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Evaluation of the Seed-borne Nature of Bean Common Mosaic Virus (BCMV) in Cowpea

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A B S T R A C T

Bean Common Mosaic Virus (BCMV) is an important viral disease in legumes, particularly the cowpea. It is a member of potyvirus, transmitted mechanically and by aphids in a non- persistent manner. Seed transmission results in the primary dispersal of the virus to new locations, often difficult to contain the spread of the virus. Hence seed transmission studies were carried out in cowpea variety C-152. It was determined by the grow out test at the two-leaf stage. The rate of seed transmission varied from 20-30 per cent in pot culture studies. Seed-borne nature of BCMV in cowpea was confirmed by DAS ELISA. Serological DAS ELISA of the cowpea seeds harvested from BCMV infected plants of both cultivars C-152 and KBC-2 revealed the presence of BCMV in embryo and cotyledon, but not in the seed coat. However, the virus titre differed between cotyledon and embryo between both the cultivars.

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.]is an important legume crop with high protein content. It is a crop of tropics and subtropical regions grown in sub-Saharan Africa, Asia, Central and South America. It is used as food, fodder, green manuring and also as a vegetable crop. It is rich in carbohydrates, proteins with traces of iron and calcium. It is also a good source of essential amino acids like lysine, leucine and phenylalanine.

In India, it is grown as a minor crop in states of Punjab, Haryana, Delhi, and West UP, along with a considerable area in Rajasthan, Karnataka, Kerala, Tamilnadu, Maharashtra and Gujarat. Pests and diseases threaten the yield of legumes. In India, cowpea occupies an area of about one million hectare with an annual production of 2.24 million tonnes and average productivity of 570 kg ha^{-1}.

Cowpea is susceptible to fungal, bacterial and viral diseases to a greater extent along with

insects. More than 140 viruses have been identified as naturally infecting cowpea (Hughes and Shoyinka, 2003). The major viral diseases are Cowpea Aphid-Borne Mosaic Virus (CABMV), Bean Common Mosaic Virus (BCMV), Cowpea Mild Mottle Virus (CPMMV), Cowpea Severe Mosaic Virus (CPSMV), Cowpea Mosaic Virus (CPMV), Cowpea Chlorotic Mottle Virus (CPCMV), Cucumber Mosaic Virus (CMV) and Cowpea Chlorotic Mosaic Virus (CCMV). Among them, BCMV is a major viral disease in cowpea resulting in significant yield losses to the tune of 70 per cent.

Reported major hosts of BCMV are *Vigna unguiculata* (mungbean), *Arachis hypogaea* (groundnut), *Glycine max* (soybean), *Vigna angularis* (adzuki bean), *Vigna mungo* (Taiwoand Gonsalves., 1982; Zhou *et al.*, 2014).

BCMV is a positive single-stranded RNA and belongs to family potyviridae. It is transmitted mechanically naturally and artificially by sap inoculation. Insect vector relationship in BCMV transmission involves aphids (*Aphis craccivora*) in a non-persistent manner.

Seed transmission plays a vital role in the epidemiology of viral crop diseases. It helps in carrying the virus from one season to the next, long-distance transport of virus, and in providing foci of primary infection (Bashir and Hampton, 1996). Plants grown from infected seeds are usually stunted and unproductive. Plants infected later in the season by aphids or mechanical transmission usually have little loss in yield, but a high percentage of the seed harvested from such plants may be infected and should not be used for seed purpose (Gillaspie *et al.*, 2007).

Disease outbreaks generally originate from contaminated seeds. Seed transmission in plant viral diseases is a major growing concern. It serves to be a primary source of inoculum of the virus. This results in transmission of the virus to new regions leading to secondary transmissions further. More viruses are transmitted through seeds of cowpea than any other crop species (Bashir, 1993). It is therefore desirable to develop an understanding of these viruses, particularly about seed-borne potyviruses. Hence seed transmission nature of BCMV was studied in controlled and field conditions in detail.

Materials and Methods

Pot culture studies

Cowpea plants of variety C-152 showing typical symptoms of BCMV were tagged in the field. Seeds were harvested from the infected plants and were grown in glasshouse in controlled conditions to determine the presence of BCMV in the seeds (Figure 2).

The leaves of the tagged plants showing BCMV symptoms were subjected to DAS ELISA (Double Antibody Sandwich Enzyme Linked Immunosorbent Assay) using BCMV specific antisera to confirm the virus to be BCMV (Table 1 and Figure 1)

To ascertain seed-borne nature of the virus

Seeds of the tagged plants were further soaked in water for four hours and later dissected into embryo, cotyledon and seed coat. The dissected parts of the seeds, along with whole seeds, were subjected to serological assays to know the exact location of the BCMV virus in the seed.

Healthy leaves and healthy seeds were taken as negative control and infected leaves of BCMV were taken as a positive control. Absorbance values were recorded at 405 nm using ELISA reader.

Preparation of buffer containing samples

Whole seeds, dissected embryo, cotyledon and seed coat of two cowpea genotypes C-152 and KBC-2 (Figure 3),infected leaf and seed samples, healthy leaf samples were ground in pestle and mortar with sample buffer in the ratio of 1:20.The ground samples were centrifuged at 8,000 rpm for five minutes. The supernatant of the samples obtained was used for the detection of BCMV by ELISA.

Detection of BCMV in cowpea by DAS ELISA

BCMV was detected in cowpea samples using DAS ELISA technique by using an anti-BCMV capture antibody and ALP labelled anti-BCMV detection antibody (Agdia, USA).

Protocol

The presence of virus in seed tissue was determined by performing the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using an Bean Common Mosaic Virus polyclonal antibody as per the manufacturer's protocol.

The capture antibody (Anti-BCMV) was diluted with carbonate coating (1:200) buffer before use. 100 µl of the prepared capture antibody was pipetted into each well. Coated plates were incubated overnight in the refrigerator (4 ºC). After incubation, the wells were emptied into a sink or waste container. The test wells were filled completely with PBST and then quickly emptied again. This step was repeated twice.

BCMV infected and healthy plant tissue was ground separately using a mortar and pestle in PBS buffer at 1:20 ratio (tissue weight in g: buffer volume in ml). The homogenate was transferred to a micro-centrifuge tube of 1.5 ml and centrifuged at 8,000 rpm for 10 minutes. Following loading diagram, 100 µl of clear supernatant was loaded into sample wells. In addition, 100 µl of positive control was dispensed into positive control wells and 100 µl of sample extraction buffer was loaded into buffer wells. Plates were kept inside the humid box and incubated for 2 hours at room temperature or overnight in the refrigerator (4 ºC).

After incubation, the plates were washed with PBST, as in step 5. The test wells were inspected for the presence of plant tissue. Enzyme conjugate (ALP labelled anti-BCMV) supplied as concentrated solution was diluted (1:200) in PBS-TPO buffer and 100 µl of enzyme conjugate solution was dispensed into test wells. Plates were kept inside the humid box and incubated for 2 hours at room temperature or overnight in the refrigerator (4 ºC).

After incubation, the plates were washed with PBST, as in step 5.The substrate solution was prepared by dissolving each PNPP tablet (5 mg) in 5 ml of substrate buffer, at a concentration of 1 mg/ml, about enough for five 8-well strips. 100 µl of PNPP substrate was dispensed into each test well. Plateswere incubated in a humid box for 60 minutes. The test wells were examined by measuring on a ELISA plate reader at 405 nm.

Results and Discussion

Among the various modes of transmission of viral diseases, seed transmission results in dissemination of the virus to new locations unknowingly. Hence it is very important to determine and confirm the seed transmission nature in of BCMV in cowpea.

The seeds harvested from the infected tagged plants of BCMV sown under glass conditions started to show typical symptoms of BCMV (Figure 2). The first symptoms of seed-borne

infection appeared on cotyledonary leaves as fine vein clearing followed by mosaic mottling and vein banding on newly formed trifoliate leaves. The results are in conformity with the reports of Udayashankar *et al.*, (2012); Puttaraju, *et al.*, (2004); Sanjeev Reddy (1997); Bashir (1993); Sekar and Sulochana (1988) and Pavitra (2013).

The symptoms were visible at two-leaf stage of the crop, sown after seven to ten days in the pots. Seed transmission rates varied from 20-30 per cent respectively, in the variety C-152. The presence of BCMV virus in the germinated seedlings was confirmed upon DAS ELISA of the leaves harvested at two-leaf stage using BCMV specific antisera.

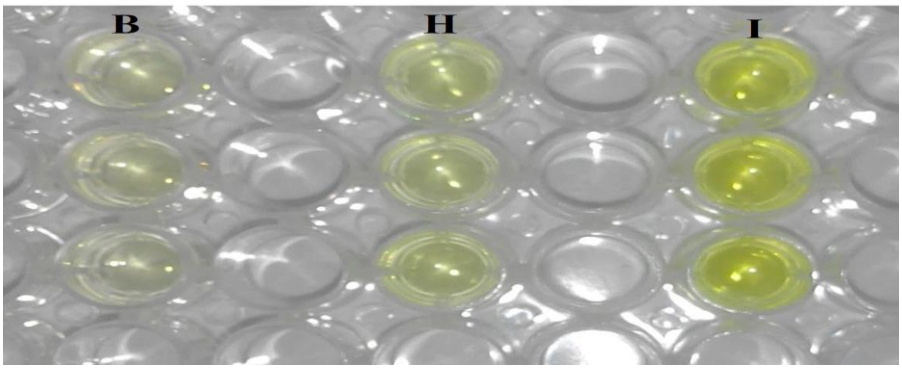
Table.1 Absorbance values at 405 nm to detect the BCMV in virus infected leaves of cowpea

Variety	Replications	Buffer (control)	Healthy leaves	BCMV Infected leaves
C-152	R_1	0.100	0.119	0.698
	R_2	0.110	0.124	0.732
	R_3	0.106	0.120	0.715
	Mean	**0.105**	**0.121**	**0.715**

Table.2 Absorbance values at 405 nm to detect the BCMV in different parts of cowpea seed

Varieties	Replications	Buffer (Control)	Healthy seed	Seed/Parts of seed from infected plant				Healthy leaf	Infected leaf
				Whole seeds of infected plant	Embryo	Cotyledon	Seed coat		
C-152	R_1	0.100	0.120	0.432	0.532	0.462	0.130	0.124	0.751
	R_2	0.115	0.116	0.420	0.562	0.445	0.115	0.129	0.749
	R_3	0.119	0.124	0.449	0.502	0.479	0.135	0.119	0.786
	Mean	**0.111**	**0.120**	**0.434**	**0.532**	**0.462**	**0.121**	**0.124**	**0.762**
KBC-2	R_1	0.119	0.118	0.507	0.425	0.533	0.120	0.130	0.691
	R_2	0.112	0.120	0.510	0.409	0.510	0.116	0.127	0.681
	R_3	0.110	0.116	0.504	0.441	0.556	0.124	0.133	0.701
	Mean	**0.113**	**0.118**	**0.507**	**0.425**	**0.533**	**0.120**	**0.130**	**0.691**

Fig.1 ELISA based detection of BCMV infecting cowpea

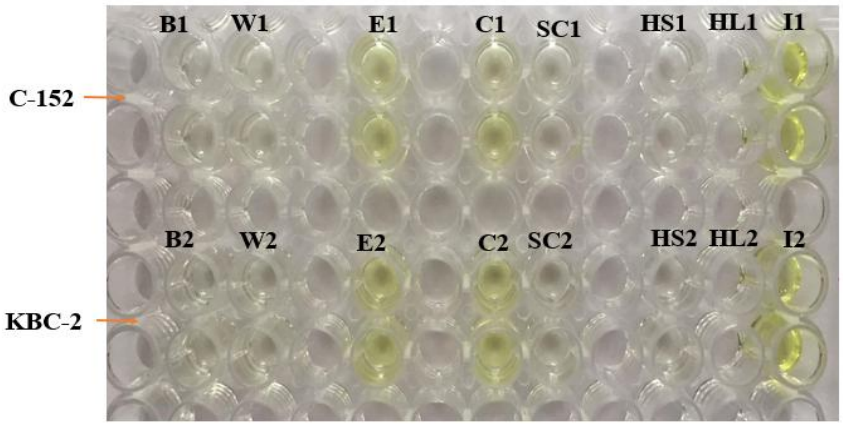


B: Buffer, H: Healthy leaves and I: Infected leaves

Fig.2 Seedlings raised from seeds harvested from BCMV infected plants showing symptoms of the virus

Fig.3 Dissected Cotyledon, seed coat and embryo, of cowpea used for ELISA

Fig.4 Serological detection of BCMV in different parts of cowpea seeds by DAS ELISA. (B1, B2: Buffer, W1, W2: Whole seeds, E1, E2: Embryo, C1, C2: Cotyledon, SC1, SC2: Seed coat, HS1, HS2: Healthy seed, HL1, HL2: Healthy leaf, I1, I2: Infected leaf)



Grow out test of the infected harvested seeds showed the presence of the virus. Pot culture studies indicated the BCMV to be seed-borne in nature. Further studies were carried over to know the location of the virus in the seed. The seeds dissected into embryo, cotyledon and seed coat along with the whole seeds were subjected to DAS ELISA (Figure 3). To check the varietal difference regarding seed transmission, along with C-152, variety KBC-2 was also subjected to DAS ELISA.

Table 2 showed the absorbance values in the whole seeds of the infected plants of both the cultivars was around four times more compared to the healthy seeds taken as negative control. Absorbance values of embryo, cotyledon of both the cultivars showed values that was found to be four to five times more compared to negative control of healthy leaves and healthy seeds.

But the absorbance values of seed coat in both the cultivars was almost on par with negative control of healthy leaves and seeds. Similarly, between healthy and infected leaves, absorbance values of infected leaves was around six times the buffer control in KBC-2 whereas, around seven times the buffer control in C-152 (Table 2 and Figure 4).

DAS ELISA confirmed the presence of virus in the embryo and cotyledon and the absence of virus in the seed coat. Serological studies confirmed the virus to be internally seed-borne in nature. Further, the presence of the virus was seen in both the cultivars. It was found in the whole infected seeds, embryo and cotyledon but absent in the seed coat. But the absorbance values differed between the two cultivars (Table 2 and Figure 4). This indicated the virus concentration also depends upon the different cultivars. The results are in accordance with the reports of Ojuederie *et al*, (2009), Puttaraju *et al.,* (2004), Bashir and Hampton (1996).

In conclusion the BCMV in cowpea results in significant yield losses. This increases further with different modes of transmission. Seed transmission plays a significant role in the disease epidemiology. Henceforth the detection of BCMV in seed in view of seed quality control assumes a great importance. The pot culture and serological studies confirmed the seed borne nature of BCMV in cowpea. This helps in early rouging of disease plants to prevent the transmission. Further the harvested seeds of cowpea can be tested for the presence of the virus through random sampling, to confirm the seed lot, to be free of virus.

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