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EFFECTIVE REGENERATION OF HIGH VALUE COFFEE PLANTLETS THROUGH DIRECT SOMATIC EMBRYOGENESIS USING SYNTHETIC EQUIVALENTS OF CHARACTERISED ENDOGENOUS BIOCHEMICALS

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ABSTRACT
Coffee is a crop commodity cultivated with great economic impact. However expansion of acreage of production is hampered by inadequate planting materials in certain regions. Somatic embryogenesis is an alternative vegetative method for rapid multiplication of desired plants. It entails induction of somatic cells to go through a series of morphological and biochemical changes that result in formation of somatic embryos that develop into true-to-type plantlets. Culture medium components have been reported to play a dominant role in development of somatic embryos. The present research determined effects of augmenting tissue culture medium with synthetic equivalents of endogenous components previously associated with direct somatic embryogenesis of coffee for plantlet regeneration. Third leaf pair explants were excised from *in vitro* germinated coffee mother plants, sterilized and cultured in half-strength Murashige and Skoog (MS) basal salts augmented with 0.2 g/L thiamine, 0.1 g/L nicotinic acid, 0.1 g/L pyridoxine, 100 mg/L myo-inositol, 100 mg/L cysteine, 1 ml/L Thidiazuron and 3 g/L gelrite plus either 20 (control), 55.8, 62, 68.2, or 74 g/L of sucrose, glucose, or fructose; 0 (control), 4.8, 5.3, 5.8, or 6.3 mg/L of chlorogenic acid (CGA), and 0 (control), 0.55, 0.6, 0.65, or 0.7 mg/L of caffeine. Five levels of each synthetic compound were evaluated in a completely randomized design with four replications (4 jars). Each jar had 5 leaf discs, measuring 1 cm^2 each, and maintained at 25 $\pm 2^oC$ and 70% humidity in darkness. Induced somatic embryos were transferred to light and cultured in MS medium with 20 g/L sucrose for shooting, then ½ strength MS rooting medium with 1 ml/L NAA, and finally top soil, sand and manure (3:2:1 v/v) mixture for weaning. The experiment was monitored for embryo induction, shooting, rooting, acclimatization and data record on counts, frequency and survival percentage. The sugars augmented, sucrose at 62 g/L resulted in the highest plantlet survival rate (75%) of induced, germinated, rooted and acclimatized coffee somatic embryos. The 4.8 mg/L CGA induced highest embryos and best shoot and root growth, as well as 100% survival. Caffeine at 0.7 mg/L induced highest embryos, whereas 0.65 mg/L promoted the best shoot and root growth, and 76% survival. Thus, the augmented sucrose, glucose, fructose, CGA and caffeine effects vary depending on concentration and stage of embryo growth and development.

KEYWORDS: Culture medium augmentation, Beverage crops, Recalcitrant plants, Somatic embryos.

INTRODUCTION

Somatic embryogenesis is the process by which somatic cells, under inductive conditions, generate embryogenic cells, which go through a series of morphological and biochemical changes that result in the formation of a somatic embryos (Yang and Zhang, 2010). The major limitation of this embryogenic process is the asynchronous production of somatic embryos, low germination and conversion of these embryos into plantlets (Etienne *et al.*, 2013). Somatic embryogenesis for all species is initiated by exposing plant tissues to the right stimulus, most often to plant hormones (Yang and Zhang, 2010) and a right balance between the external and internal factors can induce the re-programming of differentiated somatic cells. One important factor governing growth and morphogenesis of plant tissue in culture is culture medium composition. A suitable medium for initiation and maintenance can be obtained through trial and error (Allan, 1991). The major components of somatic embryogenesis culture media are carbon and energy source, inorganic macro- and micro-nutrients with potassium and iron EDTA, a level of reduced nitrogen, organic compounds such as vitamins and amino acids, and growth regulators such as auxins and cytokinins (Brown *et al.*, 1995). The recalcitrance of some species can be overcome by manipulating other media components (Birhman *et al*., 1994). Certain bioactive compounds have been identified as enhancers of somatic embryogenesis in some species (Deo *et al.*, 2010) and their efficacy in embryogenesis has been attributed to their contribution to various cellular processes such as improving cell signalling processes in various signal transduction pathways (Lakshmanan and Taji, 2000), serving as precursor molecules for certain growth regulators (Jiménez and Bangerth, 2001a; Jiménez and Bangerth, 2001b), or regulators of DNA synthesis (Kevers *et al.*, 2000; Astarita *et al.*, 2003). This paper presents results of augmenting culture media with equivalents of endogenous substances that were found to be associated with coffee direct somatic embryo development *in vitro.*

MATERIALS & METHODS

The present experiment was conducted in the laboratories and greenhouses of the Coffee Research Institute at Ruiru in Kenya. The site is situated 1.05^{o}S and 36.45^{o}E at an elevation of 1608 m above the sea level and has humic nitosol soils (Jaetzold *et al*., 2007). Explants of *Coffea arabica* cultivar Ruiru 11 were used in this experiment.

Augmentation of medium with equivalents of endogenous substances

Third leaf pair explants, excised from in vitro germinated mother plants were used. The explants were washed thoroughly under running tap water, followed by water containing Teepol detergent and finally sterile distilled water. The subsequent sterilization steps took place under a laminar flow cabinet where the explants were dipped for 30 seconds in 70% alcohol and rinsed 2-3 times in sterilized distilled water. The explants were further sterilized using 20% sodium hypochlorite for 20 minutes followed by rinsing 4 times in sterilized distilled water. The generic culture medium contained half-strength MS (1962) basal salts, supplemented with 0.2 g/L thiamine, 0.1 g/L nicotinic acid, 0.1 g/L pyridoxine, 100 mg/L myo-inositol, 100 mg/L cysteine, and 1 ml/L Thidiazuron. The synthetic components were added to it as described below. The pH was adjusted to 5.7 using 1 M NaOH or 1 M HCL and 3 g/L gelrite added before autoclaving for 15 minutes at 121^{o}C and 100 kPa. The layout for each synthetic component was completely randomized design, with four replications and 20 explants per treatment. The experiment was repeated once.

Media preparation and induction of somatic embryos

Sugars

The levels of glucose, fructose and sucrose used were established from the sucrose content that had highest induced and surviving embryos in a previous study. Glucose or sucrose (Duchefa Biochemie) at 20 (control commonly used in *in vitro* culture media), 55.8, 62, 68.2 and 74 g/L each were added to the MS (1962) basal salts medium before autoclaving. The pH of the media was adjusted to 5.7 using 1 M NaOH before autoclaving for 15 minutes at 121^{o}C and 100 kPa. Fructose (Duchefa Biochemie) at 20 (control), 55.8, 62, 68 and 74 g/L was added using a 0.22 µM filter to the autoclaved MS basal salts medium. The culture medium measuring 25 ml was dispensed into Magenta jars and 5 leaf discs (about 1 cm^{2}) cultured in each jar maintained at 25±2^{o}C and 70% humidity in a dark growth chamber for 4 months.

Chlorogenic acid

The levels of chlorogenic acid used in augmentation were established from the results of endogenous chlorogenic acid content realized in developed embryos in a previous study. Exogenous chlorogenic acid (Acros organics) at 0 (control), 4.8, 5.3, 5.8, and 6.3 mg/L was added to the MS(1962) basal salts medium with 20/L sucrose. The pH of the media was adjusted to 5.7 using 1 M NaOH followed by autoclaving for 15 minutes at 121^{o}C and 100 kPa. Culture medium (25 ml) was dispensed into each Magenta jar and 5 leaf discs (about 1 cm^{2}) cultured in each jar maintained at 25±2^{o}C and 70% humidity in a dark growth chamber for 4 months.

Caffeine

Caffeine concentrations used for augmentation were established from the highest caffeine content found in developed somatic embryos in a previous study. Exogenous caffeine (Fischer Scientific) at 0, 0.55, 0.6, 0.65 and 0.7 mg/L was added to the MS basal salts medium with 20/L sucrose. The pH of the media was adjusted to 5.7 using 1 M NaOH followed by autoclaving for 15 minutes at 121^{o}C and 100 kPa. Culture medium (25 ml) was dispensed into each Magenta jar and 5 leaf discs (about 1 cm^{2}) cultured in each jar maintained at 25±2^{o}C and 70% humidity in a dark growth chamber for 4 months. Somatic embryos induced using various equivalents of endogenous substances were transferred to germination media, containing full strength MS basal salts, 0.2 g/L thiamine, 0.1 g/L nicotinic acid, 0.1 g/L pyridoxine, 20 g/L sucrose, 100 mg/L myo-inositol and 3 g/L gelrite. The somatic embryos were incubated at 25 ± 2^{o}C and 16 h photoperiod for 6 weeks. The layout was completely randomized design, with three replications and 20 explants per treatment. The experiment was repeated once. Germinated shoots that developed from somatic embryos were transferred to rooting media, containing half strength MS basal salts, 1 ml/L naphthalene acetic acid, 0.2 g/L thiamine, 0.1 g/L nicotinic acid, 0.1 g/L pyridoxine, 100 mg/L myo-inositol, 20 g/L sucrose and 3 g/L gelrite. The germinated shoots were incubated at 25^{o}C ± 2 and 16-hour photoperiod for 6 weeks. The layout was completely randomized design, with three replications and 20 explants per treatment. The experiment was repeated once.

The rooted plantlets were removed from the rooting medium using forceps. The agar was cleaned off under running tap water and the plantlets soaked in 10% Ridomil fungicide for 1-2 hours. The potting mixture had top soil, sand and composted manure in a ratio of 3:2:1. The pots were allowed to soak water until the mixture became saturated. The plantlets were removed from the fungicide and planted in the potting mixture. The weaning pots were initially covered completely. They were opened gradually to reduce humidity and fully opened after one month. The plants were transferred individually into similar potting mixture. For induction of somatic embryos, the explants were observed weekly for the presence of somatic embryos on each explant. The total number of embryos, number of explants with somatic embryos and somatic embryos per explant were counted. After transfer of the induced embryos to germination medium, the duration taken for the embryos to shoot was recorded. The number of shoots and average shoot length were recorded after 6 weeks of culture. Following the transfer of germinated shoots to rooting media, the duration taken for the germinated shoots to root was recorded. The number of roots and average shoot length were recorded after 6 weeks in culture. Acclimatization success was recorded by counting the surviving plantlets and expressing as a percentage after the 6 weeks for all treatments.

Data values were subjected to analysis of variance by the General Linear Model for a completely randomized design using SAS 9.2 program. The linear model fitted for the each was: $Y_{ij} = \mu + T_i + \varepsilon_{ij}$, Where: i = 1, 2, 3, 4, 5; j = 1, 2, 3, 4; μ = grand mean, T_i = the i^{th} concentration of the equivalents of the endogenous components, and ε_{ij} =

random error component, normally and independently distributed about zero mean with a common variance σ^2. Differences between treatment means were separated using the LSD test at $P = 0.05$.

RESULTS

Effect of augmenting culture media with sugars on coffee somatic embryogenesis

The explants used in this study started to develop white projections at the cut ends of the leaf discs after 2 weeks in culture. Globular embryos were first observed with fructose as the main carbon source and embryos were evident after 8 weeks in culture for all the three carbohydrate sources tested. The embryo development was asynchronous; hence the cultures were maintained for 4 months to allow for maturation of embryos. On average, 47.9% of embryos regenerated with fructose were globular, 39% heart-shaped, and the rest cotyledon-shaped by the time the leaf discs turned brown (Plate 1).

PLATE 1. Fructose augmentation

PLATE 2. Glucose augmentation

PLATE 3. Sucrose augmentation

Augmenting the media with glucose resulted in only globular embryos as shown in Plate 2. About 94.5% of all embryos regenerated with sucrose as a carbon source were globular, 5.2% heart-shaped and 0.2% cotyledonary (Plate 3). Almost all the embryos formed with high levels of glucose and sucrose was globular. There were significant (P<0.05) differences in induction of somatic embryos with use of fructose as a carbon source in the induction media (Table 1).

TABLE 1: Effect of fructose, glucose and sucrose on induction of somatic embryos of Ruiru 11

Induction conc. (g/L)	Embryos on 5 explants	Embryogenic cultures (%)	Embryos on 5 explants	Embryogenic cultures (%)	Embryos on 5 explants	Embryogenic cultures (%)
	Fructose		Glucose		Sucrose	
20	20.5±3.5^{b}*	81.3±4.1^{b}	5.8±0.2^{b}*	56.7±4.3^{a}	9.1±0.9^{b}*	57.5±7.5^{b}
55.8	55.9±4.5^{a}	99.5±0.5^{a}	6.0±0.1^{b}	51.0±5.8^{a}	18.8±0.8^{a}	94.4±206^{a}
62	46.0±3.4^{a}	99.4±0.6^{a}	10.7±0.2^{a}	61.3±6.5^{a}	22.0±1.3^{a}	92.8±3.8^{a}
68.2	46.1±4.3^{a}	95.8±0.6^{a}	6.1±0.7^{b}	53.3±6.7^{a}	22.1±2.3^{a}	92.5±4.8^{a}
74	26.6±5.7^{b}	97.5±1.0^{a}	1.5±0.2^{c}	20.0±0.0^{b}	19.0±2.4^{a}	86.7±6.2^{a}
CV (%)	22.3	4.3	12.1	21.6	18.6	12.5
LSD $_{0.05}$	13.1	6.2	1.1	15.8	5.1	15.9

*Values followed by the same letter within a column are not significantly different based on the LSD test at P=0.05

Fructose at 55.8 g/L resulted in the highest number of somatic embryos developed although not significantly different from fructose concentration of 62 and 68.2 g/L. The 74 g/L fructose resulted in few embryos (26.5) induced although this concentration was not significantly (P>0.05) different from the control (20.5). There was a significant effect (P<0.05) of augmenting with fructose on embryogenic cultures. High fructose concentration of between 55.8 g/L and 74 g/L in the culture medium resulted in over 90% embryogenic cultures, although there was no significant difference in these concentrations. There were significant (P<0.05) differences in of embryo formation after addition of glucose in the culture media (Table 1). The highest number (10.65) of somatic embryos was for 62 g/L glucose. The 74 g/L glucose resulted in the lowest number of embryos (1.5). Generally, fewer embryos were induced with glucose as compared to the other carbon sources. There were significant differences (P<0.05) in the effect of glucose on somatic embryogenesis frequency. Glucose levels 20 g/L to 68.2 g/L had the highest embryogenic cultures although not significantly different from each other. The 74 g/L glucose resulted in the lowest percentage of embryogenic cultures.

Significant (P<0.05) differences in formation of somatic embryos resulted when sucrose was used as the carbon source in the induction media (Table 1). The highest number of somatic embryos resulted in culture media with sucrose concentration of 62 g/L. Sucrose concentration of 20 g/L (control) resulted in the least number and percentage of somatic embryos (9.14). Despite formation of more embryos, high sucrose content in the medium resulted in desiccated embryos. Although the 62 g/L sucrose had the highest embryogenic cultures and number of embryos, the results were not significantly different from 55.8 g/L, 68.2 g/L and 74 g/L sucrose.

Significant differences (P<0.05) resulted in shoot length and formation under the various sugars (Table 2). Embryos cultured in 20 g/L fructose had the highest shoot length of 1 cm. In addition, 20 g/L fructose had the highest frequency of shooting, although not significantly different, except from 62 g/L fructose. Root length in different fructose levels was not significantly (P>0.05) different, except for 68.2 g/L and 55.8 g/L fructose. A similar trend

was observed in frequency of rooting under different fructose levels. Most concentrations were not significantly (P>0.05) different, except 55.8 g/L and 74 g/L.

TABLE 2: Effect of fructose, glucose and sucrose on shoot growth and formation in somatic embryos

Induction conc. (g/L)	Fructose	Glucose	Sucrose	Fructose	Glucose	Sucrose
	Shoot growth (cm)			Shoot formation (%)		
20	1.0±0.1^{a}*	1.0±0.1^{b}*	0.8±0.0^{a}*	100.0±0.0^{a}	85.4±3.8^{a}	92.1±0.7^{a}
55.8	0.7±0.1^{bc}	0.8±0.0^{c}	0.8±0.1^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
62	0.9±0.0^{ab}	0.8±0.1^{c}	0.6±0.1^{bc}	87.5±6.4^{b}	100.0±0.0^{a}	61.7±0.7.3^{b}
68.2	0.7±0.1^{bc}	1.4±0.1^{a}	0.7±0.0^{ab}	100.0±0.0^{a}	89.0±11.0^{a}	100.0±0.0^{a}
74	0.7±0.0^{c}	0.0±0.0^{d}	0.5±0.0^{c}	94.4±2.9^{ab}	0.0±0.0^{b}	1000±0.0^{a}
CV %	13.7	13.0	11.9	5.6	12.0	6.2
$LSD_{0.05}$	0.2	0.28	0.2	9.9	16.4	10.3

*Values followed by the same letter within a column are not significantly different based on the LSD test at P=0.05

There were significant (P<0.05) differences in shoot growth of the various glucose levels (Table 2). Embryos induced with 68.2 g/L and germinated in 20 g/L resulted in the highest shoot length of 1.35 cm, whereas 62 g/L resulted in the shortest shoots. There was significant (P>0.05) difference on the frequency of shoot formation (Table 2). Shooting percentage was not significantly different with 20 g/L and 68.2 g/L glucose, but it was significantly different with 74% g/L. The 20 g/L glucose induced the longest root length of 1.4 cm, whereas the highest frequency of root formation was observed with 68.2 g/L glucose. No embryos germinated with high glucose content of 74 g/L in the induction media.

Significant (P<0.05) differences in shoot growth resulted for the different sucrose (Table 2). The 55.8 g/L sucrose resulted in the longest shoots, although not significantly different from 20 g/L and 68.2 g/L sucrose. In terms of shoot formation, all embryos cultured in 55.8 g/L, 68.2 g/L and 74 g/L sucrose and 92.1% cultures in 20 g/L sucrose formed shoots. Sucrose at 62 g/L resulted in the least percentage of shoots at 61.67% and this was significantly different from the other sucrose levels. There were significant (P<0.05) differences in root growth with different sucrose concentrations in the rooting medium (Table 3 & Plate 4). The 55.8 g/L sucrose had the longest roots and was significantly different from the other sucrose concentrations. The highest percentage of cultures with roots resulted in medium containing 68.2 g/L sucrose and this was significantly different from the other sucrose levels.

PLATE 4: Rooting of Ruiru 11

There were significant (P<0.05) differences in survival of plantlets regenerated under the various concentrations of the different carbon sources (Table 4). All the regenerated plantlets derived from embryos induced in media augmented with 68.2 g/L fructose survived weaning and acclimatization in the greenhouse. About 75% of the embryos induced with 62 g/L glucose and sucrose had significantly (P<0.05) greater survival than that of the other glucose and sucrose concentrations.

TABLE 3: Rooting of regenerated plantlets from various levels of fructose, glucose and sucrose

Induction conc. (mg/L)	Fructose	Glucose	Sucrose	Fructose	Glucose	Sucrose
	Root growth (cm)			Root formation (%)		
20	1.6±0.3^{a}	1.4^{a}	1.3±0.1^{b}	42.5±1.4^{ab}	30.7^{b}	21.7±1.7^{c}
55.8	1.3±0.3^{ab}	0.7^{c}	2.1±0.1^{a}	26.7±1.7^{c}	20.0^{b}	37.7±2.3^{b}
62	1.7±0.1^{a}	1.3^{ab}	0.9±0.1^{c}	37.0±1.5^{b}	55.7^{a}	40.0±1.2^{b}
68.2	0.6±0.1^{b}	1.1^{b}	1.2±0.1^{b}	40.0±2.9^{ab}	64.0^{a}	50.0±0.0^{a}
74	1.5±0.2^{a}	0.0^{d}	0.9±0.1^{c}	43.9±2.0^{a}	0^{c}	20.0±0.0^{c}
CV (%)	29.9	15.0	11.9	9.0	23.2	7.1
$LSD_{0.05}$	0.734	0.247	0.276	6.229	14.381	4.356

*Values followed by the same letter within a column are not significantly different based on the LSD test at P=0.05

TABLE 4: Survival of regenerated plantlets from various levels of fructose, glucose and sucrose

Induction conc. (mg/L)	Fructose	Glucose	Sucrose
		Survival (%)	
20	75.0±2.9[c]	60.0±2.9[b]	66.2±2.3[b]
55.8	50.0±0.0[d]	50.0±0.0[c]	50.0±0.0[d]
62	87.5±1.4[b]	75.0±2.9[a]	75±2.9[a]
68.2	100.0±0.0[a]	50.0±2.9[c]	60.0±1.2[c]
74	85.0±2.9[b]	0.0±0.0[d]	66.9±1.7[b]
CV (%)	4.2	8.2	5.2
$LSD_{0.05}$	6.1	7.0	6.0

*Values followed by the same letter within a column are not significantly different based on the LSD test at *P*=0.05

In a previous study, embryos regenerated from green leaf discs in medium containing 62.15 mg/L sucrose. This concentration proved superior to the others in regenerating somatic embryos (Tables 1), although it was not significantly different from 55.8-74 g/L. Similar levels of both exogenous fructose and glucose also resulted in high rates of somatic embryogenesis as compared to the other concentrations, although the total number of embryos formed was low. These results confirm that 62 g/L of either endogenous or exogenous sucrose, fructose or glucose promotes best somatic embryogenesis in Ruiru 11.

Effect of augmenting culture media with chlorogenic acid on coffee somatic embryogenesis

The explants started to develop white projections at the cut ends after 2 weeks in culture. Globular embryos were evident after 8 weeks in culture. The embryo development was asynchronous; hence the cultures were maintained for 4 months to allow for maturation of the inducted embryos. Of the exogenous chlorogenic acid-induced embryos, 42% were globular, 41% heart- and 17% cotyledon-shaped. The leaf explants were both green and brown and there was callus formation at the cut edges (Plate 5).

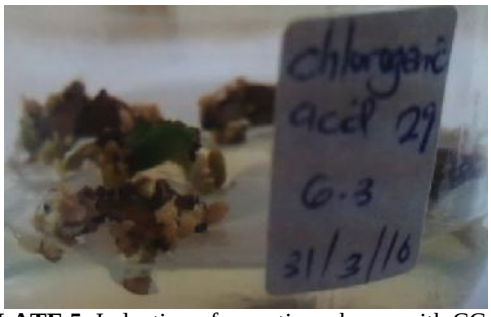

PLATE 5: Induction of somatic embryos with CGA

Significant (*P*<0.05) differences resulted among the different levels of chlorogenic acid (Table 5). The 4.8 mg/L to 6.3 mg/L CGA had a positive influence on embryo development, whereby 5.3mg/L had the highest somatic embryos (38.3) compared to the control (11). The 5.3 mg/L and 6.3 mg/L CGA had 100%, whereas 4.8 mg/L had 96% embryogenic cultures, which was significantly (*P*<0.05) different from the control (69.2%). All cultures with CGA had significantly (*P*<0.05) higher number of embryos per explant than the control.

TABLE 5: Effect of chlorogenic acid on development of somatic embryos of Ruiru 11

Induction CGA conc. (mg/L)	Embryos on 5 explants	Embryogenic cultures (%)	Shoot length (cm)	Shoot formation (%)
0	11.1±3.3[b]*	69.3±2.9[b]	0.9±0.2[a]	100.0±0.0[a]
4.8	32.2±3.0[a]	96.1±2.4[a]	1.1±0.1[a]	75.6±4.4[ab]
5.3	38.3±1.8[a]	100.0±0.0[a]	0.9±0.1[a]	91.7±2.5[ab]
5.8	32.5±1.7[a]	100.0±0.0[a]	0.9±0.1[a]	64.7±15.7[b]
6.3	36.9±3.5[a]	100.0±0.0[a]	1.0±0.1[a]	76.4±12.1[ab]
CV (%)	18.2	3.7	18.7	19.5
$LSD_{0.05}$	8.3	5.1	0.3	28.9

*Values followed by the same letter within each column are not significantly different according to the LSD test at *P*=0.05

Significant differences (*P*<0.05) were observed on the shoot length and percentage shoot formation under different chlorogenic acid levels (Table 5). Embryos induced in 4.8 mg/L had the highest shoot length (1.08 cm), although this treatment was not significantly different from the others. Plantlets induced in CGA-free media had the highest shooting (100%), although not significantly different from the other treatments, except 5.8 g/L.There were significant differences (*P*<0.05) in root length and percentage root formation under the different chlorogenic acid concentrations (Table 6). Embryos induced in CGA-free media had the shortest roots (0.4 cm) and fewest cultures with roots (8.33%) and this treatment was significantly different from the others.

There were significant (*P*<0.05) differences in survival of the regenerated plantlets for the various chlorogenic acid

concentrations (Table 6). All plantlets regenerated from embryos of 4.8 mg/L CGA and 5.3 mg/L CGA survived weaning and acclimatization, although the survival rate was not significantly different from plantlets regenerated from embryos of 6.3 mg/L CGA.

TABLE 6: Root growth and survival of regenerated plantlets induced with chlorogenic acid

Induction CGA conc. (mg/L)	Root length (cm)	Root formation (%)	Survival (%)
0	0.4±0.4[b]	8.3±8.3[b]	83.0±1.2[b]
4.8	1.7±0.2[a]	49.1±3.0[a]	100.0±0.0[a]
5.3	1.2±0.0[a]	47.4±4.0[a]	100.0±0.0[a]
5.8	1.5±0.0[a]	37.1±0.0[a]	33.3±1.9[c]
6.3	1.5±0.2[a]	38.3±0.0[a]	84.5±1.4[b]
CV (%)	29.9	29.6	2.6
$LSD_{0.05}$	0.7	19.8	3.7

*Values followed by the same letter within each column are not significantly different according to the LSD test at *P*=0.05

During quantifying equivalents of endogenous substances present during development of coffee somatic embryos in a previous study, embryos regenerated from green leaf discs in medium containing 5.3 mg/L chlorogenic acid. This CGA concentration proved effective in regenerating somatic embryos (Tables 5). Augmenting culture media with 5.3 mg/L chlorogenic acid resulted in many somatic embryos, although not significantly different from other CGA concentrations (Table 5). Chlorogenic acid at 5.3 mg/L promoted shoots and root growth, although not significantly different from the other concentrations (Table 5 and 6). These results validate that somatic embryogenesis of Ruiru 11 is promoted by 5.3 mg/L endogenous or exogenous chlorogenic acid.

Effect of augmenting culture media with caffeine on coffee somatic embryogenesis

The explants used in this study started to develop white projections at the cut ends of the leaf discs after 2 weeks in culture. Globular embryos were evident after 8 weeks in culture. Embryo development was asynchronous; hence the cultures were maintained for 4 months to allow for maturation of all those that had initiated. In total, 46% of all embryos regenerated were globular, 36% heart- and 18% cotyledon-shaped. The leaf explants were still green in colour after 4 months in culture (Plate 6).

PLATE 6: Embryos induced using exogenous caffeine

There were significant (*P*<0.05) differences in the total number of embryos formed under the different caffeine concentrations (Table 7). The 0.7 mg/L caffeine had the highest (13.3), whereas 0.65 mg/L had the least number of somatic embryos (9). Culture medium augmented with 0.55 mg/L caffeine had significantly (*P*<0.05) high percentage embryogenic cultures, although not significantly different from the control, 0.65 mg/L and 0.7 mg/L. Culture medium augmented with 0.7 mg/L caffeine had significantly (*P*<0.05) high embryos, although not significantly different from control, 0.55 and 0.6 mg/L. There were significant (*P*<0.05) effects of different caffeine concentrations on shoot growth and percent shoot formation. Embryos from cultures induced with 0.7 mg/L caffeine germinated into longest shoots of 1.17 cm, although not significantly different from 0.6 mg/L, 0.65 mg/L and 0.7 mg/L caffeine (Table 6). Germinated shoots for 0.65 mg/L induction media had the highest frequency of shoot formation of 96.7%, although not significantly different from embryos induced with 0.55 mg/L, 0.6 mg/L and 0.7 mg/L caffeine. Significant (*P*<0.05) differences also resulted during rooting of embryos from different caffeine concentrations. Cultures induced on 0.55 mg/L caffeine had the longest roots of 2 cm, although not significantly different from 0.6 mg/L, 0.65 mg/L and 0.7 mg/L caffeine. There was no significant difference in root formation for all the treatments.

There were significant (*P*<0.05) differences in the survival rates of plantlets regenerated from different caffeine concentrations (Table 8). The highest survival rate of 76% resulted for embryos induced with media augmented with 0.65 mg/L caffeine and this was significantly (*P*<0.05) different from the other caffeine concentrations.

In our previous study, embryos regenerated from green leaf discs had 0.55 mg/L caffeine. No significant difference resulted in augmenting culture media with 0.55 mg/L caffeine and the other concentrations (Table 7). Caffeine at 0.55 mg/L was effective in shoot and root

growth promotion, since there was no significant difference in shoot and root formation for this concentration and the 0.65 mg/L or 0.7 mg/L caffeine (Table 7 and 8). The results confirmed that 0.55 mg/L caffeine is optimal in somatic embryogenesis of Ruiru 11.

TABLE 7: Effect of different caffeine concentration on development of somatic embryos *in vitro*

Induction caffeine conc. (mg/L)	Embryos on 5 explants	Embryogenic cultures (%)	Shoot length (cm)	Shoot formation (%)
0	11.6±1.5^{ab}*	65.4±5.3^{ab}	1.0±0.1^{ab}	68.2±6.8^{b}
0.55	12.1±0.7^{ab}	77.2±2.9^{a}	0.9±0.0^{b}	82.2±9.7^{ab}
0.60	10.3±1.0^{ab}	64.6±4.4^{b}	1.1±0.0^{ab}	88.2±6.4^{ab}
0.65	9.0±0.5^{b}	66.1±2.8^{ab}	0.98±0.1^{ab}	96.7±3.3^{a}
0.70	13.4±2.1^{a}	71.7±4.4^{ab}	1.2±0.0^{a}	84.8±3.6^{ab}
CV (%)	23.5	11.8	12.9	13.2
$LSD_{0.05}$	4.0	12.3	0.2	20.2

*Values followed by the same letter within a column are not significantly different according to the LSD test at *P*=0.05

TABLE 8: Survival of regenerated plantlets induced with various concentrations of caffeine

Induction caffeine conc. (mg/L)	Root length (cm)	Root formation (%)	Survival (%)
0	2.0±0.4^{a}	28.3±4.4^{a}	68.3±4.4^{ab}
0.55	1.0±0.2^{b}	28.3±6.0^{a}	60.0±5.8^{bc}
0.6	1.1±0.1^{ab}	23.3±3.3^{a}	50.0 ±5.^{3c}
0.65	1.5±0.0^{ab}	25.0±2.9^{a}	76.3±1.9^{a}
0.7	1.6±0.2^{ab}	35.5±0.2^{a}	68.7±4.7^{ab}
CV (%)	26.3	26.2	12.3
$LSD_{0.05}$	0.7	13. 4	14.5

*Values followed by the same letter within each column are not significantly different according to the LSD test at *P*=0.05

DISCUSSION

Parrott and Bailey (1993) and Sculler and Reuther (1993) reported that induction of somatic embryogenesis is influenced by the carbohydrate source and that different species should have appropriate carbohydrate source for regeneration of somatic embryos. Sucrose is the main carbon source used in culture media (George, 1993), although several reports indicate that the beneficial effects of sugar on plant regeneration process appear to be species-specific (Cunha and Fernandez-Ferreira, 1999). In the present study, 62 g/L sucrose was optimal in regeneration of coffee somatic embryos. Similar findings have been reported by Iraqi *et al.* (2005) who observed that 6% sucrose was optimal in black spruce embryo production. Low amounts of sucrose in the induction media has been shown to reduce SE development in *Picea abies* (Jain *et al.*, 1988) and *Pinus strobes* (Finer *et al.*, 1989). In the present study, 20 mg/L had the least number of embryos developed. The 74 g/L sucrose had a decrease in somatic embryos formed and this was attributed to the osmotic effect of high sucrose. Carbohydrate, as a source of carbon, at high levels, can be toxic and inhibitory to growth and development of plantlets (Tiexeira, 2004).

Other carbon sources such as glucose, fructose, maltose, lactose, cellobiose, mannitol, sorbitol, and myo-inositol have proved to be less efficient as carbon sources in plant tissue culture (Lipavská and Konrádová, 2004). In most cases, the sucrose is hydrolyzed into hexoses such as glucose and fructose (George, 1993), which are then utilized (Iraqi and Tremblay, 2001). In the present study, higher number of embryos was induced with fructose as compared to sucrose and glucose. Similar results were obtained by Fuentes *et al.* (2000) who reported superior effect (3-6 fold higher) of fructose, as compared to sucrose especially in the least responsive *Coffea canephora* genotypes and the effect was attributed to a specific carbon effect. Fructose concentration of 6% was found to be the best for SE development in red spruce (Nùrgaard, 1997). The stronger reducing capacity of fructose makes it a more

powerful initiator of the glycation reaction compared to glucose (Semchyshyn *et al.*, 2011). Glycation refers to non-enzymatic reaction between reducing sugars, such as glucose, and proteins, lipids or nucleic acids. Traore and Guiltinan (2006) reported that the use of 100 mM fructose in the culture media of cacao tissue resulted in high embryogenic response.

Eapen and George (1993) reported that glucose reduced frequency of cultures with somatic embryos and the number of somatic embryos per responding culture in peanut. Fuentes *et al.* (2000) in their study on the effects of various carbohydrate sources on somatic embryogenesis in five *Coffea canephora* Pierre genotypes reported that glucose inhibited embryo formation in genotype N128 and did not significantly improve embryo production for genotypes N91 and N75. In the present study, fewer embryos were regenerated with glucose as compared to sucrose and fructose. Biahoua and Bonneau (1999) reported that higher concentrations of more than 355 mM glucose inhibited embryo formation in spindle tree somatic embryogenesis. Businge and Egertsdotter (2004) postulated that the inhibitory effect of glucose due to blocked development was not correlated to the Maillard reaction. The apparent inhibitory effect of glucose on embryo development likely involves mechanisms other than the Maillard reaction.

Generally, embryos require a higher amount of sugar than other plant tissue cultures. High sucrose in the media may cause osmotic stress, but may promote somatic

embryogenesis. High sugar content in the medium affects cell osmolarity, suggesting that the osmotic effect is what triggers development of somatic embryos. This positive effect could mimic the changes in the osmolarity that occurs in the environment within the seed.

During embryogenesis, the carbohydrate requirements may change with the stage of the culture (Thompson and Thorpe, 1987). Carbohydrate type and concentration play important roles in different stages of somatic embryogenesis. In the present study, fructose regenerated the higher embryos than glucose and sucrose. In most studies, fructose does not support embryogenesis. This is because at high temperatures especially during autoclaving of the media, fructose releases 5 hydroxymethyl-2-furaldehyde (Buah *et al.,* 2000), which is known for enhancing hyperhydricity and decreasing leaf water potential (Sumaryono *et al*., 2012). Consequently, this effect hampers subsequent developmental processes because it is toxic to the explants. The high number of embryos with use of fructose in the culture media was attributed to the filter-sterilization of fructose instead of autoclaving. Generally, it was observed that treatments that led to production of more embryos were related to the best frequencies of shoot and root growth.

Reis *et al.* (2008) inferred that somatic embryos formed in the presence of phenolic compound may not have the appropriate amounts of sucrose necessary for basic metabolic activities and therefore become arrested at the globular stage or even at earlier stages of development. This may be the reason why a higher percentage of embryos formed were globular in the present study. The results of the present study contradict those of Nic-Can *et al.* (2015), who reported that 1 µM chlorogenic acid added to the culture media at 7 days after induction reduced embryo production of *Coffea canephora* (up to 52%) and disrupted embryo development beyond the heart-shape. The 100 µM chlorogenic acid or more inhibited somatic embryogenesis completely.

The difference between the two studies is that Nic-Can *et al*. (2015) added 21 days after induction *C. canephora* explants into the medium of 7 days after induction *C. arabica* and co-cultured them together for two weeks and this resulted to a significant reduction in cell proliferation of the proembryogenic mass and a rapid phenolic oxidation around the explants. Reis *et al*., (2008) reported that inclusion of culture media with other phenolic compounds such as caffeic acid at concentrations between 140 and 560 µM, and 197.5 µM phloroglucinol significantly increased somatic embryo formation when compared to the control and resulted in 100% and 94.7±5.6% frequency of induction, respectively. The role of phenols such as chlorogenic acid during somatic embryo germination is supported by the present study, since it was found that exogenous chlorogenic acid promoted induction and germination. This result was attributed to interference of phenolics with auxin metabolism at favourable concentrations for somatic embryogenesis induction (Reis *et al.,* 2008).

Nic-Can *et al.* (2015) found 10 µM exogenous caffeine reduced somatic embryogenesis process in *C. canephora.* In addition, caffeine at the highest concentration of 1,000 µM, allowed somatic embryos to develop in *C. canephora* when compared to similar concentrations of exogenous chlorogenic acid, hydroxybenzoic acid and trans-cinnamic acid, indicating that these embryos have the ability to avoid caffeine auto-toxicity since caffeine accumulation in the cotyledons occurs after the end of cell division process. This observation may explain why embryos were induced by the various caffeine concentrations.

Da Rosa *et al.* (2006) reported that caffeine concentrations affected germination and *in vitro* development of *C. arabica* L. and *C. canephora* Pierre with significant effects on the percentage of rooting, root length, cotyledon, and fresh seedling mass. Smyth (1992) found that caffeine was the compound that most effectively inhibited rice seedling growth, with 0.05% inhibiting 50% of the elongation of the plumes and 90% of the rootlets, indicating that the roots are more sensitive than the leaves to the same concentration of caffeine.

Friedman and Waller (1983) reported that exogenous concentration of caffeine of 10 mM (4 to 6 times lower than the endogenous concentration in the seeds) inhibited growth of *C. arabica* radicles and also cell divisions during the germination of seeds, indicating that the coffee embryos have strategic ways of avoiding self-toxicity. It was observed for *C. arabica* embryos that caffeine caused highly significant effects on percentage of rooting, root length, cotyledon, and fresh seedling mass.

CONCLUSION & RECOMMENDATIONS

Augmenting culture media with equivalents of endogenous substances affected somatic embryogenesis and subsequent growth of Ruiru 11. Sucrose at 62 g/L proved to be superior in coffee somatic embryo induction, germination and rooting. Generally, glucose and fructose at 62 g/L proved to be the best in the three stages of plant development through somatic embryogenesis.

The role for phenolic compounds during somatic embryo germination was supported by the present study. Somatic embryogenesis of coffee improved after augmenting the culture media with chlorogenic acid. The 5.3 mg/L chlorogenic acid induced more embryos and enhanced both shoot and root growth. Caffeine at 0.55 mg/L was effective in shoot and root growth. Augmenting culture media with sucrose, chlorogenic acid and caffeine can be done to improve the efficiency of somatic embryo production in Ruiru 11.

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