The Pharma Innovation Journal 2023; 12(8): 69-74

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(8): 69-74

www.thepharmajournal.com
Received: 26-06-2023
Accepted: 29-07-2023

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Seed borne nature of mungbean yellow mosaic virus (MYMV) in resistant and susceptible variety of blackgram upon whitefly mediated transmission

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Abstract
Mungbean yellow mosaic virus **(**MYMV) is one of the most destructive begomoviruses, which infect legumes and cause significant yield losses. The whitefly transmission of MYMV in susceptible variety (CO_5) resulted in symptom expression in seven out of ten plants, whereas in resistant variety (Mash 114), symptom expression was absolutely absent in all the ten plants. Further, in MYMV detection through PCR using Rep region (DNA-A) and movement protein gene (DNA-B) specific primers, all the ten plants were PCR positive for DNA-A and eight for DNA-B in the case of CO_5, in which seven were symptomatic and three were asymptomatic. In the case of resistant variety Mash 114, eight out of 10 plants were PCR positive for both DNA-A and DNA-B, but were asymptomatic. The different seed parts *viz*., seed coat, cotyledon and embryonic axis from the whitefly transmitted, yellow mosaic disease infected plants exhibited the presence MYMV in all the seed parts in the case of CO_5, whereas in Mash 114, the virus was present in seed coat and cotyledon, but absent in embryonic axis. The study confirmed that the MYMV presence in embryonic axis of the susceptible variety CO_5 promotes the probability of seed transmission.

Keywords: Mungbean yellow mosaic virus**,** begomovirus, seed borne, susceptible and resistant variety, whitefly, seed borne

Introduction
Blackgram (*Vigna mungo* L.), is an important pulse crop originated from India and is grown under wide range of agro climatic condition, covering an area of approximately 4.67 million hectares with the production of 2.34 million tons. It consists of a relatively substantial amount of easily absorbable, high quality protein (24%) with low flatulence. Additionally, it has a notable iron content (40-70 ppm), rendering it an excellent option for maintaining well-rounded and healthy diets (Salam *et al*., 2009) [14]. Due to its high concentration of phosphoric acid and inclusion of 56% carbohydrates, 25% proteins, 4% minerals, 2% fat, and 0.4% vitamins, it is renowned for being nutritionally dense (Wani *et al*. 2013) [17]. However, crop is attacked by various pest and diseases which greatly affects the productivity of blackgram. Among the biotic constraints, yellow mosaic disease (YMD) is the most serious disease and major bottle neck for the blackgram cultivation (Biswas *et al*., 2012; Malathi and John, 2008) [1,9]. YMD is a result of the Mungbean yellow mosaic virus (MYMV), which is classified as a single-stranded DNA virus belonging to the genus *Begomovirus* under the family *Geminiviridae*. In India, the virus was first reported by Nariani (1960) [11] from the mungbean fields in New Delhi. The MYMV infected plants produces small yellow specks on the leaf lamina initially, which enlarge and result complete yellowing of leaves, reduction in flower and pod formation, discolored pods and seeds and size of the seeds considerably reduced in size (Malathi and John, 2008) [9]. Under favorable climate conditions, the prevalence of YMD is extensive and highly damaging. If plants are infected during their early growth stages, the disease can lead to substantial yield losses ranged from 85% to 100% (Nene 1973) [12]. The appearance of symptoms in the first trifoliate leaf of the infected plants in the field suggested that the virus may be seed borne, and which was previously investigated by Kothandaraman *et al*. (2016) [7]. The present study attempted to compare the seed borne nature of MYMV in resistant and susceptible variety of blackgram through whitefly mediated transmission.

Materials and Methods

Maintenance of MYMV inoculum

The variety CO_5 which is highly susceptible for MYMV was grown in pots under insect proof condition. The MYMV inoculum from the infected fields of blackgram (Department of Pulses, Tamil Nadu Agricultural University, Coimbatore) were brought for feeding to whiteflies with 24h acquisition access period (AAP) and 24 h inoculation access period (IAP) by using clip cages at two leaf stage and the viruliferous whiteflies were released on fifteen days old healthy plants at two leaf stage inside the munger cages (Govindan *et al*., 2014) [4]. After 24h IAP, the whiteflies were killed by spraying systemic insecticide. The plants were observed for symptom expression and the MYMV inoculum was maintained by regular transfer after every eight weeks to fresh batch of healthy plants for further experiment.

Establishment of whitefly pure culture

Whitefly egg masses were collected from blackgram plants and released on brinjal plants which were kept in the insect-proof rearing chamber for multiplication. For the determination of genotype, DNA was extracted from the whiteflies by hot shot method (Zeidan and Czosnek, 1991) [18].

Whitefly Transmission

Aviruliferous whiteflies (*Bemisia tabaci*) were collected from rearing chamber by using aspirator in clip cages (20 numbers per clip cage). Whiteflies collected in clip cages were given with the AAP for 24 h to acquire the virus from the symptomatic leaves of MYMV infected plants maintained in the insect proof chambers. Then, the viruliferous whiteflies released into Manger cage containing healthy plants of CO_5 and Mash 114 variety (Five plants per pot) in separate cages. After 24 h of IAP, whiteflies were killed by spraying systemic insecticide (Imidacloprid 200SL @ 0.4 ml/l). Plants were maintained in insect proof cage for symptom expression. After 20 days post inoculation (DPI), number of plants expressing symptoms was recorded in both the varieties, leaves were collected from 10 plants of each variety and DNA extraction was done and subjected to PCR analysis with MYMV specific primers for both DNA-A and DNA-B. Further, the plants were maintained inside the insect proof chamber until infected pods were harvested. The experiment was repeated three times to confirm the results.

Seed sample collection and DNA isolation

The pods were harvested from both the varieties and the seeds separately were surface sterilized with 1% Teepol and placed on the Petri Plate containing moistened blotter paper for 12h. Seeds were dissected into embryonic axis, cotyledon and seed coat. The DNA was extracted from the different seed parts using CTAB method (Rouhibakhsh *et al*., 2008) [13]. Hundred seeds from CO_5 and 50 seeds from Mash 114 were taken for the study. The parts from ten seeds were pooled into single sample.

Molecular confirmation of MYMV in seed parts through PCR

A set of specific primers for MYMV were used to detect the presence of the virus through PCR analysis. The primer pair (FP -5'-GCAAGCTTGCTTCCCGTACTTGACG-3' and RP 5'-TCTCTAGAGATCAGCTAGAGGAGG-3') specific for Rep region with an expected amplicon size of 500 bp for DNA-A and the primer pair specific for the movement protein gene (MPF - 5'-ATGGAGAATTATTCAGGCGCA-3' and MPR 5'-TTACAACGCTTTG TTCACATT-3') of DNA-B with an expected amplicon size of 980 bp were used for detection.

A PCR procedure was carried out using a reaction mixture of 25 µl, comprising 12.5 µl of 2X genei master mix, a ready-to-use mixture containing Taq polymerase, dNTPs, and PCR Buffer from Genei Laboratories Pvt. Ltd. (catalog number # 0667700041730). Additionally, 5 µl of template DNA and 2 µl each of forward and reverse primers (each at a concentration of 100 ng/pl) were included. PCR mix volume was made up to 25 µl by adding sterile distilled water and incubated as follows; Initial denaturation of DNA at 94 °C for 2 mins followed by 30 cycles of denaturation at 94 °C for 50s, annealing at 52 °C for 45s, extension at 72 °C for 1.30 min and final extension at 72 °C for 15 min. Finally, the PCR products were visualized by 1% agarose gel electrophoresis for 1 h at 80V. The genomic DNA isolated from MYMV infected leaf samples maintained in the insect proof chamber was used as positive control for all the PCR analysis. The positive PCR products of one embryonic axis sample for both DNA-A and DNA-B were sequenced through outsourcing (Syngenome Pvt Ltd., Coimbatore) for the further confirmation of MYMV.

Results

Whitefly mediated MYMV transmission in both susceptible and resistant variety

The results of transmission experiment revealed that the successful transmission of MYMV by *B.tabaci* viruliferous adults after 24 h of AAP and IAP was achieved and expression of typical yellow mosaic symptom was observed up to 70 percent in CO_5, whereas in Mash 114 plants, expression of symptoms was absent. The days taken for expression of characteristic yellow mosaic symptom was 15 to 20 days after inoculation in CO_5 whitefly transmitted plants (Table 1 and Fig. 1).

Table 1: Efficiency of transmission of MYMV by whiteflies in resistant and susceptible cultivar of blackgram

Variety	AAP	IAP	Number of plants infected/Number of plants inoculated	Days taken for symptom expression	Number of PCR positive samples	
					DNA-A	DNA-B
CO_5	24 h	24 h	7/10	15 to 20	10/10	8/10
Mash 114	24 h	24 h	0/10	-	8/10	8/10

Fig 1: Symptom expression in whitefly mediated MYMV transmitted blackgram plants

Molecular confirmation of MYMV in whitefly transmitted plants through PCR

After 20 days post inoculation of virus, the second trifoliate leaves were collected and subjected to PCR analysis using specific primer pairs mentioned earlier for DNA-A and DNA-B. For susceptible variety, all the ten plants were found to be positive for DNA-A and eight were positive for DNA-B (Fig. 2), in case of resistant variety, only eight plants were found to be positive out of ten plants tested for both DNA-A and DNA-B (Fig. 3), but they were asymptomatic.

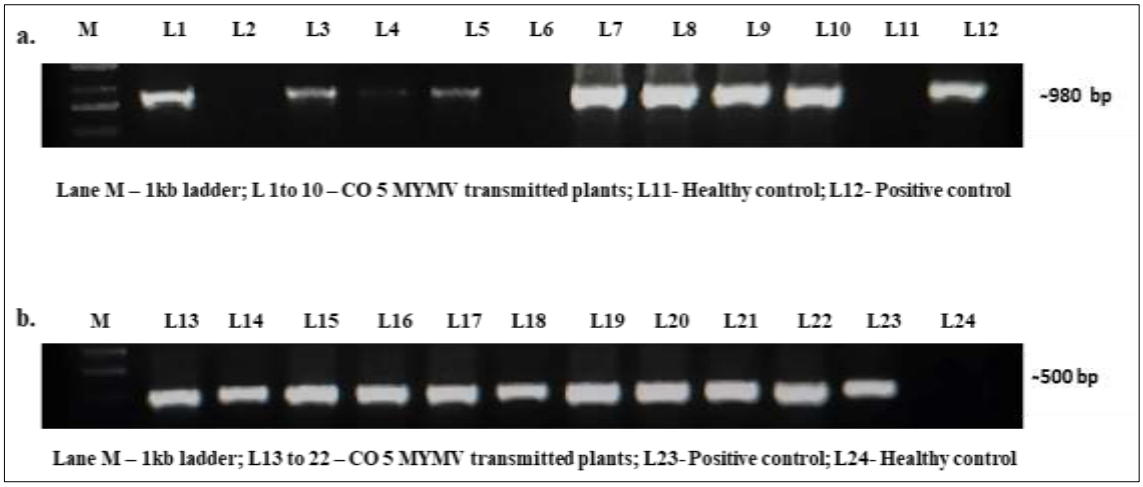

Fig 2: Detection of MYMV in CO_5 plants by PCR.
A) Agarose gel electrophoresis of PCR product obtained from MYMV transmitted CO_5 plants using movement protein specific DNA-B primer.
B) Agarose gel electrophoresis of PCR product obtained from MYMV transmitted CO_5 plants using Rep gene specific DNA-A primer

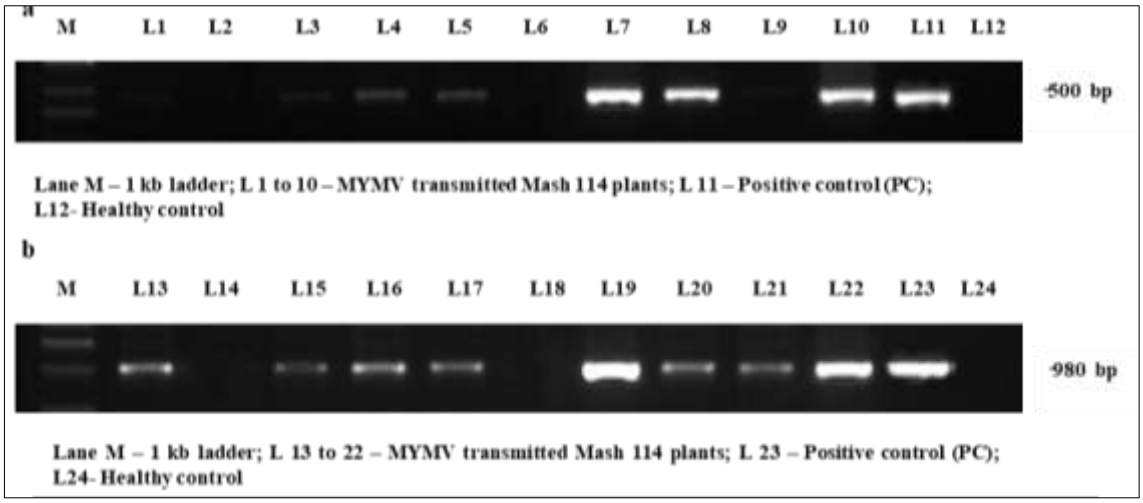

Fig 3: Detection of MYMV in mash 114 plants by PCR.
A) Agarose gel electrophoresis of PCR product obtained from MYMV transmitted mash 114 plants using Rep gene specific DNA-A primer.
B) Agarose gel electrophoresis of PCR product obtained from MYMV transmitted mash 114 plants using movement protein specific DNA-B primer

The matured pods from the virus transmitted plants of both susceptible and resistant varieties were collected. Out of 100 seeds from susceptible variety, 12 seeds were found to be misshapen, ill formed and discoloured, whereas, in resistant variety, out of 100 seeds, three were found to be misshapen and discoloured (Fig. 4).

Fig 4: Morphological abnormalities of MYMV infected seeds

PCR was performed for the detection of MYMV from different parts (seed coat, cotyledon and embryonic axis) of seeds of CO_5 and Mash 114 (Fig. 5) with Rep region specific primer for DNA-A and Movement protein specific primer for DNA-B. For DNA-A, out of 5 samples tested in Mash 114, all the five and three samples were found to be positive in cotyledon and seed coat respectively, while, in embryonic axis samples, only non-specific amplicons were observed (Fig. 6, Table 2). Whereas none of the seed part samples were positive for DNA B. Interestingly, in the susceptible variety CO_5 samples, all the 10 samples of seedcoat, cotyledons and embryonic axis were positive for DNA A, whereas in the case of DNA B, out of ten samples tested, two cotyledon, four embryonic axis and nine seed coat samples were positive (Fig. 7, Table 2). Each one PCR positive products of embryonic axis sample for DNA-A and DNA-B were sequenced for further confirmation of the MYMV. The sequence was analyzed by the BLAST program (www.ncbi.nlm.nih) and sequence of DNA-A showed 98% identity with MYMV DNA-A of Kanpur isolate (MW736055) and Vamban isolate (MW6051) and in the case of DNA B, 96% identity was observed with Coimbatore isolate (KC911724).

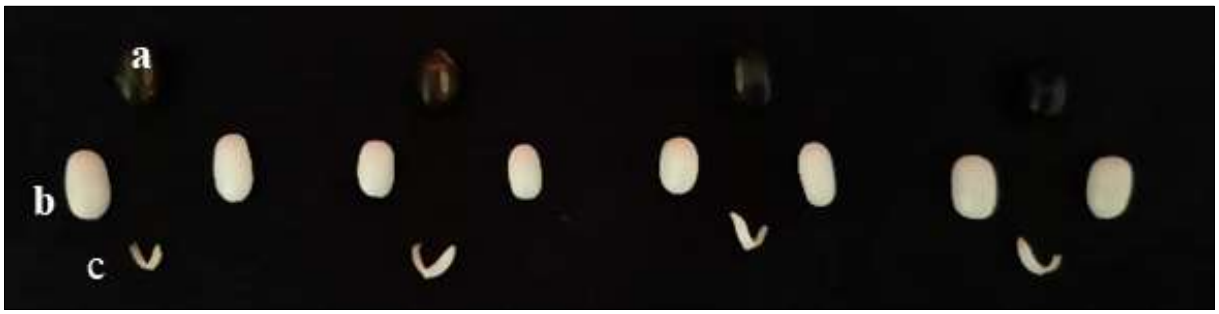

Fig 5: Different part of seeds dissected for PCR analysis a) Seed coat, b) Cotyledon, c) Embryonic axis

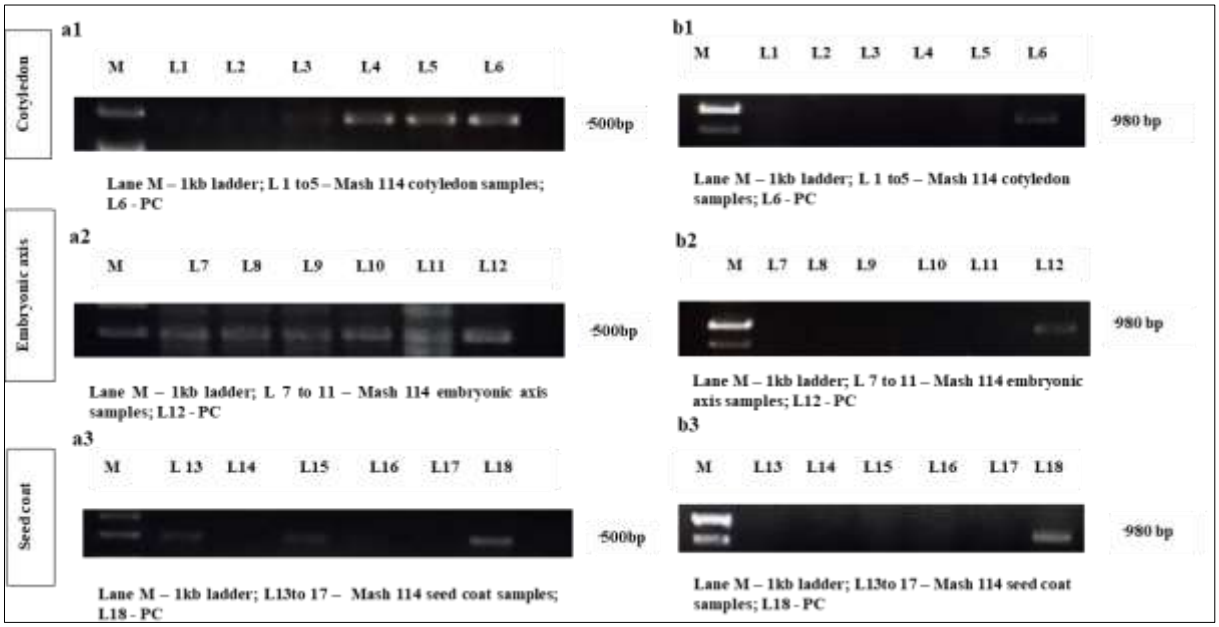

Fig 6: Detection of MYMV in different seed parts of Mash 114 seed. A1 to A3: Agarose gel electrophoresis of PCR products obtained from MYMV transmitted Mash 114 seeds parts using Rep gene specific DNA-A primer. B1 to B3: Agarose gel electrophoresis of PCR product obtained from MYMV transmitted Mash 114 seed parts using movement protein specific DNA-B primer

Table 2: Detection of MYMV DNA A and DNA B in seed parts of CO_5 (SC) and Mash 114(RC) through PCR

Different Seed parts	PCR reaction for DNA A Specific primer		PCR reaction for DNA B Specific primers	
	Number of positive reaction/total number of samples tested		Number of positive reaction/total number of samples tested	
	CO_5 (SC)	Mash 114 (RC)	CO_5 (SC)	Mash 114 (RC)
Cotyledon	10/10	5/5	2/10	0/5
Embryonic axis	10/10	-	4/10	0/5
Seed coat	9/10	3/5	9/10	0/5

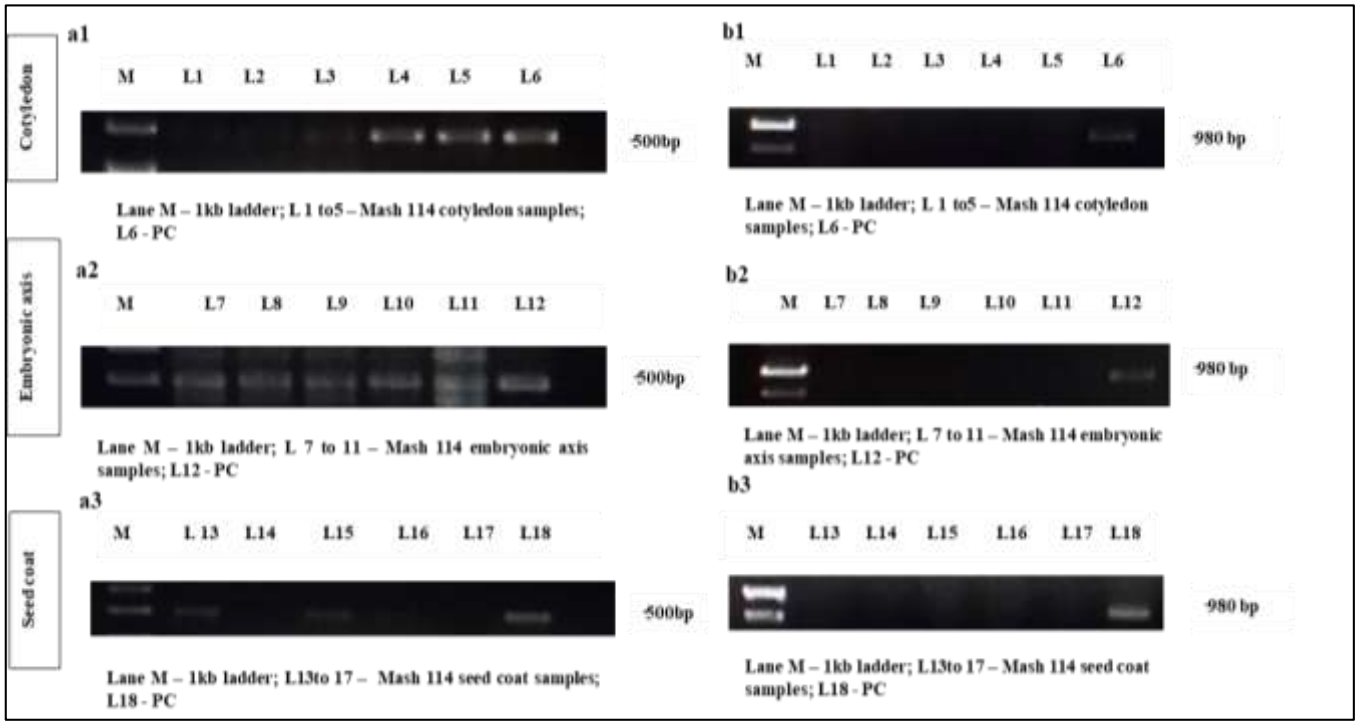

Fig 7: Detection of MYMV in different seed parts of CO_5 seed, A1 to A3: Agarose gel electrophoresis of PCR product obtained from MYMV transmitted CO_5 seed parts using Rep gene specific DNA-A primer. B1 to B3: Agarose gel electrophoresis of PCR product obtained from MYMV transmitted CO_5 seed parts using movement protein specific DNA-B primer

Discussion

New viruses are being continuously discovered, and part of them is seed borne in nature. Kim *et al.* (2015) [6] reported that the sweet potato leaf curl virus (SPLCV) was detected in the bulk of five seeds of sweet potato cultivars, Singeon-mi and Mokpo-69 with the detection rates in seeds of 13.3% to 66.7% 14.3% to 71.4% respectively. The presence of virus in endosperm with embryonic tissue was also confirmed in sweet potato seeds. Kil *et al.* (2016) [5] reported a high percentage of detection of tomato yellow leaf curl virus (TYLCV) in embryo of tomato seeds ranged from 20-100%. Detection of virus in cotyledon of seeds collected from whitefly mediated transmitted plants was high (84.6%), compared to agroinoculated plants (80.7%). Further, Perez-Padilla *et al.* (2020) investigated that TYLCV was detected as 100% in all the bulk seed samples tomato genotypes tested. Kothandaraman *et al.* (2016) [7] reported that the presence of MYMV was confirmed in seed coat, cotyledon, embryonic axis, and whole seed of susceptible variety of blackgram (CO_5) through PCR amplicon sequencing, DAS ELISA, and immunosorbent electron microscopy (ISEM). Kamesh Krishnamoorthy *et al.* (2021) [8] reported that MYMV was detected in seed coat, cotyledon, embryonic axis of seeds of blackgram. Seed borne nature of Dolichos yellow mosaic virus in lablab bean was proven by Suruthi *et al.* (2018) [16] and virus detection percentage in the seed coat, endosperm and embryo was 37.5%, 69%, and 100% respectively. Tomato leaf curl New Delhi virus (ToLCNDV) was detected in the embryos of chayote seed (Sangeetha *et al.*, 2018) [15]. Bitter gourd yellow mosaic virus (BgYMV) in whole seeds of bitter gourd was detected in 19 samples out of 24 samples tested through DAS-ELISA (Manivannan *et al.*, 2019) [10]. And, the virus also detected in seed coat, endosperm, and embryo, where, seed coat had high concentration of virus compared to endosperm and embryo. Presence of pepper yellow leaf curl Indonesia virus (PepYLCIV) in 32 seed embryos of Y-1Red, W-J1, and TMG-1chilli pepper seed samples were tested and the results revealed all 32 embryo samples were positive for both DNA-A and DNA-B of PepYLCIV (Fadhila *et al.*, 2020) [2].

In the present study, the presence of MYMV in different seed parts *viz.*, seed coat, cotyledon and embryonic axis of MYMV susceptible (CO_5) varieties of blackgram was confirmed through PCR, while, in resistant variety (MASH 114), presence of virus was detected in seed coat, cotyledon and not in embryonic axis.

Presence of MYMV in embryonic tissues is a major concern in legume cultivation, because, in future, more possibilities are there that the embryonic infection of MYMV leads to seedling infection in the field due to more permissive host virus multiplication in susceptible host under various climatic changes. Kothandaraman *et al.* (2016) [7] reported that eight seedlings revealed positive for MYMV out of 25 seedlings tested, raised from seeds of naturally infected MYMV plants,

which is 32% of the seedlings tested in grow-out tests, even though the seedlings are asymptomatic.

So, seed borne nature of MYMV in legumes is a great concern in seed supply chain and trade as well as in plant quarantine. The virus present in seed tissue of susceptible variety is a potential source for further transmission of MYMV by whiteflies, whereas in resistant variety MAS 114, embryonic axis completely free of MYMV. This important finding will help the breeders to find out the specific phenomenon blocking the virus movement to the embryonic axis in the resistant variety.

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