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Incidence and molecular characterization of cucumber mosaic virus in Pumpkin crop from Assam

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Abstract
Incidence of pumpkin mosaic disease was determined through symptomatology and leaf samples were collected following a roving survey in different pumpkin growing areas of Assam for serological assay; *viz.,* double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and molecular characterization of the virus associated with the disease. Symptomatology revealed various leaf mosaic, mottling, distortion, vein banding, vein clearing and fruit mosaic. DAS-ELISA showed 37 positive reactions out of 84 leaf samples for cucumber mosaic virus (CMV). The total genomic RNA extracted from the infected leaf samples from Jorhat district were subjected to reverse transcription-polymerase chain reaction (RT-PCR) with a pair of primers corresponding to the flanking regions of the cucumber mosaic virus coat protein (CP) gene and yielded the desired amplicon size of 593 bp. Partial sequencing and phylogenetic analysis of the PCR amplicon revealed 93.11 to 99.16 percent sequence homology with other CMV isolates worldwide.

Keywords: Cucumber mosaic virus, double-antibody sandwich enzyme-linked immunosorbent assay, reverse transcription-polymerase chain reaction, phylogeny

1. Introduction
Pumpkin (*Cucurbita moschata*; family: *Cucurbitaceae*) is one of the major cucurbitaceous crops cultivated extensively throughout the world. India ranks second after China in the production of pumpkin having 99 million hectares area and 2117 million tonnes of production in 2019-2020 (Anon, 2020) [1]. Amongst various factors, viral diseases are one of the major constraints in Pumpkin production. More than 59 different viruses have been reported from cucurbits (Lecoq and Desbiez, 1997) [5]. Cucumber mosaic virus (genus: *Cucumovirus*, family: *Bromoviridae*) is considered as one of the most economically important virus of agricultural crops having worldwide occurrence (Palukaitis and Garcia-Arenal, 2008) [9]. CMV is a major cosmopolitan virus having a wide host range, infecting more than 1200 species of plants across 100 plant families and can cause yield losses as high as 40 to 60 percent (Vasudeva and Lal, 1943; Varma and Giri, 1998; Kavyasri and Nagaraju, 2014) [11, 10, 2]. CMV is known to cause various mosaic symptoms in cucurbits including pumpkin and is transmitted by plant sap and aphids in a non-persistent manner (Lovisolo, 1981) [7]. Symptoms like mild to severe mosaic, mottling, reduced leaf size with deformed leaves and malformed fruits have been reported from pumpkin crop infected by CMV; which has become a serious threat to farmers worldwide (Khan and Begum, 2015; Nagendran *et al*., 2018) [3, 8]. In Assam, one of the major constraints in pumpkin production is the viral diseases due to which the crop suffers a huge economic loss. Although several viruses infecting cucurbits have been reported from different parts of India, however, there is limited information available regarding incidence of viral problems in pumpkin crops from Northeast India. The aim of the research was to determine the incidence of pumpkin mosaic disease and molecular characterization of the virus isolate from Assam.

2. Materials and Methods
2.1 Collection of samples and serological assay
The survey was conducted during different growing seasons of pumpkin crops from 2019 to 2021. Leaves from both symptomatic and asymptomatic pumpkin plants were collected randomly from farmer's fields of five districts of Assam; *viz.,* Jorhat, Golaghat, Karbi Anglong, Biswanath and Sivasagar districts. The leaves and twigs collected in the farmer's field were examined for virus symptoms. The collected leaf samples were stored at -20°C in the laboratory for further serological test; *viz.,* Double antibody sandwich enzyme-linked

immunosorbent assay (DAS-ELISA) using DAS-ELISA kit, Bio Reba, AG, Switzerland with CMV specific polyclonal antibody as per the manufacturer's protocol. Plants were considered infected with CMV if the ELISA reading was more than five times the negative control. The higher the disease incidence, as well as the vector population the more severe symptoms, were observed in the surveyed locations. Three leaves of selected plants were examined for the presence of aphid vectors and an average of three plants in each plot were considered. Vector (*Aphis gossypii*) population was recorded as low (<10 aphids per leaf), medium (10-15 aphids per leaf) and high (>10 aphids per leaf). The disease incidence was determined by using the following formula:

$$\% \text{ incidence of pumpkin mosaic disease} = \frac{\text{No. of ELISA-positive samples}}{\text{No. of samples tested}} \times 100$$

2.2 Molecular assay

The total genomic RNA was extracted using modified protocol of Lodhi *et al*., 1994 [6] and Landolino *et al*., 2004 [4] from the leaf samples that tested positive in the DAS-ELISA followed by Reverse-Transcription Polymerase Chain Reaction (RT-PCR) using CMV-specific primers for further molecular characterization of the virus isolate from pumpkin crop. cDNA preparation was done by using the PrimeScript™ 1st strand cDNA synthesis kit (TaKaRa-6110A) as per the manufacturer's protocol. Specific primers; *viz.,* CMVS1 (F) (5´GCCACCAAAAATAGACCG3´) and CMVS2 (R) (5´ATCTGCTGGCGTGGATTTCT3´) targeting CP gene of CMV genome with an amplicon size of 593 bp were used for PCR. Amplifications were carried out using Emerald PCR master mix (Thermo Scientific) with initial denaturation of 4 min at 94 °C followed by 30 cycles of denaturation at 94 °C for 30 sec, 53 °C for 30sec, 72 °C for 40sec and final extension of 72 °C for 8 min. The amplified products were analysed in agarose gel (1.2%) electrophoresis to confirm the presence of CMV in the assayed samples.

2.3 Phylogenetic analysis

The CMV positive sample from Jorhat district screened through RT-PCR was sent to Eurofins Genomics India Pvt. Ltd. for partial sequencing and the consensus sequence was prepared by using Bioedit software. The similarity analysis with other isolates of the virus worldwide was done using NCBI Nucleotide Basic Local Alignment Search Tool (BLASTn). The MEGA X software was used for the phylogenetic analysis of the coat protein genes through ClustaL W in the software. Phylogenetic relationships among the aligned sequences were inferred using UPGMA method and evolutionary distance was calculated using the Maximum Composite Likelihood method with the distance data matrix bootstrap re-sampled 500 times.

3. Results and Discussion

Symptomatology revealed a variety of mosaic disease symptoms; *viz.,* mild to severe mosaic, mottling, small, crinkled and deformed leaves with vein clearing and vein banding in pumpkin crops of surveyed locations. The symptoms (Fig.1) were recorded as mild to severe mosaic, mottling and wrinkled leaves with malformed fruits. The initial symptoms were characterized by yellow-green mild mosaic patterns and mottling on the growing leaves of infected plants. The intermingling yellow-green patches enlarged rapidly to cover the entire leaf surface resulting in distorted leaves. Vein banding and chlorosis were also observed from the edge of the leaf which became comparatively thick, hard and rough in appearance. There was reduction of fruit size in severely infected plants and malformation of fruits with mosaic patterns on the surface. Plants infected early in the season remain stunted and fruits became unmarketable due to pronounced rugosity and chlorotic spots on the fruit surface.

3.1 Vector population count and disease severity in the surveyed areas

The presence of aphid vectors; *viz., Aphis gossypii* and *Myzus persicae* on the pumpkin plants of surveyed locations were examined. The survey revealed severe disease symptoms as well as presence of high aphid vector population in Golaghat and Sivsagar districts; whereas, Biswanath district showed a low vector population with mild symptoms. In Jorhat district and Karbi Anglong the incidence of the vector and symptoms were in moderate range (Table 1).

Table 1: Symptom severity and aphid vector population on pumpkin in surveyed locations

Location	Symptoms observed	Aphid vector population
Jorhat	**	++
Golaghat	***	+++
Karbi Anglong	**	++
Sivsagar	***	+++
Biswanath	*	+
[(*) = Mild, (**) = Moderate, (***) = Severe, (+) = Low, (++) = Medium, (+++) = High]		

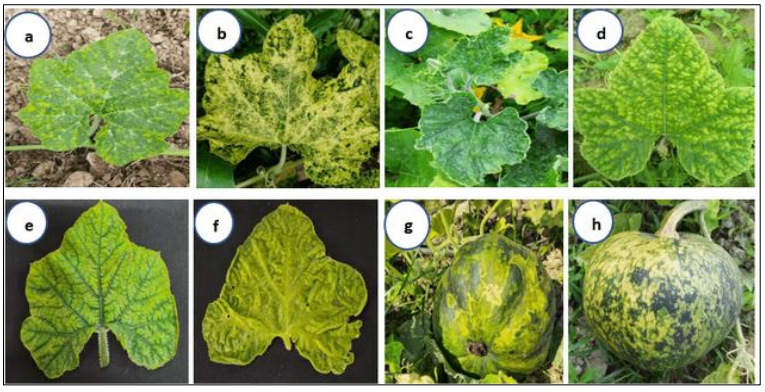

Fig 1: Characteristic symptoms observed on pumpkin crops from the surveyed locations, a. Mild mosaic, b. Severe mosaic on leaf, c. Mosaic and mottling, d. Chlorosis, e. Vein banding, f. Distorted and wrinkled leaf, g. Malformed fruit and h. Chlorotic spots on fruit.

3.2 Disease Incidence through DAS-ELISA

DAS-ELISA with CMV-specific polyclonal antibody was performed on 84 samples from two districts; *viz.*, Jorhat and Golaghat, out of which 37 samples showed positive reactions. A disease incidence of 35.71 percent was recorded for Jorhat and 52.38 percent for Golaghat.

Table 2: Pumpkin mosaic disease incidence through serological assay

Location	No. of samples tested	No. of positive samples	Disease incidence (%)
Jorhat	42	15	35.71
Golaghat	42	22	52.38

3.3 Molecular detection through RT-PCR

DAS- ELISA positive samples from Jorhat districts subjected to RT-PCR assay using specific primers for coat protein gene of CMV, resulted in amplification of desired 593 base pair products in agarose gel electrophoresis (Fig. 2) which confirmed the presence of the virus in the samples and further phylogenetic analysis was done.

3.4 Phylogenetic analysis

The virus isolate from Jorhat was designated as CMV-CP (Jorhat: Pumpkin) isolate. The partial sequencing and sequence analysis of the amplicon showed that the virus is closely related to CMV. The sequenced partial genome of the CMV isolate from Jorhat district of Assam (Table 3) was submitted under the Accession No. OL672234 in NCBI Gene Bank. After preparation of the consensus sequences for the CMV Jorhat isolate, similarity percentage of the sequence was checked with other isolates of the virus using Nucleotide Basic Local Alignment Search Tool (BLASTn) programme of National Centre for Biotechnology Information (NCBI) (Table 4). Similarity analysis of CMV-CP Jorhat: Pumpkin isolate of Assam showed maximum of up to 99.16% sequence homology with different worldwide isolates of CMV. The phylogenetic analysis of CMV Jorhat isolate was constructed by selecting twenty-two isolates of the virus from India as well as different countries worldwide; from NCBI website and the phylogenetic tree (Fig. 3) was generated using the sequence data representing the evolutionary relationship between the CMV-CP Jorhat isolate with worldwide isolates. Numbers at each node indicate bootstraps. The phylogenetic analysis of the CMV-CP Jorhat pumpkin isolate showed high sequence homology (93.11 to 99.16 percent) with other worldwide isolates. The analysis showed that the CMV-CP Jorhat pumpkin isolate belonged to a bigger cluster containing the majority of the CMV worldwide isolates whereas the Indian isolates were scattered in a different cluster.

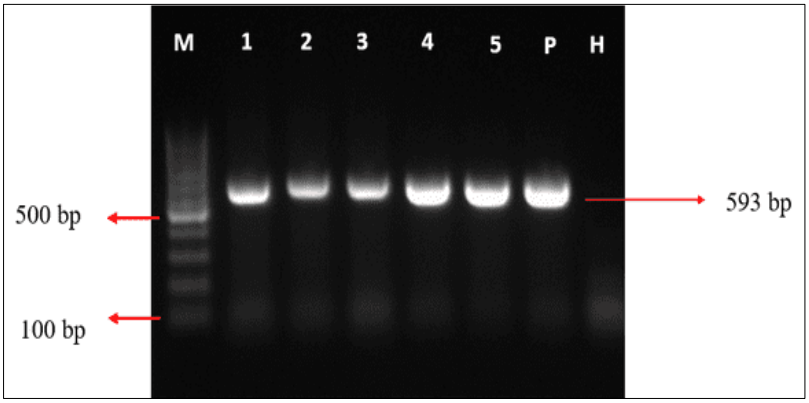

Fig 2: Agarose gel electrophoresis showing amplification of RT-PCR product. M= 1kb Ladder, 1-5=Amplified RT-PCR product of size 593 bp, P=Positive control, H=Healthy sample

Table 3: Partial genome sequence of the CMV-CP (Jorhat: Pumpkin) isolates from Assam CMV pumpkin consensus sequence

CMV-CP JORHAT: PUMPKIN (593 bp)
>AGTGAACGCTGTAGACCTGGGTACACGTTCACATCTATAACCCTTAAGCCTCCGAAAATAGACCGCGGGTCTTATTATGGTAAA
AGGTTGTTGCTTCCTGATTCAGTCACTGAGTTCGATAAGAAGCTTGTTTCGCGCATTCAAATTGGAGTTAATGATTGGCCGAAATT
TGGTTCTACCGTGTGGGTGACAGTCCGTAAAGTTCCTGCCTCCTCGGACTTATCCGTTGCCGCCATCTCTGCTATGTTTGCGGACG
GAGCCTCACCGGTACTGGTTTATCAGTACGCCGCATCTGGAGTCCAAGCCAACAACAAGTTGTTGTATGATCTTTCGGCGATGCG
CGCTGATATTGGCGACATGAGAAAGTACGCCGTCCTCGTGTATTCAAAAGACGATGCGCTCGAGACGGACGAGCTAGTACTTCA
TGTCGACATTGAGCACCAGCGTATTCCCACATCTGGGGTGCTCCCAGTCTGAACCAGTGTTTTCCCAGAACCCTCCCTCCGATTTC
TGTGGCGGGAGTTGAGTTGGTAGTGTTGCTATAAACTGCCTGAAGTCACTAAACGCTTTGCGGTGAACGGGTTGTCCATCCAG

Table 4: Sequence similarity of CMV-CP (Jorhat: Pumpkin) isolate of Assam

SL. No.	CMV worldwide isolates	Maximum identity (%) with CMV-CP Jorhat isolate	Accession number
1.	CMV isolate of China (Host: Cucumber)	99.16	MF038793.1
2.	CMV isolate of China (Host: Mustard)	99.16	MF038792.1
3.	CMV isolate of China (Host: Mustard)	99.16	MF038795.1
4.	CMV isolate of India (Host: Banana)	98.82	AY125575.1
5.	CMV isolate of India (Host: Mint)	96.28	MK482378.1
6.	CMV isolate of Iran (Host: Radish)	94.28	LC066464.1
7.	CMV isolate of India (Host: Tomato)	94.11	GU111229.1
8.	CMV isolate of China (Host: Banana)	93.95	KT302202.1
9.	CMV isolate of India (Host: Banana)	93.95	EF153734.1
10.	CMV isolate of Italy (Host: Tomato)	93.95	Y16926.1
11.	CMV isolate of Taiwan (Host: Cucumber)	93.95	D28780.1
12.	CMV isolate of Iran (Host: Radish)	93.94	LC066470.1
13.	CMV isolate of Iraq (Host: Cowpea)	93.94	MZ267789.1
14.	CMV isolate of Iraq (Host: Cucumber)	93.94	MW47781.1
15.	CMV isolate of Spain (Host: Muskmelon)	93.94	KX525737.1
16.	CMV isolate of Italy (Host: Chilli)	93.78	HE962480.1
17.	CMV isolate of China (Host: Pumpkin)	93.78	MF772327.1
18.	CMV isolate of Greece (Host: Melon)	93.60	AJ810265.1
19.	CMV isolate of India (Host: Bottle gourd)	93.27	KJ874250.1
20.	CMV isolate of India (Host: Snake gourd)	93.27	KJ874249.1
21.	CMV isolate of Thailand (Host: Cucumber)	93.11	AJ810264.1
22.	CMV isolate of Turkey (Host: Gherkin)	93.11	MN985117.1

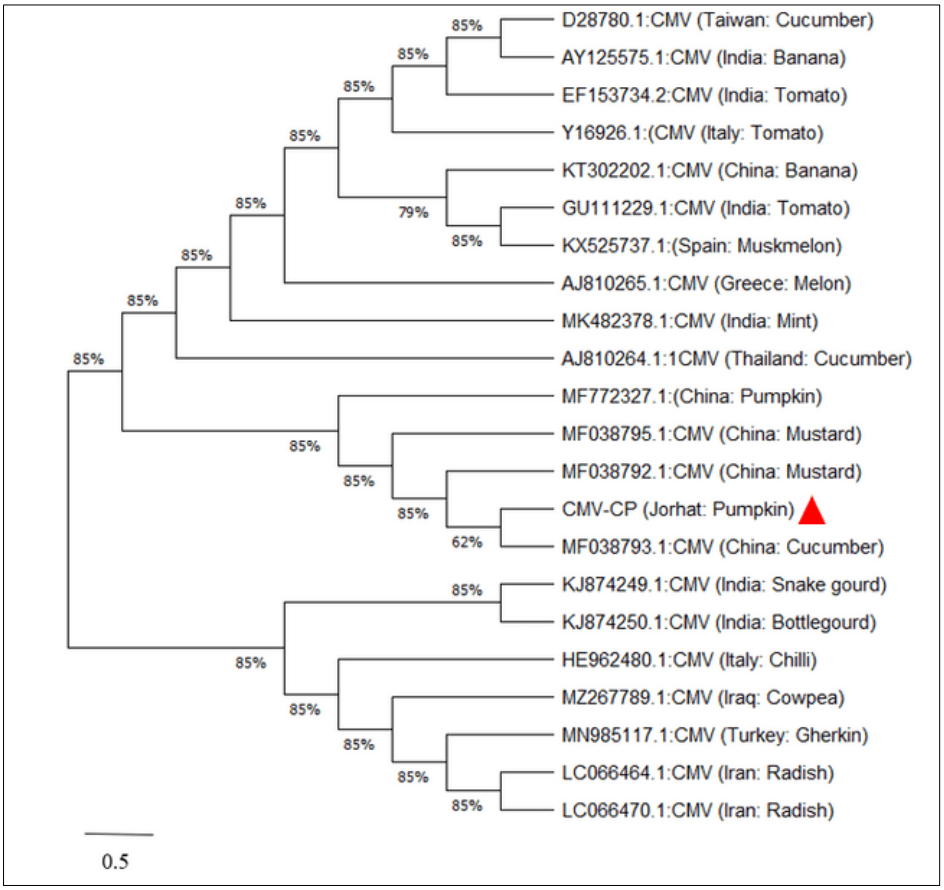

Fig 3: Phylogenetic relationship of CMV-CP (Jorhat: Pumpkin) isolate with worldwide isolates using UPGMA method. The scale bar indicates the evolutionary distances which were computed using the maximum composite likelihood method.

Conclusion

Detection of CMV in pumpkin crops of the surveyed areas indicated that the virus is widespread in the region. Symptomatology, presence of the aphid vectors and DAS-ELISA confirmed the presence of CMV causing mosaic disease of pumpkin in the surveyed locations. Molecular characterization of the CMV isolate from Jorhat district of Assam revealed coat protein sequence from the area which will pave way for further studies on the virus isolates from other pumpkin growing areas of Assam as well as formulating suitable management strategies to minimize economic loss.

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