International Journal of Bio-resource and Stress Management https://ojs.pphouse.org/index.php/IJBSM

Crossref

IJBSM April 2024, 15(4): 01-07 **Article** AR5197

Research Article *Stress Management*

DOI: HTTPS://DOI.ORG/10.23910/1.2024.5197

Screening of Beta Satellite Associated DNA-A Chili Leaf Curl Virus from Maharashtra

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ABSTRACT

The experiment was conducted during October–November 2023 at Vasantrao Naik College of Agricultural Biotechnology (Dr. PDKV, Akola), Waghapur Road, Yavatmal (445001), Maharashtra, India. The Chilli leaf curl disease (ChiLCD) has become a major disease of Chilli (*Capsicum annum* L.) throughout India. The present study aimed at precise identification and molecular characterization of Chilli leaf curl virus (ChiLCV) as it is responsible for a serious menace by causing significant loss of yield in various chilli growing districts of Maharashtra. The ChiLCD affected chilli plants were showing various symptoms such as leaf curl, smaller leaves, stunting of a plant with a smaller number of flowers and fruits. During rabi season (October 2022–February 2023) of 2022–23 sample surveys were conducted and infected samples from different regions were collected from affected fields and subjected to a polymerase chain reaction (PCR) using coat protein (CP), DNA-A, DNA-B and betasatellite specific primers. Out of 9 samples taken for the study 6 samples were amplified with 520 bp size amplicon corresponding to CP gene. The 520 bp amplicon were gel eluted and sequenced. Database searches were done by using BLAST analysis in which sequence revealed 99% similarity with ChiLCV isolates existing in GenBank database. Sequence analysis of the CP gene revealed the identity of ChiLCV associated with the ChiLCD of chilli crop in Maharashtra, India. Moreover, the phylogenetic analysis suggested the clustering of these isolates from two different regions within the same clade were found to correspond to the ChiLCV begomovirus belonging to the family Geminiviridae. The PCR results indicated that ChiLCV from Maharashtra governs monopartite DNA-A genome along with betasatellite molecule.

KEYWORDS: Chilli, leaf curl, coat protein, *Begomovirus, Geminiviridae*

***Citation* (VANCOUVER):** Shingote et al., Screening of Beta Satellite Associated DNA-A Chili Leaf Curl Virus from Maharashtra. *International Journal of Bio-resource and Stress Management,* 2024; 15(4), 01-07. HTTPS://DOI.ORG/10.23910/1.2024.5197.

Data Availability Statement: Legal restrictions are imposed on the public sharing of raw data. However, authors have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

Conflict of interests: The authors have declared that no conflict of interest exists.

RECEIVED on 02nd February 2024 RECEIVED in revised form on 28th March 2024 ACCEPTED in final form on 17th April 2024 PUBLISHED on 26th April 2024

1. INTRODUCTION

Chilli leaf curl disease is the most important constraint on Chilli production in India (Roy et al., 2023). Chilli (*Capsicum annum* L.) is an important high commercial value cash crop grown all over globe (Verma et al., 2022). Chilli leaf curl disease (ChiLCD) is caused by a cluster of related begomovirus species including *Geminiviridae* family (Jeyaraj 2023). ChiLCV from Jodhpur (Senanayake et al., 2007), Palampur (Kumar et al., 2011), Delhi (Agnihotri et al., 2018), *Tomato leaf curl* New Delhi virus (Khan et al., 2006), *Tomato leaf curl* Joydebpur virus (Shih et al., 2007) has been reported from India and also other countries like Sri Lanka (Senanayake et al., 2013) Oman (Khan et al., 2013). The ChiLCD affected chilli plants displays typical symptoms like leaf curling, leaf distortion, leaf crinkling, yellow leaf margins etc. ChiLCD causes up to 100% yield reduction through severe stunting and abortion of flowers and fruits (Borah and Dasgupta, 2012). The size of leaves and branches are reduced noticeably in severely affected plants ensuring a bushy exterior where, such plants bear only a few flowers and fruits (Kumar et al., 2012; Kumar et al., 2015).

ChiLCV is exclusively transmitted through whitefly (*Bemisia tabaci*) in a persistent-circulative manner (Nalla et al., 2023). In tropical and sub-tropical regions whiteflies are regarded as one of the most invasive and damaging species which transmit leaf curl viruses in chilli, tomato and other crops (Shingote et al., 2022; Ning and Bradley, 2015). In recent past, ChiLCV is appeared to expand their natural host range to other important hosts such as sweet pepper, chilli pepper, tomato, common bean, lisianthus and tobacco (Chattopadhyay et al., 2009; Borah and Dasgupta, 2012). Recently, epidemics of ChiLCD have been a serious problem observed in various chilli-growing regions of Maharashtra (Thakur et al., 2018). ChiLCD causes severe losses to chilli production that forced the farmers to completely abandon the chilli cultivation (Shingote et al., 2022). This pilgrim shift in chilli cultivation led to the drastic reduction in area and production of chilli in Maharashtra and central India.

Characterization of ChiLCV genome sequence from Jodhpur, Rajasthan exhibited its association with ChiLCD in India (Senanayake et al., 2007). Further, Chattopadhyay (2009) reported ChiLCV as a monopartite begomovirus. Monopartite begomoviruses are often associated with smaller DNA components, known as satellite DNAs (Kumar et al., 2015; Singh et al., 2016). There are two types of satellite DNAs namely alphasatellite and betasatellite, based on organization of their satellite DNA and their effects on the symptoms produced by the helper begomovirus (George et al., 2014; Biswas and Mandal, 2023). Alphasatellite and betasatellite both depend on helper virus for their replication and in many cases, suppress the symptoms produced by viruses (Idris et al., 2011). Kumar reported a begomovirus species associated with betasatellite from Palampur causing ChiLCD in chilli (Kumar et al., 2011). They reported that betasatellite molecule is essential for development of leaf curl symptoms whereas inoculation of only viral clone leads to stunting in chilli plants. Tahir characterized a distinct begomovirus from all previously known viruses and represents a geographically distinct, capsicum adapted, begomovirus–betasatellite complex (Tahir et al., 2010).

Although diverse virus-betasatellite complexes have been reported by various researchers in India but identification and characterization of begomoviruses associated with ChiLCD from Maharashtra and central India is hardly explored (Mansoor et al., 2003; Kumar et al., 2011; Kumar et al., 2015; Mishra et al., 2020). Thus, by keeping in mind the association of different begomovirus species with ChiLCD in India, the present study was undertaken to describe the association of a specific begomovirus with ChiLCD of chilli in Maharashtra.

2. MATERIALS AND METHODS

2.1. Sample collection

The experiment was conducted during October-November 2023 at Vasantrao Naik College of Agricultural Biotechnology (Dr. PDKV, Akola), Waghapur Road, Yavatmal (445001), Maharashtra, India. ChiLCD samples were collected from farmer's fields of district Buldhana (20.4561° N, 76.3637° E), Chandrapur (20.2095° N, 79.5603° E), Yavatmal (20.1170° N, 78.1108° E), and Jalna (19.6807° N, 75.9928° E) districts of Maharashtra, India for identification of causal agent. Surveys were conducted to observe the typical characteristic symptoms of ChiLCV in the field. The chilli fields exhibiting typical ChiLCD symptoms were examined and the leaf samples were collected for further detection study. The leaf samples were stored in the refrigerator at -20°C for its further use in different experiments. A total of 9 farmer fields, 5 from Vidarbha (two representative sample each from Chandrapur and Yavatmal, and one from Buldhana districts) and 4 from Marathwada region (Jalna district) were collected for the occurrence of the disease in chilli and recorded its incidence. For checking the severity of disease incidence ten fruits from infected as well as healthy control plants were collected in replicates. Among the surveyed places, the selected chilli plants which were affected due to ChiLCD and healthy controls were transplanted in experimental pots at Vasantrao Naik College of Agricultural Biotechnology, Yavatmal, Maharashtra to record the observations.

2.2. Total genomic DNA isolation

Total genomic DNA was extracted from the infected chilli leaf samples by method described by Porebski et al.

(1997). Quality and quantity of the isolated DNA samples was confirmed through agarose gel electrophoresis and Bio-Spectrophotometer, respectively (Eppendorf, AG, Germany).

2.3. Detection of ChiLCV by PCR

For detection of ChiLCV, PCR screening of nine chilli infected samples (five from Vidarbha region and four from Marathwada region) along with control uninfected chilli plant and water control as check were carried out using CP specific primer set (Table 1). Further the primer sets targeting DNA-A genome, DNA-B genome and betasatellite were used to explore the molecular nature of ChiLCV genome. For PCR amplification, master mix of 20 µl was prepared which comprised of 1.0 µl DNA template (30 ng μl^{-1}), 2.0 µl buffer with $MgCl_2$ (1X), 1.0 µl dNTPs (400 µM), 1.0 µl each forward and reverse primer (200 pg), 0.2 µl Taq DNA polymerase (1U), and 13.8 µl nuclease-free water to make up the volume. The temperature profile of PCR was conducted as: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, extension at 72°C for 90 sec and final extension at 72°C for 10 min. The amplified PCR products were resolved on 1.5%

Table 1: List of primers used in the present study

Sl. No.	Primer	Sequence	Targeted sequence	Amplicon size (bp)	Reference
1	1F 1R	5'GCCAATTTACAGAAAGCCAAGGAT 3' 5'GGAPTGGAGGCATGAGTACACGCC 3'	Coat protein	520	Wyatt and Brown, 1996
2	2F 2R	5'GGTACCACTACGCTACGCAGCAGCC 3' 5'GGTACCTACCCTCCCAGGGGTACAC 3'	Betasatellite	1300	Briddon et al., 2002
3	3F 3R	5' GCCCACATYGTCTTYCCNGT 3' 5'GGCTTYCTRTACATRGG 3'	DNA-A genome	1100	Rojas et al., 1993
4	4F 4R	5'GAGTTTCCGTTTGTGGAAGACCCGGAAGT 3' 5'CTAGACTTCGGTCTGTAG 3'	DNA-B genome	1120	Chowda et al., 2005

2.4. Sequencing and sequence analysis

The 520 bp amplicons obtained from the two infected chilli samples each representative from Vidarbha and Marathwada region (in triplicate) were purified by Gel Extraction Kit, Genei (GeNei®, Bangalore, India). The purified PCR products were quantified and sent for the Sanger dideoxy sequencing to the Eurofins Genomics India Pvt. Ltd. Pune. Sequences obtained were subjected to BLAST by using the nucleotide BLAST programme. BLAST was carried out against the non-redundant (nr) nucleotide database. Highly similar sequences with lowest expect value were considered for assigning the putative class to the new sequence. The phylogenentic tree was constructed using the ClustalW multiple alignment program by neighbour-joining method (1000 bootstraps) and this was conducted using MEGA X (Kumar et al., 2018).

3. RESULTS AND DISCUSSION

3.1. Symptomatology and field survey

The ChiLCD affected chilli plants were showing characterized different symptoms including reduction in leaf size, upward leaf curling, crinkling, puckering and stunting of plants in surveyed farmer's fields of Vidarbha and Marathwada regions of Maharashtra. The ChiLCD infected plants showed least fruiting while some plants were unable to bear fruits. The whole plant appears bushy and growth of plant stunted (Figure 1). However, minor variations in symptoms were observed based on that samples were collected from the different fields to verify the identity of the virus species.

In addition to this, the symptoms of ChiLCD including abaxial and adaxial curling of the leaves along with puckering

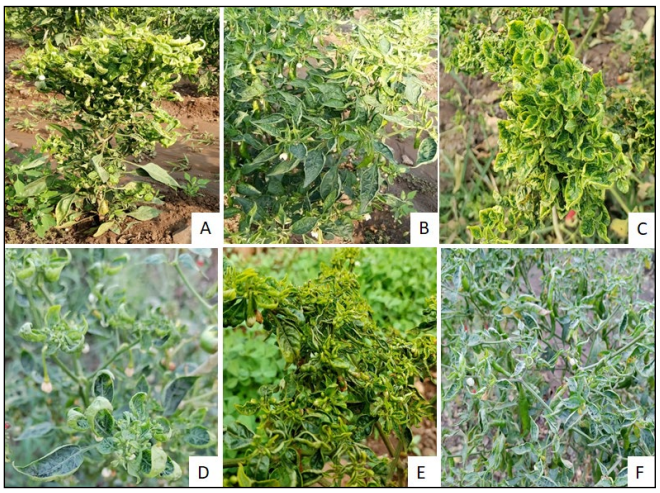

Figure 1: Symptoms of chili leaf curl disease (ChiLCD) affected chili plants. A: Reduced leaf size, B. Upward leaf curling, C. Blistering of interveinous areas and thickening and swelling of the veins, D. Upward rolling of leaves, E. Upward leaf curling with yellow leaf margin, F. Leaf curling

and blistering of interveinous areas and thickening and swelling of the veins. In advanced stages of the disease, reduced auxiliary buds produced in leaf clusters. Similar symptoms were reported and identified malformation disease of chilli in Vidarbha region of Maharashtra, India and noted three types of symptoms i.e. upward curling and crinkling, downward curling and mottling and crinkling and puckering (Moghe, 1977). Fewer flowers and fruits were developed on the diseased plants and the fruits formed were much reduced in size and curled at the styler end, reported earlier (Kumar et al., 2015). Puckering, upward curling and reduced size of leaves were also described (Senanayake et al., 2007). Typical crinkling, puckering, upward leaf curling and reduction in leaf area along with stunting of whole plants were observed (Kumar et al., 2011). Such infected plants were used for identification of ChiLCV.

Previous studies also reported the ChiLCD infected plants from 'Vidarbha' region developed fewer flowers and fruits (Kumar et al., 2015). For checking the severity of disease incidence ten fruits from infected as well as healthy control plants were collected in replicates. When we compared length of fruit, length of infected chilli fruits varied from 1 to 2 cm, whereas the length of healthy control samples ranged from 6 to 9 cm (Figure 2). Whereas, the average weight of 0.79±0.08 g was recorded in infected chilli fruits, as compared to 3.14±0.21 g in healthy control chilli fruits. In some of the ChiLCD affected plants showed no fruits developments which show the alarming threat of the disease to cause up to 100% yield losses in chilli. The results from the present study are in accordance with the previous studies (Borah and Dasgupta, 2012).

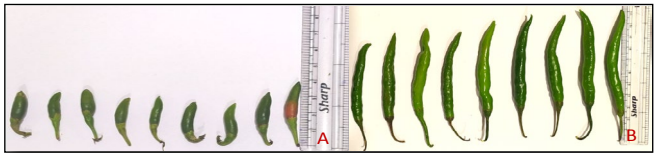

Figure 2: A. Chili fruits collected from ChiLCD affected plants, B. Chili fruits from non-infected, healthy plants

3.2. Detection of ChiLCV by PCR

Total genomic DNA was extracted from nine ChiLCD affected samples showing typical symptoms. Initial PCR screening was carried out using CP specific primer which showed amplification of expected size of 520 bp. Among the nine tested samples, six samples showed expected amplicon size of 520 bp, similar results were noted earlier (Thakur et al., 2019) (Figure 3). Out of the six PCR positive samples five were from Vidarbha (two representative sample from Chandrapur and Yavatmal, and one from Buldhana districts) and one from Marathwada regions (Jalna district) of Maharashtra. Despite repeating the PCR reactions there was no amplification in the remaining three samples. The

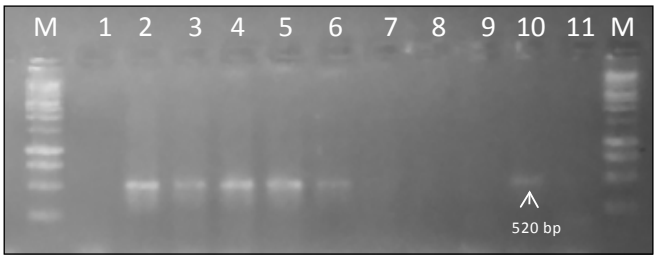

Figure 3: PCR screening of ChiLCD infected chili samples using primer 1F&R. M- 1 Kb DNA ladder, 1- Control uninfected chili plant sample, 2-6-infected chili leaf samples from Vidarbha region, 7-10-infected chili leaf samples from Marathwada region showing symptoms for ChiLCD, 11-Water control

probable cause behind non-amplification might be lesser number of viral loads, insect feeding or abiotic stresses. The selected positive samples were further screened to detect the presence of DNA-A, DNA-B and betasatellite genomic components. Each PCR reaction was repeated in triplicate to confirm the reproducibility of reaction. Selected four samples each representing respective district were successfully amplified using DNA-A and betasatellite specific primers, whereas there was no amplification in DNA-B specific primers (Figure 4). The PCR results confirmed the presence of DNA-A genome along with the betasatellite molecule. Despite several attempts there was no amplification using DNA-B specific primers, these results indicate DNA-B genome may be absent in Maharashtra isolates. Our findings were in line with previous researchers who reported ChiLCV as monopartite DNA-A genome associated with betasatellite (Kumar et al., 2011). As most of the monopartite begomoviruses are associated with a satellite molecule, which is essential for symptom development (Briddon et al., 2006). The PCR results confirmed the presence of DNA-A and betasatellite genomic components in both ChiLCV from 'Vidarbha' and 'Marathwada' regions of Maharashtra.

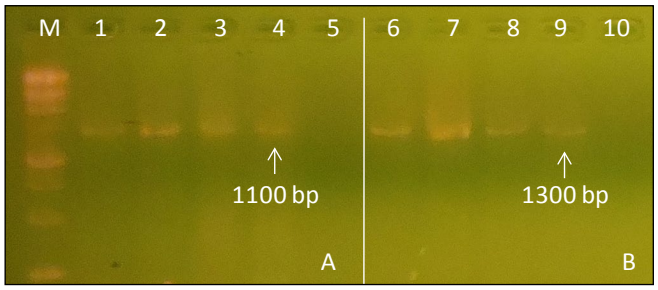

Figure 4: PCR amplification of ChiLCD infected chili samples using DNA-A and DNA-B genome specific primers; A. DNA-A specific primer 3F/5R, B. DNA-B specific primer 4F/R. M- 1 Kb DNA ladder, 1-4-infected chili leaf samples, 5- Control uninfected chili plant sample

3.3. Confirmation of ChiLCV using sequencing

For confirmation of associated virus species, out of six positive isolates confirmed with polymerase chain reaction, two representative samples each originating from Marathwada and Vidarbha regions were sequenced. The sequencing results of the two isolates showed 99.9% similarity with the ChiLCV CP genes over gene bank NCBI database. As sequence similarity was found to be very high therefore only one sequence was submitted to NCBI with accession No.MT129656.1. Based on the sequences, the isolates were found to be identical as BLAST results confirmed that this sequence represented a portion of a begomovirus DNA-A CP component. Sequence similarity results of two isolates suggested that the occurrence of same begomovirus species in 'Vidarbha' and 'Marathwada' regions of Maharashtra. The sequence information revealed 98% sequence identities corresponding to part of DNA-A genome at NCBI database ChiLCV. Based on the present criteria on the begomovirus species demarcation, both samples from different regions are similar isolates belonging to the same species. An alignment analysis of CP nucleotide sequence obtained from NCBI database through ClustalW program followed by phylogeny analysis by neighbour-joining method, exhibited highest percent similarity coverage within the leaf curl virus. *In silico* phylogenetic analysis of the Vidarbha and Marathwada isolate revealed, a phylogenetic tree branched into a same clade, and both sequences have close clustering to *chilli leaf curl virus* 210/2019 (AV1) (Figure 5). The results confirmed the association ChiLCV with the ChiLCD based on the two similar isolates originated from 'Marathwada' and 'Vidarbha' locations in Maharashtra. Thus, there is presence of same ChiLCV species in Maharashtra.

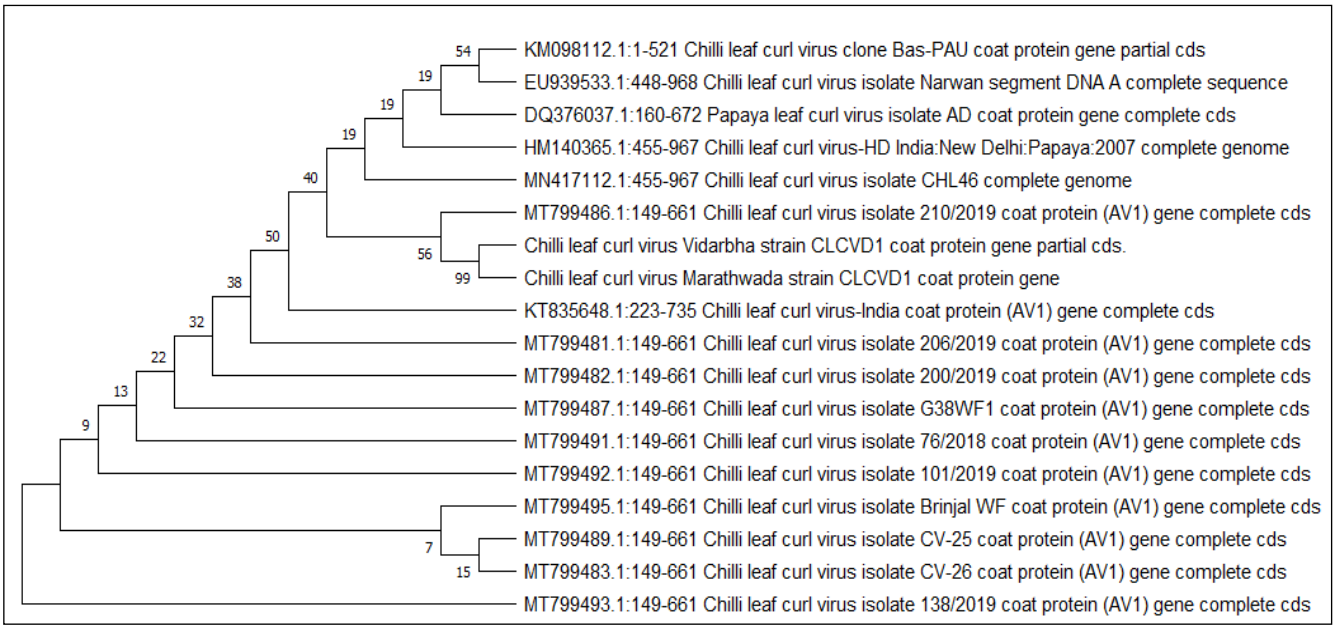

Figure 5: Phylogenetic tree of the coat protein sequence of Chilli leaf curl virus (ChiLCV) infecting chilli in Maharashtra. Bootstrap consensus tree of 17 ChiLCV and one papaya begomoviruses from DNA-A component nucleic acid sequences using the neighbor-joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 18 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 497 positions in the final dataset. Sequences were downloaded from GenBank. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018)

4. CONCLUSION

The PCR results indicated that ChiLCV from Maharashtra governs DNA-A genome along with beta satellite molecule. Though, limited samples were used to notice the occurrence of ChiLCD in Maharashtra of India, the primer set will be helpful in further studies for virus indexing of this species in chilli.

5. ACKNOWLEDGMENT

We thank Vice-chancellor, Dr PDKV, Akola for their continuous support and encouragement for this research. Our thanks are due for Dean (Agri.), Dr. PDKV, Akola for extending all the facilities to carry out the work.

7. AUTHOR'S CONTRIBUTION

Conceptualization of research work and designing of experiments (PRS, DLW and DRR); Execution of field/lab experiments and data collection (PRSand SMG); Analysis of data and interpretation (PRS and SJG); Preparation of manuscript (PRS and NDP).

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