Relationships of Selected Endogenous Factors Associated with Direct Somatic Embryogenesis of Coffee (*Coffea arabica* L.)

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ABSTRACT

Coffee is one of the most important cash crops produced in the world with great economic returns to growers and national gross domestic product. Somatic embryogenesis is a morphogenetic processes leading to plantlet regeneration and these processes are coupled with changes in the levels of primary metabolites. The present experiment established relationships of endogenous substances with direct somatic embryogenesis of coffee 'Ruiru 11'. Laboratory experiments were set up at Coffee Research Institute, Ruiru-Kenya between 2014 and 2017. The set up was in a completely randomised design, replicated three times and repeated once. 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 substances using HPLC and GCMS. Sucrose, phenolics, alkaloids, amino acids, fatty acids and their derivatives correlated positively, whereas fructose and glucose correlated negatively with the other biochemical components. Endogenous sucrose, chlorogenic acid, caffeine amino acid, fatty acids and their derivatives are potential biomarkers for coffee somatic embryogenesis, whereas endogenous fructose and glucose are inhibitors of the same. Further studies regarding the status of the biochemical components, especially in particular stages of embryo development should be conducted to establish treatments that can improve coffee direct somatic embryo development.

Keywords: Biochemical components, Biomarkers, Coffee somatic embryogenesis, Correlations, Principal components

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1. INTRODUCTION

Embryogenesis is a spatio-temporally organized developmental process that is central to the life cycles of diverse plant species (Navarro *et al.*, 2017). Somatic embryogenesis (SE) is influenced by various endogenous factors that affect biochemical pathways leading to morphogenesis (Obembe *et al.,* 2010). Growth of embryogenic cultures is usually associated with changes in synthesis and mobilization of proteins, carbohydrates and lipids. The levels of these substances vary at different stages of cell culture (Lulsdorf *et al.,* 1992), where they act in signal transduction pathway or supply substrates and energy required for cell growth and morphogenesis (Nomura and Komamine, 1995). The levels of sugars govern cellular differentiation during *in vitro* culture. Phenolic compounds are either negatively related with plant *in vitro* proliferation (Lorenzo and Angeles, 2001) or positively related to somatic embryogenesis induction (Neuenschwander and Baumann, 1992). In addition, somatic embryogenesis could be a response to stress (Dudits *et al.,* 1995) and is associated with adaptation mechanisms, such as cell reprogramming and modification in physiological and metabolic pathways (Pasternak *et al.,* 2002). Different molecules play regulatory roles in stress signalling. This paper presents relationships of glucose, fructose, sucrose, chlorogenic acid, caffeine, phyto-components and amino acids with direct somatic embryogenesis of coffee.

2. MATERIALS AND METHODS

2.1. Experimentation preliminaries

This 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. The plants were moved from the field to the laboratory and then to the greenhouse.

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

The mother plants for this experiment were obtained from germinated Ruiru 11 seeds. The resulting seedlings were transplanted into polybags filled with top soil: sand: manure (3:2:1 v/v) 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 was done in a laminar flow cabinet. The leaves were dipped for 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 4 times in sterilized distilled water. The culture medium contained half-strength Murashige and Skoog (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 for about 8 months in darkness, 25 ± 2°C and 70% relative humidity. In total, 627 culture vessels were prepared, but 183 (29%) were discarded due to fungal contamination.

2.3. Treatments

Treatments for this experiment were selected from the remaining 444 clean cultures. Culture vessels with green and brown leaf discs with and without embryos as shown in Plate 1 were used to characterise caffeine, chlorogenic acid, amino acids, sucrose, glucose, fructose and phyto-components 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.

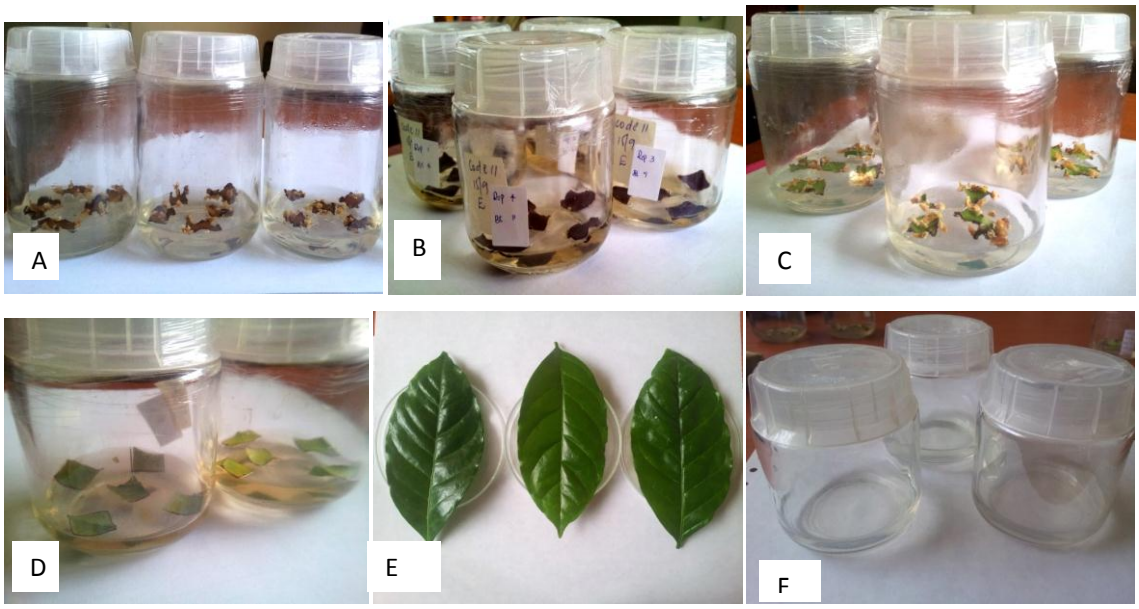

Plate 1: Treatments lay out A: Brown leaf discs with embryos. B: brown leaf discs without embryos. C: green leaf discs with embryos. D: Green leaf discs without embryos, E: Fresh leaves (control). F: Fresh media (control)

2.4. 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 the vapour to liquid and returning it back to the flasks for 25 minutes and the contents left 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).

2.5. 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 samples were 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 samples were reconstituted with 2 ml of 50% methanol, filtered through 0.45 µm micro-filters and analyzed with HPLC.

2.6. 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 the contents left to cool. After cooling, filtration was done under vacuum on celite and the filtrate recovered in 250 ml volumetric flasks. The volume was topped up with distilled water to the marks and 20 ml of the filtrates were drawn and put into 100 ml volumetric flasks. The volume was adjusted to the marks with the 20% acetonitrile. The eluates were filtered through a 0.45 µm micro-filters and analyzed with HPLC.

2.7. Extraction and evaluation of phyto-components

Phyto-components were extracted as described by Shettima *et al.* (2013) with modifications. Fifty (50) ml methanol was added to the culture vessels containing green and brown leaf discs, with and without embryos and immediately deep-frozen overnight at -20°C. The polarity of the samples was increased before partitioning against ethyl acetate, and about 1 ml of distilled water which had been adjusted to pH 8 was added. The pH of the samples was further adjusted higher than 9 with 1 M KOH to keep the phyto-components ionized and then partitioned against 100% ethyl acetate. The aqueous and organic phases were separated using a funnel and the lower aqueous phase was transferred to a new 10 ml tube. The pH of the solution was lowered to below 3 using concentrated acetic acid to conserve the phyto-components in protonated form. The acidic sample was partitioned against 100% ethyl acetate and dried by passing it through anhydrous sodium sulphate. The sample were spiked with 50 ppm internal standard (Benzophenone) and analyzed with Gas Chromatograph-Mass Spectrophotometer.

2.8. Extraction and evaluation of amino acids

Amino acids were extracted as described by AOAC (1997). Leaf disc, embryo and media from culture vessels with green and brown leaf discs with and without embryos were weighed into screw-cap Pyrex borosilicate tubes. About 2 ml of Performic acid was added and the samples were incubated at 0°C for 16 hours. About 0.84 g sodium metabisulfite and 3 mL 6N hydrochloric acid were added to the mixture. The tubes were thoroughly flushed with nitrogen, quickly capped, and placed in an oven at 110°C for 24 hours. Approximately 250 µl of the hydrolysed extracts was evaporated under a stream of nitrogen. The residue extract was re-dissolved in 1 ml 0.1 M borate buffer and filtered through 0.2 µM syringe filters and analysed with HPLC.

2.9. Data collection on the sugars

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 was 75% acetonitrile HPLC grade (SCHARLAU) and 25% distilled water at a flow rate 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 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= 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.

2.10. Data collection on caffeine and chlorogenic acid

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 wavelengths 278 nm and 324 nm for caffeine and chlorogenic acid, respectively. 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 and chlorogenic acid were identified by comparing the retention time of caffeine standard (99%) (Fischer Scientific) and chlorogenic acid standard (Acros Organics) and the sample peaks. The concentration was 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 to prepare the test solution.

2.11. Data collection on phyto-components

Identification relied on matching the mass spectrometric fragmentation pattern corresponding to the various peaks in the samples total ion chromatogram with those present in the National Institute of Science and Technology mass spectral database. Integration was done automatically for the individual peaks. The sample was spiked with 50 ppm internal standard (Benzophenone) and injected into GC-MS (Model QP2010 SE, Shimadzu) and fitted with DB 5 column. Detector used was MS and Helium was used as the carrier gas at a flow rate of 1 ml/min. The phyto-components were estimated using the following equation:

Concentration of analyte (C_1) = Peak area of analyte/peak area of internal standard x concentration of internal standard.

Content (µg/g) of the analyte = [C_1 x V]/W, Where C_1= the concentration (ppm) 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.

2.12. Data collection on amino acids

Amino acids were analyzed by injecting about 50 µl of the eluate/sample into Knauer HPLC system equipped with a super Co Discovery C-18 column at a flow rate of 1 ml/minute under ambient temperature. A Knauer fluorescent detector was used at excitation wavelength of 350 nm and emission wavelength of 450 nm. Two mobile phases were used: Mobile Phase A: was 0.01M Na_2HPO_4 at pH 8.2 which was prepared by dissolving 1.42 g Disodium phosphate heptahydrate (Na_2HPO_4) and 3.81 g Sodium borate ($Na_2B_4O_7.10\ H_2O$) in 950 ml milliQ reagent grade water then adding 32 mg sodium azide (NaN_3). The pH was adjusted to 8.2 with 5 N HCl and filled up to a volume of 1 litre. Mobile Phase B was methanol, acetonitrile and water at ratio of 45:45:10. Amino acids were identified by comparing the retention time of multi-amino acid standard mixture (99%) (Cat No. AAS18, Sigma-Aldrich) and the sample peaks. The concentration was calculated from peak areas using calibration equations, where: Concentration of analyte (C1) = Peak area of analyte/slope of the standards' calibration curve. Content (mg/g) of the analyte = [C1 x V x M x 100]/W x 1000, Where C1 = the concentration (pmol/ml mg/ml) of the analyte in the test solution, V = the volume (Dilution factor), of the test solution, and W = the weight (mg) of the sample used for the preparation of the test solution.

2.13. Data Analysis

The XLSTAT computer software was used to perform statistical correlation analysis using Pearson correlation coefficients to establish the relationships among the endogenous factors identified. The data values were subjected to Principal Component Analysis (PCA) using XLSTAT 2018 software and a distance biplot was constructed.

3. RESULTS

A total of 71 endogenous biochemical components were identified and quantified (Table 1). To narrow down which of the endogenous biochemical components present during somatic embryogenesis of Ruiru 11 could be utilized as biochemical markers for somatic embryogenesis, they were analysed to identify a core set that were consistently detected in embryogenic and non-embryogenic cultures.

Table 1: Endogenous biocomponents (µg/g) present during somatic embryogenesis

Class	Name	GE	BE	GW	BW
Alcohols	2-butyl-1-Octanol	13.21	13.88	6.9	11.7
Alcohols	2-ethyl-1-Decanol	33.74	9.34	9.36	24.25
Alcohols	1-Dodecanol	0	14.79	5.36	0
Alcohols	Z-2-Tridecen-1-ol	0	0	0	20.25
Alcohols	E-2-Tetradecen-1-ol	10.74	0	0	0
Alcohols	1-Hexadecanol	0	11.17	10.14	24.92
Alcohols	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	5.79	0	0	0
Alcohols	Z,Z-3,13-Octadecedien-1-ol	32.06	0	9.02	0
Alcohols	12-Methyl-E,E-2,13-octadecadien-1-ol	19.1	14.83	15.58	1.39
Aldehydes	3,7-dimethyl-7-Octenal	7.42	5.71	0	0
Aldehydes	2-Undecenal	51.7	8.87	0	0
Aldehydes	Dodecanal	2.22	0.23	0	0
Aldehydes	(E)-2-Tridecenal	0	0	3.93	4.83
Aldehydes	Pentadecanal	0	0	21.47	17.34
Aldehydes	(Z)-7-Hexadecenal	70.19	30.86	0	0
Aldehydes	(Z)-9-Octadecenal	31.83	15.49	0	0
Alkaloid	Caffeine	887.06	859.23	228.45	230.33
Alkane	-[(hexadecyloxy)methyl]-Oxirane	46.45	12.04	10.38	20.69

Alkane	hexadecyl-Oxirane	9.93	0	39.16	0
Alkane	1,1,3,3-tetraethyl-2,1,3-Oxadisilacyclopentane	4.95	0	0	0
Alkane	n-Octylidencyclohexane	20.75	21.36	13.46	9.24
Alkane	2-Trifluoroacetoxydodecane	1.72	0.13	0	0
Alkane	Dodecane,	28.43	10.57	7.26	6.5
Alkane	-1-iodo-Tridecane	0	0	0	20.61
Alkane	2-Cyclopropyl carbonyloxytetradecane	1.67	0	0	0
Alkane	bis(dodecyloxy)-Hexadecane	4.04	0	0	9.24
Alkane	(8)Heptadecane	13.4	30.34	4.97	4.94
Alkane	Cis 7,8 epoxy-2-methyl octadecane	253.05	0	0	0
Alkane	6-methyl-Octadecane	0	0	0	7.6
Alkane	1- sulphonyl chloride Octadecane	13.39	0	0	0
Alkane	2-methyltetracosane	38.33	14.29	6.82	9.99
Alkane	Hexatriacontane	0	0	6.41	9.3
Alkane	11,20-didecyl-Triacontane	43.04	6.64	0	0
Alkane	3,5,24-trimethyl-Tetracontane	0	23.25	9.41	9.19
Alkene	(Z)-3-Tetradecene	0	0	0	25.47

Table 1: Continued

Class	Name	GE	BE	GW	BW
Alkene	1-chloro-7-Heptadecene	0	0	4.37	0
Alkene	9-Octadecene	0	0	0	11.93
Alkene	1-Docosene	0	0	4.43	4.72
Amino acid	Alanine	3.26	6.06	0.65	1.13
Amino acid	Arginine	2.41	4.73	0.54	0.8
Amino acid	Aspartic acid	4.11	5.96	1.07	2.06
Amino acid	Glutamic acid	1.32	2.836	0.24	0.73
Amino acid	Glycine	2.54	5.66	0.74	1.43
Amino acid	Isoleucine	1.66	4.04	0.33	0.77
Amino acid	Leucine	1.3	3.51	0.24	0.35
Amino acid	Lysine	2.58	6.11	0.39	1.4
Amino acid	Methionine	1.89	4.14	0.7	0.65
Amino acid	Phenylalanine	1.66	4.53	0.34	0.82
Amino acid	Serine	1.36	2.27	0.39	0.54
Amino acid	Threonine	0.6	1.05	0.22	0.35
Amino acid	Valine	0.69	2.87	0.35	0.37
Carbohydrate	Glucose	4661	6881	7860	52385
Carbohydrate	Fructose	6701	14976	8267	62616
Carbohydrate	Sucrose	73457	37210	6648	0
Ester	2-Bromopropionic acid, pentadecyl ester	22.7	11.96	0	0
Ester	3-Chloropropionic acid, heptadecyl ester	15.98	0	0	0
Ester	3-Chloropropionic acid, tetradecyl ester	0	0	0	5.48
Ketone	9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione	27.2	34.82	1.5	9.37
Organic cpd	5-Hydroxymethylfurfural	0	12.06	0	0
Phenol	CGA	6679	3368	1380	71

SFA	Lauric acid	0	0	3.67	7.04
SFA	Caprylic acid	0	0	0	38.62
SFA	Palmitic acid	19.95	17.9	21.48	0
SFA	Margaric acid	43.08	32.17	12.12	15.49
SFA	Stearic acid	37.11	69.92	43.96	0
SFA	Nonadecylic acid	0	33.82	0	27.82
Steroid derivative	Ethyl iso-allocholate	0	23.58	0	0
UFA	Palmitoleic acid	18.99	23.28	0	0
UFA	Elaidic acid	1.99	1.13	15.01	11.12
UFA	Oleic acid	42.84	32.32	18.68	16.51
Unknown	Disparlure	0	0	0	16.97

Key: GE - Green leaf discs with embryos, BE - Brown leaf discs with embryos, GW - Green leaf discs without embryos, BW - Brown leaf discs without embryos

The PCA was done for the 71 compounds to identify the ones that were driving the separations of embryogenic and non-embryogenic cultures (Figure 1). The first two principal components (PC) accounted for 78.85% (49.67% and 29.18% on PC1 and PC2, respectively) of the variance observed. All the treatments gave positive correlation and were relevant in PC1 and the factor loading of the PCs resulted in the following equation: PC1 = 0.722GE +0.845BE + 0.796GW + 0.345BW. In PC2, green and brown cultures without embryos were positively correlated whereas green and brown cultures with embryos were negatively correlated and the PCs loading resulted in the following equation: PC2 = 0.304GW + 0.869BW - 0.535GE - 0.184BE.

Sucrose, palmitic acid and static acid clustered together and were absent in brown non-embryogenic cultures. Caprylic acid, (Z)-3-Tetradecene, -1-iodo-Tridecane, Disparlure, Z-2-Tridecen-1-ol, 1-sulphonyl chloride Octadecane and 3-Chloropropionic acid, tetradecyl ester clustered together and were present only in brown non-embryogenic cultures. Lauric acid, (E)-2-Tridecenal, 1-chloro-7-Heptadecene, 1-Docosene, Hexatriacontane and Pentadecanal also clustered together and were associated with green and brown non-embryogenic cultures. Components present in green embryogenic cultures and clustering together included 2-Cyclopropyl carbonyloxytetradecane, 1,1,3,3-tetraethyl-2, 1, 3-Oxadisilacyclopentane, E-2-Tetradecen-1-ol, 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, 1-sulphonyl chloride Octadecane and 3-Chloropropionic acid, heptadecyl ester. While grouping the endogenous biochemical components in their respective classes (excluding the carbon sources sucrose, fructose and glucose), PCA was again used to analyse the classes of endogenous biochemical components that promote or inhibit somatic embryogenesis (Figure 2). Similar to Figure 1, the first two principal components, accounted for 98.2% (84.34% and 13.86% on PC1 and PC2, respectively) of the variance observed. All the treatments gave a positive correlation and were relevant in PC1. The factor loadings of the PCs resulted in the following equation: PC1 = 0.953GE + 0.964BE + 0.993GW + 0.742BW. Brown non-embryogenic cultures were the most relevant parameter in PC2, whereas green and

brown embryogenic or non-embryogenic cultures were negatively correlated and their PCs yielded equation: PC2 = 0.67BW - 0.223GE - 0.226BE - 0.068GW.

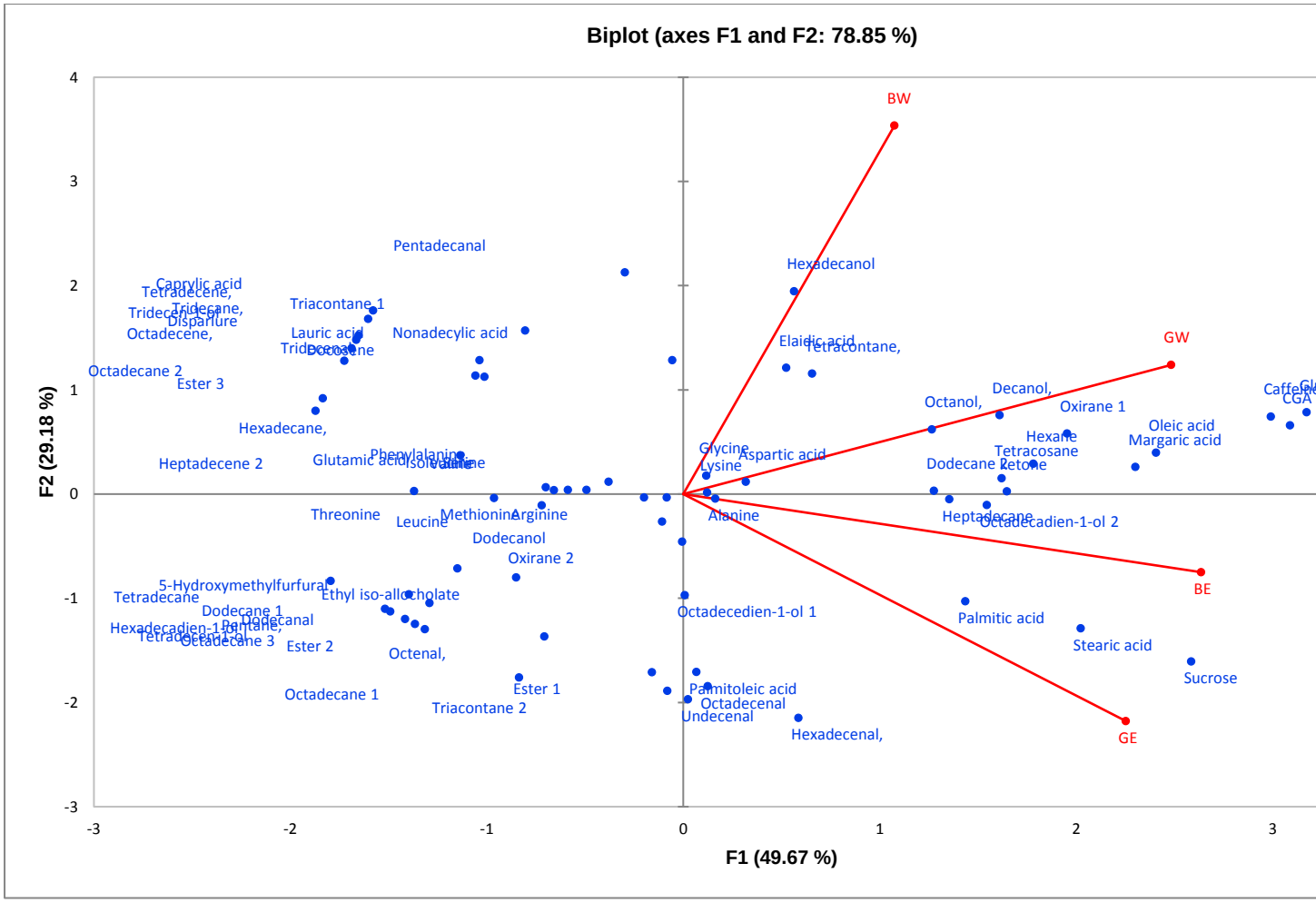

Figure 1: Principal Component Analysis biplot of the endogenous biochemical components present during somatic embryogenesis of coffee Ruiru 11.

GE - Green leaf discs with embryos, BE - Brown leaf discs with embryos, GW - Green leaf discs without embryos, BW - Brown leaf discs without embryos

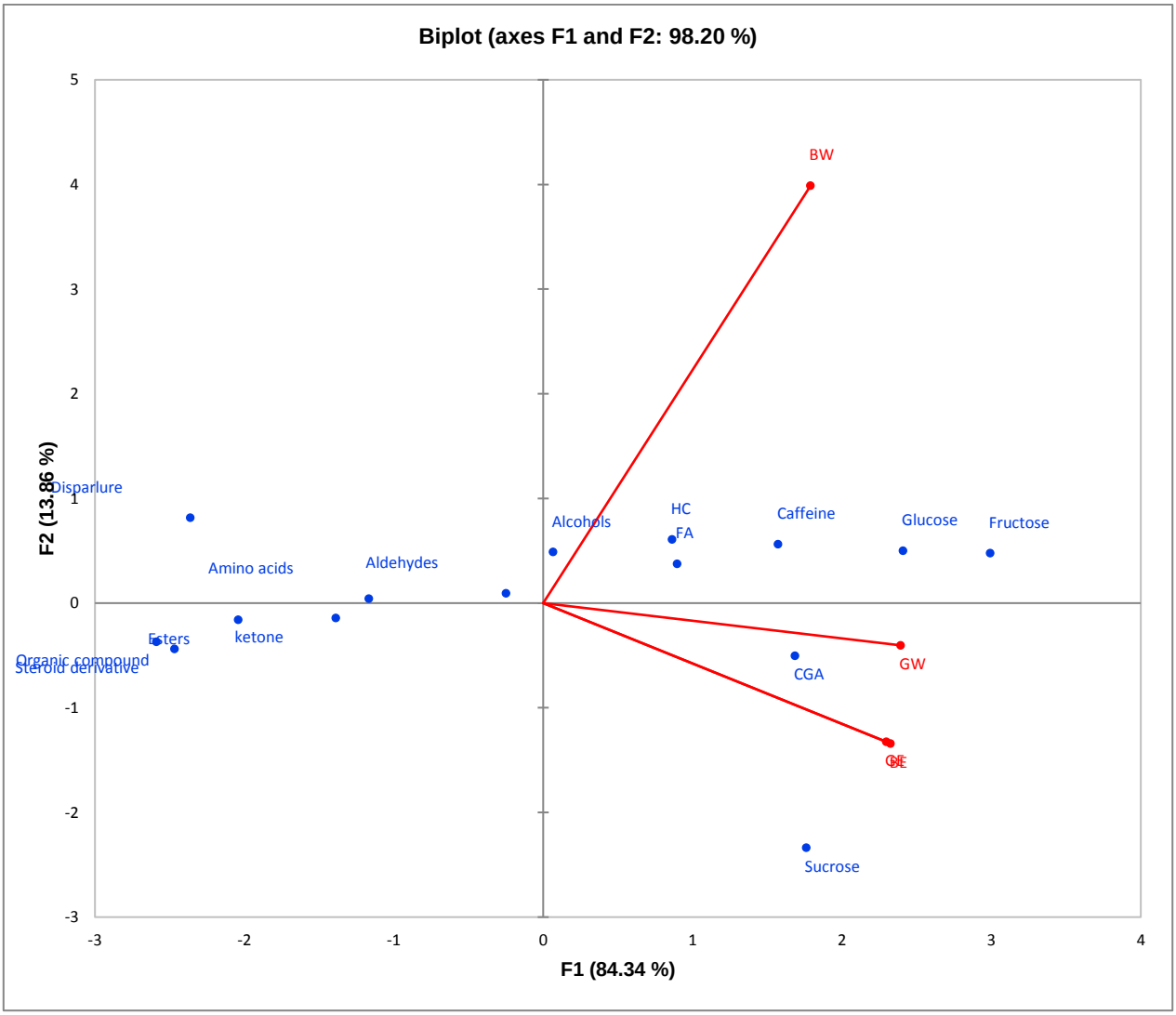

Figure 2: Principal Component Analysis (PCA) biplot of the classes of endogenous biochemical components present during somatic embryogenesis of coffee. GE - Green leaf discs with embryos, BE - Brown leaf discs with embryos, GW - Green leaf discs without embryos, BW - Brown leaf discs without embryos

From the PCA, high sucrose content was associated with embryogenic cultures and was not present in brown non-embryogenic cultures, whereas disparlure was associated with brown non-embryogenic cultures only. The 5-Hydroxymethylfurfural and steroid derivative, Ethyl iso-allocholate clustered together and was present in brown embryogenic cultures.

Esters and ketone clustered together and were not present or present in minimal amounts respectively in green non-embryogenic cultures. Endogenous alcohol, Hydrocarbons, Fatty acids and caffeine were clustered closely together in the PCA and were least in green non-embryogenic cultures. Fructose and glucose clustered closely and were high in brown non-embryogenic cultures. Correlation analysis was performed to establish relationships of the endogenous biochemical components (Table 2). Glucose and fructose were positively

correlated with each other (r=0.984, *P*=0.008) and negatively correlated with all the other classes of biochemical compounds identified during somatic embryogenesis of coffee. Sucrose was positively and significantly correlated with aldehyde (r=0.981, *P*=0.01) and chlorogenic acid (r=0.987, *P*=0.006). Positive significant correlation resulted between caffeine and ketone (r=0.904, *P*=0.049), aldehyde and alcohol (r=0.905, *P*=0.049), aldehyde and hydrocarbons (r=0.94, *P*= 0.03), aldehyde and esters (r=0.968, *P*=0.016), alcohol and hydrocarbon (r=0.994, *P*=0.003), alcohol and ester (r= 0.968, *P*=0.016), ester and hydrocarbon contents (r= 0.977, *P*=0.012) and steroid-derivative and organic acid. The organic acid and steroid derivative were also positively and significantly correlated (r=1, *P*=<0.0001). All the other correlations were either positive, or negative and insignificant.

Table 2: Correlation coefficients between various endogenous biochemical components

Variable	Glucose	Fructose	Sucrose	Caffeine	CGA	AA	FA	Aldehydes	Alcohols	Hydro carbons	Ester
Glucose	**1**										
P-value	***0***										
Fructose	**0.992**	**1**									
P-value	***0.008***	***0***									
Sucrose	-0.628	-0.602	**1**								
P-value	*0.372*	*0.398*	***0***								
Caffeine	-0.611	-0.537	0.907	**1**							
P-value	*0.389*	*0.463*	*0.093*	***0***							
CGA	-0.693	-0.676	**0.994**	0.876	**1**						
P-value	*0.307*	*0.324*	***0.006***	*0.124*	***0***						
Amino acids	-0.409	-0.293	0.498	0.817	0.445	**1**					
P-value	*0.591*	*0.707*	*0.502*	*0.183*	*0.555*	***0***					
Fatty acids	-0.734	-0.647	0.722	0.921	0.708	0.911	**1**				
P-value	*0.266*	*0.353*	*0.278*	*0.079*	*0.292*	*0.089*	***0***				
Aldehydes	-0.535	-0.519	**0.990**	0.857	**0.980**	0.406	0.626	**1**			
P-value	*0.465*	*0.481*	***0.010***	*0.143*	***0.020***	*0.594*	*0.374*	***0***			
Alcohols	-0.351	-0.365	0.901	0.663	0.894	0.127	0.354	**0.951**	**1**		
P-value	*0.649*	*0.635*	*0.099*	*0.337*	*0.106*	*0.873*	*0.646*	***0.049***	***0***		
Hydrocarbons	-0.418	-0.428	0.930	0.706	0.926	0.178	0.418	**0.970**	**0.997**	**1**	
P-value	*0.582*	*0.572*	*0.070*	*0.294*	*0.074*	*0.822*	*0.582*	***0.030***	***0.003***	***0***	
Ester	-0.387	-0.379	0.950	0.780	0.931	0.301	0.494	**0.984**	**0.984**	**0.988**	**1**
P-value	*0.613*	*0.621*	*0.050*	*0.220*	*0.069*	*0.699*	*0.506*	***0.016***	***0.016***	***0.012***	***0***
Ketone	-0.413	-0.312	0.750	**0.951**	0.692	0.928	0.903	0.696	0.477	0.516	0.627

P-value	*0.587*	*0.688*	*0.250*	***0.049***	*0.308*	*0.072*	*0.097*	*0.304*	*0.523*	*0.484*	*0.373*
Steroid der	-0.321	-0.205	0.156	0.552	0.114	0.925	0.774	0.044	-0.254	-0.200	-0.080
P-value	*0.679*	*0.795*	*0.844*	*0.448*	*0.886*	*0.075*	*0.226*	*0.956*	*0.746*	*0.800*	*0.920*
Organic cpd	-0.321	-0.205	0.156	0.552	0.114	0.925	0.774	0.044	-0.254	-0.200	-0.080
P-value	*0.679*	*0.795*	*0.844*	*0.448*	*0.886*	*0.075*	*0.226*	*0.956*	*0.746*	*0.800*	*0.920*

Bolded values are different from 0 at $P = 0.05$

4. DISCUSSION

There are different factors involved in the success or failure of the somatic embryogenesis response. An important "factor" that affects the somatic embryogenesis response is the release of organic molecules by the explants into the culture medium (Nic-Can *et al.,* 2015); some of the secreted molecules have been demonstrated to induce or modulate the somatic embryogenesis response. Other compounds, mostly secondary metabolites, have been found to inhibit the embryogenic response of the cells. Among the first biochemical observations on somatic embryogenesis was the necessity of a ''factor'' required to coordinate cell division and morphogenesis. This "factor" includes polysaccharides, amino acids, growth regulators, vitamins, low molecular weight compounds and polypeptides (Chung *et al.,* 1992). These molecules play an important role in the development of somatic embryogenesis as either inhibitors or inducers (Matthys-Rochon, 2005). Storage compounds are important markers of physiological quality of somatic embryos and a failure to produce them may affect the somatic embryo final developmental stages and conversion to plants (Cailloux *et al.,* 1996).

Variations in the endogenous components indicated that embryogenic and non-embryogenic cultures utilize their nutrients differently. A positive correlation between fructose and glucose resulted and these sugars were highest in non-embryogenic cultures compared to embryogenic cultures. In addition, these two sugars were negatively correlated with all the other biochemical compounds and clustered separately from the rest. A negative effect of accumulation of 6-carbon sugars such as fructose and glucose in embryo cells has been suggested and being of reducing nature they cannot accumulate to high levels without harmful effects, but their great advantage is a more direct entry into metabolism (Lipavská and Konrádova, 2004).

Lopez-Molina (2001) and Rolland *et al.* (2002) reported that high levels of sugars during seedling growth may be an indication of untenable growth conditions culminating in arrested development as a protection measure. This may be the reason why cultures with high fructose and glucose did not develop somatic embryos. Sucrose on the other hand was highest in embryogenic cultures and this indicated that during embryogenesis, there is increased uptake, and/or synthesis of the same as reported by Mahmud *et al.* (2014). Positive correlations between sucrose and chlorogenic acid was attributed to the fact that these compounds are considered as signaling molecules for differentiation and embryo development (Guan *et al.,* 2009; Khosroushahi *et al.,* 2011). Phenolic compounds and intermediaries of phenylpropanoid metabolism may stimulate inactivation of indole acetic acid (IAA), thereby lowering the physiological levels of IAA in the plant tissue that is undergoing differentiation and create a situation that is more favourable for organogenesis (*Jain et al.,* 2014).

Positive correlations that occurred among alcohols, aldehydes, hydrocarbons and esters may be related to the biosynthetic pathway of fatty acids. This is because the fatty acids undergo an acyl reduction pathway in which a reduction reaction occurs to form aldehyde intermediaries and can further reduce to primary fatty alcohols. In an alkane pathway, a typical reduction reaction occurs to give the aldehyde intermediaries in the acyl reduction pathway followed by decarbonylation to form odd-numbered alkanes. Often, this is the end product of the pathway but in some cases, the alkanes can carry out further downstream conversions by either hydroxylation to secondary alcohols which can then be followed by oxidation into ketones (Sin, 2012).

5. CONCLUSIONS AND RECOMMENDATIONS

Sucrose, phenolics, alkaloids, amino acids, fatty acids and their derivatives correlated positively and this implied that they are promoters or signal molecules for somatic embryogenesis in coffee. Endogenous fructose and glucose were associated with non-embryogenic cultures. In addition, these sugars were negatively correlated with the other biochemical components. Thus endogenous fructose and glucose are

inhibitors of coffee somatic embryogenesis. Endogenous sucrose, chlorogenic acids, caffeine amino acids, fatty acids and their derivatives are potential biomarkers for coffee somatic embryogenesis. Further studies regarding the status of the biochemical compounds identified, especially in particular stages of embryo development are needed in order to propose treatments to improve coffee somatic embryo development.

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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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