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Original Research Paper

Genetics

USE OF DEGENERATE PRIMERS FOR MOLECULAR IDENTIFICATION OF BEGOMOVIRUS ASSOCIATED WITH YELLOW VEIN MOSAIC AND LEAF CURL DISEASE OF OKRA IN MEERUT (UP)

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ABSTRACT The present study deals with the survey for the prevalence and collection of begomovirus infected okra samples from different regions of western Uttar Pradesh and molecular identification of the causal pathogen using begomovirus specific degenerate in primer through PCR. The result of positive PCR amplification of approximately 500bp from virus infected leaf sample of okra and absence of amplification in the healthy leaf tissue of okra clearly revealed the association of begomovirus with yellow vein mosaic virus and venation leaf curl disease of okra in western UP.

KEYWORDS : Begomovirus, okra, viral phytopathoogens.

1.INTRODUCTION

There are four major groups of plant viruses that seems to be most important among the new pathogens agents worldwide and are responsible for various newly emerging serious out breaks across the world (Varma and Malathi, 2003). Gemini viruses form the second largest family of plant viruses; the family *Gemini viridae* is a group of DNA viruses and divided into four genera: *Mastrevirus, Curtovirus, Topocovirus* and *Begomovirus* based on their genomic organization, vector transmission and host range (Varma and Malathi, 2003). They are characterized by germinate (twin shape) morphology of particles and circular single stranded genome, consisting of one or two DNA components (DNA-A and DNA-B) of ~3kb in length.

Among all known plant viruses, Gemini viruses have emerged as the most destructive pathogen and they are still evolving and so for, more than four hundred Gemini virus isolates have been demarcated. In last two decades the incidence and severity of diseases caused by Gemini viruses has increased enormously in different part of the world including Indian subcontinent. More than 80% of the known Gemini virus are transmitted by whitefly (*Aleyrodidae*) in a persistent manner and belong to genus *Begomovirus.*

In compared to *Mastrevirus, Curtovirus, Topocovirus*, the *begomovirus* have emerged as a serious problem of many economically important crops like cotton, okra, chili, tomato, cucurbits etc.

Most of the old world begomovirus consist of only one genomic component DNA-A (Monopartite) while, majority of the new world begomoviruses, are having two genomic DNA components DNA-A and DNA-B (Bipartite) (Hanley-Bowdoin et al. 1999) (Fig. 1).

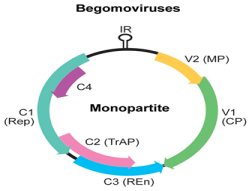

Fig. 1: Genomic Organization of Begomovirus

In recent years, a satellite DNA molecule are found to be associated with mono or bipartite begomovirus and are found to play an important role in the systemic infection and symptom developments (Bidden et al. 2001).

Okra (*Abelmoshus esculentus* (L.) Moench), also called as bhindi or lady's finger belong to the family *Malvaceae*. It is an important vegetable crop for Indian agriculture and is grown throughout the year across the Indian subcontinent.

India rank first for the production of okra with 5,784.0 thousand tones (72% of the total world production In India, Uttar Pradesh, Bihar, West Bengal, Orissa, Andhra Pradesh, Gujarat, Maharashtra, Assam, Chhattisgarh, Haryana, Karnataka, Tamilnadu and Kerala are the major okra growing states, with an area of 0.53 million hectare annual production of 6.36 million tones and a productivity of 11.9 tones/hec.

Okra is an economically important vegetable crop. The roots and stems of okra are used for cleaning the cane juice from which gur or jiggery is prepared. Okra is an important source of vitamins, calcium, potassium and other minerals, which are often missing in the plat of developing countries like India (Basu and Gosh, 1943).

Okra crop is attacked by number of pathogens namely fungi, bacteria, virus and nematode. A number of viral diseases like yellow vein mosaic, leaf curl, and venation leaf curl are known to infect okra. Among these *Okra yellow vein mosaic virus* (OYVMV) and *Okra leaf curl virus* (OLCV) and recently reported *Okra venation leaf curl virus* are the most important begomovirus diseases of okra in India and abroad (Table 1) (Singh and Dutta, 1986). The successful cultivation of okra in western Uttar Pradesh received a serious setback due to two diseases; Okra yellow vein mosaic virus (OYVMV) and Okra venation leaf curl virus (OELCV) which are transmitted persistently by white fly (Capoor and Verma, 1950; Lana, 1976) and can infect the crop at any stage of the plant.

TABLE: 1 List of Begomovirus affecting okra reported from various parts of the India.

S.No.	Begomovirus	State	Genomic component	References
1.	Bhendi yellow vein Mosaic virus	Karnataka, Maharashtra	Monopartite	Kulkarni,1924
2.	Okra venation leaf curl Virus	Karnataka	Monopartite	Singh,1996
3.	Bhendi yellow vein Maharashtra virus	Maharashtra	Monopartite	Brown et al. 2012
4.	Bhendi yellow vein Delhi virus	Delhi		Venkataravanappa et al. 2011
5.	Bhendi yellow vein Haryana virus	Haryana	Monopartite	Brown et al. 2012
6.	Radish leaf curl virus	Bihar	Monopartite	Kumar et al. 2012
7.	Cotton leaf curl Alabad virus	Haryana, Karnataka	Monopartite	Venkataravanappa et al. 2012
8.	Cotton leaf curl Bangalore virus	Karnataka	Monopartite	Venkataravanappa et al. 2013
9.	Bhendi yellow vein Bhubaneswar virus	Orissa	Monopartite	Venkataravanappa et al. 2013
10.	Okra venation leaf curl Virus	Gujarat	Monopartite	Sanwal et al., 2014
11.	Okra curl disease-associated DNA 1, isolate OBKG	(Surat) Gujarat	Alpha satellite	Chandran et al. 2013
12.	Okra yellow crinkle Cameroon alphasatellite	(Surat) Gujarat	Alpha satellite	Chandran et al. 2013
13.	Okra venation leaf curl Virus	(Surat) Gujarat	Alpha satellite	Chandran et al. 2013
14.	Bhendi yellow vein mosaic virus	Karnataka	Monopartite	Venkataravanappa et al. 2013
15.	Okra leaf Curl virus	Southern India	Monopartite	Sayed et al. 2014
16.	Bhendi yellow vein Madurai virus	Haryana, Tamil Nadu, Karnataka	Monopartite, captured DNA-B of ToLCNDV	Venkataravanappa et al. 2013
17.	Okra venation leaf curl Virus	Haryana	Monopartite	Venkataravanappa et al. 2015

It was felt desirable to undertake the present studies with the following objectives.

1. Survey for the prevalence and collection of begomovirus infected okra samples from different regions of western Uttar Pradesh.
2. Molecular identification of the causal pathogen using begomovirus specific degenerate in primer through PCR.

2. MATERIALS AND METHODS

2.1 Surveys and collection of plant material

Surveys were conducted in the okra growing areas of Meerut district of Western Uttar Pradesh during years 2016-2017. Several fields had okra plants (varieties/cultivars) showing symptoms typical of begomovirus infection, with different degrees of severity, including yellow mosaic, interveinal yellowing, upward and downward curling of leaves, venations of leaf and stunting of plants. The top leaves of these plants were sampled and brought to laboratory for detection of the causal begomovirus including few apparently asymptomatic plants. At least one plant (showed typical symptoms of yellow mosaic disease and extremely stunted) from each location was dug out with earth ball, transplanted in earthen pots and maintained in experimental field for biological and molecular assays of the associated virus.

2.2 Chemicals and Biochemical

General chemicals and reagents were purchased from companies like Hi-Media, Genetix, Merck and Spectrochem (India). Fine chemicals and biochemical of molecular biology were obtained from Sigma Chemicals Co., USA. DNA modifying enzymes were obtained from Genetix, India.

2.3 Total genomic DNA isolation by CTAB method (Porebski et al. 1997)

DNA extraction from okra plant containing sticky and resinous materials has been difficult. Polysaccharides visually evident in DNA extracted by their viscous, glue-like texture and make the DNA unmanageable in pipetting and unamplifiable in the polymerase chain reaction (PCR) by inhibiting Tag polymerase activity (Fang et al. 1992). With maturity, leaves contain increased quantities of polyphenol, tannins, and polysaccharides. Dealing with such components in nature leaves becomes necessary when younger expanding leaves and shoots are not available during the time of collection.

We are uniformly unsuccessful in our attempts to amplify Okra DNA by PCR using other reported methods, including those of Dellaporta et al. (1984) and Coen et al. (1990). As a result, we found it necessary DNA extraction from fully developed expanded leaf tissue that would yield DNA suitable for PCR. The protocol described here is relatively quick and inexpensive, and provides clean DNA, consistently amplifiable in polymerase chain reaction. Okra tissue and DNA stored for 21 months at -20^{0}C has also been shown to be stable and amplifiable.

2.4 Quantitative estimation of DNA by Pico 200 spectro photometer (Picodrop)

Double stranded DNA was analyzed on Agarose gel. The electrophoresis was carried out in submarine horizontal Agarose slab gel apparatus as described by Sambrook *et al*. (1989). Gels of different strength (0.8 to 1.5) were prepared depending on the size of DNA to be analyzed.

Appropriate amount of Agarose (0.8-1.2%, as per requirement) was dissolved in 1X TAE for running DNA samples or 0.5X TBE buffer by boiling to dissolve completely in the microwave or on electric hot plate. After cooling to about 55^{o}C, ethidium bromide (stain) was added at a concentration of 0.5 µg/ml (prepared in sterile water and mixed thoroughly) and then Agarose solution was poured on a gel casting tray sealed with microspore tape and on which a slot forming comb had been placed at a height of about 2 mm. After the gel had solidified, the comb was removed and the gel was kept in the mini horizontal electrophoresis apparatus. Enough electrophoresis buffer 1X TAE or 0.5X TBE was poured just to cover gel approximately 2-3 mm in the gel running tank. Nucleic acid samples were mixed with one-sixth volume of 6X loading dye and then loaded directly in the wells. DNA marker was also loaded to compare the size of the DNA fragments. The gel was run at 5V/cm of constant voltage till the bromophenol dye reached almost the end of the gel. Distaining was done in water for 10 min and the gel was viewed on a Fotodyne UV trans illuminator and recorded in Bio-Rad gel imager system.

The pair of begomovirus specific degenerate primers was synthesized from Genetix India Pvt. Ltd. The primers designed are capable of amplifying the partial region of coat protein. The PCR was conducted in a Genetix thermal cycler or DNA Engine, PTC-200 peltier thermal cycler (MJ Research, Inc., USA). The PCR product so obtained was checked by electrophoresis on 1% agarose gel as described earlier for DNA agarose gel electrophoresis.

3. RESULTS DISUSSION

3.1 Survey and Symptomatology:-

During surveys conducted in 2016-17, the natural occurrence of severe yellow mosaic, leaf curl and leaf curl venation was observed in various okra growing field in different locations of Meerut. The disease incidence was significant ranging from 35 - 50% in different locations which were surveyed during 2016-2017. In the present study, the incidence of venation leaf curl disease of okra in Meerut

district was found to be increasing in the two subsequent years in contrast to yellow mosaic however, in some cases the okra plant is infected by both yellow mosaic and venation leaf curl disease. However, the disease incidence in various cultivars showed variable degree of susceptibility/tolerance/resistance under field conditions in our survey (Fig. 2).The major symptoms presences were yellow vein mosaic, curling of the leaves, venations and thickening/ swelling of the veins. Infected plants showed stunted growth, less flowering and deformed fruits as compared to the healthy plants (Fig.2)Symptoms in Virus Infected Leaves.

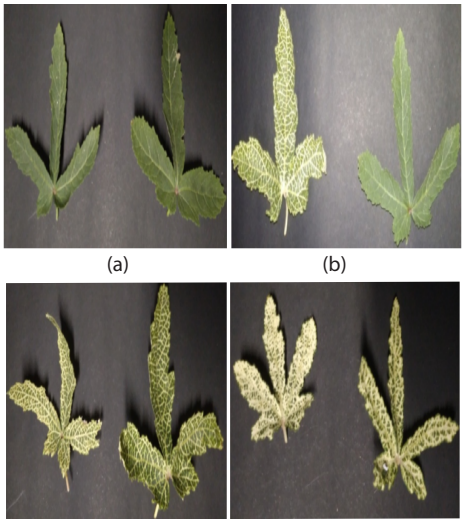

Fig. 2: (a) Uninfected Leaf of Okra, (b) Infected leaf (left) and Healthy Leaf (right), (c) Yellowing and (d) Yellow Vein Mosaic with Curling.

On the basis of symptomology, the pathogen associated with the yellow vein mosaic and leaf curl disease of okra was suspected as a Gemini virus (begomovirus). Therefore, nucleic acid based detection of the virus was attempted by PCR technique. PCR based technique is considered as most sensitive technique for the detection of virus. One okra sample from each location i.e. Gokulpur, Shisoli and Durga Bhabhi Hostel Park, CCS University were used in our study.

3.2 Genomic DNA isolation from okra

Total genomic DNA was isolated from newly emerging virus infected leaf tissue and asymptomatic leaf of healthy okra plant by using various modifications in DNA isolation protocol. In okra, the presence of high mucilage is a big problem for molecular studies because; mucilage (polysaccharides) has viscous glue like texture which makes DNA unmanageable in pipetting and also during gel electrophoresis experiment. These polysaccharides interfere with the nucleic acid and their by affect the downstream analysis. We have followed various DNA isolation protocol to optimize the DNA isolation from okra *viz* Dellaporta et al. (1983), CTAB method (Doyle, 1987). We were successful in isolating good quality of DNA (mucilage free) by using the protocol of (Porebski et al. 1997) with slight modification as described earlier in material & method.

The quality of total genomic DNA which also includes the viral DNA was checked by both-spectrophotometry using Picodrop and agarose gel electrophoresis methods. In Picodrop the OD was taken at 260 and 280 nm and concentration was recorded. The 260/280 ratio was found to be 1.8. The concentration of DNA we also checked by agrose gel electrophoresis with the help of known concentration of Lambda DNA.

3.3 Molecular identification of virus associated with yellow vein mosaic& leaf curl of okra:-

The genus begomovirus is the largest genus of Gemini-viruses, which is transmitted by whitefly, *Bemacia tabaci*. In recent past, the begomovirus have emerged as a most devastating pathogen in the world. Okra is an economically important of vegetable crops in western UP and known to be susceptible to various spp. of begomovirus of which the yellow vein mosaic and venation leaf curl disease severally effect the production and quality of okra. Old world begomovirus have both bipartite (DNA-A and DNA-B) and monopartite genome (only DNA A and lacks DNA-B). The DNA-A component is capable of autonomous replication, encodes factors required for the viral incapsidation, replication and suppression of host defense. Whereas, DNA B encodes factors essential for viral systemic movement, host range determination and symptoms expression in host plants (Rojas et al. 2005). The monopartite begomovirus are also found to be associated with satellite DNA called a DNA β (Saunders et al. 2000), which is required for the symptoms induction host range determination, replication, incapsidation, insect-transmission and movement in the plants.

Majority of the begomoviruses characterized from okra are monopartite and are associated with satellite DNA-beta molecule (Jose and Usha 2003; Saunders et al. 2000). However, reports of bipartite begomovirus are also present (Venkataravanappa et al. 2015). In India, there are several species of begomovirus either mono or bi-partite have been reported from okra and the detailed list of the begomovirus infecting okra reported from various region of India.

In southern part of India, yellow mosaic and leaf curl symptoms of okra are found to occur separately. The infected plant shows either yellow mosaic or leaf curl symptoms. However in Northern India both yellow mosaic and leaf curl symptoms are found together making it complex symptoms. This may indicate the possibility of the emergence of new viral strains in northern India from okra. In present study, the incidence of yellow mosaic and venation leaf curl disease of okra are found from okra growing region of Meerut, Western Uttar Pradesh. The disease incidence is increasing day by day, but so far, there is no report available about the molecular identification of begomovirus from okra from Meerut and Western Uttar Pradesh. Therefore, we attempted the molecular identification of begomovirus from okra applying begomovirus specific degenerate primers (Deng et al. 1994) (Table 2).

Table: 2 List of Primer used (Deng et al.1994).

S. No.	Oligo Name	Sequence (5 '-> 3 ')
1.	D1- Degenerated	TAATATTACCKGWKGVCCSC (20)
2.	D2-- Degenerated	TAATATTACCKGWKGVCCSC (20)

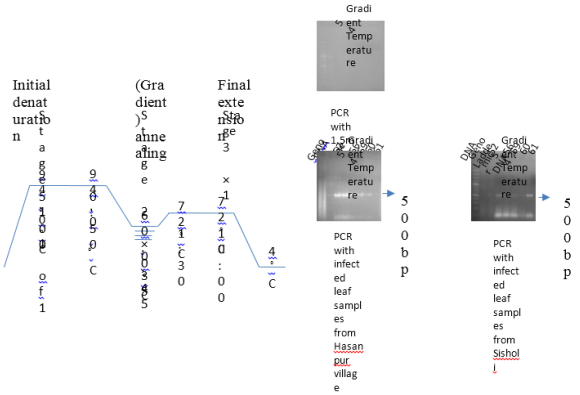

Fig. 3: Gradient PCR using D1/D2 primers and PCR-product electrophoresis gel images.

In present study, the result of positive PCR amplification of approximately 500bp from virus infected leaf sample of okra and absence of amplification in the healthy leaf tissue of okra clearly revealed the association of begomovirus with yellow vein mosaic virus and venation leaf curl disease of okra in western UP (Fig. 3). In

future the amplified PCR product will be cloned and sequenced to establish presence of any new strain of begomovirus for the yellow mosaic and venation leaf curl of okra in western UP.

4. REFERENCES:

1. Basu, K.H. and Gosh, B.D., 1943.Nutritional status in some vegetables.*Indian Journal of Medicinal Research*, *31*, pp.29-31.
2. Brown JK, Fauquet CM, Briddon RW, Zerbini M, Moriones E, Navas-Castillo J, 2012. Geminiviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, eds. *Virus Taxonomy*: Ninth Report of the International Committee on Taxonomy of Viruses. London, UK: Associated Press, pp.351–73.
3. Capoor, S.P. and Varma, P.M., 1950. Yellow vein-mosaic of Hibiscus esculentus L. *Indian Journal of Agricultural Science*, *20*, pp.217-30.
4. Chandran, S. A., Packialakshmi, R. M., Subhalakshmi, K., Prakash, C., Poovannan, K., Prabu, A. N., & Usha, R. (2013). First report of an alphasatellite associated with Okra enation leaf curl virus. *Virus genes*, *46*(3), pp.585-587.
5. Coen, E. S., Romero, J., Doyle, S., Elliott, R., Murphy, G., & Carpenter, R. (1990). floricaula: a homeotic gene required for flower development in Antirrhinum majus. *Cell*, *63*(6), pp.1311-1322.
6. Dellaporta, S. L., Wood, J., & Hicks, J. B. (1983). A plant DNA minipreparation: version II. *Plant molecular biology reporter*, *1*(4), pp.19-21.
7. Deng, D., McGrath, P.F., Robinson, D.J. and Harrison, B.D., 1994. Detection and differentiation of whitefly-transmitted geminiviruses in plants and vector insects by the polymerase chain reaction with degenerate primers. *Annals of Applied Biology*, *125*(2), pp.327-336.
8. Doyle, J. J. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin of Botanical Society of America*, *19*, pp.11-15.
9. Fang, F.C., Libby, S.J., Buchmeier, N.A., Loewen, P.C., Switala, J., Harwood, J. and Guiney, D.G., 1992. The alternative sigma factor katF (rpoS) regulates Salmonella virulence. *Proceedings of the National Academy of Sciences*, *89*(24), pp.11978-11982.
10. Hanley-Bowdoin, L., Settlage, S.B., Orozco, B.M., Nagar, S. and Robertson, D., 1999. Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. *Critical Reviews in Plant Sciences*, *18*(1), pp.71-106.
11. Jose, J. and Usha, R., 2003. Bhendi yellow vein mosaic disease in India is caused by association of a DNA β satellite with a begomovirus. *Virology*, *305*(2), pp.310-317.
12. Kulkarni, G.S., 1924. Mosaic and other related diseases of crops in the Bombay Presidency. *Poona Agriculture College Magazine*, *16*, pp.6-12.
13. Kumar, J., Kumar, A., Singh, S. P., Roy, J. K., Lalit, A., Parmar, D. & Tuli, R. (2012). First report of Radish leaf curl virus infecting okra in India. *New Dis. Rep*, *7*, pp.13-24.
14. Lana, A.F., 1976. Mosaic virus and leaf curl diseases of okra in Nigeria. *PANS*, *22*(4), pp.474-478.
15. Ouenzar, B., Hartmann, C., Rode, A., & Benslimane, A. (1998). Date palm DNA mini-preparation without liquid nitrogen. *Plant Molecular Biology Reporter*, *16*(3), pp.263-269.
16. Porebski, S., Bailey, L.G. and Baum, B.R., 1997.Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant molecular biology reporter*, *15*(1), pp.8-15.
17. Rojas, M.R., Hagen, C., Lucas, W.J. and Gilbertson, R.L., 2005. Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. *Annu.Rev. Phytopathol.*, *43*, pp.361-394.
18. Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual* (No. Ed. 2). Cold spring harbor laboratory press.
19. Sanwal, S. K., Singh, M., Singh, B., & Naik, P. S. (2014). MEETING REPORT: Resistance to Yellow Vein Mosaic Virus and Okra Enation Leaf Curl Virus: challenges and future strategies. *Current Science*, *106*(11), pp.1470-1471.
20. Saunders, K., Briddon, R.W. and Stanley, J., 2008. 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*, *89*(12), pp.3165-3172.
21. Sayed, S. S., Rana, D., Krishna, G., Reddy, P. S., & Bhattacharya, P. S. (2014). Association of Begomovirus with Okra (Abelmoschus esculentus L.) leaf curl virus disease in southern India. *SAJ Biotechnol*, *1*(102), pp.2-2.
22. Singh, S. J. (1996). Assessment of losses in okra due to enation leaf curl virus. *Indian Journal of virology*, *12*(1), pp.51-53.
23. Singh, S. J., & Dutta, O. P. (1986). Enation leaf curl of okra-a new virus disease. *Indian Journal of Virology*, *2*, pp.114-117.
24. Varma, A., & Malathi, V. G. (2003). Emerging geminivirus problems: a serious threat to crop production. *Annals of Applied Biology*, *142*(2), pp.145-164.
25. Venkataravanappa, V., Lakshminarayana Reddy, C.N., Swarnalatha, P., Devaraju, Jalali, S. and Reddy, M.K., 2012.Molecular evidence for association of Cotton leaf curl Alabad virus with yellow vein mosaic disease of okra in North India.*Archives of Phytopathology and Plant Protection*, *45*(17), pp.2095-2113.
26. Venkataravanappa, V., Prasanna, H.C., Lakshminarayana Reddy, C.N. and Krishna Reddy, M., 2015. Evidence for two predominant viral lineages, recombination and subpopulation structure in begomoviruses associated with yellow vein mosaic disease of okra in India. *Plant pathology*, *64*(3), pp.508-518.
27. Venkataravanappa, V., Reddy, C.L., Jalali, S. and Reddy, M.K., 2012.Molecular characterization of distinct bipartite begomovirus infecting bhendi (Abelmoschusesculentus L.) in India.*Virus Genes*, *44*(3), pp.522-535.
28. Venkataravanappa, V., Reddy, C.L., Jalali, S. and Reddy, M.K., 2013. Molecular characterization of a new species of begomovirus associated with yellow vein mosaic of bhendi (Okra) in Bhubhaneswar, India. *European journal of plant pathology*, *136*(4), pp.811-822.