British Biotechnology Journal
4(3): 254-269, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Influence of Type and Age of Primary Somatic Embryo on Secondary and Cyclic Somatic Embryogenesis of Cassava (*Manihot esculenta* Crantz)

Jelili T. Opabode[1*], Olufemi O. Oyelakin[2,3], Oluyemisi A. Akinyemiju[1] and Ivan L. Ingelbrecht[2,4]

[1]Department of Crop Production and Protection Obafemi Awolowo University, Ile-Ife, Nigeria.
[2]Central Biotechnology Laboratory, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria; C/O Lambourn Ltd., Carolyn House, 26 Dingwall Road, Croydon CR9 3EE, UK.
[3]Biotechnology Center, Federal University of Agriculture, Abeokuta, Nigeria.
[4]Department of Plant Biotechnology and Bio-informatics, Ghent University, K L Ledeganckstraat 35, B-9000 Ghent, Belgium.

Authors' contributions

This work is a portion of the Ph.D Thesis of the first author JTO. The authors OOO, OAA and ILI read and approved the final manuscript.

Original Research Article

Received 8th March 2013
Accepted 14th September 2013
Published 30th January 2014

ABSTRACT

This study investigated the influence of age of the cotyledons, cut from primary somatic embryos (PSE) developed from shoot meristems (SM) or immature leaf lobes (LL), on secondary somatic (SSE) and cyclic (CSE) embryogenesis of two cassava cultivars at the Central Biotech Laboratory, International Institute of Tropical Agriculture (IITA), Ibadan, between 2006 and 2010. A completely randomized design with three replicates was used for the study. Only PSE at the age of 4 weeks recorded significant ($P<0.05$) differences in SSE frequency and efficiency between the SM and LL sources. CSE production was highest using 0 to 4 weeks old SSE cotyledons, and significant ($P<0.05$) differences were only recorded between the SM and LL sources when the age of the SSE cotyledons was older than 6 weeks. The CSE frequencies from the SM source were significantly greater than that from the LL source when 8 and 10 week-old SSE cotyledons were used. The

**Corresponding author: Email: jopabode@yahoo.com;*

CSE frequencies from SM (81%, 82%) were still significantly higher than those from LL (41%, 40%) at the 5th and 6th cycles respectively while the CSE efficiency only differed at the 4th cycle, with SM (7.1) being significantly more than from LL (5.2). These results also enrich the literature by specifying the age of somatic cotyledon suitable for further somatic embryogenic cycles; 0 to 8 weeks for SSE and 0 to 5 weeks for CSE, irrespective of the cultivar (TME 12 or Kibaha) or source (SM or LL). This study further concluded that cyclic embryos should be discarded after the 4th CSE cycle and fresh starting material should be used to restart the somatic embryogenic process.

Keywords: Cassava; cyclic; cotyledon; somatic embryognenesis; secondary.

ABBREVIATIONS

PSE: Primary Somatic Embryo,
SSE: Secondary Somatic Embryogenesis,
CSE: Cyclic Somatic Embryogenesis,
LL: Immature Leaf Lobes,
SM: Shoot Meristem

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz) constitutes an important source of energy for about 600 million people in tropical and subtropical climates [1]. The increase in cassava cultivation and its important role in food security are enhanced by two factors: the unique biology of the crop and the numerous ways its starch and by-products are being used, including its use as livestock feed, source of bio-ethanol, applications in food industry, agrochemical and pharmaceutical industries [2,3].

Cassava grows well on marginal soils and is tolerant of drought and soil acidity [3]. Fresh cassava leaves serve as a good source of vegetable while its stems are utilized as stakes in yam production and as firewood. There is flexibility in harvesting, as tubers can be stored in the soil fairly long which ensures convenience in processing and marketing [4]. The starch content of cassava roots ranges from 65-91% of its total root dry weight depending on the cultivar [5]. The global demand for cassava starch is rising and it is a highly competitive and preferred starch in comparison to the conventional sources of starch like wheat, maize, rice and potato [2]. Presently, 60 million tons of starch is extracted from various sources, 10% of this comes from cassava.

Despite its potential for achieving food security and economic growth, biotic and abiotic constraints such as diseases, pests, weeds, poor soil fertility and drought are militating against cassava production [6,7]. Other problems facing cassava cultivation include postharvest physiological deterioration, high cyanide content, low protein content and fluctuating starch quality [8]. Application of conventional breeding methods for improvement of cassava against the biotic and abiotic constraints has so far recorded limited successes [4,6].

Conventional breeding of cassava is challenging due to the highly heterozygous nature of the crop which prevents a backcross scheme. The problem is exacerbated by poor flowering and limited seed set of many varieties [6]. In addition, carrying out sexual crosses in cassava is difficult and inbreeding depression is common. The problem is compounded by limited

sources of genetic resistance to most abiotic and biotic stresses [6,7]. Numerous metabolic pathways and gene networks involved in biosynthesis of starch and post-harvest physiological deterioration of tubers are bottlenecks to the effective use of conventional breeding strategies in cassava [3,7].

In the field, cassava is typically propagated clonally by stem cuttings. This propagation strategy is ideal for a bio-engineering approach to crop improvement as gene segregation through outcrossing is limited [9]. Successful genetic modification by bio-engineering requires the establishment of *in vitro* regeneration and transformation systems [9,10]. To date, the only reported means of incorporating foreign pieces of DNA into the cassava genome is via regeneration through somatic embryogenesis.

Plant regeneration via somatic embryogenesis has become an integral component of the genetic transformation system of cassava [9 -11]. Regeneration studies have shown that the frequency and efficiency of somatic embryogenesis are highly genotype-dependent, and not all cassava cultivars are amenable to somatic embryogenesis, regeneration and transformation [9-12]. Therefore, there is a need to optimize the generation of embryogenic structures for each cassava cultivar [13-19]. As a result, more than 60 African cassava cultivars, and other cultivars from South America and Asia, have been tested for their somatic embryo-producing ability. For the same reasons, somatic embryos have been induced from various cassava explants/propagules including immature leaf lobes [13-21], shoot apical meristems [13,16 21], zygotic embryos or floral tissue [21], and axillary buds [22] on several media containing various plant growth regulators.

Somatic embryogenesis in cassava is a multistage process comprising the induction and maturation of primary, secondary, cyclic and green (mature) embryos. This multistage somatic embryogenesis is affected by factors such as (1) frequency and efficiency of somatic embryogenesis, (2) regeneration system and (3) compatibility with transformation methods [20,22].

Each stage requires specific periods of time for development and the product of one stage serves as the starting material (propagule) for the next stage. Usually embryos are kept on maturation medium in the dark at 25ºC for varying period before being used as propagules (usually the cotyledons) for the next stage depending on the progress of regeneration and transformation experiments. Nutrients in the maturation medium are used by the embryos without immediate replenishment which may have a negative influence on the embryogenic competence of the cotyledon to be used for the next stage.

Many features are known to affect the efficiency of culture initiation and development of somatic embryos. Younger, more rapidly growing tissues, or tissue at an early stage of development, are most effective. Raemakers et al. [23] first noted that the secondary embryogenic competence of older mature embryos was lower than that of fresh mature embryos in cassava. A significant effect of age of somatic cotyledons on the induction of cyclic somatic embryos in Hanatee and KU 50 cassava cultivars was also reported [24]. The production of embryos from the sixth cycle of cyclic somatic embryogenesis in M.col 22 cassava cultivar was as efficient as that from the second cycle [23].

Although cassava somatic embryogenesis optimization has been reported, yet information on the influence of embryo age and its source on secondary and cyclic somatic embryogenesis efficiency is lacking for many cultivars with useful agronomic traits. Such information is essential for a complete optimization of the regeneration system in several cassava cultivars.

Similarly, information is limited on the influence of the number of cycles on somatic embryogenic frequency. In our Cassava Genetic Transformation Programme, we spent 18 months producing secondary and cyclic somatic embryos as the cultivars were initially recalcitrant to *Agrobacterium*-mediated transformation. During this period, we observed variations in the morphology of the embryos, inconsistency in the duration of development of the embryos, appearance of root-like structures in culture media, the increase in callus size and frequency, and a decrease in frequency and efficiency of secondary and cyclic somatic embryogenesis.

In order to put these problems into perspective and to optimize the available protocols, this study was conducted to increase our knowledge on the effect of age of cotyledon and source and number of cycles on SSE and CSE efficiency. The objectives of this study were therefore to ascertain the effects of (1) cultivar, source and age of somatic cotyledon on the frequency and efficiency of secondary and cyclic somatic embryogenesis and (2) cycle number on the frequency and efficiency of cyclic somatic embryogenesis.

2. MATERIALS AND METHODS

2.1 Definition of Terms

In this paper, 'primary somatic embryo' (PSE) is a somatic embryo produced originally from an immature leaf lobe (LL) or a shoot meristem (SM). 'Secondary somatic embryo' (SSE) is an embryo obtained from cultured cotyledons of a primary somatic embryos. 'Cyclic somatic embryo' (CSE) is generated from secondary somatic embryo to produce either mature somatic embryos or more cyclic somatic embryos. 'Mature somatic embryo' (MSE) is an embryo with green cotyledons which is generated from cultured cotyledons of secondary or cyclic somatic embryos; it can be used for regeneration or transformation purposes. 'Shoot meristem' is a meristem (1-2 mm) obtained from BAP-induced enlarged axillary buds. 'Pre-embryogenic structures' are developing bipolar globular embryos contained in the callused cultures. 'Somatic embryogenesis frequency' is the percentage of cultured cotyledons that produced somatic embryos. 'Somatic embryogenesis efficiency' is the number of embryos produced per cotyledon.

2.2 Plant Materials

Plantlets of the cassava cultivars TME 12 and Kibaha were obtained from the *in vitro* germplasm collection of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. The plantlets were maintained by regular subculturing at four weeks interval as *in vitro* shoot cultures on basal medium (BM). Cassava cultivars TME 12 and Kibaha are farmer-preferred cultivars in west and east Africa, respectively, which are included in our Cassava Transformation programme for improvement against biotic and abiotic stresses.

2.3 Basal Medium and Culture Conditions

Basal medium (BM) which, consisted of full-strength MS [24] salts (Sigma, USA) along with 0.8% (w/v) agar (Oxoid Ltd, England), 30 g/l sucrose (Sigma, USA) and 2µM $CuSO_4$ (Sigma, USA) was used in all experiments unless otherwise stated. The pH of the medium was adjusted to 5.8 by HCl (1 N) or NaOH (1N) priorto autoclaving at 121^0C for 15 minutes at 1.05 kg cm^{-2} pressure. Growth regulators were filter sterilized through 0.22-µM Millipore filters and added to the media after autoclaving. For all experiments, cultures were

maintained under 16 h photoperiod with 20 mol m^{-2} s^{-1} light intensity provided by cool-white fluorescent tubes at 25±2ºC.

2.4 Induction and Maturation of Primary Somatic Embryos

Primary somatic embryos were obtained from two sources: immature leaf lobes (LL) and shoot meristems (SM). The primary somatic embryos were induced from immature leaf lobes as previously described [16]. Immature leaf lobes (1-4 mm) obtained from four-week old *in vitro* plantlets were incubated in the dark on BM supplemented with 10 mg/l picloram for 14 days. Callused leaf lobes containing pre-embryogenic structures were then incubated in the dark on BM supplemented with 0.1mg/l BAP for 10 days for maturation.

For shoot meristems, the procedure as described by [21] was followed for induction of primary somatic embryos from meristems isolated from enlarged axillary buds. Nodal explants were excised from three-week old *in vitro* plantlets and incubated on BM containing 10 mg/l BAP in darkness for seven days for the enlargement of the axillary buds. Shoot meristems (1-2 mm) were isolated from the enlarged axillary buds with a sterile surgical blade and forceps using a stereo-microscope in a horizontal laminar flow bench. The isolated shoot meristems were incubated on BM supplemented with 10 mg/l of picloram for 17 days for induction of primary somatic embryos. Callused meristems with pre-embryogenic structures were then incubated in the dark on BM supplemented with 0.1 mg/l BAP for 10 days for maturation.

2.5 Effect of Cultivar and Cotyledon Source on Secondary Somatic Embryogenesis

Secondary somatic embryos were produced from primary somatic embryo cotyledons obtained from the two sources (leaf lobes and shoot meristems) of two cassava cultivars (TME 12 and Kibaha) as outlined by Hankoua et al. [16]. Cotyledons of primary somatic embryos (4-6 mm) were harvested and incubated on BM supplemented with 6 mg/l 2,4-D for induction of secondary somatic embryos for two weeks. Explants with pre-embryogenic structures were then transferred to BM fortified with 0.1 mg/l BAP. Plates were scored for the presence of secondary somatic embryos after 14 days of dark incubation. In the experiment, three hundred cotyledons were used per treatment with each Petri dish containing 30 cotyledons.

2.6 Effect of Cultivar, Cotyledon Source and Age on Secondary Somatic Embryogenesis

Harvested primary somatic cotyledons of TME 12 and Kibaha obtained from shoot meristems (SM) and leaf lobes (LL) of different (0, 2, 4, 6, 8, 10 and 12 weeks old) ages were incubated in darkness on BM fortified 12 mg/l 2, 4-D. For clarity, 0 week means cotyledons subcultured immediately after emergence and the primary cultures were three weeks old. Cotyledons with pre-embryogenic structure were placed on BM supplemented with 0.1 mg/l BAP for maturation. Plates were scored for the presence of secondary somatic embryos after 14 days of dark incubation. In the experiment, three hundred cotyledons were used per treatment with each Petri dish containing 30 cotyledons.

2.7 Effect of Cultivar and Cotyledon Source on Cyclic Somatic Embryogenesis

Secondary somatic embryos were produced from primary somatic embryo cotyledon obtained from two cassava cultivars (TME 12 and Kibaha) and two sources (leaf lobe and

shoot meristem) following the method of Hankoua et al. [16]. The Cotyledons of secondary somatic embryos (4-6 mm) were incubated on BM supplemented with 12 mg/l 2, 4-D for induction of cyclic somatic embryos for two weeks. Explants with pre-embryogenic structures were transferred to BM fortified with 0.1 mg/l BAP. Plates were scored for the presence of secondary somatic embryos after 14 days of dark incubation. Three hundred cotyledons per treatment were used with each Petri dish containing 30 cotyledons.

2.8 Effect of Cotyledon Source and Age on Cyclic Somatic Embryogenesis

Secondary somatic cotyledon of TME 12 and Kibaha originally obtained from shoot meristems and leaf lobes of different (0, 2, 4, 6, 8, 10 and 12 weeks old) ages were incubated in the dark on BM fortified with 12 mg/l 2, 4-D. Cotyledon with pre-embryogenic structures were placed on BM supplemented with 0.1 mg/l BAP for maturation. Plates were scored for the presence of cyclic somatic embryos after 14 days of dark incubation. In the experiment, three hundred cotyledons were used per treatment with each Petri dish containing 30 cotyledons.

2.9 Effect of Number of Cycle on Cyclic Somatic Embryogenesis

Secondary somatic cotyledons of TME 12 and Kibaha originally obtained from shoot meristems and leaf lobes were incubated in the dark on BM fortified with 12 mg/l 2, 4-D for six consecutive cycles. Cotyledons with pre-embryogenic structures were placed on BM supplemented with 0.1 mg/l BAP for maturation. Plates were scored for the presence of cyclic somatic embryos after 14 days of dark incubation. In the experiment, three hundred cotyledons were used per treatment with each Petri dish containing 30 cotyledons.

2.9.1 Experimental design and statistical analysis

In all experiments, treatments were arranged in a completely randomized design with four replications and experiments were repeated thrice. Percent and count data were subjected to arcsine and square root transformations to normalize variances. Data were further subjected to analysis of variance to detect differences among treatments. Means were separated by Duncan Multiple Range Test at 5% level of probability as outlined by Gomez and Gomez [25].

3. RESULTS

3.1 Induction and Maturation of Primary Somatic Embryos

We have reported the production of primary somatic embryos from shoot meristems and leaf lobes of selected cassava varieties [12]. Thereafter, secondary, cyclic and green somatic embryos were produced for this study (Fig. 1). Using *in vitro* leaf lobes as starting material, primary somatic embryos of TME 12 were obtained between 22 and 27 days after incubation on the induction medium. For TME 12, the primary somatic embryo frequency and efficiency were 22.3% and 3.5, respectively (data not shown). Kibaha leaf lobes produced primary somatic embryos faster at between 18 and 24 days after incubation. In Kibaha, the primary somatic embryo frequency and efficiency were 25.8% and 3.8, respectively (data not shown). Detail information on PSE from shoot meristems can be obtained from our previous work [12].

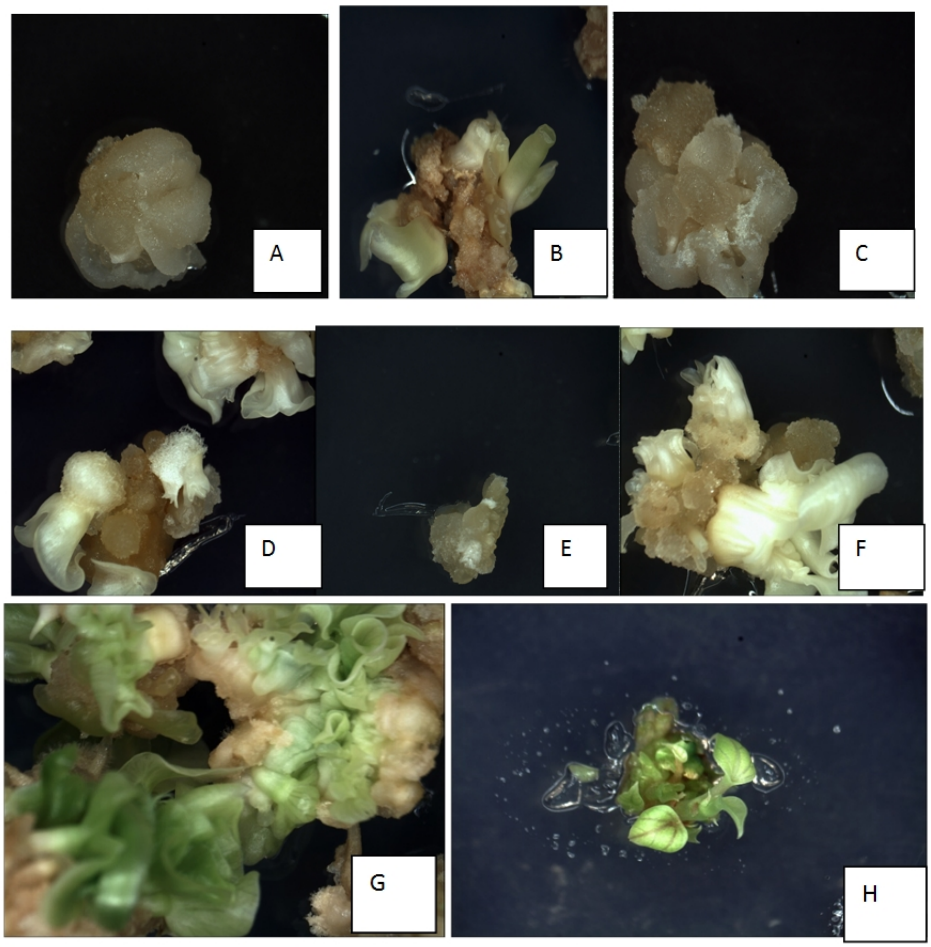


Fig. 1:Production of somatic embryos using leaf lobe as starting propagules in cassava variety TME 12. A- primary embryogenic callus (x1), B- primary somatic cotyledon (x1/2), C- secondary embryogenic callus (x1), D- secondary somatic embryos (x1/2), E-cyclic embryogenic callus (x/2), F- cyclic somatic embryos (x1), G-green somatic embryo (x1/3), E-regenerated plantlet from a mature somatic embryo (x2).

3.2 Effect of Cultivar and Cotyledon Source on Secondary Somatic Embryogenesis

Different types of somatic embryos were obtained in this study. There were significant (*P*=.05) differences in the percentage of cultures with pre-embryogenic structures, as well as the SSE frequency and efficiency between TME 12 and Kibaha cultivars (Table 1). The SSE frequency and efficiency of TME 12 were significantly higher than those of Kibaha with the SSEs of TME 12 and Kibaha mostly located at the top of PSE cotyledon while those of Kibaha were evenly distributed (Table 1). There were no significant (*P*>.05) differences in the percentage of cultures with pre-embryogenic structures and SSE efficiency between SM and LL source (Table 1). The SSE frequency of LL was significantly ($p<0.05$) higher than that from SM source. Secondary somatic embryos from the LL source were mostly located at the top of the cotyledon while those of the SM were evenly distributed on the cotyledon of Kibaha (Table 1).

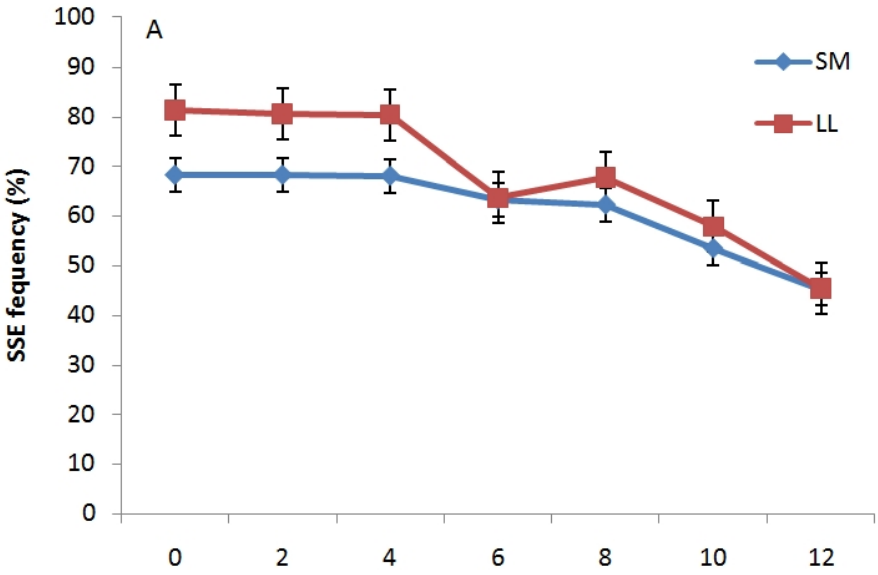
Table 1. Effect of cultivar and cotyledon source on secondary somatic embryogenesisin Cassava

Cultivar	Cotyledon source	% explant with pre-embryogenic structure	SSE frequency (%)	SSE efficiency (Number of embryo/cotyledon)	Location of embryo on explant
TME 12	SM	83.4±3.7	78.6±5.4	6.7±1.4	top
	LL	91.5±1.9	86.7±2.3	7.3±0.7	top
Kibaha	SM	74.7±4.2	72.3±5.7	6.2±1.8	even
	LL	78.3±1.5	83.4±2.1	6.1±0.4	top
LSD(0.05):	cultivar	5.32	4.80	0.32	
	source	7.43	7.25	0.81	

Values are means ± standard of the mean SM – shoot meristem, LL – leaf lobe, SSE –secondary somatic embryogenesis. LSD – Least Significant Difference at 5% level of probability

3.3 Effect of Cotyledon Source and Age on Secondary Somatic Embryogenesis

There was no significant (*P*>.05) difference in the influence of cotyledon source and age on SSE frequency and efficiency between TME 12 and Kibaha, therefore data for both cultivars were averaged and presented in Fig. 2. When the cotyledon ages were 0, 2, and 4 weeks old, SSE frequency depended on the cotyledon source. Beyond those ages (i.e between 4 and 12 weeks old), there was no significant difference in SSE frequency between SM and LL cotyledons (Fig. 2a). The SSE frequencies and efficiencies from the LL source were significantly (*P*=.05) greater than those from the SM source at 0, 2, 4 weeks of age (Fig.2b). From both the LL and SM sources, there was no marked increase in SSE efficiency between age 0 and 8 weeks, thereafter the SSE efficiency decreased sharply when the ages of explants were 10 and 12 weeks old (Fig. 2b)



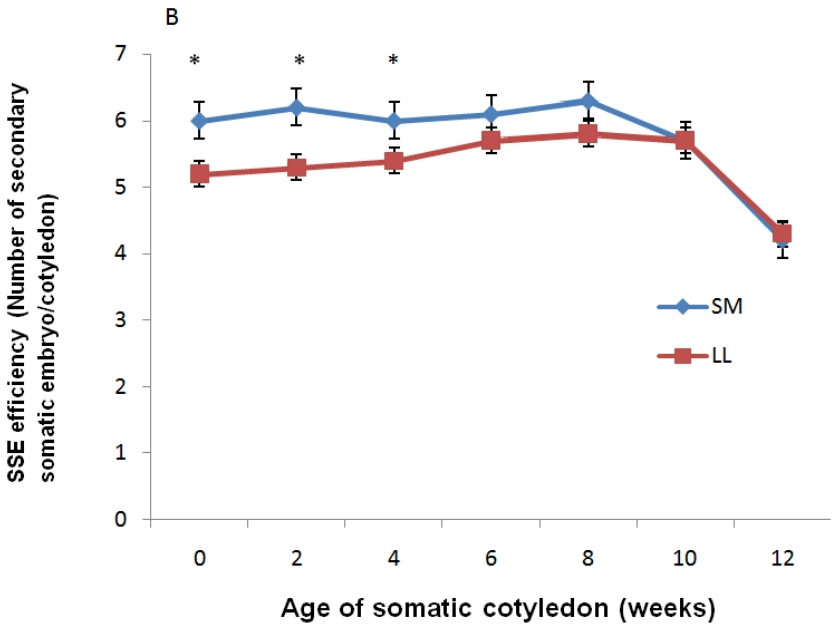


Fig. 2. Effect of cotyledon age and source on SSE frequency (A) and SSE efficiency (B) of cassava.

SM–shoot meristem, LL – leaf lobe, SSE – secondary somatic embryogenesis. Error bars represent the standard error of the mean. Asterisks indicate significant differences between cotyledon source at the P=.05 significance level. Each value is the mean of two cassava varieties

3.4 Effect of Cultivar and Cotyledon Source on Cyclic Somatic Embryogenesis

Both cultivar and cotyledon source significantly (*P*=.05) influenced CSE frequency and efficiency (Table 2). The CSE frequency and efficiency of TME 12 was significantly higher than that of Kibaha, especially from the LL source. Cyclic somatic embryos of TME 12 were mostly located at the top while that of Kibaha were evenly distributed on the cotyledon (Table 2). Similarly, CSE frequency and efficiency from the LL source was significantly higher than that from the SM source for both cultivars.

Table 2. Effect of cultivar and cotyledon source on cyclic somatic embryogenesis in Cassava

Cultivar	Cotyledon source	% explant with pre-embryogenic structure	CSE frequency (%)	CSE efficiency (Number of embryo/cotyledon)	Location of embryo on explant
TME 12	SM	85.3±3.8	92.5±4.3	6.5±0.7	top
	LL	99.2±0.8	97.3±1.4	7.4±0.2	top
Kibaha	SM	87.8±4.4	88.4±3.8	5.8±0.6	even
	LL	96.3±0.5	92.1±1.8	6.2±0.2	even
LSD(0.05):	cultivar	4.82	2.37	0.41	
	source	7.26	3.52	0.53	

Values are means ± standard of the mean SM – shoot meristem LL – leaf lobe CSE – cyclic somatic embryogenesis LSD – Least Significant Difference at 5% level of probability

3.5 Effect of Cultivar, Cotyledon Source and Age on Cyclic Somatic Embryogenesis

The influence of cotyledon source and age on CSE frequency and efficiency did not significantly depend on cultivar, therefore data for TME 12 and Kibaha were averaged and presented in Fig. 3. The CSE frequency remained high (>81%) for cotyledons 0-4 weeks old, then decreased as cotyledon age increased, moresore from the LL than from the SM source being significantly less using 8 and 10 week old cotyledons (Fig. 3a). From the SM source, CSE frequency decreased by 35.6% between age 0 and 12 weeks; within the same period CSE frequency from the LL source decreased by 43.1%. From both SM and LL sources, CSE efficiency increased as the age of cotyledon used increased up to six weeks old, thereafter CSE efficiency decreased (Fig. 3b). The CSE efficiencies from the SM source were significantly higher those from the LL sources when 8 and 12 week old cotyledons were used while from the LLsource, CSE efficiency was only significantly more when 6 week old cotyledons were used (Fig. 3b). CSE efficiency was highest from the SM source using 6 week old cotyledons.

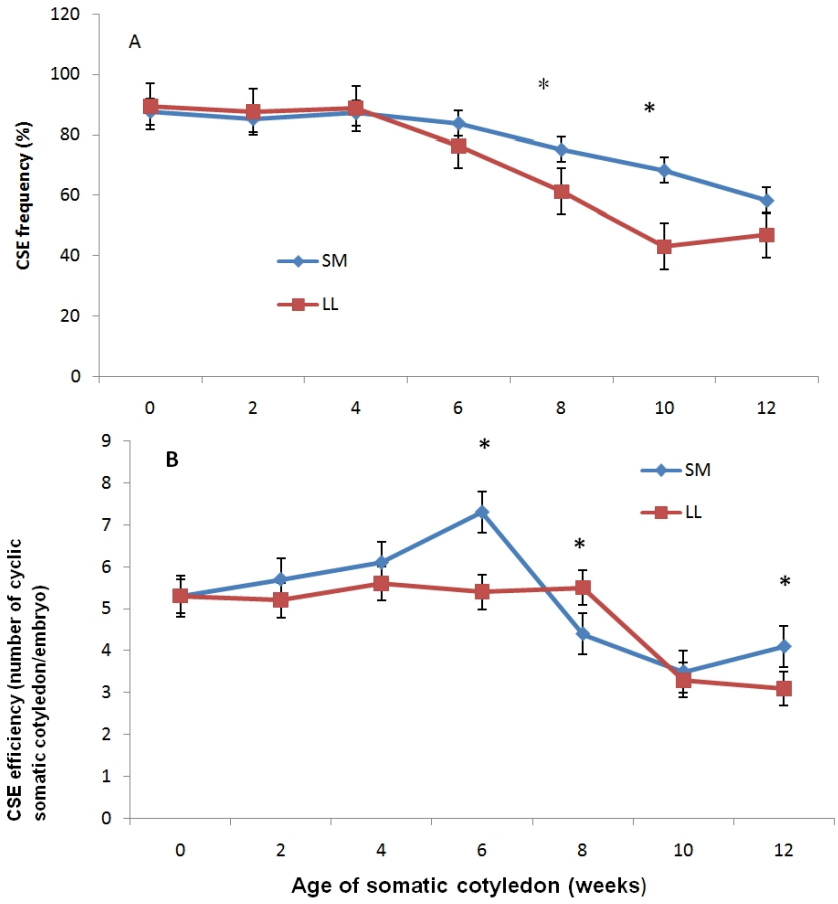


Fig. 3. Effect of cotyledon age and source on CSE frequency (A) and efficiency (B) of cassava.

SM–shoot meristem, LL – leaf lobe, CSE – cyclic somatic embryogenesis. Error bars represent the standard error of the mean. Asterisks indicate significant differences between cotyledon source at the P= 0.05 significance level. Each value is the mean of two cassava varieties

3.6 Effect of Number of Cycle on Cyclic Somatic Embryogenesis

There was no significant variety by cotyledon source interaction, therefore values presented were averages of both varieties (Fig. 4). The CSE frequencies from both the SM and LL sources increased between the 1st and 2nd cycle, thereafter it decreased slowly from the SM and rapidly from LL sources so that the at the 5th and 6th cycle, there were significant (*P*=.05) differences in CSE frequencies (Fig. 4a). However, CSE efficiency increased from both the SM and LL sources up to the 4th cycle and decreased thereafter with no significant difference between the two sources for all but the 4th cycle (Fig. 4b). At the 4th cycle, the CSE efficiency was significantly greater from the SM source than from the LL source and this was the highest CSE efficiency overall (Fig. 4b).

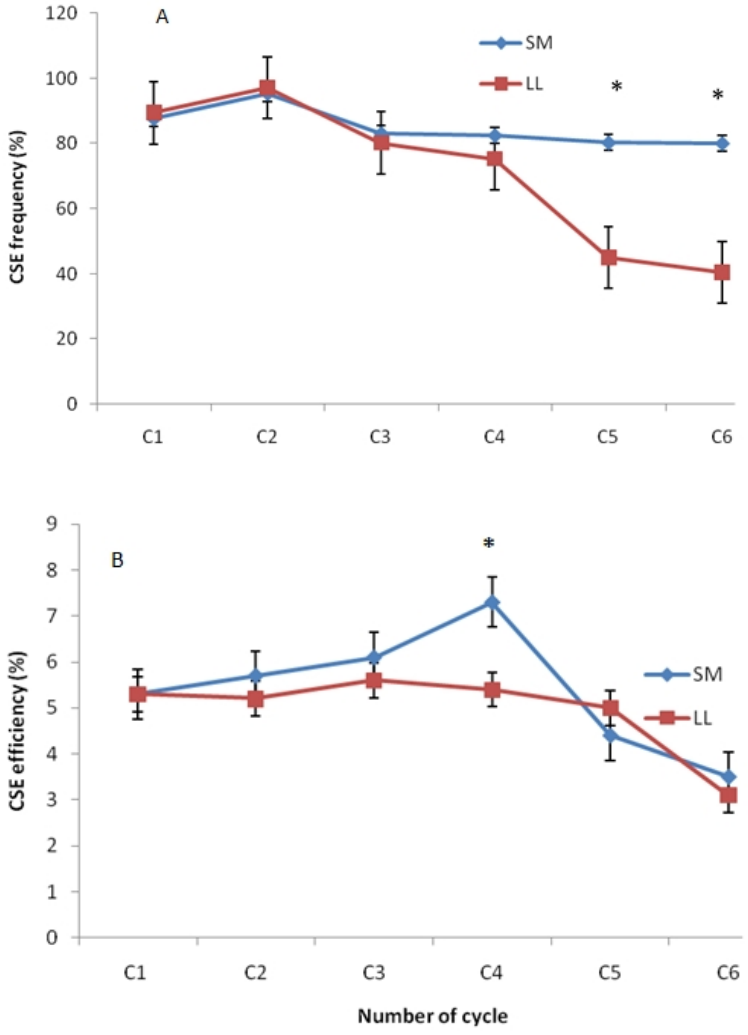


Fig. 4. Effect of number of cycles on CSE (A) and efficiency (A) of cassava.
SM – shoot meristem, LL – leaf lobe, CSE – cyclic somatic embryogenesis. Error bars represent the standard error of the mean. Asterisks indicate significant differences between explant source at the P= .05 significance level. Each value is a mean of two cassava varieties

4. DISCUSSION

The two cultivars (TME 12 and Kibaha) selected for this study were included in the cassava genetic transformation programme of International Institute of Tropical Agriculture (IITA), Ibadan, because they were farmer-preferred cultivars with agronomic traits that are cherished in Africa [12]. TME 12 dominates cassava cultivation in West Africa particularly in Nigeria where it is known as 'Tokunbo'. It is high yielding and moderately resistant to African cassava mosaic virus. Kibaha is moderately high yielding but susceptible to brown streak virus. TME 12 will be improved for enhanced starch qualities while Kibaha will be improved for resistance against brown streak virus.

Since the identification of the somatic embryo cotyledon as crucial tissue for the incorporation of foreign DNA for cassava genetic transformation, variability among cultivars for somatic embryogenesis frequency and efficiency has been frequently reported, particularly in primary somatic embryogenesis [12,16,21,23,26]. This has led to the screening of a wide range of cassava cultivars for somatic embryogenic competence using different culture media, auxins and starting materials. In the present study, cultivar and cotyledon source significantly influenced SSE production. The value of SSE frequency for TME 12 (82.7%) and Kibaha (74.9%) observed in this study (Table 1) was comparable to those of earlier reports (16,19,26). Li et al.[19] documented a range of SSE frequencies between 64% and 80% for some Latin America cassava cultivars. Similarly, Hankoua et al. [16] reported SSE frequencies of 48 to 100% for some African cultivars. Specifically, an SSE frequency of 97.5% was reported for TME 12. In another eight Latin America cassava cultivars, Feitosa et al. [26] reported a range of SSE frequencies of between 31.0 and 82.9%, and SSE efficiencies of between 1.6 and 10.6, with Rosa being the most productive cultivar.

In both varieties investigated in this study, in vitro leaf lobes were a better source than in vitro shoot merisems for production of primary somatic cotyledons as they recorded higher SSE frequencies and efficiencies. The influence of the PSE source (leaf lobes versus apical meristems) on SSE in a wide range of African varieties (TME 12 inclusive) was observed by [16] but could not be established as the statistical significance of cotyledon source on SSE was not investigated. Our data suggested that primary somatic cotyledon from the time of formation to 8-weeks old, obtained from both leaf lobe and shoot meristem cultures, were suitable as starting material for secondary somatic embryogenesis` for both TME 12 and Kibaha.

The average CSE frequency and efficiency of TME 12 (94.9%, 7.0) was significantly greater than that of Kibaha (90.3%, 6.0) (Table 1). Raemakers et al. [22] reported an 87% CSE frequency for MCol 22 cultivars while Hankoua et al. [16] reported a genotypic influence of cyclic somatic embryogenesis in eleven African cassava cultivars on picloram supplemented medium. In two Asian cultivars (KU 50 and Hanatee), Saelim et al. [23] observed a cultivar effect on establishment of cyclic somatic embryogenesis. As with SSE, the CSE frequency and efficiency from leaf lobe (94.7%, 6.8) was significantly higher than that from shoot meristem (90.5%, 6.2) (Table 1). Comparing SSE and CSE, in TME 12, CSE frequency was greater than that of the SSE frequency by 12.1% while that of Kibaha was 12.4%. CSE efficiency between TME 12 and Kibaha remained virtually the same. Cyclic somatic cotyledon of MCol 22 has been reported to produce larger number of embryos than from secondary cotyledon; for this and other reasons, the use of cyclic somatic embryos was suggested for large-scale regeneration and transformation programme [22].

Although previous studies recognized the need for using young somatic cotyledon for initiating SSE and CSE, the exact age of such cotyledon was not stated [19,22]. Raemakers et al. [22] emphasized the importance of using young somatic embryos for production of a large number of secondary embryos in cultivar Mcol. 22. For further production of somatic embryos and shoot regeneration, Liu et al. [19] suggested the use of newly developed embryos in cultivars Mcol. 505, Mcol. 22 Mper 183 and TMS 6044.

Optimization of CSE production in two Asia cultivars (KU 50 and Hanatee), indicated that the auxin concentration in the culture medium influenced the age of somatic cotyledon best used for high frequency and efficiency CSE production [23]. For example, in the KU 50 cultivar, 15-day somatic cotyledons recorded the highest CSE frequency and efficiency in picloram-based medium while 30-day-old cotyledons were best on a 2,4-D based medium. Compared with SSE, the loss of embryogenic competence set in earlier in secondary embryo cotyledon than in primary cotyledon as our results indicate that more than 5-week old secondary cotyledon was not suitable for initiating cyclic embryogenesis. These results confirmed earlier suggestions of using young materials for somatic embryogenesis and shoot regeneration. In addition, these result enrich the literature by specifying the age of somatic cotyledon suitable for somatic embryogenesis, i.e., 0 to eight weeks for SSE and 0 to five weeks for CSE for both shoot meristem and leaf lobe sourced cotyledon.

An increase in CSE frequency was reported in Mcol. 22 up to the 5^{th} cycle before decreasing using leaf lobe on 2,4-D-based medium by [22]. The authors also documented an initial increase in CSE efficiency between the 1^{st} and 2^{nd} cycle before decreasing in the same cultivar. In the present study, both cultivars (TME 12 and Kibaha) and source (leaf lobe and shoot meristem) displayed similar trends with respect to the effect of number of cycles on CSE frequency and efficiency (Fig. 4). However, the CSE frequency from the SM sources remained constant after the 3^{rd} cycle while the CSE frequency from the LL source decreased steadily as the number of cycles increased.

The results of the present study agree with the findings of [22] except that CSE frequency continued to increase up to 5^{th} cycle before declining whereas CSE frequency started falling after the 2^{nd} cycle in the present study (Fig. 4). This variation might be due to the influence of type of auxin used to induce somatic embryogenesis. In the present study picloram was used while Raemaker et al. [22] used 2,4-D. For both sources (LL and SM), deterioration sets in after the 2nd cycle, first with CSE frequency but this was compensated for by a high CSE efficiency until after the 4^{th} cycle. After the 4^{th} cycle, we observed that somatic cotyledon became small and wrinkled with light-yellow colouration. The CSE cotyledon from which embryos emerged became a large callus and wrinkled, root-like structures developed from the lower part of the callus and occupied the lower portion of plates. Based on these findings, it is suggested that cyclic embryos should be discarded after the 4^{th} cycle and fresh starting material be used to start the somatic embryogenesis cycle again.

Based on our findings, when further secondary and cyclic somatic embryogenesis in the Programme were induced from one week-old primary and secondary somatic cotyledons from both LL and SM sources in both cultivars as described in the Material and Method section, most of the problems initially experienced such as variations in the morphology of the embryos, inconsistency in the duration of development of the embryos, appearance of root-like structures in culture media, increase in callus size and frequency and a decrease in frequency and efficiency of secondary and cyclic somatic embryogenesis disappeared.

4. CONCLUSION

In conclusion, our results demonstrated that age and source of somatic cotyledon, rather than cultivar, played a significant role in the frequency and efficiency of secondary and cyclic somatic embryogenesis. In addition, cycle number also influenced the frequency and efficiency of cyclic somatic embryogenesis. We, therefore, suggest a modified protocol that involves the use of primary somatic cotyledon of age between 0 (freshly matured) and 8 weeks for induction of secondary somatic embryo and secondary somatic cotyledon of age 0 to 5 weeks for CSE, irrespective of cultivar (TME 12 or Kibaha) or source (SM or LL) in 12 mg/l of 2,4-D (Fig. 5). Furthermore, cycling of somatic embryos should not go beyond the fourth round to maximize secondary and cyclic embryogenesis for efficient regeneration system. Similar research should be conducted with improved cassava varieties because the test varieties in this study were landraces.

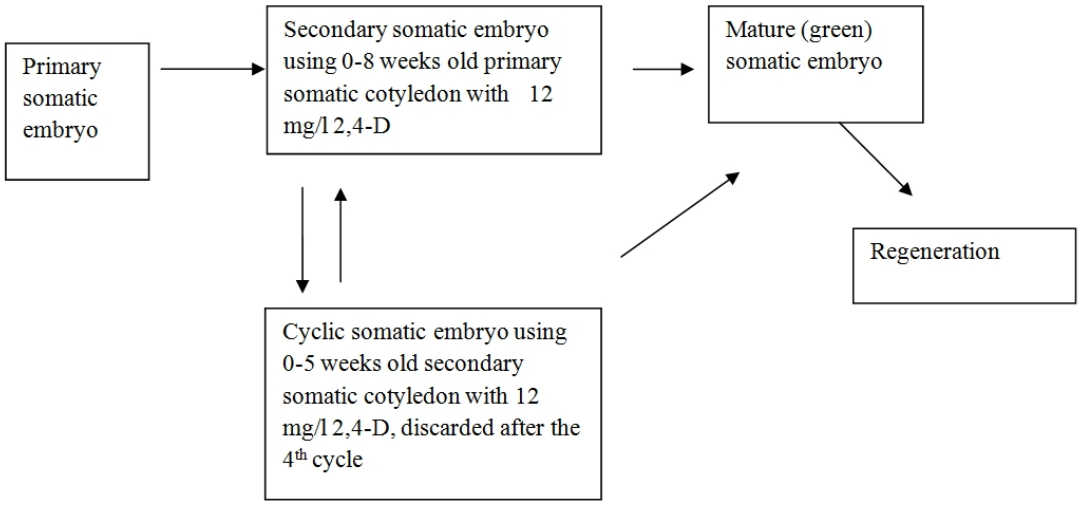


Fig. 5. Somatic embryogenesis in cassava showing modified protocol for production of secondary and cyclic embryos based on the results of this study.

ACKNOWLEDGEMENT

The financial support of Obafemi Awolowo University's Research Committee and National Biotechnology Development Agency, Abuja is acknowledged.

COMPETING INTERESTS

Authors declare that there are no competing interests between individuals and organizations that can affect the publication of this work.

REFERENCES

1. Defloor I, Dehing I, Delcour JA. Physico-chemical properties of cassava starch. Trop Sci. 1998;31:189-207

2. Nweke FI, Spencer DDC, Lynam JK. The cassava transformation, Michigan State University Press, East Lansing. 2002
3. El-Sharkawy MA. Cassava biology and physiology. Plant Mol Biol. 2004;56:481–501.
4. Nassar N, Ortiz R. Breeding cassava to feed the poor. Sci Am. 2010;302:78–84
5. Sanchez T, Salcedo E, Dufour D, Morante N, Debouck D, Moreno IX. Screening of starch quality traits in cassava (*Manihot esculenta* Crantz). Starch/Starke. 2009;61:12-19.
6. Ceballos H, Iglesias CA, Pereze JC, Dixon AGO. Cassava breeding: opportunities and challenges. Plant Mol Bio. 2004; 56:503–516.
7. Bull SE, Ndunguru J, Gruissem W, Beeching JR, VanderschurenH.Cassava constraints to production and the transfer of biotechnology to African laboratories.Plant Cell Report. 2011;30:677-679. DOI: 10.1007/s00299-010-0986-6.
8. Barceloux DG. Cyanogenic foods (cassava, fruit kernels, and cycad seeds). Dis Mon. 2009;55:336–352.
9. Taylor NJ, Chavarriaga P, Raemakers K, Siritunga D, Zhang P Development and application of transgenic technologies in cassava. Plant Mol Bio. 2004;56:671–678.
10. Osorio M, Gámez E, Molina S, Infante D. Evaluation of cassava plants generated by somatic embryogenesis at different stages of development using molecular markers. Elect J Biotech. 2012;15:(4) Available on: http://dx.doi.org/10.2225/vol15-issue 4-fulltext-3.
11. Raemakers K, Jacobsen E, Visser R. The use of somatic embryogenesis for plant.propagation in cassava. Mol Biotechnol. 2000;14(3):215-21.
12. Opabode JT, Oyelakin OO, Akinyemiju OA and Ingelbrecht IL. Primary somatic embryos from axillary meristems and immature leaf lobes of selected African cassava varieties. British Biotech. J. 2013;3:263-273.
13. Atehnkeng J, Adetimirin VO, Ng SYC. Exploring the African cassava (*Manihot esculenta* Crantz) germplasm for somatic embryogenic competence. Afr J Biotech2006;5:1324–1329.
14. Danso KE, Ford-Lloyd BV. Induction of high-frequency somatic embryos in cassava for cryopreservation. Plant Cell Rep. 2002; 21:226–232.
15. Guohua M, Qiusheng X. Induction of somatic embryogenesis and adventitious shoots from immature leaves of cassava. Plant Cell Tiss Organ Cult. 2002;70:281–288.
16. Guohua M. Effects of cytokinins and auxins on cassava shoot organogenesis and somatic embryogenesis from somatic embryo explants. Plant Cell Tiss Organ Cult.1998;54:1–7.
17. Hankoua BB, Ng SYC, Fawole I, Pouti-Kaerlas J, Pillay M, Dixon AGO. Regeneration of a wide range of African cassava genotypes via shoot organogenesis from cotyledons of maturing somatic embryos and conformity of field-established regenerants. Plant Cell Tiss Organ Cult. 2005;82:221-231.
18. Taylor NJ, Masona MV, Carcamo R, Ho T, Schöpke C, Fauquet CM. Production of embryogenic tissue and regeneration of transgenic plants in cassava (*Manihot esculenta* Crantz). Euphy. 2001;120:25–34.
19. Zhang, P. and I Pounti-Kaerlas Regeneration of transgenic cassava from transformed embryogenic tissues. Methods in Molecular Biology. 2004;286:1025-19.
20. Li HQ, Guo JY, Huang YW, Liang CY, Liu HX, Potrykus I, Puonti-Kaerlas J. Regeneration of cassava plants via shoot organogenesis. Plant Cell Rep. 1998;17:410–414.
21. Raemakers KJJM, Sofiari E, Jacobsen E, Visser RGF. Regeneration and transformation of cassava. Euphy. 1997;96:153–161.

22. Rossin CB, Rey MEC. Effect of explant source and auxins on somatic embryogenesis of selected cassava *(Manihot esculenta Crantz)* cultivars. South Afri J Bot. 2011;77:59-65
23. Raemakers KJJM, Bessembinder J. Staritsky G, Jacobsen E, Visser RG F.Induction, germination and shoot development of somatic embryos of cassava. Plant Cell Tissure Organ Culture. 1993;33:151–156.
24. Saelim L, Phansiri S, Netrphan S, Suksangpanomrung M, Narangajavana J Optimization of in vitro cyclic somatic embryogenesis and regeneration of the Asiancultivars of cassava (*Manihot esculenta Crantz*) for genetic manipulation system. Global J Biotech Biochem. 2006;1:7–15.
25. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 1962;15:473–497.
26. Gomez KA, Gomez AA. Statistical procedures for agricultural research (2nd ed). John Wiley and Sons Inc. New York. 1984;680.
27. Feitosa T, Bastos JLP, Ponte LFA, Jucá TL, Campos FAP. Somatic embryogenesis in cassava genotypes from the northeast of Brazil. Brazilian Arch Biol Techn. 2007;50-201–206.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=413&id=11&aid=3499