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Germplasm Screening for Identification of BCMV Resistance Sources from Diverse Cowpea [*Vigna unguiculata* (L.) Walp.] Germplasm using Serological and Molecular Diagnostics

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ABSTRACT

Background: Bean common mosaic disease (BCMD) caused by Bean common mosaic virus (BCMV) has been regarded as one of the most important seed-borne and aphid vector transmitted destructive disease of cowpea not only affecting its yield but also reducing its grain quality globally. Since the dawn of crop improvement, germplasm served as the source of resistance for various biotic stresses. Currently, host plant resistance is the best, reliable, economic and environmental friendly practice of virus disease management. The objective of this study was to assess the response of diverse cowpea germplasm against BCMV under natural and controlled conditions followed by serological and molecular detection.

Methods: In this study, 85 cowpea germplasm accessions and one known BCMV susceptible (C152) and one resistant (CP55) varieties are evaluated for their response to the BCMD in augmented block design (ABD). The reaction of cowpea accessions were assessed using percent disease incidence (PDI). Transmission Electron microscopy (TEM), Direct Antigen Coating-Enzyme-linked Immunosorbent Assay (DAC-ELISA) and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) were used for confirming the BCMV.

Result: Natural and controlled condition screening data analysis revealed a significant difference among diverse cowpea germplasm for BCMV diseases incidence. Based on field screening at Ranchi and New Delhi (2019 and 2020) and artificial confirmation. 24 out of 85 accessions were found to be immune and confirmed through DAC-ELISA and RT-PCR. As a consequence these accessions could be a potential new source of disease resistance for BCMV in breeding programs.Further, DAC ELISA of seed coat and Embryo revealed the presence of virus in 5% and 15% of samples tested. Accession IC418505 recorded highest disease incidence (40.0%).

Key words: BCMV resistance, Cowpea, ELISA, Germplasm screening, RT-PCR, TEM.

INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp.] is a multipurpose crop popularly grown for its long green pods as a vegetable, dry seeds as pulse and for its foliage as fodder. It is an important food legume in the tropics, sub-tropics and semi-arid regions throughout Africa and Asia, due to its tolerance for sandy soil and low rainfall (Smart 1976; Alemu *et al*., 2016). The occurrence of BCMV on common bean from India was first reported by Muniyappa 1976. Now it is distributed worldwide and causes as much as 80% and quality of harvested product (Manjunatha, 2015). Management of BCMV incidence is only possible by the way of reducing the vector *viz*., aphid population using insecticides. The application of certain insecticides, which is mostly inefficient, uneconomical, causes environmental hazard and pose a health risk. So, use of virus resistant variety is the best alternative to alleviate occurrence of BCMV disease. Identification of BCMV resistant cowpea genotypes is very much essential. Hence, it is necessary for large scale screening to identify stable resistance source among diverse cowpea genotypes. It is also used as green manure crop owing to its nitrogen fixing capacity. Being a drought-tolerant, low water footprint and warm-weather crop, it is a promising food and forage crop in a typical tropical lowland climate (Alemu *et al*., 2016; Belay *et al*., 2017). It has the highest productivity potential among *kharif* season pulses (Singh and Sharma, 1996). However, the average yield is very low due to various biotic and abiotic constraints such as insect pests, diseases, low soil fertility and drought and heat stress (Singh and Sharma, 1996; Sandhu *et al.,* 2023). Among them virus diseases are foremost restraints for successful cultivation

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of pulses and particularly BCMV frequently occurs on important pulses in tropical and sub-tropical countries (Loebenstein and Thottappilly, 2013; Sastry and Zitter, 2013; Madhumitha *et al.,* 2022). BCMV is presumed to be first originated from east or south Asia. However, owing to its seed-borne nature currently distributed worldwide (Gibbs *etal*.2008; El-Kady *et al*., 2014). Became serious as its secondary spread may take place very fast because of the activities of various aphid species active during the crop season.

BCMV belongs to the genus *Potyvirus*, family *Potyviridae* (Barnett *et al*., 1992). The genome (10 kb) of BCMV is single stranded, positive sense RNA (Bos, 1971; Dougherty and Carrington, 1988; Urcuqui-Inchima, 2001; Wylie *et al*., 2017). Under TEM BCMV will be visible as a flexuous particle. It made up of about 2000 CP monomers encapsulating the RNA genome. It measures approximately 750 nm × 15 nm (Urcuqui-Inchima, 2001). It has wide host range of 94 plant species among them 83 are leguminous species which makes it as most destructive viruses that infect the legumes. The deleterious effects of infection not only reduce yield but also diminish seed quality.

BCMV produce three types of symptoms *viz*., mosaic, systemic necrosis (black rot) and hypersensitive reaction depending on the genetic nature of the cultivar. It is a seed-transmitted disease and plants exhibits mottling, curling, blistering, stunting and malformation of the primary leaves and mosaic of trifoliate leaves (Pandey and Parmar, 2023). Infected plants bear under-developed pods and delays seed maturity. Expression of symptoms is temperature dependent (Morales and Bos, 1988).

BCMV disease incidence in *Phaseolus vulgaris* caused reduction in the yield by 53-83% (Sastry, 2013) and even symptomless BCMV infection can cause more than 50% yield losses in susceptible bean varieties (Morales, 2006). As a result of seed-borne and vector transmission causes estimated yield losses ranging from 35-98% in common bean (Galvez and Schwartz, 1980; Nalini *et al.,* 2006; Damayanti *et al*., 2008; Singh and Schwartz, 2010; Saqib *et al*., 2010; Sastry, 2013; Li *et al*., 2014).

Bean common mosaic virus (BCMV) is believed to originated from south or east Asia now it has spread worldwide wherever legumes are grown (Gibbs *et al*., 2008; El-kady *et al*., 2014). BCMV is a monopartite flexuous rod shaped virus with positive sense ssRNA genome of about 10 kb. In nature, BCMV most commonly occurs on beans and it is known to possess high degree of pathogenic variability (Manjunatha, 2015). BCMV is serious threat to bean cultivation worldwide because it is easily transmitted through seeds, pollens and aphid insect vector (Puttaraju *et al*., 2004; Kapil *et al*., 2011). Infection in the field may reach 100% (Li *et al*., 2014) and yield losses of 35%-98% have been reported (Prasad *et al*., 2007).

As currently no chemical control measure are available for virus disease management except some insecticides to control vector but it is not effective. This requires concentrated breeding efforts to develop new resistant varieties, which offer high yield as well as ensured grain quality and sustains the climatic vagaries (Upadhyaya *etal*. 2015). Thus current study was undertaken to evaluate diverse cowpea germplasm against BCMV under natural and controlled conditions.

MATERIALS AND METHODS

The experiment was carried out during 2019-2021 in the Plant Virology Laboratory, Division of Plant Quarantine, ICAR-National Bureau of Plant Genetic Resources (NBPGR) and field and artificial screening at ICAR-NBPGR farm in ICAR-IARI, New Delhi. A total of 85 cowpea accessions and one susceptible check C-152 and one resistant check C-55 were obtained from National Genebank of India located at ICAR-NBPGR New Delhi. BCMV inoculum was maintained in susceptible variety C-152 as a host under controlled conditions and further used for mechanical inoculation for artificial screening.

Natural disease screening of 85 cowpea accessions selected five accessions each from major cowpea growing area, along with a susceptible (C-152) and resistant check (CP-55) against BCMV was carried out in *kharif* season at Ranchi (2019) and New Delhi (2019 and 2020) in augmented block design (ABD). Seeds were sown in paired rows in a row of 2.5 m length with a distance of 30 cm interrow distance. To increase the disease pressure, susceptible check was planted for every two accessions and in border rows. After every 5 accessions the resistant check was randomized once. All agronomic practices were followed during experiment. Observations were recorded for the expression of various symptoms produced by BCMV every day (Tripathi *et al*., 2018).

In the same way, 10 seedlings per accession were grown in pots along with susceptible and resistant check under controlled conditions in triplicates. The seedlings were mechanically inoculated with sap containing BCMV at second trifoliate leaf stage and were observed for symptoms development every day 2 and 3 weeks post inoculation and per cent disease incidence (PDI) was calculated using the below given formula and categorized them as immune (0= no plants showing symptoms), highly resistant (1= 1-10%), resistant (2= 11-25%), moderately resistant (3= 26-40%), susceptible (4= 41-60%) and highly susceptible (5= >60% plants showing symptoms) as per Reddy *et al*. (2001).

Number of plants showing symptoms and non-symptomatic plants were recorded and per cent disease incidence (PDI) was calculated by using below given formula:

Per cent disease incidence =

$$\frac{\text{Number of plants showing symptoms}}{\text{Total number of plants}} \times 100$$

Leaf samples of natural and artificially screened cowpea were subjected to electron microscopic detection

(JEOL JEM 1011, JEOL Ltd.) by following leaf dip method (Chalam, 2008). Direct Antigen Coating-Enzyme-linked Immunosorbent Assay (DAC ELISA) was done using Agdia® ELISA kit according to manufacturer instruction to detect BCMV using seed coat and embryo.

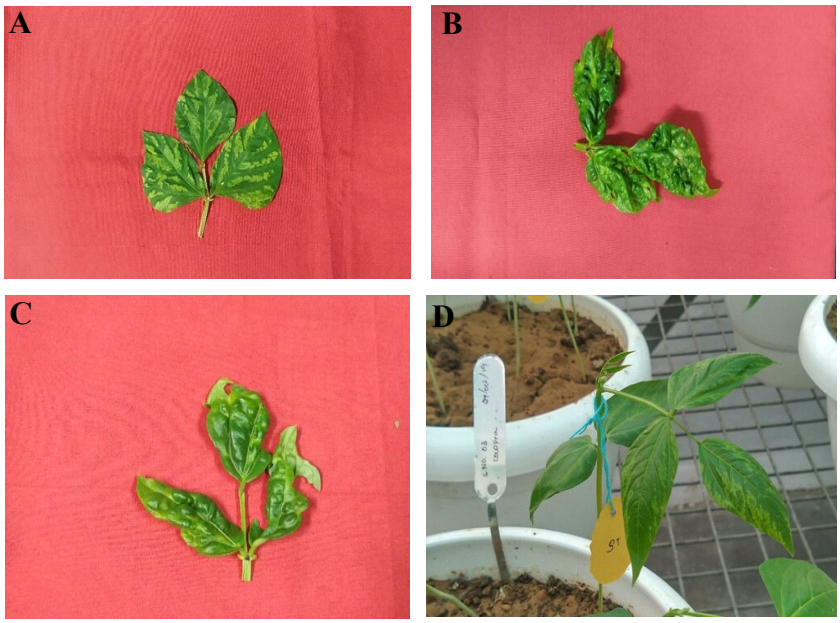
In total, 48 accessions each (32 showing symptoms and 16 not showing symptoms and 24 showing symptoms and 24 not showing symptoms) respectively from filed and controlled conditions were subjected to molecular detection using RT-PCR. The total RNA isolation from the ELISA positive BCMV infected leaf samples and healthy leaf samples (ELISA negative) was done using RNeasy® Plant Mini Kit (QIAGEN® kit catalogue No.74903). Subsequently, Thermo Scientific Verso cDNA Synthesis kit was used for the synthesis of first strand. A total of 20 µl reaction mixture was prepared by adding the ingredients such as 4 µl 5× cDNA synthesis buffer, 2 µl dNTP mix, 1 µl Random hexamer RNA primer, 1 µl RT enhancer, 1 µl Verso enzyme mix, 4 µl total RNA (template RNA) and nuclease-free water was used finally to make up the volume. The mixture was reverse transcribed at 42°C for 30 min and inactivated at 95°C for 2 min. The cDNA obtained was subjected to PCR amplification using forward 5′ TGG AAT CTG GGA AGG ACA AG 3′ and reverse primers 5′ TTT TGA AGC CGA GGA ACA AC 3′ of expected product size 157 bp. PCR amplifications were conducted in thermocycler in 20 µl reaction mixture that contained 5 µl template cDNA, 10 µl Go Taq Master Mix (2×) (Promega, Madison WI USA), 1 µl forward primer (5 µM) and 1 µl reverse primer, 3 µl nuclease free water to make up the volume. The PCR amplification was carried out in a thermal cycler with the following conditions; initial denaturation at 95°C for 4 min followed by 40 cycles of denaturation at 94°C for 30 sec., annealing at 51.3°C for 20 sec. and extension at 72°C for 45 sec., final extension at 72°C for 7 min. The amplified DNA fragments were electrophoresed in 1.2% agarose gel and analyzed.

RESULTS AND DISCUSSION

Screening under field and controlled conditions and disease scoring

Diverse cowpea germplasm accessions (85) screened under field conditions along with susceptible (C-152) and resistant check (C-55) showed various reactions to BCMV infection. The various symptoms produced by the plants were mosaic, leaf rolling, puckering and stunted growth (Fig 1). The highest disease incidence of 60% was observed in susceptible check (C-152) and no symptoms were observed in resistant check (CP-55). *Kharif* at Ranchi (2019) and New Delhi (2019 and 2020). Among the 85 accessions screened against BCMV under field conditions, 53 accessions were found to be immune, 21 accessions were highly resistant and 11 accessions were resistant (may be a table of score/category may be included).

Same set of accessions were artificially inoculated at second trifoliate leaf stage and observation on symptoms expression were recorded 10-14 days post-inoculation. The percent disease incidence ranged from 0 to 40% (Table 1). Accession IC 418505 and IC568946 recorded highest disease incidence of 40% each. Out of 85 accessions 26 accessions showed seed transmission (Fig 1D)



A. Mosaic. B. Blistering. C. Puckering. D. Seed transmission with mosaic symptoms (IC421891).

Fig 1: Cowpea accessions showing various BCMV symptoms.

and seed transmission up to 20% was observed in one accession *viz*., IC 421891. Among the 85 accessions screened for resistance against BCMV under artificial inoculation conditions along with one susceptible and one resistant check, 24 accessions were found to be immune, 31 accessions were highly resistant, 25 accessions were resistant and five were moderately resistant. Accession IC418505 recorded highest disease incidence of 40.0% followed by IC 539815 with disease incidence of 36.67%. The susceptible check (C-152) recorded 60% disease incidence and resistant check (CP-55) showed no disease symptoms (Table 1). A different level of variation among cowpea genotypes for BCMV resistance and in different legumes reported by different researchers (Jeyanandarajah and Burnt, 1993; Sharma *et al*., 2008; Hamid, 2016; Manjunatha *et al*., 2017; Deepika, 2019; Basavaraja *et al*., 2022).

Electron microscopic observation of infected samples revealed the presence of flexuous rod shaped particles of 725 nm confirmed the presence of BCMV in infected samples. However, such particles were not observed in the healthy leaf samples. Serological detection of BCMV using DAC-ELISA for accessions collected after artificial inoculation and results are in consistent with PDI of both field and artificial inoculation. DAC-ELISA is frequently used to detect BCMV by scholars (Njau *et al*., 2006; Chalam and Maurya, 2018; Feng *et al*., 2019).

RT-PCR detection of BCMV

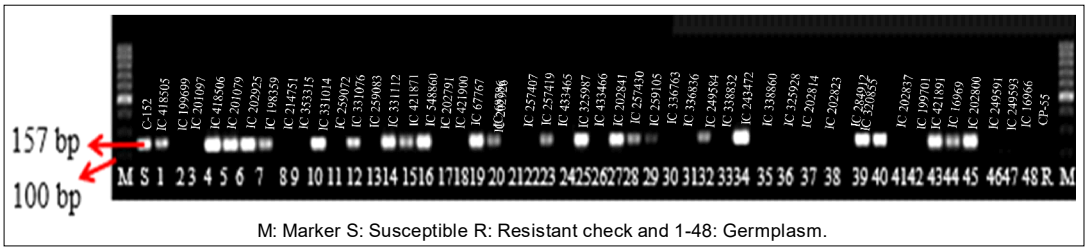
To confirm the absence of BCMV in immune accessions, 24 immune accessions and representative 24 accessions showing symptoms after artificial inoculation were tested by RT-PCR. The RNA was isolated from leaf samples of 48 accessions collected from artificially inoculated plants. cDNA was synthesized and is used as a template for amplification of product size 157 bp using BCMV 2 primer set. Amplified products were analyzed by 1.5% agarose gel electrophoresis. Bands (157 bp) were observed in 24 symptomatic samples and susceptible check C-152, indicates the presence of BCMV. In 24 immune accessions and resistant check (CP-55), no such bands were observed confirming the absence of BCMV (Fig 2) Many scholars have employed RT-PCR to detect BCMV to amplify coat protein or complete or partial coding regions (Bhadramurthy and Bhat, 2009; Wani *et al*., 2017; Manjunatha *et al*., 2017; Deepika *et al*., 2023; Feng *et al*., 2019; Nalini *et al*., 2006).

DAC-ELISA detection of BCMV in embryo and seed coat

A total of 85 embryo samples of cowpea along with susceptible check (C-152) and resistant check (CP-55)

Table 1: Grouping of cowpea accessions based on PDI score.

PDI score	Description	Reaction	No. of accessions	Accession no.
0	0% plants showing symptoms	Immune	24 + resistant check (CP-55)	IC 199699, IC 201097, IC 214751, IC353315, IC 259072, IC 259083, IC202791, IC421900, IC 202926, IC 257407, IC433465, IC433466, IC 336763, IC 336836, IC338832, IC 338860, IC 325928, IC 202814, IC 202823, IC 202837, IC 199701, IC 249591, IC 249593, IC 16966, CP-55 (resistant check)
1	1-10% plants showing symptoms	Highly resistant	31	IC214833, IC 249140, IC 202925,IC 361502, IC 398755, IC 408368, IC198359,IC 259084, IC 202804, IC331014, IC 331076,IC 344612, IC 421871, IC 548860, IC 437180, IC 68786, IC 97856, IC 198321, IC 198355, IC 433448, IC 336786, IC 325987, IC325996,IC 202886, IC 259104, IC 259105, IC 44619, IC 202709, IC 243472, IC 320849, IC 320855
2	11-25% plants showing symptoms	Resistant	25	IC 418506, IC 201079, IC 402172, IC 202800, IC 202803, IC 202807, IC 331112, IC 344662, IC 421881, IC 421891, IC 421893, IC 437178, IC 437179, IC 67767, IC 257419, IC 326042, IC 549816, IC 202841, IC 257430, IC 257452, IC249584, IC243489, IC 243501,IC 284912, IC 488080
3	26-40% plants showing symptoms	Moderately resistant	5	IC 418505, IC 522270, IC 539815, IC 259106, IC16969
4	41-60% plants showing symptoms	Susceptible	0 + susceptible check (C-152)	C-152 (susceptible check)
5	>60% plants showing symptoms	Highly susceptible	0	-



M: Marker S: Susceptible R: Resistant check and 1-48: Germplasm.

Fig 2: Gel image showing PCR amplification of BCMV in leaf samples after artificial inoculation.

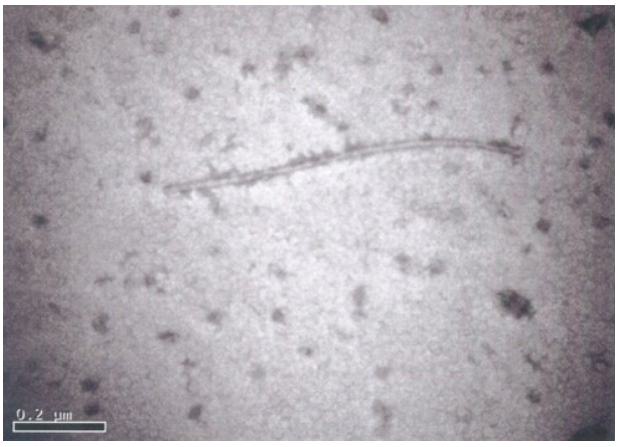


Fig 3: Electron microscopic view of flexuous particle of BCMV.

were tested by DAC-ELISA. Out of 85 samples tested, five accessions *viz*., IC 202800, IC 331014, IC 344612, IC 437178 and IC 249584 showed positive reaction (5.8%) and remaining 80 accessions did not show presence of BCMV. The resistant check (CP-55) did not showed presence of BCMV and susceptible check (C-152) showed the presence of BCMV (Fig 3). Provvidenti and Cobb (1975), explained virus is generally located in the embryo.

A total of 85 seed coat samples, one susceptible check (C-152) and one resistant check (CP-55) were tested by DAC-ELISA. Out of 85 accessions tested, 13 accessions (15%) and susceptible check showed positive results indicating the presence of BCMV in seed coat. Resistant check and 72 accessions showed negative results indicating the absence of BCMV in seed coat. Similar results were observed by Hagita and Tamada (1984).

CONCLUSION

Developing genetically resistant cultivar is the most effective, economical, eco-friendly and socially acceptable disease management and control of BCMV. Among 85 germplasm accessions screened against BCMV, 24 accessions found to be immune to BCMV infection. IC199699, IC 201097 and IC 214751 are the resistant accessions with superior agronomic value. Therefore, these accessions can be a potential source of resistant genes to BCMV. They can be utilized in cowpea improvement program to introgress resistant gene into susceptible cowpea cultivars to increase the production of cowpea. However, these need to be further evaluated under BCMV hotspots, for different strains and under multi-location to know the genotype and environment interaction to further assure the quality of resistance exhibited by the immune germplasm accessions.

Conflict of interest

The authors declare that they have no conflict of interests.

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