura stramonium*. Systemic infections *viz*., mosaic mottling, chlorotic spots, vein clearing, stunted growth, etc. were observed on *N. glutinosa*, *N. plumbaginifolia*, *Lagenaria siceraria, Cucurbita moschata, Trichosanthes cucumeria, Luffa acutangula, Citrullus lanatus, Cucumis sativus, Cucumis anguria, Benincasa hispida* and *Luffa aegyptiaca*. On *Solanum lycopersicum* curling of terminal leaves were observed at twenty-one days post inoculation (dpi) (Table 1). Transmission study revealed that the virus causing mosaic mottling on snake gourds of Tamil Nadu can be successfully transmitted to a number of test species by mechanical inoculations. According to Zhang et al. [16], subgroup I strains of CMV cause severe symptoms and disease development on tobacco. Snake gourd isolate also produced chlorotic rings, later becoming necrotic on inoculated leaves of *N. glutinosa* and severe mosaic mottling on *N. glutinosa* and *N. plumbaginifolia* on systemically infected leaves upon mechanical inoculation. Depending on the host reaction upon mechanical inoculation, the CMV isolates infecting in Tamil Nadu belongs to subgroup I. The isolate TN TNAUSG1 induced necrotic local lesions on inoculated leaves of cowpea confirming that these strains belonged to subgroup IB [8]. Also, the present isolate induced chlorotic local lesions and became necrotic on *C. amaranticolar*, severe mosaic mottling on cucumber and curling on newly developed leaves on tomato. Previously, similar symptoms were reported with subgroup I strains [4,17–19].

In order to confirm the results of ELISA and host range study, RT-PCR analysis was carried out with the CMV coat protein gene - specific primer pair. Infected samples generated amplicons of expected size (~1200 bp) in RT-PCR and the sequence data of amplified region confirmed that the virus isolates in this study associated with mosaic mottling disease of snake gourd; chlorosis and mosaic mottling on bottle gourd in Tamil Nadu as a strain of CMV. The sequence analysis by homology searches revealed that the sequences of the three isolates had 97% nucleotide identity with CMV from India (AY690621, AY690620 and AY125575). The phylogenetic analysis of coat protein gene of our CMV isolates with other known isolates belonging to sub group IA, IB and II revealed that all the CMV isolates were closely associated with sub group IB and clustered together with banana (AY125575) isolate from Kerala along with other Indian isolates and Italian isolate under sub group IB (Fig. 2). Similarly Pratap et al. [19] and Dubey et al. [20] categorized the subgroup of CMV strains based on the coat protein sequence analysis infecting tomato and gladiolus in India. Also, all these three isolates had > 92% identity with known subgroup IB CMV isolates at nucleotide level. In the pairwise alignment of amino acid sequences of coat protein genes with other known sequences of subgroup IA, IB and II revealed the three spatial changes as P25S, N31T and Y99F in subgroup IB compared to IA. Interestingly, amino acid at position 7 was substituted with Alanine (Ala) to Threonine (Thr) in both snake gourd and bottle gourd isolates. These isolates shared 91.1–98.6% identity towards subgroup I but 75.6–76.8% identity towards the subgroup II isolates with known sequences at nucleotide level. Earlier from our laboratory, we have reported CMV on snake gourd and bottle gourd for the first time in India [21]. Since there were no other reports on these crops, we attempted molecular

Table 1
Reaction of CMV isolate infecting snake gourd (TN TNAU SG1) in Tamil Nadu on different host plants through mechanical sap inoculation.

S.No.	Test host(s)	Local		Systemic	
		Symptoms	No. of days taken for symptom expression	Symptoms	No. of days taken for symptom expression
1	*Trichosanthes cucumerina* L.	Necrotic spots on inoculated leaves	2	Mosaic mottling on systemic leaves	4–5
2	*Chenopodium amaranticolor* L.	Necrotic local lesion on inoculated leaves	2–3	–	–
3	*Lagenaria siceraria* L.	Necrotic spots on inoculated leaves	3	Mosaic mottling on systemic leaves	7–10
4	*Cucurbita moschata* L.	Necrotic spots on inoculated leaves	3	Mosaic mottling on systemic leaves	7–12
5	*Luffa acutangula* L.	Necrotic spots on inoculated leaves	3–4	Mosaic mottling on newer leaves	7–10
6	*Trianthema portulacastrum* L.	Necrotic local lesion on inoculated leaves	3–4	–	–
7	*Luffa aegyptiaca* Mill.	Necrotic spots on inoculated leaves	4–5	Chlorotic circular spots and chlorosis of veins and veinlets on newer leaves	5–8
8	*Citrullus lanatus* Thunb.	Necrotic lesions with yellow halo on inoculated leaf	4–6	Mosaic on systemic infection	7–10
9	*Nicotiana glutinosa* L.	Initially necrotic lesion with yellow halo on inoculated leaves	5–7	Systemic mosaic mottling on newer leaves	> 12
10	*Vigna unguiculata* L.	Chlorotic local lesion later leads to necrotic lesions	7–9	–	–
11	*Datura stramonium* L.	Chlorotic yellow spots on inoculated leaves	10	–	–
12	*Cucumis anguria* L.	–	–	Mosaic mottling on systemic leaves	7–9
13	*Nicotiana plumbaginifolia* Viv.	–	–	Systemic mosaic mottling on newer leaves	> 8
14	*Cucumis sativus* L.	–	–	Mosaic mottling on systemic leaves	8–10
15	*Benincasa hispida* Thunb.	–	–	Chlorotic spots as systemic infection	10–14
16	*Lycopersicum esculentum* Mill.	–	–	Curling of terminal leaves	21

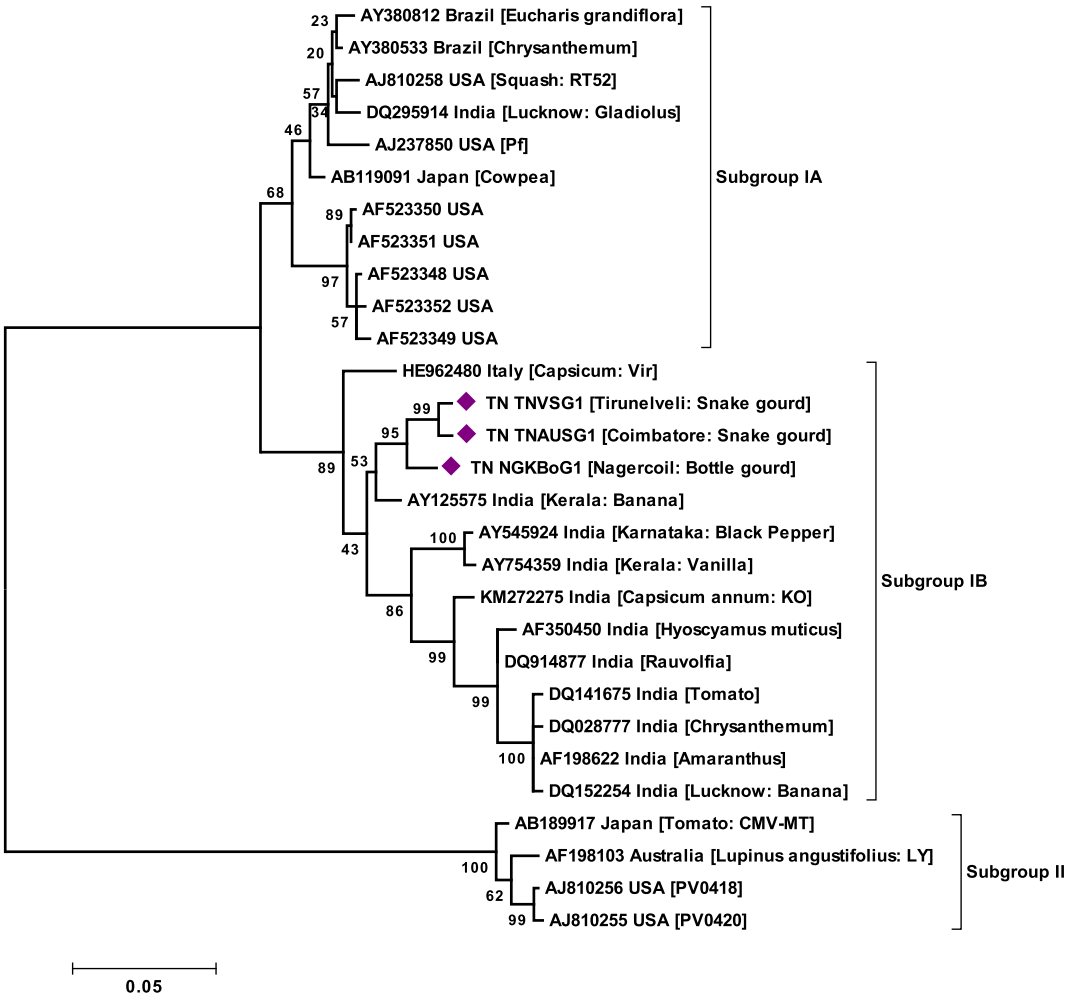

Fig. 2. Phylogenetic analysis of CMV isolates infecting snake gourd and bottle gourd in India based on coat protein sequences with other *Cucumber mosaic virus* isolates across the globe. This tree was generated using Maximum Likelihood method in MEGA 6. The bootstrap consensus tree was inferred from 1000 replicates.

characterization of coding regions of RNA1, RNA 2 and RNA 3 for authentic identification of the virus strains.

To obtain genomic fragments of CMV isolates, primer pairs were designed for the coding regions of RNA 1 (ORF 1a), RNA 2(ORF 2a and ORF 2 b) and RNA 3 (ORF 3a) using GenBank sequences (HE962478, HE962479 and HE962480). The fragments amplified by RT-PCR contained the expected size amplicons (Supplementary Table 1) and sequenced. The sequence analysis revealed that ORF 1a coding replicase protein on RNA 1 comprised of 2982 nucleotides (nt), putatively translating to 993 amino acids (aa). RNA 2 comprised of two ORFs in overlapping manner as 2a and 2 b. RNA dependent RNA polymerase (RdRP) encoded by ORF 2a consisted of 2577 nt (858 aa) and viral suppressor protein encoded by ORF 2 b comprised of 336 nt (111 aa) in RNA 2. Similarly, two ORFs were present on the RNA 3 encoding movement protein (ORF 3a) of 840 nt (279 aa) and coat protein (ORF 3 b) of 657 nt (218 aa). Both the ORFs were separated by an intergenic region (IR) of 300 nucleotides.

The gene encoding 1a protein was amplified by RT-PCR using four specific nested primer pairs and assembled sequences were submitted to NCBI GenBank database (TN TNAU SG1 – KF891356; TN TNV SG1 – KF891357; TN NGKBoG1 – KF891358). The nucleotide sequences of 1a protein shared 97–98.7% identity between snake gourd and bottle gourd isolates. Similarly, nucleotide sequences of RNA2 (TN TNAU SG1 - KJ778898; TN TNV SG 1 - KJ778899; TN NGKBoG1 - KJ778900) of snake gourd isolates shared 98.3% nucleotide identity among themselves and 92.9–93.4% identity with bottle gourd isolate. The BLAST search in NCBI revealed that the RNA1 and RNA 2 nucleotide sequences of TN TNAU SG1, TN TNV SG1 and TN NGKBoG1 shared nucleotide identity of 94–95% with *Capsicum* CMV isolate of Italy [HE962478 and HE962479]. The nucleotide sequences of RNA 3 including movement protein (3a) and coat protein (3 b) along with intergeneic region of CMV isolates (TN TNAU SG1 – KJ874248; TN TNV SG 1 - KJ874249; TN NGKBoG1 - KJ874250) shared maximum identity of 94.5–95.8% with Italy isolate from *Capsicum* (HE962480) (Supplementary Table 2). Phylogenetic analysis of 1a gene revealed that TN TNAU SG1, TN TNV SG1 and TN NGKBoG1 isolates were grouped together and formed a separate cluster along with Italy-*Capsicum* (HE962478), Malaysia-cucumber (JN054636) and India-*Capsicum* (KM272277) isolates. Similar clustering pattern and clade formation was observed in phylogenetic analysis using RNA 2 and RNA 3 (Supplementary Figs. 1a–c).

Recombination analysis using RDP4 tool showed that RNA 3 of TN NGK BoG1 was predicted to have recombination event between the position 14 and 278 nucleotide position with CMV isolate from USA (U20219) and Indian isolate (AJ831578) infecting *Lilium longiflorum* as their major and minor parent, respectively. Predicted recombination events were detected through RDP, BootScan, SiScan and LARD methods with p-value of less than 1×10^{6} (Supplementary Table 3).

Overall, both snake gourd and bottle gourd isolates shared maximum identity with Italian isolates rather than the known Asian isolates from India, Malaysia, Japan and China based on RNA1, RNA2 and RNA 3 at nucleotide level. Also all our three isolates from Tamil Nadu were shared maximum identity among themselves than any other known

isolates reported earlier. This suggests that, CMV strains infecting snake gourd and bottle gourd of Tamil Nadu sharing its origin with the isolates reported from Italy infecting *Capsicum*.

There is a report on a CMV strain causing shoestring disease in tomato from India based on sequence analysis of RNA3 [19]. There are many reports recently from India characterizing CMV isolates infecting tomato based on the RNA 3 genome [20,22,23]. We further report, the biological and molecular characterization of CMV isolates retrieved from snake gourd and bottle gourd which showed closer association towards the Italian isolate than the CMV isolates characterized previously in India. Also biological, serological and molecular analysis of ORFs involved in replication (1a and 2a), silencing suppression (2 b), movement (3a) and coat protein (3 b) reported in this study represents the confirmed report of the CMV on snake gourd and bottle gourd in India.

As CMV could be subjected to seed transmission, its entry in India through infected seeds cannot be denied. Virus transmission is thought to be due to transport of infected seed materials across the continents. Infection of CMV on cucurbits can cause yield losses up to 100% [8]. In order to prevent the severe loss, management strategies need to be standardized. Our future plan of work will focus on the diagnosis of seed materials for the virus infection and for devising the integrated strategies for the management of mosaic disease on snake gourd and bottle gourd starting from the selection of seed materials.

Compliance with ethical standards

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Conflicts of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pmpp.2018.05.010.

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