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Occurrence and Distribution of Sri Lankan Cassava Mosaic Virus in the Stems of Cassava

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Abstract

The distribution of Sri Lankan cassava mosaic virus (SLCMV) in the buds regenerated from the nodes of stem cuttings varied with different varieties. Using different diagnostic techniques (ELISA, PCR and NASH), the SLCMV distribution in 8 varieties of cassava viz., Sree Sahya, Sree Prakash, Sree Visakham, Sree Padmanabha, Sree Vijaya, M-4, H-165 and H-226 were diagnosed. Top portions (one third of total stem used for planting) of the stems in all the varieties tested had maximum virus accumulation except Sree Padmanabha, which showed high virus accumulation in bottom portion. Similar results were obtained from the diagnosis of bark samples of all the varieties with variation in the virus accumulation level.

Key words: *Sri Lankan cassava mosaic virus,* virus accumulation, cassava varieties, Enzyme-Linked Immunosorbent Assay (ELISA), Polymerase chain reaction (PCR), Nucleic Acid Spot Hybridization (NASH)

Introduction

Cassava (*Manihot esculenta* Crantz) is a semi woody perennial plant and a staple crop with great economic importance and the only edible cultivated dicotyledonous species in the genus *Manihot* (Family-Euphorbiaceae) where its evolutionary and geographical origins have remained both unresolved and controversial (Olsen and Schaal,1999). In India, cassava is grown in an area of 2.28 lakh ha with a production of 46.51 lakh tonnes (FAO 2018) for both consumption as well as starch based industrial use. Cassava Mosaic Disease (CMD) is the most important and devastatint disease in all cassava growing areas of the world. In India it causes yield loss of 17-88% depending on the cultivars grown (Malathi et al., 1985). CMD in India is caused by two viruses namely, *Indian Cassava Mosaic virus* (ICMV) and *Sri Lankan Cassava Mosaic virus* (SLCMV) in the genus *Begomovirus* (Family-Geminiviridae). They have two genomic components viz; DNA-A (encodes functions associated with viral replication and encapsidation) and DNA-B (encodes the movement protein functions). Both the components are required for infectivity (Bock,1982). Primary spread of the viruses occurs through infected cuttings, which are the usual mode of cassava propagation (Fargette et al., 1988) while secondary spread occurs in field through transmission by the whitefly *Bemisia tabaci*.

Cassava Mosaic Disease (CMD) can be effectively controlled by the cultivation of resistant cassava genotypes (Terry and Hahn 1980; Otim-Nape et al., 1994). Cell to cell or long-distance movement of virus through phloem sap is a key factor in determining viral host range and the inhibition of either method of movement is a common resistance mechanism to viral infection. Restriction of long-distance movement may be caused by the inhibition in loading of virus into the phloem and was shown at bundle sheath cell-phloem cell interfaces in some cases (Kobori et al., 2003). Cassava genotypes resistant to CMD under field conditions produce healthy plants from infected stem cuttings used for planting (Pacumbaba, 1985), a phenomenon described as reversion (Fauquet et al., 1987). Some shoots of the infected plants of these genotypes show differential

symptom expression (Jennings, 1960; Fargette et al., 1996). These observations suggest that it is due to incomplete systemic invasion of the genotypes by *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV). Restriction of virus movement into axillary buds in all the CMD resistant cassava genotypes explains the production of healthy plants in subsequent generation from ACMV infected stem cuttings (Ogbe et al., 2002). The reported reversion by the crop (Pacumbaba, 1985; Thresh et al., 1998) is therefore most likely due to the prevention of invasion of virus to the bud during the growth of plant, which serves as a self cleansing mechanism in the resistant genotypes. Because begomoviruses are moving through phloem tissue (Horvat and Verhoyen 1981), the flow of assimilate into storage root might partly enhance the downward movement of the virus leaving most of the secondary and tertiary stems uninfected (Storey and Nichols 1938).

It is essential to rapidly diagnose pathogens in planting material to obtain healthy crop. The applications of advanced techniques in molecular biology like Enzyme Linked Immunosorbent Assay (ELISA), Polymerase Chain Reaction (PCR) and Nucleic acid spot hybridization (NASH) in plant pathology has greatly improved the ability to detect the plant pathogens (Martin et al., 2000; Makeshkumar et al., 2005). The cultivation of resistant cassava genotypes or use of disease free planting material effectively overcome the problem of CMD. In the same plant, leaf to leaf variation in intensity of symptoms was very high and no uniform pattern in symptom expression was seen. Some parts of infected plants appear to be healthy and the virus titers remain low but when such plants are used as planting material, the disease will appear. In view of these facts the present study was under-taken to understand the virus distribution/accumulation pattern in the cassava stem (bottom to top) by growing them as two nodes cutting and confirming the presence or absence of virus accumulation using three molecular diagnostic techniques viz., ELISA, PCR and NASH.

Materials and Methods

The important experiments carried out for understanding the distribution of SLCMV along the full length of cassava plant shoots is as follows:

Sample collection

In order to investigate the distribution pattern of virus in the infected cassava stems grown in high disease pressure field conditions, nine months old fifteen stems (full stem) from each genotype, viz., Sree Sahya, Sree Prakash, Sree Visakham, Sree Padmanabha, Sree Vijaya, M-4, H-165 and H-226 were collected from the field of ICAR-CTCRI. Two node cuttings were made from bottom to top (as shown in Fig. 1) of each stem after eliminating the portions normally not used for planting, planted in pots containing sterile soil and maintained in an insect proof green house. After five to seven days, sprouting was observed from each bud. Similarly, to study the SLCMV accumulation in the barks of cassava stem tissues, another set of ten stems from each variety were collected. In this case the total stem was divided into three portions (as one third of total stem) from the base and named as bottom, middle and top portion (Fig.1).

Diagnosis of SLCMV distribution in plants by various methods

For the diagnosis of virus distribution along the full length of stem used for planting, leaves emerged from all the two node cuttings planted as described earlier were collected from aforementioned 8 cassava cultivars (Fig.1). These samples were subjected to nucleic acid based

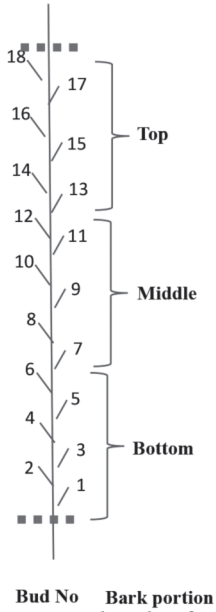

Fig.1. Bud and bark position details of SLCMV infected stem of cassava plant (after removing the portion not suitable for planting) selected for studying the virus distribution

detection (PCR, NASH) and serological detection (ELISA) for the diagnosis of SLCMV in the full length of plant stem.

Serological method

To study the SLCMV accumulation in regenerating shoots of cassava varieties in ELISA, leaves emerging from the two nodes planted as per their position were collected from the aforementioned 8 varieties grown in insect proof green house. Samples were weighed, ground in mortor, diluted 1:2 (w/v) with extraction buffer (0.05M carbonate buffer pH-9.6) and SLCMV accumulation was assayed using triple antibody sandwich enzyme linked immumosorbent Assay (TAS-ELISA) (Clark and Adams 1977) using the SLCMV specific antibody (obtained from DSMZ, Germany). Cassava plantlets derived from meristem culture, indexed for free from virus were used as virus free control. The enzyme reactions were assayed after half an hour at room temperature with an ELISA reader at 405nm. Positive reactions were considered as those with absorbance values having two times above those of the virus free controls. For detection of SLCMV accumulation in barks, the selected stem was divided into three portions ie., top (T), middle (M) and bottom (B) (Fig.1) of aforementioned 8 cassava varieties collected from the field. Single disc from each nodal portion of approximately 0.5cm in length were made from all the nodes present in that respective portion and after pooling them, two discs were randomly selected for each portion of the stem (T, M and B), then weighed, ground in mortor, diluted 1:2 (w/v) with extraction buffer (0.05M carbonate buffer pH-9.6) and SLCMV accumulation was assayed in TAS-ELISA. This was done with five stems per variety as replication.

Nucleic acid based methods

For the detection of SLCMV in collected samples, total DNA was isolated from these plant samples (leaves emerged from bud and discs made from bark) as per the protocol described by Lodhi et al., (1994) and the DNA obtained was dissolved in 50 µl of TE buffer and stored at –20°C. Polymerase chain reaction (PCR) and nucleic acid spot hybridization (NASH) technique were the nucleic acid based techniques (Makeshkumar et al., 2005) used in this study.

PCR

PCR were performed with 5 µl of total DNA isolated from the bud and bark samples of 8 cassava varieties with SLCMV coat protein gene specific primers CP-H (ATTCGAAGCGACCAGGAGAT) and CP-B (GACTGA CCGTGTTGAGCAGTC). Reactions were run in a thermal cycler with initial denaturation at 94°C for 2 minutes, followed by 30 cycles of each with denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes and extension at 72°C for 3 minutes and then final extension of 72°C for 5 minutes. At the end of reaction, the samples were analysed in 1% agarose gel electrophoresis.

NASH

Five µl of isolated total DNA of bud and bark samples of 8 cassava varieties were spotted on nitrocellulose membrane and performed NASH technique based on the protocol of Phototop Star Detection Kit-(NEB). Biotin labeled probe of full coat protein gene of SLCMV was synthesized using NEB probe labeling kit and used for detection. Coat Protein gene of SLCMV cloned in pGEM-T Easy vector was used as positive control and DNA of meristem derived and indexed plant of variety H-226 and Tris EDTA buffer were also used as a control.

Above experiments were repeated three times and pooled data are presented here.

Results and Discussion

Among the SLCMV infected cassava varieties collected from the field conditions, leaves emerged from all the two node cuttings grown from a stem of Sree Prakash, Sree Visakham, Sree Sahya, M-4 and H-165 showed symptoms of mosaic disease. Leaves emerged from two nodes cuttings of Sree Vijaya did not show symptoms of mosaic disease except the top three two node cuttings, which showed mosaic specs. Leaves emerged from two node cuttings of variety Sree Padmanabha did not show any symptom of mosaic in all the portions. In variety H-226, leaves emerged from bottom most two nodes did not show symptom, but leaves emerged from all other two node cuttings showed mosaic to distorted leaf symptoms. Leaves emerged from all the two node cuttings of Sree Prakash showed severe symptoms of mosaic and leaf distortion.

Leaf emerging from each bud were weighed and were ground with two volumes of extraction buffer and aqueous layer obtained after centrifugation was used as antigen. All the leaf samples of seven cultivars showed positive results in TAS-ELISA except the bottom most samples of Sree Vijaya (1 and 2) and H-226 (1) (Table.1). Similarly, bark samples of all the above cultivars showed positive results in TAS-ELISA except the middle region of Sree Sahya and bottom region of Sree Vijaya (Table 2).

Table 1. Reaction of different buds of stem of cassava with different diagnostic techniques for SLCMV accumulation

Variety	Stem portion	Bud sample number	Reaction observed*		
			ELISA	PCR	NASH
Sree Sahya	Bottom	1	+ + +	+	+
		2	+ + +	+	+
		3	+	+	+
	Middle	4	+	+	+
		5	+	+	+
		6	+ +	+	+
	Top	7	+ + +	++	+
		8	+ + +	++	++
		9	+ + +	++	++
Sree Visakham	Bottom	1	+	+	+
		2	+	+	+
		3	+	+	+
		4	+	+	+
		5	+	+	+
		6	+	+	+
	Middle	7	+	+	+
		8	+	+	+
		9	+	+	+
		10	+	+	–
		11	+	+	+
		12	+	+	+
	Top	13	+	++	+
		14	+ +	++	+
		15	+ + +	++	++
		16	+ + +	++	++
		17	+ + +	++	++
Sree Prakash	Bottom	1	+ + +	++	++
		2	+ + +	++	++
		3	+ + +	++	++
		4	+ + +	++	++
		5	+ + +	++	++
	Middle	6	+ + +	++	++
		7	+ +	++	++
		8	++	++	+
		9	+	++	+
		10	+	+	+
	Top	11	+	+	+
		12	+ +	++	+
		13	+ +	++	++
		14	+ +	++	++
		15	+ +	++	++
		16	+ + +	++	++

	Bottom	1	+ +	++	++
		2	+ +	++	++
		3	+ +	++	+
		4	+	+	+
		5	+	+	+
		6	+	+	+
Sree Padmanabha	Middle	7	+	+	+
		8	+	+	+
		9	+	+	+
		10	+	+	+
		11	+	+	+
		12	+	+	+
	Top	13	+	+	+
		14	+	+	+
		15	+	+	+
		16	+	+	+
		17	+	+	+
		18	+	+	+
	Bottom	1	−	−	−
		2	−	−	−
		3	+	+	−
		4	+	+	+
Sree Vijaya	Middle	5	+	+	+
		6	+	+	+
		7	+	+	+
		8	+	++	+
	Top	9	+ +	++	+
		10	+ +	++	++
		11	+ + +	++	++
	Bottom	1	+	+	+
		2	+	+	−
		3	+	+	−
M-4	Middle	4	+	++	+
		5	+	++	+
		6	+ +	++	++
	Top	7	+ + +	++	++
		8	+ + +	++	++
	Bottom	1	+	+	+
		2	+	+	+
		3	+	+	+
		4	+	+	+
		5	+ +	++	+
H-165	Middle	6	+ +	++	+
		7	+ + +	++	+
		8	+ + +	++	++
		9	+ + +	++	++
		10	+ + +	++	++
	Top	11	+ + +	++	++
		12	+ + +	++	++
		13	+ + +	++	++
		14	+ + +	++	++
		15	+ + +	++	++
		16	+ + +	++	++

H-226	Bottom	1	−	+	−
		2	+	+	−
		3	+	+	+
		4	+	+	+
	Middle	5	+ +	++	+
		6	+ +	++	+
		7	+ + +	++	+
		8	+ + +	++	+
	Top	9	+ + +	++	++
		10	+ + +	++	++
		11	+ + +	++	++

*ELISA - Absosrbance at 405nm
< 0.023: -ve; 0.024--0.100: +ve; 0.101-0.200: ++ve; > 0.201: +++ve
PCR / NASH : No band / spot : -ve; Faint band/spot: +ve; Less bright band/spot : ++ve; Bright band / spot: +++ve

Table 2. Reaction of different portion of bark samples with different diagnostic techniques for virus accumulation

Genotypes	Bark sample region	Reaction observed*		
		ELISA	PCR	NASH
Sree Sahya	Bottom	+	+	−
	Middle	−	−	−
	Top	+	+	+
Sree Visakham	Bottom	+	+	−
	Middle	+	+	+
	Top	+	+	+
Sree Prakash	Bottom	+	+	+
	Middle	+	++	++
	Top	+ +	++	++
Sree Padmanabha	Bottom	+	+	+
	Middle	+	+	−
	Top	−	+	−
Sree Vijaya	Bottom	−	−	−
	Middle	+	+	+
	Top	+	+	+
M-4	Bottom	+	+	−
	Middle	+	+	+
	Top	+	+	+
H-165	Bottom	+	+	−
	Middle	+	+	+
	Top	+	++	+
H-226	Bottom	+	+	−
	Middle	+	+	+
	Top	+	++	+

*ELISA - Absorbance at 405nm
< 0.023: -ve; 0.024--0.100: +ve; 0.101-0.200: ++ve; > 0.201: +++ve
PCR / NASH : No band / spot: -ve; Faint band/spot: +ve; Less bright band/ spot: ++ve; Bright band / spot: +++ve

Analysis of PCR product in gel electrophoresis showed accumulation of SLCMV in the form of amplification in all the samples except the bottom most samples (1 and 2) of Sree Vijaya which was negative (Fig.2). Samples from top portions of all varieties showed good amplification whereas samples from middle and bottom portion showed faint amplification except variety Sree Padmanabha in PCR analysis. In Sree Padmanabha, bottom most three samples (1-3) showed good amplification and all others showed faint amplification.

Bark samples of top portion of stems from all varieties showed faint amplification except Sree Padmanabha which showed negative amplification (absence of SLCMV accumulation), whereas variety Sree Prakash showed good amplification (Fig. 3). Bark samples of middle portion of stems showed very faint amplification in all varieties except variety Sree Sahya which was negative. Bark samples from bottom portion of stems also showed negative amplification.

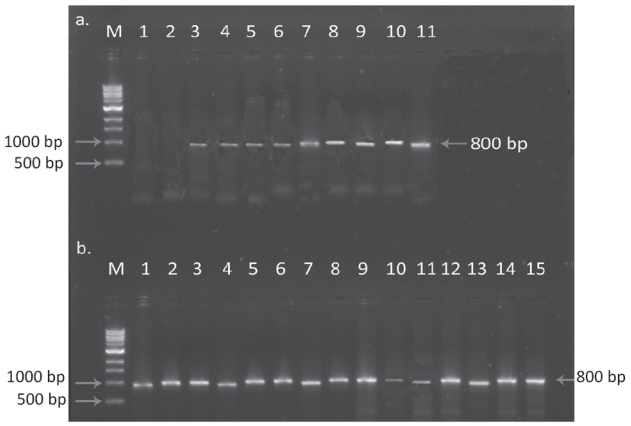

Fig. 2. PCR amplification from bud samples of different cassava varieties using SLCMV coat protein gene primer. (a). Sree Vijaya- M- 500 bp marker; Lane 1-11: Bud number 1-11. (b). Sree Prakash- M- 500 bp marker; Lane 1-15: Bud number 1-15.

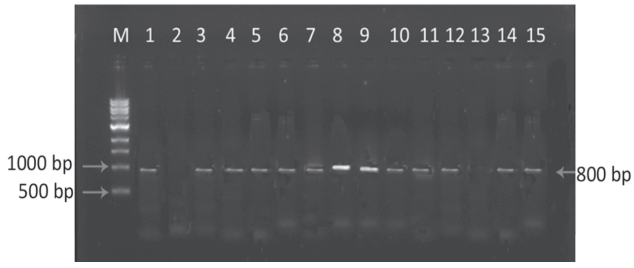

Fig. 3. PCR amplification from bark samples of different cassava varieties using SLCMV coat protein gene primer. M- 500bp marker; Lane 1-3- Sree Sahya, Lane 4-6- Sree Visakham, Lane 7-9- Sree Prakash, Lane 10-12- Sree Padmanabha, Lane 13-15- Sree Vijaya

Results of NASH, showed that the DNA from top buds of different varieties showed accumulation of SLCMV as intense hybridization in the form of dark spots (Fig.4) while a decrease in intensity of spots was observed in samples from middle and bottom portions of stems. For varieties Sree Prakash, Sree Sahya, Sree Visakham, Sree Padmanabha and H-165, bud samples from all portions showed SLCMV accumulation in the form of hybridization, whereas variety Sree Padmanabha showed faint hybridization. Bottom most three samples (1-3) of variety Sree Vijaya did not show hybridization, samples from middle portion of stem showed faint hybridization while samples from top portions of stem showed good hybridization. In variety M-4 bottom most 2 to 3 samples did not show hybridization, whereas in variety H-226 bottom most two samples (1-2) did not show hybridization (Table.1).

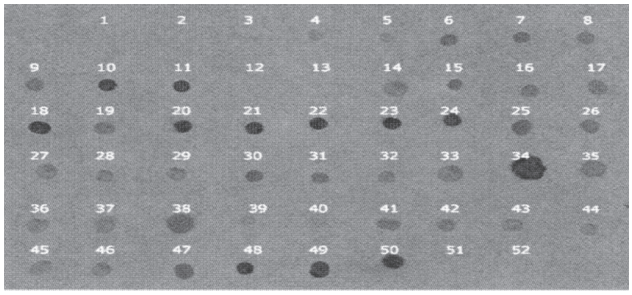

Fig. 4. Nucleic acid spot hybridization of bud samples from SLCMV infected stems of different cassava varieties using non radioactive biotinylated probe of coat protein gene of SLCMV

Spot 1 to 11- Sree Vijaya;12 to 22 -H226; 23 to 40 - Sree Padmanabha; 41 to 49 - Sree Sahya; 50 - Positive control; 51- Negative control; 52 - Buffer control

Among bark samples, from middle and top portion of stems in variety Sree Padmanabha did not show hybridization. Bark samples of bottom portion of stems of all the other varieties did not show hybridization except varieties Sree Prakash and Sree Padmanabha. Bark samples from middle and top portion stems of most of the varieties showed hybridization (Fig. 5) (Table 2).

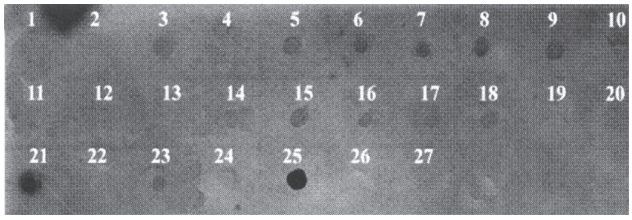

Fig. 5. Nucleic acid spot hybridization of bark samples from SLCMV infected stems of different cassava varieties using non radioactive biotinylated probe of coat protein gene of SLCMV. Spot 1-3: Sree Sahya; 4-6:Sree Visakham; 7-9: Sree Prakash; 10-12: Sree Padmanabha; 13-15: Sree Vijaya; 16-18: M-4; 19-21: H-165; 22-24: H-226; 25: Positive control; 26: Negative control; 27: Buffer control

The distribution of *Sri Lankan cassava mosaic virus* (SLCMV) in the buds from the nodes of stem cuttings varied with 8 different varieties of cassava viz., Sree Sahya, Sree Visakham, Sree Prakash, Sree Vijaya, Sree Padmanabha, M-4, H-226 and H-165. TAS-ELISA, PCR and NASH were carried out to diagnose the SLCMV distribution in 8 varieties. SLCMV was detected from the bud and bark samples of 7 cassava varieties in TAS-ELISA which indicated high virus accumulation in bud samples from

the top portion of the stem of plants. However, variety Sree Padmanabha had high SLCMV accumulation in the buds from bottom portion while bottom most bud samples of variety Sree Vijaya and H-226 had lower accumulation when compared to the healthy SLCMV indexed plants. Bark analysis of variety Sree Padmanabha also showed greater accumulation in bottom portion of stem. Cours-Darne (1968) observed that virus movement was downwards towards the root of resistant cassava genotypes. Because begomoviruses are localized in phloem tissue (Horvat and Verhoyen, 1981), the flow of assimilate into storage root might enhance the downward movement of the virus in the main stem leaving most of the secondary and tertiary stems uninfected (Storey and Nichols, 1938). PCR performed in the total DNA of buds collected from bottom to top portions of stems of 8 cassava varieties indicated high accumulation of SLCMV in top buds. The intensity of signals obtained in NASH for buds samples of different varieties also showed that SLCMV accumulates more in the top portions of the plant. The most probable reason for the increased virus content at the top portion of the plant may be due to rapid multiplication of virus occurring in young active growing area. ACMV was not detected in the first two bud samples taken just above the ground (Ogbe et al., 2002).

Distribution of SLCMV in the bark of different portion of the plant varied with different varieties. With PCR it was not able to detect SLCMV in the barks middle portion of variety Sree Sahya and bark of bottom portion of stem of variety Sree Vijaya. But all other varieties showed accumulation of SLCMV. SLCMV detection was negative in the case of bark from bottom and middle portion of varieties H-226, M-4, and Sree Vijaya in NASH. NASH test indicated SLCMV accumulation in bark of all portion of variety Sree Prakash and no SLCMV accumulation in bark of all portions of variety Sree Padmanabha except bottom portion of barks in NASH.

The SLCMV accumulation can be identified with the intensity of signals obtained in NASH and also with absorbance value obtained for ELISA. The environmental conditions play an important role to resist the virus movement in the phloem (bark tissue) of resistant genotypes (Ogbe et al., 2002). The restriction of virus accumulation in the lower part of the primary stem for resistant genotypes (Jennings, 1960 and Fargette et al., 1996) is more in bud rather than to bark (Ogbe et al., 2002) which is evident in the present study.

It is concluded that in the SLCMV infected cassava plants, virus detected in samples from bud and bark. However, the accumulation varies in different portion of the stem as well as among different cassava varieties. Hence, we could not pin point which portion of cassava stem will remain free from virus accumulation, which can be exploited for obtaining disease free plants.

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