RESEARCH ARTICLE Indian Journal of Agricultural Research, Volume 60 Issue 1: 135-139 (January 2026)

Molecular Characterization of Viruses Associated with Leaf Curl Symptoms on Capsicum (Bell Pepper) under Protected Cultivations in Dharwad, Karnataka

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10.18805/IJARe.A-5781

ABSTRACT

Background: Leaf curl or yellowing symptoms, typical of those caused by begomovirus infection, are commonly observed in capsicum (bell pepper) plants in polyhouses. Three capsicum samples with leaf curl and yellowing symptoms were collected from polyhouse at Hi-tech Horticulture, University of Agricultural Sciences, Dharwad, Karnataka, India, during 2017-2018.

Methods: Total nucleic acid was isolated from symptomatic and non-symptomatic samples by following CTAB (Cetyl trimethyl ammonium bromide) method and PCR amplified using degenerate and coat protein gene primers of chilli and tomato leaf curl virus. The virus was confirmed by gel electrophoresis with 0.8 per cent agarose. Amplified PCR product was eluted and sent to sequencing (Chromous biotech Pvt. Ltd., Bengaluru).

Result: The primer pair could amplify only *Chilli leaf curl virus* at ~500 bp but not *Tomato leaf curl virus*. Sequence homology of ChiLCV isolate form Dharwad matched with ChiLCV-Papaya-New Delhi (HM14036), ChiLCV-Chilli-Jodhpu (HM007104) and ChiLCV-Chilli-Noida (HM007114) respectively. The present study results showed that *Chilli leaf curl virus* is associated with the leaf curl and yellowing symptoms on capsicum under protected cultivation in Dharwad, Karnataka, India.

Key words: Begomovirus, *Chilli leaf curl virus,* Leaf curl, Polyhouse.

INTRODUCTION

Production of vegetables under protected cultivation system results in effective use of the land resources, besides being able to increase the production of quality vegetables both for the export and domestic markets by offsetting biotic and abiotic stresses to a great extent that otherwise is prevalent in open cultivation. Under protected cultivation, capsicum is widely grown due to higher productivity and economic feasibility (Anonymous, 2011). Observation showed that the production of this crop has been banned with viral infection. Viral diseases are the major limiting factors for successful pepper cultivation in the world (Francki *et al.,* 1979; Fujisawa *et al.,* 1986; Florini and Zitter, 1987). In order of importance are Geminiviruses.

Geminiviruses are plant viruses characterized by having single-stranded, circular DNA genomes of 2.5-3.0 kb. On the basis of genome organization, insect vector and host range, the family *Geminiviridae* is differentiated into seven genera (Varsani *et al.,* 2014) with Begomovirus being the largest genus. Symptoms are characterized by the curling and twisting of leaves followed by marked reduction in leaf size. The diseased plants look pale and stunted due to shortening of internodal length with more lateral branches resulting in a bushy appearance (Vasudeva, 1948). Members of the genus begomovirus are transmitted by whitefly (*Bemisia tabaci*) vectors. The morphology of geminivirus particles is unique and they are characterized by twin icosaheaderal capsid approximately 20×30 nm in size encapsidating a single molecule of covalently closed circular single stranded DNA (ssDNA) genomes of 2500 to 3000 bp that replicate in the nuclei of the infected cells *via* a double stranded DNA (dsDNA) intermediate (Harrison and Robinson, 1999; Verma and Malathi, 2003).

Polymerase chain reaction (PCR) using specific or degenerate primers have proved to be a rapid, accurate and efficient method of detecting and determining genetic diversity among geminiviruses (Aref *et al.,* 1994). Sequencing

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How to cite this article: Channakeshava, C., Patil, M.S., Gurupad, B., Moger, N.B. (2026). Molecular Characterization of Viruses Associated with Leaf Curl Symptoms on Capsicum (Bell Pepper) under Protected Cultivations in Dharwad, Karnataka. *Indian Journal of Agricultural Research*. **60(1):** 135-139. doi: 10.18805/IJARe.A-5781.

Submitted: 30-03-2021 **Accepted:** 14-06-2021 **Online:** 09-08-2021

of PCR fragments has contributed to the classification and phylogeny of geminiviruses (Rojas, 1992). The DNA genome of geminiviruses can be easily detected by nucleic acid hybridization visualizing geminiviral DNA-labeled digoxigenin probes (Gilbertson *et al.,* 1991). This paper aimed to identify and characterize the viruses associated with leaf curl symptoms on capsicum which are prevalent in protected cultivations at Dharwad, Karanataka, India.

MATERIALS AND METHODS

Sample collection

Leaves of capsicum plant showed typical leaf curl symptoms like, upward curling of leaf margins, yellowing, reduced lamina and short internodes were collected from Plants in a Poly-house at Hi-tech Horticulture, University of Agricultural Sciences, Dharwad, Karnataka, India, during 2017-2018. Samples were kept in cool boxes after collecting from the field and stored at -80°C and further used for DNA extraction.

Total DNA extraction and PCR detection

Total DNA extraction protocol suitable for small amount of plant tissues was followed to isolate total DNA from leaf curl and healthy leaf samples (as negative control) (Rouhibaksh *et al.,* 2008) with little modifications. Total DNA extracted was used as a template in polymerase chain reaction (PCR) to detect the occurrence of *Begomovirus*. Degenerate primers were used for detecting begomovirus are (BegomoF 5′ACGCGTGCCGTGCTGCTGCCCCATTGTCC 3′ and BegomoR 5′ ACGCGTATGGGCTGYC GAAGTTSAGAC3′), further coat protein gene specific primers of *Chilli leaf curl virus* (ChiLCV) and *Tomato leaf curl virus* (ToLCV) were used for confirming the begomovirus species (Abdul Kareem and Byadgi 2017).

PCR reaction was carried out in 20 µl volumes, which contains 1.0 µl (25 ng) of capsicum genomic DNA, 1.0 µl (2.5 pmole) of forward and reverse primers each, 1.0 µl (2.0 mM) of dNTPs, 2.0 µl of Taq buffer (10X), 1.0 µl of MgCl2 (25 mM) and 1 units of Taq polymerase. All the chemicals and plastic wares used were obtained from Genei and Tarsons respectively. The thermal cycler conditions for virus gene amplification involve 1 cycle of DNA denaturation at 94°C for 5 min followed by 35 cycles each having a denaturation at 94°C for 60 sec. annealing (71°C begomovirus, 65°C ChiLCV and ToLCV) for 60 seconds and a primer extension at 72°C for 60 sec. followed by final extension of 72°C for 10 min. The PCR products was resolved in 1.0 per cent agarose gel electrophoresis in 1X TAE buffer and an aliquot of 100ng of 100 bp DNA ladder (Himedia) used as molecular size marker and visualized by ethidium bromide staining.

Phylogenetic and homology analysis

Amplified PCR product was eluted (PCR Elution Kit, Quiagen) and sent to sequencing (Chromous biotech Pvt. Ltd., Bengaluru). The sequence results were analyzed using BLAST (Basic local alignment search tool) algorithm available at www.ncbi.nlm/gov. Phylogenetic tree was constructed by using MEGA 5.1 (Molecular evolutionary genetics analysis) software.

RESULTS AND DISCUSSION

Sampling and PCR amplification

Virus infected plants of capsicum collected from the Hi-tech horticulture polyhouse showed yellowing and leaf curling symptoms (Fig 1). The plants in early stage showed stunting and the production decreased. The relationship of a begomoviruses with the symptomatic capsicum was confirmed by PCR using begomoF/BegomoR primers as degenerate primers for begomoviruses. The symptomatic three samples gave bands of the expected size (~200 bp) (Fig 2a). Same total DNA was used to carryout PCR for coat protein gene specific primers of *Chilli leaf curl virus* and *Tomato leaf curl virus.* Upon amplification by PCR, only two symptomatic samples were amplified at ~500 bp size for *Chilli leaf curl virus* (ChiLCV-Capsicum-Dharwad) and no amplification for *Tomato leaf curl virus* coat protein gene specific primers (Fig 2b). It was clear from the results that begomovirus like *Chilli leaf curl virus* is associated with leaf

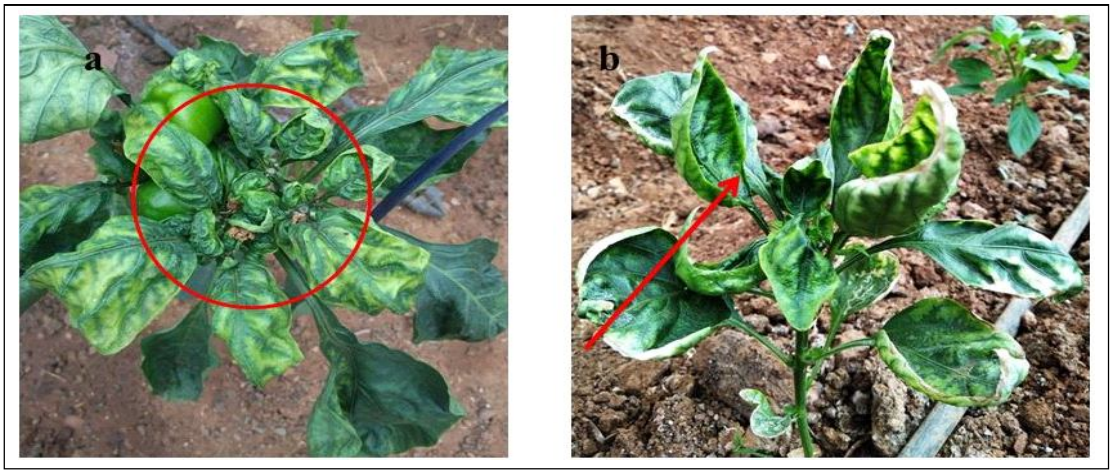


Fig 1: a and b, Leaf curl symptoms like, up word curling, puckering, reduced leaf size and stunted growth.

curl symptoms capsicum under protected cultivation in Dharwad.

Sequences analysis

Sequencing of PCR products carrying CP-genes of (ChiLCV-Capsicum-Dharwad) with oligonucleotide primers was carried out at Chromous Biotech Pvt. Ltd. Bengaluru. The results revealed ~500 bp long nucleotide sequence ChiLCV-Capsicum-Dharwad isolate was compared using BLAST programme of NCBI. The results of the BLAST search performed to identify sequence homology, clearly demonstrated that the coat protein sequence of ChiLCV matched 97-99 per cent with previously reported CP-gene sequences from different geographical locations (Table 1). Based on sequence comparison, isolate was confirmed as *Chilli leaf curl virus.*

Cluster analysis grouped the ChiLCV-Capsicum-Dharwad isolates into two main clusters with similarity co-efficient ranged from 0.000-0.015 indicating a good level of diversity. Grouping of the eighteen isolates on the basis of nucleotide relationship resulted in two major divergent groups I and II. ChiLCV-Capsicum-Dharwad infecting capsicum in Dharwad locality formed cluster I having 15 per cent diversity with cluster II. ChiLCV infecting capsicum in Dharwad found to be closely related to DNA-A of ChiLCV-Papaya-New Delhi (HM14036), ChiLCV-Chilli-Jodhpu (HM007104) and ChiLCV-Chilli-Noida (HM007114) respectively (Fig 3).

Table 1. BLAST results of nucleotide sequence of coat protein of *Chilli leaf curl virus*-Dharwad isolate.

Accession no.	*Chilli leaf curl virus* strains	Host	Origin
KJ649706	*Chilli leaf curl India virus*, DNA-A	Tomato	Sonipat, India
LN886660	*Chilli leaf curl virus,* DNA-A	Chilli	Pakistan
KF515609	*Chilli leaf curl virus*, DNA-A	Tomato	India
HM14036	*Chilli leaf curl virus*, DNA-A	Papaya	New Delhi India
DQ376037	*Papaya leaf curl virus* Coat protein gene	Papaya	India
KY800906	*Papaya leaf curl virus* isolate India, DNA-A	Papaya	New Delhi, India
JN663846	*Chilli leaf curl virus*, DNA-A	Chilli	AhmedabadIndia
HM007114	*Chilli leaf curl virus*, DNA-A	Chilli	Noida, India
HM007104	*Chilli leaf curl virus*, DNA-A	Chilli	Jodhpur India
FM179613	*Chilli leaf curl Multan virus*, segment A	Potato	Pakistan
DQ629103	*Papaya leaf curl virus*, DNA-A	Tomato	New Delhi, India
KY420149	*Pepper leaf curl Bangladesh virus* , DNA-A	Chilli	Pakistan
MF488985	*Chilli leaf curl virus*, DNA-A	Okra	Ludhiana,India
HM007100	*Chilli leaf curl Multan virus*, DNA-A	Chilli	Guntur, India
MH475358	*Chilli leaf curl virus,* DNA-A	Scrub nettle	Oman
DQ376039	*Papaya leaf curl virus,* Coat protein gene	Papaya	Gujarat, India
MF574143	*Chilli leaf curl India virus,* DNA-A	Papaya	Meerut, India
DQ629103	*Papaya leaf curl virus*, DNA-A	Tomato	New Delhi, India

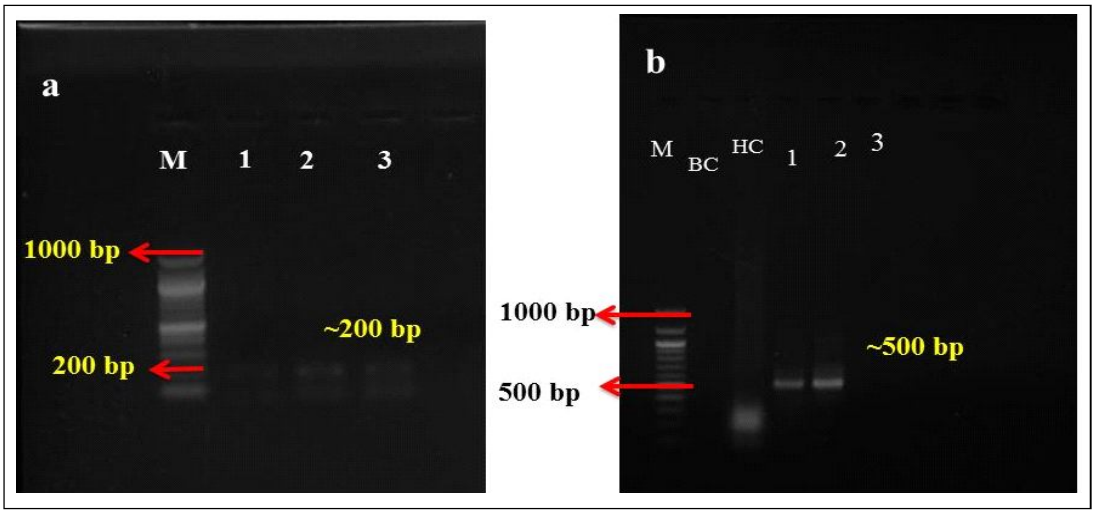


Fig 2: (a) PCR amplification for degenerated universal begomovirus primers M: 100 bp DNA ladder, 1, 2, 3 infected samples (b) PCR amplification of CP gene of ChiLCV infected capsicum samples M: 100 bp DNA ladder, BC: buffer control, HC: healthy control 1, 2, ChiLCV infected samples.

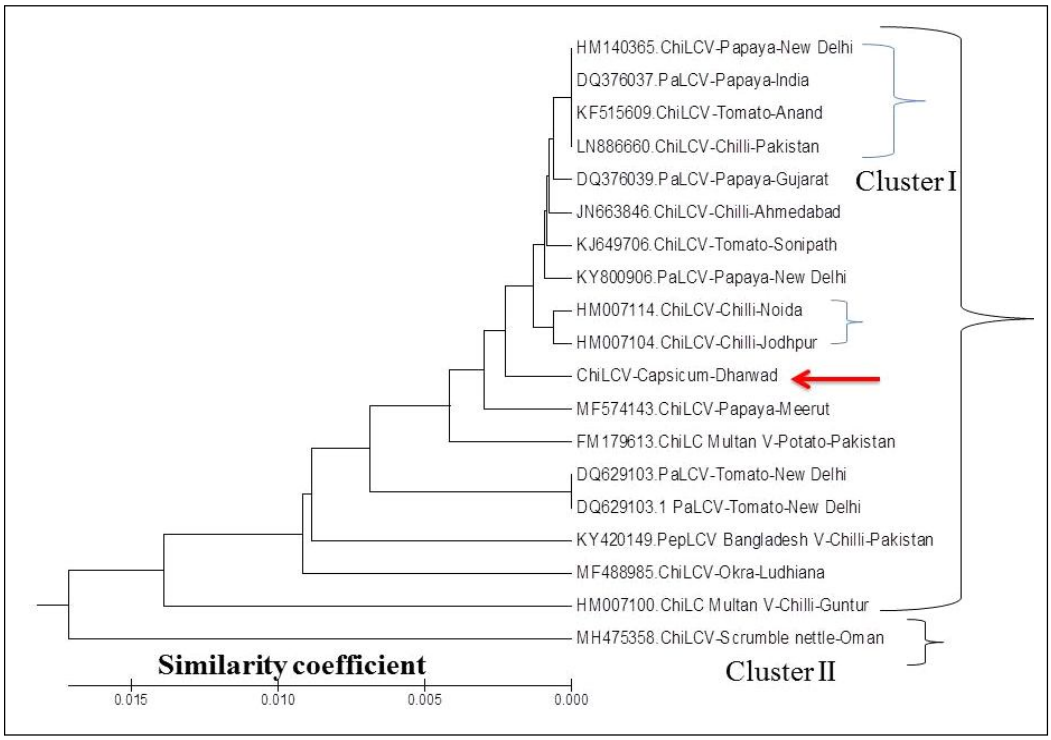


Fig 3: Phylogenetic relationship of CP gene of ChiLCV-Capsicum-Dharwad with other isolates.

The present study demonstrates the identification of begomovirus infecting capsicum under protected conditions at Dharwad, Karnataka, India, through molecular studies. There are no such reports of *Chilli leaf curl virus* infecting capsicum under protected cultivations from Dharwad, Karnataka. Kumar *et al.* (2011) found that chilli plants exhibiting leaf curl symptoms, collected from Palampur, Himachal Pradesh region of India, were associated with a Begomovirus and a betas atellite-like molecule. The nucleotide sequence of the Begomovirus genome shared maximum identity (89%) with *Pepper leaf curl Bangladesh virus*-India isolate Chhapra (PepLCBV,) Sequence analysis showed that the Begomovirus is a potential recombinant between viruses related to PepLCBV and *Chilli leaf curl virus* (ChiLCV) by Khan and Khan (2016). Hussain *et al.* (2004) reported that the presence of ToLCNDV in chilli (pepper), plants from several locations in the Punjab province of Pakistan and collected samples to confirm infection of ToLCNDV, a PCR procedure was carried out using degenerate Begomovirus.

CONCLUSION

Based on sequence comparisons and phylogenetic analysis, ChiLCV (current study) revealed high degree of similarity (~97-99%) to the begomovirus species illustrated during this study. Therefore to our knowledge, this is the first report of ChiLCV isolate associated with leaf curl symptoms on capsicum (bell pepper) under protected cultivations in Dharwad, Karnataka, India.

ACKNOWLEDGEMENT

The authors are great full to advisory committee members and department of Plant Pathology, University of Agricultural Sciences, Dharwad, Karnataka for providing facilities to conduct research.

Conflict of interest

The authors declare that they have no conflict of interest.

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