Madras Agric. J., 2022; https://doi.org/10.29321/MAJ.10.000646

RESEARCH ARTICLE

Phylogenetic Analysis of Okra Enation Leaf Curl Virus (OELCV) Transmitted by *Bemisia tabaci* Using Coat Protein Gene Fragment of OELCV in Tamil Nadu

Pasupathi E[1]*, Murugan M[2], Karthikeyan G[3], Ramalingam J[4] and Harish S[5]

[1]*Department of Agricultural Entomology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai-625 104, India

[2]Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore-641 003, India

[3&5]Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore-641 003, India

[4]Department of Plant Biotechnology, Tamil Nadu Agricultural University, Coimbatore-641 003, India

ABSTRACT

Okra enation leaf curl virus (OELCV) is a severe emerging disease of okra, *Abelmoschus esculentus* in India, causes systemic infections and has a very effective mode of transmission by the sweet potato whitefly, *Bemisia tabaci;* thus, preventing their spread has been made very complicated. It is essential to understand the OELCV isolates to study the disease reactions in plant accessions. The okra OELCV infected young leaves were collected from the Salem (Northwestern Zone) and Madurai (South Zone) of Tamil Nadu and the DNA was extracted and used to amplify the OELCV utilizing specific primers (JKOE34F, JKOE35R) targeting the OELCV coat protein gene and produced a 796 bp band in the polymerase chain reaction (PCR). The homology analysis of nucleotide sequence was carried out using BLAST programme and the results revealed that the Salem region coat protein gene sequence grouped into OELCV clone 6.2, complete genome (MK084768.1) with 99.71% of homology, and Madurai region coat protein gene sequence grouped into OELCV isolate Vijayawada coat protein (AV1) gene complete cds (KT935487.1) with a 99.38% of homology. The amplified OELCV (Salem and Madurai zone) from the study had aligned in the same cluster along with HG938362.1 and KX710156.1. This study had indicated that the OELCV pathogen alike in these zones successfully being transmitted by *B. tabaci*.

Keywords: *Bemisia tabaci; OELCV; PCR; Phylogenetic analysis*

INTRODUCTION

India is the largest producer of okra (*Abelmoschus esculentus L.* Moench) in the world and accounts for 66% of the global production (Anonymous, 2014). Okra is an important source of vitamins, calcium, potassium, and other minerals, which are often lacking in the diet of the people in developing countries. Okra has found medical application as a plasma replacement or blood volume expander and is also useful in genito-urinary disorders, spermatorrhoea, and chronic dysentery (Abidia *et al*., 2014). The most important constraint in okra production is the susceptibility of the crop to various insect pests (Acharya *et al.*, 2018) and viral diseases (Das *et al.*, 2013; Seth *et al.*, 2017). Okra harbours many insects and of which whiteflies, aphids, jassids, thrips, and mites are of great concern being a pest. Among these pests, the sweet potato whitefly, *Bemisia tabaci* Gennadius causes economic damage to okra by directly feeding on phloem sap and weakening the crop (Pasupathi *et al.*, 2019) but also transmits begomoviruses (Lazarowitz, 1992). The severe impact of whitefly mediated yellow vein mosaic virus disease (YVMV) and okra enation leaf curl virus disease (OELCV) on okra is already felt both in South and North Indian conditions (Venkataravanappa *et al*., 2014; Sanwal *et al.*, 2016; Sayed *et al.*, 2014). In India, OELCV is of recent concern in okra cultivation, even though it was first reported from Bangaluru (Karnataka) during the early 1980s; as the incidence has reached serious proportions in recent years both in Northern India (Sanwal *et al.,* 2016) and Southern India as well (Sayed *et al.*, 2014) and it is known to cause a yield loss up to 80-90% (Singh, 1996). The disease initially causes small pin-

Corresponding author's e-mail: pasupathi441@gmail.com

head enations on the undersurface of growing leaves followed by a warty and rough texture of leaves, with later leaves curling upwards. Affected plants show a twisting of the stem and lateral branches with leaves becoming thick and leathery. The curling and enations are more prevalent on leaves that develop soon after infection than in later leaves and affected plants turn severely stunted, with the developing fruits being small, deformed, and unfit for marketing and consumption (Singh, 1996; Sanwal *et al*., 2014). There is very little information available on the incidence of OELCV and its transmission by *B. tabaci* in Tamil Nadu, the Southern peninsular part of India, where okra is cultivated in small farm holdings by marginal farmers and any yield loss could hinder their livelihood. The directions of disease epidemics change with transitions of either the pathogen or its insect vector(s). Hence, it is important to record the OELCV isolates prevalent in the ecosystem to study the disease reactions in plant accessions, disease transmission capabilities by the vector insects, and also to develop ecologically sustainable management measures to contain the viral diseases.

MATERIAL AND METHODS

The molecular experiments of the present study were conducted at the laboratories of the Centre of Innovation, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai, India, 2018-19.

The OELCV infected Okra young leaves (Fig. 1) were collected from hot spot regions of its occurrence in Tamil Nadu viz., Salem (Northwestern Zone), and Madurai (South Zone), transported in frozen condition to the laboratory and used for DNA extraction and further analysised. as per Doyle and Doyle (1987) method with little modification was used to amplify the OELCV utilizing specific primers (JKOE34F, JKOE35R) and produced a 796 bp band in the polymerase chain reaction (PCR).

DNA Extraction, Amplification and Visualization

The protocol reported by Doyle and Doyle (1987) was employed with the following modification: 50-60mg of OELCV infected fresh leaves of okra were taken and ground with liquid nitrogen, and after thawing again was ground within mortar with a preheated CTAB extraction buffer (1.5ml/sample) with 0.2% 2-beta mercaptoehanol. Then the sample was incubated at 65 ^{0}C for 1 hour, and after that 1.5µL of RNase was added and incubated at 37^{0}C for 20 min. Then, it was centrifuged at 12000 rpm for 10 min to pellet the debris. Then, an equal volume of Chloroform: Isoamyl alcohol (24:1 v/v) was added to the supernatant, gently vortexed for 10 min, and centrifuged at 13000 rpm for 10 min. The supernatant was transferred into 0.7 volume of ice-cold isopropanol and 0.15 volume ammonium acetate to precipitate DNA at -20^{0}C for 30 min. The precipitate was washed twice by adding 500 µL of 70% chilled ethanol to remove ions and then absolute ethanol. The centrifugation was done at 13000 rpm for 1 min to pellet the DNA, then air dried and re-suspended in 50µL of TE buffer. The quantity of extracted DNA was checked using Eppendorf Biospectrometre® (Kinetic Germany).

For detection of OELCV, 100 ng of total DNA was used for PCR reaction using specific primers (JKOE34F - 5'-AAGAATTATGTCGAAGCGTCCTGCTT-3' and JKOE35R 5'-AAGAATCGTAGAAGTAACTCCTAACTT-3') (Rakesh Kumar, 2016), synthesized by Eurofins Genomics India (Bangalore, India), which targeted the OELCV coat protein (CP) gene. The reaction mixture was given a short spin thorough mixing of the cocktail components. The PCR was conducted in a fast PCR machine (Medline, U.K) as programmed with the amplification reaction of 4 min initial denaturation at 94^{0}C, 30 seconds denaturation at 94^{0}C, 30 seconds annealing at 50^{0}C, and 45 seconds extension at, 72^{0}C. A final extension step at 72^{0}C for 20 min was performed after 35 cycles.

Agarose gel (1.2%) electrophoresis was performed to visualize the DNA fragments in the amplified products of OELCV. Fifteen microlitre of PCR amplified product was loaded. The voltage was maintained at 100 volts for 45 min for DNA separation and at 100 volts for a maximum of 1 h for PCR, the voltage was maintained. The staining was done with ethidium bromide solution (10 mg/mL) separately after the completion of gel electrophoresis and the bands were visualized and documented in the gel documentation system (Bio-rad, USA).

PCR Product Purification and Sequencing

The amplified products of the target gene of interest purified using HiPurATM PCR product purification kit (Himedia Laboratories, Mumbai) by following the manufacturer's instructions and sending for sequencing.

In a 2.0 ml capped collection tube, 5 volumes of PCR Binding Solution (SPB) to 1 volume of the PCR sample was added and mixed well by pipetting. Then, transferred the mixture to the HiElute miniprep spin column (capped) and centrifuged for 1 min at 12,000 RCF. The flow-through was discarded and the column was replaced in the same collection tube. After that, 700 µL diluted Wash Solution (HPE) was added to the column and centrifuged for 1 min at 12,000 RCF. The flow-through was discarded and the column was replaced in the same collection tube. Then, was centrifuged for 1 min at 12,000 RCF to remove excess ethanol and the column was transferred to a clean 2.0 ml uncapped collection tube, pipetted 50 µl of elution buffer (ET) to the center of the column, and then incubated at room temperature (15-25°C) for 1 min. After centrifugation for 1 min at 12,000 RCF the eluted product was transferred to a fresh capped 2 ml collection tube for longer DNA storage. The purified PCR amplification product present in the eluate was ready for immediate use. For long-term storage, it was stored at -20°C or lower temperature (-80°C). By using the above method, the full-length sequences of specific coat protein genes of OELCV were processed.

The eluted purified PCR fragments were sent for sequencing through outsourcing at Agrigenome, Kochi, Kerala, India, where the Sanger sequencing (Sanger *et al*., 1977) was done with forward and reverse primers of the target sequence. The coloured peaks in the chromatogram obtained that corresponded to the nucleotide in that location of the sequence (Obenrader, 2003) were used to delineate the nitrogenous base pair information, and sequence manual editing was carried (Bio-Edit version 7.1) to obtain the correct sequence information.

Bioinformatics Analysis

The manually edited sequences were subjected to homology analysis BLAST programme in with the National Center for Biotechnology Information (NCBI) (Anonymous)http://www.ncbi.nlm.nlh.gov) programme.

The phylogenetic tree was constructed using nine sequences of the coat protein gene of OELCV available with the NCBI (www.ncbi.nlm.nlh.gov) (Table 1) and the difference in the sequences were determined using the sequence alignment editor [Bio-Edit version (7.1)]. The alignment was further analyzed using the MEGA 6.0 program, using the neighbor joining method with a bootstrap value of 1000 based on the >70% majority rule and confidence limits placed at the tree's major nodes.

RESULTS AND DISCUSSION

Amplification of OELCV using coat protein gene-specific primers yielded a 796 bp band (Fig. 2). The sequencing yielded good quality sequences of 743bp OELCV CP gene information which was aligned using NCBI BLAST and there was >99% homology with the OELCV CP gene sequences already deposited in NCBI.

On blasting the sequence (Salem Region) using BLASTN program the virus showed highest identity (99.71%) (Table 2) with Okra enation leaf curl virus clone 6.2, complete genome (MK084768.1), Okra enation leaf curl virus clone 6.1, complete genome (MK084767.1), Okra enation leaf curl virus clone S9C4, complete genome (MK069435.1), Okra enation leaf curl virus clone 1(1), complete genome (MK069433.1), Okra enation leaf curl virus clone SZ157 pre-coat protein (V2), coat protein (V1), replication enhancer protein (V3), and transcriptional activator protein (C2) genes, complete cds (KX710156.1), Okra enation leaf curl virus isolate Matara segment DNA-A, complete sequence (KX698092.1), Okra enation leaf curl virus isolate Puttalam segment DNA-A, complete sequence (KX698091.1), Okra enation leaf curl virus isolate Surat AV2, coat protein, AC5, Ren, TrAP, Rep, and AC4 genes, complete cds (KC342220.1), Okra enation leaf curl virus isolate India:Gandhinagar:2012 clone 3 segment DNA-A, complete sequence (KC019308.1).

On blasting the sequence (Madurai Region) using BLASTN program the virus showed the highest identity (99.38 %) (Table 3) with Okra enation leaf curl virus isolate Vijayawada coat protein (AV1) gene, complete cds (KT935487.1), and (97%) with Okra enation leaf curl virus isolate OK92-HR segment DNA A, complete sequence (KT390310.1), Okra enation leaf curl virus isolate DAVav02, complete sequence (9MN389529.1), Okra enation leaf curl virus isolate OK143-PUNJ segment DNA A, complete sequence (KT390317.1), Okra enation leaf curl virus segment DNA-A, isolate OELCuV-PKS, clone Neo 15 (HG518793.2).

Our results of coat protein gene (Salem region) sequence grouped into OELCV Okra enation leaf curl virus clone 6.2, complete genome (MK084768.1) with 99.71% of homology, and results of coat protein

gene (Madurai region) sequence grouped into Okra enation leaf curl virus isolate Vijayawada coat protein (AV1) gene, complete cds (KT935487.1) with 99.38% of homology. Phylogenetic analysis had revealed that both the amplified OELCV from the present study had aligned in the same cluster along with HG938362.1 and KX710156.1 (Fig. 3), which were reported in Pakistan. This indicated that the OELCV pathogen were alike in the study regions and were successfully transmitted by *B. tabaci.*

Venkataravanappa *et al.* (2014) examined, leaf curl associated with enation (OELCV) symptom that was noted in okra originating from Haryana state, India. Complete genome sequences were determined by Venkataravanappa *et al*. (2014) for seven isolates and a comparison to begomovirus sequences available in the NCBI databases showed the highest levels of nucleotide sequence identity (84.5 to 87.1 %) with OELCV. In a similar study, Rakesh Kumar *et al*. (2019) reported that the OELCV CP genes of Varanasi, Hyderabad and Vijayawada OELCV isolates formed varied clustering patterns along with other isolates of OELCV reported from India, and all the three isolates of that study clustered together along with Surat (KC342220) and Gandhi Nagar (KC019308) isolates reported from Gujarat, while the Sonipat (GU111997) and Munthal (GU112000) isolates reported from Haryana, aligned together in another cluster suggesting differences in OELCV isolates infecting okra. As there are varied isolates reported in the country, it is pertinent to screen the okra germplasms to major isolates of OELCV to bring sustainable management of the pathogen on okra to save the livelihood of a small and marginal farming community.

Table 1. GenBank accession numbers of selected Begomovirus sequences used in this study for analysis

S. No	Accession No.	Begomoviruses	Reporting Place & Country
1.	HG938362.1	Okra enation leaf curl virus Rep gene, CP gene, V2 gene, REn gene and TrAP gene, segment DNA-A, isolate NEO-32 OELCuV-PKS, clone Neo-32	Sindh : Pakistan
2.	KX710156.1	Okra enation leaf curl virus clone SZ157 pre-coat protein (V2), coat protein (V1), replication enhancer protein (V3), and transcriptional activator protein (C2) genes, complete cds;	Burewala : Pakistan
3.	JX242520.1	Mesta yellow vein mosaic virus isolate Okra:Tirupati:2010 pre-coat protein (V1) gene, partial cds; JX242515.1 Mesta yellow vein mosaic virus isolate Okra:ASR:2010 pre-coat protein (V1) gene, partial cds;	Tirupati : India
4.	JX242515.1	Mesta yellow vein mosaic virus isolate Okra:ASR:2010 pre-coat protein (V1) gene, partial cds	ASR : India
5.	JX242517.1	Mesta yellow vein mosaic virus isolate Okra:Ludhiana:2010 pre-coat protein (V1) gene, partial cds	Ludhiana : India
6.	KT935487.1	Okra enation leaf curl virus isolate Vijayawada coat protein (AV1) gene, complete cds	Vijayawada : India
7.	KT898974.1	Okra enation leaf curl virus isolate Varanasi coat protein (AV1) gene, complete cds.	Varanasi : India

Table 2. Details of OELCV sequences with their accession numbers from NCBI, GenBank database used for sequence analysis and phylogenetic comparison of the amplified OELCV (Salem District, Tamil Nadu) coat protein gene from field collected okra plants expressing enation symptoms

S. No	Description	Accession number	Homology of nucleotide sequence
1	Okra enation leaf curl virus clone 6.2, complete genome	MK084768.1	99.71%
2	Okra enation leaf curl virus clone 6.1, complete genome	MK084767.1	99.71%

3	Okra enation leaf curl virus clone S9C4, complete genome	MK069435.1	99.71%
4	Okra enation leaf curl virus clone 1(1), complete genome	MK069433.1	99.71%
5	Okra enation leaf curl virus clone SZ157 pre-coat protein (V2), coat protein (V1), replication enhancer protein (V3), and transcriptional activator protein (C2) genes, complete cds	KX710156.1	99.71%
6	Okra enation leaf curl virus isolate Matara segment DNA-A, complete sequence	KX698092.1	99.71%
7	Okra enation leaf curl virus isolate Puttalam segment DNA-A, complete sequence	KX698091.1	99.71%
8	Okra enation leaf curl virus isolate Surat AV2, coat protein, AC5, Ren, TrAP, Rep, and AC4 genes, complete cds	KC342220.1	99.71%
9	Okra enation leaf curl virus isolate India:Gandhinagar:2012 clone 3 segment DNA-A, complete sequence	KC019308.1	99.71%
10	Okra enation leaf curl virus clone 5.2, complete genome	MK084766.1	99.56%

Table 3. OELCV accession numbers from NCBI, GenBank database used for sequence analysis and phylogenetic comparison of the amplified OELCV (Madurai District, Tamil Nadu) coat protein gene from field collected okra plants expressing enation symptoms

S. No	Description	Accession number	Homology of nucleotide sequence
1	Okra enation leaf curl virus isolate Vijayawada coat protein (AV1) gene, complete cds	KT935487.1	99.38%
2	Okra enation leaf curl virus isolate OK92-HR segment DNA A, complete sequence	KT390310.1	97.36%
3	Okra enation leaf curl virus isolate DAVav02, complete sequence	MN389529.1	97.05%
4	Okra enation leaf curl virus isolate OK143-PUNJ segment DNA A, complete sequence	KT390317.1	97.05%
5	Okra enation leaf curl virus segment DNA-A, isolate OELCuV-PKS, clone Neo 15	HG518793.2	97.05%
6	Okra enation leaf curl virus isolate OK91-HR segment DNA A, complete sequence	KT390309.1	96.89%
7	Okra enation leaf curl virus Rep gene, CP gene, V2 gene, REn gene and TrAP gene, segment DNA-A, isolate NEO-32 OELCuV-PKS, clone Neo-32	HG938362.1	96.89%
8	Okra enation leaf curl virus clone 6.2, complete genome	MK084768.1	96.73%
9	Okra enation leaf curl virus clone 6.1, complete genome	MK084767.1	96.73%
10	Okra enation leaf curl virus clone S9C4, complete genome	MK069435.1	96.73%

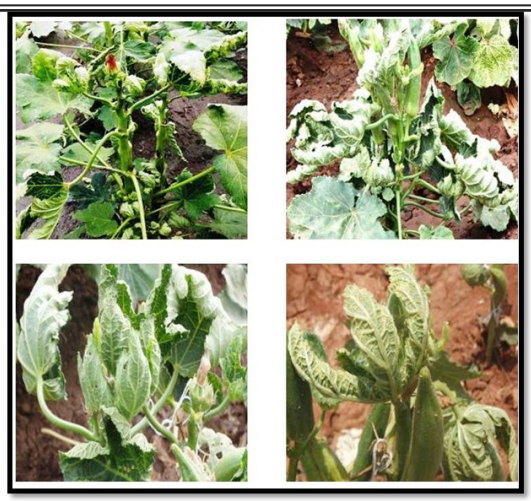

Figure 1. Symptoms exhibited by Okra enation leaf curl virus infected okra (*Abelmoschus esculentus*) plants in the field

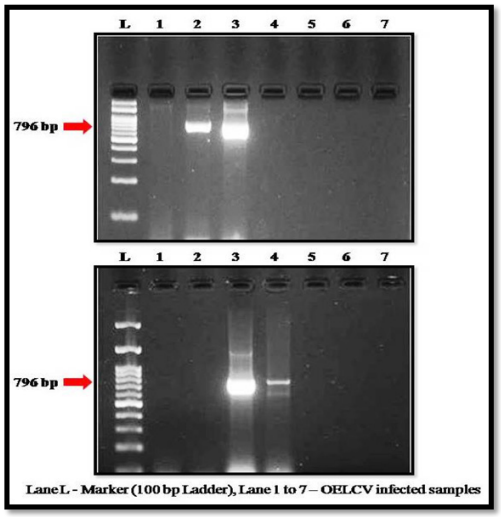

Figure 2. Polymerase chain reaction amplification of coat protein gene of OELCV using specific primers in okra (Salem & Madurai Region)

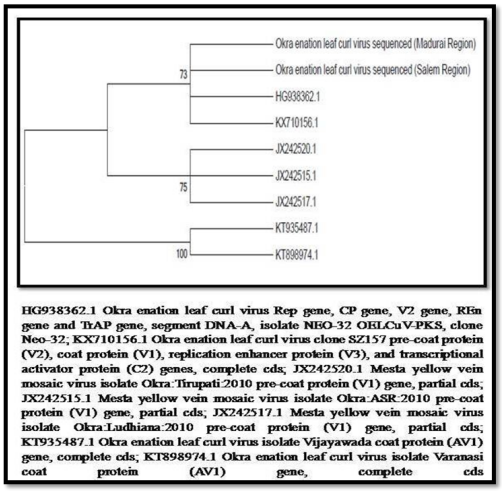

Figure 3. Phylogenetic analysis for Okra enation leaf curl virus (OELCV) using coat protein gene

CONCLUSION

This disease is going to be the future menace of okra cultivation and needs a strategic breeding program to evolve resistance against OELCV. Identifying resistant genotypes, understanding the interactions between plant viruses and their insect, and utilizing biotechnological tools to develop okra varieties, preferably with multiple resistance/ tolerance to YVMV and OELCV diseases, are the need of the moment. Additionally, further studies are required to determine the actual geographical extent of the OELCV reported from Tamil Nadu in other Southern regions of the Country. Hence, it is required to use interdisciplinary approaches to tackle the severe challenges of OELCV management in okra.

Ethics statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

All the authors agreed to publish the content.

Competing interests

The authors declare no conflict of interest in publication of this content.

REFERENCES

Abidia, A. B., PriyaSingha., VarunChauhana., Brahm Kumar Tiwaria., Shubhendra Singh Chauhan., SobitaSimonb. and S. Bilalc. 2014. An overview on okra (Abelmoschus esculentus) and its importance as a nutritive vegetable in the world. Intl. J. of Pharmacy and Biological Sci., 4(2): 227-233.

Acharya, J., Chatterjee, S., Konar, A., Chattopadhyay, A., Mandal, A. K. and S. Dutta. 2018. Host plant resistance through physicochemical characters against major insect pests of okra occurring in the Gangetic plains of eastern India. Int. J. Pest Manag., 65: 137-146.

Anonymous. “http://www.ncbi.nlm.nlh.gov.”

Anonymous. 2014. Agricultural Statistics at a Glance, Directorate of Economics and Statistics, Ministry of Agriculture, Govt. of India.

Das, S., Chattopadhyay, A., Dutta, S., Chattopadhyay, S. B. and P. Hazra. 2013. Breeding okra for higher productivity and yellow vein mosaic tolerance.

Intl. J. Veg. Sci., 19: 58-77.

Doyle, J. J. and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue.

Lazarowitz, S. G. 1992. Geminiviruses: genome structure and gene function. Critical Reviews in Plant Sci., 11: 327-349.

Pasupathi, E., Murugan, M., Harish, S., and C. Chinnaiah. 2019. Screening of okra germplasm for resistance to whitefly, Bemisia tabaci and okra enation leaf curl virus (OELCV) under field conditions. J. of Pharmacognosy and Phytochemistry, 8(5): 2306-2313.

Rakesh Kumar, V. R. 2016. Global prospectives in virus disease management, International conference: virocon. Bengeluru, India.

Rakesh Kumar., Ramchandran Esakky. and Sairam Reddy Palicherla. 2019. The emergence of okra enation leaf curl virus an important begomovirus, infecting okra in several states across India. Archives of Phytopathology and Plant Protection., 52 (1-2): 234-238.

Sanwal, S. K., Singh, M., Singh, B., Naik, P. S. 2014. Resistance to yellow vein mosaic virus and okra enation leaf curl virus: challenges and future strategies. Current Science, 106: 470-1471.

Sanwal, S. V., Venkataravanappa. and B. Singh. 2016. Resistance to bhendi yellow vein mosaic disease: a review. Indian J. Agr. Sci., 86: 835-843.

Sayed, S., Rana, D., Krishna, G., Reddy, P. and P. Bhattacharya. 2014. Association of Begomovirus with Okra (Abelmoschus esculentus L.) leaf curl virus disease in southern India. SAJ Biotechnol., 1(1).

Seth, T., Chattopadhyay, A., Dutta, S., Hazra, P. and B. Singh. 2017.Genetic control of yellow vein mosaic virus disease in okra and its relationship with biochemical parameters. Euphytica, 2: 13-30.

Singh, S. J. 1996. Assessment of losses in okra due to enation leaf curl virus. Indian J. of Virology, 12: 51-52.

Venkataravanappa, V., Reddy, C. L., Jalali, S., Briddon, R. W. and Reddy, M. K. 2014. Molecular identification and biological characterisation of a begomovirus associated with okra enation leaf curl disease in India. European J. of plant patho., 141(2): 217-235.