DETECTION OF YELLOW VEIN MOSAIC DISEASE OF OKRA IN GUJARAT

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ABSTRACT

Yellow vein mosaic disease is the major limitation in the production of bhendi or okra (Abelmoschus esculentus), an important vegetable crop of India. Direct Antigen Coating ELISA (DAC-ELISA), African Cassava mosaic virus antisera could efficiently detect yellow vein mosaic virus in all four dilutions where average A 405 nm ranged from 0.554 to 0.919. Healthy samples of okra leaves showed absorbance values ranging from 0.204 to 0.219. The diseased sample was subjected to Polymerase Chain Reaction (PCR) to diagnose and confirm geminiviral infection. Oligonucleotide primers designed to anneal to the conserved sequences of numerous range of Whitefly Transmitted Geminiviruses (WTGs) were employed to amplify the genomic template DNA of virus. The primer pair B1.f (Virion Sense) and Bcp2.r (Common Region Sense) consistently yielded amplicons of 2.7Kb fragment size for the genomic DNA.

KEY WORDS: DAC- ELISA, Geminiviruses, Okra, PCR, Primers, YVMV

INTRODUCTION

The commercial cultivation of okra has received a great setback under Indian conditions due to the attack of many fungal, viral and nematode diseases *viz*., powdery mildew, leaf spots, yellow vein mosaic, enation leaf curl, okra leaf curl, okra mosaic and root-knot nematodes. Among them, yellow vein mosaic virus (YVM) is the most destructive virus disease and has become limiting factor in successful cultivation of the crop. The disease is characterized by typical yellow network of veins and veinlets. In severe cases, the interveinal areas of the leaves become chlorotic and the leaf turn yellow. Fruits are dwarfed, malformed and become yellowish green in colour, thereby reducing their market value. The virus causing YVM of okra is known as yellow vein mosaic virus (YVMV). The virus is neither sap transmissible nor seed and in nature the transmission occurs only through insect vector, whitefly (*Bemisia tabaci*). The disease is caused by a complex consisting of monopartite begomovirus bhendi yellow vein mosaic virus (BYVMV, Geminiviridae) and a small satellite DNA-β component. The causal agent of yellow vein mosaic disease of okra was diagnosed as a geminivirus, 18 x 30 nm in size, which showed a close relationship to Indian cassava mosaic bigeminivirus (ICMV) in ELISA tests using ICMV polyclonal antiserum. The virus, named bhendi yellow vein mosaic virus, was more closely related to ICMV than Indian cassava mosaic bigeminivirus.

MATERIALS AND METHODS

Enzyme Linked Immunosorbent Assay

Reaction of OYVMV to other antisera of geminivirus was evaluated in indirect ELISA (DAC- ELISA) as per the procedure described by Hobbs *et al*. (1987) and Mowat and Dowson (1987). For the source of antigen, 10 days old okra plants were inoculated with OYVMV using 15 viruliferous whiteflies per plant. On the initiation of symptoms on leaf samples from inoculated plants were collected at 15 days after inoculation. Polyclonal antibody raised in rabbits was taken. Alkaline phosphatase conjugated goat antirabbit immunoglobulin.

Reagents

A. *Coating buffer (Carbonate buffer, pH 9.6) for antigen extraction and dilution*

Sodium carbonate (Na_2CO_3)	1.59 g
Sodium bicarbonate ($NaHCO_3$)	2.93 g
Sodium diethyl dithiocarbamate (DIECA)	0.01 M
Polyvinyl Pyrrolidone (PVP)- MW.40000	20.0 g
Distilled water	1.0 lit
Stored at 4^0C	

B. Phosphate buffered saline – Tween (PBS-T, pH 7.4) washing solution

Sodium hydrogen phosphate dibasic $Na_2HPO_4.2H_2O$	1.44 g
Potassium dihydrogen orthophosphate (KH_2PO_4)	0.2 g
Potassium chloride (KCL)	0.2 g
Sodium chloride (NaCl)	8.0 g
Tween - 20	0.5 g
Distilled water	1.0 lit
Stored at room temperature	

C. Antibody / conjugate dilution buffer (PBS-TPO)

PBS-T	1.0 lit
PVP	20.0 g
Ovalalbumin	2.0 g

D. Substrate buffer, pH 9.8

Diethanolamine	97.0 ml
Distilled water	800.0 ml

The pH was adjusted to 9.8 with 1.0 N HCL and the volume was made up to 1000 ml with distilled water. The substrate buffer was stored at room temperature to prevent its solidification in cold. Substrate was prepared freshly just before use by dissolving p- nitrophenyl phosphate (PNP) to a concentration of 0.5 mg per ml of diethanolamine substrate buffer.

Procedure

Leaf samples of okra, ageratum, croton and sida were extracted in carbonate buffer, pH 9.6 containing 0.01M Diethyl dithiocarbamate and 0.2 % polyvinyl pyrrolidone. The homogenates were centrifuged at 10,000 rpm for 10 minutes at 0^0 C. Then the antigens were pippeted into duplicate wells (100 µl per well) of tarsons make immunological plates. The treatments were replicated twice. After incubation and washing the antigens were exposed to polyclonal antisera against ACMV (diluted to 1: 1000 with antibody buffer) and incubated. The alkaline phosphatase conjugated antirabbit immunoglobulin (100 µl) was loaded to each at a dilution of 1:

2000 with antibody dilution buffer. Then the substrate (p- nitrophenyl phosphate diethanolamine buffer, 0.5 mg per ml) was loaded to the wells at 150 µl per well and incubated for a period of 30 minutes at room temperature after which 50 µl of 3M NaOH was added to each well for stopping the enzyme – substrate reaction. Suitable control wells were maintained using extracts of healthy plant and the antigen extraction buffer. The absorbance of the well content was read at 405 nm in ELISA reader (BIO-RAD model 550).

Polymerase chain reaction

Extraction of viral genomic DNA

With a motive of extracting viral genomic DNA from samples, citrate method done by Jose and Usha (2000) was applied which has been systematically envisaged as under:

DNA extraction protocol

1 g of okra leaves was taken and ground in pestle and mortar using liquid nitrogen. Then finely ground powder was transferred in flask containing 6 ml of extraction buffer. 2 ml of chloroform: isoamyl alcohol was added in flask and mixed thoroughly for formation of an emulsion. It was transferred in centrifuge tubes and spun at 15,000 g for 15 minutes at 4^0C. Top aqueous phase was transferred in a beaker containing 0.2 M NaCl and PEG 6000 (7%) and stirred it for 3 hrs at 4^0 C using magnetic stirrer. Undissolved mucilage was spool out and solution was transferred to polypropylene tubes and spun at 18,000 g for 25 minutes at 4^0 C. Supernatant was drained off and to avoid any trace of PEG, the pellet was dried and taken and was dissolved in 600 µl of 0.1M citrate buffer pH 6.0 without β-mercaptoethanol. Quick spun was given at 1,100 g to remove the insoluble materials. The above solution was equally divided into three micro centrifuge tubes and 400 µl of freshly prepared 1 % SDS and 0.2M NaCl solution was added in each tubes, mixed gently and kept on ice for 15 minutes. 300 µl of 3 M sodium acetate (ph 5.2) was added to each tubes, mixed gently and kept on ice for 30 min. It was spun at 10,000 g for 10 min at 4^0 C. 2.5 volumes of absolute alcohol were added to the supernatant, mixed by inversion and solution was freezed at -20^0C for 2 hrs. It was again spun at 12,000 g for 10 min at 4^0 C to pellet the nucleic acids. Supernatant was poured off and a pellet was washed with cold 70 % ethanol. Tubes were vacuum dried and each pellet was resuspended in 20 µl of H_2O. Aqueous phase (60 µl) was transferred into a new micro centrifuge tube and one half volume of 7.5 m ammonium acetate and 2.5 volume of absolute alcohol was added, it was mixed and left for overnight at -70^0 C. It was spun at 12,000 g for 10 min at 4^0C. Supernatant was aspirated and pellet was washed with cold 70 % ethanol. Pellet was vacuum dried; it was resuspended immediately in 15 µl of distilled water. It was used for PCR study.

Quantitative assay

The quantitative assay was done through the Nano Drop-1000 N.D. version 3.3.1.

Qualitative assay

This was achieved by subjecting sample DNA suspension to Horizontal Gel Electrophoresis. A 1.2 percent Agarose (Agarose LE, Bangalore GENEI, India) solution was prepared by dissolving 1.2 g Agarose in 100ml 0.5X TBE buffer and melting the same in a microwave oven for 3 minutes at 60 per cent of total Watt Power of the machine. The Agarose powder after weighing in a precision standard electronic digital balance was sprinkled on the ice chilled buffer for better adsorption and kept at room temperature for at least 30 minutes before melting. The gel was prepared on a slab gel tray by sealing the free ends with the help of an adhesive tape framed on a center-balanced gel preparing stage.

Polypropylene combs were also fixed before pouring in melted gel solution of Agarose onto the slab; care was taken to remove the entrapped air bubbles in gel to facilitate uniform electrical conductivity. Before pouring in the Agarose solution 2µl of Ethidium Bromide was added and mixed thoroughly. This was done with an objective of visualizing nucleic acid bands after electrophoresis them in a Horizontal Submarine Electrophoresis Apparatus (Bangalore GENEI, India).The combs were removed after about 40 minutes once the Agarose Gel had already been solidified. Due care had been taken while removing the comb from gel to avoid any possible cracking of the well which otherwise could create undesirable hindrance on electropolation of sample DNA. The samples were loaded in wells of the slab gel prepared, immersed up to 3cm in 0.5X TBE running buffer in the apparatus with the help of a loading syringe (Hamilton, Germany). Before loading the samples, 1µl of Bromophenol Blue dye solution was properly mixed with 6µl of DNA suspension. The apparatus was then set to 80 volts, 45 minutes, 40 watts and 3mA by a power pack (BIORAD, USA). After electropolation, the migrated sample-DNA was viewed under Bio Imaging system (Gene Genius, London) and the gel was snapped for a record.

Preparation of reaction mixture

The reaction mix was prepared in a 0.5ml microfuge tube; resting on a chilled Minicooler or ice pad. 33.3 µl Ultra pure Autoclaved water 8.0 µl PCR master mix, 1.0 µl Primer I (Virion sense) B1.f, 1.0 µl, Primer II (Common Region Sense) Bcp2.r and 7.2 µl Template DNA were added sequentially under aseptic condition in a Laminar Air Flow Cabinet. The master mixture devoid of template DNA was freshly prepared and then briefly centrifuged at 10,000rpm for 1 minute in a microfuge and then 7.2 µl template DNA was added to each 200µl microfuge tube to make a final volume of 50µl of PCR reaction mix. Afterwards, it was again centrifuged briefly for 1 minute at 3000 rpm, once the reaction mixture was pippeted into a 0.2 ml PCR tube.

Thermal cycling

The thermal cycling for amplification was accomplished in a minichip based thermal cycler (*T-personal*, Biometra, Germany) programmed as depicted below:

Preheating temperature	Nil
Lid Temperature	105°C
Denaturing temperature	94°C for 1 minute
Annealing temperature	55°C for 2 minute
Extension temperature	72°C for 2 minute
Repeated 30 times for	30 cycles
Final extension temperature	72^0C for 10 minutes Holding at 4^0C for indefinite time period

Analysis of PCR products

The PCR products were subjected to Agarose Gel Electrophoresis in a Horizontal Submarine Apparatus (Bangalore GENEI, India). The gel contained 1.2 per cent Agarose 1000 (capable of segregating 50bp segments; Bangalore GENEI, India) was prepared as described earlier. Bromophenol Blue Dye, 1µl was added to 6µl sample (PCR product) and loaded on wells framed in gel with the help of a loading syringe (Hamilton, Germany). Markers were

provided by loading 1µl 1Kb plus DNA ladder (Bangalore GENEI, India) along with 1µl Bromophenol Blue dye Solution. Samples were now electrophoresed at 80 volt, 40 watt and 3mA for 45 to 60 minutes in 0.5X TBE running buffer in the Horizontal Electrophoresis Submarine Apparatus. Ethidium Bromide was used to stain the nucleic acid as described earlier. The gel was illuminated under Bio imaging system (Gene Genius, London) to visualize the amplicons of the Geminivirus DNA and photographed.

Extraction by citrate method

100 mM sodium citrate, pH 6.0, Chloroform : isoamyl alcohol 24:1 (v/v), PEG 6000 2 M NaCl, β-mercaptoethanol (50 µl/10 ml). TBE Buffer was prepared as Stock (5X) by mixing 54 g Tris base,, 27.5 g Boric Acid, 20 ml 0.5 M EDTA (pH 8.0) and 1 litre Autoclaved Distilled water.0.5 X Working solution was prepared by adding 1 part of 5X TBE 0.045 M Tris and 9 part autoclaved distilled water . 1.2 g agarose solution was prepared**.** 6X Loading Buffer (stored at 4°C) was prepared by mixing 25 mg Bromophenol Blue and 3 ml Glycerol and 10 ml autoclaved distilled water was added. Ethidium Staining Solution (1: 1000) was made as 20 µl ethidium bromide solution and 20 ml and autoclaved distilled water.

RESULTS AND DISCUSSION

Enzyme Linked Immunosorbent Assay

African cassava mosaic virus (ACMV) polyclonal antibody conjugated with alkaline phosphatase was used to detect OYVMV in crude sap of okra. p-nitrophenylphosphate (0.5mg/ml) in 10 per cent diethanolamine was added as substrate, After adding substrate yellow colour started developing in 30 minutes indicated presence of yellow vein mosaic virus, whereas known sample did not produce any colour up to certain (Autophoto degradation of p-nitrophenylphosphate brings extra yellow colour) period. Two hour after adding substrate plate was read at A 405nm by BIO-RAD ELISA plate reader. ACMV conjugate could efficiently detect yellow vein mosaic virus in all four dilutions where average A 405nm ranged from 0.554 to 0.919. Healthy okra leaf extract showed absorbance values ranging from 0.204 to 0.219. The absorbance value for infected leaf extract was 2 to 4 times higher than the comparable with diseased leaf extract (Table 1).

Polymerase chain reaction

Quantitative and qualitative assay of template DNA

Sample DNA extracts used as template DNA in PCR reaction was testified by Nano Drop-1000 N. D. Version 3.3.1. The data pertaining to the same have been depicted in Table 2. The data for quantitative measure unveil that there had been appreciable quantity of template DNA (1.81 ng/µl) in the nucleic acid extracts of okra (by Extraction buffer Protocol). 1.78 ng/µl quantity of viral DNA obtained from *Althea rosea* whereas very much low quantity of viral DNA (0.335 ng/µl) obtained from *Ageratum conyzoides*

Preparation of reaction mixture

The reaction mix prepared for the PCR had been differential in context to visualization of nucleic acid bands, as it is focused from the digitized image of representative agarose gel. The reaction mixture containing 1.5µl $MgCl_2$ had been found to yield steep and clear nucleic acid bands (exempted from spurious products) rather than to that of higher concentration of $MgCl_2$ (2.0µl).

Thermal cycling

A thermal gradient reaction to optimize the annealing temperature and thus to better the specificity of reaction had been attempted, digitized image of the representative agarose gel supports that an increased annealing temperature (58.7^0C)

for the thermal cycling in PCR gave very specific band of amplicons devoid of spurious products and any artifact which are not uncommon under low temperature annealing reactions.

Reaction of oligonucleotide primers to the template DNA

The samples that were found positive in reaction to the oligonucleotide primers, designed to react with diverse range of characterized, incompletely characterized and even uncharacterized Geminiviruses have been envisaged in the (Table. 3). The primer pair B1.f (Virion Sense) and Bcp2.r (Common Region Sense) consistently yielded amplicons of 2.7Kb fragment size for the genomic DNA-A (Plate 1).

OYVMV inoculated plant species by *B. tabaci* in glasshouse were tested by DAC-ELISA. A network of serological relationship exists among whitefly transmitted geminiviruses (Harrison, 1985). Harrison *et al.* (1991) provided evidence that OYVMV was a geminivirus. The present study also revealed an antigenic similarity between OYVMV and ACMV. Detection of OYVMV in sap extracts of infected okra leaves using polyclonal antibody to ACMV was also reported by Handa and Gupta (1993). The polymerase chain reaction, to detect and confirm the presence of Geminiviruses was found to be best resolved with a $MgCl_2$ concentration of 1.5µl per 50µl reaction mix. Whereas, it was 0.5 to 2.5µl per 50µl reaction mix, as used by Rojas *et al.* (1993). The concentration of $MgCl_2$ is a crucial decisive factor in PCR, higher concentrations of the same shall lead to the formation of many an unspecific spurious products and the sensitivity of the test is altered, adversely (Deng *et al.*, 1994). The annealing temperature was standardized at 58.7^0C for 2 minute during the reaction to finish up with a very specific and steep band under resolution of the primed amplicons. However, it was 50 to 55^0C at 1 and 2 minutes, respectively (Rojas *et al.*, 1993). The annealing temperature was excelled to even 60°C at times to furnish with a very specific product after PCR (Deng *et al.*, 1994). The role of annealing temperature in a PCR is indispensably decisive in priming with greater specificity and avoiding spurious products and artifact effects. Generally, the priming specificity is directly proportional to the increase in annealing temperature within the temperature range of $55\text{-}60^0$ C, declining or exceeding which may not catalyse priming in PCR. The oligonucleotide degenerate primers designed from the conserved sequence of the viral genome identified from the nucleotide or amino acid sequence alignments to amplify the fragment of the DNA. The primer combination B1.f and Bcp2.r had been consistent in amplifying a fragment length of approximately 2.7 Kb from genomic DNA of the WTGs. The result of it was conformity with that of Jose and Usha (2000).

CONCLUSION

In ELISA, YVMV diseased leaf extract in four dilutions were positively reacted with the African cassava mosaic virus antisera. The strong reaction was observed in 1:10 antigen dilution.The DNA was extracted by extraction buffer method and 1.81 ng/µl and 1.78 ng/µl DNA was obtained from Okra and hollyhock, respectively. This extracted DNA was subjected to polymerase chain reaction using the primer pair B1.f (Virion Sense) and Bcp2.r (Common Region Sense) which consistently yielded amplicons of 2.7Kb fragment size for the genomic DNA. Serologically yellow vein mosaic virus is closely related to African cassava mosaic virus. PCR result evident begomovirus belongs to family geminiviridae of subgroup III.

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Table 1: Serological reaction of YVMV of okra against African cassava mosaic virus

Antigen Dilution	O. D. at A 405nm	
	Okra Healthy Leaf Extract*	*YVMV Diseased Leaf Extract**
1:10	0.219	0.919
1:20	0.212	0.809
1: 50	0.207	0.680
1:100	0.204	0.554

African cassava mosaic virus dilution 1: 1000
**** Mean values of 12 wells.***

Table 2: Quantitative and qualitative assay of viral genomic DNA by nanodrop meter

Sr. No.	Sample	Absorbance		Ratio (λ_1/λ_2)	DNA Concen-tration	Agarose Gel Electro-phoresis (Bands Evident)
		At 260 nm (λ_1)	At 280nm (λ_2)			
1	Okra infected	0.362	0.275	1.3145	1.81	Yes
2	*Althea rosea*	0.356	0.2732	1.3015	1.78	Yes
3	*Ageratum conyzoides*	0.067	0.011	1.4842	0.335	No

Absorbance values read in Nano Drop-1000 N. D. Version 3.3.1

Table 3: Polymerase Chain Reaction (PCR) using oligonucleotide degenerate primers against yellow vein mosaic virus of okra

Primer	Sequence (5′ to 3′)	Molecular Weight (μg/μ mole)	Milli molar Extinction Coefficient (OD/ μmol)	μg per OD	nmoles per OD	Primer Length	Scale of Synthesis (n mol)	Per Cent GC
B1.f	AAT TAA TAA AGT TTG AAT TTT ATA TC	7981	146	5.7	18.3	26	0.01	11.5
Bcp2.r	TCA ATT CGT TAC AGA GTC	5474	147	5.3	26.9	18	0.01	38.9

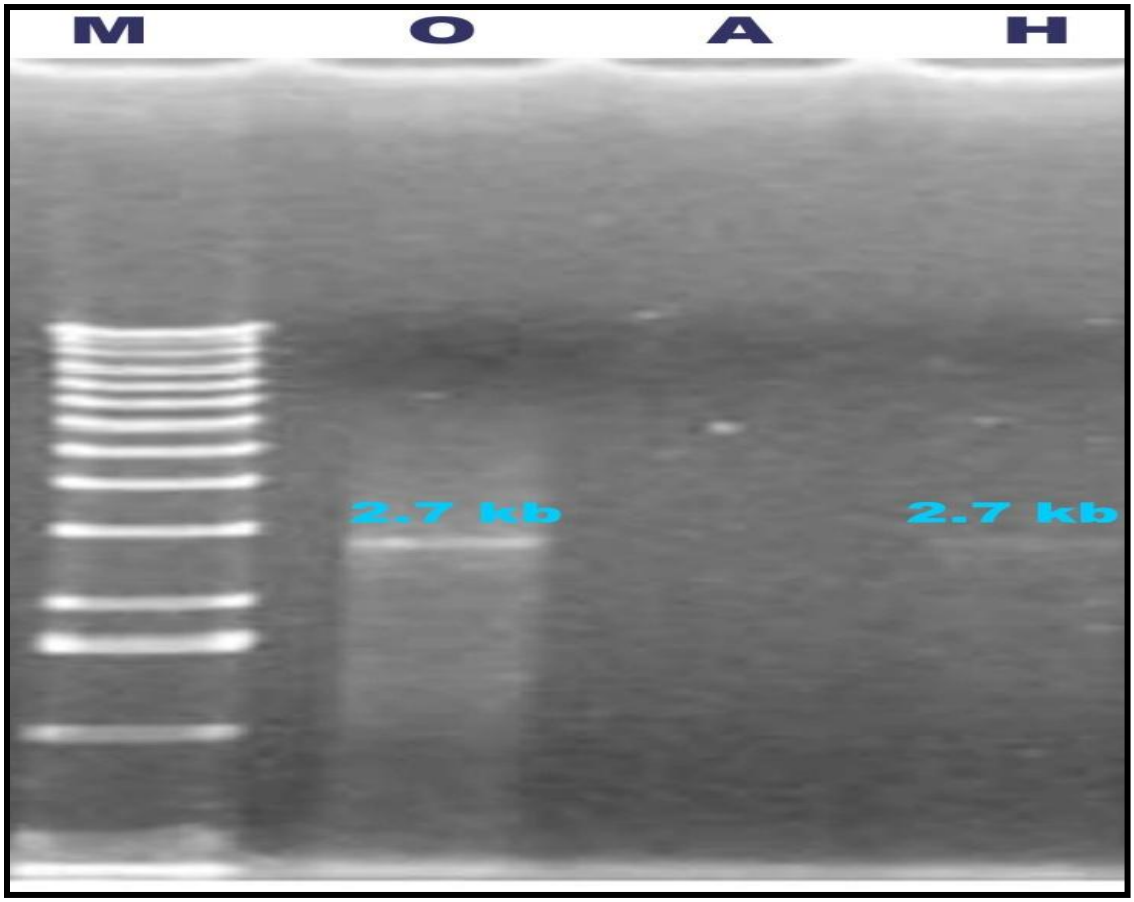

Plate 1: PCA amplification of 2.7 kb viral DNA

(Where: M: Marker O : Okra A : Ageratum conyzoides H : Hibiscus rosa-sinensis)

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