MOLECULAR CHARACTERISATION OF YELLOW MOSAIC VIRUS INFECTING BLACK GRAM AND GREEN GRAM IN COIMBATORE DISTRICT

ABSTRACT

YELLOW MOSAIC VIRUS (YMV) IS INDEED ONE OF THE MOST DESTRUCTIVE DISEASES OF MUNGBEAN (*VIGNA RADIATA (L.) WILCZEK*) AND URDBEAN (*VIGNA MUNGO L. HEPPER*). *MUNGBEAN YELLOW MOSAIC VIRUS* INFECTING URDBEAN AND BLACK GRAM CAUSE GREAT YIELD LOSS. THE VIRUS BELONGS TO THE GENUS *BEGOMOVIRUS* OF THE FAMILY *GEMINIVIRIDAE* TRANSMITTED BY WHITE FLY. INFECTED SAMPLES WERE COLLECTED AND SCORED ACCORDING TO DISEASE SCALE. DISEASED SAMPLES WERE COLLECTED FROM INFECTED PLANTS FROM THE FIELD OF COIMBATORE DISTRICT AND PERCENT DISEASE INDEX WAS CALCULATED. THE SYMPTOMS OF YELLOW MOSAIC VIRUS RANGES FROM SMALL YELLOW SPOTS WHICH DEVELOP AS MOSAIC YELLOW PATTERNS. LATER THE DISEASE LEADS TO COMPLETE YELLOWING OF THE LEAF SAMPLES. THE DNA WAS ISOLATED FROM BOTH BLACK GRAM AND GREEN GRAM SAMPLES THROUGH CTAB METHOD. BOTH THE QUALITY AND QUANTITY OF THE DNA WAS EVALUATED THROUGH GEL ELECTROPHORESIS AND NANODROP READING. THE DNA WAS MOLECULARLY CONFIRMED WITH UNIVERSAL PRIMER WHICH SHOW AMPLIFICATION AROUND 1200 BP THROUGH POLYMERASE CHAIN REACTION (PCR). POLYMERASE CHAIN REACTION OF YELLOW MOSAIC VIRUS INFECTING BLACK GRAM AND GREEN GRAM SAMPLES USING MYMV-CP-F/MYMV-CP-R PRIMERS WERE AMPLIFIED AND THE EXPECTED PRODUCT OF SIZE 1000 BP FROM THE OBTAINED INFECTED SAMPLES.

Keywords: [blackgram ,greengram, mosaic, virus, disease scale, coat protein}

1. INTRODUCTION

[Pulses are an essential source of protein and fibre for the world's population. They are high in protein, calories, dietary fibre, a variety of minerals, and bioactive compounds. India is the world's top producer and consumer of pulses. Mungbean (*Vigna radiata (l.) Wilczek*) and urdbean (*Vigna mungo l. Hepper*) are the most major pulse crops grown in the country. Mungbean is also known as green gram or moong, while urdbean is known as black gram or urd (Mishra et.al., 2020) Central Asia is said to be the birthplace of black gram and green

gram (Kumar and Kumar. 2014). India accounts for 70% of global output of black gram and 65% of global production of green gram, respectively

Mungbean yellow mosaic virus, tobacco streak virus, and urdbean leaf crinkle virus are the most common viruses that infect black gram and green gram. Yellow mosaic virus is considered the most devastating viral disease affecting urdbean, green gram causing yield losses ranging from 5 to 100 percent (Nene, 1972; Singh et al., 1980; Rathi, 2002). Nariani (1960) discovered mungbean yellow mosaic in an experimental field at the Indian Agricultural Research Institute in New Delhi. Nene (1968) called it *Mungbean Yellow Mosaic Virus*. *Honda et al.,* (1983) purified the virus initially. *Mungbean Yellow Mosaic Virus* (MYMV) isolates cause infection in the southern and western parts of the country, whereas *Mungbean Yellow Mosaic India Virus* (MYMIV) isolates cause infection in the central, northern, and eastern part (Islam et al., 2012; Malathi and John,2008; Ilyas et al., 2010; Islam et al., 2012; Shahid et al., 2012; Tsai et al., 2013).

The MYMV disease is attributed to begomoviruses, commonly known as geminiviruses, which belong to the prominent and most important genus in the Geminiviridae family. These viruses are characterized as single-stranded DNA viruses that specifically infect plants. They possess typical geminate incomplete icosahedral particles (*Karthikeyan et al.,* 2018). The genome organisation of MYMV consists of 2 circular ssDNA component - DNA-A and DNA-B which are roughly 2.8 kb in size (Borah & Dasgupta 2012). MYMV is transmitted by whitefly *Bemisia tabaci* in persistent manner. It is transmitted by grafting not by seed or soil. *Thongmeearkom et al.* (1981) reported the virus gains entry into the host's phloem cells via the proboscis of the whitefly. Approximately two days before the appearance of symptoms, the viral clusters become visible within the nuclei of the host cells.

In the field, the virus causes yield losses of ranges between 10-90 percentage. The symptoms begin as scattered yellow spots and progress to irregular green and yellow patches that alternate with each other. Yellow spots enlarge, resulting in yellowing, wilting, and withering. Infected plant pods will be smaller in size than normal plant pods.

2. MATERIAL AND METHODS

2.1 Collection of samples

Mungbean Yellow Mosaic Virus (MYMV) infected plants of both black gram and green gram were collected from the experimental field of Department of Pulses, Tamil Nadu Agricultural University, Coimbatore. Leaves showing specific and prominent symptoms were collected. Virus infected leaves showing varied level of symptoms were collected and evaluated. Collected leaves were classified based on disease severity rating from 0-5 disease rating

scale adopted by *Akhtar et al., (2009)* with slight modification (Table 1). Sixteen samples were collected from the field and classified accordingly.

Table 1. scale for assessing the YMV incidence with modification (0-5)

Symptoms	**Rating/Disease severity**
Complete absence of symptoms	0
Small yellow specks or spots on few leaves seen	1
Bright yellow specks or spots common on leaves and some are coalesced	2
Mostly coalesced bright yellow specks or spots common on leaves covering 30% leaf area	3
Plants showing coalesced bright yellow specks or spots covering 50%	4
Yellowing or chlorosis of all leaves on whole plant followed by necrosis, shortening of internode	5

Per cent disease index was computed using the pattern given by Wheeler (1969)

$$Percent\ Disease\ Index = \frac{Sum\ of\ all\ the\ numerical\ ratings}{Number\ of\ observations \times Maximum\ disease\ rating} * 100$$

2.2 Isolation of genomic

Genomic DNA was isolated from MYMV infected green gram and black gram leaves, along with healthy uninfected plants. Four samples belonging to different disease score were selected for DNA extraction. Both green gram and black gram samples were used. Extraction method described by *Rouhibakhsh et al. (2008)* with some modifications was followed. 100 mg of young fresh leaves were collected and pulverised in a pre-chilled sterile pestle and mortar thoroughly with 1.5 ml of pre-warmed (65°c) DNA extraction buffer. Then the content was transfered to Eppendorf tube, incubated at 65°c for 30 minutes in a water bath with periodic mixing. The supernatant (750 µl) was mixed with an equal volume of chloroform: isoamyl alcohol (24:1) by vertexing, followed by a 10-minute centrifugation at 10000 rpm. The aqueous layer was transferred to a fresh eppendorf tube and mixed with 300 µl of ice-cold isopropanol and 30 µl of 7.5 M ammonium acetate (stored at -200°c) and kept it at 40°c overnight. The next day the contents was centrifuged for 10 minutes at 10,000 rpm. Pellets were rinsed with 70% ethanol and centrifuged at 10000 rpm for 10 minutes. The

supernatant was removed, and the pellet was air dried for 30 minutes before being re-suspended in 40 µl of TE buffer or double distilled water and kept at -20°c. All of the DNA extracts were further diluted in single distilled water (sdw) from 1:5 to 1:10 before being used for PCR amplifications.

2.2.1 Quantitative and qualitative analysis of extracted DNA

The DNA quality was tested using gel electrophoresis on a 0.8% agarose gel. 0.8g of agarose was melted in 100 ml of TAE buffer and gel for 80 mV for 45 minutes after adding ethidium bromide. Reaction mixture was prepared and checked for the DNA presence. Reaction mixture contain distilled water- 2 µl, DNA sample - 2 µl, 6X loading buffer - 2 µl.

The concentration of the DNA present was quantified using nanodrop. The concentration was noted in ng/ µl while the protein contamination was checked by 260/280 nanodrop reading.

2.3 Molecular confirmation of virus using universal primer

Preliminary molecular confirmation of the virus was done using the begomovirus universal primers. Forward and reverse primer are PARIC 772 and PALIC 1960 respectively (Table 3). Reaction mixture was prepared using distilled water, forward and reverse primers along with master mix. The reaction mixture of 10 µl was prepared and kept for PCR reaction. Reaction condition at 94° for 2 min for initial denaturation, followed by denaturation at 94°c for 1 minute, annealing at 55°c for 2 minutes and extension at 72°c for 3 minutes which was repeated for 35 cycles and the final extension step was carried out at 72°c for 10 minutes.

2.4 Confirmation of virus by amplifying the coat protein region

For further confirmation of the sample for MYMV, PCR amplification was done for coat protein region of the virus. Coat protein of DNA A is used for the primer given by Rangaswamy (2018). The primer sequence for forward primer is – 5’ ATG GG (T/G) TCC GTT GTA TGC TTG 3’ and reverse primer is 5’ GGC GTC ATT AGC ATA GGC AAT 3’ (Table 2). The PCR condition is similar as that of universal primer.

Table 2. Primers used for molecular confirmation of virus

Sl.no	Primer	Sequence	References
1	Universal primer – forward primer-	5’GGNAARATHTGGGATGGA3’	*Rojas et.al .,* (1993)

	PARIC 772		
2	Universal primer – reverse primer - PALIC 1960	5’ACNGGNAARACNATGTGGGC3’	
3	Coat protein specific primer – forward primer	5’ATGGG(T/G) TCCGTTGTATGCTTG 3’	Rangaswamy (2018)
4	Coat protein specific primer – reverse primer	5’ GGCGTCATTAGCATAGGCAAT 3’	

Table 3. Content of reaction mixture prepared for PCR amplification

Sl..no	Content	Quantity (µl)
1	Master mix	5
2	Distilled water	2
3	Forward primer	1.
4	Template DNA	1
5	Reverse primer	1

Table 4. PCR conditions for amplifying the viral DNA

Step 1	Initial denaturation	94° c for 2 minutes
Step 2	denaturation	94° c for 1minutes
Step 3	Annealing	55° c for 2 minutes
Step 4	Extension	72° c for 3 minutes
Step 5	Steps 2 to 4 repeated for a total of 35 cycles	

Step 6	Final extension	72° c for 10 minutes

2.5 Agarose gel electrophoresis

The amplified PCR product was examined through gel electrophoresis using 1% of agarose gel stained with ethidium bromide in 1x TAE buffer for 45 min in 85 V with 1 kb ladder. Then the amplified product was examined under del documentation unit. The expected amplicon of product for rojas primer would be around 1200 base pair and that of specific primer would be of 1000 base pair

3. RESULTS AND DISCUSSION

Black gram and green gram field was found infected with *Mungbean Yellow Mosaic Virus* infection in different range. The entire field appear as mosaic patches. The visible signs are scattered yellow-colored dots on the young leaves, which eventually transform into a yellow mosaic pattern and result in total yellowing. Individual plant shows MYMV symptoms with low to high intensity.

Fig 1. Appearance of MYMV in field as mosaic patches

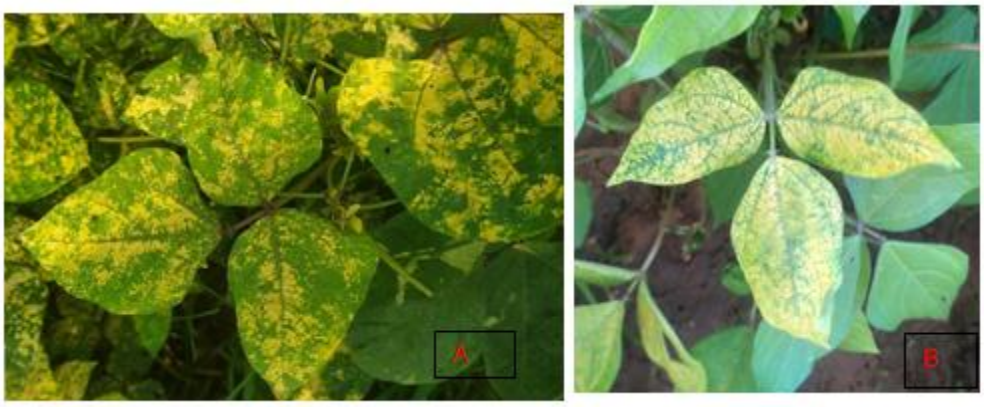

Fig 2. MYMV symptoms collected from field

A- MYMV symptoms from green gram b – MYMV symptoms from black gram

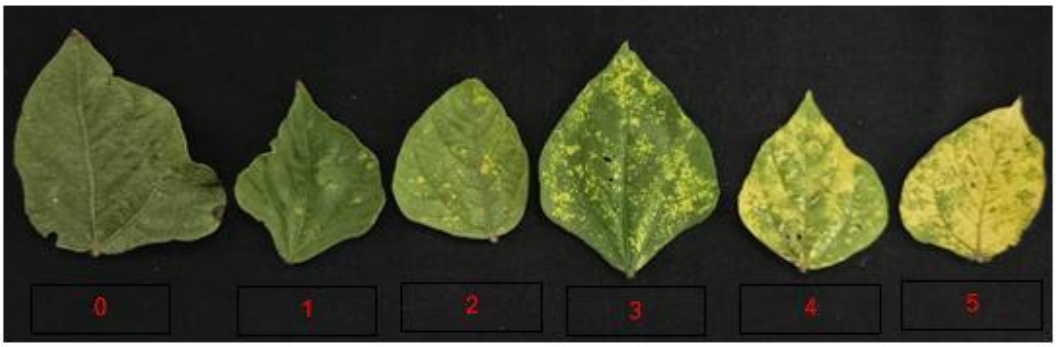

Fig 3a. Disease score for each leaf sample of MYMV infected in green gram. Leaf sample 1- score 0, leaf sample 2- score 1, leaf sample 3- score 2, leaf sample 4– score 3, leaf sample 5 -score 4, leaf sample 6 – score 5

Fig 3b. Disease score for each leaf sample of MYMV infected in black gram. Leaf sample 1- score 0, leaf sample 2- score 1, leaf sample 3- score 2, leaf sample 4– score 3, leaf sample 5 -score 4, leaf sample 6 – score 5

Table 5. classifying the collected leaf sample based on disease score

Disease score	Frequency of observation	Sum of observations
0	1	0
1	1	1
2	4	8
3	4	12
4	4	16
5	2	10

Total	16	47

$$Percease\ Index = \frac{47}{16 \times 5} \times 100$$

$$= 58.75\ \%$$

Collected leaf samples were grouped based on disease score and percent disease incidence was calculated. PDI is 58.75% indicating that more than 50% of the cultivated area is infected by MYMV. It implies that the disease severity is high in the green gram and black gram field.

Isolated DNA shows a clear band when gel electrophoresis at 0.8 % agarose which was done to check the quality of DNA. Quantity of DNA was assessed using nanodrop which shows a reading which implies good concentration of the DNA in infected samples. The 260/280 absorbance in nanodrop reading implicate the quality of DNA which will indicate the RNA or protein contamination. The value of range 1.6-1.9 indicating the quantity of DNA is good.

Table 6. concentration of extracted DNA

Sample	260/280 reading	Concentration of DNA (ng/ µl)
Gg1	1.715	1022.2
Gg2	1.817	1380
Gg3	1.720	1571.6
Gg4	1.962	1705.6
Bg1	1.634	980.2
Bg2	1.842	1164.3
Bg3	1.658	1274.7
Bg4	1.795	1633.7

The isolated DNA was diluted in 1:10 ratio and PCR was done to confirm the virus using rojas primer. All the samples are amplified with expected amplicon size of 1200 bp. The DNA samples were further amplifed with using coat protein specific primer from DNA A which show a amplicon at 1000bp.

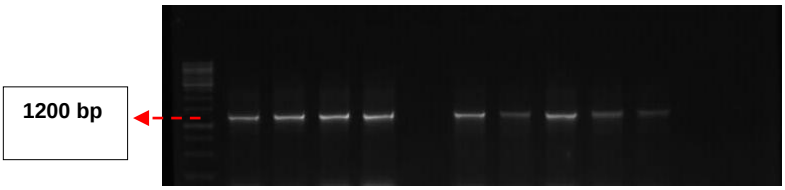

Fig.4 Band image of infected plant genomic DNA amplified with Universal primers through gel electrophoresis. Lane1- 1Kb Ladder, Lane2- Gg1, Lane3- Gg2, Lane4- Gg3, Lane5- Gg4, Lane6-blank, Lane7-Bg1, Lane8- Bg2, Lane9- Bg3, Lane10- Bg4, Lane11-Positive control Lane12- negative control

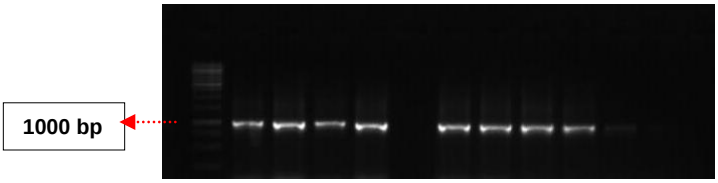

Fig .5 Band image of infected plant genomic DNA amplified with coat protein specific primers through gel electrophoresis. . Lane1- 1Kb Ladder, Lane2- Gg1, Lane3- Gg2, Lane4- Gg3, Lane5- Gg4, Lane6-blank, Lane7-Bg1, Lane8- Bg2, Lane9- Bg3, Lane10- Bg4, Lane11-Positive control Lane12- negative control

4. CONCLUSION

The disease samples were collected from black gram and green gram field infected with Mungbean Yellow Mosaic Virus (MYMV). The samples were scored based on the disease score and calculated the Percent Disease index. The PDI value implies that the disease severity is more than 50% indicating that disease incidence is high. DNA from the infected samples were extracted using CTAB method. Good and high amount of DNA was obtained and purity was assessed. The extracted DNA was amplified using Rojas and Coat protein specific primer to confirm the presence of DNA. Bands were observed for Rojas and Coat protein specific primer at 1200 bp and 1000 bp respectively indicating the presence of Mungbean Yellow Mosaic Virus (MYMV) in all infected samples.

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