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Screening and identification of resistant sources against *Cowpea mild mottle virus* (CPMMV) disease in soybean

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Abstract

A distinct strain of *Cowpea mild mottle virus* (CPMMV) infecting soybean was reported from India in 2013. Until now there are no resistant sources against CPMMV disease. In the present study 133 genotypes were screened and three sources of resistance against CPMMV in soybean were identified. The resistance was confirmed by sap inoculation and the presence of the virus was also confirmed through RT-PCR by designing primer specific to coat protein gene and NaBp region of CPMMV genome. The lines which were found to be resistant were further used to develop mapping population.

Key words: *Glycine max,* disease incidence, RT-PCR, CPMMV

Introduction

In India, soybean is the leading oilseed crop. The area of soybean is increasing year by year but the productivity still remains low. The three major constraints in soybean production in India are drought, weed infestation and location specific biotic stress (Lal and Sapra 2013). Among biotic stresses, three major viruses have been reported from India namely, *Mungbean yellow mosaic virus* (MYMV), *Soybean mosaic virus* (SMV) and *Groundnut bud necrosis virus* (GBNV) (Lal et al. 2005). A strain of *Tobacco mosaic virus* (TMV) infecting soybean in India was also reported in 2013 (Kumar et al. 2013). However, recently a distinct strain of *Cowpea mild mottle virus* (CPMMV) infecting soybean plants from India was reported by Yadav et al. (2013). They screened 27 cultivars of soybean for resistance against CPMMV. All the cultivars were found to be susceptible to CPMMV. Therefore the purpose of present study was to identify sources of resistance against CPMMV amongst soybean genotypes available in India.

Materials and methods

Plant material

One hundred and thirty three diverse genotypes of soybean selected based on their agro-morphological characters like plant height, pods/plant, seeds/pod, days to 50% flowering, days to 50% maturity, seed weight and single plant yield as given by International board of plant genetic resource (IPGBR 1984). During *kharif* 2013 and *kharif* 2014 these genotypes were raised in the experimental fields of Indian Agricultural Research Institute (IARI,28^{o}382'N, 77^{o}802'E) in Augmented design in plots of 2 rows each of 2m length, to know range of variation between the genotypes coefficient of variation, range and critical difference were calculated using Agristat software. Principal component analysis and grouping of genotypes into clusters were done using R software and screening for CPMMV disease in the field was done based on morphological symptoms, which include systemic mottling, leaf mosaic and distortion (Fig. 1). Occurrence of symptoms was observed from V1 stage (vegetative stage1) and for every 15 days interval disease symptoms were recorded. Disease incidence was calculated by taking number of plants with symptoms out of total number of plants in 2 rows of particular genotype (Arogundade et al. 2007; Bediako et al. 2014). The genotypes were classified into different class as per the criteria given in Table 1 (Anonymous. 2012). None of the genotypes were found to be absolutely resistant or highly resistant. Hence genotypes with moderate resistance were subjected to mechanical inoculation in an insect proof polyhouse

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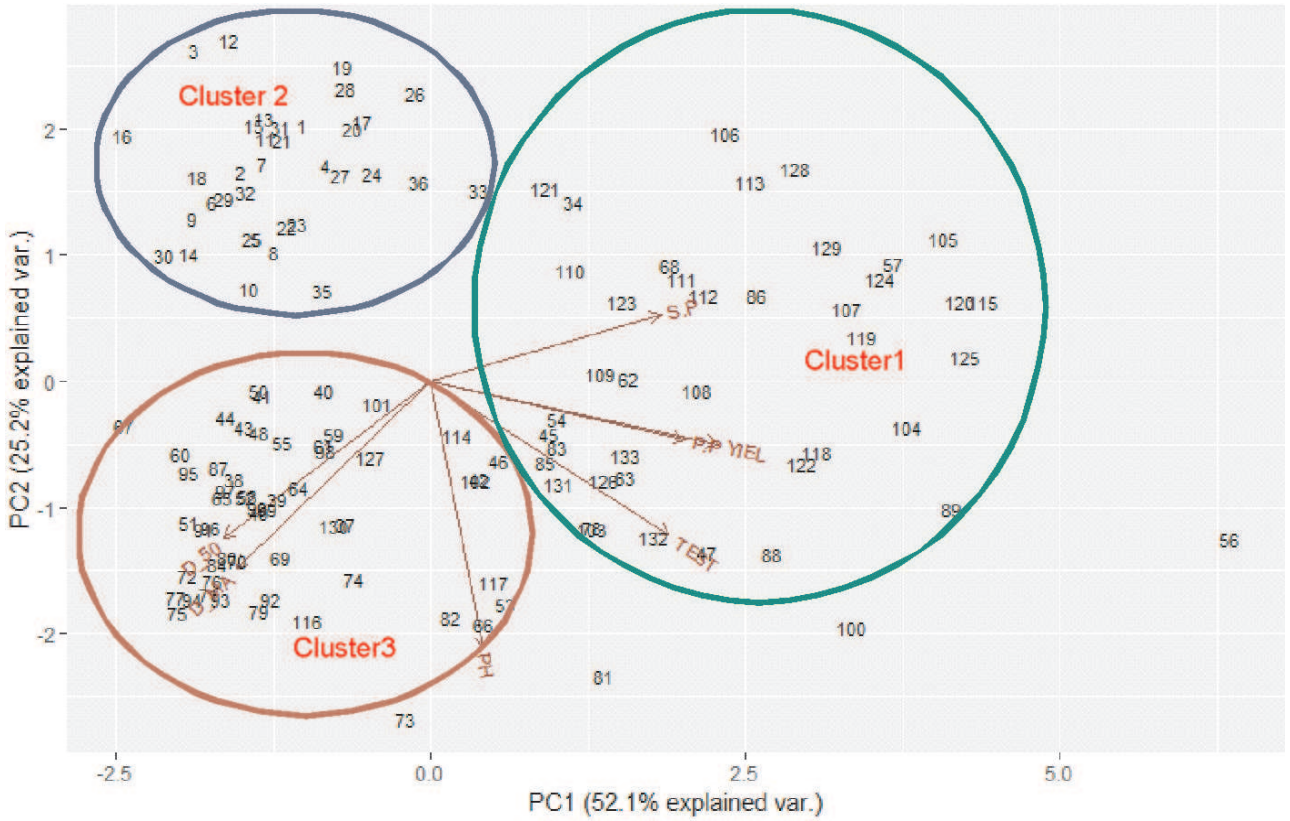

Fig. 1. PCA for morphological traits (SS = Seeds/pod, PP = Pods/plant, Test Wt = 100 seed weight, PH = Plant height, DMAT = Days to maturity, D50 = Days to 50% flowering)

Table 1. Classification of genotypes based on percentage of infected plants

Score	Resistant category
0%	Absolutely resistant (AR)
0.01-11.11%	Highly resistant (HR)
12.22-33.33%	Moderately resistant (MR)
34.44-55.55%	Moderately susceptible (MS)
56.6-77.77%	Susceptible (S)
78.88-100%	Highly susceptible (HS)

and presence of CPMMV was checked through electron microscopy and RT-PCR by designing primer specific to CPMMV isolate available in IARI fields.

Sap inoculations

The resistant genotypes along with the susceptible check were planted in soil mixture (1:1:1 soil, sand and peat) in 6 inches earthen pots. Inoculums consisted of extracts from infected leaves of susceptible soybean plants maintained in the polyhouse on different host plants that was prepared by homogenizing infected leaves in chilled 0.025M KPO_4 buffer, pH 7.2, plus 0.01M sodium sulphite with sterilized pestles and mortars and prepared inoculum was used to apply on carborandum-dusted leaf surfaces. Plants were inoculated with CPMMV, 7-10 days after planting at first trifoliate leaf stage. Two to three weeks after inoculation, trifoliate leaves were examined for systemic virus symptoms. Plants showing systemic mottling, leaf mosaic and distortion were recorded as susceptible and with mild to no symptoms on to as resistant.

Primer designing and testing

A primer was designed based on the distinct strain of CPMMV sequence available in NCBI database (Accession no. JX524198.1) submitted by Yadav et al. (2013) from India. Using software Primer 3 forward and reverse primer were picked from the sequence (Table 2) which amplifies partial coat protein gene and NaBp from 3' terminal region giving a product of 1065bp.

Table 2. CPMMV specific primer sequence

Primer	Sequence	Start	Length	Tm	GC%	3'	Hairpin
Left primer	GAAAGAAAAGCCAGGGGTGC	51	20	65.00	55	0	0
Right primer	AATACCCGGGACACACTTCA	1115	20	65.00	50	0	0

RT-PCR

Infected leaf samples were collected immediately after symptom appearance and RNA was extracted using *RNASure* Plant kit (Genetix Biotech Asia Pvt. Ltd.) and cDNA was prepared using *RevertAid* First Strand cDNA synthesis kit (Fermentas Company). A 20ul PCR reaction was set with CPMMV specific primers having annealing temperature of 61^{o}c for 35 cycles. The PCR products were run on 1.2% agarose gel and the PCR product was sequenced (SciGenome Company) and checked through nucleiotide blast for further confirmation of virus.

Results and discussion

Descriptive statistics (mean, range, maximum, minimum, coefficient of variation and critical difference) of the morphological traits were calculated. High amount of variation was observed between genotypes (Table 3).

Principal component analysis

First two PC's explained 77% of total variation and 23% variation are explained by the remaining 5 PCs. The contribution of both PC1and PC2 to the variation is 52% and 25% with Eigen values 3.6491 and 1.7630 respectively (Table 4). As the variation explained by Eigen values is greater than one the Eigen vectors needed to be study for each PC (Peric et al. 2016). In PC1 yield, pods per plant, 100 seed weight, seeds per pod were contributed to variation and in PC2 maximum variation is contributed by plant height, days to 50% flowering and maturity in negative direction (Table 5).Thus PC1 is mainly related to yield related characters and PC2 is for vegetative and reproductive characters. Similar kind of variation was observed in soybean by Iqbal et al. (2008) and in okra by Gangopadhyay et al. (2016) when they carried out multivariate analysis for different morphological traits where yield and vegetative traits contributed high

Table 3. Descriptive statistics for morphological characters of 133 genotypes

S. No.	Character	Max.	Min.	Range	Mean	CV%	SE (d)	CD (0.05)
1	Plant height (cm)	94	28.00	66.00	64.96	24.68	13.9679	27.50
2	Pods/plant	96	6.60	92.70	36.02	10.86	5.6236	11.07
3	Seeds/pod	03	1.00	2.00	2.71	28.84	8.4828	16.70
4	Days to 50% flowering	64.5	38.00	26.50	55.28	14.08	0.3131	0.61
5	Days to maturity	127	100.00	27.00	114.76	3.53	1.5922	3.13
6	100 seed weight (g)	14.08	3.20	11.88	6.81	2.45	2.2925	4.51
7	Yield/plant	45.331	0.054	45.27	7.4302	52.64	3.1935	6.28

Max. = Maximum; Min. = minimum

Table 4. Importance of components in Principle component analysis

Importance of components	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Eigen values	3.6491	1.7630	0.4909	0.4507	0.3315	0.2643	0.0502
Standard deviation	1.9103	1.3270	0.7006	0.6713	0.5757	0.5141	0.2242
Proportion of variance	0.5213	0.2519	0.0701	0.0643	0.0473	0.0377	0.0071
Cumulative variance	0.5213	0.7732	0.8433	0.9076	0.9550	0.9928	0.9938

variation of PC1 and PC2 respectively. These genotypes were clustered into three groups; cluster 1 consists of 26 genotypes having high no. of pods/ plant, seeds/pod and test weight. Fourty eight genotypes in cluster 2 having less number of pods/ plant, no. of seeds/pod, test weight, pods/plant with similar days to maturity and flowering as cluster1 and in third cluster, 49 genotypes were grouped having taller plant height and maximum days to flowering and maturity (Table 5). Genotypes in the cluster 1 have characters related to yield with early maturity and flowering.

Field screening and disease incidence

Data was recorded on number of infected plants and disease incidence was calculated as % incidence for each genotype (Arogundade et al. 2007). In our experiments disease incidence ranged between 19.08% to 100%. Likewise Yadav et al. (2013) reported a very high incidence of CPMMV during 2011 and 2012, ranging from 25.9 to 71%. Based on infection index the genotypes can be classified into four distinct clusters, highly susceptible (90%-100%), susceptible (60.76%-77.69%), moderately susceptible (37.63%-48.60%) and moderately resistant (19.08-20.54%) (Fig 2). Three genotypes DS12-5 (19.08%), SL 958 (20.18%) and SL 900 (20.54%) were identified as moderately resistant based on field screening.

Table 5. Loadings of principle component for PC1 and PC2

Loadings	PC1	PC2
Plant height	0.092	-0.654
No. of pods/plant	0.436	-0.144
No. of seeds/pod	0.395	0.165
Days to 50% flowering	-0.353	-0.384
Days to 50% maturity	-0.332	-0.457
100 seed weight	0.408	-0.376
Yield	0.492	-0.154

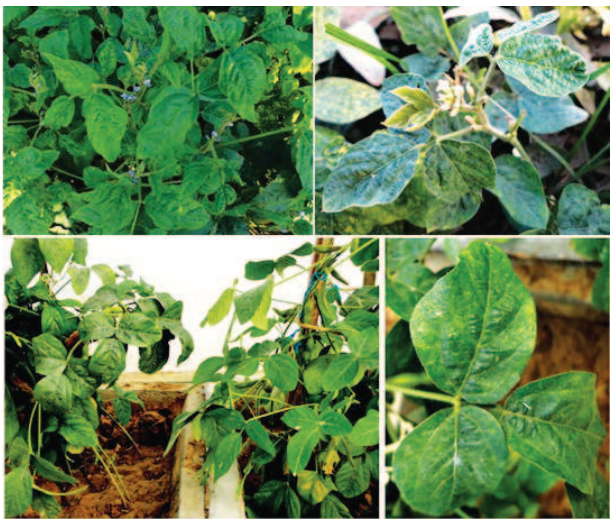

Fig. 2. Plants showing mottling, leaf mosaic and distortion in field and in polyhouse

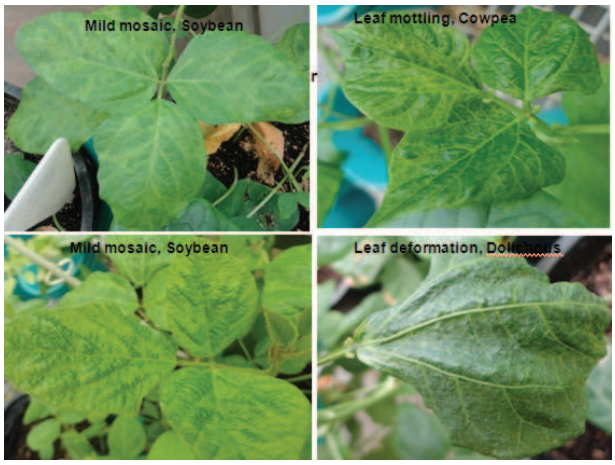

Fig. 3. Virus culture on different host plants

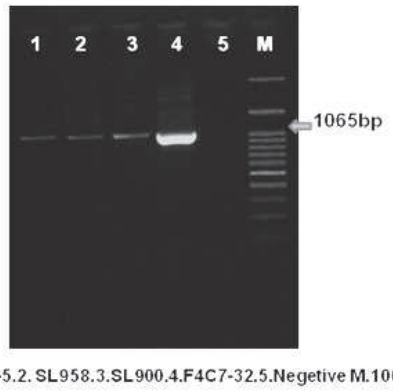

Fig. 4. RT-PCR with CPMMV specific primers

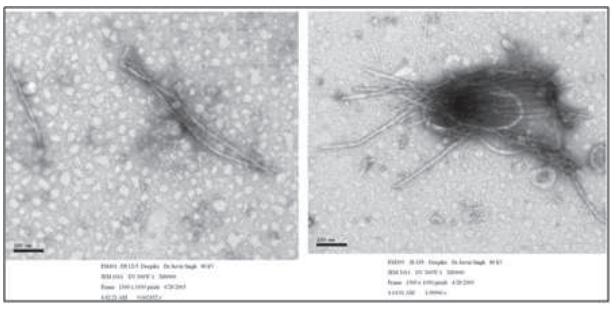

Fig. 5. Electron microscopy of soybean leaf samples infected with CPMMV

Artificial screening

These three lines and a susceptible line JS335 as control were further tested for resistance through mechanical inoculation by using CPMMV culture maintained on soybean, cowpea and dolichus in insect

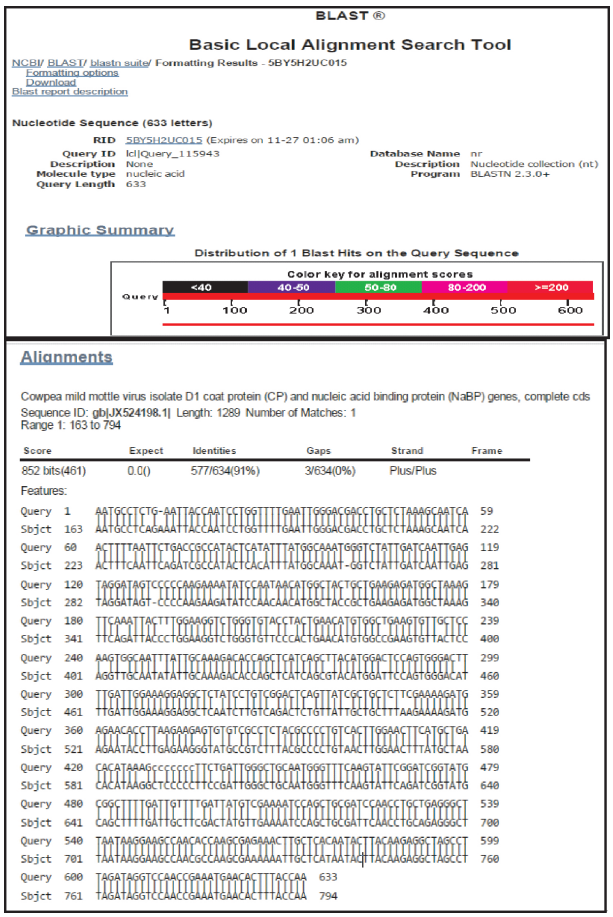

Fig. 6. BLAST results of CPMMV showing 91% identity with sequence with JX 5241980.1

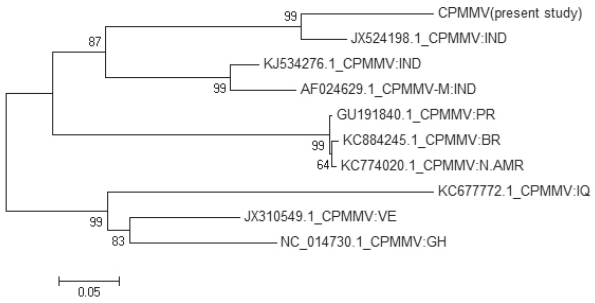

Fig. 7. Phylogenetic tree of CPMMV (Present study) when compared with sequences from IND (India), PR (Puerto Rica), BR (Brazil), N.AMR (North America), IQ (Iraq), VE (Venezuela) and GH (Ghana)

poof net house (Fig. 3). After 2-3 weeks of inoculation mild mosaic symptoms were developed on susceptible line JS 335 but the three moderately resistant lines remained free from the severe disease symptoms at the later stages of plant development. In susceptible line JS 335 severe leaf distortion was observed at plant maturity stage (Fig. 2).

Confirmation of CPMMV virus

The presence of virus in these four lines was confirmed by RT-PCR and electron microscopy. In the RT-PCR 1065bp band which include partial coat protein gene and NaBp region of CPMMV genome was observed in both susceptible and moderately resistant lines but with varying band intensities (Fig. 4). The presence of faint band in the moderately resistant line may be due to presence of low concentration of virus in the plants due to mechanical inoculation and these samples were further given for electron microscopy. Electron micrograph show the expected particle size 620-650 x 12-15nm (Fig. 5) in the samples. The 1065bp band amplified in RT-PCR was sequenced and the sequence information was used for nucleotides BLAST in NCBI database to check similarity with CPMMV strains. The results show that the CPMMV strain used in the present study has 91% identity with CPMMV strain Accession no. JX524198.1 submitted by Yadav et al. (2013) (Fig. 6). Phylogenetic analysis revealed that the sequence identified in present study was closely related to the same sequence which was earlier reported by Yadav et al. (2013) from India; whereas as it with the other isolates from India, Puerto Rico, Brazil, North America, Iraq, Venezuela and Ghana; it shared 77% to 80% identity (Fig. 7). The genotypes DS12-5 and SL 958 were used as parents in developing mapping populations. The inheritance of the resistance against CPMMV in soybean was worked out and the gene has been tagged (Cheruku et al. 2017).

Authors' contribution

Conceptualization of research (SKL, BM, DC); Designing of the experiments (SKL, DC, AT); Contribution of experimental materials (SKL); Execution of field/lab experiments and data collection (DC, SKL, PY, KPS, SK); Analysis of data and interpretation (SKL, DC); Preparation of manuscript (SKL, DC, BM, AT).

Declaration

The authors declare no conflict of interest.

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References

Anonymous. 2012. Proceedings and Technical Programme (2011-12) of the 41st Annual group meeting of All India coordinated Research Project on Soybean, held at University of Agricultural Sciences, Bangalore, India. pp. 71.

Arogundade O., Balogun O. S. and Fawole O. B. 2007. Incidence and severity of common viral and fungal diseases of dry season tomato crop in a Southern Guinea Savannah Agro-Ecology. Agrosearch., **9**(1&2): 55-62.

Asare Bediako E., G. C. Van der Puije, K. J. Taah, E. A. Abole and A Baidoo. 2014. Prevalence of Okra Mosaic and Leaf Curl Diseases and Podagrica spp. Damage of Okra (*Albelmoschus esculentus*) Plants. Int. J. Curr. Res. Aca. Rev., **2**(6): 260-271.

Deepika Ch, Lal S. K., Talukdar A. and Mandal B. 2017. Inheritance and mapping of resistance against Cowpea mild mottle virus (CPMMV) strain D1 in Soybean. Plant Breed., **136**(2): 155-160

Gangopadhyay K. K., Singh A., Bag M. K., Ranjan P., Prasad T. V., Roy A. and Dutta M. (2017). Diversity analysis and evaluation of wild *Abelmoschus* species for agro-morphological traits and major biotic stresses under the north western agro-climatic condition of India. Genet. Resour. Crop Evol., **64**(4): 775-790.

Lal S. K. and Sapra R. L. 2013. Advances in Soybean Breeding. In V. L. Chopra (Ed.) Breeding Field Crops 2: Advances, **2**: 165-194.

Lal S. K., Rana V. K. S., Sapra R. L. and Singh K. P. 2005.Screening and utilization of soybean germplasm for breeding resistance against Mungbean yellow mosaic virus. Soybean Genet. News Letter, **l32**.

Mahmood T and Waheed A. 2008. Evaluation of Soybean [*Glycine max* (L.) Merrill] Germplasm for Some Important Morphological Traits Using Multivariate Analysis, **40**(6): 2323-2328.

Yadav M. K., Biswas K. K., Lal S. K., Barnwal V. K. and Jain R. K. 2013. A distinct strain of Cowpea mild mottle virus infecting soybean in India. J. Phytopathol., **161**: 739-744.

Peric V., Nikolic A., Babic V., Sudaric A., Srebric M., Dordevic V. and Mladenovic Drinic S. 2014. Genetic relatedness of soybean genotypes based on agro morphological traits and rapid markers. Genetika, **46**: 839- 854.