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Production of friable embryogenic callus and regeneration of Ugandan farmer-preferred cassava genotypes

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Generation of embryogenic callus is a key step in genetic engineering of many crop species, including cassava. Protocols for generation of friable embryogenic callus (FEC) have been lacking for Ugandan cassava genotypes, thereby delaying their genetic engineering for agronomic and other desirable traits. The objective of this study was to determine conditions suitable for production and regeneration of FEC in the Ugandan cassava genotypes; Aladu, Bukalasa and Ebwanateraka, and control cultivar 60444. Immature leaf lobe explants were established on Murashige and Skoog (MS) based media for initiation of organized embryogenic callus (OES). To produce FEC, resulting OES were established on Gresshoff and Doy based callus induction media with varying levels of sucrose, maltose, tyrosine, tryptophan, naphthalene acetic acid (NAA) under light and dark conditions. Subsequently, FEC was subcultured to MS-based embryo maturation and embryo regeneration media. All genotypes produced OES. All genotypes produced FEC except Bukalasa. The amino acid tyrosine favoured production of FEC in Aladu and Ebwanatereka, but not in 60444, while 20 g/L of sucrose trigged production of FEC in Aladu and 60444, but 40 g/L of sucrose was superior for Ebwanatereka. Media supplemented with 1 ml/L naphthalene acetic acid NAA facilitated embryo regeneration in Ebwanatereka and 60444, while Aladu responded better to 5 ml/L NAA. Light, tyrosine and sucrose were essential for FEC production in Uganda cultivars while NAA was required for regeneration of somatic embryos. Ability to produce FEC in these genotypes lays a foundation for their improvement through genetic transformation for the desired and agronomic traits.

Key words: Cassava (*Manihot esculenta* Crantz), somatic embryogenesis, amino acids, carbon sources.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) of the family Euphorbiaceace, is the second most important staple food crop grown by low income African farmers (FAOSTAT, 2011). In Uganda, annual production of cassava is approximately 4.9 million metric tonnes (MT) (FAOSTAT, 2012), with about 80% of the population depending on the crop as a source of starch and dietary energy (ASARECA, 2002). In addition, the crop is sold locally by small scale farmers to generate income (Otim-Nape et al., 2001) and used for animal feed, production of beverages and various industrial applications such in the manufacture of textiles (Tonukari, 2004).

The area under cassava cultivation in Uganda has increased from an estimated 379,000 ha in 2006 to 426,000 in 2012. However, average yields have dropped from approximately 123,000 Hg/ha to 115,500 Hg/ha over the same period (FAOSTAT, 2012). This decline is in part due to the impact of Cassava brown streak disease (CBSD). CBSD is caused by two Ipomovirus; Cassava brown streak virus (CBSV) and Ugandan cassava brown streak virus (UCBVS) (Alicai et al., 2007; Mbanzibwa et al., 2009; Patil et al., 2010) and is now considered to be one of the most important threats to food security in the tropics (Patil et al., 2014). Sources of effective resistance to CBSD within known farmer preferred varieties are limited. In addition, the heterozygous nature and long breeding cycle of the crop, present a challenge to development and delivery of CBSD resistance materials to farmers via conventional breeding systems (Kawano, 2000; Liu et al., 2011).

Recently, transgenic approaches to control CBSD by RNAi technology have been demonstrated under controlled growth conditions (Yadav et al., 2011; Van der et al., 1992) and within confined field trials (Ogwok et al., 2012). In order to exploit these advances for development of virus resistant Ugandan planting materials, capacity for transgenic modification must be expanded into a wider range of genetic backgrounds. Such ability would also allow application of biotechnology for other traits such as enhanced nutrition value (Sayre et al., 2011) modified starch quality, improved post-harvest shelf life, as well as resistance to pests and diseases (Taylor et al., 2004; Nutti, 2004; Zhao et al., 2011).

Recovery of genetically modified cassava is based on production of totipotent embryogenic tissues that act as the target for transgene integration via Agrobacterium or direct gene transfer technologies (Taylor et al., 2004; Liu et al., 2011). Lack of efficient regeneration systems for cassava has been a limiting factor for application of cassava biotechnology to improve Ugandan cassava genotypes.

Somatic embryogenesis was originally reported for regeneration of cassava plants via zygotic cotyledons (Stamp and Henshaw, 1987), and then from immature leaves, repetitive cycling of somatic embryos (Szabados et al., 1987) and via friable embryogenic callus (FEC) (Taylor et al., 1996, 2001, 2012). The direct use of somatic embryos for recovery of transgenic plants has not been encouraging, because these structures are multi-cellular encouraging, because these structures are multi-cellular and highly organized, leading to increased chances of recovering chimeras when targeted for transgene integration (Raemaker et al., 1997; Quiroz-Figueroa et al., 2006). Conversely FEC produced from organized embryogenic structures (OES) is highly disorganized and regenerates via a single cell origin (Taylor et al., 2004, 2012). As a result, FEC has proven to be an efficient target tissue for transgene integration (Bull et al., 2009; Taylor et al., 2012). Until recently, most reports were restricted to recovery of transgenic plants in the West African cassava model cultivar 60444. However this capacity has now been expanded into a range of West Africa farmer-preferred germplasm (Sayre et al., 2011; Zainuddin et al., 2012; Nyaboga et al., 2013). As an initial step towards establishing capacity to apply biotechnology to the improvement of Ugandan cassava, we report here, factors affecting induction of somatic embryogenesis, production of FEC and plant regeneration in three Ugandan farmer preferred cassava genotypes. Cultivars like Aladu, Ebwanatereka and Bukalasa were selected for this study representing popular cultivars from major cassava growing regions of Uganda. Ebwanatereka is commonly grown in Soroti, Kaberamaido, Busia and Iganga, districts of the Eastern and North Eastern region while Aladu is grown in Lira and Apac, districts of the Northern region and Bukalasa grown in Masindi, Kibale and Mityana, districts of Western and Central region.

These cultivars have other good attributes that include being sweet, mealy, soft, high dry matter content, produces good cassava flour, good for waragi, ready market for fresh roots, high yielding and stores long in soil (Aladu and Bukalasa) and early maturing (Ebwanatereka). All these cultivars are susceptible to cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) (Orone, Cassava Breeder, NaCRRI, Uganda, personal communication).

MATERIALS AND METHODS

Four genotypes were used in this study; the three Ugandan farmer preferred genotypes Ebwanatereka, Bukalasa and Aladu, and the West African model cultivar 60444. Stem cuttings of the Ugandan genotypes were transported from the National Crops Resources Research Institute (NaCRRI) to the Donald Danforth Plant Science Center (DDPSC), St Louis, MO, USA. Fifty stakes of each genotype were planted in 12 cm pots containing Farad 51, transferred to growth chamber and maintained at 28±2°C for 16 h in light and 8 h dark at 2100 $umolm^{-2}s^{-1}$ for two years.

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Abbreviations: OES, Organised embryogenic structures; **FEC,** friable embryogenic callus; **MS,** Murashige and Skoog Basal media; **GD,** Gresshoff and Doy basal media; **NAA,** naphthalene acetic acid; **BAP,** benzylaminopurine; **CBSD,** cassava brown streak disease; **CBSV,** cassava brown streak virus; **UCBVS,** Ugandan cassava brown streak virus; **UFPCG,** Ugandan farmer preferred cassava genotypes; **GD250P,** Gresshoff and Doy basal medium supplemented with the auxin picloram; **MS2 50P,** Murashige and Skoog basal medium supplemented with the auxin picloram.

Preparation of stock solutions and basal media

Murashige and Skoog (Murashige and Skoog (1962) and Gresshoff and Doy (Gresshoff and Doy (1972) basal media were prepared as described by Taylor et al. (2012). Fifty (50) ml of 10 mM stocks of either tyrosine or tryptophan were added to 1 litre of GD basal media supplemented with sucrose or maltose at 20 g/L or 40 g/L with 50 µM picloram (GD2 50P) after autoclaving. The explants were established and maintained at 28 ± 2ºC for 16 h in light and 8 h dark at 2100 umolm^{-2}s^{-1} for 42 days

Initiation of nodal cuttings from the screen house into tissue culture

New stem growth each carrying approximately 10 nodes was removed from soil grown plants. Individual nodes were excised using a single edged razor and placed in 250 ml conical flasks. Approximately 20 individual nodes were added to 100 ml of water containing 15% v/v sodium hypochlorite plus two drops of Tween-20 in a 500 ml conical flask and agitated at 150 rpm on an orbital shaker for 30 min. Individual nodes were then rinsed five times with sterile distilled water and necrotic tissues carefully sliced off using a scalpel in a laminar flow hood. Four individual nodal cuttings were placed on Nunc 25 x 100 mm Petri dish containing MS basal media supplemented with 20 g/L sucrose (MS2) and solidified with 2.3 g/L of phytagel. Approximately 100 nodal cuttings of each cultivar were transferred to the growth-room and maintained for approximately three weeks. Shoots developing from the nodal cuttings were excised above the original nodal tissue and established on MS2 basal media solidified with 8 g/L noble agar at seven shoots per Petri dish.

Induction of embryogenic tissues

Leaf-lobe explants 2 to 6 mm in length were excised from *in vitro* mother plants using a hypodermic needle and placed on MS2 medium supplemented with 50 µM picloram (MS2 50P) in the manner described by Taylor et al. (1996, 2012). After 28 days, organized embryogenic structures (OES) developing from the adaxial surface were excised and sub-cultured onto fresh MS2 50P. The resulting proliferating clumps of OES were then repeatedly sub-cultured onto fresh MS2 50P every four weeks for a total of 12 weeks. Production of OES was assessed as the percentage of leaf explants seen to produce these structures and as the amount of OES produced per responding explant. A scale of 1-5 described an area of the explant surface covered by the embryogenic structures where; 1 = 1-20%, 2 = 21-40%, 3 = 41-60%, 4 = 61-80%, and 5 = 81-100%. The data obtained was transformed with ARCSINH function using Microsoft Excel 2010 package. Mean values of surface area coverage of OES on leaf lobes and frequencies of OES produced were subjected to analysis of variance (ANOVA) using statistical package Genstat Release 14.1 (2011) at 5% significance level.

OES was used as starting material for FEC production. OES was excised away from associated non-embryogenic callus with the use of a hypodermic needle (Taylor et al., 2012), collected and crushed through a 1 mm^2 pore-sized steel wire mesh using a spatula. One ml of Gresshoff and Doy (GD) liquid medium supplemented with 50 µM picloram was applied over the crushed OES to allow easier manipulation. Nine OES fragments, each 1 mm^2 in size were placed on 15 × 100 cm Petri dish containing GD2 50P medium modified by addition of either an amino acid tyrosine or tryptophan at 500 µM. The sugar type and concentration of sucrose or maltose at 20 or 40 g/L were used to assess production of FEC, from 1 mm^2 OES pieces. Five Petri dishes per treatment were set up each containing nine 1 mm^2 OES fragments. OES pieces were cultured under light in growth room conditions as described above.

Experiments were conducted three times and recorded for production of non-embryogenic callus (NEC), OES and FEC produced at 28, 35, and 42 days after initiation. In order to quantify FEC production, the FEC was spread to form a monolayer on the agar surface and a grid scale placed under the Petri dish to determine the surface area. Mean values for production of NEC, OES and FEC produced were subjected to ANOVA using statistical package Genstat Release 7.

Regeneration of cotyledon stage embryos into plantlets

To determine its regenerative ability, FEC was transferred as 0.4 x 0.4 cm sized colonies. Five colonies were placed each in 100 × 25 mm Petri dish containing MS2 media supplemented with 5 ml/L naphthalene acetic acid (NAA) with 20 g/L sucrose. Five petri dishes with five colonies each were set up for the cultivar Aladu, Ebwanatereka and 60444. Twenty five (25) colonies per treatment per genotype were established for each experiment. The same was done for the two NAA levels (1 and 0.1 ml/L) with sucrose for the three cultivars. Similar treatments were established for the three cultivars with the three levels of NAA at 20 g/L of maltose. The experiment was repeated three times. Approximately three weeks later developing cotyledon-stage embryos were transferred as individuals to MS basal medium supplemented with 2 ml/L benzylaminopurine (BAP), at six cotyledon-stage embryos per 100 × 25 mm Petri dish (Taylor et al., 2012). Eight weeks later, regenerated plants with well-developed root systems were selected and transferred to MS2 basal media. The number of the embryos produced per cultivar, types of embryos (one cotyledon, two cotyledon and trumpet-shaped embryos) and percentage of embryos regenerating to produce plants were recorded. Mean values for all parameters recorded above were subjected to ANOVA using statistical package Genstat Release 14.1 (2011) at 5% significance level.

Acclimatization of the regenerated plants from *in vitro* to the screen house

A total of 50 plants per cultivar (Aladu, Ebwanatereka and 60444) were transferred to the screen house for acclimatization. The plants were cleaned with distilled water to remove any agar on the roots. To vermiculite, 0.5 g/L of Jacks professional fertilizer containing nitrogen, phosphorus and potassium in the ratios of 9: 45: 15 respectively as well as fungicide called Ridomil (2.5 g/L) were added. The cleaned plants for each genotype were transferred to vermiculite. A total of 150 plants were established in the screen house. A month later, the surviving plants were moved to a composition of vermiculite and soil in the ratio of 2:1 respectively. The number of surviving weaned plants was counted.

RESULTS

Production of embryogenic tissues

In the present study, the capability of three Ugandan cassava genotypes to produce organized embryogenic callus (OES) in the presence of the 50 uM picloram was demonstrated using leaf lobes (Figure 3A). Mean percentages of OES (Figure 3B) production in this study for Aladu was 40.1%, Bukalasa 44.0% and Ebwanatereka 37.9%, compared with control genotype

Table 1. Frequency and mean production of Organised embryogenic structures (OES) from leaf lobes of different cassava cultivars on MS medium supplemented with 50µM Picloram.

Cultivar	Frequency of OES (%)	Mean production of OES (%)
60444	76.84 ± 2.11	36. 42 ± 3.91
Aladu	78.95 ± 1.95	40.1 ± 3.79
Bukalasa	88.95 ± 1.47	44.0 ± 3.38
Ebwanatereka	84.74 ± 1.37	37.0 ± 2.69

Percentages of OES produced from 190 explants. OES production frequencies were recorded by calculating the ratio of OES clusters/cultured explants. Mean OES production were recorded by calculating the amounts of OES produced by each explant/the number of explants. Data represents means ± SD of three independent experiments.

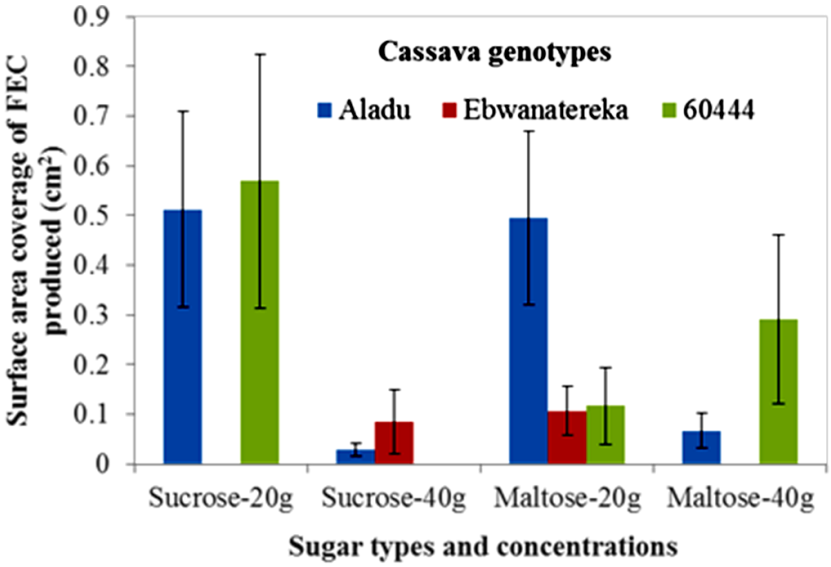

Figure 1. Mean production of friable embryogenic callus (FEC) from cassava genotypes on Gresshoff and Doy (GD) basal medium supplemented with two sugar types at different concentrations

60444 in which had an average of 36.4% (Table 1) produced embryogenic structures under the same conditions. However using the frequencies, the production of OES from the genotypes 60444 was 77%, Aladu 79%, Bukalasa 89% and Ebwanatereka 85% (Table 1). The OES produced was used for production of friable embryogenic callus (FEC) (Figure 3C) as demonstrated in the subsequent experiments using carbon sources and amino acids.

Effect of sugar types and concentration on FEC production

The genotypes responded to production of FEC using the both sucrose and maltose at concentrations of 20 or 40 g/L. Response to these culture conditions varied between cultivars. Significant differences were observed with respect to mean amounts of FEC produced in the presence of sugar types and concentrations for Aladu ($P = 0.019$), while no significant differences were observed for Ebwanatereka ($P = 0.168$) and control 60444 ($P = 0.088$) (Figure 1i). After 42 days, FEC production was greatest in Aladu when OES was cultured on 20 g/L of sucrose (0.51 ± 0.19 mm^2) or 20 g/L of maltose (0.49 ± 0.17 mm^2) and significantly greater than either sugar type at 40 g/L of maltose (0.07 ± 0.03 mm^2) (Figure 1). Conversely, FEC production from Ebwanatereka was highest at 20 g/L of maltose (0.12 ± 0.05 mm^2) followed by 40 g/L of sucrose (0.08 ± 0.06 mm^2) and no FEC was produced at 20 g/L of sucrose and 40 g/L of maltose (Figure 1). In the case of 60444, highest amounts of FEC were produced on medium containing 20 g/L of sucrose (0.57 ± 0.26 mm^2) followed by 40 g/L of maltose (0.29 ± 0.17 and lowest at 20 g/L of maltose (0.12 ± 0.08 mm^2) (Figure 1).

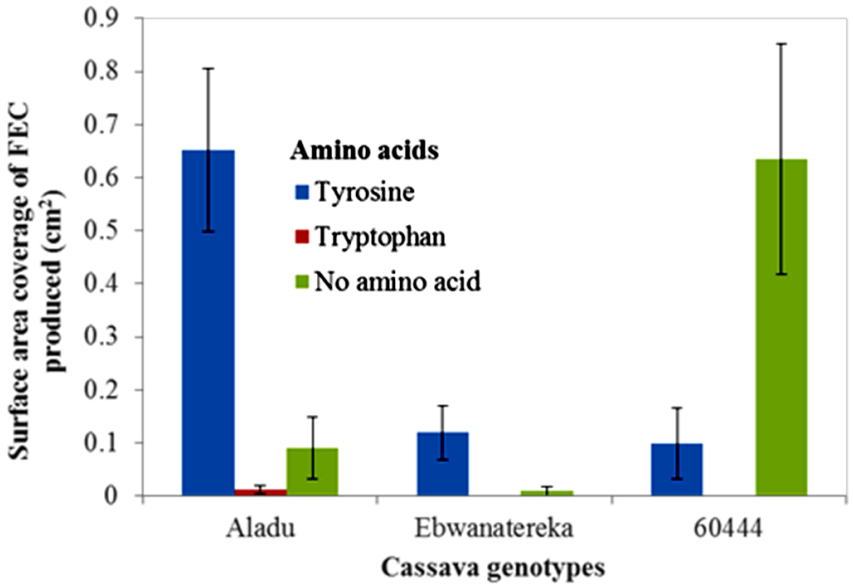

Figure 2. Mean production of friable embryogenic callus (FEC) from cassava genotypes on Gresshoff and Doy basal medium supplemented with amino acids at the same concentration.

Effect of amino acids on FEC production

The Ugandan genotypes responded to production of FEC using the amino acid tyrosine. Recently, Nyaboga et al. *(*2013) reported the beneficial effects of tyrosine in the induction of FEC from OES tissues in cassava. In the present study the aromatic amino acids tyrosine and tryptophan were investigated for their ability to stimulate FEC production in the Ugandan cultivars Ebwanateraka and Aladu. Significant differences were observed with respect to mean amounts of FEC produced in the presence of amino acids for Aladu (P = <.001), Ebwanatereka (P = 0.030) and control 60444 (P = 0.003) (Figure 2). These results were obtained from tissues after one culture cycle of 42 days. All genotypes produced FEC in the presence of tyrosine. The highest mean amounts of FEC in the presence of tyrosine were produced by Aladu (0.65 ± 0.15 mm^2) followed by Ebwanatereka (0.0.12 ± 0.05 mm^2) and the least by 60444 (0.09 ± 0.07 mm^2) (Figure 2). However, only Aladu (0.01 ± 0.01 mm^2) produced FEC in the presence of tryptophan (Figure 2). In the absence of the amino acids, the control genotype 60444 (0.63 ± 0.22 mm^2) produced the highest mean amounts of FEC followed by Aladu (0.09 ± 0.06 mm^2) and the least by Ebwanatereka (0.01 ± 0.01 mm^2) (Figure 2).

Effect of naphthalene acetic acid (NAA) on germination of FEC into cotyledon embryos

The Ugandan cassava genotypes; Aladu, Ebwanatereka and the control cultivar 60444, were capable of producing friable embryogenic tissue (FEC) (Figure 3C). The single celled embryo (Figure 3D) formed a torpedo shaped embryo (Figure 3E). Torpedo shaped embryo progressed to form a heart shaped embryo (Figure 3F). Heart shaped embryo formed a cotyledon embryos (Figure 3G). Significant differences in the number of embryos produced were observed among cassava genotypes with respect to levels of NAA (P = 0.003). The highest mean number of germinated embryos was observed at 1 µM of NAA followed by 5 µM and the least at 0.1 µM (Table 2). The highest mean number of germinated FEC into cotyledon embryos was observed at 1 µM of NAA for 60444 (12.24 ± 3.88) and Ebwanatereka (12.34 ± 9.12) while 5 µM of NAA facilitated embryo germination for Aladu (7.44 ± 1.58) (Table 2). The second best option for germination of the FEC into cotyledon embryos was observed at 5 µM of NAA for 60444 (6.58 ± 3.22) and Ebwanatereka (8.35 ± 4.18) while 1 µM of NAA favoured germination of FEC for Aladu (5.82 ± 2.57) (Table 2). In all genotypes used, 0.1 µM of NAA resulted in the least response of germination of the FEC into cotyledon embryos for Aladu (2.94 ± 2.42) followed by Ebwanatereka (3.34 ± 3.46) and 60444 (3.4 ± 2.48) (Table 2).

Effect of sugar types on germination of FEC into cotyledon embryos

The FEC from the three genotypes germinated progressively as explained in the preceding section in the

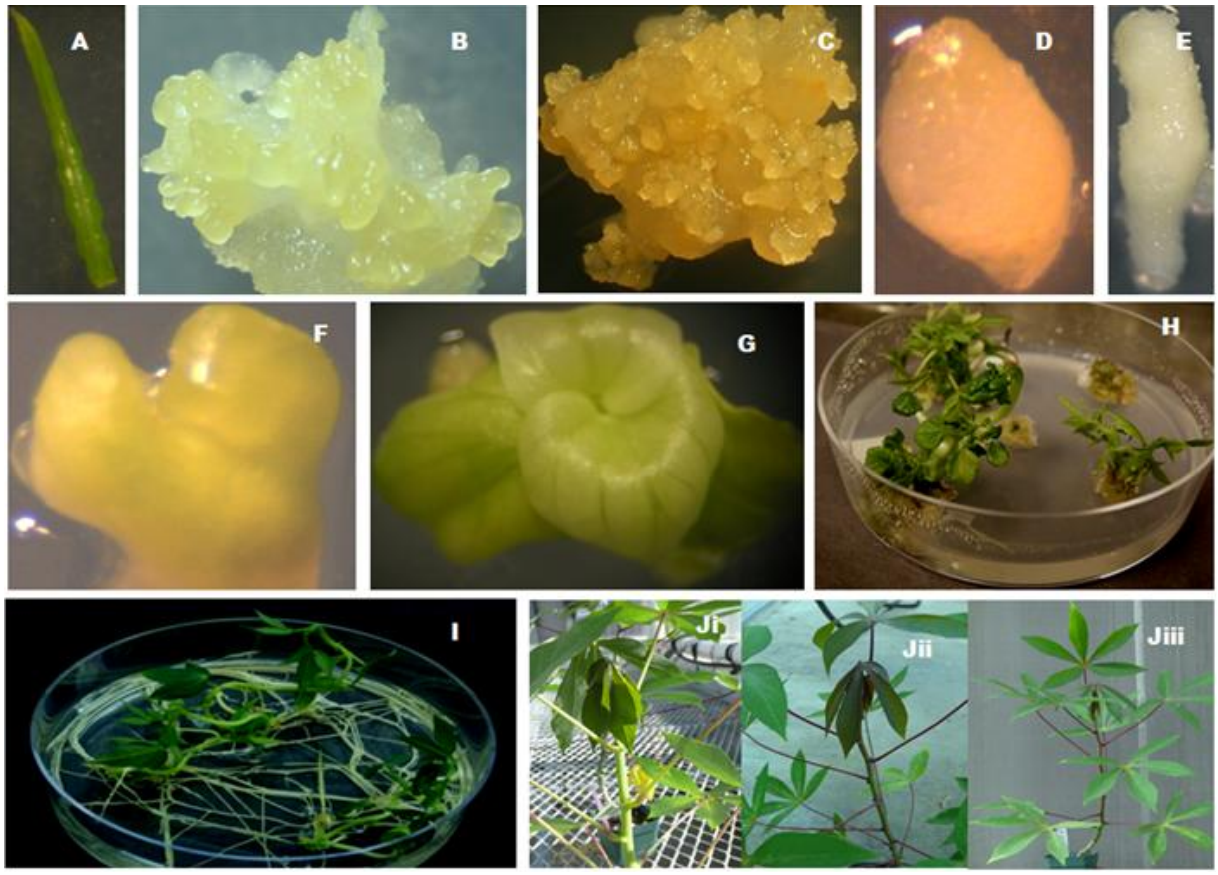

Figure 3. Production and regeneration of friable embryogenic callus (FEC) from Ugandan cassava cultivars into plants demonstrated. A) Leaf lobe established on picloram based induction media, B) Organised embryogenic structures (OES) of cassava genotype 60444, C) FEC produced by the genotype Aladu, D) Bulging single celled embryo, E) Early globular stage of the embryo, F) Late heart stage of the embryo, G) Mature cotyledon embryo H) A mature cotyledon embryo germinating into a plant, I) Fully regenerated plants in vitro and Ji, Jii, Jiii) Acclimatized in vitro regenerated plants for 60444, Aladu and Ebwanatereka respectively.

Table 2. Mean number of embryos produced on media with varying levels of Naphthalene acetic acid (NAA) on two carbon sources.

Cultivar	Mean number of embryos produced on media with different NAA levels			Mean number of embryos produced on media with sucrose or maltose at 20 g/L	
	5 µM	1 µM	0.1 µM	Sucrose	Maltose
Aladu	7.44 ± 1.58a	5.82 ± 2.57a	2.94 ± 2.42a	5.72 ± 2.25a	5.08 ± 3.41a
Ebwanatereka	8.35 ± 4.18b	12.34 ± 9.12b	3.34 ± 3.26b	11.85 ± 7.89b	4.17 ± 3.24b
60444	6.58 ± 3.22c	12.24 ± 3.88c	3.4 ± 2.48c	8.0 ± 5.76c	6.81 ± 3.73c

Values are mean ± SD of three independent experiments. Values in a column followed by different letters are significantly different from each other at $p \leq 0.05$

presence of sugars too. Significant differences in the number of embryos that germinated from the FEC as were observed in the presence of sugars (P = <0.001) for the three genotypes. The mean number of cotyledon embryos produced by the different genotypes in media with 20 g/L of sucrose was highest in Ebwanatereka (11.85 ± 2.04) followed by 60444 (8.0 ± 1.49) and least in Aladu (5.72 ± 0.58) (Table 2). In media supplemented with 20 g/L of maltose, the highest number of cotyledon embryos were produced in 60444 (6.81 ± 0.96) followed by Aladu (5.08 ± 0.58) and least with Ebwanatereka (4.17 ± 0.84) (Table 2). Sucrose was a suitable sugar for germination of FEC into cotyledon embryos for all the genotypes.

Table 3. Mean number of embryos produced due to interaction between Naphthalene acetic acid (NAA) and two carbon sources (sucrose and maltose).

Cultivar	Mean number of embryos produced at different NAA levels in media with 20 g/L of Sucrose			Mean number of embryos produced at different NAA levels in media with 20 g/L of Maltose		
	5 µM	1 µM	0.1 µM	5 µM	1 µM	0.1 µM
Aladu	7.52 ± 1.61a	5.12 ± 1.07a	4.52 ± 2.53a	7.36 ± 1.56a	6.52 ± 3.33a	1.36 ± 0.57a
Ebwanatereka	9.42 ± 5.09b	20.24 ± 6.19b	5.88 ± 3.23b	7.28 ± 2.59b	4.44 ± 1.77b	0.8 ± 0.76b
60444	8.72 ± 2.82c	13.72 ± 3.88c	1.56 ± 1.32c	4.44 ± 1.92c	10.76 ± 3.25c	5.24 ± 1.94c

Values are mean ± SD of three independent experiments. Values in a column followed by different letters are significantly different from each other at $p \leq 0.05$.

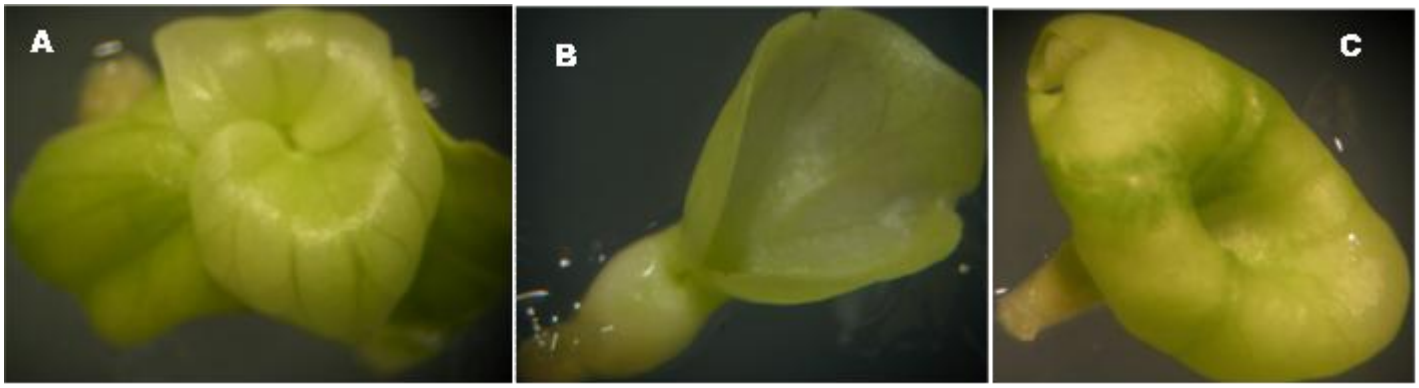

Figure 4. Types of cotyledons produced during the maturation phase of the somatic embryos. (A) Two cotyledon embryo, (B) one cotyledon embryo and (C) trumpet shaped cotyledon embryo.

Effect of the interaction of sugars and naphthalene acetic acid (NAA) on germination of FEC into cotyledon embryos

The FEC from the three genotypes germinated in interaction of the sugars and the NAA as described in the preceeding sections. Significant differences in the number of embryos that germinated from FEC were observed due to the interaction of the NAA and sugars on each genotype (P = <0.001). In media with 20 g/L of sucrose, the highest mean number of germinated cotyledon embryos were observed in Ebwanatereka (20.24 ± 6.19) with 1 µM NAA followed by 60444 (13.72 ± 3.88) with 1 µM NAA and least were observed in 60444 (1.56 ± 1.32) with 0.1 µM NAA (Table 3). In media with 20 g/L of sucrose, the highest mean number of germinated cotyledon embryos for Aladu were observed in media with 5 µM NAA (7.52 ± 1.61) followed by 1 µM NAA (5.12 ± 1.07) and least 0.1 µM NAA (4.52 ± 2.53) (Table 3). For Ebwanatereka in media with 20 g/L of sucrose, the highest mean number of germinated cotyledon embryos were observed in media with 1 µM NAA (20.24 ± 6.19) followed by 5 µM NAA (9.42 ± 5.09) and least 0.1 µM NAA (5.88 ± 3.23) (Table 3). The highest mean number of germinated cotyledon embryos in 60444 were observed in media with 20 g/L of sucrose with 1 µM NAA (13.72 ± 3.88) followed by 5 µM NAA (8.72 ± 2.82) and least with 0.1 µM NAA (1.56 ± 1.32) (Table 3).

In media with 20 g/L of maltose, the highest number of cotyledon embryos that germinated in media with varying levels of NAA was observed in 60444 (10.76 ± 3.25) with 1 µM NAA followed by Aladu (7.36 ± 1.56) with 5 µM NAA and least in Ebwanatereka (0.8 ± 0.76) with 0.1 µM NAA (Table 3). In media with 20 g/L of maltose, the highest mean number of germinated cotyledon embryos for Aladu were observed in media with 5 µM NAA (7.36 ± 1.56) followed by 1 µM NAA (6.52 ± 3.33) and least was observed with 0.1 µM NAA (1.36 ± 0.57) (Table 3). For Ebwanatereka in media with 20 g/L of maltose, the highest mean number of germinated cotyledon embryos were observed in media with 5 µM NAA(7.28 ± 2.59) followed by 1µM NAA (4.44 ± 1.77) and the least with 0.1µM NAA (0.8 ± 0.76) (Table 3). The highest mean number of germinated cotyledon embryos in 60444 were observed in media with 20 g/L of maltose with 1µM NAA (10.76 ± 3.25) followed by 0.1 µM NAA (5.24 ± 1.94) and the least with 5 µM NAA (4.44 ± 1.92) (Table 3).

Effect of sugar types on regenerative ability of the different cotyledon embryos into plants

The genotypes Aladu, Ebwanatereka and 60444, all produced the three types of cotyledons categorized as two cotyledon embryo (Figure 4A), one cotyledon embryos (Figure 4B) and trumpet shaped embryo (Figure

Table 4. Mean number of cotyledons type embryos produced from the three cassava cultivars and the percentage regeneration.

Cultivar	Two cotyledon		One cotyledon		Trumpet shaped		Mean number of regenerated embryos into plants (%)	
	Sucrose	Maltose	Sucrose	Maltose	Sucrose	Maltose	Sucrose	Maltose
Aladu	1.91 ± 1.14a	2.67 ± 1.90a	1.08 ± 0.83a	1.27 ± 1.0a	0.15 ± 0.22a	0.12 ± 0.19a	46.77 ± 19.08a	51.27 ± 17.85a
Ebwanatereka	5.63 ± 3.99b	1.81 ± 1.72b	1.75 ± 1.41b	1.04 ± 0.79b	0.17 ± 0.31b	0.20 ± 0.31b	67.42 ± 17.07b	56.83 ± 17.62b
60444	4.36 ± 2.50c	3.81 ± 2.24c	2.00 ± 1.47c	0.96 ± 0.60b	0.08 ± 0.09c	0.07 ± 0.12c	74.14 ± 15.85c	68.40 ± 17.51c

Values are mean ± SD of three independent experiments. Values in a column followed by different letters are significantly different from each other at $p \leq 0.05$.

4C). Significant differences were observed for two cotyledon embryos (P = <.001) and one cotyledon embryo (P = 0.01) while no significant differences were observed for trumpet shaped embryos (P = 0.856). Generally Ebwanatereka produced the highest (5.63 ± 3.99) and least (1.81 ± 1.72) mean number of two cotyledon embryos (Table 4). The highest mean number of two cotyledon embryos in media with sucrose at 20 g/L were observed in Ebwanatereka (5.63 ± 3.99) followed by 60444 (4.36 ± 2.50) and least in Aladu (1.91 ± 1.14) while in media with maltose at 20 g/L, The highest mean number of two cotyledon embryos were produced by 60444 (3.81 ± 2.24) followed by Aladu (2.67 ± 1.90) and least in Ebwanatereka (1.81 ± 1.72) (Table 4).

Overall, the highest (2.00 ± 1.47) and least (0.96 ± 0.60) mean number of one cotyledon embryos were observed in 60444 (Table 4). The highest mean number of one cotyledon embryos in media with 20 g/L of sucrose were observed in 60444 (2.00 ± 1.47) followed by Ebwanatereka (1.75 ± 1.41) and least in Aladu (1.08 ± 0.83) (Table 4). In media with 20 g/L of maltose, the highest numbers of one cotyledon embryos produced were observed in Aladu (1.27 ± 1.0) followed by Ebwanatereka (1.04 ± 0.79) and least in 60444 (0.96 ± 0.60) (Table 4). The highest mean number of trumpet shaped embryos were produced by Ebwanatereka (0.2 ± 0.31) and the least number were produced by 60444 (0.07 ± 0.12) (Table 4).

The highest mean number of trumpet shaped embryos in media with 20 g/L of sucrose were observed in Ebwanatereka (0.17 ± 0.31) followed by Aladu (0.15 ± 0.22) and least in 60444 (0.08 ± 0.09) (Table 4). The highest mean number of trumpet shaped embryos in media with 20 g/L of maltose were observed in Ebwanatereka (0.2 ± 0.31) followed by Aladu (0.12 ± 0.19) and the least number were produced in 60444 (0.07 ± 0.12) (Table 4). Among the cotyledonary embryos produced, the two cotyledon embryo (Figure 3A) or those with one cotyledon embryo (Figure 3B) that eventually developed a second cotyledon matured as shown in Figure 3H and these fully regenerated phenotypically normal looking plants (Figure 3I) which were acclimatized as shown in Figure 3J, for the three genotypes; 60444, Aladu and Ebwanatereka represented as Figure 3Ji, Jii, Jiii, respectively. Fully regenerated plants as shown in Figure 3I, were obtained in media with sucrose and maltose. Regenerability of two cotyledon embryos into plants was highest in control genotype 60444 followed by Ebwanatereka and least in Aladu (Table 4). In media with sucrose, the control genotype 60444 had the highest percentage of regenerability (74.14 ± 15.85) followed by Ebwanatereka (67.42 ± 17.07) and least in Aladu (46.77 ± 19.08) (Table 4). In media with maltose, the highest regenerability was in the control genotype 60444 (68.40 ± 17.519) followed by Ebwanatereka (56.83 ± 17.62) and least in Aladu (51.27 ± 17.85) (Table 4). The fully regenerated phenotypically normal looking plants of the genotypes 60444, Aladu and Ebwanatereka were established in the greenhouse as described by Taylor et al. (2012) to ensure that these plants are true to type (Figure 3J).

DISCUSSION

Results indicate that the cassava cultivars investigated were able to produce organized embryogenic structures (OES), friable embryogenic callus (FEC) and regenerate to normal looking plants. The results generally indicated that production of OES was highest in Bukalasa, followed by Ebwanatereka, 60444 and lastly Aladu. Results also indicate that response of cultivars to *in vitro* conditions was cultivar dependent. Previous reports on production of OES from 60444 have resulted in frequencies as high as 80% (Taylor et al., 1996). Lower induction rates in terms of means production of OES reported here could be attributed to the fact that the mother plants used in the experiments were older than 12 weeks old (Taylor et al.,1996).

Bukalasa was not used in subsequent experiments for production of friable embryogenic callus (FEC) because preliminary experiments indicated a failure of the somatic embryos to transit to FEC. The OES produced from Aladu, Ebwanatereka and 60444 were used in experiments to generate FEC.

In *in vitro* manipulation, sucrose is the most common carbon source used (George et al., 2008). .George et al., 2008 stated that sucrose has consistently been found to be the best carbohydrate for *in vitro* growth of cultures. The inversion of sucrose to glucose and fructose is possible in the presence of an enzyme invertase found in the plant cell wall. The degree to which this inversion occurs varies from genotype to genotype, explaining the differences in the way the different genotypes utilize the different sugar concentrations (George et al., 2008). Maltose is known to serve as a carbon source and an osmoticum. It is a reducing sugar that is broken down to give two glucose molecules. In comparison to sucrose, the rate of extracellular hydrolysis is slower. Lower concentrations of maltose favored production of FEC in all the genotypes in comparison to higher concentrations which is in agreement with findings for alfalfa and rice (George et al., 2008, Seul et al., 2013).

In plants, amino acids serve as precursors for a variety of plant hormones such as auxin and salicylate as well as a wide range of aromatic secondary metabolites such as flavonoids, cell wall lignin, phenylpropaniods, anthocyanins and other metabolites (Dixon, 2001; Galili and Tzin, 2010). Ebwanatereka and 60444 did not produce any FEC in the presence of tryptophan while low amounts of FEC were produced by Aladu (Figure 4). Absence of amino acids in the media favoured mean production of FEC in the control genotype 60444 (Figure 4). It has been reported that presence of amino acids in medium is important because it partially replaces the ammonium ions, therefore increasing the levels of reduced nitrogen which is necessary for development of somatic embryos (George et al., 2008; Anthony Ceaser and Ignacimuthu, 2010). For genotype like 60444 which responded in the absence of the amino acid, indicates that the available ammonium ions in that medium were enough to trigger the embryogenic pathway. The ability to produce FEC in Ugandan farmer preferred cassava genotypes (UFPCG) has been made possible by the existence of optimized procedures for genotype 60444, with the adjustment of some conditions to favour UFPCGs.

Tyrosine was noted as more suitable amino acid than tryptophan in triggering FEC production in the UFPCG (Figure 4). Hankoua et al., 2006 also suggested that addition of tyrosine to media facililtated generation of prolific FEC. Galili and Tzin (2010), noted that tyrosine and tryptophan are both involved in the shikimate pathway. Ebwanatereka and Aladu produced FEC in the media with 500 µM tyrosine. This could be attributed to the fact that tyrosine is a precursor of secondary metabolite tocochromanol. Tocochromanols, which include tocopherols and tocotrienols are lipid soluble molecules belonging to the group of vitamin E compounds known to play an essential role as antioxidants in plants (Falk and Munné-Bosch, 2010). Studies on *vte1* and *vte2* A. thaliana and *sxdl* maize mutants indicate that tocopherols may be involved in the regulation of photoassimilate export in leaves, therefore affecting carbohydrate metabolism, source-sink relationships and growth (Sattler et al., 2003; Falk and Munné-Bosch, 2010).

Also tocochromanols are potent hydrogen donors regulating membrane associated signaling pathways and modulation of gene expression (Brigelius-Flohe et al., 2002; Falk and Munné-Bosch, 2010). This may explain why tyrosine facilitated production of FEC. Tryptophan on the other hand, is known as a precussor of auxins such as indole-3-acetic acid (IAA) (Ostin et al., 1998; Galili and Tzin, 2010), indole glucosinolates (Halkier, 1999; Galili and Tzin, 2010) which are beneficial to the plant. However, results in this study indicate that Ebwanatereka and 60444 failed to produce FEC in the media with 500 µM tryptophan while Aladu produced FEC in low quantities. According to Makwarela et al. (2004), two South African cassava genotypes T200 and T400 produced FEC at concentration of 125 µM tryptophan. This may imply that the tryptophan concentration used was too high, or that different genotypes respond differently.

Regeneration potential of FEC investigated by varying the auxin levels while maintaining the cytokinin levels shows that the auxins and cytokinins aid in cell division, differentiation and shoot proliferation. Taylor et al. (2012) demonstrated the ability of FEC to regenerate for the genotype 60444. It is important to note that the callus should not be older than 20 weeks or 4-5 cycles for the regenerative potential of the FEC to be realized (Taylor et al., 2012).

The effect of auxins is remarkable because it induces cellular responses such as cell elongation, which impacts directly on timing and orientation of cell divisions and thus contributes to patterning (Thomas et al., 2004). Results indicate that the carbon sources and auxin levels had a significant impact on the regeneration potential of the genotypes (Table 1). An auxin level of 1 µM of NAA proved suitable for regeneration of the embryogenic tissue from the cassava genotypes, 60444 and Ebwanatereka with sucrose at 20 g/L being the best carbon source. For Aladu, the carbon source maltose at 20 g/L favoured the regeneration process. On the other hand, Aladu required 5 µM of NAA, to produce substantial amounts of embryos, indicating that different genotypes respond differently to the auxin NAA at different concentrations.

The ability of competent embryo cell to go through progress changes in development are closely associated with cell to cell communication, of which extracellular signals and cell surface bound receptors are said to be

involved (Thomas et al., 2004). During maturation process, the competent embryo progressed to globular pattern formation eventually forming heart shaped embryos (George et al., 2008), resulting in the production of phenotypically normal looking plants. Thomas et al., 2004 suggested that cellular auxin response in different parts of the embryo and at different stages play a significant role in its progressive development, because cells are already predetermined therefore the presence of the auxin triggers the preset pathway. The regeneration process took three to four months, with continuous subculture to obtain a fully developed plant as demonstrated in the control genotype 60444 from friable embryogenic callus (Taylor et al., 2012). The percentage regeneration from each genotype varied depending on the carbon source utilized in the MS-based medium. Both sugar types supported plant regeneration from the mature cotyledon embryos to phenotypically normal looking plants.

Conclusion

Production and regeneration of friable embryogenic callus (FEC) has been tested and proven in the Ugandan cassava genotypes Ebwanatereka and Aladu. Tyrosine is important for production of FEC. Naphthalene acetic acid (NAA) and sugars were necessary for maturation and regeneration of FEC into phenotypically normal looking plants. Ability to produce FEC in these genotypes lays a foundation for their improvement through genetic transformation for the desired and agronomic traits.

Conflict of interests

The authors did not declare any conflict of interest.

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