DOI: https://doi.org/10.54393/fbt.v3i02.45

FUTURISTIC BIOTECHNOLOGY

https://fbtjournal.com/index.php/fbt

Volume 3, Issue 2 (Jul-Sep 2023)

Original Article

Detection of Cotton Leaf Curl Disease using Betasatellite-based Molecular Marker

Umer Sohail Meer[1*]

[1]International School Lahore, Pakistan

ARTICLE INFO

Key Words:

Betasatellites, Cotton Leaf Curl Disease, Begomoviruses, Geminiviruses, PCR

How to Cite:

Sohail Meer, U. (2023). Detection of Cotton Leaf Curl Disease using Betasatellite-based Molecular Marker: Detection of Cotton Leaf Curl Disease. Futuristic Biotechnology, 3(02). https://doi.org/10.54393/fbt.v3i02.45

***Corresponding Author:**

Umer Sohail Meer
International School Lahore, Pakistan
umersohailmeer101@gmail.com

Received Date: 11th July, 2023
Acceptance Date: 20th September, 2023
Published Date: 30th September, 2023

ABSTRACT

Cotton leaf curl disease (CLCuD) caused by whitefly-transmitted begomoviruses has hampered cotton production across the Punjab and Sindh provinces of Pakistan and northeastern India. Eight species of begomoviruses in association with a single betasatellite "Cotton leaf curl Multan betasatellite (CLCuMB)" have been reported to cause CLCuD. **Objective:** To detect early and efficiently Cotton leaf curl disease (CLCuD) using betasatellite-based molecular marker. **Methods:** 3-7 samples leaves were collected from symptomatic cotton fields in selected five areas of cotton production in Pakistan. Total DNA was extracted from collected leaves using the Cetyl trimethylammonium bromide (CTAB) method. Primers were designed by MUSCLE alignment tool and target region was amplified by PCR and amplification confirmed by performing gel electrophoresis. After DNA sequencing Phylogenetic analysis of the was carried out using software MEGA-X. **Results:** Amplified target region of483bp was observed by running 1% agarose gel. Comparison of DNA sequences revealed two nucleotide substitutions in DNA sequence from samples collected from Multan, Sakrand, Rahim Yar Khan and, while four nucleotide substations in sample collected from Vehari. High nucleotide substation in DNA sequence from Vehari as compared to other regions. **Conclusions:** In conclusion all of eight distinct begomoviruses causing CLCuD with CLCuMB is indicative of the fact that CLCuMB based molecular marker can be developed for detection of the disease. Early detection of disease will help the breeders and farmers to manage the disease.

INTRODUCTION

Cotton leaf curl disease (CLCuD) is caused by members of whitefly transmitted genus Begomovirus, family Geminiviridae [1]. Begomoviruses contain circular, single-stranded DNA genome, which may be monopartite or bipartite based on genomic components [2]. Monopartite begomoviruses have betasatellite and alphasatellite along with DNA-A, the main genomic component. Betasatellites are known to be involved in the repression of plant host defenses and therefore are linked to pathogenicity. Although the role of alphasatellites is incompletely understood, evidence suggests that they can modulate symptom severity and reduce betasatellite accumulation [3-5]. Begomovirus infection results in a variety of symptoms including vein thickening, vein yellowing, mosaic, leaf curling, and stunting [6]. Begomoviruses cause substantial losses to agricultural crops, and so pose a major threat to food and fiber production to global agriculture, including Pakistan [7, 8]. In Asia, economically important diseases caused by geminiviruses were mostly recorded in the Indian subcontinent. Indian cassava mosaic virus, Mungbean yellow mosaic virus, tobacco leaf curl virus, tomato leaf curl virus and cotton leaf curl virus are among the most common Geminiviruses, which cause severe damage to several crops [9]. Cotton production has been hampered by CLCuD in Pakistan. The typical symptoms of disease include leaf curling, enations, stunting of the plant resulting in reduced yield and quality. Eight species of distinct begomoviruses along with Cotton leaf curl Multan betasatellite (CLCuMB) have been reported to cause CLCuD. In Africa only Cotton leaf curl Gezira virus

DOI: https://doi.org/10.54393/fbt.v3i02.45

(CLCuGeV) has been reported to infect cotton [10]. From central Africa to Jordan many plant species including hollyhock, cotton, okra, and Sida species have been infected by CLCuGeV [11]. The situation of CLCuD in Pakistan and India is more complex than that in Africa. During first epidemic Cotton leaf curl Multan Virus (CLCuMV) was reported as causative agent of CLCuD in 1990. The most recent epidemic was caused by Cotton leaf curl Burewala virus (CLCuBuV) that was able to break resistance in varieties developed against CLCuMV [12]. During both the epidemics of CLCuD the disease started from Multan and Burewala and then spread eastwards into the cotton-growing states of India [13, 14]. The risk of global spread of CLCuD is increased due to global transport of plant material, especially ornamentals. The present study reports the development and testing of primers for detection and validation of CLCuD. Instead of designing primers and validation of PCR detection for eight different species of begomoviruses responsible for CLCuD, detection on the basis of betasatellite proved more convenient and practical for early detection of the disease. First the PCR was tested on already cloned betasatellites and then suspected cotton plants collected from different cotton growing regions of the country. So, the molecular marker based on consensus sequence of all variants of CLCuMB will help to identify CLCuD due to any begomovirus. The screening of plant imports using molecular diagnostics will greatly reduce the introductions and spread of exotic begomoviruses and will help to boost agricultural output of cotton and other crops throughout the world.

METHODS

Field collections

Plant samples (young leaves) were collected from symptomatic cotton fields in selected five major areas of cotton production in Pakistan (Multan, Sakrand, Rahim Yar Khan, Vehari and Lodhran). Leaves (3-7) were collected from new growth of each plant, labeled and stored in a cool container.

DNA Isolation

Total DNA was extracted from collected leaves using the Cetyl trimethylammonium bromide (CTAB) method [15]. Collected leaf samples were crushed by adding liquid nitrogen. Leaf powder was transferred to 1.5 mL tubes, and 650 µL of 2% CTAB buffer was added. The mixture was incubated at 65 °C for 30 min in a water bath. After adding and mixing 650 µL of chloroform: iso-amyl alcohol (24:1) the sample was centrifuged at 10,000 rpm for 12 mins. The upper aqueous layer was carefully transferred to a fresh tube, and a double volume of cold isopropanol was added to each sample and then samples were incubated at -20 °C for 2 hours. The mixture was centrifuged at 10,000 rpm for 10 min and the resultant pellet was washed with 70% ethanol, air-dried for 30 mins, and re-suspended in 50 µL of sterilized distilled water.

Primer Design

All different variants of CLCuMB genomes were downloaded from NCBI GenBank (https://blast.ncbi.nlm.nih.gov/) and multiple alignments of these sequences was created in MEGA-X software. Potential primers were designed from a MUSCLE alignment of the downloaded sequences, that were predicted to anneal conserved regions of sequences. Primer pair Beta-F/Beta-R (Beta-F 5' ccatggaactggctgattccggcat 3'; Beta-R 5' gaattctgtacttgaaaacccagagatattg 3') was designed for amplification of betasatellite and the expected product size was 483bp.

Polymerase Chain Reaction (PCR)

Target region of CLCuMB from extracted DNA and from cloned betasatellite (Positive control) was amplified by PCR. Each reaction contained 25 ng of template DNA to amplify 401 bp of the CLCuMB using Beta-F/Beta-R primer pair. The conditions for PCR were set as initial denaturation at 95 ºC for 2 min, and then, at 95 ºC for 90 sec, 50 ºC for 45 sec, 72 ºC for 1min for 35 cycles, and final extension for 5 mins at 72 ºC. The amplification was confirmed by performing gel electrophoresis.

DNA Sequencing and Phylogenetic Analysis

Thirteen DNA samples from five locations were amplified and out of them five samples (one from each location) were submitted for DNA sequencing to commercial laboratory (Macrogen, Korea). The DNA sequences were used to blast on NCBI for finding and downloading of closely related sequences. Multiple alignments of the DNA sequences were performed. Phylogenetic analysis of the DNA sequences was carried out using the Maximum Likelihood method [16] in software MEGA-X [17]. Bootstrap test with 1000 replications was included during tree construction.

RESULTS

Total genomic DNA was extracted from fourteen viral infected samples of cotton. Results were checked by running 1 % agarose gel. All thirteen cotton samples from five areas and positive control (1019) had a good quality of DNA with minute differences in final concentration. (Figure 1).

DOI: https://doi.org/10.54393/fbt.v3i02.45

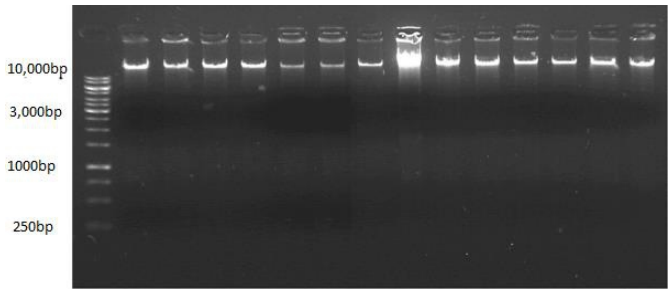

Figure 1: Genomic DNA profile of fourteen cotton samples (1% agarose gel electrophoresis)

First lane is 1kb ladder (Thermo Fisher Scientific). Four lanes after ladder include the samples taken from different cotton growing regions (Multan, Sakrand, Rahim Yar Khan, Vehari and Lodhran) of Pakistan. DNA quantification was done using spectrophotometer as quantification is necessary to check the quality and purity of DNA. DNA concentration of all the samples was in the range of 857.5 ug/ml to 1570 ug/ml. Out of thirteen DNA samples from five different locations, one sample from each of five locations were selected for further processing in PCR, DNA sequencing and phylogenetic analysis. Presence of CLCuMB was confirmed in each of five samples by PCR. Amplified target region of CLCuMB (483bp) was observed by running 1% agarose gel (Figure 2).

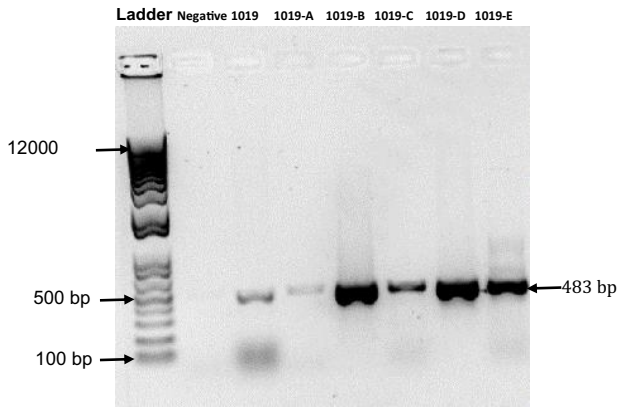

Figure 2: 1% agarose gel showing the results for CLCuMB amplified PCR product

Lane 1 DNA ladder (1kb plus DNA Ladder, Invitrogen), Lane 2 negative control, Lane 3 positive control (1019) and the remaining are amplicons from DNA of field plants (1019-A from Multan, 1019-B from Sakrand, 1019-C from Rahim Yar Khan, 1019-D from Vehari and 1019-E from Lodhran). PCR product for CLCuMB were excised and purified from agarose gel using Expin gene cleaning kit. After gel elution of amplified PCR product, quality and size of product was checked by running 1% agarose gel and all five samples were submitted for DNA sequencing. DNA Sequencing was done by sanger sequencing through a commercial facility. DNA sequences were edited and assembled using BioEdit and DNAStar software. Then sequences were used to blast on NCBI Genbank to download the closely related sequences. The evolutionary history of DNA sequences was inferred by using the Maximum Likelihood method based on the Tamura &Nei model [16]. These multiple sequences were aligned for homology analysis by using Muscle method in MEGA-X software. All five sequences were aligned with six most closely related sequences downloaded from GenBank, using Clustal W algorithm in MegAlign (DNASTAR Lasergene) software. Pairwise nucleotide sequence distance was calculated. All the five sequences reported in present study shown homology at 100% with each other and 99.6-99.8% pairwise sequence homology with Burewala strain of CLCuMB sequences available in GenBank (Figure 3).

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11		
1		100.0	100.0	100.0	100.0	99.8	99.8	99.8	99.8	99.8	99.8	1	1019_A
2	0.0		100.0	100.0	100.0	99.8	99.8	99.6	99.8	99.8	99.8	2	1019_B
3	0.0	0.0		100.0	100.0	99.8	99.8	99.6	99.8	99.8	99.8	3	1019_C
4	0.0	0.0	0.0		100.0	99.8	99.8	99.6	99.8	99.8	99.8	4	1019_D
5	0.0	0.0	0.0	0.0		99.8	99.8	99.6	99.8	99.8	99.8	5	1019_E
6	0.2	0.2	0.2	0.2	0.2		99.6	99.8	100.0	99.6	99.6	6	LN886539
7	0.2	0.2	0.2	0.2	0.2	0.4		99.3	99.6	100.0	100.0	7	HE601940
8	0.4	0.4	0.4	0.4	0.4	0.2	0.7		99.8	99.3	99.3	8	EU384579
9	0.2	0.2	0.2	0.2	0.2	0.0	0.4	0.2		99.6	99.6	9	HF564598
10	0.2	0.2	0.2	0.2	0.2	0.4	0.0	0.7	0.4		100.0	10	MW722447
11	0.2	0.2	0.2	0.2	0.2	0.4	0.0	0.7	0.4	0.0		11	FN554723
	1	2	3	4	5	6	7	8	9	10	11		

Figure 3: Pairwise nucleotide identity of CLCuMB sequen-ces used in this study with closely related sequences from GenBank.

Sequences were aligned using Clustal W method in MegAlign (DNASTAR Lasergene) software. Five CLCuMB genome sequences from present study, fifteen previously described CLCuMB genome sequences, and four other genomes of betasatellites, available from the NCBI GenBank database were used in phylogenetic analysis. Based on the phylogenetic analysis CLCuMB sequences reported in this study clustered together with Cotton leaf Curl Multan betasatellite- Shahdadpur strain (CLCuMBSha) (99.8% bootstrap value). Phylogenetic analysis revealed that CLCuMB reported in this study falls in the same clade as the previously reported CLCuMB from Indo-Pak subcontinent (Figure 4).

DOI: https://doi.org/10.54393/fbt.v3i02.45

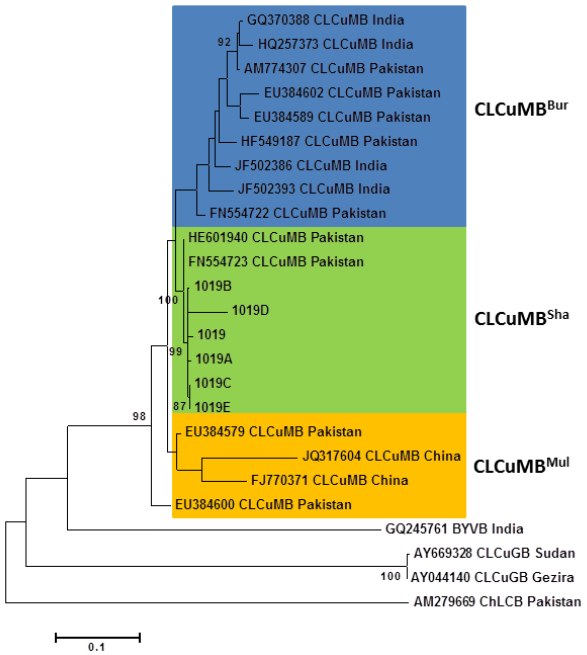

Figure 4: Phylogenetic analysis of CLCuMB with selected betasatellite DNA sequences

DISCUSSION

Rich diversity of begomoviruses has hampered the agricultural economy of Punjab province of Pakistan [11, 18-20]. Primers were designed for PCR-amplification of the most abundant CLCuD related betasatellite, the CLCuMuB. Comparison of DNA sequences revealed two nucleotide substitutions in DNA sequence from samples collected from Multan, Sakrand, Rahim Yar Khan and, while four nucleotide substations in sample collected from Vehari as compared to previously reported DNA sequences used in analysis. High nucleotide substation in DNA sequence from Vehari as compared to other regions can be corelated with Vehari region including city of Burewala is considered as "hot spot" of CLCuD [21]. Although cotton verities grown in Vehari and other regions of Punjab are same, the reason for high CLCuD incidence maybe different haplotype of whitefly or climatic conditions of the region. The results of the test for CLCuMuB primer specificity indicated that the primers successfully amplify three different strains of CLCuMuB. No amplification was observed for heterologous betasatellite species. If these finding could be verified in more samples, then it might have a potential for early detection of CLCuD. Previous studies have reported a number of universal and species-specific primers for the detection of CLCuD related begomoviruses but this study reports a novel method to amplify CLCuMB for detection of CLCuD. Association of multiple begomoviruses to single betasatellite indicates that CLCuMB based detection of CLCuD might be an efficient tool. Betasatellites are involved in the repression of plant host defenses and therefore are linked to pathogenicity that might be the reason for presence of only single beatasatellite in CLCuD. There are reports which suggest that betasatellite accumulation is related to symptom severity and disease [3-5, 22]. Many universal, degenerate primers have been used to amplify betasatellites in previous studies but this study reports CLCuMB specific primers for detection of CLCuD [23]. Association of single betasatellite with CLCuD, makes CLCuMB a good candidate for development of PCR primers and their validation for CLCuD detection. Furthermore, this primer pair can be used to test the presence of CLCuD related begomoviruses in ornamental plants, prior to export, that can be a potential source of CLCuD spread to other parts of the world, including Australia, Uzbekistan, Brazil, and USA. Trade of ornamental plants may be a source of CLCuD spread because they can serve as an alternate host of begomoviruses and often show only mild symptoms or symptomless, and so are not screened during exports, and can lead to global spread. In 2009, first report of CLCuD came from China and possibly the disease was spread due to trade of ornamental plants from Pakistan [24]. CLCuD outbreaks are devastating to cotton growers and the textile industry. Monitoring CLCuD and Whitefly strains will allow the government/private industry to recommend measures to prevent the occurrence of disease outbreaks. The information will allow knowledge-based decisions on resistant gene deployments including the necessary vector or Whitefly control measures that will ensure a sustainable harvest for the growers.

CONCLUSIONS

Recently, Cotton leaf curl Gezira virus (CLCuGV), associated with CLCuD in Africa, has been reported from Sindh province in association with CLCuMB, instead of the cognate Cotton leaf curl Gezira betasatellite (CLCuGB) from limited number of samples. The results of recent studies showed that Cotton leaf curl Burewala virus (CLCuBuV) is the major virus infecting cotton in Pakistan and India. Association of all of the eight distinct begomoviruses causing CLCuD with CLCuMB is indicative of the fact that CLCuMB based molecular marker can be developed for detection of the disease. So, the molecular marker based on consensus sequence of all variants of CLCuMB was developed and validated to identify CLCuD due to any begomovirus. In this study, betasatellite specific primer pair is developed and validated for more efficient and reliable detection of CLCuD in early stages. Early detection of disease will help the breeders and farmers to manage the disease.

DOI: https://doi.org/10.54393/fbt.v3i02.45

Authors Contribution

Conceptualization: USM
Methodology: USM
Formal analysis: USM
Writing-review and editing: USM

All authors have read and agreed to the published version of the manuscript.

Acknowledgement

Authors express their gratitude to goat farmers for their invaluable support in supplying blood samples from their animals.

Conflicts of Interest

The authors declare no conflict of interest.

Source of Funding

The authors declare that they did not receive any financial support for the research, authorship, and/or publication of this article.

REFERENCES

[1] Gill RJ and Brown JK. Systematics of Bemisia and Bemisia relatives: can molecular techniques solve the Bemisia tabaci complex conundrum–a taxonomist's viewpoint. Bemisia: Bionomics and Management of a Global Pest. 2010: 5-29. doi: 10.1007/978-90-481-2460-2_1.

[2] Briddon RW, Bull SE, Amin I, Mansoor S, Bedford ID, Rishi N et al. Diversity of DNA 1: a satellite-like molecule associated with monopartite begomovirus–DNA β complexes. Virology. 2004 Jul; 324(2): 462-74. doi: 10.1016/j.virol.2004.03.041.

[3] Idris AM, Shahid MS, Briddon RW, Khan AJ, Zhu JK, Brown JK. An unusual alphasatellite associated with monopartite begomoviruses attenuates symptoms and reduces betasatellite accumulation. Journal of General Virology. 2011 Mar; 92(3): 706-17. doi: 10.1099/vir.0.025288-0.

[4] Mansoor S, Briddon RW, Bull SE, Bedford ID, Bashir A, Hussain M et al. Cotton leaf curl disease is associated with multiple monopartite begomoviruses supported by single DNA β. Archives of Virology. 2003 Oct; 148: 1969-86. doi: 10.1007/s00705-003-0149-y.

[5] Saunders K, Briddon RW, Stanley J. Replication promiscuity of DNA-β satellites associated with monopartite begomoviruses; deletion mutagenesis of the Ageratum yellow vein virus DNA-β satellite localizes sequences involved in replication. Journal of General Virology. 2008 Dec; 89(12): 3165-72. doi: 10.1099/vir.0.2008/003848-0.

[6] Briddon RW and Markham PG. Complementation of bipartite begomovirus movement functions by topocuviruses and curtoviruses. Archives of Virology. 2001 Sep; 146: 1811-9. doi: 10.1007/s007050170067

[7] Brown JK, Frohlich DE, Rosell RC. The sweetpotato or silverleaf whiteflies: biotypes of Bemisia tabaci or a species complex? Annual Review of Entomology. 1995 Jan; 40(1): 511-34. doi: 10.1146/annurev.en.40.010195.002455.

[8] Mansoor S, Zafar Y, Briddon RW. Geminivirus disease complexes: the threat is spreading. Trends in Plant Science. 2006 May; 11(5): 209-12. doi: 10.1016 /j.tplants.2006.03.003.

[9] Karthikeyan A, Shobhana VG, Sudha M, Raveendran M, Senthil N, Pandiyan M et al. Mungbean yellow mosaic virus (MYMV): a threat to green gram (Vigna radiata) production in Asia. International Journal of Pest Management. 2014 Oct; 60(4): 314-24. doi: 10.1080/ 09670874.2014.982230.

[10] Idris AM and Brown JK. Molecular analysis of Cotton leaf curl virus-Sudan reveals an evolutionary history of recombination. Virus Genes. 2002 Jun; 24: 249-56. doi: 10.1023/A:1015380600089.

[11] Tahir MN, Amin I, Briddon RW, Mansoor S. The merging of two dynasties-identification of an African cotton leaf curl disease-associated begomovirus with cotton in Pakistan. PLoS One. 2011 May; 6(5): doi: 10.1371/journal.pone.0020366.

[12] Sattar MN, Kvarnheden A, Saeed M, Briddon RW. Cotton leaf curl disease–an emerging threat to cotton production worldwide. Journal of General Virology. 2013 Apr; 94(4): 695-710. doi: 10.1099/vir.0.049627-0.

[13] Rajagopalan PA, Naik A, Katturi P, Kurulekar M, Kankanallu RS, Anandalakshmi R. Dominance of resistance-breaking cotton leaf curl Burewala virus (CLCuBuV) in northwestern India. Archives of Virology. 2012 May; 157: 855-68. doi: 10.1007/s00705-012-1225-y.

[14] Zaffalon V, Mukherjee SK, Reddy VS, Thompson JR, Tepfer M. A survey of geminiviruses and associated satellite DNAs in the cotton-growing areas of northwestern India. Archives of Virology. 2012 Mar; 157(3): 483-95. doi: 10.1007/s00705-011-1201-y.

[15] Doyle JJ and Doyle JL. Isolation of plant DNA from fresh tissue. Focus.1990; (12)13-15. doi: 10.2307/2419362.

[16] Tamura K and Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution. 1993 May 1;10(3):512-26.

DOI: https://doi.org/10.54393/fbt.v3i02.45

[17] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution. 2013 Dec; 30(12): 2725-9. doi: 10.1093/molbev/mst197.

[18] Haider MS, Tahir M, Saeed A, Ahmed S, Parveen R, Rashid N. First report of a begomovirus infecting the ornamental plant Vinca minor L. Australasian Plant Disease Notes. 2008 Nov; 3(1): 150-1. doi: 10.1007/BF03211274.

[19] Hussain K, Hussain M, Mansoor S, Briddon RW. Complete nucleotide sequence of a begomovirus and associated betasatellite infecting croton (Croton bonplandianus) in Pakistan. Archives of Virology. 2011 Jun; 156: 1101-5. doi: 10.1007/s00705-011-0993-0.

[20] Shahid MS, Mansoor S, Briddon RW. Complete nucleotide sequences of cotton leaf curl Rajasthan virus and its associated DNA [beta] molecule infecting tomato. Archives of Virology. 2007 Oct; 152(11): 2131. doi: 10.1007/s00705-007-1043-9.

[21] Ashraf KH and Hanif M. Screening of cotton germplasm against cotton leaf curl virus. Pakistan Journal of Botany. 2010; 42(5): 3327-42.

[22] Cui X, Tao X, Xie Y, Fauquet CM, Zhou X. A DNAβ associated with Tomato yellow leaf curl China virus is required for symptom induction. Journal of Virology. 2004 Dec; 78(24). doi: 10.1128/JVI.78.24.13966-13974.2004.

[23] Sharma D, Kulshreshtha A, Roshan P, Hallan V. Molecular characterization and infectivity analysis of a bipartite begomovirus associated with cotton leaf curl Multan betasatellite naturally infecting Rumex nepalensis in northern India. Journal of Plant Pathology. 2019 Nov; 101: 935-41. doi: 10.1007/s42161-019-00295-6.

[24] Cai JH, Xie K, Lin L, Qin BX, Chen BS, Meng JR, Liu YL. Cotton leaf curl Multan virus newly reported to be associated with cotton leaf curl disease in China. Plant Pathology. 2010; 59(4): 794-5. doi: 10.1111/j.1365-3059.2010.02266.x.