Journal of Root Crops, 2017, Vol. 43 No. 1, pp. 23-33
Indian Society for Root Crops
ISSN 0378-2409, ISSN 2454-9053 (online)

Comparative Potential of Somatic Embryogenesis and Friable Embryogenic Callus Production in Farmer Preferred Indian Cassava Cultivars

J. Dhanya[1], N. A. Leen[1], D. C. Deepthi [1], M. Moushmi[1], M. R. Beena[2], M. N. Sheela[1], and T. Makeshkumar[1]

[1] ICAR- Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram 695 017, Kerala, India

[2] Leibniz Institute- DSMZ, Germany

Corresponding author: T. Makeshkumar, e -mail: makeshkumar.t@icar.gov.in

Received: 27 February 2017; Accepted: 02 April 2017

Abstract

The most crucial step towards developing farmer preferred qualities in cassava is to successfully produce stable and re-generable friable embryogenic callus (FEC). FEC production in four indigenous cassava cultivars viz., H226, H165, Sree Vijaya, Sree Sahya in comparison with model cultivar, TMS 60444, were attempted. The focus was also on production and maintenance of virus free *in vitro* mother plants, optimization of somatic embryogenesis using two different explants, axillary bud (AB) and immature leaf lobe (ILL), other than induction of FEC and evaluation of its regeneration potential. All the varieties produced moderate to optimum OES (organized embryogenic structures) when the explants were cultured in MS medium supplemented with 12 mg l^{-1} picloram, in which the highest frequency of OES induction of 97.49 % and 95.64 % were obtained in H226 and H165, respectively using ILL. The number of healthy plantlets regenerated from 50 mg of FEC were 14, 11, and 17 from H165, H226 and TMS 60444 respectively. These results could be a pointer towards the production of FEC in other indigenous cassava varieties, which are highly susceptible to CMD with suitable modification in culture media for developing resistance.

Key words: Cassava, friable embryogenic callus, indigenous cultivar, somatic embryogenesis

Introduction

Cassava (*Manihot esculanta* Crantz) is an important tropical crop cultivated in an area of 23.9 million ha, producing 268 million tonnes with a productivity of 11.2 t ha^{-1} (FAOSTAT, 2015). It plays an important role as a cash crop for small-holder farmers and serves as famine reserve crop. It is utilized and consumed as a secondary staple food as cooked or processed products, as animal feed and in starch industry. In Asia, it is cultivated in an area of 4.1 million ha producing 89.8 million tonnes with a productivity of 21.9 t ha^{-1} while in India, it is cultivated in an area of 0.23 million ha producing 8.1 million tonnes with a productivity of 35.6 t ha^{-1} (FAOSTAT, 2015). Cassava mosaic disease in India is one of the major devastating diseases that would seriously affect the crop yield if left unattended. It is caused by single or multiple infections of distinct whitefly transmitted begomoviruses namely *Indian cassava mosaic virus* (ICMV) and/or *Sri Lankan cassava mosaic virus* (SLCMV) (Malathi et al., 1985; Patil et al., 2005; Anitha et al., 2011). The disease is mainly perpetuated through indiscriminate use of infected planting material. Even though the use of virus eliminated meristem derived plants has shown increase in yield, it is prone to re infection by these viruses from farmers field itself (Malathi et al., 1985; Thankappan et al., 1997).

Various surveys on cassava mosaic disease (CMD) incidence conducted in India during different periods revealed yield losses ranging from 25-88% depending on the cultivar grown in Kerala, Tamil Nadu and Andhra Pradesh (Malathi et al., 1985). A low to high incidence

of 44.5 to 96.75% was recorded in South Kerala during a survey conducted in 2005-2006 (Anitha et al., 2011) and more than 90% incidence was recorded in Tamil Nadu during 2005-2006 survey (Rajanimala et al., 2011). A recent study, conducted in Kerala during 2015-2016, revealed mixed infections of SLCMV and ICMV (in nine districts), single infection of SLCMV (in four districts) and ICMV (in five districts) (Jayakrishnan and Makeshkumar, 2016). Production of Indian genotypes resistant to cassava mosaic disease should be given importance in such circumstances either through conventional breeding or through biotechnological approaches.

Even though development of resistant varieties through conventional breeding has its own limitations, field evaluation trials of selected newly developed CMD resistant clones using three different CMD resistance sources are being carried out successfully by breeders at ICAR-CTCRI (Sheela et al., 2016). It was reported that screening of available cassava germplasm for resistance, identification and evaluation by molecular analyses and grafting takes around 10 years, which is a major constraint. Due to the non-availability of resistance genes in farmer preferred cultivars and production of improved farmer preferred lines by conventional breeding is time consuming and tedious process, researchers in cassava development programme have been focussing on genetic transformation technology for transferring resistance genes into existing conventionally susceptible/ tolerant/ improved cultivars for deployment to farmers and breeders (Makeshkumar et al., 2012).

Development of efficient, regeneration or tissue culture system is a crucial requirement for the success of any transgenic technology but some bottlenecks remain, the main being varying *in vitro* morphogenic responses between even closely related cultivar. Different explant sources like shoot apices, axillary buds, somatic cotyledon, somatic embryos, embryogenic clusters, protoplasts, embryogenic suspension derived tissues, friable embryogenic callus (FEC), FEC derived protoplasts have been used till date for efficient regeneration and transformation studies in cassava, and several successful procedures for somatic embryogenesis have been reported in African, South American and Asian cassava cultivars (Hankoua et al., 2005; Raemakers et al., 2001; Liu et al., 2011; Beena et al., 2014; Chavarriaga et al., 2016). The efficiency for production of organized embryogenic structures (OES), somatic embryo induction and its regeneration; FEC production and its regeneration and their transformation efficiencies are highly genotype dependent (Hankoua et al., 2005; Atehnkeng et al., 2006).

One of the main constraints in engineering resistance against CMD is lack of an efficient explant for *Agrobacterium*-mediated transformation system in farmers' preferred Indian cassava varieties. Moreover, the protocol for the generation of FEC have been lacking in Indian farmer friendly cultivars because of the recalcitarant nature of the crop, and difficulty in inducing FEC thereby delaying the option for use of transgenic approach to control geminivirus infection. An approach for FEC production in Indian varieties and its regeneration potential have not been reported considerably when compared to African cassava varieties (Nyaboga et al., 2013).

In the present study foremost importance was given in the production of FEC, one of the most widely used regeneration systems or target tissue for genetic transformation in cassava. As FECs are originated from a single cell, it has an advantage of producing non-chimeric plants compared to other explants. Raemakers et al. (2001) reported the genotypic effect on FEC production of a range of Asian cassava genotypes. Similarly, the present work is carried out to analyse the existence of differential response in farmer preferred indigenous cultivars like H226, H165, Sree Vijaya, Sree Sahya because of their useful traits *viz.*, short duration, drought tolerance, and industrial importance specially starch content. The induced OES was used as the starting material for production of FECs and its regeneration potential was also evaluated for analysing the quality of FEC.

Materials and Methods

Production of virus free *in vitro* mother plantlets

Indigenous cassava varieties *viz.*, H226, H165, Sree Vijaya and Sree Sahya grown on the experimental field at ICAR-CTCRI, Sreekariyam, Thiruvanathapuram, Kerala, and *in vitro* multiplied culture of model cultivar, TMS 60444, obtained thankfully from Dr. Herve Vanderschuren, ETH, Zurich, Switzerland were used in the present study. Field-grown cultivars showed severe symptoms of CMD, which indicated the presence of ICMV/SLCMV. Hence meristem culture was utilized for the establishment of virus free *in vitro* mother plants. The absence of virus in the *in vitro*

cultures was confirmed through Polymerase Chain Reaction (PCR) analysis using virus specific primers (Makeshkumar et al., 2005; Anitha et al., 2011).

Apical shoot tips (2 cm length) were collected from these field grown plants and surface sterilization was done according to Nair and Chandra Babu (1996) with minor modifications. Apical shoot tips were dipped in laboline solution for five minutes after removing the leaves from the shoots and washed under running tap water for 30 minutes or until the foam were completely washed out. The shoot tips were then treated with fungicide (Bavistin) solution (1% w/v) for three minutes. Rest of the treatment was done inside the sterile hood. Explants were treated with 0.1% $HgCl_2$ for 2 minutes followed by 70% ethanol wash for one minutes and finally air-dried in sterile filter paper. In between every treatment, the explants were rinsed 2-3 times in sterilized distilled water. Apical meristematic domes (0.2-0.4 mm size), devoid of leaf primordia, were excised under stereo microscope and inoculated on cassava meristem media [(MS, Murashige and Skoog, 1962) media supplemented with 2% sucrose (w/v), benzyl adenine (BA, 0.1 mg l^{-1}), naphthalene acetic acid (NAA, 0.2 mg l^{-1}), gibberellic acid, (GA_3, 0.04 mg l^{-1}) (Kartha, 1974)], pH was adjusted to 5.8 and the medium was solidified with 0.7% (w/v) plant agar. After 3-4 weeks, the juvenile plants having leaves were sub-cultured to MS basal medium (MS media including vitamins supplemented with 2% (w/v) sucrose, adjusted to pH 5.8-5.9 and solidified using 0.7% (w/v) plant agar). All the cultures were maintained at 26± 2° C under 16/8 hour photoperiod. Total DNA was isolated from 2-4 leaf stage plantlets using CTAB method (Doyle and Doyle, 1987). The concentration of the DNA was checked using NanoDrop UV-Spectrophotometer. The virus free mother plants were selected and multiplied on MS medium and sub-cultured every 3-5 weeks.

Study on different concentration of picloram on SE induction

Nodes with axillary bud (1 cm length) were placed horizontally on MSC medium (MS media including vitamins supplemented with 0.3 mg l^{-1} $CuSO_4$) were incubated for 3-4 days at 28° C and under dark conditions. Under a stereo microscope, the un opened leaf-lobes were aseptically dissected using a syringe needle and sterile pointed forceps and placed at the abaxial side and midrib of young leaf lobe touching the MSCP (MSC medium containing different concentrations of picloram viz., 6, 8, 10, 12 and 14 mg l^{-1}) medium for 2 weeks at 28° C and dark conditions. The effect of different concentrations of picloram on somatic embryo induction in the above mentioned cultivars were studied to analyse the most effective concentration for inducing optimum number of somatic embryos to use for further studies. The number of somatic embryos induced per explant was evaluated within 35 days. Each treatment comprised 48 explants.

Somatic embryogenesis and friable embryogenic callus induction

Effect of different explants on SE

Two different explants, immature un opened leaf lobes (ILL) (3-4 mm size) and axillary bud (AB), isolated from 30-40 days old virus-free *in vitro* plants, were used for the establishment of starting material for OES. The AB and ILL explants were harvested and cultured as explained by Beena et al. (2014). The comparative potential of somatic embryogenesis from ILL and AB was evaluated based on the frequency of OES induction (%), number of SE produced per explants and the efficiency of SE production for both ILL and AB explants.

Comparison of OES induction frequency

The frequency of OES induction was recorded by calculating the ratio of OES clusters responded / cultured explants x 100 (Nyaboga et al., 2013). The regeneration capacity of OES induced was also analysed to evaluate the capacity of somatic embryo to regenerate into a complete healthy plant. OES were placed on MSNB (MS + 0.2 mg l^{-1} NAA + 0.5 mg l^{-1} BAP) media for maturation of somatic embryos and were sub-cultured every 10 - 12 days to fresh MSNB for several cycles till the emergence of young cotyledons. The green cotyledons started developing within three weeks and were sub-cultured to fresh regeneration medium, CEM and the regenerated shoots were further sub-cultured to MSC medium solidified with 0.6% (w/v) plant agar for *in vitro* plantlet propagation (Bull et al., 2009).

Comparison on efficiency (Scores)

The amount of somatic embryo produced per OES cluster (efficiency) for each variety and for different explant were scored according to 0-5 scale system developed by Nyaboga et al. (2015), where 0 = no SE obtained, 1 = very low SE, up to 10% of the OES cluster, 2 = low S, 11–25% of the OES, 3 = medium SE, 26–50% of the

OES, 4 = high SE, 51–75% of the OES, and 5 = very high SE with mostly structures embryogenic on entire OES cluster. Each treatment comprised 64 explants.

FEC induction and regeneration potential

FEC production was done based on the Bull et al. (2009) protocol with slight modification. After monitoring the developmental stage of starting material (OES) produced per variety, the sub-culture duration of OES and its cleaning was done at different phases on MSCP before transferring to GD (Gresshoff and Doy, 1974). The OES produced were slightly chopped using scalpel blade or separated using syringe needle to small pieces and placed as clusters of 0.5 cm diameter on GD medium comprising 12 mg l^{-1} picloram (GDP). In some of the varieties (H226 and Sree Sahya), washing of embryogenic structure (ES) using liquid GD medium was done during subculture to fresh GDP medium, for removing the NFEC (non-friable embryogenic callus) and viscous substance surrounding the ES. The presence of FEC initials were checked every week. Once the FEC were observed, they were immediately transferred to fresh GDP for multiplication and sub-cultured every two weeks.

For evaluating the regeneration potential of induced FEC, 0.5 cm dia. clumps (approximately 50mg) were placed on MSN media (MS with vitamins containing 1 mg l^{-1} NAA), sub-cultured every 10 days for maturation of somatic embryos, for several cycles till the emergence of cotyledon. The green cotyledons developed were sub-cultured to fresh regeneration medium (Bull et al., 2009) and the regenerated shoots were further sub-cultured to MSC medium solidified using 0.6% plant agar for *in vitro* plantlet propagation.

Statistical analysis

The comparative potential for somatic embryogenesis (SE) of four cassava varieties from ILL/AB explants, effect of different concentration of picloram and the regeneration potential of FEC produced were studied by counting the cotyledonary stage embryos (CSE) recovered, average germination of CSE and number of plants regenerated within 30-35 days from 50 mg FEC were statistically analyzed through one way analysis of variance (ANOVA) at $p < 0.05$. All the statistical analyses were carried out using SAS system version 9 (SAS 2010).

Results and Discussion

Farmer favoured four indigenous cultivars, *viz.*, three CMD susceptible varieties (H226, Sree Vijaya and Sree Sahya), one CMD tolerant variety (H165) and TMS 60444 as control, were selected for the present study for evaluating their potential for somatic embryogenesis. H165, a popular variety cultivated in Kerala, Andhra Pradesh and especially in Tamil Nadu under rain-fed conditions and H226, a hybrid cultivar (Magoon et al., 1970), predominantly cultivated in Tamil Nadu and Kerala, are among the first three high yielding varieties of cassava released from ICAR-CTCRI in 1971. Sree Sahya is a multiple hybrid, highly resistant to drought (Jos et al., 1981) and Sree Vijaya, one of the early maturing varieties are cultivated in Kerala, Assam, West Bengal, Bihar, Orissa, and Andhra Pradesh (Abraham et al., 2000). Even though all these cultivars are preferred because of their useful agronomic traits like high yield, short duration, drought tolerance, industrial importance, they are always prone to primary and secondary viral infection. Hence they were preferred as the target cultivars for creating transgenic plants with CMD resistance. As a pre-requisite for genetic transformation for resistance, this study was conducted to evaluate the FEC production and regeneration capabilities of the above-mentioned chosen cultivars.

Production of virus free *in vitro* plants

Pathogen multiplication or their invasion in the meristematic zone is limited due to absence of differentiated conducting tissues; hence meristem culture is most widely and frequently used technique for the elimination of invading pathogens in infected plants (Berbee et al., 1973; Kartha et al., 1974; Kartha and Gamborg, 1975; Kaiser and Teemba, 1979; Adejare and Coutts, 1981). Meristematic domes of size (0.2-0.4 mm), isolated from the shoot apices of the new sprouts in the field grown plants responded well in cassava meristem media. Care was taken to remove the leaf primordia covering the meristematic dome prevented the growth of unwanted callus around the meristematic zone. Since the size of the initial explant was too small, there was delay in initial culture establishment. Slight bulging of meristem dome and initiation of juvenile, clustered thin leaves on bulged shoot appeared only after 14-18 days. Elongated shoots with young unopened leaves started emerging

within 5 weeks on MS basal medium and about 90 % of explant regenerated into a complete plantlet.

Various detection techniques including PCR and nucleic acid hybridization techniques have been reported earlier and routinely applied for the detection of cassava mosaic virus in different cassava landraces all over the world (Makeshkumar et al., 2005; Otono et al., 2015). All the plantlets derived out of meristem cultures were not devoid of virus. Virus indexing revealed that about 16.67 % of them still carried the virus (Fig. 1). Meristem derived virus free plants can be used as stock material for planting in field. It has been reported early that meristem derived plants yielded 20-30% more than diseased one and their rate of reinfection was comparatively much lower (initially nil and upto 38-41% at 8th month) than using normal infected planting material (65-73% at 8th month) (Nair et al., 1990; Deepthi and Makeshkumar, 2016. Other than meristem culture, as an alternative method the use of somatic embryogenesis for elimination of *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV) from cassava have been achieved (Nkaa et al., 2013). In the present study, meristem derived virus free *in vitro* material was mainly used for the production of initial explants (ILL and AB) which were further utilised for the production of other tissue types like somatic cotyledon, embryogenic tissues and FEC.

Effect of different concentration of picloram on SE induction

The fate of a somatic plant cell to transit into a totipotent embryogenic state is under the influence of many indigenous or exogenous factor including *in vitro* tissue culture conditions, wounding, oxidative stress inducers, pH, plant growth regulators especially auxins where they dedifferentiate, activate their cell division cycle and finally results in induction of somatic embryogenesis. The frequency of induction is also dependent on the genotype, tissue or organ (Attila et al., 2003). In most of the plant species, 2,4-D has been widely used initially for the induction of primary and secondary somatic embryogenesis (Joseph et al., 2004; Pinheiro et al., 2013). In cassava, it was evident from the previous reports that incorporation of an artificial auxin *viz.*, picloram to MS medium recapitulates the embryogenic potential of mitotically quiescent somatic cell and gave better response to SE induction and FEC production than 2,4-D (Hankoua et al., 2006; Vidal et al., 2014).

Study on evaluating the best picloram concentration for inducing maximum somatic embryogenesis revealed that regardless of the picloram concentration, somatic embryo was induced in all the varieties but only varied in the number of somatic embryo produced per explants between different treatment within and between the varieties. For H165, maximum embryo was induced on 12mg l^{-1} and 14 mg l^{-1} and mean values of SE induced per explant were on par with each other while there was significant difference between the different treatments on embryo induction for H226 and Sree Vijaya, gave maximum induction at 12 mg l^{-1} concentration. Similar to H165, for Sree Sahya also, showed maximum embryo induction on 12 mg l^{-1} and 14 mg l^{-1} and the mean values were similar, hence the lowest concentration (12 mg l^{-1}) was selected for further induction studies. Data on evaluating the best picloram concentration for inducing maximum SE per explant revealed that, H165 and H226 produced highest number of embryos per explant (33.02 and 28.3 respectively) but not as high as control cultivar, TMS 60444 (48.33). Sree Sahya and Sree Vijaya also produced comparatively less number of embryos (11.06 and 13.33 respectively) at the same concentration. From Table 1, it is evident that the concentration of picloram at which optimum number of embryo induced per explant was on MS media supplemented with

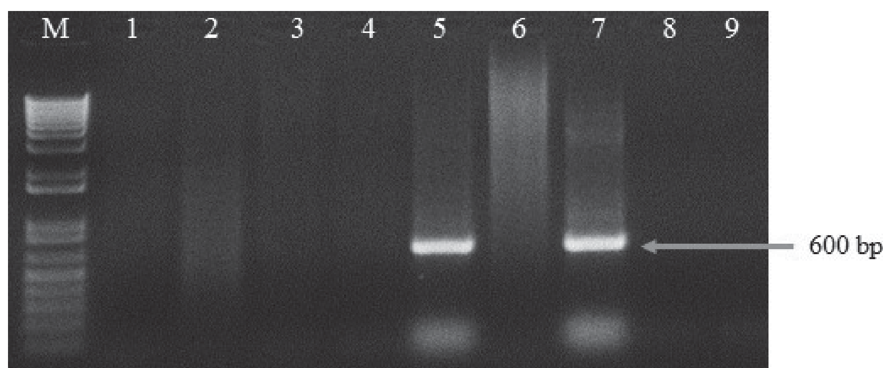

Fig. 1. Virus indexing of meristem derived plants with multiplex primers. Lane1: M,1Kb plus ladder, Lane 2-6: meristem derived cassava samples, Lane 7: SLCMV positive field sample; Lane 8: non template control, Lane 9: negative control

picloram at concentration 12 mg l^{-1} and $CuSO_4$ (0.3 mg l^{-1}). Similarly, few previous reports on somatic embryogenesis demonstrated the frequent use of picloram (12 mg l^{-1}) than with 2,4 D for the induction of embryogenic callus in Asian varieties (Saelim et al., 2006; Beena et al., 2014; Anuradha et al., 2015) and in African varieties (Hankoua et al., 2005, 2006; Atehnkeng et al., 2006; Vidal et al., 2014; Ubalua and Mbanaso, 2014).

Effect of different explant source on SE induction

For the generation of high quality OES, axillary bud (enlarged, bulb-like, devoid of leaf primordia) and young, unopened leaf lobe of size 2-3 mm were selected. Embedding the explants in media helped nutrient absorption and induced initial bulging of explant within 5-7 days. Pro-embryogenic callus started initiating from AB within 10-12 days and within 10 days on both sides of the midrib of very young leaf lobe. OES proliferation was observed more after 16-20 days for AB and took 18-21 days for ILL when placed on MS medium supplemented with picloram (12mg l^{-1}) and $CuSO_4$ (0.3 mg l^{-1}) (Fig. 2). Alfred et al. (2014) observed OES from young leaf lobe between 14 and 21 days on DKW2 50P medium. Atehnkeng et al. (2006) reported that young leaf lobe of size 1-6 mm gave better response than axillary meristem in seven cultivars out of eleven African cultivars and took a long period between 27-35 days to produce pro-embryos.

The pro-embryogenic structures were cleaned by removing the non-embryogenic structures (NES) or white viscous mucilaginous substance surrounding the embryogenic callus, before subcultured to fresh MSCP media (subculture every 2 weeks depending on cultivar) and finally resulted in production of high quality OES. There was significant difference (pd"0.05) between the varieties

Table 1. Effect of different picloram concentrations on cassava somatic embryogenesis

Picloram (mg l^{-1})	Cassava cv				
	H165	H226	Sree Sahya	Sree Vijaya	TMS 60444
6	12.45[hi]	6.50[lm]	6.52[lm]	5.125[m]	41.29[c]
8	10.18[ijk]	21.56[fg]	6.41[lm]	8.27[jkl]	42.79[cb]
10	20.31[g]	10.35[ijk]	7.95[kl]	9.43[jk]	45.22[b]
12	33.02[d]	28.39[e]	11.06[hij]	13.33[h]	48.33[a]
14	23.91[ef]	12.45[hi]	8.93[jkl]	10.08[ijk]	40.27[c]

Values in the table represent mean no. of somatic embryos produced per explant from three replicates.
Values indicated by different letters are significantly different from each other at pd" 0.05.

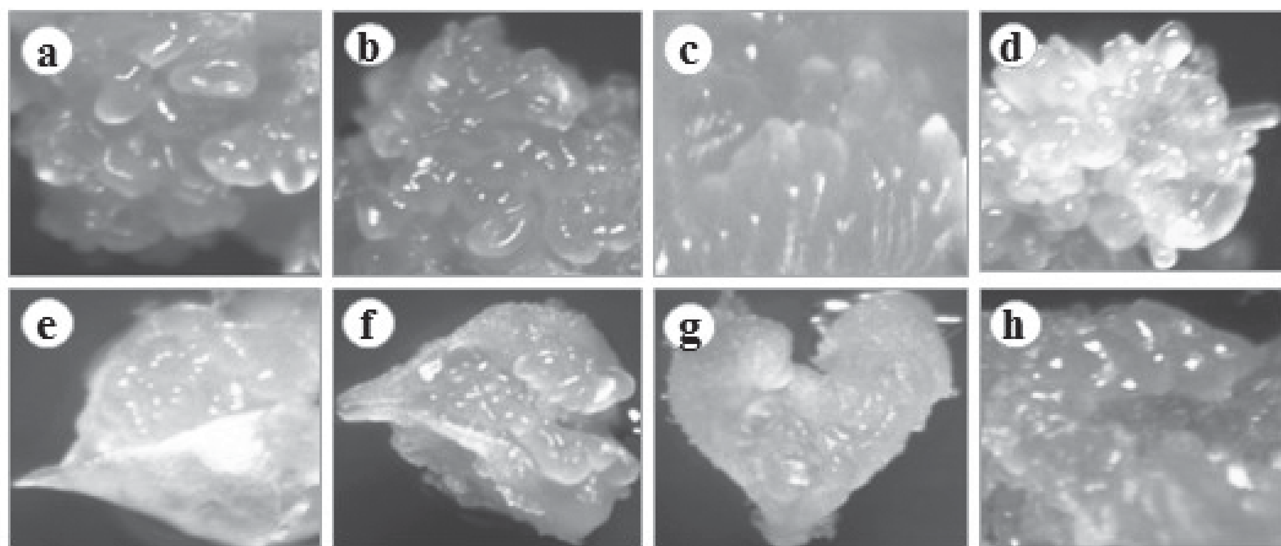

Fig. 2. Induction of organised embryogenic structures in four indigenous cassava varieties: H165, H226, Sree Sahya, Sree Vijaya from AB (a-d) and ILL (e-h) respectively.

for different variables studied including effect of explant source (AB/ILL) on somatic embryogenesis; frequency of OES induced, mean number of somatic embryo produced per explant and efficiency of somatic embryo production between different varieties (Fig. 3). For H165 and H226, ILL was found to be the best explant for inducing somatic embryo when compared to AB, and the results coincide with Beena et al. (2014). In contrast to this, AB was effective in inducing maximum SE for varieties, Sree Vijaya and Sree Sahya. Rossin et al. (2010) demonstrated AB as the best explant to induce higher SE compared to ILL for all the African cultivars selected (African ,CIAT, IITA elite varieties) except one variety and Nyaboga et al. (2015) also reported of best response by using AB as the initial explant for the induction of OES.

Comparison of frequency of induced OES

OES was successfully induced in all the varieties tested at a moderate to high efficiency from both explants, AB and ILL, ranging from 50.97 to 89.06% and 45.94 to 97.49 %, respectively. There was significant difference between the two explants, AB and ILL showing that they induced somatic embryos at varying frequencies between the selected varieties and their genotypic dependency agree with other reports on cassava somatic embryogenesis (Hankoua et al., 2006; Atehnkang et al., 2006; Szabados et al., 1987).

In this study, a good quality OES was induced from the initial explant on MS medium supplemented with picloram (12 mg l^{-1}) and $CuSO_4$ (0.3 mg l^{-1}) in all the varieties. Similar results have been found earlier by Taylor et al. (1996), where different tissue types were induced from organised embryogenic structures of cassava on various basal media supplemented with picloram (12 mg l^{-1}). Similar results were also noticed in African cultivars on embryogenic medium (Atehnkeng et al., 2006; Rossin et al., 2011).

The highest frequency of OES induction of 97.49% and 95.64% were obtained in H226 and H165, respectively using ILL as explant and was on par with each other. Hankoua et al. (2005) also showed higher induction rate in ILL and also used axillary meristem (AM) instead of AB which is used in the present study. The cultivar Sree Vijaya performed equally well using AB and ILL as explant producing moderate OES induction frequencies of 71.81% and 69.81%, respectively. The lowest OES induction frequency was obtained for Sree Sahya was 50.97% while using AB as initial explant (Fig. 3). The colour of the OES induced was cream colour in all the varieties except Sree Vijaya producing light yellow coloured embryogenic structures. OES was achieved and evaluated within 35 days for all the cultivars from ILL and AB and OES was not kept for induction beyond this time limit since it resulted in poor quality of OES or reduced FEC induction.

Frequency of somatic embryogenesis

The amount of somatic embryo produced per OES cluster (efficiency) for each variety and for different explant varied and were scored according to 0-5 scale system developed by Nyaboga et al. (2015). Satisfactory induction (frequency > 80% and score > 2.5) of SE was observed in H165 and H226 while the score was less for Sree Sahya and Sree Vijaya (frequency < 80% and score < 2.5) (Fig. 3).

When comparing the scores for SE induction frequency in the selected varieties, it was analysed that in H165, high frequency of OES (82.50, 95.64%) and high score (2.5, 3.0) were obtained, and it was also in the case of H226 with high frequency (89.06, 95.64%) and high score

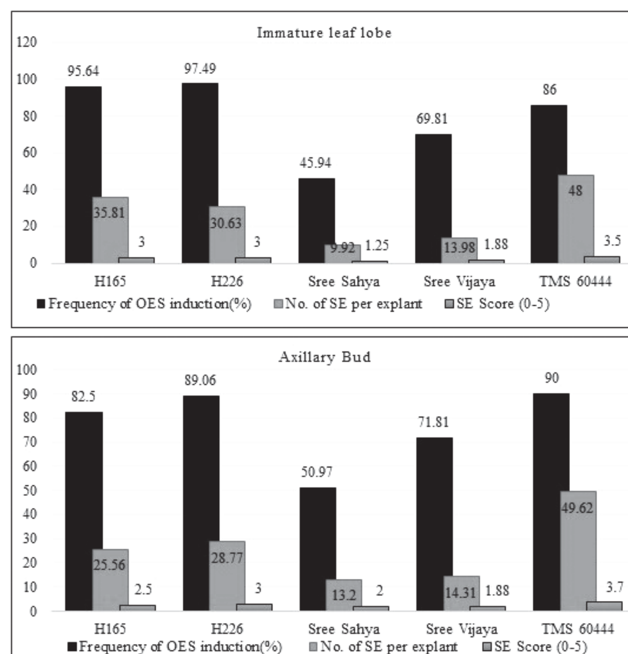

Fig. 3. Comparative potential of somatic embryogenesis of selected cassava varieties from immature leaf lobe and axillary bud. Value represents mean of three independent experiments

(3.0, 3.0) from AB and ILL respectively for both varieties. Sree Sahya and Sree Vijaya showed low frequency (< 80%) of SE from AB (50.97, 71.81%) and ILL (45.94, 69.81%) and low score from ILL (1.25-2.0) and AB (2.0, 1.88), respectively. Thus frequencies and scores correlate for these varieties.

Production and regeneration of FEC

All the four varieties produced high frequencies of OES induction but FEC induction potential varied between the varieties and was induced only in two of the indigenous varieties studied, H165 and H226 along with TMS 60444, which produced more quality proliferating FECs (Fig. 4). In H165, fragmentation or chopping of OES clusters using syringe needle or scalpel blade, cleaning and placement of OES as small clusters (0.5 cm diameter) onto MSCP (2 cycles, 4 weeks), followed by three cycles of subculture on GDP favoured initiation of high quality FEC induction much faster. Our result harmonize with other reports, that wounds created on OES clusters during chopping process favored FEC induction (Taylor et al., 2012). Other methods like crushing of OES through 1-2 mm metal wired mesh also enhanced FEC induction much faster (Nyaboga et al., 2015).

In case of H226, cleaning of OES was inevitable after two cycles (4 or 5 weeks) on MSCP ie., NFEC were discarded, the white mucous embedding the ES was washed with sterile distilled water 2-3 times, then with GDP liquid medium. This enormously helped in production of quality of OES cluster. Chopping or separating of OES cluster during subculture to third cycle on MSCP and further over seven subcultures to GDP solid media (15weeks) resulted in induction of FEC. TMS 60444 produced highly proliferating, good quality and creamy white textured FEC clusters. FEC for different cultivars were induced at different time points; for H165 within 7-9 weeks, H226 (15-17 weeks) and TMS 60444 (9-11 weeks) from 5-6 week old high quality OES (Fig. 4). It was observed that after 3-4 cycles of subculture on GDP solid medium, the texture and friable nature of H226 FEC slightly started changing and each unit changed to maturation phase during further subculture indicating reverse process of transition back to somatic plant cell from embryogenic stage. Sree Vijaya and Sree Sahya failed to produce FEC and can be correlated with low frequency of SE induction and low scores when compared with other varieties that produced FEC. Formation of different types of tissues in selected cassava cultivars were also recorded (Table 2).

OES induced from all the varieties used in the current study were able to produce cotyledon on somatic embryo maturation media (MSNB) within three weeks and regenerate into a healthy plantlet on MS medium devoid of growth regulators (Fig. 4). Similarly, the regeneration potential of the FEC produced were also studied by counting the cotyledonary stage embryos (CSE) recovered, average germination of CSE and number of recovered plants per 50 mg FEC by placing the FEC clusters in MSN medium over a period of 5-6 cycles, followed by

Table 2. Formation of different tissues in selected cassava cultivars

Cultivar	OES formation	FEC development (%)	Average degree of FEC development (scale 0 to 5)	Degree of NFEC	Degree of dark brown callus	Duration of FEC development from OES (weeks)
H165	+++++	42.33[b]	1.5[b]	+	-	7-9
H226	+++++	20.83[c]	0.75[c]	++	+	15-17
Sree Sahya	++	-	-	+++	+	-
Sree Vijaya	++	-	-	++	-	-
TMS 60444	++++	90.17[a]	3.4[a]	+	-	9-11

Values in the table represent mean of three replicates. Degree of tissue formation assessed from + = minimum (up to 10% of total tissue produced) to +++++ = maximum (greater than 75% of total tissue produced).
Values in column followed by different letters are significantly different from each other at $p \leq 0.05$.

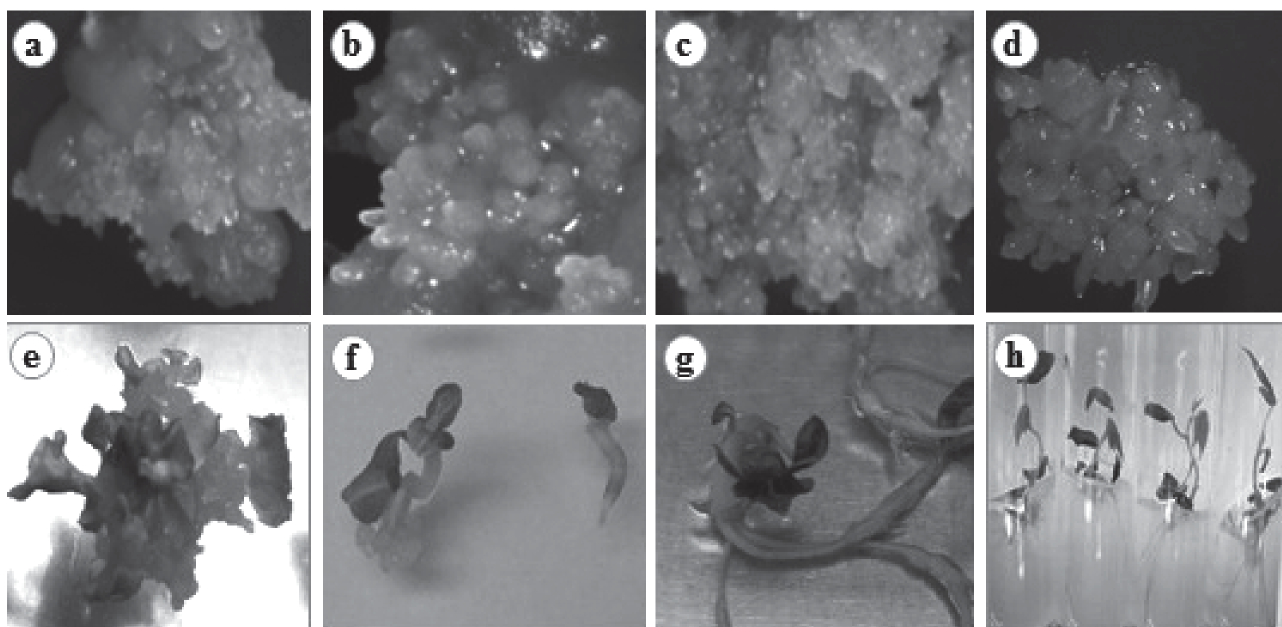

Fig. 4. Production and regeneration of friable embryogenic callus (a) H165 (b) H226 (c) TMS 60444 and (d-h) H165 regeneration: (d) embryo maturation (e) cotyledon maturation (f-h) regeneration into complete plantlet.

transferring to shoot elongation medium (Nyaboga et al., 2013).

The FEC on further culturing on MSN, started maturing by changing the friable nature and develop into swollen, tube-like light green early-CSE within 14 days (Fig. 4(d)) and upon subculture to same media, the cotyledon matured. About 29-51% of the CSE were recovered per 50 mg FEC over a period of 6-7 weeks and about 37-53% of the matured cotyledon germinated on CEM and started root initiation within 2 weeks. The regenerated plants were healthy and finally resulted in the production of 14, 11 and 17 average number of plants from H165, H226 and TMS 60444, respectively (Table 3).

The study on evaluating the somatic embryogenesis was considered as crucial for each variety because several reports on African cassava varieties demonstrate that in certain varieties somatic embryos failed to regenerate into complete plantlet (Atehnkeng et al., 2006; Nkaa et al., 2015) and presently it has been observed that some varieties showed high frequency of OES but low efficiency score and vice versa. A further study on other cassava varieties that failed to induce FEC is required, by manipulating the culture conditions favoring stable FEC production and also for maintaining its friable nature. Most of the genetic transformation system depends on the regeneration of transformed tissue which has to be standardized for each cultivar which may differ from the reported conditions. Source of explant, age of explant, percentage of response for OES induction, mean number of SE produced, auxin concentration, processing of OES cluster and their sub-culturing duration, all these factors influenced the potential for somatic embryogenesis and FEC induction in the present study.

Table 3. Regeneration of complete plantlet from FECs of H165, H226 and TMS60444

Cultivar	Cotyledonary stage embryos recovered*	Average germination of embryos (%)	No. of plants regenerated
H165	32.33[b]	40.00[b]	14.83[b]
H226	29.16[c]	36.86[c]	11.50[c]
TMS 60444	50.67[a]	53.17[a]	17.83[a]

*50 mg of FEC for each variety was used in each experiments. Values in column followed by different letters are significantly different from each other at $p \leq 0.05$.

Conclusion

Organized embryogenic cultures were produced from different cassava cultivars (H226, H165, Sree Vijaya, Sree Sahya and TMS 60444) in MS medium supplemented with 12 mgl^{-1} picloram using immature leaf lobe as explants. Plantlets were successfully developed from these embryogenic cultres. These findings shows the potential for using them in transgenic approach for creating disease resistance as well as in understanding different molecular mechanism of cassava for important characters.

Acknowledgement

The authors are grateful to the Director, ICAR-CTCRI for extending all facilities. This work was initiated under the financial support of ICAR Network Projects on Transgenics in Crops and completed with the support of Indo Swiss Cassava Network Project (No. BT/IC-2/ISCB/ Phase IV/01/Cassava/2014).

References

Abraham, K., Nair, S. G. and Naskar, S. K. 2000. Cassava breeding and varietal dissemination in India - Major achievements during the past 25-30 years. *In Sixth Asian Cassava Research Workshop*,. 21-25 February 2000. Vietnam. pp 176-181.

Adejare, G. O. and Coutts, R. H. 1981. Eradication of cassava mosaic disease from Nigerian cassava clones by meristem-tip cultivation. *Plant Cell, Tiss. Org. Cult.,* **1**: 25-32.

Anitha J., Makeshkumar, T. and Edison, S. 2011. Survey of cassava mosaic disease in Kerala. *J. Root crops,* **37**(1): 41-47.

Anuradha, T., Kumar, K. K. and Balasubramanian, P. 2015. Cyclic somatic embryogenesis of elite Indian cassava variety, H226. *Indian J. Biotechnol.,* **14**: 559-565.

Atehnkeng, J., Adetimirin, V.O. and Ng, S.Y.C. 2006. Exploring the African cassava (*Manihot esculenta* Crantz) germplasm for somatic embryogenic competence. *Afric. J. Biotechnol.*, **5**: 1324-1329.

Beena, M. R., Winter, S. and Makeshkumar, T. 2014. Influence of age of explants and genotypes on somatic embryogenesis in African and Indian cassava cultivars. *J. Root crops,* **40**(2): 21-27.

Berbee, F.M., Berbee, J.G. and Hildebrandt, A.C., 1973. Induction of callus and virus symptomless plants from shoot tip cultures of cassava. *In vitro*, **8**: 421.

Bull, S.E., Owiti, J.A., Niklaus, M., Beeching, J.R., Gruissem, W., and Vanderschuren, H. 2009. Agrobacterium-mediated transformation of friable embryogenic calli and regeneration of transgenic cassava. *Nat. Protocol*, **4**: 1845 -1854.

Chavarriaga-Aguirre, P., Brand, A., Medina, A., Prías, M., Escobar, R., Martinez, J., Díaz, P., López, C., Roca, W. M. and Tohme, J. 2016. The potential of using biotechnology to improve cassava: a review. *In Vitro Cell. Dev. Biol. Plant*, **52**(5): 461-478.

Deepthi, D.C. and Makeshkumar, T. 2016. Elimination of cassava mosaic disease through meristem culture and field evaluation for yield loss assessment in cassava genotype. J. Root Crops, **42**: 45-52

Doyle, J. J. and Doyle, J. L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem.*, **19**: 11-15.

Food and Agricultural Organisation. 2015. *Cassava area harvested, yield and production data*, 2014.

Gresshoff, P. and Doy, C. 1974. Derivation of a haploid cell line from *Vitis vinifera* and the importance of the stage of meiotic development of anthers for haploid culture of this and other genera. Z. Pflanzen physiol., **73**:132–141.

Hankoua, B.B., Ng, S.Y.C., Fawole, I., Puonti-Kaerlas, J., Pillay, M. and Dixon, A.G.O. 2005. Regeneration of a wide range of African cassava genotypes via shoot organogenesis from cotyledons of maturing somatic embryos and conformity of the field-established regenerants. *Plant Cell Tiss. Org. Cult.*, **82**: 221-231.

Hankoua, B. B., Taylor, N. J., Ng, S.Y.C., Fawole, I., Puonti-Kaerlas, J., Padmanabhan, C., Yadav, J.S., Fauquet, C.M., Dixon, A.G.O. and Fondong, V.N., 2006. Production of the first transgenic cassava in Africa via direct shoot organogenesis from friable embryogenic calli and germination of maturing somatic embryos. *Afric. J. Biotechnol.*, **5**: 1700–1712.

ICAR-CTCRI, Annual Report 2015 - 2016, ICAR-Central Tuber Crops Research Institute, Thiruvananthapuram, Kerala, India, pp.168.

Jayakrishnan, J.T. and Makeshkumar, T. 2016. Molecular phylogeography of cassava mosaic viruses in Kerala. In*: National Conference on Tropical Tuber Crops for the Sustenance and welfare of tribal communities- 2016, Conference document.* Sheela Immanuel (Eds). St. Josephs Press, Thiruvananthapuram, p. 234.

Jos, J.S., N. Hrishi, S. B. Maini and R. G. Nair. 1981. Two high yielding hybrids of cassava. *J. Root Crops*, **6**: 1-6.

Joseph, R., Yeoh, H. H. and Loh, C. S. 2004. Induced mutations in cassava using somatic embryos and the identification of mutant plants with altered starch yield and composition. *Plant Cell Rep.*, **23**(1/2): 91-98.

Kaiser, W. J. and Teemba, L. R. 1979. Use of tissue culture and thermotherapy to free East African cassava cultivars of African cassava mosaic and cassava brown streak diseases. *Pl. Dis. Rep..*, **63**: 780-784.

Kartha, K. K. and Gamborg, O. L. 1974. Elimination of cassava mosaic disease by meristem culture. *Phytopath.*, **65**: 826-828.

Kartha, K. K., Gamborg, O. L., Constabel, F. and Shyluk, J. P. 1975. Regeneration of plants through shoot apical meristems. *Plant Sci. Lett.*, **2**: 107-113.

Liu, J., Zheng, Q., Ma, Q., Gadidasu K.K., Zhang, P. 2011. Cassava genetic transformation and its application in breeding. *J. Integr. Plant Biol.*, **53**: 552–569.

Magoon, M. L., S. G. Appan, R. Krishnan and R. C. Mandal. 1970. Some promising high yielding hybrids and selections of cassava. *SABRAO Newsletter* **2**: 19-26.

Malathi, V. G., Nair, N. G. and Santha, P. 1985. Cassava mosaic disease. *Central Tuber Crops Research Institute, Technical bulletin series*, **5**: 18.

Makeshkumar, T., Anoopsankar, Nair, R. R. and Edison, S. 2005. Detection of Indian cassava mosaic virus through Polymerase chain reaction and Nucleic acid Hybridisaton techniques. *J. Root crops*, **31**(1): 1-6.

Makeshkumar, T., Dhanya J., Beena, M. R., Prakash Krishnan, B.S. and Winter, S. 2012. Studies to develop RNAi mediated resistance against cassava mosaic viruses in India. In: Second Scientific Conference of Global Cassava Partnership for the 21st Century, Oral and Poster presentation - GCP21, Kampala, Uganda.

Murashige, T., Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*., **15**(3): 473-497

Nair, N. G. 1990. Performance of virus - free cassava (*Manihot esculenta* Crantz.) developed through meristem tip culture. *J. Root Crops*, **16**(2): 123 – 131.

Nair, N. G. and Chandrababu, S. 1996. *In vitro* production and micropropagation of three species of edible yams. In: *Tropical Tuber Crops: Problems, Prospects and Future Strategies*. Kurup, G. T., Palaniswami, M.S., Potty, V.P., Padmaja, G., Kabeerathumma, S. and Pillai, S. V. (Eds.). Oxford and IBH Publishing, New Delhi. pp. 211-215.

Nkaa, F. A., Ene-Obong, E. E., Taylor, N. J., Fauquet, C. M. and Mbanaso, E.N.A. 2013. Elimination of *African cassava mosaic virus* (ACMV) and *East African cassava mosaicvirus* (EACMV) from cassava (*Manihot esculenta* Crantz) cv. 'Nwugo' via somatic embryogenesis. *Am. J. Biotechnol. Mol. Sci*., **3**(2): 33-40.

Nkaa, F. A., Ene-Obong, E. E., Taylor, N. J., Fauquet, C. M. and C. N. Egesi. 2015. Evaluation of ten elite Nigerian cassava (*Manihot esculenta* Crantz) cultivar for somatic embryogenesis and regeneration potentials. *J. Adv. Biol. Biotechnol*., **2**(2): 133-143.

Nyaboga, E., Njiru,J., Nguu, E., Gruissem, W., Vanderschuren, H., and Tripathi, L. 2013. Unlocking the potential of tropical root crop biotechnology in east Africa by establishing a genetic transformation platform for local farmer- preferred cassava cultivars. *Front. Plant Sci*., **4**: 526.

Nyaboga, E., Njiru, J., and Tripathi, L., 2015. Factors influencing somatic embryogenesis, regeneration, and Agrobacterium-mediated transformation of cassava (*Manihot esculenta* Crantz) cultivar TME14. *Front. Plant Sci*., **6**: 411.

Otono, F. B., Ndofunsu, A. D., Nakweti, R. K., Ntumbula, A. M., Hity, M., Kasali, L., Ndiku, L., Gladys, R. and Philippe, L. 2015. PCR and ELISA detection of cassava mosaic virus in a Congolese cassava landrace. *Intern. J. Biotechnol. Food Sci*., **3**(1):10-16.

Patil, B. L., Rajasubramaniam, S., Bagchi, C. and Dasgupta I. 2005. Both Indian cassava mosaic virus and Sri Lankan cassava mosaic virus are found in India and exhibit high variability as assessed by PCR-RFLP. *Arch Virol*., **150**: 389-397.

Pinheiro, M. V. M. 2013. Maturation of *Anthurium andraeanum* cv. Eidibel somatic embryos from nodal segments. *In Vitro Cell. Dev. Bio. Plant*. **49**(3): 304–312.

Raemakers, K., Schreuder, M., Pereira, I., Munyikwa, T., Jacobsen, E. and Visser, R. 2001. Progress made in FEC transformation of cassava. *Euphytica*, **120**: 15-24.

Rajanimala, N., Rabindran, R., Mohan, S., and Sethuraman, K. 2011. Survey for the occurrence of Cassava mosaic Disease in Tamil Nadu. *J. Root Crops*, **37**(2): 197-199.

Rossin, C.B. and Rey, M.E.C. 2011. Effect of explant source and auxins on somatic embryogenesis of selected cassava (*Manihot esculenta* Crantz) cultivars. *South Afri. J. Bot*., **77**: 59–65.

Saelim, L., Phansiri, S., Netrphan, S., Suksangpanomrung, M. and Narangajavana, J. 2006. Optimization of *in vitro* cyclic somatic embryogenesis and regeneration of the Asian cultivars of cassava (*Manihot esculenta* Crantz) for genetic manipulation system. *Global J. Biotechnol. Biochem*., **1**: 7-15.

SAS. 2010. SAS/STAT Software Version 9.3, SAS Institute Inc. Cary, NC.

Sheela, M. N., Abhilash, P. V., Dharshan, S., Prakash Krishnan, B. S, Arun, P., Makeshkumar, T., Asha, K.I., Mohan, C., Sunitha, S., Krishna Radhika, N., Asha Devi, A. and James George. 2016. Breeding for varieties resistant to cassava mosaic disease using different source of resistance. In*: National Conference on Tropical Tuber Crops for the Sustenance and welfare of tribal communities- 2016 Conference document.* Sheela Immanuel (Eds.). St. Josephs Press, Thiruvananthapuram. pp. 42.

Szabados, L., Hoyos, R. and Roca, W. 1987. *In vitro* somatic embryogenesis and plant regeneration of cassava. *Plant Cell Rep*., **6**(3): 248-251.

Taylor, N.J., Edwards, M., Kiernan, R.J., Davey, C.D., Blakesley, D. and Henshaw, G.G. 1996. Development of friable embryogenic callus and embryogenic suspension culture systems in cassava (*Manihot esculenta* Crantz). *Nat Biotechnol*., **14**(6): 726-730.

Taylor, T., Gaitán-Solís, E., Moll, T., Trauterman, B., Jones, T., Pranjal, A., Trembley, C., Abernathy, V., Corbin, D. and Fauquet, C. M. 2012. A high-throughput platform for the production and analysis of transgenic cassava (*Manihot esculenta*) plants. *Trop. Plant Biol*., **5**: 127-139.

Thankappan, M., Makeshkumar, T. and Edison, S. 1997. Management of virus diseases in tropical tuber crops. *Proceedings of the International Conference on Integrated Plant Disease Management for Sustainable Agriculture*, Indian Phytopathological Society, pp. 1173-1175.

Ubalua, A. O. and Mbanaso, E. 2014. Somatic embryogenesis in two Nigerian cassava cultivars (Sandpaper and TMS 60444). *J. Evol.Biol. Res*., **6**(3): 9-12.

Vidal, Á. M., de Carvalho C. M. A. P., da Silva, S.A., de Almeida, W. A. B., and Souza, F. V. D. 2014. *In vitro* regeneration and morphogenesis of somatic embryos of cassava. *Revista Ciência Agronômica*, **45**(3): 558-565.