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SERIODIAGNOSIS AND ELECTRON MICROSCOPY OF BEAN COMMON MOSAIC VIRUS (BCMV) INFECTING COWPEA IN SOUTHERN KARNATAKA, INDIA

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ABSTRACT
A Survey was carried out in eight districts of southern Karnataka, India to assess the incidence of bean common mosaic virus-black eye cowpea strain on cowpea (BCMV). Plants showing virus like symptoms were serologically tested with direct antigen coating enzyme- linked immune sorbent assay (DAC-ELISA) against potyvirus antisera, to confirm the virus. The virus was readily transmitted by sap inoculation as well as through Aphids to the cowpea cv. C-152 under glass house conditions. We observed systemic infection in green gram, black gram, soybean, French bean and groundnut and necrotic local lesions on *Chenopodium amaranticolor*. This virus is long flexuous filamentous particles approximately measuring 952nm in size.

INTRODUCTION

Cowpea [*Vigna unguiculata* (L) Walp.] is an important nutritive grain and fodder legume grown in many tropical and subtropical countries. It provides major sources of protein when consumed as grains for vegetarians in India. Hundred grams of green tender pods contain 4.3 g protein, 2.0g fibre, 8.0 g carbodydrates, 74 mg phosphorus, 2.5 mg iron, 13.0 mg vitamin-C and 0.9 mg minerals. Due to its high protein content (25%) in grains, cowpea has been referred to as "poor man's meat".

The main production constraints of pulses in general and cowpea in particular are biotic and abiotic stresses. Diseases are prominent among biotic stresses know to affect the productivity. Fungi, bacterial and viral diseases are considered as major limiting factors for the production of cowpea in the tropical and subtropical countries (Mali and Thottappilly, 1986).

More than 20 viruses are reported from various cowpea growing areas worldwide. Viral diseases were significantly contributing to the reduced yield of cowpea in Asia, Africa and Latin America. The effects of viruses could be devastating and were a major constraint to the production of cowpea (Thottappilly and Rossel, 1992). The major viruses affecting cowpea include bean common mosaic virus, black eye cowpea mosaic Potyvirus (BICMV), cowpea aphid-borne mosaic virus (CABMV), cowpea chlorotic mottle virus (CCMV), cowpea mild mottle virus (CPMMV), southern bean mosaic virus (SBMV), cowpea mosaic virus (CPMV), cucumber mosaic virus (CMV), cowpea chlorotic mosaic virus (CPCMV) and cowpea severe mosaic virus (CPSMV) (Thottappilly and Rossel, 1985; Hampten and Gubba, 1997).

Among all the viruses Bean common mosaic virus (BCMV) is the most widespread and frequent viruses of cowpea. It was first reported by Iwanovski (1894) from Russia and the virus described from U.S.A. by Stewart and Reddick (1917). The virus belongs to the genus Potyvirus (family Potyviridae) and is transmitted by aphids in a non-persistent manner. While the viruses could cause significant yield loss (Galvez *et al*., 1989), the seed-borne nature of these viruses would be an important hazard to seed increase and dissemination programs.

Keeping all this in view, present paper deals with seriodiagnosis and electron microscopy of Bean common mosaic virus (BCMV) infecting cowpea.

MATERIALS AND METHODS

Roving survey was conducted during *Kharif* 2012-13 in different districts of southern Karnataka to assess the status of *Bean common mosaic virus* - black eye cowpea strain. A minimum of five fields were selected randomly in each locations for assessing the disease status. Observations were also recorded on variety grown, stage of crop, rainfed and irrigated

and symptoms *etc*. The percent disease incidence (Shoyinka *et al.,* 1997) was calculated using the following formula

$$\% \text{ disease incidence } \frac{(\text{Total no. of infected plants})}{(\text{Total no. of plants observed})} \quad 100$$

Leaf samples from infected cowpea plants showing mosaic symptoms were also collected during survey and tested for virus by using DAC-ELISA.

Sap, aphid and seed transmission

Young leaves of 15-20 days old showing characteristic mosaic symptoms were collected from infected cowpea plants washed in tap water to remove the dust particles adhering to them and dried between the folds of blotting paper. The leaves were then macerated in chilled mortar and pestle using potassium phosphate buffer (pH 7.0, 0.05M) at the rate of 1ml/gm of leaf tissue. The resultant extract was squeezed through absorbent cotton and the extract thus obtained was used as standard inoculum.

Aphid (*Aphis craccivora*) was multiplied on cotton. Apterous adult aphids were collected from the host plant in a petriplate and starved for 30 minutes. For acquisition of the virus, young virus infected leaves were taken in another petriplate. Petioles of the leaves were embeded in moist cotton to keep them fresh and to prevent drying. The starved aphids were transferred on to these leaves and allowed to feed for one hour. After the acquisition feeding period, aphids were transferred to test plants at the rate of twenty aphids per plant for 24 hours inoculation feeding. After allowing for 24 hours of inoculation feeding, the aphids were killed by spraying the plants with 0.2 per cent Imidacloprid. The inoculated plants were kept in the insect proof glass house for twenty days for symptom expression. 25 plants in five replications were used to study aphid transmission. Seeds collected from cowpea (C-152) infected with mosaic disease were sown in seed pans at the rate of 20 seeds per pan in six replications along with 10 seeds from healthy plant as control and kept in the insect proof glasshouse. The plants were observed for disease symptoms upto a period of four weeks. The percent germination and rate of seed transmission were recorded (Bashir, M. and Hompton, R.O., 1994).

Host range, DAC-ELISA and electron microscopy

Studies were undertaken to know the host range for the virus. Plants of each species were raised in polyethylene bags. Plants were inoculated at primary leaf stage with standard extract of virus by mechanical sap inoculation as described earlier. In each plant species, ten plants were inoculated and one set of un-inoculated plants were maintained as control. Inoculated plants with were kept in the insect proof glass house and examined periodically for symptom expression. The plant species inoculated, were re-inoculated to *Chenopodium amaranticolor* plants to confirm the presence of virus. The symptoms expressed by the different plant species were recorded.

The final viral suspension that was obtained by purification was taken for electron microscopic studies. The formavar coated grids were floated on purified viral suspension for 10 minutes. Then, stained the grids with 2 per cent phosphotungstic acid (PTA) for five minutes and allowed to dry. After drying the grids were observed under JEOL 100S transmission electron microscope and taken the picture of viral particles (Bashir and Hampton 1995).

A direct antigen coating enzyme linked immuno sorbent asssay (DAC-ELISA) was performed using goat anti rabbit IgG-alkaline phosphatise conjugate to detect the virus causing mosaic disease on Cowpea. Cowpea leaves infected with mosaic were harvested and tissue was ground in coating buffer (1gm tissue in 1ml carbonate buffer) with the help of pestle and mortar. The extract was filtered through a layer of muslin cloth and transferred to a test tube. The filtrate thus obtained was treated as 1:1 and serial dilution of 1:10 and 1:100 was made out of the filtrate with coating buffer (Bashir *et al*., 1999).

ELISA plates (microtitre plates) was coated with test antigen 200μL per well in buffer and incubated for one hour at 37ºC temperature. After one hour incubation, excess antigen was removed by washing the plate thrice in PBS-T at three minutes intervals. The residual liquid was removed by gently tapping the plates against the pad of filter papers. All the unoccupied sites of wells were blocked with PBS-T and inoculated at 37ºC for one hour. The plates were washed in PBS-T as in step-2. Two hundred microlitre of antiserum in antibody buffer (1:500) was added to each well and inoculated at 37ºC for one hour. The plates were washed in PBS-T as in step-2. The substrate freshly prepared by dissolving p-nitrophenyl phosphate (1mg/mL) in substrate buffer was added at the rate of 200μL/ well and the substrate container was covered with aluminium foil to avoid auto-photo degradation of p-nitrophenyl phosphate. Absorbance was recorded at 30 minutes after adding the substrate at 405nm by Biotek microplate reader (EL 309).

RESULTS AND DISCUSSION

The results of the roving survey on the incidence of mosaic virus disease of cowpea crop in the 8 districts of southern Karnataka are presented in Table 1. The highest and lowest percent disease incidence ranged from 36.66 to 17.76 percent. Similar types of surveys were earlier documented by Puttaraju and Santhosan (2000). The field incidence of *Black eye cowpea mosaic virus* in Karnataka, India was found to be widespread and was recorded in 18 fields out of 21 fields surveyed. Disease incidence in the field varied from 1-70 per cent, based on weather condition and susceptibility of the variety.

The sap transmission per cent varied from 88.00 to 96.00 (Table 2). The inoculated plants exhibited symptoms such as vein clearing, vein banding and mosaic mottling on the newly formed subsequent leaves 12-20 days after inoculation (fig.1). The aphid was found transmitting the virus successfully to an extent ranges from 44.00 to 64.00 per cent (Table 2). The level of seed transmission of the virus varied from 33.33 to 47.07 per cent, with an average of 38.72 per cent (Table 3). The plant developed symptoms 7-10 days after sowing. The first symptom of seed borne infection appeared on cotyledonary leaves as fine vein clearing followed by mosaic mottling and vein banding on newly formed trifoliate leaves. Mali and Kulthe (1980) reported sap transmissibility of a potyvirus resembling *Black eye cowpea mosaic virus* causing mosaic in cowpea. Further Sharma *et al*. (2009) reported successful sap-transmission of *Black eye cowpea mosaic virus*

Table 1: Incidence of *Bean common mosaic virus* - black eye cowpea strain in different districts of Southern Karnataka during *Kharif* 2012-13

Sl.No	Districts	Name of the Location	Variety/Line	Per cent disease incidence	PDI (mean)
1	Bengaluru ruralNelamangala	Hesaragatta	Local variety	23.33	24.99
		IIHR	KBC-2	50.00	
		Shivakote	C-152	18.00	
		Aralasandra	Local variety	15.00	
		Doddabelekere	Local variety	18.66	
2	Bengaluru Urban	ZARS, GKVK	C-152	50.00	36.66
	Bengaluru north		Horticulture farm, GKVK	KBC-1	23.33
3	MysoreNanjangud	Badanavalu	Local variety	18.00	20.91
		Mullur	Local variety	17.33	
		Devanur	Local variety	24.66	
		Kalale	Local variety	26.66	
		Naveelure	Local variety	17.92	
	T.Narsipura	Talkadu	Local variety	23.00	19.45
		Madapura	Local variety	19.00	
		Hiriyur	Local variety	21.33	
		Jajur	Local variety	20.62	
		Kodihalli	Local variety	13.33	
4	ChamarajnagarGundlupet	Manchalli	Local variety	16.00	17.76
		Berambadi	C-152	17.33	
		Savakanahalli palya	Local variety	18.12	
		Terakanambi	C-152	17.77	
		Hangala	C-152	19.58	
5	KolarChinthamani	Devapalli	C-152	17.00	18.33
		Munganahalli	C-152	20.00	
		Dalsanoor palya	Local variety	18.67	
		Golahalli	Local variety	21.00	
		Nagdenahalli	Local variety	15.00	
6	RamanagarKanakpur	Narayanapura	C-152	20.66	17.20
		Kallahalli	C-152	17.64	
		Jakkasandra	Local variety	18.58	
		Kaggalahalli	C-152	17.50	
		Veeraiahandoddi	Local variety	11.66	
7	TumkurTiptur	Aralaguppe	C-152	20.00	19.05
		Honnavalli	C-152	18.00	
		Nuggehalli	Local variety	21.42	
		Adinaikanahalli	Local variety	16.80	
8.	Mandya	Shivahalli	C-152	16.66	18.22
		V.C.Farm	MFC-09-15	24.09	
		Dyapasandra	Local variety	16.00	
		Holalu	Local variety	16.15	

Table 2: Sap and Aphid transmissibility of *Bean common mosaic virus* - black eye cowpea strain in Cowpea

Experiment No.	Sap transmission No. of plants inoculated	No. of plants infected	Per cent transmission	Aphid transmission No. of plants inoculated	No. of plants infected	Per cent transmission
1.	25	22	88.00	25	11	44.00
2.	25	23	92.00	25	15	60.00
3.	20	18	90.00	20	9	45.00
4.	25	24	96.00	20	8	40.00
5.	30	28	93.33	25	16	64.00
Average	25	23	91.87	23	11.8	50.60

Cowpea variety – C-152; Number of aphids per plant - 20; Pre acquisition starvation – 30 min; Acquisition access period – 1 hr; Inoculation feeding period - 24 hr

(BlCMV).

The transmission studies of virus by aphid species revealed that the aphid transmitted the virus to an extent of 44.00 to 64.00 per cent. The inoculated plants exhibited symptoms such as vein clearing, vein banding and mosaic mottling on the newly formed subsequent leaves 10-20 days after inoculation. These results were similar to the reports of Murugesan and Janaki (1972) who studied relationship of BlCMV and *Aphis craccivora* and found single viruliferous aphid transmitting the virus to healthy cowpea plants. Mali and Kulthe (1980b) reported *Aphis gossypii* as a vector of the seed borne potyvirus causing mosaic on cowpea.

Table 3: Seed transmission of *Bean common mosaic virus* - black eye cowpea strain in Cowpea (C-152)

Experiment No.	No. of seeds sown	No. of seeds germinated	Germination(%)	No. of plants infected	Transmission(%)
1.	20	15	75.00	5	33.33
2.	20	18	90.00	7	38.88
3.	20	16	80.00	6	37.50
4.	20	19	95.00	7	36.84
5.	20	17	85.00	8	47.07
Average	20	17	85.00	6.6	38.72

Table 4: Host range of *Bean common mosaic virus* - black eye cowpea strain in cowpea

Sl.No.	Name of the host	No. of plants inoculated	No. of plants infected	Transmission (%)	Symptoms observed
	Leguminosae				
1.	*Vigna mungo* (L) Hopper	10	7	70.00	Mosaic mottling and vein clearing
2.	*V. radiata (L)* Wilzek	10	4	40.00	Mosaic mottling and leaf distortion
3.	*Arachis hypogaea* L.	10	5	50.00	Mosaic pattern and chlorotic lesions
4.	*Macrotyloma uniflorum* Lam	10	0	0.00	No symptoms
5.	*Cajanus cajana* (L.) Millsp	10	0	0.00	No symptoms
6.	*Cicer arietinum* L.	10	0	0.00	No symptoms
7.	*Vigna unguiculata subsp. s esquipedalis* (L.) Walp. [(L.) Verdc.	10	0	0.00	No symptoms
8.	*Phaseolus lunatus* L.	10	0	0.00	No symptoms
9.	*Phaseolus vulgaris* L.	10	9	90.00	Mosaic mottling and leaf distortion
10.	*Glycine max* (L.) Merr.	10	8	80.00	Chlorotic spot and mosaic pattern
	Cucurbitaceae				
11.	*Cucumis sativus* L.	10	0	0.00	No symptoms
12.	*Benincosa hispida* Thumb.	10	0	0.00	No symptoms
13.	*Cucurbita moschata* Duchsne	10	0	0.00	No symptoms
14.	*Memordica charantia* L.	10	0	0.00	No symptoms
	Solanaceae				
15.	*Nicotiana tobaccum* L.	10	0	0.00	No symptoms
16.	*Nicotiana glutinosa* L.	10	0	0.00	No symptoms
17.	*Lycopersicon esculantum* Mill.	10	0	0.00	No symptoms
18.	*Solanum nigrum* L.	10	0	0.00	No symptoms
	Chenopodiaceae				
19.	*Chenopodium amaranticolor* L.	10	10	100.00	Chloratic and necrotic local lesions

Table 5: Detection of mosaic causing virus in cowpea by DAC- ELISA

Treatment	OD values at 405 nm				
Buffer control	0.298	0.193	0.229	0.189	0.212
Healthy	0.304	0.218	0.445	0.302	0.301
Infected	0.741	0.423	0.909	0.826	0.628

The virus was found to be transmissible through seeds of cowpea cultivar C-152 was obtained from the infected plants. The per cent seed transmission ranged from 33.33 to 47.07 per cent. The plants developed symptoms after 7-10 days of sowing. 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 Sharma and Verma (1986), Bashir and Hompton (1994) studied seed transmission of some isolates of black eye cowpea mosaic, potyvirus.

Among the test plant species inoculated with the crude sap, green gram, black gram and ground nut showed systemic symptoms like mosaic mottling, vein clearing and vein banding after ten days of inoculation. Localized chloratic followed necrotic lesions were observed on leaves of *Chenopodium amaranticolor* four to five days after inoculation. The results are presented in Table 4. In host range studies, virus produced systemic symptoms on three species of Leguminosae family out of nine *viz., Vigna mungo* (L.), *Vigna radiata* (L.) and *Arachis hypogaea* Severe mosaic mottling, leaf distortion and chlorotic lesions are the major symptoms were observed in all the three species seven to ten days after inoculation. Localized chlorotic spots followed by necrotic lesions were observed on leaves of *Chenopodium amaranticolor* four to five days after inoculation.

The results on host range of virus are in conformity with the findings of Mali and Kulthe (1980a) and Murphy *et al.* (1987) who reported that the host range of *Black eye cowpea mosaic virus* infecting plant species of Leguminosae includes black gram, green gram and groundnut. They also reported that potyvirus resembling black eye cowpea mosaic virus produced necrotic lesion on *Gomphrina globosa* and chloratic and

Figure 1: Transmission studies of mosaic disease on cowpea a) Symptoms on Mechanically inoculated cowpea plants (severe mosaic with green patches). b) Symptoms on cowpea plants inoculated with viruliferous aphids (alternate green and yellow patches) (*Aphis craccivora,* Koch.).

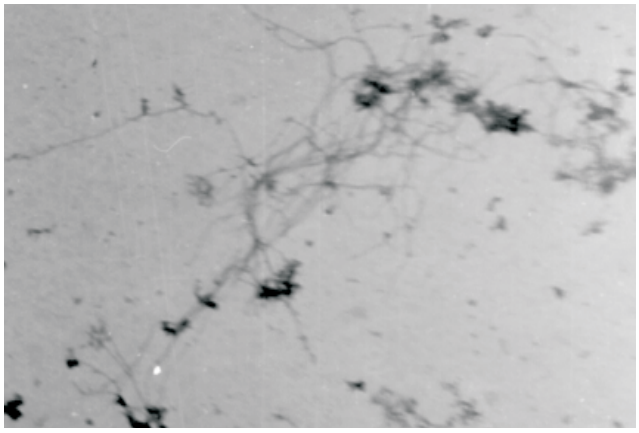

Figure 2: Flexuous virus particle as seen under TEM (magnification of 10,000X)

necrotic lesions on *Chenopodium amaranticolor*. Providentii (1986) observed that *Black eye cowpea mosaic virus* infected *Vigna mungo* producing diffused green mottle on newly formed leaves.

Electron microscopic studies revealed the presence of rod shaped long flexuous particles measuring approximately 952 nm in length in diseased samples of cowpea which were not found in healthy samples. These particles have morphology similar to those of potyviruses. Sharma and Chalam (2009) reported that sap-inoculated cowpea leaves revealed the presence of flexuous particles of 750-950 nm indicating the presence of a Potyvirus.

The use of Alkaline-phosphatase labelled Potyvirus specific antisera has greatly facilitated the identification of the virus by DAC ELISA. The direct antigen coating enzyme linked immune sorbent assay (DAC-ELISA) results revealed that the virus showed positive reaction to potyvirus antisera. Dijkstra *et al*. (1987) used alkaline phosphatise double antibody sandwich ELISA (DAS-ELISA) to establish serological relationship among strains of Black Eye cowpea mosaic virus followed by Gubba (1994).

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