ENDOGENOUS CHLOROGENIC ACID AND CAFFEINE ASSOCIATED WITH IN VITRO SOMATIC EMBRYOGENESIS OF COFFEE (*Coffea arabica* L.)

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ABSTRACT
Coffee plays a central role in Kenyan economy through foreign exchange and income earnings. However, its production has been constrained by factors like high production cost, inappropriate technology and inadequate seedlings. Coffee somatic embryogenesis is another vegetative propagation method, besides cuttings and grafting. Its main use is for F1 hybrid propagation to avoid manual hybrid seed production. Developmental processes and factors related to coffee somatic embryogenesis are not well established, resulting in poor induction or few embryos and low subsequent seedling regeneration. This study identified and quantified endogenous chlorogenic acid (CGA) and caffeine phenols and alkaloids associated with *Coffea arabica* 'Ruiru 11' somatic embryogenesis. Third leaf pairs of greenhouse-grown mother plants were cultured in half-strength Murashige and Skoog, 1962 media. Both green and brown leaf discs with and without embryos constituted treatments, and embryos with fresh culture media and leaf explants constituted controls. A completely random design replicated thrice and repeated once in 2014 was used. Chlorogenic acid and caffeine were extracted and analyzed using a Knuer HPLC system and identified by comparing their retention time with that of standards. Fresh leaves had high 6.51 mg/g FW CGA and 1.14 mg/g FW caffeine. Chlorogenic acid amounting 5.34 mg/g FW was significantly (P<0.05) high in embryos on green leaf discs. Embryos on brown leaf discs had significantly (P<0.05) high 0.5778 mg/g FW caffeine. Green leaf discs with embryos had 0.004 mg/g FW highest endogenous CGA. Culture media with embryos on green leaf discs had 0.6935 mg/g FW highest caffeine. Generally, higher CGA was in non-embryogenic brown and green leaf discs, compared to embryogenic brown and green leaf discs. Embryogenic capacity seemed to be associated with a balance of phenolics. High caffeine in embryos formed on brown leaf discs was a result of accumulation during embryogenesis. The inference that high caffeine in culture media allowed development of somatic embryos when embryos avoided caffeine auto-toxicity through space and time separation will be presented and discussed.

Keywords: *Alkaloids, Coffee seedlings, Leaf explants, Phenolics, Plant propagation*

INTRODUCTION

Coffee is the second most valuable traded commodity in the world, with sales estimated to be US$ 90 billion annually. In addition, production of coffee forms the economic backbone of many countries worldwide (FAO, 2003). Presently in Kenya, the coffee industry contributes about KES 10 billion per year to the national economy and is the fourth largest foreign exchange earner after tea, tourism and horticulture (Karanja and Nyoro, 2002). Plant tissue culture plays an important role in agricultural biotechnology and plant propagation. It allows *in vitro* regeneration and multiplication of plants under aseptic conditions through a process known as micropropagation. *In vitro* regeneration of coffee is mainly through somatic embryogenesis because it presents the highest rate of multiplication (Fernandez-Da Silva et al., 2005). Coffee is a relevant model species for fundamental research in molecular biology, biochemical and biotechnological research (Santos-Briones and Hernández-Sotomayor, 2006) particularly for investigation of release of inhibitors and stimulation of compounds *in vitro.*

Phenolic compounds are predominantly present as a family of esters formed between quinic acid and hydroxycinnamic acids, collectively known as chlorogenic acids (CGA) (Clifford, 1985). The up to 14% CGAs in green coffee seeds have a marked influence in determining coffee quality and contribute immensely in the formation of flavour (Farah et al., 2006). The relatively high levels of chlorogenic acids and other related phenols in coffee seeds give emphasis to their physiological importance for the coffee plant. Phenolic compounds are often associated with somatic embryo formation. It was observed that embryogenic calli developed only after browning of the initial coffee explant through a necrosis-like process which does not impair somatic embryo formation (Neuenschwander and Baumann, 1992).

Caffeine is the most abundant and important purine alkaloid derived from several important crops, such as coffee, tea, cocoa and guarana (Pompelli et al, 2013). The importance of caffeine in the coffee beverage seems to be solely as a stimulant. Caffeine is known to have a toxic effect on insects and fungi (Indu, 2004). Frischknecht et al (1986) postulated that this may be defense strategy since as the leaflet emerges from the stipules that confer mechanical protection, caffeine increases as a chemical defense. Caffeine in coffee plants is actively biosynthesized during leaflet emergence and then decreases when leaves reach their optimal photosynthetic capacity (Zheng and Ashihara, 2004). Stimulation of caffeine production in cell cultures of *C. arabica* is achieved by application of stress (Frischknecht and Baumann (1985).

Despite great progress in development of somatic embryogenesis, recalcitrant crops such as coffee have difficulty regenerating somatic embryos. Factors particularly those involved in regulation of the induction and development of coffee somatic embryos are not clear. Therefore, it is imperative to determine the endogenous inhibitors and stimulators present during somatic embryogenesis of coffee so as to counteract and augment them, respectively.

MATERIALS AND METHODS

This research was conducted in laboratories and greenhouses of the Coffee Research Foundation at Ruiru in Kenya using *Coffea arabica* cultivar Ruiru 11. Third leaf pair explants were excised from 6-month-old greenhouse-grown mother plants between March and April, 2014. 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 in a laminar flow cabinet. The explants were dipped quickly for approximately 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 thoroughly 4 times in sterilized distilled water. The culture media contained half-strength Murashige and Skoog (MS 1962) inorganic basal salts, supplemented with vitamins namely 0.2g/L thiamine, 0.1g/L nicotinic acid and 0.1g/L pyridoxine 30 g/L sucrose, 100 mg/L myo-inositol, 100 mg/L cysteine, and 1 ml/L Thidiazuron. The pH of the media 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 at 100 kPa. Culture media measuring 25 ml was poured into Magenta vessels (Sigma Chemical Co.) and 5 leaf discs measuring approximately 1 cm^2 cultured in each vessel that was maintained in the dark at 25^{o}C ± 2 and 70% humidity.

After 6-8 months of culture, treatments were applied as follows: brown leaf discs with and without embryos, green leaf discs with and without embryos, fresh culture media and leaf explants excised from greenhouse-grown mother plants served as controls (Figure 1). The treatments were used to identify and quantify endogenous CGA and caffeine in the leaf discs, developed embryos and culture media, as appropriate. A completely randomized design, replicated three times and repeated once was used.

Sample Preparation, Extraction and Analysis of Chlorogenic Acid

Extraction of chlorogenic acid was done as described by Kathurima and Njoroge (2012). Thus, leaf disc, embryo and culture medium, were weighed into a 100 ml conical flask and their weights noted. About 50 ml of 96% ethanol (AR) and 10 ml acetone were added to the samples, which were first homogenized at approximately 4^{o}C, and then transonicated using an ultrasonic bath for 10 minutes. The homogenate was filtered using Whatman 42 filter paper. The filtrate was recovered in a 100 ml round bottomed flask and evaporated under 40^{o}C. The sample was reconstituted using 2 ml of 50% methanol. The sample was filtered through a 0.45 µm Chromafil micro-filter. About 50 microlitre of the sample extract was injected into Knuer HPLC.

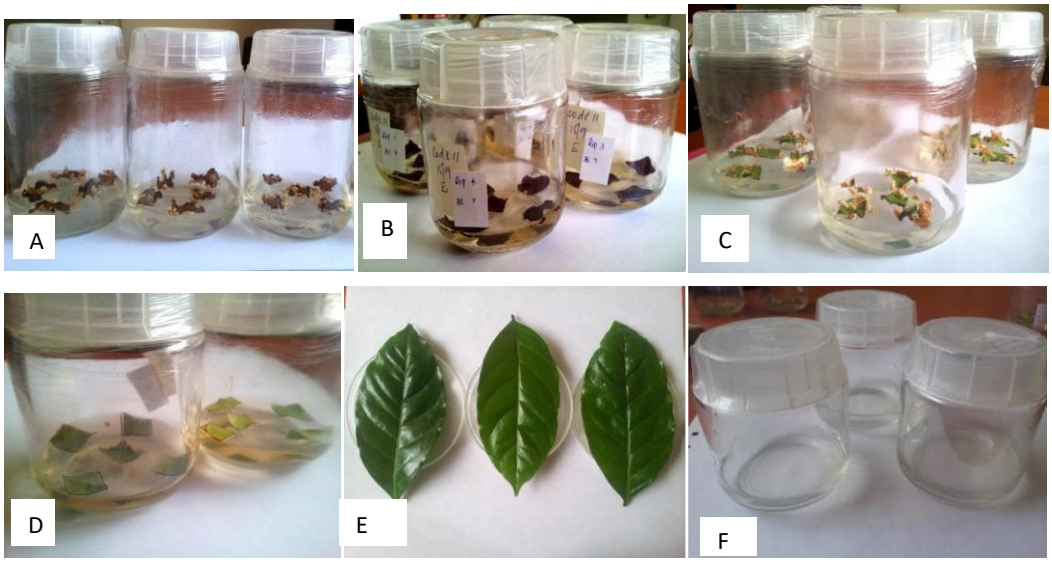

Figure 1. Treatments used for biochemical analysis. **A**: Brown leaf discs with embryo. **B**: Brown leaf discs without embryos. **C**: Green leaf discs with embryos. **D**: Green leaf discs without embryos. **E**: Fresh leaves (control). **F**: Fresh leaves (control).

Chlorogenic acid was analyzed using a Knauer HPLC system equipped with a BDS HYPERSIL C-18 for chlorogenic acid. The detector was diode array at wavelengths 324 nm for chlorogenic acid. The mobile phase was methanol HPLC grade (PANCREAC) 35%, distilled water 65%, acetic acid (PROLABO) 0.1%, at a flow rate of 1 ml/minute under ambient temperature. Chlorogenic acid was identified by comparing the retention time of 99% Chlorogenic acid standard (Acros Organics) and concentration calculated from peak areas using calibration equations where: Concentration of analyte (C_1) = Peak area of analyte/slope of the standards' calibration curve. Content (mg/g) of the analyte = [C_1 x V x 1000^{-3}]/W, Where C_1= the concentration (mg/L) of the analyte in the test solution, V= the volume (mL), of the test solution, and W= the weight (g) of the sample used for the preparation of the test solution.

Sample Preparation, Extraction and Analysis of Caffeine

Caffeine was extracted as described by Kathurima and Njoroge (2012). The culture medium, each leaf disc and embryo were weighed into 250 ml flat-bottomed flasks with a round neck. Thereafter, 0.5 g magnesium oxide (Merck) and 200 ml distilled water were added. Refluxing was done for 25 minutes and the contents left 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 with distilled water to the mark, and 20 ml of the filtrate was drawn and put into a 100 ml volumetric flask. The volume was adjusted to the

mark with 20% acetronitrile. The eluate was filtered through a 0.45 µm Chromafil micro-filter and about 50 microlitre of the sample extract was injected into Knauer HPLC.

Caffeine was analysed using a Knauer HPLC system equipped with a super Co Discovery C-18 column for caffeine. The detector was diode array at wavelengths 278 nm for caffeine. The mobile phase was methanol HPLC grade (PANCREAC) 35%, distilled water 65%, acetic acid (PROLABO) 0.1%, at a flow rate of 1 ml/minute under ambient temperature. Caffeine was identified by comparing the retention time of 99% caffeine standard (Fischer Scientific) and concentration calculated from peak areas using calibration equations where: Concentration of analyte (C_1) = Peak area of analyte/slope of the standards' calibration curve. Content (mg/g) of the analyte = [C_1 x V x 1000^{-3}] W, Where C1= the concentration (mg/L) of the analyte in the test solution, V= the volume (mL) of the test solution, W= the weight (g) of the sample used for the preparation of the test solution.

Data Analysis

The SAS 9.2 computer software was used to analyse data. Data were subjected to analysis of variance (ANOVA) and significantly different means were separated using the least significance difference (LSD) test at P=0.05. Chlorogenic acid and caffeine content in the leaf discs, embryos and culture media were correlated using Pearson correlation coefficients.

RESULTS

There were significant differences in the endogenous CGA content in all the leaf discs (Table 1). Fresh leaves had the highest (P<0.05) CGA content of 6.5106 mg/g FW. Chlorogenic acid content of 5.34 mg/g FW was significantly (P<0.05) high in embryos developed on green leaf discs. There were significant (P<0.05) differences in CGA content in the culture media. Green leaf discs with embryos had the highest endogenous CGA content of 0.004 mg/g FW. Significant (P<0.05) differences resulted in endogenous caffeine content among the sample leaf discs tested (Table 1). 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) higher caffeine content of 0.5778 mg/g FW. The highest (P<0.05) caffeine content of 0.6935 mg/g FW resulted in the culture media that had green leaf discs with embryos. Chlorogenic acid content in the leaf discs showed negative non-significant (P>0.05) correlations with CGA content in embryos and the culture media (Table 2). Significant positive correlation (P<0.0001) resulted for CGA content in the embryos and media. The caffeine content in the leaf discs showed negative correlations with caffeine content in embryos and media, although these correlations were not significant(P>0.05) (Table 2). Caffeine content in embryos was negatively correlated with caffeine content in the media and the correlation was also not significant (P>0.05). Figures 1 and 2 showed that the number of somatic embryos had a positive, but insignificant correlation with either chlorogenic acid or caffeine content (R^2 = 28%).

Table 1: Chlorogenic acid and caffeine contents in leaf, embryo and culture media

	Chlorogenic acid (mg/g FW) specimen			Total no. of embryos	Caffeine (mg/g FW) specimen			Total no. of embryos
Treatment	Leaf	Embryo	Media		Leaf	Embryo	Media	
CM	na	na	0.0000000e	na	na	na	0.00000c	na
CL	6.5106a*	na	na	na	1.14461a	na	na	na
GW	1.3777b	na	0.0021277c	na	0.2734b	na	0.01643bc	na
GE	1.3334b	5.3411a	0.0046457a	147	0.21545bc	0.03717b	0.69350a	191
BW	0.0699c	na	0.0013140d	na	0.21202bc	na	0.01488bc	na
BE	0.0589c	3.3057b	0.0038589b	144	0.15639c	0.57780a	0.03503b	124
CV (%)	13.48	15.37892	21.84		19.61	29.09	13.25	na

LSD $_{0.05}$	0.3737	0.3943	0.0008	0.1164	0.0530	0.02980	na

* Values followed by the same letter within a column are not significantly different at *P = 0.05*. BW = Brown leaf discs without embryos, BE = Brown leaf discs with embryos, GE = Green leaf discs with embryos, GW = Green leaf discs without embryo, CL = Fresh leaves (Control), CM = Fresh media (Control), na = not applicable

Table 2: Correlation coefficients between chlorogenic acid and caffeine contents in leaf, embryo and media

Variables for chlorogenic acid content (mg/g FW)			Variables for caffeine content (mg/g FW)		
Leaf	Leaf		Leaf	Leaf	
Embryo *P-value*	-0.21045 0.4019	Embryo	Embryo *P-value*	-0.08583 0.7349	Embryo
Media *P-value*	-0.36459 0.1369	0.86568 <0.0001	Media *P-value*	-0.22375 0.3721	-0.09792 0.6991

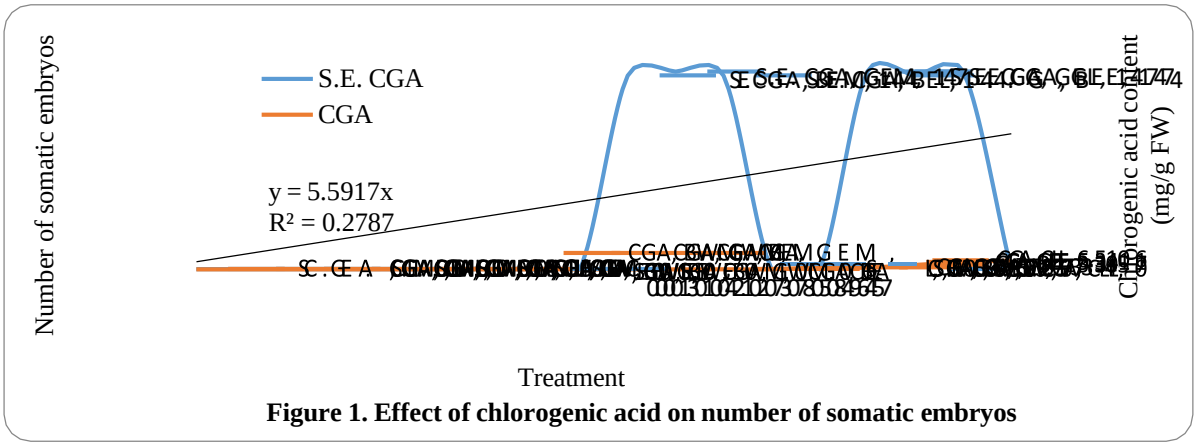

Figure 1. Effect of chlorogenic acid on number of somatic embryos

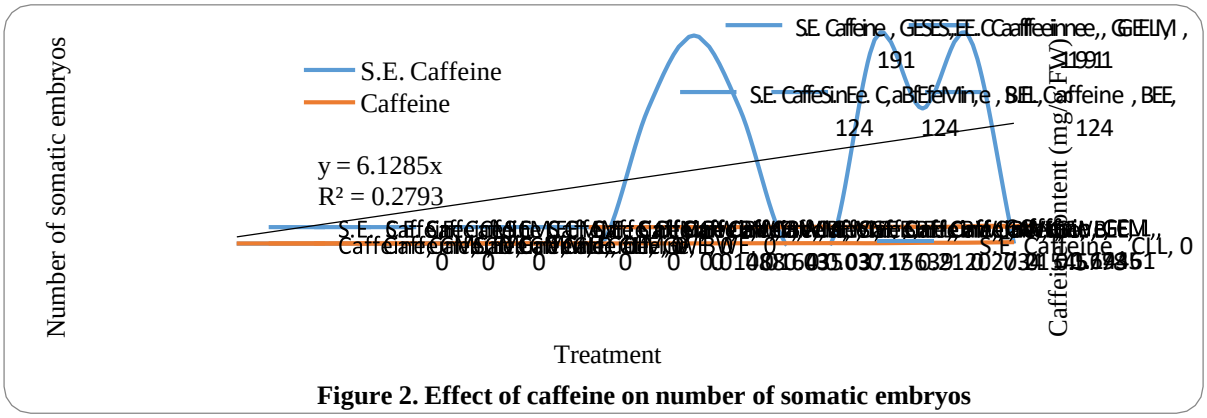

Figure 2. Effect of caffeine on number of somatic embryos

DISCUSSION

When the origin of the leaf node and the growth stage were taken into account, the concentration, nature and localization of chlorogenic acid varied during leaf development, with juvenile leaves clearly showing to be highly accumulating organs (Mondolot et al., 2006). In this study, fresh leaves had the highest CGA content. This was in agreement with previous analyses of *C. arabica* and *C. pseudozanguebariae* leaves (Aerts and Baumann, 1994). Sartor and Mazzafera (2000) reported contents of 5.64 mg/g and 16.78 mg/g chlorogenic acid, in *C. arabica* and *C. dewervei*, respectively. Baumann et al. (1991) reported a concentration of 4.55 mg/g of chlorogenic acid in leaves of *C. arabica*. Generally, higher concentration of chlorogenic acid resulted in non-embryogenic brown and green leaf discs as compared to embryogenic brown and green leaf discs. Similar trend was observed in cocoa where high concentration of phenolic compound hydroxycinnamic acid was associated with non-

embryogenic response (Alemanno et al., 2003). Embryogenic capacity seemed to be associated with a balance of phenolics.

Neuenschwander and Baumann (1992) found that embryogenic calli developed only after browning of the initial explant. In some instances, the browning of explants did not affect somatic embryo formation in their experiment. In this 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. Khosroushahi et al. (2011) observed a positive correlation between phenolics and callus growth of *Taxus brevifolia* and this indicated that phenolics lead to cell proliferation and increase in callus growth instead of secondary metabolite such as paclitaxel production. The results of this study are in agreement with a study by Liu et al. (2015), who reported that the polyphenol content in *Fraxinus mandshurica* (1%) was not significantly higher than that in *Syringa reticulate var. mandshurica* (0.7%), but the browning percentage of *F. mandshurica* was significantly higher than that of *S. reticulata var. mandshurica*, which indicated that explant browning of *F. mandshurica* might not be greatly related to accumulation of polyphenols.

In coffee plants, caffeine is actively biosynthesized during leaflet emergence and then decreases when leaves reach their optimal photosynthetic capacity (Frischknecht et al., 1986). Caffeine content of 1.4 mg/g FW in the explant reported in the present study was in agreement with previous reports. Ashihara et al. (1996), reported caffeine content of 2.1 mg/g FW in mature leaves. Sartor and Mazzafera (2000) obtained 9.29 mg/g DW caffeine content in *C. arabica*. In addition, Mazzafera (2004) indicated that the control of caffeine levels in plants is a function of the balance between rates of synthesis and degradation although this balance seems to vary depending on the plant species and the tissue developmental stage. Indu (2004) reported that plantlets (i.e. cotyledonary embryos) 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 in embryos formed from the brown leaf discs can be as a result of the accumulation of caffeine 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 since time immemorial (Keller et al., 1972; Waller et al., 1983). Caffeine formation in *Coffea 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 (Waller et al., 1983)). In this study, the green leaf discs had the least caffeine content in the leaf discs and embryos and had the highest amount of caffeine in the medium. These results were in agreement with previous reports by Waller et al. (1983), who reported up to 49.9 + 4.8 µmoles/mg DW caffeine released into the culture media. Nic-Can et al. (2015), in their study to identify secreted compounds by *Coffea arabica* explants, reported caffeine content of 11.042 µmole/flask and 10.982 µmole/flask in low molecular mass of conditioned medium. These authors reported that caffeine in media allows somatic embryos to develop in *C. canephora*. Similar results have also been observed during the development of zygotic embryos in coffee (Friedman and Waller, 1983). These authors inferred that the embryos have the ability to avoid caffeine autotoxicity, suggesting that the accumulation of caffeine is separated by space and time as a way of avoiding autotoxicity. This may be the reason why somatic embryos were formed in media that had high caffeine content. The mechanism of phenolics action in promoting somatic embryo formation is unclear. However, it is supposed 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 concentrations are accompanied by accumulation of chlorogenic acid as a pathway to a complex to store the caffeine in the vacuole (Mösli and Baumann 1996).

CONCLUSION AND RECOMMENDATIONS

The results indicated 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. Therefore, augmentation of media with balanced phenolics is recommended to enhance coffee somatic embryo regeneration.

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