Journal of Root Crops, 2016, Vol. 42 No. 1, pp. 45-52
Indian Society for Root Crops
ISSN 0378-2409, ISSN 2454-9053 (online)

Elimination of Cassava Mosaic Disease through Meristem Culture and Field Evaluation for Yield Loss Assessment in Cassava Genotypes

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Received: 28 May 2016; Accepted: 29 June 2016

Abstract

The presence of *Sri Lankan cassava mosaic virus* (SLCMV) and *Indian cassava mosaic virus* (ICMV) and other cassava mosaic viruses which cause cassava mosaic disease is a major threat to cassava production worldwide. Therefore it is highly advantageous to use virus free cuttings for multiplication and cultivation. Here we report meristem culture for cleaning of five different cassava cultivars infected with SLCMV. Polymerase chain reaction (PCR) was employed to index meristem derived plants using specific primers. The meristem derived plantlets were free of symptoms and further confirmed the absence of virus through PCR using virus specific primers. Meristem derived plants were included in field study to determine the effect of SLCMV infection in yield. Meristem derived virus free planting materials increases yield but such advantage soon lost due to rapid re-infection with virus on field grown plants through efficient whitefly transmission.

Key words: Cassava, meristem culture, SLCMV, PCR, yield

Introduction

Cassava *(Manihot esculenta* Crantz; Euphorbiaceae) is the only species in its genus that is cultivated as a food crop. Cassava is a shrub 1-5 m high which is cultivated for its starch containing tuberous roots (Cock, 1985). The storage root provide more dietary energy per hectare and less working hours than any other staple crops, making it the mainstay of small holders in tropics with limited access to agricultural inputs. The crop has been cultivated in India for more than a century. One of the major constraints in cassava production is Cassava Mosaic Disease (CMD). In India, this disease is caused by two viruses-*Indian Cassava Mosaic begomovirus* (ICMV) and *Sri Lankan Cassava Mosaic begomovirus* (SLCMV) (Thresh et al., 1998; Saunders et al., 2002; Dutt et al., 2005; Anitha Jose et al., 2011; Legg et al., 2014). They are transmitted mainly through whitefly, *Bemisia tabaci*. There are many literatures available on the viruses transmitted by *B. tabaci* (Bedford et al., 1994; Harrison and Robinson, 1999; Malathi and Varma, 2003; Fauquet et al., 2003; Antony et al., 2006). Cassava Mosaic viruses are also disseminated through stem cuttings which are used routinely for vegetative propagation. Dissemination by stem cuttings can lead to the introduction of CMD to new areas and accounts for the occurrence of the disease in areas where there is little or no spread by the whitefly vector (Hillocks and Thresh, 2000). Presence of these viruses can cause losses up to 40% to 50% of total yields in cassava throughout the continent (Thresh et al., 1994; Otim-Nape et al., 1996; Malathi et al., 1985). Cassava mosaic disease is prevalent and causing serious losses in cassava in southern India and in many parts of Sub-Saharan Africa (Thresh et al., 1998).

The most widely used and unique technique for elimination of various pathogens including viruses, viroides, phytoplasma, bacteria and fungi is plant meristem

culture (Walkey, 1978; Pierik, 1989; Bhojwani and Razdan, 1996). Meristem tip culture takes advantage of the fact that many viruses fail to invade the meristematic region. Meristems are frequently devoid of systemic pathogen due to the absence of differentiated conducting tissues. In addition, the use of planting material derived from pre-existing meristems has been proposed to generate more genetically uniform plants and to reduce the amount of variation among the propagules to retain genetic integrity (Villordon and LaBonte, 1996). There are reports on successful elimination of virus from infected plants through meristem culture and virus indexing of cassava (Kartha et al., 1974; Berbee et al., 1973; Kaiser and Teemba, 1979; Adejare and Coutts, 1981). In cassava (Nair et al., 1979), sweet potato (Jeeva et al., 2004), yam (Rajitha et al., 2011) and elephant foot yam (Kamala and Makeshkumar, 2014) successful elimination of disease causing agents viz., *cassava mosaic virus, sweet potato feathery mottle virus, yam mild mosaic virus* and *Dasheen mosaic virus* respectively were reported through meristem tip culture. Alam et al., (2004) also produced virus free seeds of tomato using meristem culture.

Meristem culture will not automatically guarantee total freedom from plant virus (Thottappilly and Rossel, 1988 and Thottappilly Rossel, 1992). Virus detection is important for virus free planting material production and safe movement of germplasm. Before the intercontinental transfer of planting materials, the meristem derived plants should be tested for the presence of virus. ELISA is a convenient method to detect presence of virus, but the use of this technique is limited in detection sensitivity with very low concentration of virus such as in tissue cultured plants. For routine diagnosis of meristem derived plantlets, fast, reliable and inexpensive method such as PCR would provide a possible alternative. Various diagnostic protocols for the detection of *cassava mosaic virus* were developed for routine diagnosis (Makeshkumar et al., 2005; Raji et al., 2015, 2016).

Adequate assessment of yield loss was not carried out because of the difficulty in maintaining a healthy field grown plants for a complete growth period for comparison with an infectious plant. The storage root yield loss at 69% for the susceptible genotype TME 117 and 32% the resistant genotype TMS 30395 were field-grown plants for seven months were recorded by Terry and Hahn (1980). Storage root yield losses of between 36 and 40% were observed on 12-month, field-grown, moderately resistant genotypes, whose health status were assessed for five months after planting (Ogbe, 2001). Planting of cuttings derived from *in vitro* virus free plantlets of cassava in a high CMD infection pressure area gave higher tuberous yield and number of stem cuttings as compared to plants derived from standard cuttings. Yield increase of 50% to 100% were also reported by planting cuttings derived from *in vitro* plantlets in Congo compared to conventional field cuttings (Mabanza et al., 1995).

Hence the present work was developed to eliminate *cassava mosaic virus* through meristem tip culture and indexing of meristem derived plantlets using PCR with specific primers. Another objective of this study was to test the yield effects of meristem derived virus free cassava cultivars against SLCMV infection.

Materials and Methods

Plant material and source of explants

Cassava mosaic resistant (CMR) lines showing recovery phenotypes viz, CMR 117, CMR 1, CMR 123 and CMR 102 and highly susceptible variety H 226 were used in the present study to establish meristem culture.

Culture media composition and preparation

Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal media are prepared. 20 g^{-1} sucrose was added and pH was adjusted to 5.8 with 1N NaOH. Agar added (8 g^{-1}) before autoclaving at 121ºC for 20 minutes at 15 lbs pressure. 1 μM NAA, 0.1 μM GA3 and 0.5 μM BAP was supplemented with MS basal media for meristem culture.

Meristem culture

Selected cassava genotypes were maintained at ICAR-CTCRI field from which explants were collected. Excised shoot tips were collected from actively growing branches and washed under running tap water and disinfected with fungicide, bavistin (carbendazim 0.05%), followed by approximately 0.02% tween-20 [polyoxyethelene (20) sorbitan, oleate]. Further sterilization was done under running laminar air flow cabinet. The explants were treated with 0.1% mercuric chloride solution for 3 minutes. Treated explants were washed four to five times with sterile distilled water to remove the effect of surface sterilizing agent. After that the explants were washed with 70% alcohol. The shoot apex consisting of apical dome

with one or two leaf primordia was isolated with sterile needle and scalpel under dissection microscope. Meristematic region in the apex was uncovered by gradually removing of remaining leaf primordia and the isolated shoot apical meristem of 0.1 to 0.5 mm in size was transferred to culture tubes with sterilized MS medium supplemented with NAA, BAP and GA3. After 3-4 weeks the developed meristem was subcultured to MS basal medium including vitamins for shoot and root regeneration. The developed plantlets were further multiplied using nodal segments in MS medium. The developed plants in 3-4 leaf stage were virus indexed. After indexing only virus negative plants, were mass multiplied for field trial.

Virus indexing of meristem derived plants using PCR

Fully opened leaf lobes of *in vitro* raised plants were excised using sterile forceps and blade. DNA was extracted from 0.1mg tissue using CTAB method (Doyle and Doyle., 1987). The concentration of extracted DNA was determined with Nanodrop (Denovix, USA) and quality was checked by running 3 μl sample on 1% agarose gel. The DNA extracted was subjected to PCR assay using virus specific primers CP (F) (GGA TCC ATG TCG AAG CGA CCA) and CP (R) (AAG CTT TTA ATT GCT GAC CGA) (Makeshkumar et al., 2005). The DNA of virus infected plants from field served as positive control. The PCR reaction was performed in a 25 μL total reaction consisting of 2 μL DNA, 10 picomole each of virus specific primers 2.5 μL of 10X reaction buffer (50 mM Tris-HCl (pH 9.0 at 25 °C), 1.5 mM $MgCl_2$, 15 mM $(NH_4)_2SO_4$ and 0.1 % Triton® X-100), 1 μL of 10 mM dNTPs, 0.5 μL of (1U/ μL) Taq DNA polymerase. The PCR was performed in Eppendorf Mastercycler with the following thermal programme: one cycle at 94ºC for 2 min, 30 cycles of 94 ºC for 1 min, 55 ºC for 2 min,72 ºC for 3 min and final extension step of 72 ºC for 5 min. The amplified product was analyzed on 1% agarose gel, stained with ethidium bromide and photographed under UV-gel doc system (Alpha imager, USA).

Hardening and Acclimatization for field trial

Virus indexed plants were hardened for field study. The plants when attained 3-5 cm height with well developed roots were hardened and survived plants were transferred to net house. Stem cuttings from these plants were used for open field study for yield loss assessment. No insecticide was used to control viral vectors in open field.

Observations and statistical analysis:

In the field trial, meristem-derived plantlets of T0 and T1 generation were planted in field. The average tuber number, tuber length, tuber girth, tuber weight, total biomass and harvest index from three randomly selected plants were evaluated to test their performance. Analysis of variances was performed for these yield-related characters using SAS Statistical Package version 9.1 (SAS Institute, Cary, NC, USA).

Results and Discussion

For the primary establishment of meristem culture from field grown plants, surface sterilization was optimized for different concentration and time with mercuric chloride solution. Among different concentrations and time periods tested, 0.1% mercuric chloride for 3 min was found to be effective for surface sterilization. About 90 % of the explants were found healthy and free of contamination, when treated with 0.1% mercuric chloride for 3 min. At 0.05% mercuric chloride most of the explants were contaminated and at concentration 0.15% tissue killing was observed. Percentage of contaminated explants was decreased with increase in sterilization period in all varieties. But an increase in sterilization period also resulted a decrease in survivability of non contaminated explants (Table 1). Many researchers previously reported the use of different sterilization methods for cassava nodal and meristem culture. Sterilizing cassava nodes with an initial wash using 70% ethanol for 5 min followed by 10% NaOCl for 20 min was found to be best (Cacai et al., 2013).

Table1. Effect of concentration and time of surface sterilization on cassava explants

Surface sterilant	Concentration (%)	Treatment time (minute)	Survived explants (%)
Mercuric Chloride	0.05	1	40
		3	60
		5	50
	0.1	1	60
		3	90
		5	50
	0.15	1	40
		3	50
		5	30

For the primary establishment of meristem, MS medium supplemented with growth regulators are used. MS medium supplemented with 0.1 μM NAA, 0.1 μM GA3 and 0.5 μM BAP was used as cassava meristem culture medium (Nair et al., 1979). This combination induced plant regeneration in 100% of the meristems. Plant development from isolated meristems usually requires exogenous hormone supplements. Meristem derived tiny shoots were transferred to MS basal medium supplemented with sucrose and agar, for shoot and root development. Spontaneous rooting was observed in all cases. Those plants were multiplied by nodal cuttings for mass multiplication (Fig.1).

Virus Indexing by PCR

When the DNA samples from meristem derived and positive control plants were tested for the presence of SLCMV with PCR, an amplified PCR product of 800 bp was obtained only from positive control. There was no amplification in meristem derived plants, which indicated that all plants were virus free. Virus indexing technology is the essential component of virus free planting material production. Early detection of virus in tissue culture plants plays an important role in its control (Mahadev et al., 2013). The use of techniques ELISA and PCR for detection of *cassava mosaic virus* was reported earlier (Makeshkumar and Nair, 2001; Makeshkumar et al., 2005; Hegde et al., 2010; Otono et al., 2015).

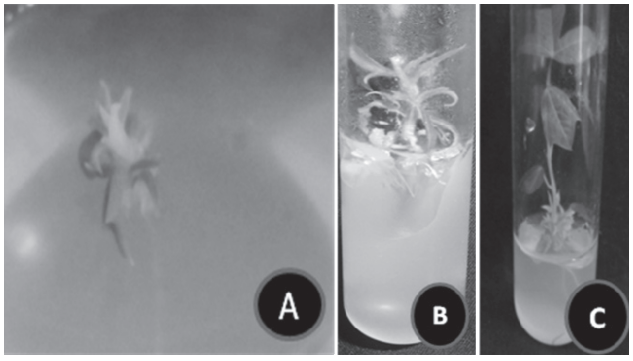

Fig.1. CMD elimination through meristem culture in cassava. A&B. Establishment of isolated meristem in meristem culture medium. C. Rooting of tiny shoots in basal MS medium

Hardening and acclimatization

Young well rooted virus free plants obtained from meristem culture were successfully acclimatized, established and grew satisfactorily in a green house (Fig. 3). Well rooted plants having 3-4 cm height found to be better for hardening. Plantlets with more than 5cm height were unsuitable for hardening because it caused shoot damage during extraction from culture tubes (Shiji et al., 2014). The physiological status of roots is critical for plant survival during the first few days of acclimatization. As cassava is a delicate plant to harden, huge losses occur during transfer from *in vitro* laboratory to *ex vitro* field conditions, which requires care and media optimization (Jorge, 2002). In the present study successful acclimatization was achieved when coir pith used for the first two weeks of acclimatization. 1:1 proportion of soil and coir pith found to be better for the further establishment (Fig. 4). According to Ogero et al., (2012) the *in vitro* regenerants of the two sweet potato varieties adapted well when they were transplanted onto a mixture of red soil and rice husks. Bernardo et al. (2002) used a sterilized mix of milled and sieved black soil and washed and sieved coarse sand (1:3 ratio) as substrate for cassava hardening. The

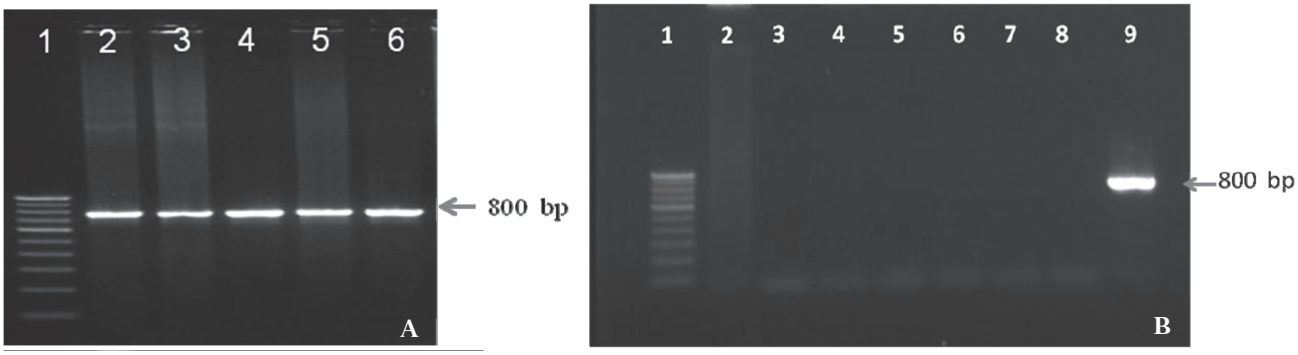

Fig.2. Virus indexing with PCR using CP gene specific primers. A: Lane 1- 100 bp ladder, Lane 2-6 cassava mother plants used for meristem culture isolation. B: Lane 1- 1kb plus ladder, Lane 2-6 meristem derived cassava samples, Lane 7- negative control, Lane 8- Non Template Control, Lane 9- Positive control

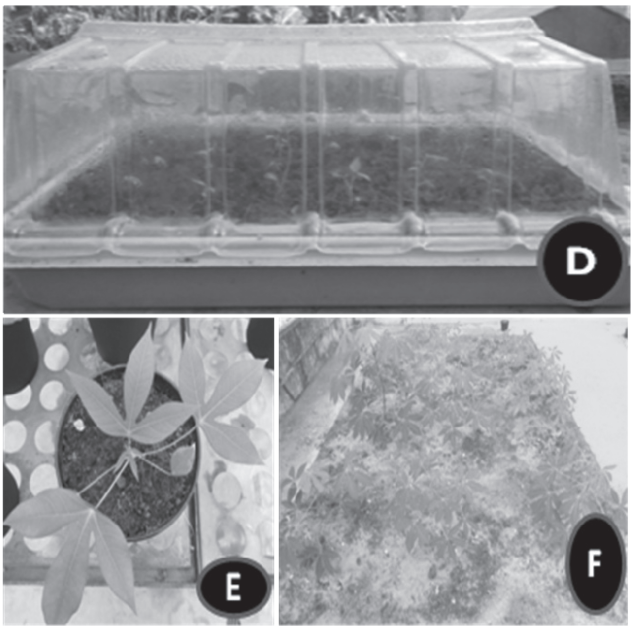

Fig.3. D. Acclimatization of meristem derived plantlets, E. Hardened plants after 4 weeks, F. meristem derived plants in net house

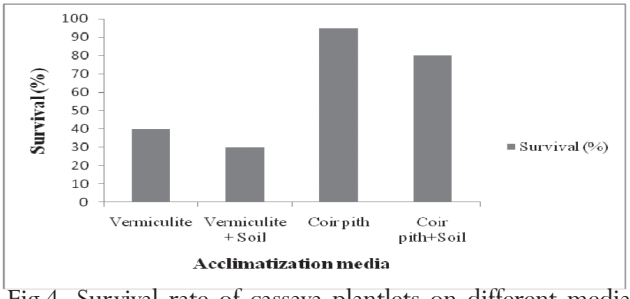

Fig.4. Survival rate of cassava plantlets on different media

success of plantlet acclimatization and survival depends on the development of good root and shoot system and care which was taken during transplantation (Ogero et al., 2012).

Field Trial of T0 and T1 plants:

The results of field evaluation of T0 and T1 plants were presented in Table 2. The data of storage root yield of T0 and T1 plants were subjected to analysis of variance using the general linear model (GLM) procedure of the Statistical Analysis System (SAS, 2003). There was significant difference between control and test plants. The yield characteristics are higher for T1 plants in open field compared to T0 plants in net house. Disease and viral incidence were much lesser in T0 plants maintained in net house.

Regarding total tuber number, CMR 123 produced more number among varieties. T1 plants in open field produced more number of tubers than T0 plants and control in all varieties except H226. Total tuber yield described almost same tendency as tuber number except for CMR 1 and H165. In contrast, total biomass of all genotypes had high value for control than T0 and T1 plants except for CMR 102. But in relation to harvest index, all varieties except

Table 2. Field evaluation of virus free cassava plants for their performance

Variety	Plant material source	Tuber no. (Avg)	Tuber length (Cm) (Avg)	Tuber girth (Cm) (Avg)	Tuber weight (Kg) (Avg)	Total biomass (Kg) (Avg)	Harvest index (Avg)
H226	Net house (T0)	7.75^{EFG}	8.70^{E}	6.25^{D}	3.65^{EFG}	5.71^{HI}	0.64^{A}
	Open field (T1)	7.75^{EFG}	8.15^{E}	5.35^{D}	4.35^{CDEF}	6.95^{FGH}	0.62^{AB}
	Control	8.45^{DEF}	8.25^{E}	5.12^{D}	4.45^{BCDE}	8.98^{BCD}	0.50^{FG}
H 165	Net house (T0)	5.25^{GH}	12.60^{CD}	11.00^{BC}	2.20^{H}	3.93^{J}	0.56^{CD}
	Open field (T1)	6.40^{FGH}	13.70^{BC}	12.85^{AB}	2.31^{H}	$4.18I^{J}$	0.55^{DE}
	Control	4.75^{H}	9.60^{DE}	6.30^{D}	2.95^{GH}	6.41^{GH}	0.46^{G}
CMR 123	Net house (T0)	11.05^{BCD}	8.75^{E}	5.60^{D}	4.40^{BCDEF}	7.40^{DEFG}	0.60^{BC}
	Open field (T1)	13.00^{AB}	8.10^{E}	5.55^{D}	5.05^{ABC}	8.28^{CDEF}	0.61^{AB}
	Control	11.70^{BC}	8.45^{E}	4.60^{D}	4.25^{CDEF}	8.55^{CDE}	0.50^{FG}
CMR 1	Net house (T0)	9.50^{CDE}	7.80^{E}	5.65^{D}	3.90^{DEFG}	6.95^{FGH}	0.56^{CD}
	Open field (T1)	12.50^{AB}	8.85^{E}	5.60^{D}	4.90^{ABCD}	9.29^{BC}	0.53^{DEF}
	Control	8.25^{EF}	8.90^{E}	4.90^{D}	5.40^{AB}	10.38^{AB}	0.52^{EF}
CMR 102	Net house (T0)	9.50^{CDE}	16.00^{AB}	10.50^{C}	3.40^{FG}	6.60^{GH}	0.52^{EF}
	Open field (T1)	12.50^{AB}	15.50^{AB}	13.60^{A}	5.88^{A}	11.13^{A}	0.53^{DEF}
	Control	8.25^{EF}	16.85^{A}	13.96^{A}	3.65^{EFG}	7.37^{EFG}	0.49^{FG}

Numbers with same letters as superscript in each column are not significantly different at $p > 0.05$ level

CMR 123 showed higher harvest index for T0 plants. For CMR 123 T1 plants showed high value.

T0 plants in net house showed little infection with virus and give better yield. But the T1 plants when planted in open field re-infected with virus and the yield performance was poor. Significant yield effects seen in control plants showed that virus infection is one of the key factors causing yield loss. Meristem derived virus free planting materials increase yield but such advantage soon lost due to rapid re infection with virus on field grown plants through efficient whitefly transmission. In a number of field trials using virus free planting materials of different cultivars demonstrated that yield improvement can be achieved through virus free planting materials (Msogoya et al., 2012., Garcia et al., 1993). The return of virus free planting material is clear from this and other studies. But in areas with abundant virus vectors (whitefly) and other sources of virus inoculums, the benefits of virus free planting material could soon be lost due to rapid re-infection (Bryan et al., 2003, Akano et al., 1997, Thresh et al., 1997; Thankappan et al., 1996, 2000).

Conclusion

The present study revealed a novel technique for elimination of *cassava mosaic virus* and production of virus free planting material. In view of all experimental results, it is robustly recommended the use of meristem culture to produce virus free planting material in cassava. The virus free planting material showed best performance in field in response to yield compared to conventional planting material. From the practical view point of our study, it is desirable to maintain the field free of insect vectors in order to reduce rapid re-infection in virus free planting material.

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