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Biological relationship of *Bean common mosaic virus* (BCMV) infecting cowpea with leguminous plant species

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Abstract: *Bean common mosaic virus* (BCMV) associated with cowpea mechanically inoculated to different leguminous plants. Out of nineteen including cowpea Var.C-152, the virus was easily transferred to ten different leguminous hosts. All other hosts assessed for the presence of BCMV were found to be uninfected. The number of days taken for symptom expression and symptoms were varied within plant species. Pole bean expressed mosaic symptom after long incubation period (15-18 days) whereas, shorter incubation period was observed in common bean and rice bean (7- 10 days). BCMV produced chlorosis, mosaic, leaf distortion, puckering, vein banding, vein clearing and vein netting on cowpea(C-152). A typical virus symptom, mosaic was observed in green gram, common bean, lime bean, rice bean and yard long bean, whereas, leaf rolling and leaf distortion was observed in black gram, pole bean and snap bean. The virus-host relationship was confirmed by back inoculation test to *C. amaranticolor*. Further symptomatic plants were subjected for Reverse Transcriptase polymerase chain reaction (RT-PCR) for molecular confirmation using BCMV coat protein (CP) specific primer pair. A PCR fragment size of 439bp was amplified for the symptomatic plants. The results generated indicated the ability of a plant to support virus expression and host specificity of BMCV within the leguminous plant species.

Keywords: Biological relationship, Cowpea, Leguminous plants, Sap inoculation, Virus

INTRODUCTION

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 *et al*., 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).
The occurrence of BCMV on common bean has been reported from India since long (Manjunatha *et al*., 2016) but not much work has been done on biological relationship of BCMV with other leguminous plants. The identification of potential biological relationship or host range of a particular plant virus is the first prerequisite to understand epidemiology and to design suitable management strategy (Morris *et al*., 2006). Further a comparison of host range of plant viruses might lead to knowledge of certain differences in diseases expression by plant viruses.
Several researchers employed mechanical sap inoculation to transfer BCMV to other leguminous plants (Morris *et al*., 2006; Bhadramurthy and Bhat, 2009; El -Kady *et al*., 2014) to study its biological relationship. But, mechanical sap inoculation alone is not reliable method to judge host range of particular virus because it failed in distinguish between symptoms induced by virus and abiotic factors such as stunting, leaf rolling and cupping of infected plants (Robert *et al*., 1991) Recent phylogenetic data revealed that BCMV is well associated with number of leguminous and non-leguminous plants of the different parts of the world (Hosseini and Hosseini, 2014).
The combination of mechanical sap inoculation and sensitive methods like PCR, ELISA have been made accurate determination of host range, strain identification and cultivar differentiation to different virus groups (Bhadramurthy and Bhat, 2009).With this idea the present study, has been conducted to find out the biological relationship of BCMV infecting cowpea with other legumes crops using mechanical sap inoculation and Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR) using coat protein (CP) specific

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degenerated primers.

MATERIALS AND METHODS

Plant material and virus inoculation: BCMV infected leaves of cowpea were collected from research plots of GKVK, UAS, Bengaluru. The collected samples were mechanically inoculated to *Chenopodium amaranticolor* and cowpea Var. C-152 to get pure virus culture. These plants were further maintained as source of virus inoculum. A total 19 different legume plant species were raised from seeds in polyethylene bags under insect proof condition and plants were mechanically inoculated at primary leaf stage. Mechanical inoculation was carried out in pre-chilled pestle and mortar by sap in cold 0.1 M phosphate buffer (pH7.2) containing 0.1% (v/v) Beta-mercaptoethanol. The extracted sap was inoculated on the leaves of healthy test plants dusted with Celite and then washed off with tap water after 2-3 min. In each plant species, 10 plants were inoculated and one set of un-inoculated plants were maintained as control. The inoculated plants were kept in the insect proof glass house and examined periodically for symptom expression.

Confirmation of virus etiology: The presence of virus in symptomatic and asymptomatic plant leaves was confirmed by back inoculation test and molecular detection (Manjunatha *et al*., 2015)

Back inoculation test: The back inoculation test was carried out using propagation hosts such as *Chenopodium amaraticolor* and cowpea Var. C-152. The infected leaves of leguminous plants were collected and back inoculated to propagation hosts mechanically to confirm of viral etiology.

Molecular detection

RNA extraction and cDNA synthesis: Total RNA was extracted from healthy and infected plant samples using RNA Extraction kit (Sigma). The obtained total RNA was used for synthesis of cDNA. A total of 20 µl RT mixture was prepared by adding 2 µl of 10X RT buffer,1.0 µlof 25 µl mM $MgCl_2$, 2.0 µl of 10 mM dNTP mixture, 2.0 µl of 10 µM Reverse Primer (*5'AGGCATGTACGGCTTCTCG*3'), 1.0µl of Reverse transcriptase (100 units/µl), 5.0 µl of isolated RNA and finally volume was made withsterile distilled water. Reaction mixture containing RNA (5.0 µl) + Reverse primer BCMV (2.0 µl) was incubated at 65 ^{0}C for 15 min and then quenched on ice. The RT-PCR mixture was reverse transcribed at 42 ^{0}Cfor 60 min and then at 94 ^{0}C for 5 min to synthesize cDNA.

Reverse transcriptase PCR (RT-PCR): The c-DNAs' obtained were subjected to PCR amplification using forward primer (5'CGCAGGCTCCAAAGG AAAAG 3') designed to amplify of coat protein of BCMV. A total of 25 µl reaction mixtures that contained 2.0µl of cDNA, 12.5 µl of master mix, 2.0 µl of forward primer and 8.5µl of sterile water was amplified in thermocycler. The PCR amplification was carried out with the following conditions; initial denaturation at 94°C for 3 min followed by 35 cycle reaction profile involving 1min of denaturation at 94 ^{0}C, 1 min of annealing at 54 °C and extension for 2 min at 72 °C followed by a final extension for 10 min at 72 °C, finally hold at 4 °C. The reaction products (25 µl) were analyzed on 1% agarose gel along with 100 bp DNA ladder (Fermentas, USA). The DNA bands were visualized and photographed using UV illuminator and a gel documentation unit.

RESULTS AND DISCUSSION

Bean common mosaic virus (BCMV) has been identified as major constraint on cowpea production. In this study biological relationship of BCMV with other leguminous plant species was determined. Nineteen different plant species belonging to *Leguminosae* family were inoculated with pure virus inoculum and results are presented in Table 1. Out of nineteen, BCMV produced systemic symptoms on nine species *viz*., French bean (*Phaseolus vulgaris*), Yard long bean (*V. unguiculata* subsp. *sesquipedalis*), Green gram (*Vigna radiata*), Black gram (*Vigna mungo*), Rice bean (*Vigna umbellata*), Pole bean (*Phaseolus coccineus*), Lima bean (*Phaseolus lunatus*), (*Phaseolus* sp.) and Cowpea (*Vigna unguiculata*).Systemic symptoms were observed in all nine plant species, within 10 to 12 days after inoculation. These results indicated that the hosts of the virus isolate mainly restricted to *Phaseolus* spp. and findings appear to be in line with results of (Bhadramurthy and Bhat, 2009; El-kady *et al*., 2014). The restriction could be due to certain host clades having lost or gained immune or cellular components that affect susceptibility to a given pathogen called as 'phylogenetic clade effect' (Longdon *et al*., 2014)

The time taken for symptom expression was varied within plant species. Pole bean took longer incubation period (15-18 days) for symptom expression. Whereas, shorter incubation period (7-10 days) was observed in

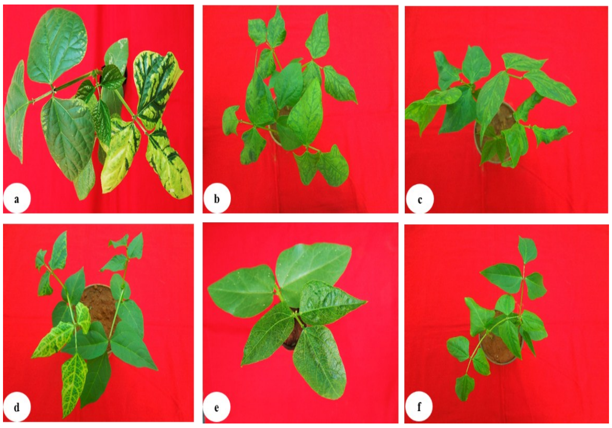

Fig. 1. *Different types of symptoms induced by BCMV on cowpea (cv. C-152): a. leaf chlorosis; b. mosaic; c. leaf distortion and puckering; d. vein clearing; e. vein netting and f. vein banding.*

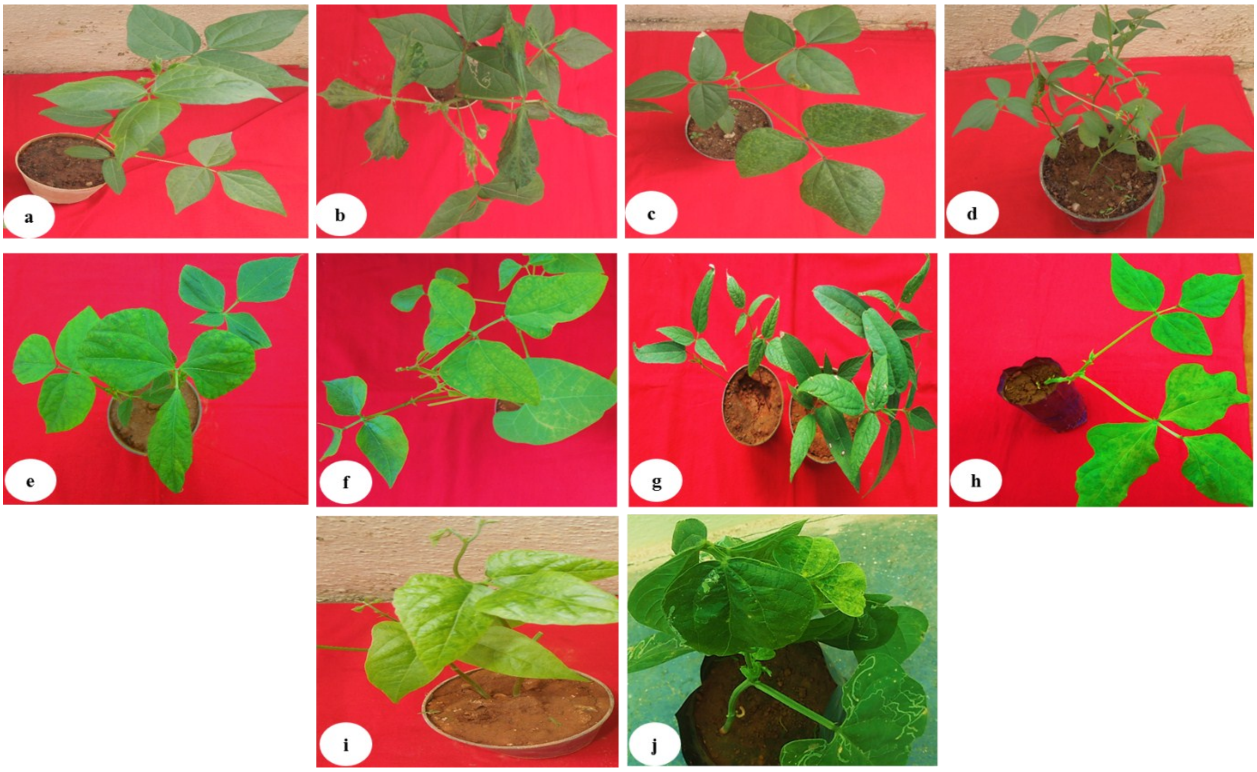

Fig. 2. *Different types of symptoms induced by BCMV on leguminous plant species upon mechanical sap inoculation : Black gram plants with mosaic (a), leaf distortion and leaf puckering (b); Green gram plants with mosaic (c) and leaf rolling (d);Common bean (e) and yard long bean (f) with mosaic; Rice bean with mosaic(g) and pole bean with mosaicand leaf distortion(h);Lima bean with mosaic and chlorosis (i) and Snap bean with mosaic, leaf rolling and yellowing (j).*

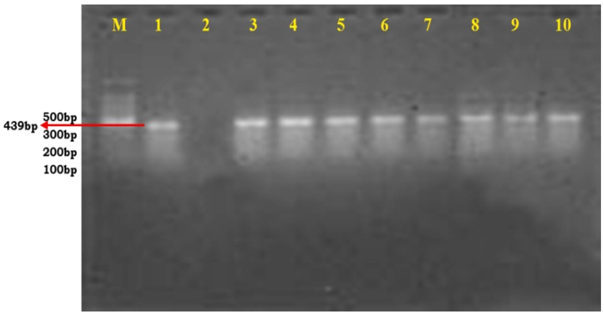

Fig. 3. *RT-PCR amplification of coat protein (CP) gene from BCMV infected leaf samples of different leguminous plant species;M= 100bp ladder; Lane 1: Infected leaf sample from cowpea;Lane 2: Healthy leaf sample from cowpea;Lane 3-10:Infected leaf sample from Green gram, Blackgram, Lima bean, Snap bean, Pole bean, Rice bean, Yard long bean and common bean respectively.*

common bean and rice bean. The replication and movement requires different viral protein host interactions (Chung *et al*., 2015). The difference in incubation period for symptom expression might be due to poor response of host for virus multiplication, speed of movement and developmental stage of host plant (Zhang *et al*., 2012). Even systemic invasion of virus in some hosts depended on temperature, virus concentration in the inoculum, virus strain and type of host (Feil and Purcell, 2001; Zitter and Murphy, 2009).

BCMV induced different type symptoms on tested hosts. The virus induced chlorosis, mosaic, leaf distortion, puckering, vein banding, vein clearing and vein netting on cowpea(C-152) as shown in the figure 1.A typical mosaic symptom was observed in green gram, common bean, lime bean, rice bean and yard long bean, whereas, leaf rolling and leaf distortion was observed in black gram, pole bean and snap bean (Fig. 2). Mangeni *et al*. (2014) experimentally showed symptoms of BCMV such as severe mosaic, curling of the leaves, vein banding, mottled and malformed pods on infected cowpea plants. The type of symptoms induced by virus depends on the interaction between host resistance and virus pathogenicity genes (Brunt *et al*., 1996; Chung *et al*., 2015). It was also studied, symptomology of plants under attack of BCMV depends on crop variety, plant age, viral strain and climatic conditions such as temperature (Chellappan *et al*., 2005; Szittya *et al*., 2003). The mosaic pattern, dark green bands along the main veins and lighter green interveinal tissue (vein banding)in BCMV infected common bean plants was by Morales, (1998) under varied temperature. In later, stages leaves exhibited downward curling with longer and narrower than healthy ones. BCMV isolate obtained from lima bean plant expressed mosaic symptoms and venial necrosis on mature leaves (Melgarejo *et al*., 2007).

BCMV failed to infect *Leguminosae* family species *viz*., (*Macrotylom acuniflorum*), red gram (*Cajanus*

Table 1. Symptomology and host range of *Bean common mosaic virus* (BCMV) infecting cowpea.

S. N.	Plant species inoculated	No. of plants inoculated	No. of plants infected	Incubation period (dpi)	Per cent Infection	Symptoms	PCR Confirmation of infectivity
1	Chickpea (*Cicer arietinum*)	10	0	-	0.0	-	-
2	Peas (*Pisum sativum*)	10	0	-	0.0	-	-
3	Common bean (*Phaseolus vulgaris*)	10	7	7-10	70.0	Mc	+
4	Lablab bean (*Lablab purpureus*)	10	0	-	0.0	-	+
5	Yard long bean (*V. u.* ssp. *Sesquipedalis*)	10	3	12-14	30.0	Mc	+
6	Moth bean (*Vigna aconitifolia*)	10	0	-	-	-	-
7	Rice bean (*Vigna umbellata*)	10	4	7-10	40.0	Mc	+
8	Pole bean (*Phaseolus coccineus*)	10	2	15-18	20.0	Mc, Ld	+
9	Lime bean (*Phaseolus lunatus*)	10	5	12-14	50.0	Mc, Chl	+
10	Winged bean (*Psophocarpus tetragonolobus*)	10	0	-	0.0	-	-
11	Snap bean (*Phaseolus* sp.)	10	3	12-14	30.0	Mc, Lr, Y	+
12	Cluster bean (*Cyamopsis tetragonoloba*)	10	0	-	0.0	-	-
13	Soy bean (*Glycine max*)	10	0	-	0.0	-	-
14	Ground nut (*Arachis hypogaea*)	10	0	-	0.0	-	-
15	Greengram (*Vigna radiata*)	10	8	8-12	80.0	Mc, Lr	+
16	Blackgram (*Vigna mungo*)	10	8	8-12	80.0	Mc,Ld, Lp	+
17	Redgram (*Cajanus cajani*)	10	0	-	0.0	-	-
18	Horsegram (*Macrotyloma uniflorum*)	10	0	-	0.0	-	-
19	Cowpea (*Vignaunguiculata*)	10	9	7-10	90.0	Mc, Y, Ld, Lr	+

Note: Mc- Mosaic, Ld- leaf distortion, Lp- leaf puckering, Lr- Leaf rolling, Y- Yellowing + (Positive reaction with BCMV specific CP primers); dpi: Days after inoculation

cajana), chickpea (*Cicer arientinum*), cluster bean (*Cyamopsis tetragonoloba*), soyabean (*Glycine max*), groundnut (*Arachis hypogaea*), *winged bean* (*Psophocarpus tetragonolobus*), *lablab bean* (*Lablab purpureus*), *pea* (*Pisum sativum*). Results were cleared that BMCV has its host specificity. Even, Grisoni *et al*. (2004) failed to transmit, BCMV isolates of *Vigna tahitensis* to *V. marina* and *Macroptilum lathyrodoides.* The plants may be non-susceptible to viruses because a corresponding protein fails to function in the viral replication complex (Dietzgen *et al*., 2016). It was also discussed that non-susceptibility of host is due to lack of recognition system between host and virus at molecular level (Garcia-Arenal and Fraile, 2013). In many cases plants' insusceptibility to viruses results from a lack of cell-to-cell movement (Zhao *et al*., 2016)

The biological relationship of BCMV with different leguminous plants was confirmed through bioassay by back inoculation test to *Chenopodium amaranticolor* and cowpea Var. C-152 plants. Further symptomatic plants were subjected for RT-PCR for molecular confirmation using BCMV coat protein (CP) specific primer pair. A PCR fragment size of 439bp (Fig. 3) was amplified for the symptomatic plants and asymptomatic plants were found virus free.

Use of molecular technique, RT-PCR with bioassay method would help in finding out biological relationship of BCMV even under latent infection and mixed infections (Morris *et al*. 2006). The polymerase chain reaction (PCR) method employed by various workers for the detection and identification of potyviruses and rely on degenerated primers designed to conserved sequences of WCICEN box or QMKAA motif in coat protein (CP) gene. BCMV and *Bean common mosaic necrosis virus* (BCMNV) were simultaneously detected by the different size of RT-PCR products by designing two virus specific primer pairs (Xu and Hampton, 1996; Melgarejo *et al*., 2007; Udaya Shankar *et al*., 2009). Bhadramurthy and Bhat (2009) confirmed the association of BCMV with Vanilla in India based on host reaction and coat protein amplification.

Conclusion

Our experimental results indicated that BCMV can be easily transferred to other related leguminous plant species through mechanical sap inoculation and cause different type of symptoms. The incubation period for symptom production is also varied with host species. The identified new hosts (black gram, green gram, rice bean and pole bean) in this experiment which may influence epidemiology of BCMV in India as virus reservoirs. Hence, the information generated in present work could help in predicting disease emergence in leguminous hosts and to frame suitable management strategy.

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