The Pharma Innovation Journal 2022; 11(9): 2153-2160

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; 11(9): 2153-2160

www.thepharmajournal.com
Received: 15-07-2022
Accepted: 21-08-2022

Prateek Ranjan Behera
Ph.D., Scholar, Department of Plant Pathology, Odisha University of Agriculture & Technology, Bhubaneswar, Odisha, India

Dr. MK Mishra
Professor, Department of Plant Pathology, Odisha University of Agriculture & Technology, Bhubaneswar, Odisha, India

Dr. Sandeep Kumar
Assistant Professor, Department of Plant Pathology, Odisha University of Agriculture & Technology, Bhubaneswar, Odisha, India

Dr. AK Senapati
Professor, Department of Plant Pathology, Odisha University of Agriculture & Technology, Bhubaneswar, Odisha, India

Corresponding Author:
Prateek Ranjan Behera
Ph.D., Scholar, Department of Plant Pathology, Odisha University of Agriculture & Technology, Bhubaneswar, Odisha, India

Mechanical and seed transmission studies of yellow mosaic virus disease in greengram

Prateek Ranjan Behera, MK Mishra, Sandeep Kumar and AK Senapati

Abstract
Yellow mosaic virus disease (YMV) infecting mungbean is one of the most important cause responsible for vast scale yield loss in India and across the globe. In the present study, the transmissibility of YMV through sap and seed were examined. Mechanical transmission of the YMV either using 2% celite with carborundum powder or β mercataethanol along with virus extract in three healthy mungbean varieties was found to be unsuccessful as there was no symptom development 20-30 days after incoculation and the leaf samples tested negative in PCR amplification. There was 16.7 % reduced germination in the mungbean seeds collected from YMV infected plants. Although the grow-out test of seeds collected from the YMV infected mungbean plants showed no development of symptoms, there was detection of YMV in 20% plants selected arbitrarily 30 days after sowing in PCR test using the coat protein genome specific of the legume infecting begomoviruses. PCR amplification of different parts of germinated seeds showed the presence of the virus in cotyledon, cotyledonary leaves, epicotyl except seed coat.

Keywords: Mechanical, seed transmission, yellow mosaic virus, greengram

1. Introduction
The yellow mosaic disease in greengram is caused by viruses belonging to the genus *begomovirus* and family *Geminiviridae*. The *begomoviruses* are transmitted by whitefly in a circulative, persistant and non-propagative manner. The genome can be either monopartite having only the DNA-A component or bipartite, having both DNA-A and DNA-B (Fauquet *et al*, 2003; Kings *et al*, 2011) [1, 6]. The virus appears as a twin (Gemini) particle in the Transmission Electron Microscope (TEM) and hence it falls under the family *Geminiviridae*. Reviews have been found establishing the conclusive evidence about the distribution of the yellow mosaic viruses that the viruses are mostly limited to the cambium and phloem parenchyma except in few begomoviruses where they exit from the phloem tissues in to the mesophyll tissue (Rojas *et al*, 2005) [10]. The mungbean plants infected by the yellow mosaic viruses produces lesser number of pods, yellow discoloured and poorly filled seeds, smaller size, lesser weight than that of the healthy seeds (Varma *et al*, 1992). It has also been reported that development of yellow speak symptom even in the primary trifoliate leaf at the seedling emergence stage which acts as suggestive evidence on the seed borne nature of the virus (Nene, 1973 and Kothandaraman *et al*, 2015) [8, 7]. Highest rate of mechanical transmission of Thailand isolates of MYMV was achieved by employing the 0.1M sodium or potassium buffer, p^H 7.8 (Honda *et al*. 1983 and Shad *et al*, 2005) [3, 12]. In 1983, Phosphate buffer p^H 8.0 resulted better than the sodium phosphate and citrate phosphate buffer for mechanical transmission of MYMV in greengram (Chowdary *et al*, 1983) [1]. In the present study, seeds were collected from yellow mosaic virus infected greengram plants grown in agricultural farm Odisha University of Agriculture & Technology, Bhubaneswar, Odisha. Transmission studies of YMV were carried out through seed and sap. The presence of the virus, if any was confirmed by molecular detection.

2. Materials and Methods
2.1 Mechanical inoculation of yellow mosaic virus in mungbean
YMV Infected mungbean leaves samples were collected from LGG-460 variety of greengram and used for mechanical transmission in three YMV susceptible varieties *i.e*., Bananai Local, LGG-460 & TMB-136. There was eight test mungbean plants in each variety were employed for mechanical transmission.

2.1.1 Raising and maintenance of healthy mungbean plants

The seeds of YMV susceptible varieties (Bananai Local, LGG-460 & TMB-136) for the test plant were procured from the Centre for Pulses Research (CPR) Berhampur, OUAT, Ganjam, Odisha. The seeds were sown on the plastic pots filled with soil and FYM (2:1), which were kept in insect free chamber covered with muslin cloth. For each variety there were 8 plants grown in different pots. Those healthy mungbean seedlings of 10 days old were utilized for mechanical transmission of yellow mosaic virus.

2.1.2 Preparation of the 0.1M Potassium Phosphate buffer p^H 7.6

13.60 g of Potassium dihydrogen phosphate (KH_2PO_4) was added to 100 ml distilled water & 17.41g of Di potassium hydrogen phosphate (K_2HPO_4) was added to 100 ml distilled water to get 1M solution each. 13.4 ml of KH_2PO_4 was added to 86.6 ml of 1M K_2HPO_4 to get 100 ml 0.1M potassium phosphate buffer p^H 7.6. The p^H also was confirmed by p^H meter and stored in refrigerator at 4 ºC for further use.

2.1.3 Procedure for the mechanical transmission

Yellow mosaic infected symptomatic leaves of mungbean were freshly collected and washed with the distilled water. Then the leaves were dried by using the tissue paper. One gram of fresh leaf tissue was taken in a sterilized mortar and pestle, macerated with liquid nitrogen after adding 0.1M phosphate buffer p^H 7.6 @ 5:1 *i.e.*, 5 ml of buffer taken per 1 g fresh leaf tissue. The extract was stirred continuously and filtered through double layered muslin cloth. Three different methods of mechanical inoculation were carried out in three different varieties *i.e.*, Bananai Local, LGG-460 & TMB-136 as follows.

Treatments
T1- Virus extract+ 2% celite and carborundum powder
T2- Virus extract+ β-mercaptoethanol
T3- Virus extract only
T4- Control

The inoculation was conducted by rubbing the upper surface of the healthy leaves of greengram with cotton swab immersed in respective virus extract completely. Indicator plant *Nicotiana benthamiana* was also inoculated mechanically with above virus extract by mixing with 2% celite and carborundum powder. The leaves were given support by flattened surface of palm from below to make sure the uniformity in pressure during the rubbing. The inoculated leaves of respective varieties were left as it is for few minutes before washed with sterilized water. The inoculated plants were stored in insect proof muslin chamber for the recording of the symptom expression.

2.2 Seed transmission of the YMV in mungbean

2.2.1 Collection of the seeds from healthy and naturally infected mungbean plants

The healthy and infected seeds were collected from greengram (LGG-460) cultivated in OUAT farm, Bhubaneswar, Odisha, India. The seeds were allowed to sundry to minimize the moisture percentage for proper germination. The dried seeds were used for the transmission study.

2.2.2 Effect of the virus infection on seed germination

Germination percentage of seeds collected from healthy and infected mungbean plants, was carried out by roll towel method as per the protocol prescribed by the International Seed Testing Association (ISTA 1976) [4]. During the test, the seeds were exposed to 90 ± 3% relative humidity (RH) and 25 ± 1 ºC and the germination was recorded (Kanimozhi *et al*, 2009) [5].

2.2.3 Grow-out test of the mungbean seeds independently inside insect-proof house

One hundred fifty number of seeds (Plate 1) collected from the naturally YMV infected mungbean plants were sown in plastic pots filled with soil and FYM (2:1) and kept in an insect free muslin house for further observation and recording of the symptoms expression.

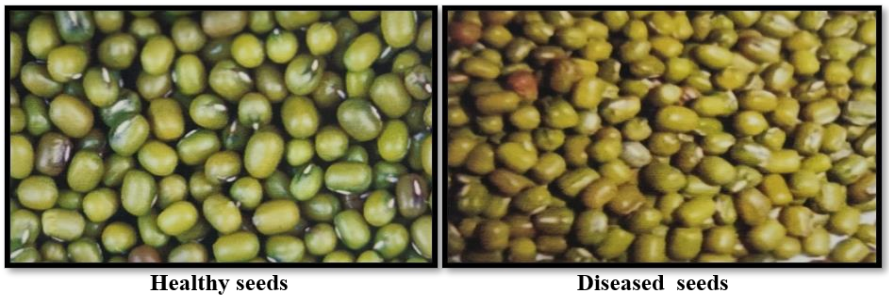

Plate 1: Seeds collected form asymptomatic mungbean plants (Healthy seeds) & seeds collected form symptomatic mungbean plants (Diseased seeds)

2.3 Molecular detection of YMV from leaves samples collected from sap inoculated and different parts of infected seeds

2.3.1 Molecular detection of the YMV from sap inoculated leaves

The inoculated leaves were taken from the mungbean plants 15 days after inoculation (DAI) *i.e.*, 25 days old for the molecular detection through PCR tests. The freshly plucked leaves were collected and weighted accurately 150 mg with the help of electronic weighing machine for the extraction DNA explained below.

2.3.2 Molecular detection of the YMV from seed

Seeds collected from naturally infected mungbean plants (LGG-460) were taken in ten numbers. The seeds were soaked in water over night (6-7 Hrs.) and kept for germination for 5 days. Different portions of germinated seed viz. Cotyledon, cotyledonary leaves, Epicotyl and seed coat were

collected from all ten germinated seeds and kept separately for DNA isolation. Also, DNA was isolated from the freshly collected leaf of 25 days old grow-out test mungbean plants.

2.3.3 Extraction of the total genomic DNA

The total DNA was extracted from the healthy and symptomatic leaves of inoculated mungbean seedlings according to the method reported by Rouhibakhsh *et al*. (2008) [11]. 150 mg of leaf tissue and tissue collected from different portions of germinated seeds were macerated separately employing sterile mortar and pestle with liquid nitrogen and then the ground tissues were shifted into 1.5 ml eppendorf tubes separately. 1500 µl of extraction buffer (CTAB) pre-warmed up to 65 °C was mixed with the ground samples present in 1.5 ml eppendorf tubes. Incubation of the whole crude saps were done for ½ hr at 60 °C in a water bath with intermittent mixing. 750 µl of the supernatant were shifted into new 1.5 ml eppendorf tubes and mixed with same volume (750 µl) of Phenol: chloroform: isoamyl alcohol (25: 24: 1) by vertexing. All the sample was allowed to a micro centrifuge for centrifugation at 1300 rpm for 10 minutes, followed by the collection of supernatants was collected in new 1.5 ml eppendorf tubes by discarding the solid palette settled at the bottom of the Eppendorfs. Mixture of 30 µl of 7.5 M ammonium acetate + 300 µl of chilled isopropanol was mixed with aqueous supernatant by inversion to get precipitated DNA. The Eppendorf tubes were centrifuges for 10 minutes at 13000 rpm and the pellet formed were washed with 70% ethyl alcohol followed by dried in a vacuum drier for 10 min until alcohol odour disappeared. Then these were resuspended with 40 µl of $T_{10}E_{0.1}$ buffer (0.1 mM EDTA of pH 8.0 and 10 mM Tris-HCl of pH 8.0) each and stored in deep freezer at -20 °C. All the extracted DNA were additionally diluted from 1:10 to 1:40 with double distilled water (DDW) before amplification employing the thermocycler. The quality and quantity of DNA were examined at 280 and 260 nm by employing UV spectrometer and the ratio of OD_{260} to OD_{280} was found to be 1.9-2.0 range.

2.3.4 Primers used for the amplification

RUGEMF1 and RUGEMR1 have been used which is specific to the conserved region of Coat protein (AV-1) region of the yellow mosaic viruses belonging to begomoviruses (Ramesh *et al*, 2016) [9]. The sequence of the primers was detailed below.

Primers	Sequence (5'- 3')
RUGEMF1	TGTGAGGGACCATGTAAAGTTC
RUGEMR1	GCATGAGTACATGCCATATAC

2.3.5 Master mixture of PCR

The master mixture needed was prepared from the Banglore Genei and the Eppendorf, Germany was used. The reaction mixture was dispensed into 0.2 ml of PCR tubes and the total volume was made up to 20 µl with double distilled water (DDW) as detailed below.

Components	Volume (µl)
Taq buffer (10 X)	2.00
dNTP (1mM)	1.00
Forward Primer (5 pM)	1.00
Reverse Primer (5 pM)	1.00
DNA template (40 ng)	1.00
Taq polymerase (5 u/µl)	0.50
Double distilled water	13.50
Total	20.00

2.3.6 Thermal cycler program for PCR amplification

The programme for PCR consists of single cycle of initial denaturation of DNA at 94 °C for 5 min followed by 35 cycles each having denaturation at 94 °C for 40 sec, annealing at 57 °C for 30 sec and an extension of primer at 72 °C for 30 sec. The final extension at 72 °C for 5 min was given for 1 cycle and finally hold at 4 °C for 30 min. After PCR amplification, the PCR tubes were collected and stored at -20 °C until the gel electrophoresis was completed.

2.3.7 Agarose gel electrophoresis

One gram of agarose was taken in a 250ml conical flask having 100ml of 1X TAE buffer followed by heating of the solution was done in a hot air oven by uniform stirring in order to dissolve the agarose completely. The solution was allowed to cool to bring its temperature to about 50 °C followed by 2-3 drops of ethidium bromide (0.5 µg/ml) was mixed with the solution. The solution was poured onto the gel casting plate after placing the comb in the trough and care was taken to ensure the solution remain bubble free. The agarose solution was allowed to solidify and then the comb was removed after keeping the casting plate in electrophoretic apparatus comprising enough 1X TAE (Stock 50X TAE) buffer to cover the wells thoroughly. The amplified PCR products (5 µl) to be examined were loaded carefully into the wells present on the casting plate after mixing with loading dye (6x) by using the micropipette. During the electrophoresis, voltage was fixed at 80 volts for 45 minutes. The DNA bands stained with the Ethidium bromide were seen under the UV-transilluminator and documented employing gel documentation system (UviTec Cambridge, England)

Note: 20 ml of 1X TAE was made up to 1 lit (1000 ml) by adding sterile distilled water

3. Result and Discussions

Transmission studies conducted with relation to seed and sap and subsequent confirmation by molecular test are described below.

3.1 Mechanical transmission of YMV in mungbean

Observations for the appearance of YMV symptoms in mungbean after sap transmission were taken after 30 days after inoculation. There was no symptoms development in any of the treatment of all the three varieties even after 30 days after inoculation (Plate 2). Leaf distortion and puckering symptoms were observed on the indicator plants, *Nocotiana benthamiana* 20 days after inoculation (Plate 3). PCR amplification tests of mechanically inoculated leavesalso revealed negative results in all three varieties. Presence of YMV was confirmed in *N.* benthamiana by PCR test (Table 1). The sap transmission of YMV from greengram to greengram was also reported unsuccessful by Grewal, 1988 [14]. However, the Thailand isolates were reported to be successful in mechanical transmission in greengram (Honda *et al*, 1983) [3]. The molecular confirmation of the successful

transmission of the YMV was done according to the protocol described in Materials and Methods. Two samples from every treatment and one sample from control treatment were utilized for the PCR tests using the primer pair targeting the conserved region of the yellow mosaic disease causing begomoviruses. All the samples were found to be negative as there was no band in the Gel electrophoresis image. However, there was a band in the gel image of the sample taken from the symptomatic indicator plant (*N. benthamiana*) at 450bp (Plate 4). The findings proved that unsuccessful transmission of the virus in greengram which was found similar with the findings given by Grewal, 1988 [14].

Table 1: Molecular detection of mechanically transmitted YMV in greengram using different extraction medium

Treatments	Greengram varieties		
	Bananai local	**LGG-460**	**TMB-136**
T1- Virus extract+ 2% celite and carborundum powder	Negative*	Negative	Negative
T2- Virus extract+ β-mercaptoethanol	Negative	Negative	Negative
T3- Virus extract only	Negative	Negative	Negative
T4- Control (no virus inoculation)	Negative	Negative	Negative
Nicotiana benthamiana (Virus extract+ 2% celite and carborundum powder)	Positive		

* It denotes presence or absence of YMV in PCR amplification

3.2 Germination percentage of greengram seeds collected from YMV infected plants

Germination percentage of seeds collected from YMV infected greengram plants was recorded 75% as compared to 90% in the seeds collected from healthy mungbean plants. Germination percentage was reduced to 16.7% in the seeds infected with YMV (Table 2). Similar result was reported by Kanimozhi *et al*, 2009 [5] with 76.50% germination in of *Mungbean Yellow Mosaic Virus* (MYMV) infected seeds and 92% in healthy seed of greengram.

Table 2: Reduction in germination of YMV infected greengram seeds

Type of seed	Germination per cent	Per cent reduction
Seeds collected from YMV infected greengram (LGG-460)	75	16.7
Seeds collected from YMV healthy greengram (LGG-460)	90	-

3.3 Presence of YMV in different parts of germinated greengram seeds collected from YMV infected plants

Seed transmission study was carried out in two ways. First one was the grow-out test of the infected seeds collected from naturally infected greengram plants as described in the Materials and methods earlier. There was no expression of the symptom by the grow-out plants even 30 days after sowing (Plate 5). The result showed similarity with the findings given by Kothandaraman *et al*, 2015 [7] with no symptom development in the grow-out blackgram plants. There was no development of the symptoms on the plants grown from the infected seeds, the leaf samples were taken arbitrarily from 25 different plants for further confirmation with PCR. Twenty per cent of the samples had shown amplification band at 450 bp in gel image. Similar result was reported by Kothandaraman *et al*, 2015 [7] with 32% of the seedlings grown from the infected seeds showing positive in PCR test. They also concluded that the metabolic pathways may also be interfered by the genomic products of virus and hence it resulted as no symptom expression. The tissues isolated and gathered from 10 germinated seeds (Plate 6) were further allowed for the PCR tests and positive reaction was found in cotyledon, cotyledonary leaves and epicotyl. But the seed coat was tested negative in PCR (Table 3; Plate 7). Hence this confirmed the seed borne nature of the YMV in greengram. The failure of symptom expression of YMV in grown up plants may be due to presence of very less amount virus particle in different parts of germinated seeds. The greengram seeds may serves as an indirect source of inoculum for the yellow mosaic virus disease. This result is in consensus with the finding given by Kothandaraman *et al*, 2015 [7].

Table 3: Detection of YMV in different parts of the germinated mungbean seeds collected from YMV infected LGG-460

Plant part	PCR test
Cotyledon	+ ve
Cotyledonary leaves	+ ve
Epicotyl	+ ve
Seed coat	- ve

+ ve: Present; - ve: Absent

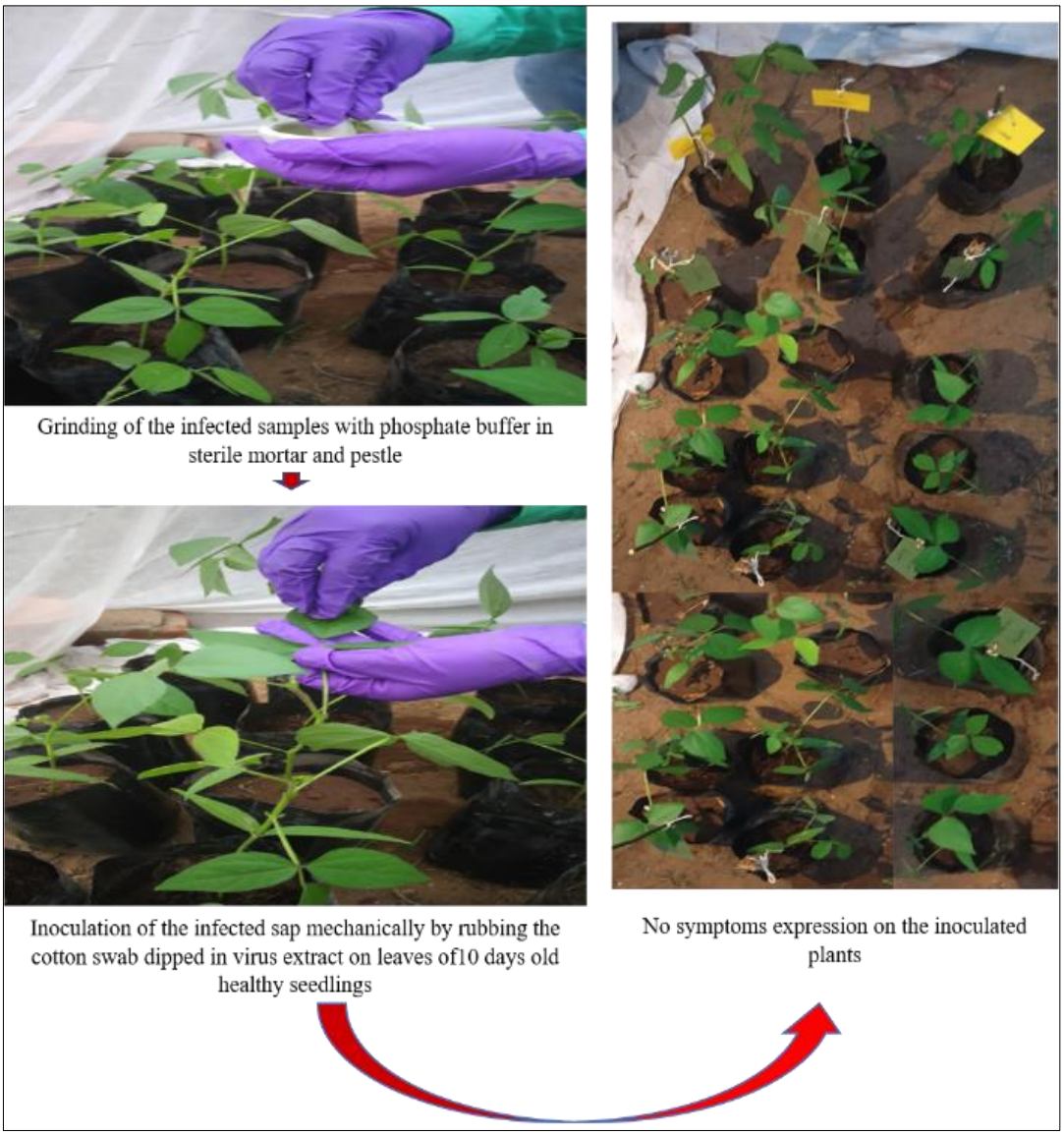

Plate 2: Mechanical inoculation of the YMV on healthy mungbean seedlings

Plate 3: *Nicotiana benthamiana* showing the symptoms after sap transmission of YMV from greengram

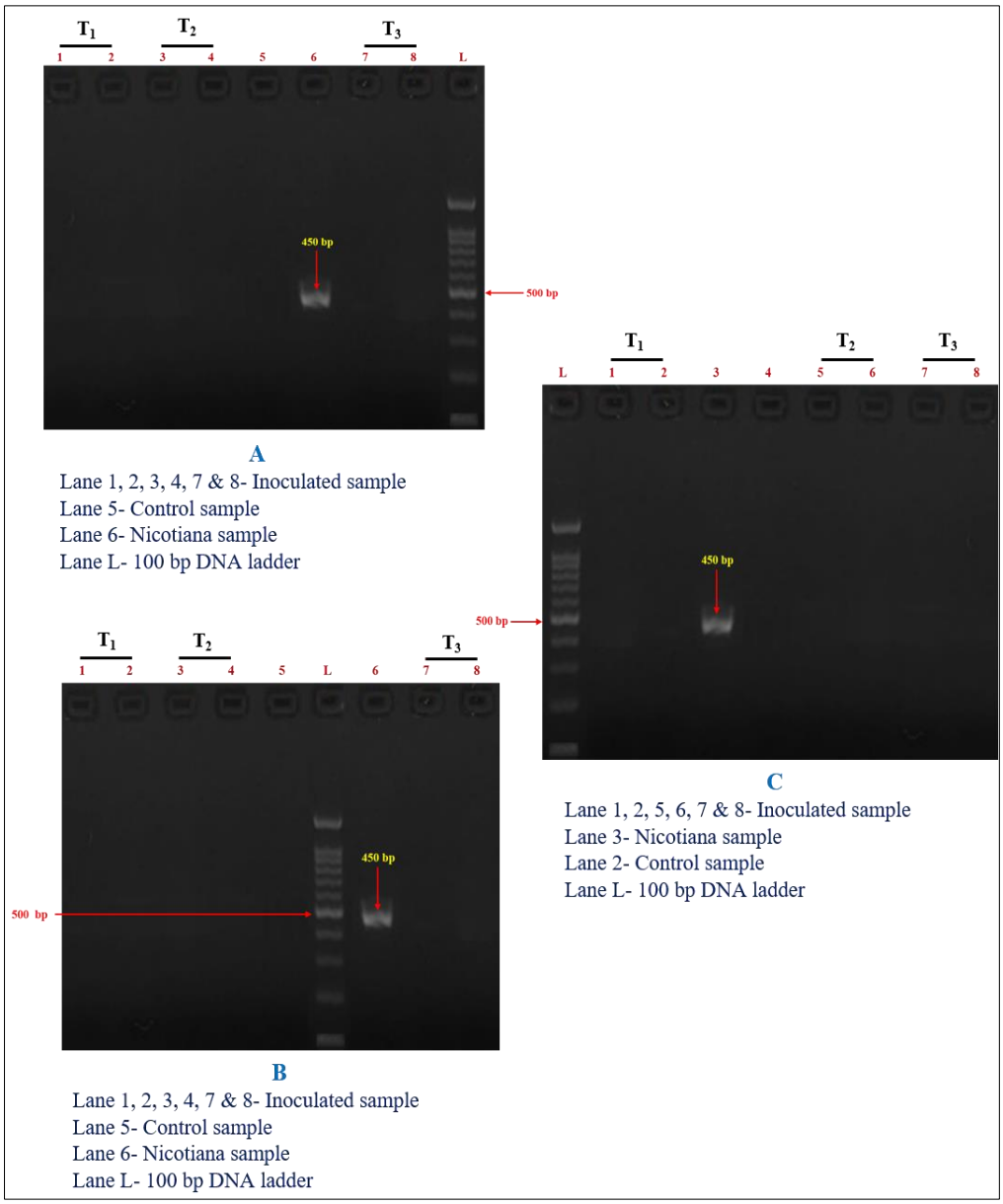

Plate 4: Gel electrophoresis image showing band at 450bp of the leaf samples of treatments applied mungbean plants (A. Bananai local B. LGG-460 & C. TMB-136) after sap inoculation

Plate 5: Plants showing no symptoms of yellow mosaic disease after 30 days after sowing

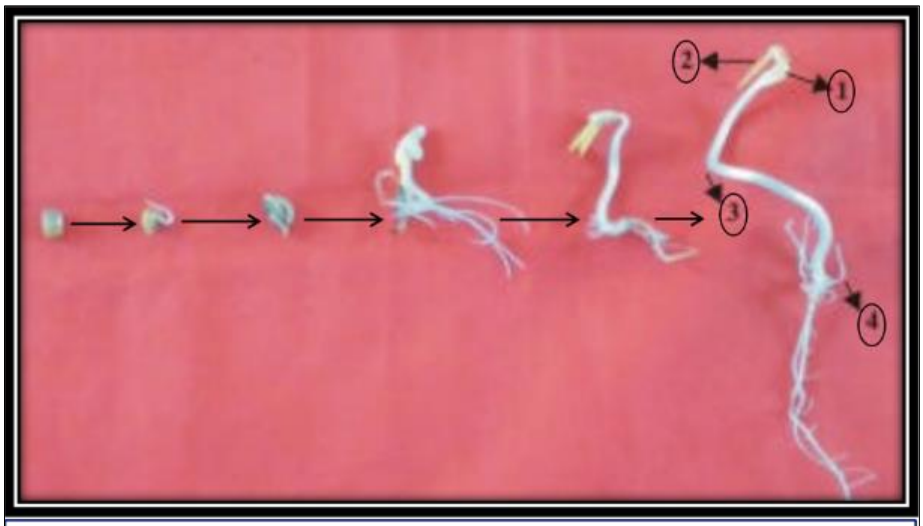

Plate 6: Different stages of germination of infected mungbean seed

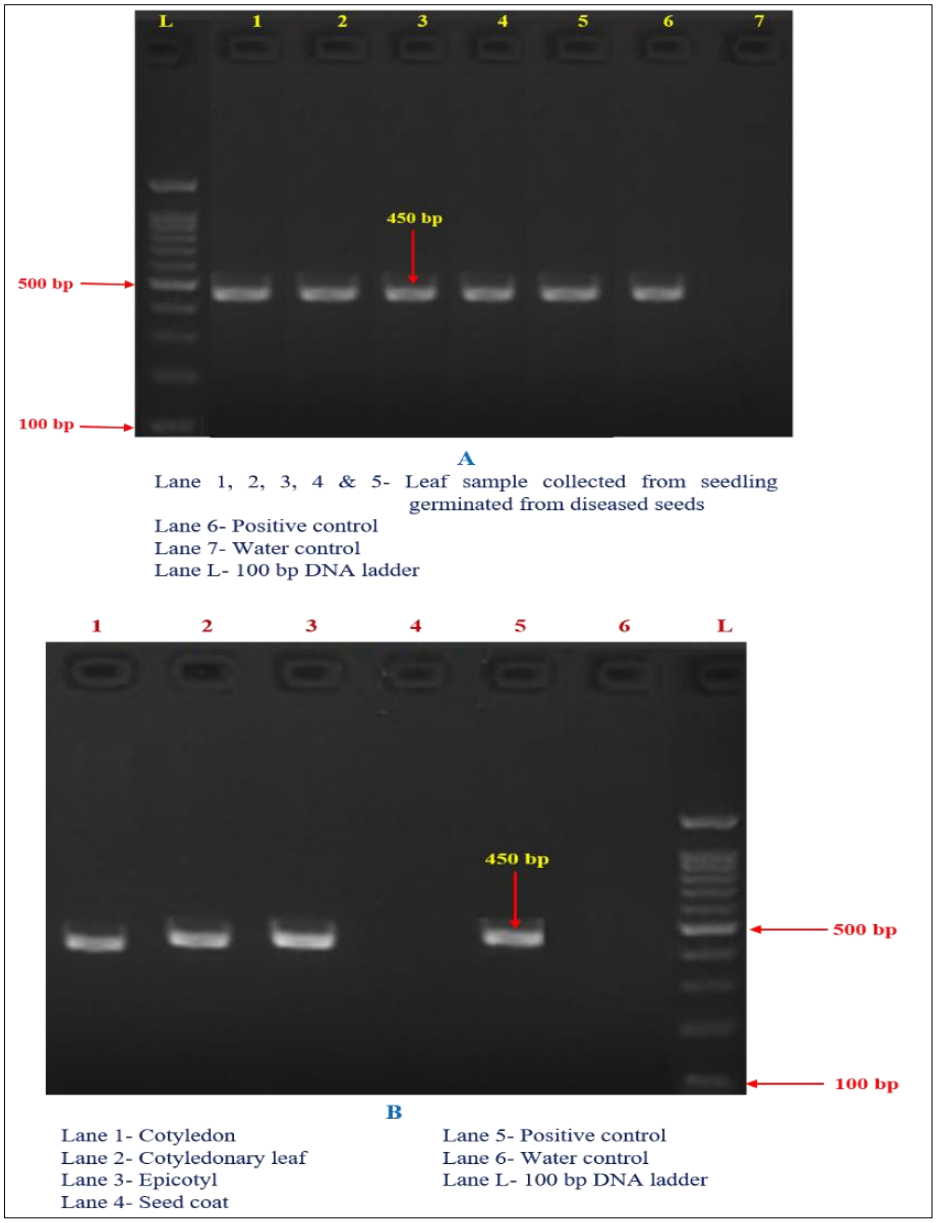

Plate 7: Gel images showing an amplification band at 450 bp of leaf samples (A) & Different plant parts of diseased seeds (B)

Conclusion

Investigations on transmission of YMV of greengram revealed no mechanical transmission. Germination of seeds collected from YMV infected plants was also reduced. There was no symptom expression also in grown up plants, though latent infection was there. Though the seed transmission of YMV couldn't produce any symptom in grown up plants latent infection of the virus was found in the preliminary stage of the seedlings.

Appendix

CTAB (Extraction buffer)

CTAB	: 4 %
Nacl	: 1.4 M
EDTA (p^H 8.0)	: 20 mM
Tris Hcl	: 100 mM

Stock solution (100 ml)

372.24 g (molecular weight) in 1000 ml = 1M EDTA
37.224 g in 1000 ml (3.72 g/ 100 ml) = 0.1 M EDTA
186.12 g in 1000 ml (18.612 g/100 ml) = 0.5 M EDTA
Dissolved the chemical in 50 ml of distilled water and adjusted the p^H and the volume made up to 100 ml.

Loading dye (6X)

Dissolve the sucrose (40 g) completely in 80 ml of distilled water and add 0.25 % of Bromophenol blue and finally made volume up to 100 ml and stored at 4 °C.

50X TAE Buffer (1000 ml)

0.5 M EDTA	: 100 ml
Glacial acetic acid	: 57 ml
Tris base	: 242 g
Distilled water	: 840 ml

References

1. Chowdary AK, Chowdhury NK. Effect of buffers on different PH on the infectivity of urdbean leaf crinkle virus. Indian Phytopathology. 1983;12:477-478.
2. Fauquet CM, Bisaro DM, Briddon RW, Brown J, Harrison BD, Rybicki EP, *et al.* Revision of taxonomic criteria for species demarcation in the family Geminiviridae, and an updated list of begomovirus species. Archives of Virology. 2003;148:405-421.
3. Honda Y, Iwaki M, Saito Y. Mechanical transmission, purification and some properties of whitefly-borne mungbean yellow mosaic virus in Thailand. Plant Disease. 1983;67:801-804.
4. ISTA. International rules for seed testing. Seed Science Technology. 1976;4:23-28.
5. Kanimozhi S, Ganapathy T, Rajinimala N. Seed transmission of ULCV in mungbean and Urdbean plants infected with both MYMV and ULCV. Archives of Phytopathology and Plant Protection. 2009;42(5):401-408.
6. Considerable AMQ, Adams MJ, Carstens EB, Lefkowitz EJ. Virus taxonomy. Ninth report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego; c2011.
7. Kothandaraman SV, Devadason A, Ganesan MV. Seed-borne nature of a begomovirus, Mung bean yellow mosaic virus in black gram. Applied Microbiology and Biotechnology. 2015;100(4):1925-1933.
8. Nene YL Viral diseases of some warm weather pulse crops in India. Plant Disease Reporter. 1973;57:463-467.
9. Ramesh SV, Chouhan BS, Gupta GK, Ramteke R, Chand, Husain SM. Molecular Diversity of Coat Protein Gene Encoded by Legume Begomoviruses and PCR Assay to Detect Yellow Mosaic Viruses Infecting Soybean in India. British Biotechnology Journal. 2016;12(3):1-10.
10. Rojas MR, Hagen C, Lucas WJ, Gilbertson RL. Exploiting chinks in the plant's armor: Evolution and emergence of geminiviruses. Annual Review of Phytopathology. 2005;43:361-394.
11. Rouhibakhsh A, Priya J, Periasamy A, Haq QMI, Malathi VG. An improved DNA isolation method and PCR protocol for efficient detection of multicomponents of begomovirus in legumes. Journal of Virological Methods. 2008;147:37-42.
12. Shad N, Mughal SM, Bashir M. Transmission of mungbean yellow mosaic Begomovirus (MYMV). Pakistan Journal of Phytopathology. 2005;17(2):141-143.
13. Varma A, Dhar AK, Mandal B. MYMV transmission and control in India. In: Green SK, Kim DH. (Eds) Mungbean yellow mosaic disease. Proceedings of an International Workshop, Bangkok. Publication. 92–373 AVRDC, 1992, Shanhua, Tainan, Taiwan. 1991, Pp. 8-27.
14. Grewel JS. Diseases of pulse crops: An over view. Indian Phytopathology. 1988;41(1):1-14.