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Research Article

Evaluation of *Vigna* germplasms for resistance to *mungbean yellow mosaic virus* using agro inoculation

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Abstract
A total of twenty two germplasm lines of *Vigna* spp. were subjected to agroinoculation to confirm their level resistance/susceptibility against Mungbean Yellow Mosaic Virus (MYMV) using *Agrobacterium* construct of MYMV strain VA 239. The results showed that twelve mungbean and one urdbean germplasms were found to be highly susceptible (HS) and the rest from both mungbean and urdbean showed susceptible (S) to strain VA 239. One rice bean germplasm (TNAU Yellow) showed complete resistance. The accumulation of the viral genome in the host was confirmed by PCR amplification by using gene-specific primer. The screening results concluded that the resistant germplasm can be utilized as a donor in disease resistance breeding programme for generating MYMV resistant progenies in future.

Keywords
Mungbean, Urdbean, *Vigna* spp., Germplasms, Screening, MYMV, Agroinoculation, Resistance

Introduction

Pulses popularly known as poor man's meat are the important sources of proteins, vitamins and minerals that overlays a major contribution to the national's nutritional security (Singh *et al.,* 2015). India has found to be the world's largest producer of pulses contributing nearly 13-15 million tons of the global productivity with the production of wide range of pulse crops (Solanki *et al.,* 2011). Despite of its importance, *Vigna* spp. undergoes several production constrains due to climatic changes, emergence of new species / strains of pests and diseases and increasing deficiency of macro and micronutrients in the soil. In spite of the above mentioned factors, the annual production of these pulses are greatly limited due to yellow mosaic disease (YMD) caused by *mungbean yellow mosaic virus* (MYMV), particularly in tropical and subtropical countries (Varma and Malathi, 2003).

MYMV belongs to genus *Begomovirus* of the family *Geminiviridae* with characteristic bipartite genome (Dhakar *et al*., 2010)and are transmitted by whitefly in a circulative persistant manner (Sidhu *et al*., 2009). MYMV infected plants were subjected to show severe reduction in the quality of seeds and causes heavy yield losses. Use of disease resistant varieties regarded as an economical and durable method for controlling the MYMV losses. The genetic basis for this resistance could be broadened through introgression of unexplored wild genes into cultivated varieties (Monika *et al.,* 2001; Bisht *et al.,* 2005; Pandiyan *et al.,* 2008; Naimuddin *et al.,* 2011). Screening for MYMV resistance on field conditions remains failure due to environmental conditions, host nature and viral load. Hence, a good deal of research efforts has been directed towards screening of mungbean germplasms through agroinoculation, a novel strategy which uses the tumor - inducing plasmid of *Agrobacterium tumefaciens* for introducing the infectious viral clones into the plants to express the MYMV symptoms through encapsidation and replication and this method of efficient screening have been successfully reported by some workers (Biswas and Varma 2001; Sudha *et al.,* 2013). With this background of knowledge, twenty two *Vigna* germplasms originated from India were screened against MYMV using agroinoculation technique.

Materials and Methods

The experiment was conducted to screen the *Vigna* species for resistance or susceptibility to MYMV at the Department of Plant Biotechnology, Tamil Nadu Agricultural University, Coimbatore. A total of fifteen germplasm of mungbean (*Vigna radiata*)

namely PUSA- 118, PUSA-101, SML119, PLS-316, LM 469, Pabatla, NDM 5-3, AVT/RMI-6/1, Maduramoong, PUSA- 9871, VRM(Gg)1, VBN(Gg) 2, CO 8, CO 7 and CO 980, one germplasm of rice bean *(Vigna umbellata)* namely TNAU Yellow and six germplasms of urdbean *(Vigna mungo)* namely C0 5, VBG -70, IPU 99-247, L120-135, VBG 92 and NB -3 originated from different parts of India were selected and used in the present study for agroinoculation screening.

Agro inoculation was done on 2 days old sprouted seeds of selected germplasms by protocol suggested by Jacob *et al.* (2003) through MYMV infectious clone VA 239 (KA30 DNA A + KA27 DNA B) constructed by Balaji *et al.* (2004) and the protocol were as follows.

Briefly, *A. tumefaciens* harboring VA 239 (KA30 DNA A + KA27 DNA B) was grown in AB minimal medium (pH 7.0) at 28°C to an optical density of 1 at 600 nm. Later the grown cultures were centrifuged at 1,100 × g for 10 min at 25°C. The pellet was resuspended in AB minimal medium (pH 5.6) supplemented with 100 μM acetosyringone. 2 days old germinated *Vigna* seeds were immersed in the respective culture after puncturing the hypocotyl region with a 30-G needle. The infection was carried out at 25°C for 12 h in the dark. Subsequently, the seedlings were washed with sterile single distilled water and sown. After agroinoculation, the plants were maintained in a growth chamber at 25 ^{0}C with proper relative humidity of 60–70% and photoperiod of 16/18 h. Hoagland's solution was sprayed twice a week for proper growth and development of the plants. The symptom observation in the trifoliate leaves was recorded after 15thday from inoculation and the plants were identified as susceptible or resistance based on the presence or absence of yellow mosaic symptoms in a given time. The uninoculated plants of each accession without agroinoculation were maintained as control. The leaves with yellow mosaic symptoms were harvested 24 days after inoculation for DNA analysis.

Leaf samples were collected from both control and inoculated plants after 30 days and were used for further analysis. DNA was extracted after symptom expression from the inoculated plants of selected germplasms along with their respective uninoculated controls using the protocol described by Sudha (2009).The symptomatic leaf tissues (50 – 100 mg) were ground in sterilized pestle and mortar with 600μl of CTAB buffer and were transferred into an eppendorf tube. After the addition of pinch of polyvinyl pyrrolidone (PVP) and mercaptoethanol (0.5 μL) the tubes were incubated at 65°C (Water bath) for 30 min with occasional mixing. The incubated tubes were removed from the water bath and are allowed to cool at room temperature. Equal volume of chloroform: isoamyl alcohol mixture (24: 1) was added, inverted two or three times to mix well and incubated at room temperature for 15 min. It was centrifuged at 12000 rpm for 20 min and the clear aqueous phase was transferred to a new sterile tube. To the supernatant equal volume of phenol: chloroform (1:1) was added and centrifuged at 12000 rpm for 20 min and the clear aqueous phase was transferred to a new sterile tube. 700μl of ice-cold isopropanol and 1/10 volume of 3M sodium acetate was added to the collected supernatant and incubated at -20°C overnight. On the next day, the tubes were centrifuged at 12000 rpm for 20 min to pelletize the DNA and the supernatant was discarded. The DNA pellet was washed with 70 % ethanol. After washing with 70% ethanol the DNA pellet was air dried completely. Depending upon the size of the pellet, DNA was dissolved in 100-200 μl of 1X TE buffer (pH 8.0) and stored at -20º C.

For checking purity and quality, the genomic DNA was electrophoresed on 0.8 % agarose gel stained with ethidium bromide (EtBr) following method suggested by Sambrook J and Russell (2001). The gel was visualized in a gel documentation system (Alpha Imager TM 1200, Alpha Innotech Corp., CA, USA). DNA was quantified using Nano - Drop spectrophotometer (ND-1000 Spectrophotometer, NanoDrop Technologies, USA). 1 μl of total DNA was diluted to 1 ml with deionized water. The absorbance for all samples were measured at 260/280 nm. DNA has maximal absorbance at 260/230 nm. An optical density (OD) of 1.0 corresponds to 50 μg / ml for double stranded DNA.

After the necessary quantity and quality checks the DNA concentration was normalized to 50 ng / μl for PCR. The infectivity was checked by PCR assay using coat protein gene specific primer (FP1 5'GCGGAATTACGATACCGCC3' and RP1 5'GATGCATGAGTACATGCC3') (Maheswari, 2008) and the temperature cycles were placed as follows: 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C. The final elongation step was extended to 10 min at 72°C and finally maintained at 4°C. Amplified products were analyzed by electrophoresis in 1.2% agarose gels in TBE buffer and visualized by staining with ethidium bromide and recorded with an Alpha Imager 2000 (Alpha Innotech, San Leandro, CA) and the size of the PCR products was determined.

Results and Discussion

The majority of commercially available pulse varieties were prone to incidence of MYMV and hence it necessitates the development of MYMV

resistant varieties through disease resistant breeding programme. The screening for MYMV resistance in disease resistant breeding is previously determined based only on the visual morphological observations i.e., symptoms that occur on the plants once after infection. The symptomless genotypes were often assumed to be resistant, however on contrast MYMV symptoms may not always appear in the field due to the influence of some factors such as environmental changes, whitefly genotypes, host factors *etc*., which makes the complexity/ failure in the production of symptoms under field conditions for the identification of the true MYMV resistant lines. Unfortunately maintaining the natural inoculum of the virus isolates were also encountered as a hurdle since MYMV is vector transmitted by the whitefly, *Bemisia tabaci*, in a persistent (circulative) manner. On this context, advancement in genetic engineering provides a new inoculation technique called agroinoculation (Grimsley *et al*., 1986) through which the viral constructs can be artificially inoculated into the plants and were effectively used to evaluate resistance of the genotypes at the molecular level. Since then the agro infection method which is popularly known as agroinoculation has been utilized extensively to deliver viruses inside plants for transient RNA interference (RNAi) and virus-induced gene silencing (VIGS) studies for imparting resistance.

To prove infectivity and to identify the resistant source against viruses, agroinoculation or biolistic inoculation methods were effectively used to deliver viral constructs into the plants. Mechanism behind agroinoculation is such that, once *Agrobacterium* strain harboring the viral DNA delivers its T-DNA into the plants, which in turn also releases the viral genome into the plant and later it replicates through homologous recombination (Rogers *et al*., 1986) or replicational release (Stenger *et al*., 1991) and hence, were effectually used for screening. Many efforts have been made to improve agro infection efficiencies of Gemini viruses. Besides the convenience of cloning and achieving a high frequency agroinfection, Jacob *et al.* (2003) followed a single strain strategy of Agroinfection by co delivery of MYMV DNA A and DNA B from one *Agrobacterium* strain and observed 100% agroinfection. Similarly in earlier studies several successful reports for agroinoculation screening strategy to identify the MYMV virus-resistant lines were observed (Balaji *et al.,* 2004; Usharani *et al.,* 2005; Karthikeyan *et al.,* 2011; Sudha *et al.,* 2013; Madhumitha *et al.,* 2019).

In the present study, an attempt was made to screen mungbean, Rice bean and Urdbean germplasm entries for MYMV through agroinoculation for which *Agrobacterium C 58* strain harboring the partial dimers of the MYMV strains were used (obtained from Madurai Kamaraj University namely VA239 (KA30 DNA A and KA27 DNAB)) to study the extent of resistance/susceptibility to MYMV. All the selected germplasms of *Vigna* species namely Mungbean, Rice bean and Urdbean after agroinoculation were monitored for the occurrence of *yellow mosaic virus* and the symptoms were recorded. Interestingly among the fifteen mungbean germplasm entries, twelve were found to be highly susceptible with typical yellow mosaic symptoms in the trifoliate leaves upon agroinoculation, the other three were found to be susceptible with mild mosaic symptoms. Rice bean variety, TNAU yellow were found to be consistently resistant to MYMV across replications and no symptoms were seen till the maturity stage. It is also evident from soybean agroinoculation that few varieties did not develop symptoms even after three repetitions (Usharani *et al*., 2005). The strain VA 239 had observed for very severe effects on the morphology that includes puckering accompanied with stunting of plants in addition to the development of typical yellow mosaic symptoms in urd bean. Similarly Sudha *et al.,*(2013) observed for stunted plants with shortened petioles and leaf curling on agroinoculated symptomatic plants. In urdbean, among the lines which were screened for MYMV resistance through agroinoculation five germplasm lines were found to be susceptible and one germplasm CO 5 was found to be highly susceptible (Table 1). In all the germplasms, symptoms were observed 15 – 17 days of post agroinoculation and the data was scored for the presence of resistance/susceptibility (Fig 1). Corroborating to the above findings Jacob *et al.* (2003) followed a co delivery of MYMV DNA A and DNA B from one *Agrobacterium* strain and yielded 100% agroinfection. Usharani *et al.* (2005) conducted infectivity analysis of MYMV through agroinoculation in soybean isolate and obtained infectivity of about 70–95 percent. Similarly, Sudha *et al.* (2013) and Madhumitha *et al.*(2019) reported that the level of MYMV infectivity on agroinoculated plants ranged between 0–100 percent even for the field-resistant MYMV genotypes.

The presence of viral DNA in agroinoculated plants in both symptomatic and asymptomatic plants were verified with expected amplicon size by amplification of viral DNA using coat protein specific primers of DNA A in the host. One germplasm from mungbean and urdbean which is susceptible and one resistant rice bean were taken and PCR was confirmed. The expected amplicon size of 703 bp was obtained in all the infected

susceptible samples. The resistant (TNAU Yellow) and samples from control plants (healthy) of both resistant and susceptible did not show any amplification (Fig 2). The absence of viral genome in agroinoculation plants gives a clear picture on the absence of viral particles inside the host. The presence of viral genome on the susceptible plants clearly indicated the multiplication of virus inside the cell after integration and on contrast some mechanism inside the cell prevented the further multiplication of the virus in resistant plants. It is possible that some tissue specific host tolerance mechanism is operating inside the plant resulting in resistance in rice bean. The earlier study of Maheswari (2008) revealed that CP gene sequences had shown the homology of isolated virus to MYMV. Wyatt and Brown (1996) reported CP gene as the most highly conserved gene in the family *Geminiviridae.* This gene sequence, which effectively predicts discrete strains, species and taxonomic lineages of begomoviruses, was accepted by the ICTV as a desirable marker for virus identity when a full length genomic sequence is not available (Rybicki, 2000).These results are in accordance with the reports of Usharani *et al.* (2005), Sudha *et al.* (2013) and Madhumitha *et al.,*(2019) who observed for the presence of viral DNA only in agroinoculated symptomatic plants.

The results of the outcome suggested that the level of accuracy and infectivity obtained through the agroinoculation technique was very effective and the technique can be exploited further for the identification of new resistant sources. The identified resistant sources of *Vigna* spp. can be further utilized under crop resistant breeding programme as a donor for the development of MYMV resistant plants.

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Table 1.Agroinoculation screening of mungbean, rice bean and urdbean germplasms for MYMV resistance

Crop	Germplasms	Scale	Disease Rating (across replications)	No of days for symptom development	Per cent infection Mean*
Mungbean	PUSA- 118	9	HS	17	74.0
	PUSA-101	9	HS	15	79.3
	SML119	9	HS	17	90.2
	PLS- 316	9	HS	18	88.3
	LM 469	9	HS	17	91.4
	Pabatla	9	HS	16	87.5
	NDM 5-3	9	HS	17	79.0
	AVT/RMI-6/1	9	HS	18	87.3
	Maduramoong	9	HS	15	88.0
	PUSA- 9871	9	HS	18	89.5
	VRM(Gg)1	9	HS	17	90.2
	VBN(Gg) 2	9	HS	17	89.3
	CO 8	7	S	16	85.1
	CO 7	7	S	15	78.3
	CO 980	7	S	16	80.5
Rice bean	TNAU Yellow	1	R	-	-
Urdbean	C0 5	9	HS	18	89.6
	VBG -70	7	S	17	85.3
	IPU 99-247	7	S	15	82.3
	L120-135	7	S	16	75.8
	VBG 92	7	S	17	82.9
	NB -3	7	S	18	80.5

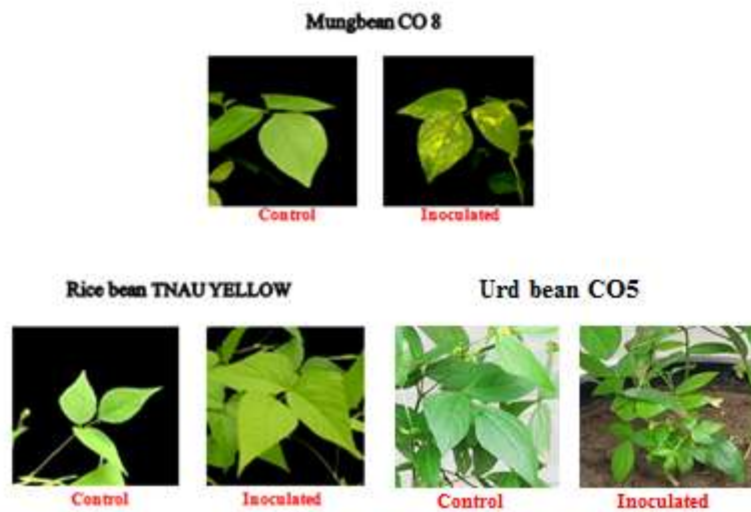

Fig. 1. Symptoms appearance on uninoculated control and agroinoculated plants of Mungbean, Rice bean and Urdbean

Fig 2. PCR assay on different germplasms (Rice bean, Urdbean, Mungbean) using coat protein gene specific primers

From left to right, Lane 1– 7, 1.1kb ladder, 2.TNAU Yellow uninoculated control, 3. TNAU Yellow inoculated, 4. CO 5 uninoculated control, 5. CO 5 inoculated, 6.CO 8 uninoculated control, 7.CO 8 inoculated.

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