Original Research Article

Detection of Cucumber Mosaic Virus (CMV) in Banana Cv. Karpura Chakkarakeli (AAB) by DAC-ELISA Method

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A B S T R A C T

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Cucumber mosaic disease, caused by *cucumber mosaic virus* (CMV), is a serious disease that affects the productivity of banana to a greater extent. The production of virus-free planting material is an efficient method in controlling this disease and increase productivity. Effective control of their spread depends on robust detection of these viruses in propagation stock, planting material, infected nursery plants and through strict quarantine. In vitro propagation, especially meristem tip culture has played a key role for obtaining a large number of virus free, homogenous planting materials in plantains and bananas (*Musa* spp.). In the present study mother plants of banana cv. Karpura Chakkarakeli (AAB) utilized for direct regeneration from immature male floral bud meristems for mass propagation were virus indexed by DAC-ELISA was found very effective and detected 10% CMV infected mother plants. The reliability and convenience of this assay makes it useful for plant quarantine and indexing plants for propagation.

Introduction

Bananas and plantains (*Musa* spp.) belongs to family Musaceae and the order Zingiberales, are large perennial herbs vital to food security in many tropical and subtropical countries (D'Hont *et al.,* 2012) across the world. They are the sixth-ranked food crop produced worldwide following maize, rice, wheat, potatoes and cassava with 139 million tons produced in 2012 (Kumar *et al.,* 2003). India ranks first in the world in banana production, with a total annual production of 30.80 million tons and it contributes to a lion's share of 34.4% of the total fruit production obtained from an area of 8.83 lakh ha. Andhra Pradesh is one of the leading producers of banana in India and is grown in

an area of 88.96 thousand ha with a production of 5.003 million tons and productivity of 56.24 tons ha^{-1} (National Horticulture Board, 2018). Bananas are propagated by suckers, division of the rhizome (corm) or by micropropagation (Israeli *et al.,* 1995).

The use of suckers and rhizomes as planting stock, however, is responsible for the spread of many pests and pathogens, especially viruses (Kumar *et al.,* 2015).

Banana bunchy top virus (BBTV), b*anana bract mosaic virus* (BBrMV), *banana streak viruses* (BSVs) and *cucumber mosaic virus* (CMV) are frequently reported infecting bananas globally (Thiribhuvanamala and Doraisamy 2001; Kumar *et al.,* 2009; Adegbola *et al.,* 2013; Amrita *et al.,* 2014; Boloy *et al.,* 2014; Wang *et al.,* 2014).

Cucumber mosaic virus causes a mosaic-like chlorosis and heart rot of bananas. It belongs to the type species, *Cucumber mosaic virus*, genus Cucumovirus in the family Bromoviridae. CMV has a segmented, tripartite, linear, single-stranded, positive-sense RNA genome composed of RNA1 (3.4 kb), RNA2 (3.1 kb), and RNA3 (2.2 kb) (Hu *et al.,* 1995).

Although resistant cultivars are the most convenient and effective way to control many plant diseases, banana plants with a high resistance to these viruses are not currently available (Kumar *et al.,* 2015).

Effective control is possible only through early identification and elimination of virus-infected plants followed by propagation and planting of virus-free material (Hu *et al.,* 1995). To aid in the rapid, convenient, sensitive and reliable identification of virus infections, Direct Antigen Coating - Enzyme-Linked Immuno Sorbent Assay (DAC-

ELISA) is one of the effective serological ways to identify the presence of the virus at earlier stages (Khan *et al.,* 2012).

Materials and Methods

In this experiment mother plants of banana cv. Karpura Chakkarakeli (AAB) were examined for major viral strain *cucumber mosaic virus* (CMV) which was most prevalent in the coastal regions of Andhra Pradesh.

Direct Antigen Coating - Enzyme Linked Immuno Sorbent Assay (DAC-ELISA) test was conducted to detect the presence of virus particles in the explants [mother plants of Karpura Chakkarakeli (AAB)] (Engvall and Perlmann, 1971) before selecting for *in vitro* culturing.

Materials required for ELISA testing:

ELISA plates

Several brands were available. Among them 'Nunc-Maxisorp' plates were selected.

Micropipettes

1-40 µl, 40-200 µl and 200-1000 µl single channel pipettes and 40-200 µl multichannel pipettes. Those with adjustable volumes were preferable.

ELISA plate reader

It is either manual or automatic fitted with 405 nm filter.

Polyclonal (rabbit) antibodies

Polyclonal antiserum to *cucumber mosaic virus* (CMV) was procured from National Research Centre for Banana (NRCB) Tiruchirapalli, Tamil Nadu, India.

Mortars and pestles, Muslin cloth, pH meter, Light box and Incubator.

Substrate solution

Dissolved 0.5 mg/mL p-nitro phenyl phosphate (PNPP) in 10% diethanolamine and adjusted the pH to 9.8 (for each 15 mg of tablet required 30 mL of substrate buffer). This solution was prepared as fresh prior to use.

DAC-ELISA procedure

Freshly dissected leaves of the 40 banana samples (designated as plant sample 1 to plant sample 40) were ground separately by using carbonate coating buffer in the ratio of 1:10 (w/v), in the homogenizer machine.

The homogenates were centrifuged at 3000 rpm for 5 min at 4°C.

The supernatant from above constituted the test samples, on which the DAC-ELISA was performed by using polyclonal antiserum to CMV.

Protocol for DAC-ELISA

Antiserum (antibody) of CMV was diluted in the ratio of 1:1000, with the coating buffer (pH 9.6).

The diluted antiserum was loaded (100 μl per well) in the microtiter plate.

Incubated the plate overnight at 4°C under moist humid chamber at 37°C for 1h.

After incubation, discarded the reagents quickly and washed the plate with three changes of PBS-T (pH 7.4), by allowing 3 min gap for each wash.

Added the test sample (100 μl per well) in the microtiter plate (each sample was added in

the duplicate wells). Added positive (antigen from infected source) and negative controls (antigen from uninfected source) of BBrMV in the respective wells. Also added carbonate buffer (no antigen) controls were included with all the experiments.

Covered the plates and were placed it in a humid chamber and incubated at 37 °C for 1 h or in a refrigerator at 4°C for overnight.

Discarded the reagents quickly and washed the plates with three changes of PBS-T (pH 7.4) by allowing 3 min gap for each wash.

Added 100 μl of PNPP substrate into each well, covered the plates and incubated under dark conditions at room temperature.

The plates were observed under X-ray film light box for recording colour changes. Results recorded after long intervals (>4 h) may not be accurate. In case of positive the colourless-substrate will turns to light yellow and then to deep-yellow colour. Light yellow colour indicates week positive and deep-yellow indicates a strong positive.

The reaction was stopped by addition of 50 μl of 3M NaOH per well.

Measured the absorbance at 405 nm in an ELISA plate reader.

Sample was considered infected by virus if its optical density (OD) value at 405nm, was twice or more of the mean value of the negative control.

Results and Discussion

Mother plants of banana cv. Karpura Chakkarakeli (AAB) were tested by DAC-ELISA in two batches against the commercially available polyclonal antisera of *cucumber mosaic virus* (CMV). The mean optical density (OD) values were recorded at

405 nm for two replicates of each of the forty samples (sample 1 to sample 40). The readings of buffer control (BC), positive control (PC) and negative control (NC) were recorded and were presented in Table 1.

The OD value of the test samples twice the negative control (NC) value were considered as positive for presence of virus in the sample. Visually, the virus infected samples had exhibited distinct yellow coloration in the substrate buffer aliquots held in micro titre plates.

Theoptical density (OD) values of the forty samples (sample 1 to sample 40) tested were less than twice the negative control (NC) value except for the plant samples 7(0.623), 15(0.577), 26(0.598) and 38(0.531) (Table 1) based on DAC-ELISA test.

Table.1

| Location of work	:	Horticultural Research Station, Kovvur
Technique used for indexing	:	Direct Antigen Coating - Enzyme Linked Immuno Sorbent Assay (DAC - ELISA)
Type of plant material used for indexing	:	Newly emerged 3rd or 4th leaf

Table.2

Name of Buffer	pH	Chemical	Formula	Amount (per 1000 mL)
Carbonate coating buffer (1X)	9.6	Sodium carbonate	Na_2CO_3	1.59 g
		Sodium bi-carbonate	$NaHCO_3$	2.93 g
Dissolved in 1L water, adjusted pH to 9.6 and stored at 4°C				
Wash Buffer Phosphate Buffer Saline with Tween (PBS-T) (1X)	7.4	Sodium chloride	NaCl	8.0 g
		Disodium phosphate	Na_2HPO_4	0.2 g
		Potassium dihydrogen orthophosphate	$KH_2 PO_4$	1.15 g
		Potassium chloride	KCl	0.2 g
		Tween-20		0.5ml
Dissolved in 800mL distilled water, adjusted pH to 7.4, made final volume up to 1000ml and stored at 4°C				
Enzyme conjugate buffer (1X) (PBS-TPB)	7.4	Polyvinyl Pyrrolidone MW 24-40,000	PVP	20.0 g
		Bovine Serum Albumin	BSA	2.0 g
Dissolved in 800ml PBS-T, adjusted pH to 7.4, made final volume to 1000 mL with PBS-T and stored at 4°C.				
Substrate Buffer	9.8	Magnesium chloride hexahydrate	$MgCl_2.6 H_2O$	0.1 g
		Diethanolamine		10 g
Dissolved 10g of diethanolamine in 50 mL of distilled water made up to 100 mL and adjusted the pH to 9.8 with concentrated HCl and stored at 4°C.				

Table.3 Indexing of banana cv. Karpura Chakkarakeli (AAB) for Cucumber Mosaic Virus (CMV) by using DAC-ELISA method.

Plant sample	OD of Sample	Cucumber Mosaic Virus (CMV)			ELISA Results
		OD of PC	OD of NC	OD of BC	
Plant Sample 1	0.253	0.931	0.227	0.194	Negative
Plant Sample 2	0.206	0.931	0.227	0.194	Negative
Plant Sample 3	0.238	0.931	0.227	0.194	Negative
Plant Sample 4	0.222	0.931	0.227	0.194	Negative
Plant Sample 5	0.144	0.931	0.227	0.194	Negative
Plant Sample 6	0.178	0.931	0.227	0.194	Negative
Plant Sample 7	0.623	0.931	0.227	0.194	**Positive**
Plant Sample 8	0.098	0.931	0.227	0.194	Negative
Plant Sample 9	0.087	0.931	0.227	0.194	Negative
Plant Sample 10	0.111	0.931	0.227	0.194	Negative
Plant Sample 11	0.103	0.931	0.227	0.194	Negative
Plant Sample 12	0.122	0.931	0.227	0.194	Negative
Plant Sample 13	0.107	0.931	0.227	0.194	Negative
Plant Sample 14	0.112	0.931	0.227	0.194	Negative
Plant Sample 15	0.577	0.931	0.227	0.194	**Positive**
Plant Sample 16	0.157	0.931	0.227	0.194	Negative
Plant Sample 17	0.106	0.931	0.227	0.194	Negative
Plant Sample 18	0.097	0.931	0.227	0.194	Negative
Plant Sample 19	0.129	0.931	0.227	0.194	Negative
Plant Sample 20	0.238	0.931	0.227	0.194	Negative
Plant Sample 21	0.177	0.931	0.227	0.194	Negative
Plant Sample 22	0.163	0.931	0.227	0.194	Negative
Plant Sample 23	0.151	0.931	0.227	0.194	Negative
Plant Sample 24	0.091	0.931	0.227	0.194	Negative
Plant Sample 25	0.068	0.931	0.227	0.194	Negative
Plant Sample 26	0.598	0.931	0.227	0.194	**Positive**
Plant Sample 27	0.111	0.931	0.227	0.194	Negative
Plant Sample 28	0.124	0.931	0.227	0.194	Negative
Plant Sample 29	0.137	0.931	0.227	0.194	Negative
Plant Sample 30	0.063	0.931	0.227	0.194	Negative
Plant Sample 31	0.117	0.931	0.227	0.194	Negative
Plant Sample 32	0.100	0.931	0.227	0.194	Negative
Plant Sample 33	0.073	0.931	0.227	0.194	Negative
Plant Sample 34	0.122	0.931	0.227	0.194	Negative
Plant Sample 35	0.112	0.931	0.227	0.194	Negative
Plant Sample 36	0.234	0.931	0.227	0.194	Negative
Plant Sample 37	0.147	0.931	0.227	0.194	Negative
Plant Sample 38	0.531	0.931	0.227	0.194	**Positive**
Plant Sample 39	0.132	0.931	0.227	0.194	Negative
Plant Sample 40	0.111	0.931	0.227	0.194	Negative

OD: Optical Density, **PC:** Positive Control, **NC:** Negative Control, **BC:** Buffer Control, **DAC-ELISA:** Direct Antigen Coated - Enzyme Linked Immuno Sorbent Assay

Plate.1 ELISA plate showing results of banana mother plant (cv. Karpura Chakkarakeli (AAB)) samples for *cucumber mosaic virus* (CMV). Samples showing positive results (yellow wells) for CMV: **(A)** Streaks on leaf lamina of banana plant infected with *cucumber mosaic virus* (CMV).
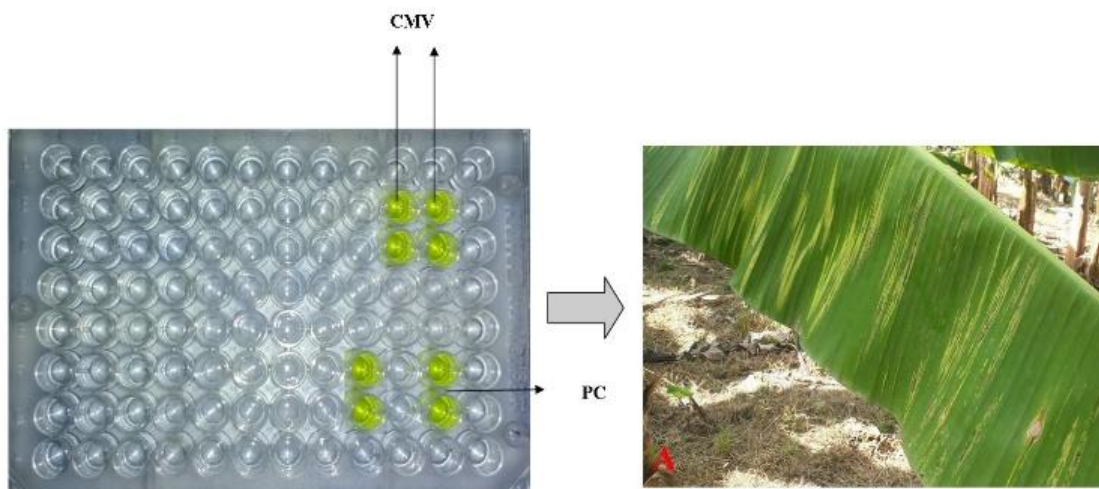
Of the forty plant samples tested 10% of samples recorded infection (plant samples 7, 15, 26 and 38) with CMV and were discarded from micropropagation studies (Plate 1). No yellow colour of the aliquots was noticed except for the plant samples (7, 15, 26 and 38) and the positive control (PC), visually recognized as positive for CMV.

The potential applicability of DAC-ELISA test on a large scale for indexing of banana cultivars to viral infections were earlier reported by Kiranmai *et al.,* (1996), Thottappilly *et al.,* (1998), Agindotan *et al.,* (2003), Dhanya *et al.,* (2007), Rajasulochana *et al.,* (2008), El-Dougdoug and El-Shamy (2011), Manoranjitham *et al.,* (2019) and Fidan and Koc (2019) (Table 2 and 3).

Devising an efficient method of diagnosing the infection of *cucumber mosaic virus* (CMV) is very important and development of such protocol will be of great help to the banana growers.

In the present study DAC-ELISA was found effective, reliable and economical method to detect CMV infected mother plants of banana cv. Karpura Chakkarakeli (AAB).

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the author.

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