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Molecular Detection of *Mungbean Yellow Mosaic Virus* and Their Associated Satellite DNA from Field Samples of Tamil Nadu

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Pulses are the important source of protein in vegetarian diet and they are affected by several viral diseases. Among the virus diseases, yellow mosaic disease (YMD) caused by *Mungbean yellow mosaic virus* (MYMV) belonging to the genus *Begomovirus* (family: *Geminiviridae*) is a serious malady in cultivation of grain legumes in India and cause severe yield loss. YMD has very narrow host range within legumes and cause biologically indistinguishable symptoms, so the identification of virus is difficult. The molecular detection of MYMV from infected blackgram plants showing yellow mosaic symptoms was carried out. PCR with begomovirus specific primers detected the presence of begomovirus from the infected samples with amplicons of ~1100bp. The PCR with MYMV-specific coat protein primers detected the presence of MYMV from the samples collected from three districts of Tamil Nadu with the amplicons of ~1000bp. The dot blot hybridization showed positive response with the infected samples and negative response with healthy samples. The associated satellite DNA components were detected using universal abutting primers. Hence, molecular detection of MYMV seems simple and very sensitive method.

Key words: Mungbean Yellow Mosaic Virus, Polymerase Chain Reaction, Dot blot hybridization

Pulse crops also known as grain legumes are the second most important group of crops grown essentially in South Asia. In India, they form the important constituent in the vegetarian diet and are cultivated under different agroclimatic conditions. They are cultivated as a short duration crop either as sole crop or in intercropping system round the year in all the four crop seasons. India holds the first rank in pulses production and consumption in the world. India grows the largest varieties of pulses in the world accounting for about 32 per cent of the area and 23 per cent of the world production. It is grown in an area of 10.84 million hectares in India with a production of 17.09 million tonnes (productivity – 699 kg/ha) (GOI Bulletin, 2012). Among pulses, blackgram (*Vigna mungo* L. Hepper) occupies a prominent place in India, covering an area of about 3.29 million hactares with the production of 1.83 million tonnes and a productivity of 555 kg/ha (AICRP report, 2012-13).

Viruses belonging to Como, Gemini, Ilar Poty and Tospovirus groups are known to infect pulses under natural conditions in various parts of the world. Major damage is due to the yellow mosaic disease, caused by Mungbean Yellow Mosaic Virus (MYMV) belonging to the genus Begomovirus of the family Geminiviridae. This disease is a serious malady of the pulses and is the major threat to cultivation of pulses in India (Malathi, 2007). The yellow mosaic disease was reported in mungbean by Nariani (1960). Detection and diagnosis of MYMV and their associated satellite DNA components are essential for managing the disease caused by them and in studying the epidemiology of the viruses. Most of the yellow mosaic viruses infecting grain legumes in India are not sap-transmissible, share a very narrow host range within legumes and cause biologically indistinguishable symptoms, making specific identification of the viruses difficult.

Recently, there has been a shift from identification of the virus based on biological characteristics like symptoms, host range and transmission characters to serological and nucleic acid based techniques. Because of the difficulty in purifying begomovirus virion particles, nucleic acid based approaches like PCR and hybridization with radiolabelled probes are being widely preferred for their diagnosis in the place of serological techniques. Roy and Malathi (2004) developed nucleic acid hybridization technique to detect the *Cowpea Golden Mosaic Virus* (CPGMV) in many crops and weed species. This study showed that CPGMV DNA A probe detected all the begomoviruses whereas, CPGMV DNA B probe is specific to CPGMV. Deng *et al*. (1994) designed the degenerate primers to amplify the virus belonging to the family *Geminiviridae*. Rojas *et al*. (1993) designed the degenerate primers from the highly conserved region of DNA A component to amplify the virus belonging the genus *Begomovirus*. Naimuddin *et al*. (2011a) designed the specific primers to amplify both the DNA components of MYMV and MYMIV and the

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primers were designed from the coat protein region DNA A component, whereas for DNA B, the primers were designed from the movement protein region.

Keeping the importance of accurate identification of virus and their associated DNA components in view, the present study was carried out to identify the viruses associated with yellow mosaic disease of blackgram at Tamil Nadu.

Materials and Methods

Sample collection

Blackgram plants showing severe yellow mosaic symptoms were collected from distantly situated districts of Tamil Nadu *viz*., experimental fields at TNAU, Coimbatore (Coimbatore), farmers field at Vamban (Pudukottai) and Panpozhi (Tirunelveli) (Table 1), where the disease incidence was 90-100 per cent. Young leaf samples showing yellow mosaic symptoms were collected and used for this research work.

Extraction of genomic DNA from infected samples

Total DNA was extracted from young leaves of blackgram and greengram showing yellow mosaic symptoms collected from Coimbatore, Vamban and Panpozhi by GEM-CTAB method (Rouhibakhsh *et al.,* 2008) using 2 % β-mercapto ethanol. The symptomatic fresh young leaves were collected from field in the early morning. One hundred milligram of leaves were ground to fine powder using liquid nitrogen. Pre-warmed DNA extraction buffer (N-cetyl-N,N,N trimethyl ammonium bromide (2 %), 100 mM Tris HCl (pH: 8.0), 1.4 M NaCl, 20 mM EDTA (pH: 8.0) and β-mercaptoethanol (2%)) was added to the ground leaves and incubated at 65°C for 30 min followed by the addition of 0.7-0.8 vol. of chloroform and isoamylalcohol (24:1 v/v). The contents were gently mixed by inverting the tube for 10 min and then centrifuged at 10,000 rpm for 10 min. The upper aqueous phase containing DNA was transferred to a new 1.5 ml micro centrifuge tube and added with equal volume of isopropanol and mixed well by inverting the tube to precipitate the nucleic acid and centrifuged at 10,000 rpm for 10 min. The supernatant was decanted and the DNA pellet was washed with 70% ethanol and air dried. The DNA pellet was resuspended in 50 µl of sterile double distilled water. The genomic DNA was checked by 0.8% agarose gel electrophoresis and stored at -20°C for further use.

Detection of YMV through dot blot hybridization

The presence of begomovirus from the infected samples was verified by dot blot hybridization. The total genomic DNA was spotted on nitrocellulose membrane and was fixed onto membrane by baking the membrane at 80ºC for 1-2 h following standard protocol (Sambrook *et al*., 1989). The hybridization was done with a (∂^{32}P)-dCTP labelled probes specific for CP gene of MYMV-Bg (GenBank Accession no.DQ400848). The probes were prepared by random primer labelling method (Feinberg and Vogelstein, 1983). Hybridization signal detection was carried out using a Storage Phosphor System Cyclone® Plus (Perkin Elmer, Shelton, CT, USA). The hybridization signal intensity was analyzed through densitometric analysis using Opti Quant Version 5.0 (Perkin Elmer, Shelton, CT, USA).

Detection of begomovirus through PCR

The purified genomic DNA was amplified by Polymerase Chain Reaction (PCR) using universal Begomovirus-specific primers PALIc1960 (5'-ACNGGNAARACNATGTGGGC-3') and PALIr772 (5'-GGNAARATHTGGATGGA-3') (Rojas *et al.,* 1993). Reactions were performed in 20 µl mixture containing approximately 50 ng of genomic DNA, 5 mM each dNTPs, 20 pmol of each PALIc1960 forward primer and PALIr772 reverse primer (Table 3) and 0.5 U of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The reaction was carried out in Eppendorf epgradient S Master cycler (Eppendorf, Hamburg, Germany) programmed with initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 3 min, and a final extension step at 72°C for 10 min. Amplicons were analyzed by electrophoresis in 1.5% agarose gels in TAE buffer and visualized by staining with ethidium bromide and recorded with an Alpha Imager 2000 (Alpha Innotech, San Leandro, CA). The sizes of the PCR products were determined by comparison with standard 1 kb molecular marker (Fermentas).

Detection of MYMV through PCR

To detect the presence of MYMV, the PCR was performed using MYMV-specific coat protein primers (forward primer, 5'-ATGGG(T/G) TCCGTTGTATGCTTG-3' and reverse primer, 5'-GGCGTCATTAGCATAGGCAAT-3') (Naimuddin *et al*., 2011).

Detection of associated satellite DNA component

Detection of alpha and betasatellite

The purified genomic DNA was amplified by PCR using universal betasatellites specific primers β01 (5'-GTAGGTACCACTACGCTACGCAGCAGCC-3') and β02(5'-GTGGTACCTACCTACCCTCCCAGGGGTAC AC-3') (Briddon *et al*., 2002) and alphasatellites were amplified using universal abutting primers UN101 -AAGCTTGCGACTATTGTATGAAAGAGG-3') / UN102 (5'- AAGCTTCGTCTGTCTTACGAGCTCGCTG -3') (Bull *et al.* 2003).

Results and Discussion

Disease incidence, symptoms and sample collection

Leaves of blackgram showing severe yellow mosaic symptoms were collected from three hot spot areas of Tamil Nadu, *viz*., Coimbatore (Coimbatore), Vamban (Pudukottai) and Panpozhi (Tirunelveli) which represents three districts of Tamil Nadu. The affected plants showed yellow patches intermingled

with green areas, which later coalesced and showed complete yellowing of leaves (Figure 1). In severe cases pods turned yellow and contained small and

Fig 1. Yellow mosaic disease affected blackgram plants showing severe yellow mosaic symptoms

shrivelled seeds. The seeds were yellow coloured, small, slightly shrunken compared to seeds from healthy plants, which appeared shiny and jet black. Young leaves samples were collected from infected plants from each district.

Table 1. Sample collected from different location in the year 2011-2013

Crop	Location	No. of samples	Symptoms
Black gram	Coimbatore, Coimbatore	45	Yellow mosaic, complete yellowing, bleached appearance of leaf, necrosis, leaf puckering
	Vamban, Pudukottai	27	
	Panpozhi, Tirunelveli	4	
Total number of samples		76	

These samples were used for the present study.

Preliminary diagnosis for the presence of MYMV through dot blot hybridization

Out of 74 samples, 47 samples (63.51%) showed positive response in the hybridization, whereas healthy samples showed no hybridization (Figure 2). Mandal *et al*. (1997) detected the MYMV using dot blot hybridization. Roy and Malathi (2004) developed nucleic acid hybridization technique to detect the *Cowpea Golden Mosaic Virus* (CPGMV) in many crops and weed species.

Detection of MYMV through PCR

The results were further confirmed through PCR using degenerate primers PAR1v772/ PAL1c1960 (Rojas *et al*., 1993). The expected amplification of 1.1 kb was observed in 22 samples (29.72%)

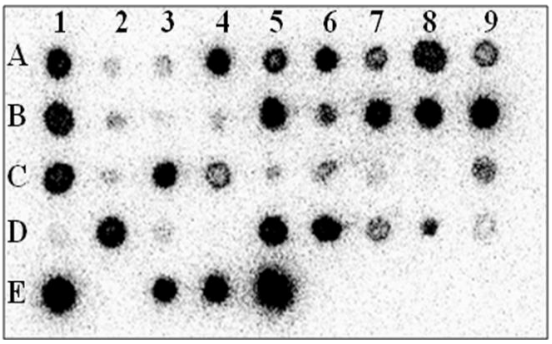

Fig 2. Preliminary diagnosis of *Mungbean Yellow mosaic virus* from yellow mosaic disease affected samples through dot blot hybridization using the probe to MYMV-Vam, DQ400848. Row A,B,C,D and E1-E5- Infected field samples; Row E6-9: Helathy samples

out of 74 samples tested (Figure 3) confirming the presence of begomovirus. In order to detect the MYMV, the PCR was performed using the MYMV-

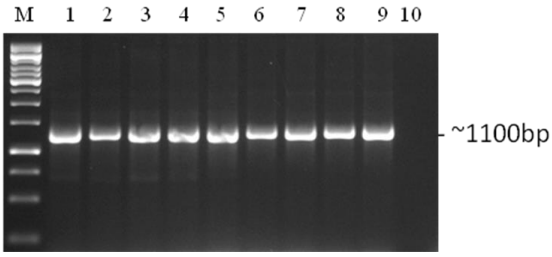

Fig 3. Agarose gel electrophoresis showing presence of begomovirus from yellow mosaic disease affected samples. The amplicons ~1100bp representing the presence of begomovirus. Lane M- 1Kb ladder, Lane 1-9: YMD affected plant samples; Lane 10: Healthy samples.

specific coat protein primers and the results revealed that the expected amplicons of ~1000 bp (Figure 4) indicating the presence of MYMV. The failure to get PCR amplicons may be due to low concentration of the viral DNA and the interference due to polyphenol

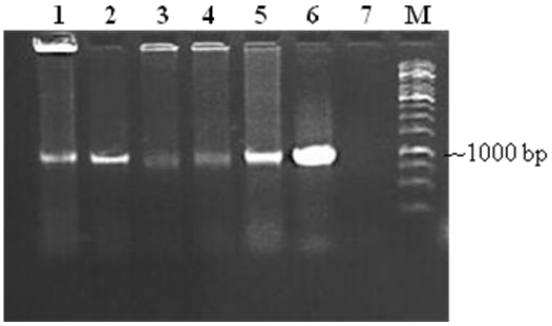

Fig 4. Agarose gel electrophoresis showing presence of MYMV from yellow mosaic disease affected samples through PCR using MYMV-specific Coat protein primers. The amplicons ~1100bp representing the presence of MYMV. Lane M- 1Kb ladder, Lane 1-6: YMD affected plant samples; Lane 7: Healthy samples.

and tannin affecting quality of DNA, the problems experienced by many research workers. Swanson *et al*. (1992) reported the biggest hurdle in the extraction of high quality DNA from legume hosts, which are rich in phenols and polysaccharides and also with very low concentration of virus. Varma *et al*. (1992) reported that the detection of viral genome in the legume host such as mungbean is very difficult

even in dot blot hybridization tests. The association of a begomovirus was confirmed by PCR using primer pairs specific to MYMIV and MYMV that commonly infect cultivated species of Vigna in different parts of India (Karthikeyan *et al*., 2004; Usharani *et al.,* 2004; Naimuddin *et al*., 2011b).

Detection of associated satellite DNA component

DNA was isolated from field samples of blackgram showing severe yellow mosaic along with leaf puckering symptoms. When genomic DNA was subjected to PCR using betasatellite - specific primers, the amplification was seen in the YMD affected plant samples (Figure 5). In recent years, leaf curl and leaf distortion symptoms are observed

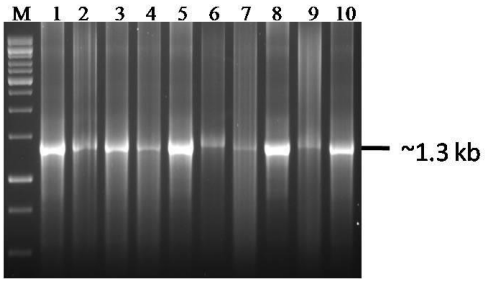

Fig. 5. Detection of betasatellites through PCR using betasatellite specific primers. Lane M- 1Kb ladder, Lane 1-10: YMD affected plant samples

in blackgram and greengram plants in farmers' field in Tamil Nadu. The yellow mosaic symptoms are severe and trifoliate leaves exhibit asymmetry and leaflets look distorted. The results revealed the association of betasatellites with MYMV. In recent years, the begomovirus-betasatellite complex have been reported in legumes. They are tomato leaf curl betasatellite associated with MYMIV in cowpea in northern India (Rouhibakhsh and Malathi, 2005), another Tobacco leaf curl betasatellite associated with MYMIV in cowpea in Pakistan (Ilyas *et al*., 2010), Mungbean yellow mosaic India betasatellite associated with MYMIV in cowpea from India (JX443646, www.ncbi.nlm.nih.gov), the Papaya leaf curl betasatellite from cowpea (DQ118862, www. ncbi.nlm.nih.gov). A new monopartite begomovirus, French bean leaf curl virus (Kamaal *et al*., 2012) has been identified in French bean and it is associated with French bean leaf curl betasatellite (FbLCB).

DNA was isolated from field samples of blackgram which showed severe yellow mosaic along with leaf puckering and leaf distortion symptoms. When genomic DNA was subjected to PCR using alphasatellite-specific primers, the amplification was seen in all the samples tested (Figure 6). Association of alphasatellite with the monopartite begomovirus and betasatellite is well known in many hosts plants (Briddon *et al*. 2004). Recently, Idris *et al*. (2011) showed that an unusual alphasatellite (DNA 2) associated with Tomato yellow leaf curl Oman virus ameliorated symptoms and significantly reduced betasatellite DNA accumulation. In the case of yellow mosaic symptoms, lot of variations are seen in

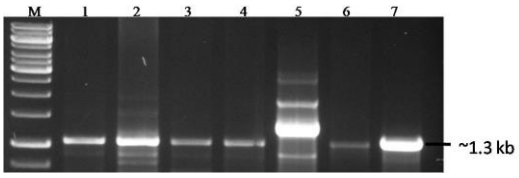

Fig. 6. Detection of alphasatellites through PCR using alphasatellite specific primers.Lane M- 1Kb ladder, Lane 1-7: YMD affected plant samples

symptom severity ranging from well restricted yellow or chlorotic area to, completely bleached yellow leaf, green and yellow patches distributed either randomly or closely; and a very severe necrotic spots on the leaf.

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