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Detection and molecular characterization of *Bean Common mosaic virus* in mungbean

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Abstract
Bean common mosaic virus (BCMV) is an important seed borne pathogen. The virus caused downward leaf rolling, thickening of leaves, mosaic, leaf deformation, necrosis of tissues of abaxial side of leaves, necrosis of apical stem portion. *Vigna mungo*, *Punicum granatum*, *Vigna unguiculata* and *Ricinis communis* gave positive reaction to BCMV antisera and potyvirus antisera. The infected leaves of IR-16, Meha and K-851 gave strong positive reaction with BCMV specific antisera with O.D. value of 3.99, 3.91 and 3.37, respectively as compared to positive control with OD value of 2.20.Transmission electron microscope studies revealed the presence of BCMV as flexuous particles of 823 nm long.The RT-PCR amplified product when run on 1 per cent agarose revealed the presence of predicted ~1300 bp product.

Keywords: Mungbean, BCMV, seed transmission, electron microscope, ELISA and RT-PCR

Introduction
Bean common mosaic virus (BCMV) is one of the most serious and widespread virus in beans world area and belongs to the Potyvirus group, which is largest group of plant viruses (Shukla *et al*., 1994) [29]. BCMV was first isolated from mungbean (*Vigna radiata*) in Iran by Kaiser *et al*. (1968) [14] with the name mungbean mosaic virus. The BCMV in India was reported in 1963 simultaneously by Yaraguntaiah and Nariani and Nagaich and Vashisht. BCMV is economically important disease in Africa, Europe, North America and Latin America. However, infection levels may reach 100% with yield losses ranging from 35-98% (Galvez, 1980) [8]. Yield reductions in bean crop due to BCMV ranged from 53 to 68% in Oregon, USA, depending on disease severity (Hampton, 1975) [10], whereas, Up to 98% yield losses have been reported due to BCMV infection by Varma, (1988) [31] and Hampton *et al*, (1982) [11]. In present study, we report the occurrence of BCMV on mungbean in India on the basis of molecular characterization.

Materials and Methods
Collection of BCMV infected plants
Young infected plants of susceptible mungbean cultivars, IR-16 and K-851 and wild hosts with distinct BCMV symptoms growing under field condition were collected for recording the symptoms and studies.

Symptomatology
To study the symptoms of BCMV infection as part of doctoral research work, seeds collected from infected plants of mungbean variety IR-16 and K-851 were sown in the field in 5x1.8 m^2 with plant to plant spacing of 10 cm and row to row spacing of 30 cm as well in 10 earthen pots of 30 cm diameter containing sterilized mixture of sandy loam soil and compost (1:1) in glasshouse conditions. For studying the symptomatology of virus infection in sap inoculated plants, 10-14 days old healthy seedlings of mungbean were inoculated by mechanical sap inoculation from diseased plants. The inoculated plants were kept in the glasshouse and symptom expressions were recorded periodically.

Sap Transmission

Preparation of inoculum

Young leaves of 15-20 days old plants showing characteristic BCMV symptoms were collected from infected mungbean plants, washed in tap water to remove the dust particles adhering to them and dried between folds of blotting paper. The leaves were macerated in chilled mortar and pestle using potassium phosphate buffer (pH 7.2, 0.05M) at the rate of 1ml/gm of leaf tissue. The resultant pulps were squeezed through double layered muslin cloth and the extract thus obtained was used as standard inoculum.

Method of inoculation

After thorough grinding, the whole leaf pulp is passed through double layers of muslin to filter standard extract of the leaves. Carborundum powder was added to allow wounding of the cells of healthy plants of IR-16 and K-851 varieties of mungbean. The sap was applied by rubbing the leaves gently with a cheese cloth, finger supporting the leaf from the ventral surface with a piece of card board, in one direction only, i.e. from petiole to the apex of the leaf.Five plants each in 6 replications were maintained to test mechanical inoculation and symptom expression. The inoculated leaves were washed for 1-2 minutes after inoculation to remove the excess of inoculum with a fine jet of distilled water from a squeeze bottle and plants were kept under observation for 15-20 days in the glass house.

Seed transmission

For Seed transmission study of virus were conducted under insect-proof cover green net, seeds of mungbean verities i.e. IR-16, K-851 and Meha from diseased plants were collected from the field. Genotype of LGG 460 seeds were also collected which was found resistant in field. Seeds collected from healthy plants of the varieties served as control. The plants were observed keenly for expression of disease symptoms upto a period of 4 weeks. The percent seed germination and rate seed transmission of the BCMV were recorded.

$$\text{Per cent Seed Transmission} = \frac{\text{Total number of infected plant due to BCMV}}{\text{Total plant stand}} \text{ X } 100$$

Host range

Pure culture of the virus maintained on IR-16 and K-851 varieties of mungbean were used as source of virus inoculum. A total of 10 seedlings of each of the *Phaseolus vulgaris*, *Vigna mungo*, *Vigna radiata*, *Vigna unguiculata*, *Chenopodium album*, *Glycine max*, *Datura stramonium*, *Solanum nigrum*, *Capsicum annum* and *Nicotiana benthamiana* at the 3-5 leaf stage were inoculated by mechanical inoculation with sap extracted in 0.01 M phosphate buffer, pH 7.0. The inoculated plants were observed for symptom expression and the symptoms were recorded weekly after sap inoculation. Symptomatic hosts were confirmed with DAS-ELISA.Besides, surveys were conducted for BCMV symptomatic hosts plants around the experimental field and nearby places of Anand. The plants showing symptomatic hosts of BCMV were confirmed with DAS-ELISA.

Serology

Serological characterization of BCMV was done to detect the virus. The Double Antibody Sandwich-ELISA (DAS-ELISA) was adopted for the detection of the BCMV as described by Clark and Adams, (1977) [4]. The leaf samples were collected from diseased plants and were grinded in an Indirect Sample Extraction Buffer (1:20). Samples were loaded to antibody coated plate. After a time of incubation, 200µl of enzyme labeled I_gG of BCMV was diluted in 1:200 of enzyme conjugate buffer and added to each well of the microtitre plate and incubated at 30^0C for 2 hours. After washing of plate, 200µl of p-nitrophenyl phosphate substrate (1mg/ml of substrate buffer) was added and incubated for 2 hours at room temperature. Spectrophotometric readings at 405 nm were performed after different times of substrate reaction.

Transmission electron microcopy

A drop of 1:100 dilution of purified virus preparation was placed on carbon coated grids, incubated for 30 seconds, washed with distilled water and stained with 2% uranyl acetate. Excess stain was removed by filter paper and grids were examined under transmission electron microscope. Similarly, for leaf dip preparation, infected mungbean leaves were ground in a few drops of 0.05 M phosphate buffer (pH = 7.0). Sap thus extracted was centrifuged at low speed in an Eppendorf centrifuge for 2 min. A drop of resultant supernatant was placed on carbon coated grids and the same procedure was followed as with purified preparation.

Polymerase Chain Reaction (PCR) using family specific universal primers

RNA Isolation

Total RNA from the fresh virus infected leaf of mungbean was isolated using Trizol LS reagent (Invitrogen). Hundred (100) mg of virus infected leaves were crushed with liquid Nitrogen in a chilled sterilized pestle and mortar for 30 min. 750 µl of Trizol LS reagent (Invitrogen Life Technologies, USA) was added in the crushed sample and again crushed it till it become liquid. Homogenized sample was then taken in 2 ml Eppendorf tube and then keep it horizontal on ice for 10 min.200 µl of chloroform was added to the homogenized sample and tubes were vigorously shaken in vortex mixer (Genei, Bangalore, India) for 15 seconds. Tube was incubated again on ice for 5 min. After incubation it was centrifuged at 12,000 rpm for 15 min. in refrigerated centrifuge (Remi Instruments, India) at 4^0C. After completion of above step, the top aqueous phase was transferred to a new Eppendorf tube with the help of micropipette, Thermo electron Corporation, Finland) and then 500µl of chilled isopropyl alcohol was added. Contents were gently mixed and incubated for 10 minutes at room temperature. Again sample was centrifuged at 12,000 g for 10 minutes at 4^0C. Pellet thus obtained was washed with 1000 µl of chilled 75 per cent ethanol. For this, sample was vortexed and then centrifuged at 7500 g for 5 minutes at 4^0C. Supernatant was thrown and pellet was dried by tapping the tube on blotting sheet. After drying, 50 µl RNase free water was added in Eppendorf and incubated at 55-60^0C for 10 minutes. RNA obtained was stored at -20^0C in deep freeze (Blue Star Ltd., Bombay, India).

RNA check

Purity of isolated RNA was checked by electrophoresis. 3 µl of RNA was mixed with 2µl of gel loading dye and loaded on 1 per cent agarose gel containing Ethidium Bromide (0.5 mg/ml) and electrophoresed in 0.5X TAE buffer at 80 volts for 2 hours. Then the gel was viewed under Gel Documentation System using UV transilluminator. High quality and purity RNA was then used for cDNA synthesis.

cDNA synthesis

First strand cDNA synthesis was initiated at the polyadenylated 3' terminus of the RNA with oligodT_{18} primer by reverse transcription performed in a 25 µl reaction mixture containing 1-2 µg of total RNA mixed with 5 µl of 5X reverse transcription buffer, 5 µl of 20 mMdNTP mix (Fermentas, USA), 10 µM of oligodT primer, 20 units of RNase inhibitor (0.5 µl) (Biogene), 200 units of Moloney murine leukemia virus (M-MuLV reverse transcriptase (1 µl)) and RNase free water added to make final volume. The reaction mixture was incubated at 37^0C for 75 minutes and then heated for 5 minutes at 70^0C to inactivate reverse transcriptase. RT-PCR was carried out using BCMV coat protein (CP) gene specific primers (Sharma *et al*., 2009) [25]. Composition of RT reaction is presented in Table 1.

Primers used for PCR amplification of CP gene of BCMV

BCMV CPF TGGCTGCTTGAGAGAGATGA Product size 1.3kb BCMV CPR ATCACTCTGCATGTCCTCAC The above reaction mixture was vortexed and centrifuged briefly to collect the mixture at the bottom of the PCR tube (0.2 ml). cDNA synthesis was carried out at 37^0C for 75 minutes followed by denaturation at 70^0C for 5 minutes using thermal cycler (Gene Amp PCR System 9700, Applied Bio systems, USA). The PCR tubes were then immediately placed on ice and then stored in -20^0C (Blue Star Ltd, Bombay, India) for further amplification.

PCR amplification of viral cDNA was carried out in thermal cycler by using 10.0 µl cDNA, 2.0 µl of forward primer (F), 2.0 µl of reverse complimentary primer (R), 5.0 µl buffer, 2.5 µl of $MgCl_2$, 3.0 µl dNTP mix (Fermentas), 1.0 µl of Taq DNA polymerase and 24.5 µl of water. Total reaction mixture becomes 50.0 µl. Then it was subjected to PCR amplification. The PCR reaction mixture is presented in Table 2 and conditions during thermal cycling is presented in Table 3.

The above reaction mixture was vortexed and centrifuged briefly to collect the mixture at the bottom of the PCR tube (0.2 ml). cDNA synthesis was carried out at 37 ^{0}C for 75 minutes followed by denaturation at 70 ^{0}C for 5 min. using thermal cycler (Gene Amp PCR System 9700, Applied Biosystems, USA). The thermal cycler was programmed for 35 cycles with one cycle of initial denaturation and steps 2-4 were repeated 35 times and final elongation was carried out at 72^0C for 30 minutes. The PCR tubes were then immediately placed on ice and then stored in -20^0C (Blue Star Ltd, Bombay, India) for further amplification.

Analysis of RT-PCR product by Agarose gel Electrophoresis

After completion of PCR amplification, 10 µl of PCR product mixed with 4 µl of gel loading dye was loaded in wells/slots and electrophoresed in 1.2 per cent agarose gel containing ethidium bromide (0.5 mg/ml) in 0.5X TAE using a submarine horizontal agarose slab gel (SAIPLAS, UK) at 80 volts for 2 hours. DNA marker, Lambda DNA / *Eco R1-Hind III* double digest 1000 bp DNA ladder (MBI Fermentas, USA) was used as standard. For size determination, the gel was viewed under Gel documentation system using UV light and image was captured.

Result and Discussion

Symptomatology

Symptomatology study aids in providing information about the symptoms produced by the virus and helps for diagnosis of the disease in the field conditions. Symptoms of BCMV vary with mungbean variety, virus strain, environmental conditions and stage of plant growth at the time of infection. The disease plants under field were observed weekly starting from germination to harvesting of crops. The first symptom of the disease was observed at trifoliate leaf stage. The plants infected by BCMV showed reduction and downward rolling of leaf lamina, necrosis of veins, leaf deformation and discolorations of interveinal area of leaf (Plate 1). As the disease progressed mosaic and puckering of leaves with severe necrosis were observed. Further severe necrosis with reduced lamina of growing leaves at apical region of infected plants was observed. Leaves became leathery and distorted severely at maturity. The diseased plants produced less pods with necrosis which were shriveled and produced a few light weight small and discolored seeds. Similar symptoms such as leaf curling, blistering, dwarfing, and chlorosis, etc. were described by other workers (Pierce, 1934; and Morales and Bos, 1988) [23, 21]. Systemically infected plants may have smaller and fewer pods and infected pods sometimes is covered with small, dark-green spots which matured later than uninfected pods have been described by some others (Kapoor *et al*., 2009; Bhadramurthy and Bhat, 2009; Deepti and Chalam, 2009 and Pathania *et al.*, 2012) [15, 2, 6] in mungbean as well as in other leguminous hosts.

Sap Transmission

Transmission is fundamental property of plants viruses as being obligate parasites they must be transmitted from one susceptible host to another as well as need to be introduced in living cell for their survival and continuity of life cycle. To study whether the virus is sap transmissible or not?, mechanical transmission studied were undertaken. For this, purpose sap was extracted from newly infected symptomatic leaves in 0.01M phosphate buffer (pH-7.0) and was inoculated on healthy plants of mungbean variety IR-16 and K-851 through leaf rub method under insect proof glass house conditions. Result of present study revealed the appearance of first symptom after 16 days of sap inoculation. All inoculated plants showed characteristic mosaic symptoms. After 28 days of inoculation, downward rolling of leaves lamina was observed in all inoculated plants. However, leaves inoculated with plain water (control) did not produce any symptoms. The result is in conformity with the results of Meiners *et al.*, (1977) [19] who reported that *Bean common mosaic virus* was transmitted by sap and produce severe mosaic symptoms.

Characteristics mosaic and leaf deformation symptom was observed in inoculated plants after 30 days of inoculation. Kapil *et al*., (2011) [16] observed mosaic symptoms along with leaf deformation on susceptible cultivar 'Jawala' of french bean due to BCMV.

Seed transmission

Seed transmission is a means of distant spread of viruses and the exotic strains from one region to other and one continents to another. It aids in the infection of plants at the young seedling stage. The rate of seed transmission *i.e.* the percentage of seeds that produce infected plants depends on the stage of plant growth at the time of infection, the virus strain and bean variety. Plants that are infected after flowering generally develop a little or no seed infection.

Seed transmission of virus was studied in the seeds collected from tagged healthy and infected plants in the field as well as in net house. Seed borne infection was observed after 16 days of sowing of infected seed. Initially malformation of leaves and mosaic symptoms were observed which later developed

into severe mosaic, downward curling of leaf lamina and smaller leaves. Interveinal discoloration in the form of bronzing was also observed on lower leaf surface at later stages. In the present investigation the seed transmission rate of the virus was recorded as 24.00, 26.00 and 22.00 per cent in IR-16, K-851 and Meha varieties of mungbean, respectively (Table 4). Genotype LGG 460 did not express any symptoms of BCMV. However, no symptoms were observed in seeds which were collected from healthy plants. Similar results were also obtained by Kaiser and Mossahebi, (1974) [13]. Kaiser *et al.,* (1968) [14] reported 8 to 32 per cent seed transmission of BCMV in 12 mungbean lines. While Kumar *et al.,* (2011) [18] reported 3.37 to 9.18% of seed-transmission of BCMV in mungbean and urdbean. Seed transmission of BCMV was observed up to 8-10% in T-9 variety of urdbean by Pathania *et al.,* (2012) [24]. However, in guar line PI 340385 seed transmission of 94% was reported by Ben-Moshe *et al*., (1991) [1]. Similarly, BCMV is transmitted through host seed up to 93% as reported by Schmidt, (1992) [28]. Morales and Bos, (1988) [21] observed that the rate of BCMV transmission through seeds varies between genotypes of common bean and virus strains which may ranging from 0 to 83%. Sharma *et al*., (2009) [25] reported that BCMV was sap (90%), seed (75%) and aphid (70–80%) transmissible.

Host range

Twenty various hosts were studied for the host of BCMV and Potyvirus. Symptomatological and serological techniques (DAS ELISA) were used for detection of the virus. The detection result of the virus revealed urdbean (*Vigna mungo*) and marigold (*Calendula officinalis*) gave strongly positive reaction to BCMV with O.D. value of 3.890 and 3.513, respectively with BCMV antisera. Whereas, urdbean (*V. mungo*), pomegranate (*Punicum granatum*), cowpea (*V. unguiculata*) and castor (*Ricinis communis*) gave positive reaction as compared to positive control to potyvirus antisera. Our result is in agreement with *V. mungo*, while *Calendula officinalis* (Marigold) as a host of BCMV forms the first report in India. However, other host with their O.D. values were soybean (*Glycine max*) (2.268), *Solanum lycopersicum* (2.886), *V. unguiculata* (2.678), *Chenopodium album* (2.672), *Amaranthus viridis* (2.873), *Phaseolus vulgaris* (2.984) and *Ricinis communis* (2.872) which detected positively to BCMV as compared to positive control (Table 5). Similarly results were also found by Zaumeyer and Thomas, (1957) [34] on *Vigna unguiculata* and *Glycine max* by Verma *et al*., (1962) [32]. In present study Caster (*Ricinis communis*) and Tomato (*Solanum lycopersicum*) from the first report against the BCMV While, *Abelmoschus esculentus, Sesamum indicum, Arachis hypogaea, Lamiumam plexicaule, Azadirachta indica, Parthenium hysterophorus* and *Catharantus roseus* gave negative results as compared to positive control. Present results are in agreement with the findings by Galvez, (1980) [8]; Boswell and Gibbs, (1983) [3] and Morales and Bos, (1988) [21] for leguminous hosts of Potyvirus. In present investigation it was found that BCMV has close serological relationship with Potyvirus and *Punicum granatum* and *Ricinis communis* were detected for the first time as hosts of potyvirus.

Serology

Serological assays are the most reliable and highly sensitive methods for the detection of viruses. Among this "Enzyme Linked Immunosorbent Assay" (ELISA) is best suited for detection of plant viruses. DAS-ELISA technique was employed for the detection of the virus present in the mungbean. The infected leaves of IR-16, Meha and K-851 gave strong positive reaction with BCMV specific antisera with O.D. value of 3.99, 3.91 and 3.37, respectively (Table 6& Plate 2) as compared to positive control with OD value of 2.20. Similarly, the diseased plants of mungbean varieties *viz*., K-851, IR-16 and Meha showed high ELISA O.D. values of 3.25, 3.17 and 3.12, respectively, in comparison to positive control value of (2.50) and negative value of control (0.32) with potyvirus specific antisera (Table 7& Plate 3). While healthy leaves gave negative reaction with O.D. value of 0.13. The DAS-ELISA test confirmed the association of *Bean common mosaic virus* (BCMV)-a Potyvirus with the disease in mungbean.

Similar detection results were obtained for identifying BCMV with serotypes by indirect Enzyme-linked immunosorbent assay (ELISA) (Puttaraju *et al*., 2004) [26]. Potyvirus and BCMV could be detected by ELISA (Puttaraju *et al*., 1999; Khetarpal *et al*. 1994) [27, 17]. Polyclonal antiserum of Potato virus Y (PVY) has also been used for the detection of BCMV (Mishra *et al.*, 1997) [20]. Gnutova *et al.,* (2000) [9] developed indirect and "sandwich"- variants of ELISA to detect *Bean common mosaic virus*. Kapoor *et al*., (2009) [15] tested the presence of *Bean common mosaic virus* in French bean germplasm showing symptoms such as leaf rolling, leaf distortion, mottling, puckering, vein banding, stunted growth, etc by Double Antibody Sandwich–Enzyme Linked Immunosorbent Assay (DAS-ELISA).

Transmission electron microcopy

Detection of virus in plants, seeds, vegetative propagating materials is essential for developing disease management strategies. Histopathological investigations using Transmission Electron Microscopy (TEM) helps in determining shape and size of virus and detection of virus. Transmission electron microscope is used for detection of filamentous plant virus of BCMV. In present study the TEM was used to detect the BCMV in mungbean. The virus particles 823 nm long were detected as flexuous particles (Plate 4). Earlier workers have reported BCMV infecting mungbean as flexuous rod-shaped particles with an average length of 750 to 760 nm in Korea and elsewhere (Hong-SooChoi *et al*., 2006; Damayanti *et al.*, 2008 and Morales and Bos, 1988) [12, 5, 21]. However, Udayashankar *et al*., (2011) [30] reported that the particles of *bean common mosaic virus* infecting cowpea were flexuous rods measuring 750 – 925 x 15 nm.

Detection of BCMV through Polymerase Chain Reaction (PCR) using specific universal primers

RNA of BCMV was purified from heavily infected leaf samples of mungbean using Trizol reagent and optimal density of the RNA and quantity of RNA ng/µl were checked by Nanodrop (Table 8). cDNA strand was synthesized from this RNA using oligodT (dT_{18}) primer and reverse transcriptase enzyme and then cp gene/region was amplified by PCR using downstream and upstream primers. The RT-PCR amplified product when run on 1 per cent agarose revealed the presence of predicted ~1300 bp product (Plate 5). Sharma *et al*., (2009) [25] isolated viral RNA from BCMV infected leaves by trizol reagent. RT-PCR amplification of total genomic RNA generated an amplicon of ~1000bp with BCMV degenerate primers.

Flores-Estevez, *et al*., (2003) [7] used RNA as a template for RT-PCR (reverse transcription followed by polymerase chain

reactions) using specific primers directed toward the coat protein cistron and obtained the products of 890 bp and 740 bp for BCMV and BCMNV, respectively. Amplification of an 890 bp fragment was detected in ELISA positive of butter bean and kidney bean genotypes as well as some ELISA negative plants.

The studies confirmed the presence of BCMV in the symptomatic mungbean plants, with about 1300 bp product of cp gene.

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