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Detection and biophysical characterization studies of cucumber mosaic virus causing infectious chlorosis disease of banana

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Abstract
Cucumber mosaic virus (CMV) causing infectious chlorosis disease of banana was subjected to virus indexing and characterized on basis of biophysical properties. The present investigation results have revealed the occurrence and association of *Cucumber mosaic virus* with infectious chlorosis disease based on pathogenicity assay, serology (DAC-ELISA) and electron microscopy (EM). The virus developed necrotic localized symptomatic lesions on local lesion host, cowpea (*Vigna unguiculata*). DAC-ELISA was performed with crude sap, specific antiserum and alkaline phosphatase-linked secondary antibodies (1:10000 dilutions). The mean absorbance at 405 nm for negative and positive samples was 0.1255 and 0.413 respectively, while virus infected samples recorded two times more than the values of negative controls with values ranging between 0.322-0.413. The titre of antisera (1:1000) used in this study gave higher reactivity and it could give clear difference between healthy and infected samples. EM done by leaf dip method showed isometric particles of 28 nm in diameter. Biophysical properties of CMV isolate under investigation for Thermal inactivation point (TIP) is approximately between 65-70°C, the Dilution end point (DEP) ranged between 10-4 to 10-5, longevity *in vitro* got inactivated from 3rd day onwards at room temperature (28°C) and from 7th day onwards in refrigerated conditions (4°C).

Keywords: *Cucumber mosaic virus*, enzyme-linked immunosorbent assay, indexing, biophysical properties

1. Introduction
Banana (*Musa spp*.) the poor man's apple is native to the tropical region of south east Asia. Owing to its greater socio-economic significance and multifaceted uses, they are referred as 'Kalpatharu' (Plant of Virtues) and Kalpavriksh. It is one of the world's most important fruit crops and plays a critical role in the income security of many tropical and sub-tropical countries. In India, the crop is mainly grown in Tamil Nadu, Karnataka, Andhra Pradesh, Maharastra, Gujarat, Kerala, Assam, West Bengal, Bihar, Madhya Pradesh, Odisha, Chhattisgarh and Uttar Pradesh with the total area of 8.8 lakh ha producing 30 lakh tones. In Kerala, banana is grown in almost all districts with an area of 1.85 lakh ha and production of 11.6 lakh tones (DAC & FW, 2015) [8]. Diseases, particularly virus borne are considered as major threat due to the abundance of insect vectors, easily available collateral hosts and vegetative method of propagation. These are the biotic agents affecting the banana cultivation causing economic losses and threat to farming community. The viral diseases known to infect banana are banana bunchy top, banana streak, banana bract mosaic and infectious chlorosis disease. Of these, infectious chlorosis disease caused by *Cucumber mosaic virus* which is now emerging as a serious threat, affecting yield and wide spread in Kerala (Estellita *et al*., 1996) [11]. Intercropping banana with cucurbitaceous vegetables is a common practice in Kerala which led to an increase in the incidence of the disease. The potential effect of climate change also aggravated the spread of viruses through the vector. This disease is also known as heart rot, virus sheath rot, cucumber mosaic and banana mosaic (Stover, 1972) [37].
Infectious chlorosis diseased plants showing symptoms such as mosaic, leaf distortion, stunting of whole plant caused by *Cucumber mosaic virus* was first reported during 1943 in Jalagaon district in Maharashtra (Kamat and Patel, 1951) [17]. Since then, it was reported from Gujarat, Tamil Nadu, Karnataka, Andhra Pradesh, Uttar Pradesh and Kerala (Joshi and Joshi, 1976; Mohan and Lakshimanan, 1988; Estelitta *et al.*, 1996; Ramesh, 2009; Khan *et al.*, 2011; Ali *et al.*, 2012) [16, 30, 11, 33, 18, 1]. During 2015-2017, purposive sampling surveys were conducted in Thrissur District (Kerala). Banana plants exhibiting pale yellow or chlorotic discontinuous linear mosaic patches associated with prominent spindle or diamond shaped chlorotic lesions on leaf lamina,

young leaves were deformed and tapered towards leaf apex giving strap like appearance and leaf and fruit deformations accompanied by stunted plant symptoms were load, growth stage of plant and other factors influence the symptom expression in plants. Due to change in climatic conditions and nutritional deficiency**,** masking of visible symptoms occurs indicating Symptomology cannot be the sole criterion in identifying infected plants. However confirmed evidences were still lacking to confirm the causal agent causing infectious chlorosis disease of banana in Thrissur (Kerala). In this regard, the present investigations were undertaken with objective of identification of virus for their identity by performing such as serological assay, Transmission electron microscopy and biophysical characterization. These studies will provide more insight to study the detailed characterization of the virus causing infectious chlorosis disease of banana.

2. Methodology

The present investigation were undertaken to identify the causal agents based on serological testing and electron microscopic analysis. The research work was carried out at Banana Research Station, Kannara, Kerala Agricultural University, Thrissur, Kerala, India.

2.1 Collection and maintenance

The banana plants infected with infectious chlorosis disease showing various symptoms like mosaic, leaf distortion and stunting of plant were maintained at insect proof net house BRS, Kannara.

2.2 Pathogenicity assay

Extract was prepared by grinding symptomatic leaves of banana in pre chilled pestle and mortar with 0.1 M Potassium phosphate buffer (pH 7.2) (5ml/g tissue). The homogenate was filtered through double layered muslin cloth. Carborandum powder (600 mesh size) was uniformly dusted on the upper side of leaves and the filtrate was inoculated on local lesion host, cowpea (*Vigna unguiculata)* at two seedling stage and on two month old tissue culture of banana *var*. Nendran. The inoculated leaves were washed after 2-3 min using a wash bottle in order to remove any excessive inoculum and extraneous particles. One set of cowpea and banana plants were mock-inoculated with buffer as a negative control. Following inoculation, seedling were placed in insect proof net house and observed for the symptom development.

2.3 Detection of virus

2.3.1 *Serology (DAC-ELISA)*

A total of four plants maintained at insect proof net house showing variation in symptoms were indexed for presence of CMV**.** DAC-ELISA for detecting CMV was carried out in polystyrene plates (Cp-Star) using protocol described by Clark ad Adams, (1977) [7]. Antigen preparation included homogenization of leaf tissue in (10x) coating buffer (pH 9.2) using chilled pestle-mortor followed by centrifugation at 8000 rpm for 10 min at 4°C. The supernatant was loaded into ELISA plates and incubated for 2 h at 37°C. After incubation the plates were washed thrice with phosphate buffer saline with tween-20 (PBS-T). 100 µl blocking solution was added and incubated at 37°C for 1 h followed by washing with PBS-T. CMV specific antiserum was used at 1:1000 dilution was loaded into the well using antibody buffer to incubate overnight at 4°C. The plates were then washed with PBS-T and 100 µl of secondary antibody conjugated with alkaline phosphatase (1:10000) was loaded. After 2 h of incubation at 37°C plates were washed with PBS-T and freshly prepared 100 µl substrate buffer with *p*-Nitrophenyl phosphate (1 mg ml-1) was added in dark to incubate for 30 min at 37°C. The color reaction was read at 405 nm using ELISA plate reader (Vesamax micro titre plate reader). Buffer and healthy banana samples served as negative controls in all tests. Each sample was loaded in duplicate wells and the average A405 values obtained for three replicates was used in the analysis. The A405 values obtained for the samples were deducted from A405 values obtained for healthy sample. The resulting A405 values were then considered to be positive if its absorbance was twice the mean of two negative control wells.

2.3.2 Electron microscopy

To study the morphology of the virus particle associated with infectious chlorosis disease, infected plant samples were analyzed at Indian Agricultural Research Institute (IARI), New Delhi using JOEL-100 EX-II Transmission electron Microscope facility available at Advanced Centre for Plant virology, Department of Plant Pathology.

2.4 Biophysical properties

Biophysical properties of CMV isolate under investigations for Thermal Inactivation Point (TIP), Dilution End Point (DEP) and Longevity *in vitro* (LIV) were determined as per standard methods described by Noordam (1973) [31]. The indicator plant used for bioassay was cowpea (*Vigna unguiculata*).

2.4.1 Thermal Inactivation Point (TIP)

Aliquot of 5ml standard virus infected leaf extract was taken in eleven glass test tubes and each test tube was then individually exposed to temperature of 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75°C maintained in water bath for 10 min. The treated sap from each test tube was mechanically inoculated on set of ten cowpea seedlings at two leaf stage for each temperature treatment. The control was maintained by inoculating the leaves of test plant with unheated standard virus infected leaf extract kept at room temperature (28°C). The inoculated plants were kept in insect proof net house and periodically observed for the development of local lesions and recorded the number of local lesions developed on each.

2.4.2 Dilution End Point (DEP)

The experiment was conducted to determine infectivity of the plant sap of the virus infected plants after serial dilution. A series of dilutions *viz.*, 1: 10-1, 1: 10-2, 1: 10-3, 1: 10-4, 1: 10-5, 1: 10-6, 1: 10-7, and 1: 10-8 were prepared from standard leaf extract using distilled water. These diluted saps were inoculated on cowpea seedlings at two leaves stage starting from the lowest dilution to highest dilution and undiluted standard crude sap served as control. The inoculated plants were kept in insect proof net house and observed regularly for the expression of symptoms. The observations on number of local lesions were recorded.

2.4.3 Longevity *In vitro* (LIV)

Standard leaf extract from the disease plants was kept in two different conditions *viz.*, room temperature (24±2°C) and at cool temperature (40C) which were used to inoculate at fixed intervals *i.e.* 2 hrs, 3 hrs, 4 hrs, 6 hrs, 8 hrs, 10 hrs, 12 hrs, 24 hrs, 2 days, 3 days, 4 days, 6 days, 7 days, 8 days, and 9 days. Inoculation made soon after preparation of crude extract (0 h) served as control. The inoculated plants were labeled and kept

in insect proof net house and observations of local lesion development were recorded.

3. Results and Discussion

3.1 Collection and maintenance

In January 2016, banana plants from various fields showing symptoms such as linear discontinuous chlorotic lesions, inter-veinal chlorosis, spindle shaped chlorotic lesion, mosaic with extreme distortion, reduction of leaf lamina and Stunting of plants were observed (Fig. 1 -6) respectively and collected to maintain at insect proof net house, BRS, Kannara. The salient symptoms recorded were different patterns of mosaic, linear discoloration on midrib and petiole and various degrees of leaf deformation. Similar type of symptoms was also reported by Magee (1940) [26]. This was one of the early reports of the disease. Subsequently other researchers also reported similar symptoms on leaf lamina such as yellow streak like mosaic patterns, reduction in size and inward curling of leaf lamina and necrosis of leaves (Yot-Dauthy and Bove, 1966; Capoor and Varma, 1968; Capoor and Varma, 1970; Stover, 1972; Mali and Deshpande, 1975; Mali and Rajegore, 1979; Bouhida and Lockhart, 1990; Jones, 1991; Jones and Lockhart, 1993; Lepcha *et al.*, 2017; Vishnoi *et al.*, 2013) [4, 5, 39, 37, 28, 29, 3, 14, 23, 15, 38]. These plants were categorized into four different isolates according to the symptoms as mentioned (Table. 1).

3.2 Pathogenicity assay

The virus in the present investigation was found to develop localized symptoms of necrotic local lesion on cowpea (*Vigna unguiculata*) (Fig. 7) within 48 h after sap inoculation with 100 per cent of virus transmission. However, the transmission of CMV from banana to banana was not successful by mechanical transmission. Mechanical inoculation method has been tried from banana to banana by earlier workers with negative results (Capoor and Varma, 1968; Mali and Rajagore, 1980) [4]. The failure of mechanical transmission by the leaf rub method has been attributed to the production of tannins and phenols in the inoculated test plants due to the injury caused during the process which activates the host response at molecular level to synthesis the overall general chemical substance to act as defense against any foreign substance (Benigno, 1963; Yot-Dauthy and Bove, 1966) [2, 39].

3.3 Detection of virus

3.3.1 Detection of CMV by ELISA

In the present study, attempts were made for serodiagnosis of the virus from infected and apparently healthy banana plants were validated using commercially available antiserum. Enzymed linked immunosorbant assay (ELISA) indexing protocols, using polyclonal antiserum which is a sensitive test for detection and identification of virus. The positive reaction of crude sap from disease plants was obtained from the dilution 1:1000 diluted antibody. This method was found to be very efficient in detecting symptom less plant. The results revealed that positive plants recorded a mean absorbance (A405 values) of 0.413 which recorded more than twice the mean absorbance of negative control (0.1225). Results of four plants showing different infectious chlorosis symptoms from insect proof net house recorded positive with little variation in absorbance value. In general higher titers of virus was recorded in positive plant followed by plant showing Chlorotic lesion, mosaic with extreme distortion and reduction of leaf lamina recording absorbance value of 0.358 (Table 1). Similar findings were reported by earlier workers (Kiranmai *et al.*, 1996; Selvarajan *et al.*, 2011; Kouadio *et al.*, 2014; Khan *et al.*, 2012; Lepcha *et al.*, 2017; Dheepa and Paranjothi, 2010; Ali *et al*., 2012; Khan *et al*., 2011) [20, 36, 22, 18, 23, 9, 1, 19] reported the similar reactions of CMV to the antibody.

3.2 Electron microscopy

The morphology of the virus associated with infectious chlorosis disease of banana was studied by taking electron photomicrographs and revealed the presence of spherical isometric virus particles of size 28.5 nm (Fig. 8). This confirms that the pathogen is *Cucumber mosaic virus* which belonged to genus Cucumovirus, Family Bromoviridae. Similar particle size of 28-30 nm, and morphology for CMV (Banana) was reported earlier by many workers (Lockhart and Fischer, 1976; Holcomb and Valverde, 1991; Kiranmai *et al*., 1997; Doomar *et al*., 1990; Vishnoi *et al*., 2013; Madhubala *et al.*, 2005; Rostami *et al.*, 2014; Samad *et al.*, 2008 and Ali *et al.*, 2012) [24, 12, 20, 10, 38, 25, 34, 35, 1].

Table 1: Different isolates of CMV collected on basis of symptoms

Isolate no.	Symptom	Reaction to CMV antisera	OD Value at 405 nm	Mean absorbance
1	LDCL and IC	+	0.322	0.333
2	LDCL, IC and R	+	0.325	
3	MD	+	0.330	
4	SSCL	+	0.358	
+ ve	CL, MD and R	+	0.413	0.413
Control	-	-	0.1225	0.125

LDCL = linear discontinuous chlorotic lesions, CL = Chlorotic lesion, IC = Interveinal chlorosis of leaf, SSCL = Spindle shaped Chlorotic lesions of leaf, MD = Mosaic with extreme distortion and R = Reduction of leaf lamina

3.4 Biophysical properties

3.4.1 Thermal inactivation point

In the present study, it was observed that the plants inoculated with crude sap (28°C) showed 100% transmission producing maximum number of local lesions 58 (Fig. 9). The sap exposed to 30°C and 35°C temperature showed 97 and 90 per cent transmission respectively. Then with the increase in temperature there is gradual decline in the number of local lesions which showed the loss of infectivity of the virus particle at higher temperature. The crude sap subjected to 65°C was found to be least infective with only 5 percent transmission. Beyond 65°C there was no infectivity. From the results it is concluded that the thermal inactivation point of the virus ranged between 65-70°C.

3.4.2 Dilution end point

The undiluted crude sap recorded 100 per cent transmission with maximum number of local lesions (54) the sap remained its infectivity up to the dilution of 10-4. From this experiment it was concluded that the dilution end point of the virus is 10-4 (Fig. 10).

3.4.3 Longevity *in-vitro* (LIV) at room temperature (24-28°C)

The studies on LIV done at room temperature revealed that maximum 100 per cent transmission was observed when inoculated with crude sap immediately after extraction from infected leaves. Gradual decrease in per cent transmission was observed when the crude sap was kept at room temperature for different periods. The results showed that the virus could retain infectivity up to 3 days and got inactivated from 3rd day onwards when kept at room temperature (Fig. 11).

3.4.4 At refrigerated conditions (4°C)

When stored at refrigerated conditions (4°C), the infectivity of the sap was retained upto seven days. A gradual decline in the per cent transmission was observed by storing the extract in refrigerator (4°C). However, over the period of 4 hours of storage under refrigerated conditions, the crude sap retained the infectivity with maximum transmission of 94%. The infectivity of the virus was retained up to 7 days (Fig. 12).

For the above experiment it was observed that the LIV of the crude sap was 2 and 7 days at room temperature and refrigerated conditions respectively.

Fig 1: Linear discontinuous chlorotic lesions

Fig 2: Interveinal chlorosis

Fig 3: Spindle shaped chlorotic lesion

Fig 4: Mosaic with extreme distortion

Fig 5: Reduction of leaf lamina

Fig 6: Stunting

Fig 7: Local lesion host Cowpea (*Vigna unguiculata*)

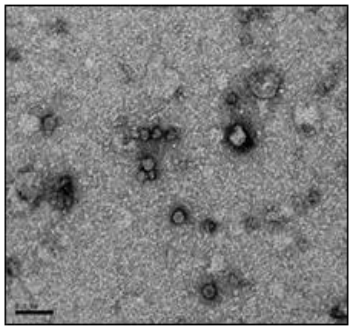

Fig 8: Electron micrograph showing isometric particals of CMV (28.5 nm) (120000 X magnification)

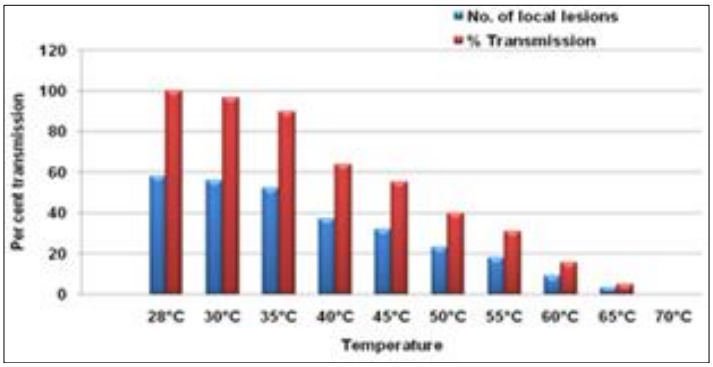

Fig 9: Effect of temperature on per cent transmission of the virus (Thermal inactivation point)

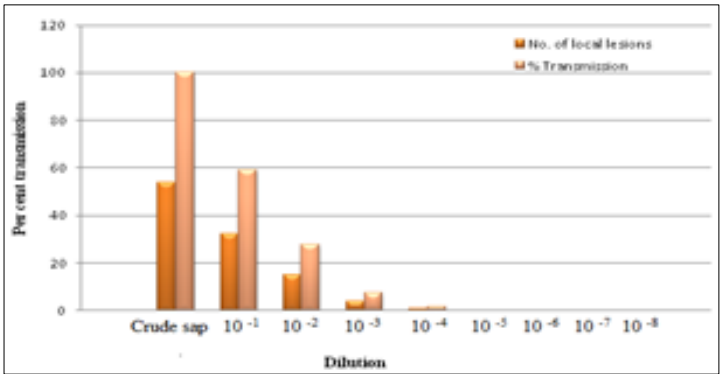

Fig 10: Effect of dilution on per cent transmission of the virus (Dilution end point)

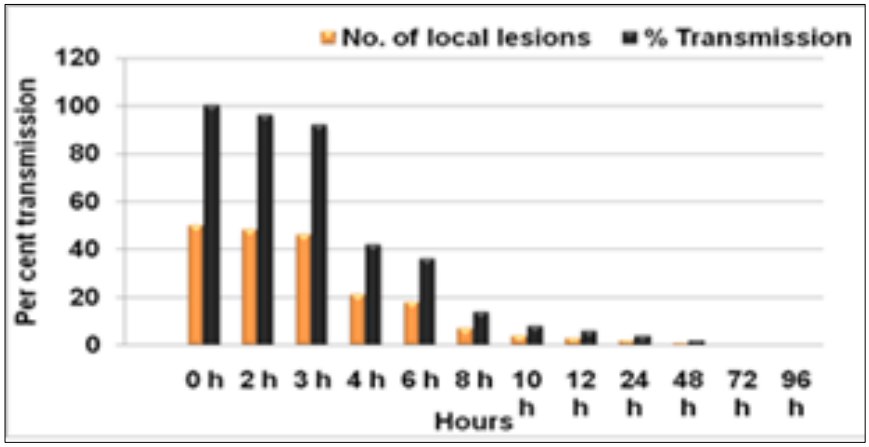

Fig 11: Effect of storage time on per cent transmission of the virus at room temperature (Longevity *in-vitro* point).

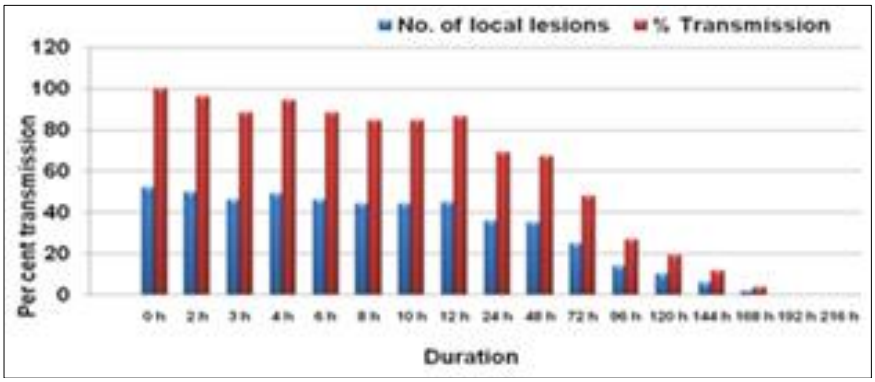

Fig 12: Effect of storage time on per cent transmission of the virus at refrigerator conditions (Longevity *in-vitro* point).

All the physical properties *viz*. TIP, DEP and LIV of CMV fall in the range of those reported earlier for CMV strains elsewhere (Mali and Rajagore, 1980; Hull, 2002, Parvin *et al*., 2007; Chandankar *et al*., 2013) [13, 32, 6]. However, differences or a similarity was found in DEP, LIV and TIP among the present CMV isolate elsewhere. The TIP, DEP and LIV of the present CMV have dissimilarity to those reported earlier. This might be due to high humid and temperature conditions.

4. Conclusion

In this present investigation based on pathogenicity assay, Detection of virus by pathogenicity assay, DAC-ELISA, electron microscopic studies clearly indicates that the occurrence and association of *Cucumber mosaic virus* with infectious chlorosis disease of banana. concluded that the virus causing infectious chlorosis disease of banana in this tract may be caused by spherical isometric shaped virus particles of size 28 nm confirming that it is a strain of CMV and belongs to genus: Cucumovirus and family: Bromoviridae. However further molecular characterization will be carried for confirmation.

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