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Quantification of a legume begomovirus to evaluate soybean genotypes for resistance to yellow mosaic disease

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ABSTRACT

Mungbean yellow mosaic India virus (MYMIV) infecting soybean and other legumes causes yellow mosaic disease (YMD). Evaluation of soybean genotypes for YMD resistance involves field screening at disease hot spots or in a protected environment using infectious clones or viruliferous whiteflies as sources of virus inocula. Development of efficient virus inoculation and quantification protocols to screen soybean genetic stocks against YMD is imperative for breeding resistant varieties. Binary plasmids harbouring complete, tandem dimeric genomic components DNA A and DNA B of MYMIV-soybean isolate were engineered. The infectivity of the clones was demonstrated in soybean genotypes JS335 and UPSM534 that display contrasting YMD resistance. As a follow-up, soybean germplasm lines, breeding lines, and representative cultivars that were initially screened at an YMD hotspot were then subjected to *Agrobacterium*-based infection with MYMIV. Quantitative real time polymerase chain reaction (qRT-PCR) based copy number analysis of MYMIV genomic components allowed soybean genotypes to be classified into three discrete categories; resistant, moderately resistant and susceptible to the viral infection. Thus, a soybean germplasm disease screening system based on agro-infection and qRT-PCR based quantification of MYMIV was developed to facilitate breeding YMD resistant soybean. The implications of this study for obtaining YMD resistant soybean cultivars are discussed.

1. Introduction

The family *Geminiviridae* comprises a large group of phytopathogenic viruses (Fauquet and Stanley 2003; Fauquet et al., 2008; Brown et al., 2015; Zerbini et al., 2017) including begomoviruses infecting legumes (Ilyas et al., 2010; Qazi et al., 2007). Legume begomoviruses are transmitted by *Bemisia tabaci* and cause yellow mosaic disease (YMD) and are collectively known as yellow mosaic viruses (YMVs). *Mungbean yellow mosaic India virus* (MYMIV) which infects legumes that include soybean belongs to the genus *Begomovirus*, family *Geminiviridae* and causes YMD. YMV infection in legumes causes an estimated annual crop loss of US $ 300 million (Varma and Malathi, 2003).

Begomoviruses characteristically have circular, single stranded DNA (ssDNA) genomes encapsidated in twinned icosahedral virions of 18 nm × 30 nm (Stanley et al., 2005). The genomes of YMVs infecting soybean have two ssDNA genomic components, known as DNA A and DNA B, each approximately of 2750 nts in length. The genomic components encode proteins in virion-sense and complementary-sense strands (Usharani et al., 2004; Ramesh et al., 2013). DNA A encodes the coat protein (CP) (AV1) and the pre-coat protein (AV2) on the virion-sense whereas genes (AC1, AC2 and AC3) on the complementary-sense strand code for the replication associated protein (Rep), transcriptional activator protein (TrAP), replication enhancer protein (REn), respectively. Two further genes in the complementary-sense strand, AC4 and AC5, code for proteins that are symptom determinants and suppressors of RNA silencing (Li et al., 2015). Thus, genomic component DNA A, encodes proteins for viral ssDNA encapsidation, replication and suppression of RNA silencing. Component DNA B encodes the nuclear shuttle protein encoded in the virion-sense and the movement protein in the complementary-sense which are involved in intracellular and cell-to-cell movement of virus (Sanderfoot and Lazarowitz, 1996; Stanley and Gay, 1983; Ingham et al., 1995).

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Soybean cultivation in central India is affected by severe stresses such as late onset of monsoon, long dry spells, and intense rainfall over a short time span (Anon., 2016). YMV infection has aggravated the problems for soybean cultivation. YMD in soybean has been confined to the plains of north India. In 2015 the spread of disease into central India caused severe yield losses (Anon., 2015) and MYMIV has been shown to be the causative agent of YMD of soybean grown in central India (Ramesh et al., 2016).

Disease resistant cultivars forms an integral component of plant protection measures and resistant soybean cultivars are indispensable to manage YMD. To develop resistant varieties, breeders are dependent on YMD hot spots (plains of north India where natural infection of YMD occurs) to screen the soybean genotypes (Ramesh et al., 2016; Ramteke and Gupta, 2005; Ramteke et al., 2007). Since YMVs are not sap-transmissible, the efficacy of virus transmission depends on species of whiteflies (Lapidot et al., 2007). At least 40 cryptic species of whiteflies have been identified to be associated with diseased caused by begomoviruses (Jiu et al., 2017). Experimental insect transmission of virus is a time consuming process as it requires whiteflies to be reared and maintained on susceptible plants. Introduction of viral nucleic acids into plants can be achieved by agroinoculation (Grimsley et al., 1986). The advantages of agroinoculation are that it is season-independent and provides reproducible levels of infectivity.

Development of efficient and effective virus inoculation and quantification protocols to screen soybean genetic stocks against MYMIV is of prime importance. The study here shows the utility of rolling circle amplification (RCA) (Inoue-Nagata et al., 2004; Bang et al., 2014) in developing MYMIV infectious clones and efficacy of these infectious clones in screening soybean genotypes. Real-time PCR has become the method of choice for detection and quantification of plant pathogenic viruses (Mason et al., 2008; Harper et al., 2011; Quito-Avila et al., 2012; Ammara et al., 2017; Shafiq et al., 2017). To complement the utility of MYMIV infectious clones, a quantitative real-time PCR (qRT-PCR) based MYMIV titre assay has been developed. The outcome of field screening for YMD resistance at a hot spot and agroinoculation based assessment of soybean genotypes for MYMIV resistance have been compared. The combined utility of infectious clones and qRT-PCR based viral copy number analysis of MYMIV was used a tool to screen the soybean genotypes for YMD resistance.

2. Materials and methods

2.1. Rolling circle amplification (RCA) and restriction digestion enzymes

Symptomatic leaves from soybean plants grown on a research farm at the Indian Council of Agricultural Research-Indian Institute of Soybean Research (ICAR-IISR) were collected during the 2015 cropping season. Total DNA was isolated from the healthy/non-symptomatic and infected soybean leaves expressing characteristic symptoms associated with YMD (Doyle and Doyle, 1987). RCA of viral genomic components was performed following the protocol of Ramesh et al. (2017). Restriction digestion map of genomic sequences of MYMIV infecting soybean grown in the central India state of Madhya Pradesh (KC852204 and KP828155) was generated using BioEdit sequence alignment editor [v7.0.0] (Hall, 1999). Restriction mapping identified unique restriction enzyme recognition sites in the viral genome. Among the many single cutter enzymes, *Xba* I was chosen as it cuts the DNA A genomic component at nucleotide co-ordinate 2541. Similarly, analysis of the DNA B sequence identified *Bam* HI as a restriction enzyme of choice as it cuts the nucleotide position 1342.

2.2. Construction of infectious MYMIV plasmids

In order to generate head to tail tandem repeats of MYMIV, 1 µg of RCA product was partially digested using 0.02 units of enzymes *Xba* I and *Bam* HI, in two separate reactions, to obtain ~5.4 kb restriction fragments of DNA A and DNA B respectively. The restriction digestion reaction was terminated by heat inactivation of enzymes (65 °C for 20 min) and the DNA fragments were gel eluted using QIAquick Gel Extraction Kit™(Venlo, Limburg, Netherlands). The binary plasmids harbouring viral genomic components were developed by following standard molecular cloning procedures (Sambrook and Russell, 2001). *Escherichia coli* strain DH5α was used for performing sub-cloning procedures. Briefly, following gel extraction, both the viral derived DNA fragments (~5.4Kb each) corresponding to DNA A and DNA B genomic components of MYMIV were inserted in to the *Xba* I and *Bam*HI sites, respectively, of a modified pFGC5941 plasmid which was altered by removing the chalcone synthase A gene (*chsA*) intron to yield pFGC5941ΔchsA (Supplementary Fig.1 Fig. 1). Thus, binary plasmids harbouring head to tail tandem repeats of DNA A (NRCS-A2X) and DNA B (NRCS-B2X) were developed and inserts of the plasmids were sequenced. The nucleotide sequences of the MYMIV isolate used were deposited with GenBank under the accession numbers MH181823 and MH181824 for the DNA A and DNA B, respectively.

2.3. Agro-infection of soybean lines and virus detection

The binary plasmids NRCS A2X-7 and NRCS B2X-1 were separately mobilized into *Agrobacterium tumefaciens* strain LBA 4404 by the freeze-thaw method (Chen et al., 1994). *A. tumefaciens* harboring the binary plasmids were grown overnight in LB medium in an orbital incubator shaker set at 200 rpm and 28 °C. The culture was centrifuged, and the cell pellet was plated on LB agar medium containing kanamycin (50 µg/mL) and incubated at 28 °C overnight. *Agrobacterium* cells harvested from the lawn were suspended in 500 µl of G5 Gamborg B5 medium (Duchefa Biochemie) containing 100µM acetosyringone. For large scale agro-inoculation of soybean lines, liquid cultures of the *Agrobacterium* cells were used. Seeds of selected soybean genotypes were germinated on filter paper and seedlings were grown in potting mix in a glasshouse. Seedlings at three-leaf stage were pricked at the stem nodes to generate wounds and agroinoculation was carried out in the stem nodes using 30-gauge needles. Soybean plants inoculated with *Agrobacterium* harbouring an empty pFGC5941ΔchsA were used as mock/control. Plants were maintained in an insect-free growth chamber with16 h light/8 h dark. Total DNA was extracted from the leaves of soybean plants that showed characteristic yellow mosaic symptoms and from the leaves of mock inoculated plants (Doyle and Doyle, 1987). PCR detection of conserved genomic region of legume begomoviruses (Girish and Usha, 2005) and MYMIV (Ramesh et al., 2016) were performed.

2.4. Field evaluation of soybean

Soybean genotypes (n = 92) were grown at the research farm of Punjab Agricultural Research University (PAU), Ludhiana (Table 1). Field grown soybean lines were evaluated based on the occurrence and intensity of characteristic symptoms associated with YMD. The disease scoring of the genotypes was done at pod filling, using the standard 0–9 scale (Suman et al., 2015; Khan et al., 2013). Briefly, scoring is based on the percent area of leaf and pods symptomatic due to YMD, where 0 denotes no symptoms or completely immune and 9 denotes > 75% of plant parts are covered due to yellow mosaic or highly susceptible (Suman et al., 2015).

2.5. Standard curves for virus quantification and statistical analysis

Standard curves were generated for qRT-PCR based absolute quantification of MYMIV using binary plasmids harbouring full-length MYMIV DNA A (NRCS A1X) and DNA B (NRCS B1X) genomic components. The standards were prepared by following tenfold serial dilution of plasmids in the DNA extracted from healthy soybean plants. The plasmids dilutions were made to attain 10 to 10^7 copies of viral genome per sample. The viral pre-coat protein (AV2) and movement protein

Table 1
Evaluation of soybean genotypes for YMD resistance in the field.

Disease severity scale	Soybean Genotypes	Number of genotypes	Category	Description
0	SL958	1	Immune	No symptoms or very minute yellow specks on the leaves
1-2	Bragg, DS 228, PS 1024, PS 1029, PS 1042, SL 295,	6	Highly resistant	Yellow specks covering up to 5% leaf area
3-4	DS 97–12 (Pusa 9712), KHSb-2, PK 416, PK 471, PK 472, PS 564, PS 1092, PS 1347, Pusa 20, SL 525, UPSM 534	11	Resistant	Yellow mottling symptoms affecting upto 15% leaf area
5-6	Co 1, Kalitur, MAUS 71, NRC 12, TAMS 38, TAMS 98–21,	6	Moderately resistant	Symptoms of yellow mottling followed by discoloration up to 30% leaf area
7-8	ADT-1, Alankar, Ankur, Birsa Soya 1, Co2, DS 98–14, DS 98–14, Gaurav, Guj. Soya 1, Hara Soya, Indira Soya 9, Imp. Pelican, JS 2, JS 71–05, JS 76–205, JS 79–81, JS 80–21, JS 97–52, Lee, MACS 13, MACS 57, MACS 58, MACS 124, MACS 450, MAUS 1, Monetta, NRC 2, NRC 7, NRC 37, Palam Soya, PK 308, PS 1225, PS 1241, Pusa 22, Pusa 24, Pusa 37, Pusa 40, PRS 1, RKS-18, RAUS 5, SL 688, Type 49, VL Soya 1, VL Soya 2, VL Soya 21, VL Soya 47, VL Soya 63	47	Susceptible	Symptoms of prominent yellow mottling and discoloration of leaves, pods, leaf size reduction covering upto 75% of foliage and stunted plants
9	Co3, Durga, Guj. Soya 2, Hardee, JS 75–46, JS 90–41, JS 93–05, JS 95–60, JS 335, KB -79, LSb-1, MAUS 2, MAUS 32, MAUS 47, MAUS 61, MAUS61-2, MAUS 81, PK 262, Punjab -1, Pusa 16, Shilajeet, Shivalik, VL Soya 59,	23	Highly susceptible	Severe yellow discoloration of leaves covering > 75% of foliage, stunted plants and reduction in pod size

(BV1) genes were detected and quantified in the DNA extracted from control and virus inoculated soybean plants using the primers AV2F(ATGTGGGATCCATTGTTGAACG)/AV2R(TCAATCTCCTCCGTGCATTCG) and BV1F(TTAAGCAAACGCCTAATGGG)/BV1R(AGGTCGAAGACTCCAACGTG), respectively. Real time PCR was performed in a Light Cycler R 480 II (Roche) and the amplification conditions were: 95 °C for 2 min, 40 cycles of 95 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s. Ct values obtained from three technical replicates (each plasmid dilution point) were used for generating the standard curves. Linear regression analysis of Ct values over the log of total DNA content in each sample was conducted to produce the standard curve. To determine if the differences of viral titres among the various groups of soybean genotypes were statistically significant a one-way analysis of variance (ANOVA) was performed.

3. Results

3.1. MYMIV infection in contrasting soybean lines

The binary vectors developed to infect soybean with MYMIV were used to ascertain the efficiency of infection. Initially, two soybean genotypes, JS335 (susceptible to MYMIV) and UPSM534 (resistant to MYMIV), were used for infectivity analysis. In the susceptible variety, typical yellow mosaic symptoms started appearing 7–9 days post inoculation whereas a small number of plants of UPSM534 showed very mild symptoms (Fig. 1A). Of the 40 inoculated JS335 plants 35 (87.5%) developed yellow mosaic symptoms, whereas 6 out of 40 plants (15%) of the resistant genotype UPSM 534 showed very mild symptoms. Symptomatic JS335 and UPSM 534 plants were confirmed infected by PCR with primers for legume begomoviruses (Girish and Usha, 2005) and primers amplifying the MYMIV coat protein gene (Ramesh et al., 2016) (Fig. 2B). In addition, RCA products produced from the extracted DNA samples yielded ˜2.7 kb products when digested with restriction enzymes which linearize the DNA A and DNA B genomic components (Data not shown). All symptomatic JS335 and UPSM 534 plants showed the presence of MYMIV. PCR amplifications with DNA extracted from non-symptomatic plants did not result in amplification products showing the absence of MYMIV (Fig. 1B).

3.2. Resistance status of soybean genotypes

Screening of soybean genotypes in the field at PAU, Ludhiana showed differing responses (Table 1). None of the genotypes evaluated was completely immune to the viral disease. Seventeen genotypes that were bred mostly in the northern part of the country were found to be either highly resistant or resistant to MYMIV with a disease severity score of 1–3 (Table 1). Some varieties, such as Co1, Kalitur and MAUS 71, displayed moderate resistance with a disease severity score of 5–6. Field screening of soybean varieties under natural conditions of YMV infections showed that most were either highly susceptible or susceptible to the disease.

3.3. Resistance status of soybean lines based on Agroinoculation

Selected soybean genotypes were agroinoculated with MYMIV (Table 2). Infectivity of MYMIV in resistant genotypes was lower than in susceptible soybean lines. All inoculated susceptible soybean genotypes developed characteristic disease symptoms (Fig. 2). Soybean genotypes that were categorized as resistant did not develop visible symptoms within 9 days post inoculation (dpi). However, mild symptoms were observed in a few genotypes (Fig. 2). Diagnostic PCR detected MYMIV infection in all the symptomatic genotypes and not in the non-symptomatic plants. At 15–20 dpi the inoculated plants were scored for disease severity (Table 2). The disease severity scores for agroinoculated soybean genotypes correlated with those for plants screened in the field, demonstrating that the methods produce comparable results.

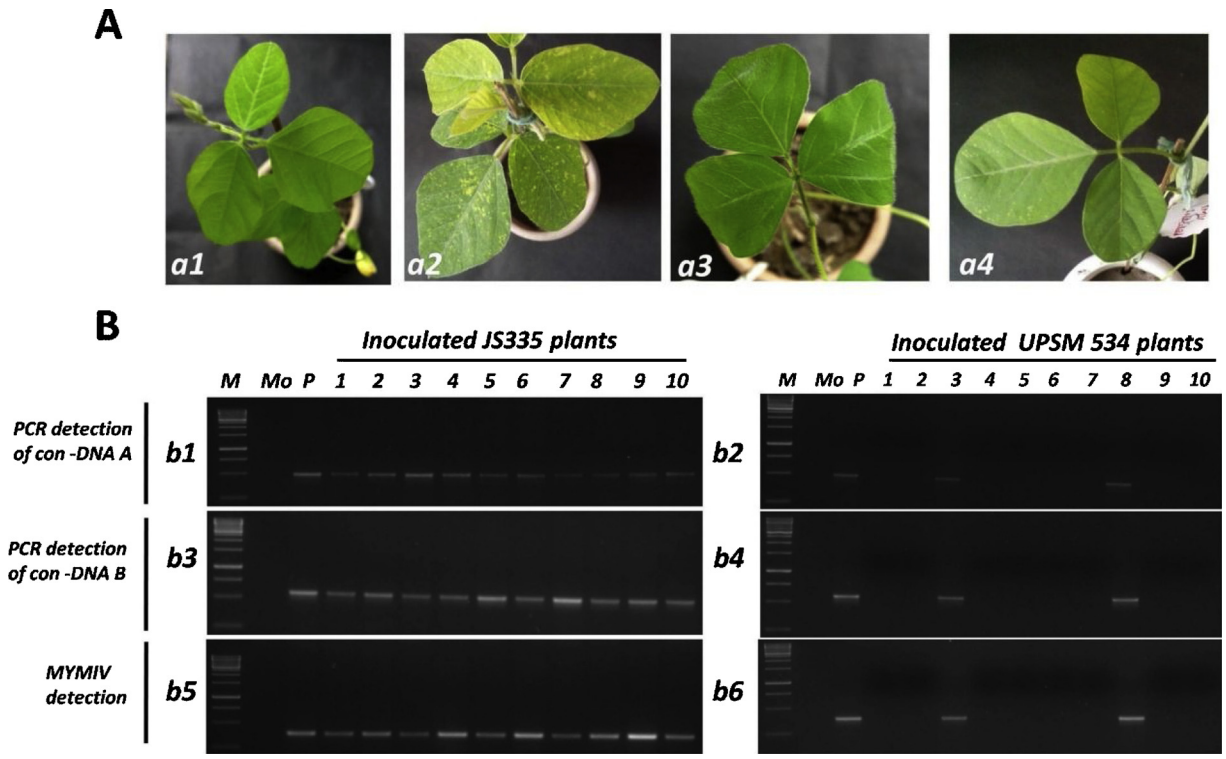

Fig. 1. Agro-infection, virus detection and infectivity analysis of soybean genotypes using binary plasmids (NRCS-A2X and NRCS-B2X) harbouring MYMIV genome. A) Typical yellow mosaic symptoms associated with MYMIV infection was observed in (a2) JS335 plants 9 days post inoculation (dpi) whereas (a4) UPSM534 showed little visible symptoms. Mock-inoculated JS 335 (a1) and UPSM 534 plants (a3) showed no visible changes.
B) PCR assay to detect conserved DNA A and DNA B genome sequences of legume begomoviruses (b1 through b4; expected amplicons size is ~500bp) and MYMIV infection (b5 through b6) in mock (Mo) and agroinoculated soybean plants (1–10) (expected size fragment is 391bp). M- 1 kb DNA molecular size marker; P-Positive control.

However, the genotypes PS1042 and SL295, with a disease score of 2 in field screening, had a score of 3 for the *Agrobacterium*-mediated inoculation with MYMIV. Genotype SL958 was notable in apparently being immune to YMD under field screening but showed symptoms with a disease score of 3 for agroinoculation.

3.4. MYMIV titre in soybean genotypes

Following the YMD scoring of soybean lines based on the field and agroinoculation screening, absolute quantification of MYMIV was performed using total DNA extracted from the agro-infected plants. The equation for the standard curve was produced by plotting Ct values against log viral DNA A quantity [$Y = -3.13X + 36.86$; $R^2 = 0.998$ with a calculated efficiency of 108%] (Supplementary Fig. 2a). Similarly, a standard curve was developed for quantification of MYMIV DNA B [$Y = -3.267X + 34.83$; $R^2 = 0.999$ with the efficiency of 98.15%] (Supplementary Fig. 2b). Absolute quantification of MYMIV in agro-infected plants allowed the categorization of the soybean genotypes into three different classes in accordance with field and agro-infection based screening. Highly resistant or resistant soybean genotypes such as UPSM534, Bragg, PS1042, PK471, SL295, PS1029, SL525, DS228, DS1024 and PK416 showed very low levels of both DNA A and DNA B ($< 10 \times 10^5$ genomic units/200 ng DNA) in the agro-infected plants. YMD resistant genotypes, NRC125, JS97-52, JS20-69, NRC124, JS20-29, SL958, SL 979, and NRC117 exhibited medium levels of MYMIV DNA A and DNA B copy numbers (10–100 $\times 10^5$ genomic units /200 ng DNA). Genotypes that were characterized as susceptible to YMD (NRC117, JS9560, JS335, JS9305, VLS59, MAUS 2, Co3, NRC86) showed relatively high virus titres ($> 100 \times 10^5$ genomic units/200 ng DNA) of DNA A and DNA B (Table 3). Viral titres of the various categories of the soybean genotypes showed statistically significant differences for both the DNA A (*f*-ratio value = 12.32405 and *p*-value 0.000257) and DNA B (The *f*-ratio value = 18.24956 and *p*-value 0.000021)

4. Discussion

Infectious clones of MYMIV have been developed using complete-or-partial dimeric viral DNA in a binary vector (Mandal et al., 1997; Usharani et al., 2005; Yadav et al., 2009). The study here reports rapid construction of MYMIV infectious clones using RCA and restriction enzymes which obviates the multiple sub-cloning procedures (Yadav et al., 2009; Jin et al., 2012). The rapid appearance of yellow mosaic symptoms in 9 dpi using these clones augurs well for up-scaling the system to efficiently screen a large number of soybean genotypes. In the absence of infectious clones, breeders have to screen soybean genotypes for YMD resistance under field conditions where MYMIV occurs (Ramteke and Gupta, 2005; Ramteke et al., 2007). The use of MYMV infectious clones to screen legume genotypes for virus resistance has been shown previously (Biswas and Varma, 2001; Sudha et al., 2015). YMD continues to be a serious hurdle in realizing the production potential of soybean in India due to sudden outbreaks in central India (Anon., 2015). It is imperative to have YMD resistant soybean genotypes suitable for central India. Hence, the second objective of this work involved screening soybean genotypes that are adapted to the central India against YMD.

Field evaluation of soybean genotypes at PAU, Ludhiana revealed that a few genotypes of soybean were resistant. However, none of the varieties bred for central India were resistant. A total of seventeen genotypes were categorized as highly resistant and resistant to YMD (Table 1). These genotypes could be used as parent lines in breeding programmes to develop the progenies resistant to YMV in central India.

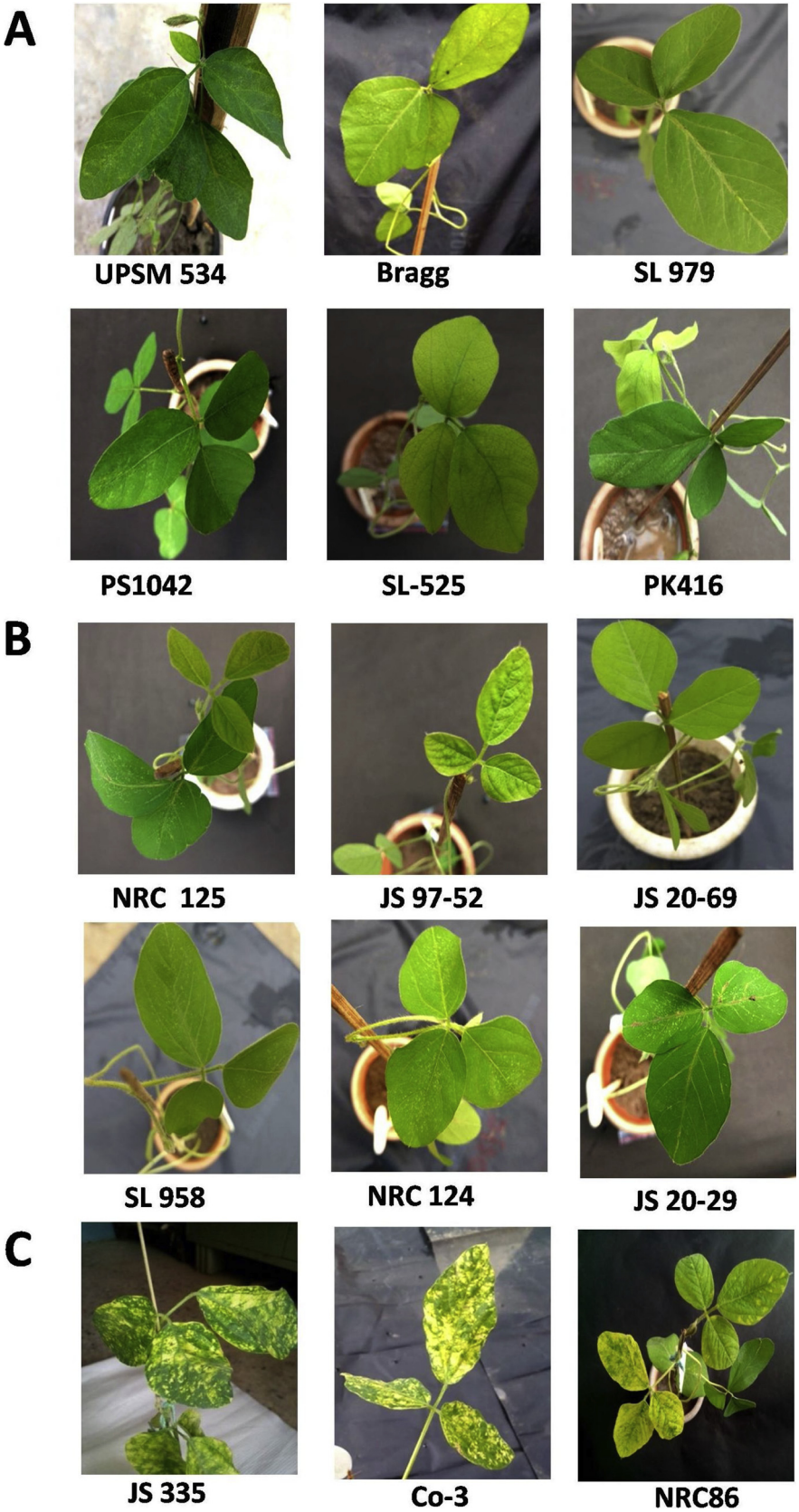

Fig. 2. Agro-infection of select soybean genotypes, for absolute quantification of MYMIV. Various categories of soybean genotypes were agroinoculated with MYMIV. No visible symptoms (A) or slight symptoms (B) were observed in resistant and moderately resistant category genotypes respectively 9 days post inoculation whereas typical yellow mosaic symptoms (C) were observed in susceptible genotypes.

Table 2
Evaluation of soybean genotypes for YMD by agro-inoculation.

Soybean Genotypes	Pedigree	Disease score[#]	
		Field screening	Agro-infection screening
UPSM534	PI 171,443 (YMD resistant germplasm)	3	3
Bragg	Jackson × D 49-2491	2	2
SL979	SL525 × DS 98-14	1	1
PS1042	Bragg × PK 416	2	3
PK471	Hardee × Punjab-1	2	2
SL295	PK416 × PS 564	2	3
PS1029	PK262 × PK 317	2	2
SL525	PK416 × PK 1023	3	3
DS228	–	2	2
PS1024	PK 308 × PK 317	3	3
PK416	UPSM 534 × S38	3	3
NRC 125	EC546882 × PS1044	–	3
JS 97-52	PK 327 × L129	–	3
JS20-69	JS 97-52 × SL 710	–	4
NRC 124	JS97-52 × (EC389148 × PS1042)	–	3
JS20-29	JS 97-50 × JS 95-96	–	4
SL958	SL525 × SL706	0	3
NRC117	JS 97-52 × (EC389148 × PS1042)	–	4
JS9560	Selection from PS 73-22	–	9
JS 335	JS 78-77 × JS71-5	9	9
JS9305	Secondary selection from PS 73-22	–	9
VLS59	(Pb1 × VLS2) × EC361336	9	9
MAUS 2	Selection from SH 84-14	9	9
Co3	UGM 69 × JS335	9	9
NRC86	RKS 15 × EC 481,309	–	9

Representative soybean genotypes were selected based on the field screening for YMD. Promising and contrasting breeding lines and germplasm accessions were also evaluated through agro-inoculation procedure. Agro-infection was carried out using clones NRCSA2X-7 and NRCS B2X-1. #Disease scoring was based on the field screening and agro-inoculation studies against YMD and MYMIV respectively.

Earlier, screening of soybean genotypes under field conditions identified three exotic resistant lines along with some moderately resistant genotypes whereas most of the lines were susceptible to the disease (Koranne and Tyagi, 1985). Ramteke et al. (2007) identified four cultivars and the genotype UPSM534 that are resistant to YMD based on field screening. Kumar et al. (2014) identified 21 genotypes which showed resistance to YMD. Of these UPSM 534 and SL525 were found to be resistant based on agro-inoculation.

In order to validate the findings of field evaluation, MYMIV infectious clones were used for screening the soybean genotypes developed at ICAR-IISR and elsewhere. The outcome of agro-infection experiments was in accordance with disease scoring observed in the field screening against YMD validating the utility of this technique. Genotypes PS1042 and SL295 showed a disease severity score of 3 in agro-inoculation experiment compared to the score of 2 in field evaluation. In contrast, cultivar SL958 was rated as immune by field screening yet characterized as highly resistant by agro-inoculation. This inconsistency can be explained by possible SL958 having resistance to the whitefly infestation. Although overall correlation in the outcome of field and agro-inoculation based screening shows the usefulness of agro-infection technique, the likely resistance of SL958 to insects suggests that combined screening in the field and agro-inoculation is required to identify vector resistant genetic sources.

The utility of real-time PCR to quantify plant pathogens including pathogenic viruses (Mumford et al., 2000; Korimbocus et al., 2002) and viroids (Boonham et al., 2004) has been demonstrated because of its improved sensitivity compared to the nucleic acid hybridization and serological methods (Mason et al., 2008). Complementing the field and

Table 3
qRT-PCR based MYMIV titre quantitation post agroinoculation in select soybean genotypes.

Soybean genotypes	MYMIV absolute copy number	
	DNA A ($\times 10^5$ genomic units/200 ng DNA)	DNA B ($\times 10^5$ genomic units/200 ng DNA)
Resistant		
UPSM534	5.03 ± 0.44	2.78 ± 0.23
Bragg	0.31 ± 0.01	0.32 ± 0.01
SL979	2.05 ± 1.24	9.19 ± 1.01
PS1042	0.34 ± 0.01	0.82 ± 0.07
PK471	1.48 ± 0.12	3.50 ± 0.17
SL295	0.52 ± 0.02	1.30 ± 0.08
PS1029	0.57 ± 0.05	0.81 ± 0.01
SL525	0.18 ± 0.01	0.55 ± 0.03
DS228	0.91 ± 0.05	1.77 ± 0.08
PS1024	0.45 ± 0.07	1.28 ± 0.02
PK416	0.29 ± 0.01	0.31 ± 0.06
Moderately resistant		
NRC125	26.47 ± 1.78	15.39 ± 1.01
JS97-52	15.57 ± 0.98	16.23 ± 0.09
JS20-69	41.60 ± 1.99	35.90 ± 0.06
NRC124	28.77 ± 0.07	18.55 ± 1.13
JS20-29	36.16 ± 0.76	27.75 ± 1.08
SL958	51.85 ± 0.03	42.77 ± 0.93
Susceptible and highly susceptible		
NRC117	178.39 ± 0.23	102.11 ± 2.97
JS9560	167.61 ± 0.56	110.53 ± 1.57
JS335	325.53 ± 2.12	508.40 ± 2.39
JS9305	183.31 ± 0.08	147.00 ± 1.87
VLS59	148.21 ± 1.08	131.67 ± 0.04
MAUS 2	816.80 ± 2.23	312.47 ± 1.67
Co3	270.70 ± 0.65	398.75 ± 1.65
NRC86	161.26 ± 0.78	161.75 ± 0.96

Equal amount of extracted DNA (μg) from the three plants was mixed and 200 ng DNA from this mixture was used for quantification studies. A one-way analysis of variance (ANOVA) to compare the mean viral titres of the various categories of the soybean genotypes revealed statistically significant differences in the viral copy numbers of DNA A (f-ratio value = 12.32405 and p-value 0.000257) and DNA B (The f-ratio value = 18.24956 and p-value 0.000021).

Agrobacterium screening, qRT-PCR based viral titre estimation has helped in categorizing the soybean genotypes into three discrete classes based on absolute MYMIV copy numbers. A good correlation was observed between the disease severity in field conditions and virus titre measured through qRT-PCR. The combined effectiveness of agro-infection and sensitivity of qRT-PCR was used to evaluate the promising breeding lines which revealed that JS97-52 and its derivatives (such as NRC124, NRC125) showed moderate resistance. However, NRC117 and NRC 86 showed susceptible reaction to YMD. Consequently, the results suggest that the promising genotype JS97-52 and its derivatives would show field level moderate resistance to YMD.

Shafiq et al. (2017) showed the significance of qRT-PCR assay in quantifying the virus levels and compared it to the disease severity in cotton plants affected by cotton leaf curl disease (CLCuD). For CLCuD affecting cotton the relationship between virus levels and disease severity was not absolute (Shafiq et al., 2017). However, for YMD affecting soybean qRT-PCR assay has demonstrated that the severity of symptoms under field conditions generally corresponds to the virus titre levels. This strategy of MYMIV agro-infection and qRT-PCR quantification could aid the process of fast-forward breeding wherein field evaluation of promising lines could be restricted to only ascertaining the economic yield levels. However, field screening of YMD is essential for identifying of vector- resistant genetic sources. Besides screening of soybean germplasm for resistance against YMD, infectious clones in combination with qRT-PCR can be used in investigations such as tagging and fine mapping of YMD resistant loci in soybean. MYMIV

infectivity in soybean under controlled conditions is required for deciphering molecular events defining the host-virus interactions and also in functional genomics studies.

Author contributions

SVR-conceived and designed the research; SVR and BSC-conducted the experiments, including the development of infectious clones; MS and SG-selection of soybean genotypes for agroinoculation; BSC, MS and SG-agro-inoculation of select soybean genotypes; RR &BSG-field screening of soybean genotypes; SP-designed qRT-PCR experiment; BSC, SG & AS- performed qRT-PCR ; SVR, VSB, MS & SP-provided reagents and analysis tools; SVR, MS, SP, RR and SC-wrote the manuscript; All authors read and approved the final version of manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.03.002.

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