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Seed Borne Nature of *Mungbean yellow mosaic virus* (MYMV) in Blackgram in Tamil Nadu

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The yellow mosaic disease of blackgram caused by *Mungbean yellow mosaic virus* has emerged as a serious threat to pulses production especially in the South Eastern Asia. Seed borne nature of MYMV in blackgram seeds was determined using the seeds harvested from a MYMV resistant (either VBN-6 or VBN-8) and susceptible blackgram (CO-5) varieties grown in three different agroclimatic zones of Tamil Nadu in India for three consecutive cropping seasons namely, *Rabi* 2018 (October- December), Summer 2019 (March-May) and *Kharif* 2019 (June- August) at three different time intervals *viz*., 20, 40 and 60 days after sowing (DAS). Seed borne nature of MYMV was observed only in the susceptible variety CO-5 and was absent in the resistant varieties. Transmission of MYMV from infected plant to seeds was observed in all the three parts of the seeds viz., seed coat, cotyledon and embryo. Seeds from infected plants also showed abnormalities like shrinking, discolouration, ill filling inside pods and misshapen appearance.

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Keywords: Blackgram; mungbean yellow mosaic virus; seed borne nature.

1. INTRODUCTION

Yellow mosaic disease of legumes have emerged as a serious threat to production of a variety of grain legumes like Frenchbean (*Phaseolus vulgaris*), blackgram (*Vigna mungo*), cluster bean (*Cyamopsis tetragonoloba*), groundnut (*Arachis hypogea*), horsegram (*Macrotyloma uniflorum*), hyacinth bean (*Lablab purpurea*), moth bean (*Vigna aconitifolia),* mungbean (*Vigna radiata*) Lima bean, pigeonpea *(Cajanus cajan*) and soybean *(Glycine max*) [1-3]. The disease is also a major constraint in other countries like Bangladesh, Pakistan and Sri Lanka [4-5]. In blackgram YMD is caused by *Mungbean yellow mosaic virus* and Mungbean yellow mosaic India virus. Both these virus belong to the genus Begomovirus. Intial symptoms of the disease occur as small yellow flecks in the veinlet of young emerging leaves. Consequently developing leaves exhibit more conspicuous and irregular yellow and green patches which alternate each other. There is no much effect on leaf size, however in susceptible genotypes affected plants bear only few pods which are smaller and deformed in size. The symptoms also extend to the seed where seeds from diseased plants are ill-filled and there is a reduction in seed size and weight. Seeds also tend to be misshapen exhibiting a yellow discoloration [1,3]. Under extreme conditions, the remaining plant parts completely turn yellow in colour. The disease leads to a decrease in the photosynthetic efficiency which affects the crop yield [6].

The appearance of YMD symptoms in mungbean and urdbean around two weeks after sowing gave an impression of it being seed borne or caused by some factors present in the soil. In addition to this the presence of yellow patches on the seed coat of seeds formed within the susceptible blackgram and greengram genotypes created an interest in discovering the possibility of seed transmission of YMD. Seeds of mungbean genotype 44 showing yellow patches of seed coat were subjected to PCR using primers specific for MYMIV. The results showed that the virus was present in the seeds, however the virus was absent in the seedlings raised from MYMIV positive seeds which indicated that the virus might be lost during the process of germination resulting in failure of seed transmission [7].

Confirmation of seed borne nature of MYMV helps to determine their potential as a virus inoculum source by which will be of great concern in quarantine and trade related aspects [8]. Previous studies on the distribution pattern of begomovirus inside plants have proved that in many of the host virus combinations, the viruses are restricted only to the phloem parenchyma and cambium. Exit of the virus from phloem to other mesophyll parenchymatous tissue takes place only in a few cases due to which it was concluded that these boegomovirus specifically are not capable of entering into the seed parts as the vascular supply to the seed is limited partially only till the hilum portion of seed coat [9-10]. When a virus is seed transmitted, the developing embryo might get infected prior to fertilization or get infected by gametes carrying the viruses [11].

2. MATERIALS AND METHODS

2.1 Collection of Seeds

To determine the seed borne nature of MYMV in blackgram seeds, the seeds were harvested separately from infected and healthy plants of a blackgram susceptible (CO-5) and resistant (either VBN-6 or VBN-8) varieties. The plants were grown in three different agroclimatic zones of Tamil Nadu, India: Coimbatore, Vamban and Panpozhi across three different seasons namely Kharif, Rabi and Summer. The seeds were collected from fully matured plants prior to harvest. Four symptomatic and asymptomatic plants were chosen randomly in the field to perform seed collection which were posteriorly labeled and stored separately.

2.1.1 Excision of seed parts

To determine the spread of the virus, seeds were surface sterilized with Teepol 1% to remove the traces of any superficial virus. These seeds were then allowed to imbibe in water for 12 h. The seed parts (seed coat, cotyledon and embryo) were excised separately using a fine forceps and needle and used for the purpose of DNA extraction.

2.1.2 DNA extraction from seeds

The seeds were soaked overnight in water under laboratory conditions after which the seed parts (seed coat, cotyledon and embryo) were excised for DNA extraction. Gem-CTAB method was used for isolation of genomic DNA Rouhibakhsh et al. A group of five seed parts (either seed coat, cotyledon or embryo) were ground to a fine powder using liquid nitrogen. Pre-warmed CTAB buffer (100mM Tris-HCl pH 8, 10 mM EDTA, 1.4-2.0 M NaCl, 2% CTAB and 2-5% mercaptoethanol) was poured onto the ground seed parts and the solution transferred to 1.5ml eppendorf tubes. Contents of the tube were finely mixed two to three times by gentle inversion and incubated at 65^0C for 1h. For removal of protein 0.8 volume of chloroform:isoamyl alcohol (24:1) was added and mixed gently by inversion for 10min in such a way that a fine emulsion was formed. Then the tubes were centrifuged at 10,000rpm for 10 min which was followed by transferring the top aqueous layer to a new tube and the above step was repeated. Approximately 0.7 volume of isopropanol was added to the top aqueous layer obtained and this mixture was incubated at -20°C for 30 min or overnight to allow precipitation of the nucleic acid. This was then centrifuged at 10,000 rpm for 10 min to collect the precipitate. The precipitated DNA pellet was washed with 70 per ethanol followed by air drying until the ethanol completely evaporated. The dried pellet was dissolved in 50 µL sterile distilled water.

2.1.3 Determination of MYMV in blackgram seeds using Polymerase Chain Reaction (PCR)

The genomic DNA obtained from sees of the symptomatic and asymptomatic blackgram plants was used as a template for the Polymerase chain reaction (PCR) using MYMV DNA A coat protein specific primer pairs (5’-ATGGGKTCCGTTGTATGCTTG-3’ and 5’-GGCGTCATTAGCATAGGCAAT-3’) Akram et al. The amplicon size obtained from these primer correspond to size of 1000bp. PCR was performed for 25 µl reaction volume containing 12.5 µl of 2X Taqara EmeralAmp GT PCR Mastermix (readymade mixture of Taq DNA polymerase, optimized buffer and dNTP mixture), 5 µl of template DNA and 1 µl each of forward and reverse primer. PCR reaction was carried out in Eppendorf epgradient S Master cycler with the reaction cycles of initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 3 min,. A final extension step at 72°C for 10 min was performed and the product was held at 4°C.

2.1.4 Agarose gel electrophoresis

The PCR amplicons were analyzed by electrophoresis in 1.5% agarose . The gels were prepared in 1X TAE (Tris acetate EDTA) buffer, pH 8.8, containing ethidium bromide (0.5 µg/mL). Electrophoresis was performed at ~85V for 1 h using 1X TAE as electrophoresis buffer. The gel was visualized and results were documented in a UV-Gel Documentation system. (Protein simple, Alphaimager, USA).

3. RESULTS

3.1 Morphological Abnormalities in Seeds from Infected Plants

In the present study, we collected blackgram seeds collected from three locations across seasons in Tamil Nadu, India to evaluated the seed borne nature of MYMV. It was observed that the seeds collected from infected blackgram plants showed visually noticeable symptoms such as ill filling of seeds in pods, yellow discoloration of seeds and misshapen, shrunken and shriveled seeds.

3.2 Detection of MYMV in Seeds through PCR in Different Locations across Seasons

The agarose gel electrophoresis of PCR products revealed the expected amplicon size of 1000 bp in products obtrained from the DNA of seed coat, cotyledon and embryo. The amplification was absent in DNA of seed parts extracted from seeds obtained from healthy plants. In addition, an enhanced concentration of amplicons were visualized in PCR products from seed coat and embryonic regions.

In the case of Coimbatore and Panpozhi, the blackgram plants showed the presence of latent infection despite the absence of visual symptoms of MYMV. However PCR amplification of DNA obtained from seeds of these plants did not yield any amplification using primers specific for MYMV.

Table 1. Seed borne nature of MYMV in blackgram in Coimbatore across seasons

Croppping season	Seed part	Variety	Nature of Plant selected	Number of samples	Number of samples showing positive amplification at 1000 bp*	Percentage of samples
Kharif	Seed Coat	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	0	0
		VBN-6	Asymptomatic	6	0	0
	Cotyledon	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	0	0
		VBN-6	Asymptomatic	6	0	0
	Embryo	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	0	0
		VBN-6	Asymptomatic	6	0	0
Rabi	Seed Coat	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
	Cotyledon	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
	Embryo	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
Summer	Seed Coat	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
	Cotyledon	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
	Embryo	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0

*As it is observed that the performance pertaining to the above data contains only 0 and 100 values, only descriptive statistics has been used. *Mean of three replications sampled from three field replications*

Table 2. Seed borne nature of MYMV in blackgram in Vamban across seasons

Croppping season	Seed part	Variety	Nature of Plant selecected	Number of samples	Number of samples showing positive amplification at 1000 bp*	Percentage of samples
Kharif	SeedCoat	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
	Cotyledon	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
	Embryo	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
Rabi	SeedCoat	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
	Cotyledon	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
	Embryo	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
Summer	SeedCoat	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
	Cotyledon	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
	Embryo	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0

*As it is observed that the performance pertaining to the above data contains only 0 and 100 values, only descriptive statistics has been used. *Mean of three replications sampled from three field replications*

4. DISCUSSION

In the present study, seed borne infection of MYMV was present in the seeds obtained from MYMV infected plants irrespective of the time interval of infection. Amplification using PCR was absent in seeds obtained from healthy plants where latent infection was suspected in seeds.

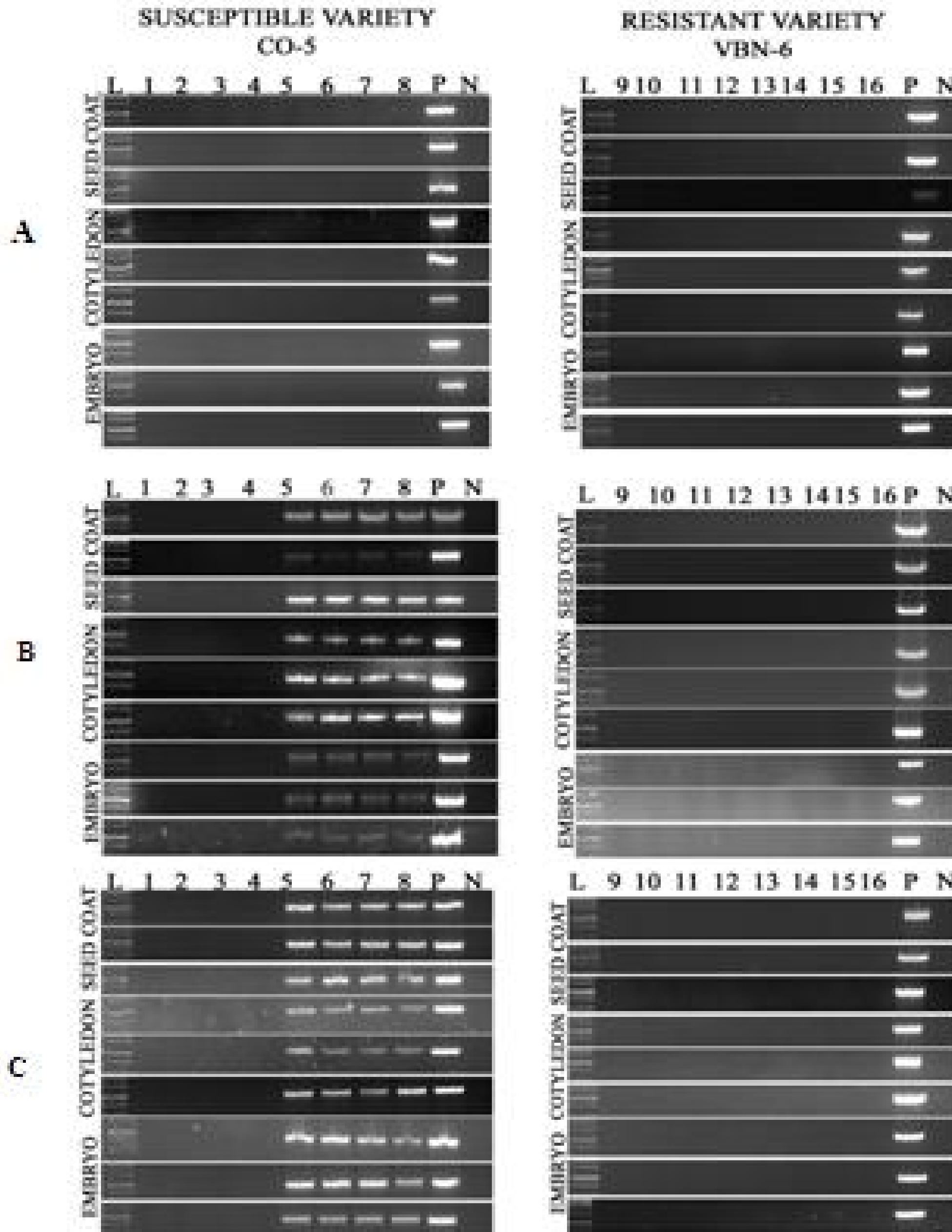

Fig. 1. Agarose gel electrophoresis of PCR amplicons to observed MYMV presence in three parts of seeds (seed coat, cotyledon, and embryo) from susceptible (CO-5) and resistant (VBN-6) varieties of black gram (*Vigna mungo*) collected atthree different cropping seasons, Kharif (A), Rabi (B) and Summer (C) at Coimbatore at different days after sowing. Lane 1-4 and 9-16: PCR amplicons from asymptomatic sample, Lanes 5-8: PCR amplicons from symptomatic sample, L: 1 kb ladder, P: positive control, N: negative control

PCR performed using universal begomovirus specific primers for the seed parts from MYMV infected plants showed the presence of amplicons in seed coats, cotyledons and embryonic axes which were separated from a group of ten seeds. Amplification was absent when PCR was performed from seeds of healthy plants .The presence of the virus in seeds was also confirmed through rolling circle amplification where 2.7 kb replicative circular form of DNA was found in the seed tissue of infected plants. Grow out tests of seeds collected from infected plants showed no symptoms in young seedlings despite viral DNA being detectable in 32% of the seedlings by PCR [8].

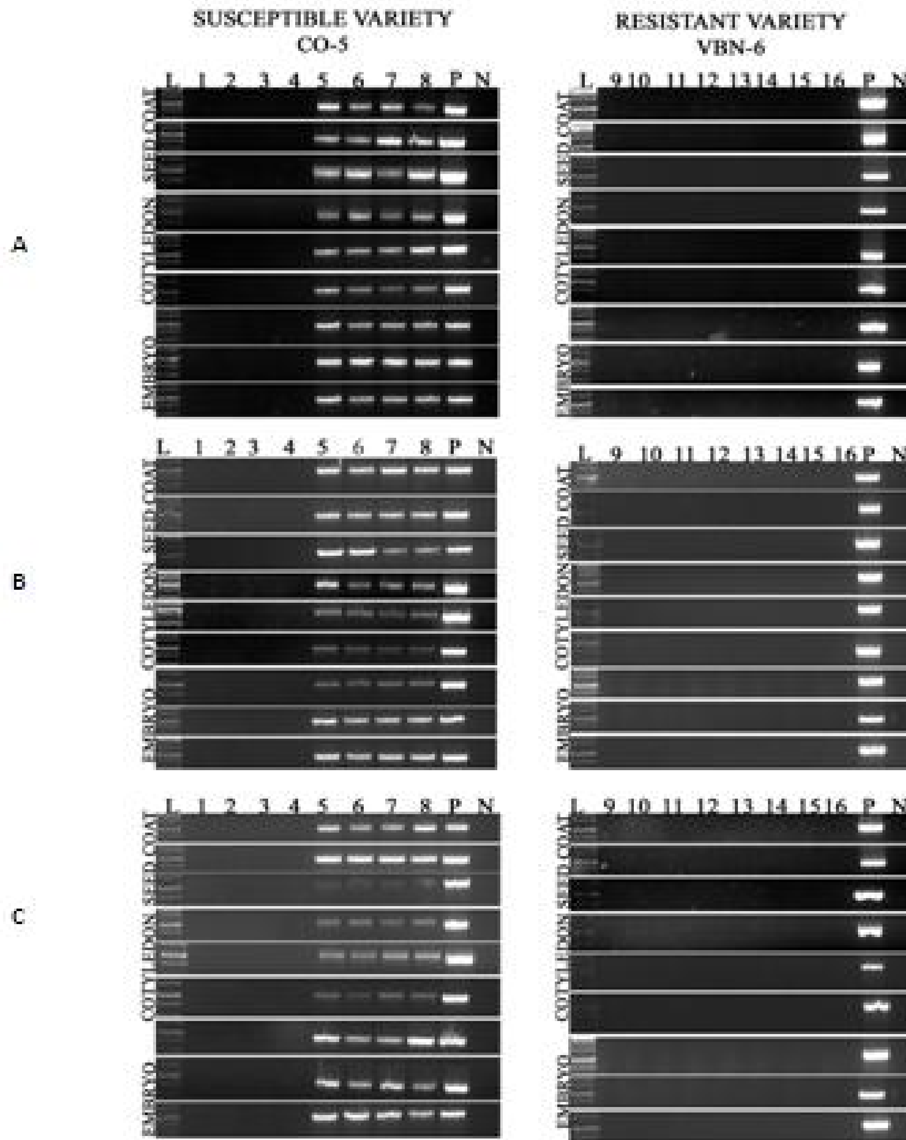

Fig. 2. Agarose gel electrophoresis of PCR amplicons to observed MYMV presence in three parts of seeds (seed coat, cotyledon, and embryo) from susceptible (CO-5) and resistant (VBN-6) varieties of black gram (*Vigna mungo*) collected atthree different cropping seasons, Kharif (A), Rabi (B) and Summer (C) at Vamban at different days after sowing. Lane 1-4 and 9-16: PCR amplicons from asymptomatic sample, Lanes 5-8: PCR amplicons from symptomatic sample, L: 1 kb ladder, P: positive control, N: negative control

Approximately 50 whole seeds collected from yellow mosaic infected field bean plants were subjected to DAS-ELISA. The results revealed all of them as positive in DAS–ELISA with an OD value of 0.206. PCR analysis confirmed the presence of Dolichos yellow mosaic virus (DoYMV) in different parts of the seed where the virus was present up to 100 % in the embryonic region followed by endosperm (69.23%). Seed coat contained the lowest amount of virus (37.5%). This was the first evidence of seed transmission of DoYMV [12].

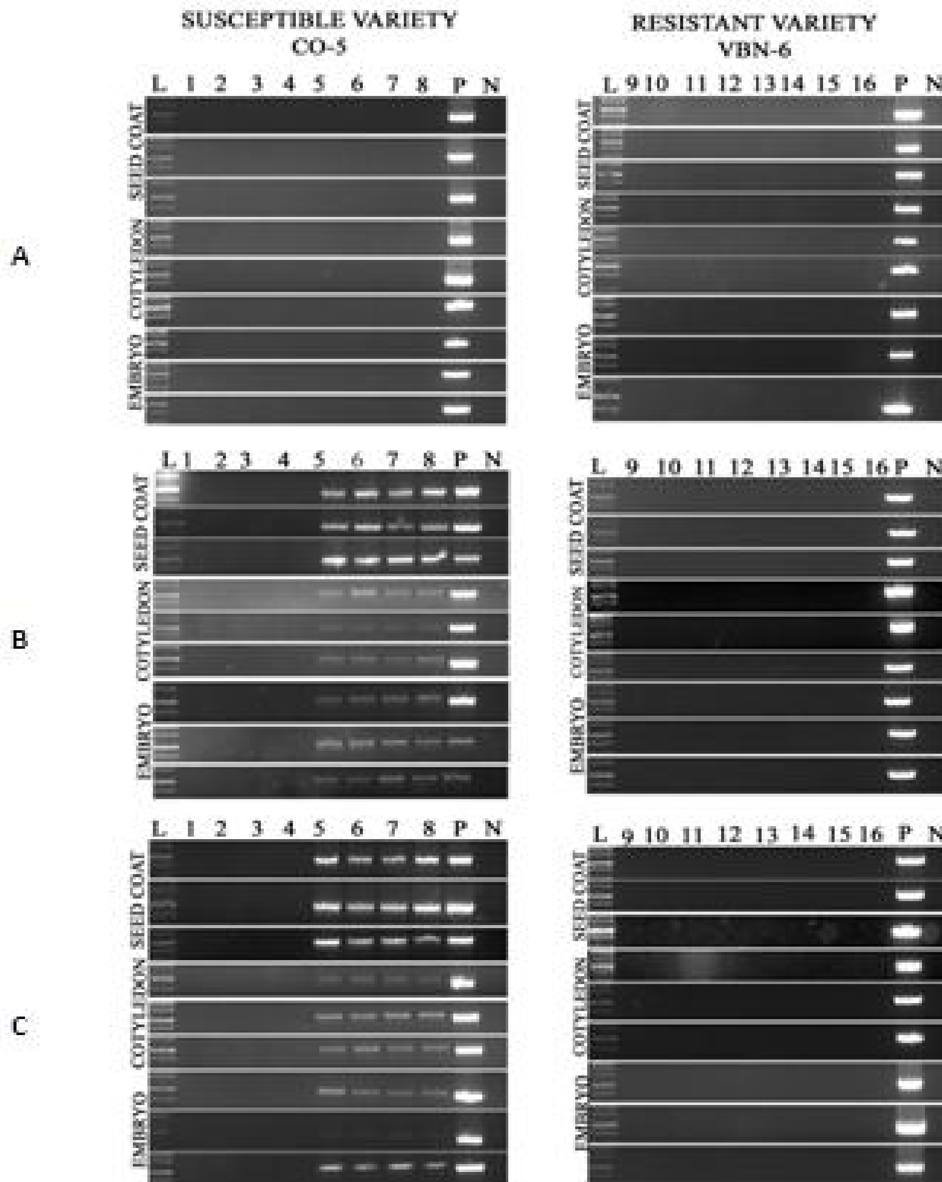

Fig. 3. Agarose gel electrophoresis of PCR amplicons to observed MYMV presence in three parts of seeds (seed coat, cotyledon, and embryo) from susceptible (CO-5) and resistant (VBN-6) varieties of black gram (*Vigna mungo*) collected atthree different cropping seasons, Kharif (A), Rabi (B) and Summer (C) at Panpozhi at different days after sowing. Lane 1-4 and 9-16: PCR amplicons from asymptomatic sample, Lanes 5-8: PCR amplicons from symptomatic sample, L: 1 kb ladder, P: positive control, N: negative control

Table 3. Seed borne nature of MYMV in blackgram in Panpozhi across seasons

Cropping season	Seed part	Variety	Nature of Plant selecected	Number of samples	Number of samples showing positive amplification at 1000 bp*	Percentage of samples
Kharif	Seed Coat	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	0	0
		VBN-6	Asymptomatic	6	0	0
	Cotyledon	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	0	0
		VBN-6	Asymptomatic	6	0	0
	Embryo	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	0	0
		VBN-6	Asymptomatic	6	0	0
Rabi	Seed Coat	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
	Cotyledon	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
	Embryo	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
Summer	Seed Coat	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
	Cotyledon	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0
	Embryo	CO-5	Asymptomatic	6	0	0
			Symptomatic	6	6	100
		VBN-6	Asymptomatic	6	0	0

*As it is observed that the performance pertaining to the above data contains only 0 and 100 values, only descriptive statistics has been used. *Mean of three replications sampled from three field replications*

5. CONCLUSION

In the present study seed borne nature of MYMV was observed in the seeds obtained from MYMV symptomatic black gram plants across different agroclimatic zones and seasons in Tamil Nadu, India. The seed borne nature of seeds from infected plants was irrespective of time of infection of the plants.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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