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Evidence of Association of Begomovirus with the Yellow Vein Disease of an Ornamental Plant Pot Marigold (*Calendula officinalis*) from Western Uttar Pradesh

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Begomoviruses are among the most damaging pathogens causing epidemics in economically important crops particularly in tropical and subtropical regions. During February 2015, 20 samples of Calendula with yellow vein disease were collected from the campus of S. V. Patel University of Agriculture & Technology, Meerut, Uttar Pradesh, India. Total genomic DNA was isolated from the symptomatic and asymptomatic leaf samples and subjected to PCR using coat protein gene specific primer of begomovirus. The PCR amplification of ~770 bp was obtained from the 13 plants out of 20 collected plants. The PCR amplicon from coat protein gene was cloned, sequenced and submitted to GenBank, with accession number KT833850. The sequence data was further analyzed by BLAST analysis and phylogenetic tree was constructed using MEGA5.0 software which revealed close similarity of sequences with coat protein gene (AV1) components of other potato begomoviruses, which are all tentative strains of Tomato Leaf Curl New Delhi Virus (ToLCNDV). The result also indicated that Calendula spp. plants infected with Tomato Leaf Curl New Delhi Virus may act as an alternate host (reservoir) for other economically important plants.

Keywords: Begomovirus, PCR, ToLCNDV, *Calendula officinalis.*

Begomoviruses (genus Begomovirus, family *Geminiviridae*) are a group of plant viruses transmitted by the white ûy *Bemisia tabaci* (Aleyrodidae) to a large variety of cultivated and uncultivated plant species. They possess a circular single-stranded DNA genome encapsidated in twinned icosahedral virus particles (Rojas et al., 2005). Huge economic losses have been observed in India and other countries due to geminivirus infection in cotton (Briddon & Markham; 2000), tomato (Moffat; 1999), cassava (Thresh; 1998) and grain legumes (Verma; 1992). Begomovirus are an outsized varied family of plant viruses (Mansoor et al., 2003) which infects an expansive assortment of plants such as ornamentals, weeds and crops and causes a noteworthy loss to agriculture and horticulture worldwide (Lima et al., 2013). More than 80% of the known geminiviruses are

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transmitted by whiteflies and belong to the genus Begomovirus.Most of these viruses have bipartite genome designated as DNA-A and DNA-B and infect dicotyledenous plants. Ornamental plants are extensively scattered worldwide and have high environmental adaptability. Ornamental plants are considered as a foundation of new viruses and are considered as reservoirs of unidentified economically imperative viruses which are often neglected during virus diversity study (Urbino et al., 2013). Many scientific reports have demonstrated that ornamental plants serve as reservoir or alternative hosts for begomovirus survival (Raj et al., 2007) and spread in the absence of the main crops (Iiyas et al., 2013). Thus, there is a pressing need for additional information on the diversity and distribution of begomovirus in ornamental plants.

Calendula ofûcinalis L. (Asteraceae) is an important annual ornamental plant grown in gardens during the winter season and has an aesthetic beauty of bright yellow colored flower. It belongs to the family, Asteraceae, and is commonly known as Pot Marigold. The plant is native to Central and Southern Europe, Western Asia and USA. In addition, it has a considerable importance to the cosmetic/pharmaceutical industry because of it's used in the manufacture of antiseptic creams.

Plant viruses affect the aesthetic value of ornamental Calendula by reducing its rate of growth as well as the quality and quantity of its ûowers. *Calendula officinalis* plants have been found to be affected by Cucumber mosaic virus (Lisa & Della-Valle, 1979; Naqvi & Samad, 1985), Turnip mosaic virus (Lisa et al. , 1979) and Tobacco mosaic virus (Hristova et al ., 1994). A rosette disease transmitted by whiteûies and grafting was recorded on *C. ofûcinalis* by Gupta & Verma (1983). In the present investigation PCR and nucleic acid sequence based molecular identification and characterization of begomovirus associated with leaf yellow vein disease of *Calendula officinalis* was done.

MATERIALS AND METHODS

Sample Collection

The twenty *Calendula officinalis* plants showing vein yellowing, shortening of leaves and petioles, stunting of plants, reduction in growth, number and size of flowers and asymptomatic were randomly collected from the S. V. Patel University of Agriculture and Technology, Meerut, Uttar Pradesh during February 2015 - 2016. The samples were sealed in plastic zip bags and labelled to distinguish the identity of each sample and stored at -20ºC for further use.

DNA extraction, PCR and cloning

The total genomic DNA was extracted from symptomatic and asymptomatic leaves of calendula plant using CTAB method (Dellaporta et al., 1983). DNA extract was allowed for PCR based detection of virus using primers (TLCVAV1-F 5 ' C G A A C C G A C C A G C A G A T A T C A 3' and TLCVAV1-R 5'TTTGATGCAT GAGTACAGGCCA 3') from the CP region of leaf curl virus (Singh et al., 2013). PCR amplification were carried out using a MJ Mini, Bio- Rad thermal cycler in a 25 µl reaction containing 2.5 µl 10X PCR buffer, 0.5 µl, 25 mM $MgCl_2$, 2.5mM each dNTPs, 20mM 1.25 µl each primers, 0.1 µl Taq DNA polymerase (Merk Bioscience Pvt. Ltd., Bengaluru, India) and 2 µl template DNA. The DNA was amplified by initial denaturation of 94 °C for 10 min followed by 35 cycles of 94 °C for 30 s denaturation, 67 °C for 45 s, Primer annealing, 72 °C for the 1 min. primer extension and final extension at 72 °C for 10 min. The PCR amplicons obtained were electrophoresed through 0.8% (w/v) agarose gel in 1X TAE and visualized under UV light after staining with ethidium bromide (0.5 ug mL-1). PCR amplified products of expected sizes were purified (GeneJET Gel Extration Kit, Lithuania) and ligated into the pTZ57R/T vector (Fermentas, Arlington, Canada). The recombinant vector was transformed into *E.coli.* strain DH5a. Selected recombinant clones were screened by PCR using same set of primers as described earlier. Restriction digestion of plasmid DNA of recombinant clones was carried out to further confirm the presence of insert in the vector.

Nucleic acid sequencing and data analysis

The selected positive clones were sequenced at the automated DNA sequencing facility, Department of Biochemistry, Delhi University, South campus, New Delhi using M13 and T7 primers (Chromus Biotech Pvt. Ltd., India). The sequence data obtained after sequencing was then validated by performing BLAST [www.ncbi.nih.gov/BLAST] (Altschul et.al., 1990) analysis.

BLAST programme of NCBI was used to analyse the sequence data and to find related begomovirus sequences for *in-silico* analysis (Altschul *et al.,* 1990). Nucleotide and amino acid sequence homologies were viewed using Bioedit software 7.3. (Hall, 1999). Sequence alignments were produced using Clustal W programme (Thompson *et al.,* 1994). Phylogenetic trees were constructed based on matrices of aligned sequences using neighbour-joining algorithm of Mega 5.0 software (Tamura *et al*., 2011).

RESULTS

Detection of begomovirus

The symptoms of infected *Calendula ofücinalis* plants observed during field survey in campus of S. V. Patel University of Agriculture & Technology, Meerut were vein yellowing, shortening of leaves and petioles, and stunting of plants, reduction in growth, number and size of flowers (Fig. 1 A & B). To identify the begomovirus associated with the disease, PCR was performed using the total DNA of infected and healthy samples with a pair of primers specific to the coat protein (CP) gene of the genus Begomovirus (Singh 2013). The electrophoresis of PCR products on 0.8% agarose gel showed the expected size (770 bp) amplicons in infected samples only (Fig. 2 and 3). However, no such amplicons were obtained in healthy samples. The PCR amplicon was cloned and sequenced and the data obtained from clones was submitted to GenBank and Accession number KT833850 was assigned.

Fig. 1. A. Healthy Calendula plant; B. Naturally infected *Calendula officinalis* plant showing yellow vein net disease in field; a close view of leaf of infected plant showing severe yellow vein net symptoms

Sequence Analysis

BLAST search analysis of nucleotide sequence KT833850 revealed 95–98% sequence identity with various strains of Tomato leaf curl New Delhi virus (ToLCNDV) infecting Tomato (AB976527), Chilli (DQ029202), Pumpkin (JN129254), Luffa (HM989845), Poppy (KC513822), Potato (KC205270) from India and Black nightshade (AJ620187), Field bindweed (KC960492), Bitter gourd (AM747291) and Tomato (DQ116883) from Pakistan and KC207815 on Luffa from South Korea. Pair-wise alignment of nucleotide (nt) and deduced amino acid (aa) sequences of the CP gene of the virus isolate in study (Accession no KT833850) with CP gene sequences of selected ToLCNDV isolates from diverse plant species, and other begomoviruses was calculated using Bioedit programme. Pair-wise alignment of the virus isolate in study revealed the 95.3–98.4% identity at the nt level and 86.3 to 91.7% at aa level with various isolates of ToLCNDV (Table 1).

The highest identity was observed with several isolates of ToLCNDV reported from different geographical locations and different plant hosts such as tomato (AB976527), Chilli (GU831539), potato (KC205213), Tomato leaf curl New Delhi on Luffa (KC207815) from South Korea. Moreover, the phylogenetic analyses nucleotide sequences of the virus isolate showed a close relationship with various strains of

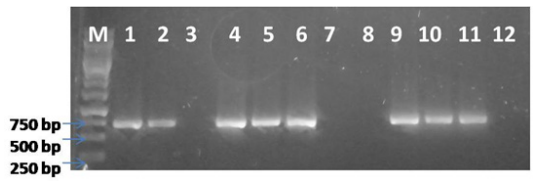

Fig. 2. Detection of ToLCV through PCR in infected calendula plants using TLCAV1-F/R. M- Molecular marker (1 Kb ladder), Lane – 1, 2, 4, 5, 6, 9, 10 & 11 : 770 bp DNA specific to coat protein region, Lane – 3, 7, 8, 12: no amplification

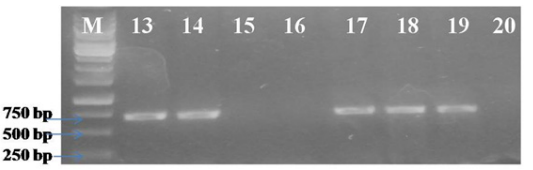

Fig. 3. Detection of ToLCV through PCR in infected calendula plants from different locations using TLCAV1-F/R. M- Molecular marker (1 Kb ladder), Lane – 13, 14, 17, 18 &19: 770 bp DNA specific to coat protein region , Lane – 15, 16 & 20- no amplification

ToLCNDV from tomato, poppy, luffa bitter guard etc. (Fig. 4).

DISCUSSION

Begomoviruses have been reported from different plant species in India. In the present study, initial survey for the incidence of yellow vein disease in calendula plants showed the 65% incidence (13 out of 20 plants) in PCR assay using coat protein (CP) gene primer pair (Fig. 2 and 3). The CP gene primers were reported in successfully detection of begomovirus by PCR in several plants species (Shorab et al. 2006; Singh et al., 2007; Tiwari et al., 2012, Singh et al, 2013). Similarly, Hallan (1998) reported begomovirus infection in tomato plants by extracting DNA followed by amplification of coat protein region using specific primer pair. The nucleotide sequence of virus isolate was BLAST searched to identify the homologous sequences in the NCBI databases and subjected to construct a phylogenetic tree tree using Mega 5 programme (Tamura et al., 2011). The coat protein gene of ToLCV isolates showed homology of more than 95-98% with all other reported geminiviruses coat protein gene sequences form different plat hosts (Table 1). The highest homology was observed with several isolates of ToLCNDV reported from different geographical locations and variety of plant hosts such as tomato (AB97657) and potato (KC205213).

The pairwise nucleotide sequence identity

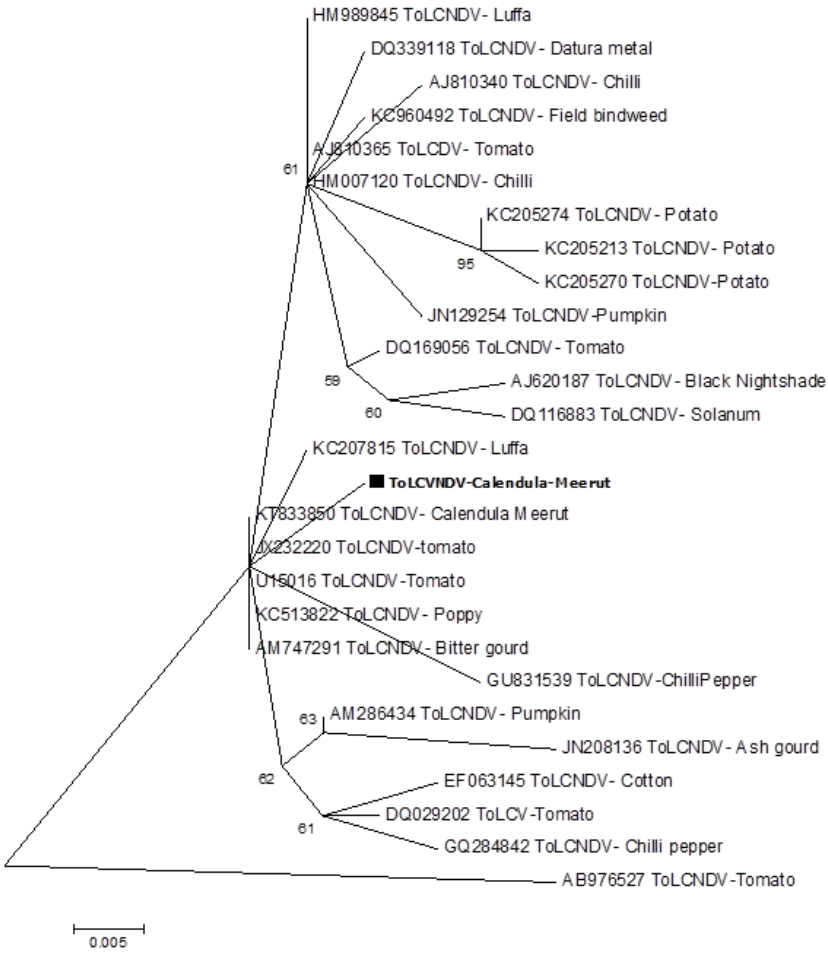

Fig. 4. Phylogenetic tree depicting the relationship of various begomoviruses based on alignment of coat protein gene nucleotide sequences. Tree was constructed using Mega 5 programme with 1000 bootstrap values

Table 1. Coat protein gene sequence analysis of the virus isolate (EF123060) based on the Basic Local Alignment Search Tool (BLAST) and sequence identity

Accession Number	Virus	Host Name	Origin	Nucleotide %	Amino acid %
AB976527	Tomato leaf curl New Delhi virus (AV1)	Tomato	India	98.4	86.3
DQ029202	Tomato leaf curl virus coat protein gene	Tomato	India	97	91
GU831539	Tomato leaf curl New Delhi virus isolate DHO1	Chilli	India	96.4	90.2
HM007120	Tomato leaf curl New Delhi virus pChTumB2 DNAA	Chilli	India	96.3	91.4
AJ620187	Tomato leaf curl New Delhi virus segment A	Black nightshade	Pakistan	96.3	90.2
KC960492	Tomato leaf curl New Delhi virus segment DNA A	Field bindweed	Pakistan	96.1	91
JX232220	Tomato leaf curl virus segment DNA-A	Tomato	India	96.1	91.7
JN129254	Tomato leaf curl New Delhi virus segment DNA A	Pumpkin	India	96	90.6
AM286434	Tomato leaf curl New Delhi virus- segment DNA-A	Pumpkin	India	96	91.4
DQ141676	Tomato leaf curl New Delhi virus - coat protein gene	Chilli pepper	India	95.9	91
HM989845	Tomato leaf curl New Delhi virus-JLX10	Luffa	India	95.7	91.4
GQ284842	Tomato leaf curl New Delhi virus- cp (AV1) gene	Chili pepper	India	95.7	90.6
KC207815	Tomato leaf curl New Delhi virus- at protein (AV1) gene	Luffa	South Korea	95.6	91.4
AJ810365	Tomato leaf curl virus AV1 gene for cp-isolate 26	Tomato	India	96.3	91.4
JN208136	Tomato leaf curl New Delhi virus segment DNA-A	Ash gourd	India	95.6	89.8
EF063145	Tomato leaf curl New delhi virus	Cotton	India	95.6	91
KC513822	Tomato leaf curl New Delhi virus-segment DNA-A	Poppy	India	95.4	91.7
AM747291	Tomato leaf curl New Delhi virus - complete sequence	Bitter gourd	Pakistan	95.4	91.7
KC205274	Tomato leaf curl New Delhi virus-isolate KAL-6 (AV1)	Potato	India	95.3	90.2
DQ339118	Whiteflytransmitted Indian begomovirus (AV1) gene	Datura metal	India	95.3	91
KC205270	Tomato leaf curl New Delhi virus- isolate JOR-2 (AV1)	Potato	India	95.3	89.8
U15016	Tomato leaf curl New Delhi virusMild coat protein	Tomato	USA	96.1	91.7
AJ810340	Tomato leaf curl virus AV1 gene for cp	Chilli	UK	95.7	90.6
DQ116883	Tomato leaf curl New Delhi virus	Solanum	Pakistan	95.7	90.2
DQ169056	Tomato leaf curl New Delhi virus segment DNA A	Tomato	India	95.4	91
KC205213	Tomato leaf curl New Delhi virusisolate HSM20	Potato	India	95.3	90.1

(Table 1) and phylogenetic study (Fig. 4) confirmed the causal agent of yellow vein net symptoms of *Calendula officinalis* as a strain of Tomato leaf curl New Delhi virus. Study also revealed that CP gene of present begomoviruses illustrates a high degree of sequence similarity and closeness with different important crop-infecting begomoviruses. However, virus isolate did not reveal a close identity to Cotton leaf curl Rajasthana virus (NC 003199) used earlier for cross hybridisation (Chatterjee et al. 2005) and with Mesta yellow vein mosaic virus (DQ298138) (Paul et al. 2006) (Raj et al. 2007). Yellow vein net disease of Calendula associated with Cucumber mosaic virus has been reported previously (Naqvi & Samad, 1985) which belongs to a plant pathogenic *virus* in the family Bromoviridae. The study of the CP gene of begomovirus complex isolates from northern India and their similarity with other important crop-infecting begomoviruses reveals the broadened host range in India.

Khan et al., 2005 observed yellow vein net disease on several *Calendula officinalis* plants in Aligarh and Lucknow, region of India with similar disease symptoms consisting of vein yellowing, shortening of leaves and petioles and stunting and the infecting virus isolate was identified as a Begomovirus. Khan et al., 2007 analyzed nucleotide sequence of coat protein gene from calendula which shared maximum identities of 96–97% with four strains of Tobacco curly shoot virus (ToCSV) and an Ageratum ernation virus (AgEV) during BLAST analysis of sequence data.

Similarily, Naturally infected plants showing yellow leaf vein netting symptoms accompanied by excessive yellowing and curling of leaves and stunting of the whole plant were reported on *Ageratum houstonianum* (Srivastava et al., 2015), *Amaranthus cruentus* (Raj et al., 2008), *Hibiscus cannabinus L* (Raj et al., 2007).

The geminivirus disease complexes have wide host range within dicots plants, including vegetables and fibre crops, ornamental plants and weeds indicating there is little, if any, natural resistance in their germplasm. Widespread distribution and diversity, coupled to the global movement of plant material and dissemination of the whitefly vector, suggest that geminivirus disease complexes pose a serious threat to tropical and sub-tropical agro-ecosystems worldwide (Mansoor et al., 2003). Hence, nucleotide sequence information of the viral genes can be used for developing suitable diagnostic techniques for detection of virus at early stage in plants. Moreover, cloned modified CP components can be used to develop transgenic potato against the virus disease.

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