Evaluation of Endogenous Sugars, Chlorogenic Acid and Caffeine Associated with Direct Somatic Embryogenesis of Coffee (*Coffea arabica* L.)

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ABSTRACT
Coffee is one of the most important cash crops cultivated in the world with great economic importance. During the induction of somatic embryogenesis, there are different endogenous compounds involved in the success or failure of the somatic embryogenesis response and these compounds determine the specificity of cellular responses. This present experiment identified and quantified endogenous sugars, chlorogenic acid and caffeine present during somatic embryogenesis of 'Ruiru 11'. Laboratory experiments were set up at Coffee Research Institute, Ruiru-Kenya between 2014 and 2016. Third leaf pair explants were excised from 8-month-old greenhouse-grown mother plants and cultured in half strength Murashige and Skoog basal salts augmented with Thidiazuron. Once embryos had developed, the cultures were analysed for endogenous sugars, caffeine and chlorogenic acid using HPLC. Generally, green embryogenic cultures contained more and higher quantities of the compounds. Glucose and fructose were highest (38.95 mg/g and 45.43 mg/g respectively) in leaf discs of brown non-embryogenic cultures. Sucrose was highest (62.15 mg/g) in embryos of green embryogenic cultures. Embryos of green embryogenic cultures had the highest chlorogenic acid (5.3 mg/g), whereas caffeine was highest (0.55 mg/g) in embryos of brown embryogenic cultures. Endogenous fructose and glucose inhibited embryogenesis, while sucrose, chlorogenic acids and caffeine promoted embryogenesis and are potential biomarkers for embryogenesis. Other biochemical compounds such as organic acids should be identified and their role in coffee somatic embryogenesis determined.

Keywords: Coffee, Endogenous biochemical compounds, Somatic embryogenesis, Tissue culture

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1. INTRODUCTION

There are different factors involved in the success or failure of the Somatic Embryogenesis (SE) response. One factor that affects the SE response is the release of organic molecules by the explants into the culture medium and these molecules induce or modulate the SE response (Nic-Can *et al.,* 2015) have been classified as polysaccharides, amino acids, growth regulators, vitamins (Matthys-Rochon, 2005). In general, somatic embryos have high levels of starch, protein, and soluble carbohydrates producing excellent growth and more vigorous plants (Attree *et al.,* 1992). The accumulation of reserves is vital for the development of somatic embryos and their conversion into plantlets (Flinn *et al.,* 1993). Carbohydrates are energy sources for cells, carbon frameworks for biosynthetic processes and osmotic agents (Tremblay and Tremblay, 1991). Soluble sugars, such as glucose and sucrose, are involved in regulation of developmental processes occurring from embryo development to seed maturation (Gibson, 2005). In tissue culture studies, phenolic substances, especially oxidized phenolics generally affect *in vitro* proliferation negatively (Arnaldos *et al.,* 2001). Phenolics are normally viewed as deleterious compounds during *in vitro* culture, since their exudation and oxidation negatively affect explants by causing browning and necrosis, especially when mature explants of woody plants are used. In contrast, Mucciarelli *et al.* (2000) suggested that many phenolic compounds control auxin catabolism probably by raising or lowering the level of indole acetic acid (IAA) through enzymatic reactions to permit embryo formation. Alkaloid accumulation observed during production of secondary plant metabolites is attributed to the level of the alkaloid biosynthesis and is strongly influenced by cellular differentiation (Ivanov *et al.,* 2012) .This paper reports glucose, fructose, sucrose, chlorogenic acid and caffeine identified, quantified and analysed during development of coffee somatic embryos *in vitro*.

2. MATERIALS AND METHODS

The experiment was conducted in the laboratories and greenhouses of the Coffee Research Institute at Ruiru in Kenya. The site is situated 1.05°S and 36.45°E at an elevation of 1608 m above the sea level and has humic nitosol soils (Jaetzold *et al.*, 2007). The *Coffea arabica* cultivar Ruiru 11 planted at this site was used in the experiment. The plants were moved from the fields to laboratories and then to greenhouses.

2.1. Plant material, surface sterilization and induction of somatic embryos

The mother plants for this experiment were obtained from germination of Ruiru 11 seeds. The resulting seedlings were transplanted to polybags filled with top soil: sand: manure (3:2:1 v/v) potting mixture and maintained in the greenhouse for about 8 months.

Third leaf pair explants were excised from the greenhouse-grown mother plants between March and April, 2014. The leaves were washed thoroughly under running tap water followed by water containing Teepol detergent and finally sterile distilled water. The subsequent sterilization steps were done in a laminar flow cabinet. The leaves were dipped quickly (approximately 30 seconds) in 70% alcohol and rinsed 2-3 times in sterilized distilled water. The leaves were sterilized further using 25% sodium hypochlorite for 25 minutes followed by rinsing thoroughly (4 times) in sterilized distilled water. The culture medium contained half-strength Murashige and Skoog (MS 1962) inorganic basal salts, supplemented with 0.2 g/L thiamine, 0.1 g/L nicotinic acid, 0.1 g/L pyridoxine, 30 g/L sucrose, 100 mg/L myo-inositol, 100 mg/L cysteine, 3 g/L gelrite, and 1 ml/L Thidiazuron. The pH of the medium 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°C and 100 kPa. Culture medium (25 ml) was poured into Magenta vessels (Sigma Chemical Co.) and 5 leaf discs (1 cm^2) cultured in each vessel maintained in a dark, 25 ± 2°C and 70% relative humidity growth chamber for about 8 months. A total of 627 culture vessels were prepared. Out of these 627 vessels, 183 (29%) were discarded due to fungal contamination.

2.2. Treatments

From the remaining 444 culture vessels, treatments for this experiment were selected. Culture vessels with green and brown leaf discs with and without embryos as shown in Plate 1 were used to characterise glucose, fructose, sucrose, chlorogenic acid, and caffeine in the leaf discs, embryos and medium. Fresh culture media and leaf explants excised from greenhouse-grown mother plants were used as the controls. The experimental

layout was a completely randomized design, with three replications and six culture vessels per treatment. The experiment was repeated once.

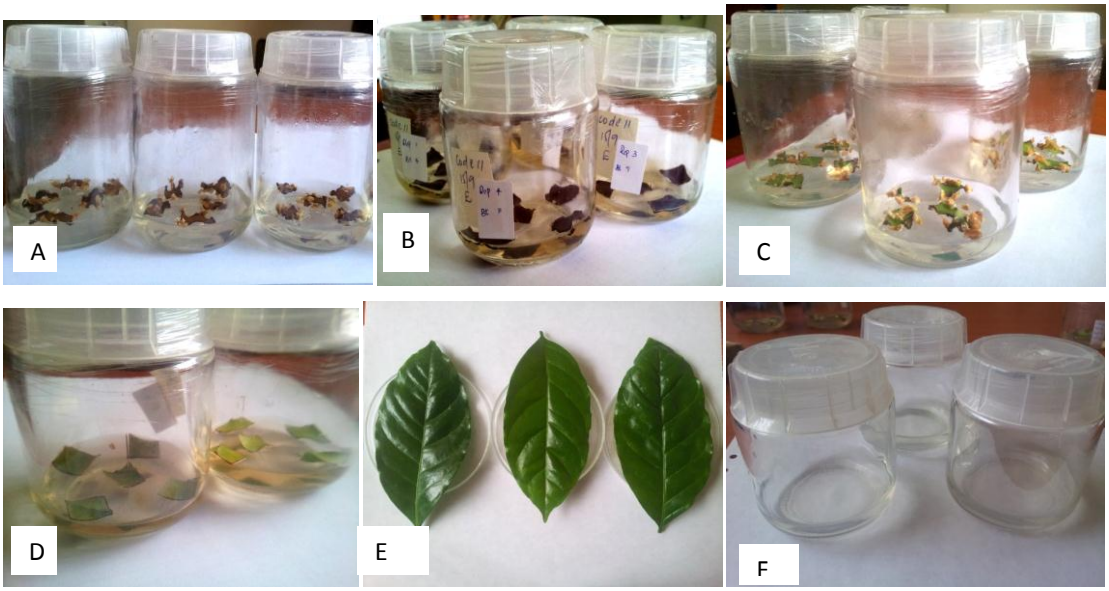

Plate 1: Treatments used for biochemical analysis. A: Brown leaf discs with embryos. B: brown leaf discs without embryos. C: green leaf discs with embryos. D: Green leaf discs without embryos, E: Fresh leaves (control). F: Fresh media (control)

2.3. Extraction and evaluation of sugars

The sugars sucrose, fructose and glucose were extracted as described by Osborne and Voogt (1978) with modifications. The leaf discs, somatic embryos and culture medium from culture vessels with green and brown leaf discs, with and without embryos were separately weighed and placed into round bottomed flasks. About 100 ml of 96% ethanol was added into each flask. The extraction of the sugars was done for one hour under reflux by boiling the leaf discs, somatic embryos, or media with the ethanol, while continually cooling of the vapour to liquid and returning it back to the flasks for 25 minutes and leaving the contents to cool. The extract was filtered and evaporated to dry. The extract was reconstituted to 2 ml for the leaf and 5 ml for the embryo and media using mobile phase acetonitrile: distilled water at a ratio of 80:20. The extract sample was filtered through a 0.45 µm micro-filter (Chromafil) and analyzed with High Performance Liquid Chromatography (HPLC).

2.4. Extraction and evaluation of chlorogenic acid

Extraction of chlorogenic acid was done as described by Kathurima and Njoroge (2012). Leaf discs (1 cm^2), somatic embryos and culture medium from culture vessels with green and brown leaf discs, with and without embryos were weighed into 100 ml conical flasks and the weights recorded. About 50 ml of 96% ethanol (AR) and 10 ml acetone were added to each sample. The sample was first homogenized at 4°C and then transonicated using an ultrasonic bath for 10 minutes. Filtering was done using Whatman No. 42 (12.5 cm) paper. The filtrate was recovered in a 100 ml round bottomed flasks and evaporated at 40°C. The sample was reconstituted with 2 ml of 50% methanol, filtered through 0.45 µm micro-filters and analyzed with HPLC.

2.5. Extraction and evaluation of caffeine

Caffeine was extracted as described by Kathurima and Njoroge (2012). Leaf discs (1 cm^2), somatic embryos and culture medium from culture vessels with green and brown leaf discs, with and without embryos were weighed into 250 ml flat-bottomed flasks with round necks. Thereafter, 0.5 g magnesium oxide (Merck) and 200 ml distilled water were added into the flasks. Refluxing was done by boiling while continually cooling the vapour to liquid and returning it back to the flasks for 25 minutes and leaving the contents to cool. After cooling, filtration was done under vacuum on celite and the filtrate recovered in 250 ml volumetric flask. The volume

was topped up using distilled water to the mark and 20 ml of the filtrate drawn and put into 100 ml volumetric flask. The volume was adjusted to the mark using 20% acetonitrile. The eluate was filtered through a 0.45 µm micro-filters and analyzed with HPLC.

2.6. Data collection on the sugars

Glucose, fructose and sucrose were analyzed by injecting about 50 µl of the extract/sample into Knauer HPLC equipped with a Eurospher 100-5 NH_2 column and a reflective index detector. The mobile phase had 75% acetonitrile HPLC grade (SCHARLAU) and 25% distilled water at a flow rate of 1 ml/minute under ambient temperature. Glucose, fructose and sucrose were identified by comparing the retention time with that of sucrose standard (Fischer Scientific) and their concentration was calculated from peaks using calibration equations, where: Concentration of analyte (C_1) = Peak of analyte/slope of the standards' calibration curve. Content (mg/g) of the analyte = $[C_1 \times V \times 1000^{-3}]/W$, Where C_1= concentration (mg/L) of the analyte in the test solution, V = volume (mL) of the test solution, and W = weight (g) of the sample used for preparation of the test solution.

2.7. Data collection on chlorogenic acid and caffeine

Caffeine and chlorogenic acid were analyzed by injecting about 50 µl of eluate sample into Knauer HPLC equipped with a super Co Discovery C-18 column for caffeine and BDS HYPERSIL C-18 column for chlorogenic acid. The detector was diode array at 278 nm and 324 nm wavelengths for caffeine and chlorogenic acid, respectively. The mobile phase had 35% methanol HPLC grade (PANCREAC), 65% distilled water, and 0.1% acetic acid (PROLABO) at a flow rate of 1 ml/minute under ambient temperature. Caffeine and chlorogenic acid were identified by comparing the retention time of caffeine standard (99%) (Fischer Scientific) and chlorogenic acid standard (Acros Organics) with the sample peaks. The concentration was calculated from peaks using calibration equations, where: Concentration of analyte (C_1) = Peak of analyte/slope of the standards' calibration curve. Content (mg/g) of the analyte = $[C_1 \times V \times 1000^{-3}]/W$, Where C_1 = concentration (mg/L) of the analyte in the test solution, V = volume (mL), of the test solution, and W = weight (g) of the sample used to prepare the test solution.

2.8. Data analysis

The SAS 9.2 computer software was used to analyze data. Data values were subjected to analysis of variance using the General Linear Model for a completely randomized design. The linear model fitted to the CRD data was: $Yij = \mu + Ti + \varepsilon ij$, Where: µ is grand mean, Ti is i^{th} concentration of the endogenous factor; i = 1, 2, 3...10, 11, 12; j = 1, 2, 3; and εij is random error component, normally and independently distributed about zero mean with a common variance σ^2. Differences between treatment means were separated using LSD test at $P = 0.05$.

3. RESULTS

3.1. Evaluation of endogenous sugars

The glucose, fructose and sucrose contents obtained are shown in Table 1. There were significant differences in the glucose content of the samples on fresh weight basis (FW) and dry weight basis (DW) (Table 1). Brown leaf discs without embryos had endogenous glucose content of 38.95 mg/g FW and 273.97 mg/g DW. No glucose was detected in green leaf discs without embryos. Glucose was significantly ($P<0.05$) high in embryos developed from brown leaf discs at 2.76 mg/g FW and 19.54 mg/g DW. There were significant differences in glucose content in the culture media. Brown leaf discs without embryos had endogenous glucose content of 13.43 mg/g FW, whereas the green leaf discs without embryos had endogenous glucose content of 580 mg/g DW.

Significant ($P<0.05$) differences resulted in endogenous fructose content (Table 1). Brown leaf discs without embryos had fructose content of 45.43 mg/g FW and 277.55 mg/g DW. Embryos that had developed from brown leaf discs had the significantly ($P<0.05$) higher fructose content of 4.14 mg/g FW and 29.28 mg/g DW. The highest ($P<0.05$) fructose content of 17.19 mg/g FW and 620.77 mg/g DW resulted in the culture media where the leaf discs had turned brown and no embryos formed.

Significant differences resulted in the endogenous sucrose content in the treatments tested (Table 1). Freshly harvested leaves had the highest (P<0.05) sucrose content of 19.26 mg/g FW and 125.67 mg/g DW. Embryos developed from green leaf discs had the highest (P<0.05) sucrose content of 62.15 mg/g FW and 512.05 mg/g DW. Sucrose content of 16.22 mg/g FW and 648.82 mg/g DW was detected in control culture media.

Table 1: Glucose, fructose and sucrose contents in leaves, embryos and culture media

Treatment	FW (mg/g)	DW (mg/g)	FW (mg/g)	DW (mg/g)	FW (mg/g)	DW (mg/g)
	Glucose in leaves		Glucose in embryos		Glucose in media	
CM	0.0±0.0^{d}*	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{c}	3.5±0.4^{c}	138.2±15.6^{d}
CL	16.3±0.1^{b}	106.4±1.0^{b}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{e}	0.0±0.0^{e}
GE	0.2±0.0^{d}	1.2±0.3^{d}	2.1±0.0^{b}	16.9±0.3^{b}	2.4±1.7^{d}	178.2±13.2^{c}
GW	0.0±0.0^{d}	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{c}	7.9±0.1^{b}	580.4±7.1^{a}
BE	4.0±1.8^{c}	24.3±11.2^{c}	2.8±0.1^{a}	19.6±0.7^{a}	0.1±0.0^{e}	4.94±1.0^{e}
BW	39.0±1.0^{a}	274.0±6.2^{a}	0.0±0.0^{c}	0.0±0.0^{c}	13.4±0.5^{a}	485.2±19.0^{b}
CV (%)	15.0	14.8	9.0	8.6	10.7	8.8
LSD (0.05)	2.7	16.2	0.1	0.9	0.9	36.2
	Fructose in leaves		Fructose in embryos		Fructose in media	
CM	0.0±0.0^{d}*	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{c}	3.3±0.0^{c}	132.9±1.5^{c}
CL	3.4±0.1^{c}	22.3±0.3^{c}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{d}	0.0±0.0^{d}
GE	0.4±0.0^{d}	2.2±0.2^{d}	2.7±0.3^{b}	22.5±2.8^{b}	3.6±0.0^{c}	266.9±14.2^{b}
GW	0.0±0.0^{d}	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{c}	8.3±0.0^{b}	610.4±6.4^{a}
BE	10.3±2.0^{b}	62.7±12.3^{b}	4.1±0.3^{a}	29.3±2.1^{a}	0.6±0.0^{d}	21.1±7.5^{d}
BW	45.4±0.8^{a}	277.6±5.0^{a}	0.0±0.0^{c}	0.0±0.0^{c}	17.2±0.5^{a}	620.8±19.0^{a}
CV (%)	15.5	15.5	28.0	28.7	7.8	6.6
LSD (0.05)	2.7	16.7	0.6	4.4	0.8	32.35
	Sucrose in leaves		Sucrose in embryos		Sucrose in media	
CM	0.0±0.0^{d}	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{c}	16.2±0.2^{a}	648.8±6.5a
CL	19.2±0.3^{a}*	125.7±2.2^{a}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{b}	0.0±0.0^{b}
GE	11.3±0.2^{b}	69.3±1.3^{b}	62.2±2.0^{a}	512.1±16.5^{a}	0.0±0.0^{b}	0.0±0.0^{b}
GW	6.7±0.5^{c}	40.7±2.9^{c}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{b}	0.0±0.0^{b}
BE	0.0±0.0^{d}	0.0±0.0^{d}	37.2±0.8^{b}	263.2±6.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}
BW	0.0±0.0^{d}	0.0±0.0^{d}	0c	0^{c}	0.0±0.0^{b}	0.0±0.0^{b}
CV (%)	7.0	6.9	9.3	9.6	4.3	4.3
LSD (0.05)	0.8	4.8	2.7	22.1	0.2	8.2

*Values followed by the same letter within each column are not significantly different according to the LSD test at P = 0.05. **Key**: BW - Brown leaf discs without embryos, BE - Brown leaf discs with embryos, GE - Green leaf discs with embryos, GW - Green leaf discs without embryos, CL - Fresh leaves (Control), CM -Fresh media (Control).

3.2. Evaluation of chlorogenic acid

There were significant differences in chlorogenic acid content in the samples (Table 2). Fresh leaves had the highest (P<0.05) chlorogenic acid content of 6.51 mg/g FW and 42.56 mg/g DW. Chlorogenic acid content of 5.34 mg/g FW and 44 mg/g DW was significantly (P<0.05) high in embryos developed from green leaf discs. There were significant (P<0.05) differences in chlorogenic acid content in the culture media. Green leaf discs with embryos had the highest amounts of chlorogenic acid content of 0.004 mg/g FW and 0.34 mg/g DW.

Table 2: Chlorogenic acid content in leaves, embryos and media

Treatment	FW (mg/g)	DW (mg/g)	FW (mg/g)	DW (mg/g)	FW (mg/g)	DW (mg/g)
	CGA in leaves		CGA in embryos		CGA in media	
CM	0.0±0.0^{c}*	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{e}	0.0±0.0^{d}
CL	6.5±0.0^{a}	42.6±0.2^{a}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{e}	0.0±0.0^{d}
GE	1.3±0.2^{b}	8.2±1.4^{b}	5.3±0.1^{a}	44.0±0.7^{a}	0.005±0.0^{a}	0.3±0.0^{a}

GW	1.4±0.2^{b}	8.4±1.1^{b}	0.0±0.0^{c}	0.0±0.0^{c}	0.002±0.0^{c}	0.1±0.0^{b}
BE	0.1±0.0^{c}	0.4±0.0^{c}	3.3±0.3^{b}	23.4±2.1^{b}	0.004±0.0^{b}	0.2±0.0^{b}
BW	0.1±0.0^{c}	0.4±0.0^{c}	0.0±0.0^{c}	0c	0.001±0.0^{d}	0.04±0.0^{c}
CV (%)	13.5	12. 9	15.4	14.2	21.84	16.1
LSD (0.05)	0.4	2.3	0.4	2.8	0.001	0.03

*Values followed by the same letter within each column are not significantly different according to the LSD test at *P* = 0.05. **Key**: BW - Brown leaf discs without embryos, BE - Brown leaf discs with embryos, GE - Green leaf discs with embryos, GW - Green leaf discs without embryos, CL - Fresh leaves (Control), CM - Fresh media (Control)

3.3. Evaluation of caffeine

Significant (*P*<0.05) differences resulted in endogenous caffeine content (Table 3). Fresh leaves had the highest caffeine content of 1.14 mg/g FW and 4.0867 mg/g DW. Embryos that had developed from brown leaf discs had significantly (*P*<0.05) higher caffeine content of 0.58 mg/g FW and 4.09 mg/g DW. The highest (*P*<0.05) caffeine content of 0.69 mg/g FW and 51.21 mg/g DW resulted in the culture media with green leaf discs with embryos. Significant (*P*<0.05) differences resulted in endogenous caffeine content among the leaf discs tested (Table 3). Fresh leaves had the highest caffeine content of 1.14461 mg/g FW. Embryos that had developed from brown leaf discs had significantly (*P*<0.05) high caffeine content of 0.58 mg/g FW. The highest (*P*<0.05) caffeine content of 0.69 mg/g FW resulted in the culture media that had green leaf discs with embryos.

Table 3: Caffeine content in leaves, embryos and culture media

Treatment	FW (mg/g)	DW (mg/g)	FW (mg/g)	DW (Mg/g)	FW (mg/g)	DW (mg/g)
	Caffeine in leaves		Caffeine in embryos		Caffeine in media	
CM	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{b}	0.0±0.0^{b}	0.00±0.0^{c}	0.0±0.0^{b}
CL	1.1±0.1^{a}	7.5±0.4^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.00±0.0^{c}	0.0±0.0^{b}
GE	0.2±0.0^{c}	1.0±0.2^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.70±0.0^{a}	51.2±1.7^{a}
GW	0.2±0.0^{bc}	1.3±0.2^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.02±0.0^{bc}	1.2±0.0^{b}
BE	0.3±0.0^{b}	1.7±0.3^{b}	0.6±0.0^{a}	4.1±0.3a	0.04±0.0^{b}	1.3±0.0^{b}
BW	0.2±0.0^{bc}	1.3±0.2^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.01±0.0^{bc}	0.5±0.0^{b}
CV (%)	19.6	19.4	29.1	28.8	13.3	13.7
LSD ($_{0.05}$)	0.1	0.7	0.1	0. 4	0.03	2.2

*Values followed by the same letter within each column are not significantly different according to the LSD test at *P* = 0.05. **Key**: BW - Brown leaf discs without embryos, BE - Brown leaf discs with embryos, GE - Green leaf discs with embryos, GW - Green leaf discs without embryos, CL - Fresh leaves (Control), CM - Fresh media (Control)

4. DISCUSSION

4.1. Sugars

It is generally accepted that explant browning results in a decline in culture competence, with eventual loss of totipotency or even death of explants (Benson, 2000). Explant browning is usually caused by oxidase. Polyphenol oxidase (PPO) oxidizes phenols to produce brown-coloured ubiquinols, which accumulate in explants and are released into the medium (Liu *et al.,* 2015). However, explant browning can also be caused by environmental stress or other adverse conditions, including programmed cell death (PCD) and natural death (Liu *et al.*, 2015). In the present experiment, brown explants also generated somatic embryos. Although the mechanisms for the PCD induction of SE are not clear, two waves of programmed cell death occurred during SE of Norway spruce, which indicated that PCD played important roles in formation and development of somatic embryos (Filonova *et al.,* 2000). This might be the explanation for the development of somatic embryos in browned explants and non-browned explants.

Lipavska and Konrádova (2004) suggested the negative effect of accumulation of 6 carbon sugars such as fructose and glucose in embryo cells. In plants, endogenous fructose and glucose is derived from hydrolysis of sucrose, which occurs in reactions catalyzed by enzymes β-fructofuranosidase (EC 3.2.1.26) or sucrose synthase (EC 2.4.1.13) (Sturm and Tang, 1999). Generally, higher fructose content than glucose content was present in the leaf discs and this may be attributed to the fact that plant cells would uptake the glucose preferentially which competitively inhibits the uptake of fructose. The preference of glucose than fructose by cells was caused by its ability to be catabolized directly through glycolysis, whereas fructose has to be converted into glucose and sucrose prior to glycolysis (Dijkema *et al.,* 1988). Businge *et al.,* (2012) reported that in Norway spruce somatic embryogenesis, non-embryogenic cell line 06:22:02 had high fructose, indicating that relatively high levels of endogenous fructose prior to maturation was associated with the subsequent aberrant embryo development.

Higher sucrose content than glucose and fructose contents in mature coffee somatic embryos were observed in the present experiment. Similar observations have been reported in somatic embryogenesis of avocado (Sanchez-Romero *et al.,* 2002) where initially the hexose/sucrose levels were high in small embryos measuring 7–8 mm long, but with further development of up to 25 mm, a switch in the hexose/sucrose ratio took place due to a decrease in hexose levels and an increase in sucrose level. The trend continued in the following developmental stages reaching the lowest hexose/sucrose ratio in embryos measuring 38–40 mm long (Sanchez-Romero *et al.,* 2002). Pescador *et al*. (2008) also reported the amounts of glucose and fructose was conspicuously lower than that of sucrose during somatic embryogenesis of *Acca sellowiana*. Glucose and fructose contents showed similar trends, although glucose was always lower than fructose. Glucose is probably preferentially used over fructose to meet metabolic demands (Treat *et al.,* 1989). Low glucose content observed in embryos could be attributed to its utilization as a carbon source for synthesis of sucrose and starch that begin their accumulation at this stage, showing a similar trend to that observed in Norway spruce somatic embryos (Lipavská *et al.,* 2000). One of the known causes of low endogenous hexose content is α-amylase synthesis and starch catabolism (Yu *et al.,* 2000), which is a prerequisite for organ formation and somatic embryo differentiation. The reducing glucose and fructose cannot accumulate to high levels without harmful effects, but their great advantage is a more direct entry into metabolism (Lipavská and Konrádova, 2004).

Sucrose is frequently used as a carbon source in plant tissue culture media. Its hydrolysis into glucose and fructose has been demonstrated in a wide variety of plant cell and tissue cultures (George, 1993). In the present experiment, no sucrose content was detected in the media for all treatments, except the control. Akita and Takayama (1994) also reported that in potato microtuber jar fermentar, total sucrose degraded into glucose and fructose after 10 weeks of *in vitro* growth. Sucrose may have been split into glucose and fructose by exogenous enzymes before uptake (Nùrgaard, 1997). For optimal plantlet growth, sucrose sustainability is necessary and if it is rapidly hydrolyzed into glucose and fructose, it makes the long-term maintenance of desirable sucrose level difficult. Autoclaving contributes to sucrose hydrolysis and also a large amount of it breaks down during growth of plantlets (Kanabus *et al*., 1986).

4.2. Chlorogenic acid

The most abundant phenolic compounds in coffee are hydroxycinnamic acids which exist mainly in the esterified form. An example is chlorogenic acid (5-caffeoylquinic acid) (CGA) which is the most widespread and formed between caffeic and quinic acids (Molgaard and Ravn, 1988). When origin of the leaf node and growth stage were considered, the concentration, nature and localization of CGA varied throughout leaf development with juvenile leaves being the highly accumulating organs (Mondolot *et al.,* 2006). In the present study, fresh leaves had the highest CGA content, similar to analyses of *C. arabica* and *C. pseudozanguebariae* leaves (Aerts and Baumann, 1994; Bertrand *et al.,* 2003). Sartor and Mazzafera (2000) reported contents of 5.64 mg/g and 16.78 mg/g CGA in *C. arabica* and *C. dewervei,* respectively.

Generally, higher CGA resulted in non-embryogenic brown and green leaf discs than in embryogenic brown and green leaf discs, although not significantly different. Similar trend was observed in cocoa where high

hydroxycinnamic acid was associated with non-embryogenic response (Alemanno *et al.,* 2003). Embryogenic capacity therefore seemed to be associated with balanced concentration of phenolics. When explants are cut, the contents of the cytoplasm and vacuoles mix and come out from the explant and phenolic compounds can readily become oxidized by air. Oxidized phenolic compounds inhibit enzyme activity and result in darkening of the culture medium and subsequent lethal browning of explants or rooting inhibition (Arnaldos *et al*., 2001; Ozyigit *et al.,* 2007). Residual phenolics are also important in browning and thus the phenolics synthesized by the explants during any period of organogenesis play an important role in browning and rooting problems. The natural rate of formation of some phenolic compounds depends on the rate of growth of cultured tissues (Barz, 1977). In the present study, brown leaf discs had the lowest chlorogenic acid content, implying that explant browning ended in culture growth and competence decline, with eventual loss of totipotency.

Neuenschwander and Baumann (1992) observed embryogenic calli develop only after browning of the initial explant. In some instances, browning of explants did not affect somatic embryo formation. In the present study, somatic embryos also developed after browning of leaf explants. These results demonstrate that somatic embryogenesis induction is not incompatible with phenolic compound production during *in vitro* culture. Excessive accumulation of phenols that cause browning of the tissues is necessary for the somatic embryogenesis process in coffee (Quiroz-Figueroa *et al*., 2001). It is possible that these phenolic compounds act as signals to induce the differentiation process. Mucciarelli *et al.,* (2000) reported that phenolic compounds probably act by raising or lowering the level of indole acetic acid through enzymatic reactions.

Khosroushahi *et al*., (2011) reported a positive correlation between phenolic amounts and callus growth of *Taxus brevifolia* and this result indicated that phenolics lead to cell proliferation, and thus increase in callus growth instead of secondary metabolite production such as paclitaxel. In addition, an increase of phenolics might facilitate cell wall generation and subsequently enhance cell proliferation (Khosroushahi *et al*., 2011). Phenolic compounds produced as a response of explants to stress conditions can create a chemical environment appropriate for somatic embryo formation and development. Eldin and Ibrahim (2015) reported that phenols produced during somatic embryogenesis of date palm reacted with hydrogen peroxide produced during IAA degradation, thereby protecting the cell from its toxic effects.

An alternative explanation is that due to the chelating properties of these compounds, some inhibitors present in the embryogenic cultures get inactivated. The results of the present study agreed with a study by Liu *et al*. (2015) that the *Fraxinus mandshurica* (1%) polyphenol was not significantly higher than that in *Syringa reticulate var. mandshurica* (0.7%), but browning of *F. mandshurica* was significantly higher than that of *S. reticulata var. mandshurica*, indicating that the *F. mandshurica* explant browning was not greatly related to accumulation of polyphenols. Liu *et al*. (2015) predicted that explant browning might be a manifestation of necrosis caused by stress or differential response of explants to the stress, which resulted in a hypersensitivity response that induced PCD and browning of explants. Increase in phenolic content is normally associated with increase in enzymes that regulate synthesis of phenolic compounds, while the intensity of browning is related with the hyperactivity of oxidative enzymes (Cochrane, 1994). Laukkanen *et al*., (1999) cultured calli from shoot tips of mature Scot pine and examined phenol oxidase development (POD) and activity (PPO). Subsequently, brownish green calli were obtained in 14 days, greenish brown in 28 days and totally brown in 42 days. The PPO activity increased rapidly during culture, but slowed down after 28 days.

The mechanism of action of phenolics is difficult to explain and is somewhat ambiguous; however, it is one factor involved in determining the capacity of plant tissues to regenerate (Cvirková *et al*., 1999). An increase in phenolic compounds has been associated with a decrease in growth and a decline in protein synthesis. Beruto *et al*., (1996) observed a higher level of phenols in non-regenerating callus of *Ranunculus asiaticus* compared to regenerating callus. Hrubcová *et al*. (1994) and Cvirková *et al*. (1999) showed that non-embryogenic suspension of *Medicago sativa* produced more phenolic compounds than embryogenic suspension. It is known that high concentration of caffeine accompanies considerable accumulation of chlorogenic acid to form a complex that store the caffeine in the vacuole (Mösli and Baumann, 1996). It was also observed that during coffee germination, the concentration of chlorogenic acid decreases and caffeine is released, whereas during

leaf expansion accumulation of both compounds occurs (Aerts and Baumann 1994). Chlorogenic acid could be required to reinforce the cell wall, since together with its derivatives, it is necessary in the lignification process (Aerts and Baumann 1994). This effect is achieved probably through modification of cell plasticity and interference with the embryogenic response (Mounir and Ismail, 2004). Similarly, Eldin and Ibrahim (2015) reported that phenol concentration was at its lowest level in embryogenic callus and then increased during the subsequent developmental stages of date palm somatic embryogenesis. Eldin and Ibrahim (2015) reported gradual increase in the phenol concentrations from 67.8 to 94.7 mg/100 g fresh weight in pro-embryos and mature somatic embryos of date palm somatic embryos and postulated that the high concentration of phenols found in the mature somatic embryos may be used during their conversion to complete plantlets. Abdallah *et al.,* (2001) concluded that large amount of phenols observed in plantlets form the basic material used during subsequent secondary wall formation. These results imply that increase of phenolics facilitates cell wall generation and subsequently enhances cell proliferation (Khosroshahi *et al.,* 2011). Many reports have recommended that the role of phenolic compounds in *in vitro* cultures should be analyzed more carefully since in some systems, phenols promote *in vitro* morphogenic processes. It is not yet clear how phenolic compounds affect somatic embryogenesis induction and development. A possible interference of phenolic compounds with auxin metabolism and, as a consequence, with the levels of this plant growth regulator has been suggested (Gross *et al*., 1977; Pressey, 1990). The study of Ozyigit *et al.,* (2007) indicated that the role of the phenolic compounds depends on its chemical structure, plant species, the biological process studied (organogenesis or somatic embryogenesis), and its developmental stage.

4.3. Caffeine

In coffee plants, caffeine is actively biosynthesized during leaflet emergence and then decreases when leaves reach their optimal photosynthetic capacity (Frischknecht *et al.,* 1986). The 1.4 mg/g FW and 7.48 mg/g DW caffeine obtained in the explant in the present study was in agreement with previous reports. Ashihara *et al*., (1996) reported that caffeine biosynthesis from adenine and guanine was only found in young leaves, but conversion of theobromine and caffeine was found in mature and aged coffee leaves. Mazzafera *et al*., (1994) studied metabolism of caffeine in two cultivars of *C. arabica*, 'Mundo Novo' (commercial cultivar) and 'Laurina' (mutant) and found 8-10 mg/g and 7 mg/g caffeine, respectively. Ashihara *et al.,* (1996) found 2.1 mg/g FW caffeine in mature leaves, whereas Sartor and Mazzafera (2000) found 9.29 mg/g DW caffeine in *C. arabica*. Mazzafera and Magalhães (1991) found 21.9 g/kg caffeine in young leaves, which was higher than values reported in literature for *C. arabica*. However, the result was expected since it has been demonstrated that younger leaves have higher alkaloid content than older leaves (Frischknecht *et al.,* 1986). High endogenous caffeine found in *C. arabica* leaves may be due to the rate limiting step in purine alkaloid catabolism during the conversion of caffeine to theophylline (Indu, 2004). In a study of caffeine and its metabolites during somatic embryogenesis of *C. canephora* variety CxR, Indu (2004) reported that cotyledonary embryo plantlets formed highest amounts of theobromine and caffeine as compared to callus, globular and torpedo embryos, indicating that caffeine accumulates during embryogenesis. In the present study, high caffeine content in the embryos formed from brown leaf discs was attributed to accumulation during the embryogenesis process.

The ability to produce coffee cell cultures from callus cultures which maintain the ability to produce caffeine and theobromine, and release these purine alkaloids into the medium, has existed for over 40 years most likely due to diffusion (Keller *et al*., 1972; Waller *et al*., 1983). Waller *et al*., (1983) reported that caffeine formation in *C. arabica in vitro* paralleled tissue growth; however, as the callus grew older, less caffeine remained in the tissue and more was found in the medium. In the present study, the green leaf discs had the least caffeine content and in their embryos and the highest in the medium, agreeing with a previous report by Waller *et al.,* (1983), who found up to 49.9+4.8 µmoles/mg DW caffeine in the culture media.

Nic-Can *et al*., (2015) studied *C. arabica* explant secreted compounds and found 11.042 µmole/flask and 10.982 µmole/flask caffeine in low molecular mass of conditioned medium (LmmCM). The caffeine in media allowed somatic embryos to develop in *C. canephora*. Similar results have been observed during development of zygotic embryos in coffee (Friedman and Waller, 1983), indicating that the embryos had the ability to avoid

caffeine autotoxicity through space and time separation. This may be the reason why somatic embryos developed in media with high caffeine content. The mechanism of phenolic action in promoting somatic embryo formation is unclear. It is postulated that there is a factor involved in determining the capacity of plant tissues to regenerate (Cvirková *et al.,* 1999). It is known that high caffeine content is accompanied by accumulation of chlorogenic acid as a pathway to a complex to store the caffeine in the vacuole (Mösli and Baumann, 1996).

5. CONCLUSION AND RECOMMENDATIONS

Several biochemicals were identified during development of coffee somatic embryos. Generally, green embryogenic cultures contained higher quantities of the identified biochemicals. Glucose and fructose were highest in brown non-embryogenic cultures. Sucrose was highest in green embryogenic cultures. Since glucose is preferentially used to meet metabolic demand, its decrease was ascribed to its role in the process of embryo growth and development. High endogenous sucrose in the embryo is an indication that embryo maturation is connected with storage matter accumulation. Green embryogenic cultures had the highest chlorogenic acid content, whereas caffeine was highest in brown embryogenic cultures, indicating that high CGA and caffeine in culture media allows development of somatic embryos through space and time separation-avoidance of CGA and caffeine auto-toxicity. Embryogenic capacity seemed to be associated with a balance of phenolics. Other biochemical compounds such as organic acids should be identified and their role in coffee somatic embryogenesis determined.

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Dr. Rose N. Mayoli was born in 1978 in Machakos-Kenya, and received both her B.Sc. in Horticulture in 2003 and M.Sc. in 2008 from Egerton University, Kenya and Ph.D. in Plant Biotechnology in 2018 from Chuka University, Kenya. Dr. Mayoli's work experience has included: July 2003-August 2003 Quality Controller, Equator Flowers Kenya Limited, August 2003-August 2004 Assistant Production Manager, Carzan Flowers Kenya Limited; January 2009-July 2009 Senior Quality Control Supervisor, Primarosa Flowers Kenya Limited; September 2009-date, Research Officer and Head of Plant Physiology Unit, Coffee Research Institute, Kenya Agricultural and Livestock Research Organization. Dr. Mayoli's research interests concern horticulture, floriculture, beverage crops, plant growth regulators, plant tissue culture, plant biotechnology, molecular biology, and plant physiology.

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