


# Endogenous sugars associated with development of somatic embryos of coffee (*coffea arabica* l.)

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**ABSTRACT**
Plant tissue culture allows rapid *in vitro* regeneration of plants. Processes and factors related to development of coffee somatic embryos are not well established, resulting in poor induction or few embryos and hence low regeneration of coffee seedlings. This research identified and quantified endogenous sugars (glucose, fructose and sucrose) associated with somatic embryogenesis in *Coffea arabica* cultivar Ruiru 11. Third leaf pair of greenhouse-grown mother plants was used as explants in half-strength Murashige and Skoog (MS 1962) media. Both green and brown leaf discs cultures with and without embryos were used to characterise the sugars. Embryos with fresh culture media and leaf explants were used as controls. A complete random design replicated thrice and repeated in two seasons in 2014 was used. Sucrose, fructose and glucose were extracted and analyzed using a Knuer HPLC and identified by comparing retention time with that of sucrose standard. Glucose content was significantly ($P$<0.05) high in brown leaf discs without embryos in both seasons (40.15 mg/g and 37.75 mg/g FW, respectively). Fructose content was significantly ($P$<0.05) high in brown leaf discs without embryos in both seasons (48.4mg/g and 42.4 mg/g FW). Sucrose content was significantly ($P$<0.05) high in fresh leaves in both seasons (18.87 mg/g and 19.57 mg/g, respectively) whereas in season 2, the sucrose content was significantly ($P$<0.05) high in embryos on green leaf discs (58.43 mg/g). Harmful effects of high accumulation of fructose and glucose which are reducing in nature resulted in no embryo development in the brown leaf discs. High sucrose which resulted in brown and green leaf discs with embryos implied that embryo maturation is associated with storage material accumulation and is accompanied with an increase in sucrose to hexose ratio.
**Keywords**: *Coffea arabica*, Somatic embryogenesis, Endogenous sugars

## INTRODUCTION

The coffee industry has continued to play a central role in the economy of Kenya through foreign exchange and income earnings. Presently, 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). Coffee contributes about 10% of the total agricultural export earnings, about 3.2% of the country's export earnings and up to 30% of the total labour force employed in agriculture. The industry is estimated to support 15% of the country's population either directly or indirectly. The sub-sector contributes to food security and source of livelihoods of an estimated 5 million Kenyans. Therefore, global coffee market conditions have important implications for growth and poverty reduction in Kenya. Coffee production in Kenya has been constrained by many factors which include; high cost of production, inappropriate technology and mismanagement of coffee co-operatives (Karanja and Nyoro, 2002). Major diseases that attack coffee include; Coffee Berry Disease (CBD) caused by *Colletotrichum kahawae*, Coffee Leaf Rust (CLR) caused by *Hemileia vastatrix* and to a lesser extent, the Bacterial Blight of Coffee (BBC) caused *by Pseudomonas syringae pathovar garcae* (Omondi et al*.,* 2001). New coffee varieties (Ruiru 11 and Batian) that combine CBD and CLR resistance with improved yield and quality have been developed at the Coffee Research Foundation of Kenya. The cultivar Ruiru 11 is a composite of about 60 F1 hybrid sibs each derived from a cross between a specific female and male population (Omondi et al*.,* 2001).

Plant tissue culture is an important technique in agricultural biotechnology. It allows in vitro regeneration and multiplication of plants under aseptic conditions through a process known as micropropagation. To date, micropropagation is the most common application of tissue culture. High cost of tissue culture equipments, however, limits its application mostly to high-value ornamental, plantation and forestry plant species (Santana-Buzzy et al*.,* 2007).

Many *Coffea* species have difficulty regenerating somatic embryos in tissue culture, in spite of the great progress accomplished in development of embryogenic cell induction protocols (Berthouly and Etienne (1999). This difficulty results in regeneration of few embryos during the induction process (Landey, 2013) and subsequently fewer coffee seedlings that don't meet farmers' demands of new disease resistant

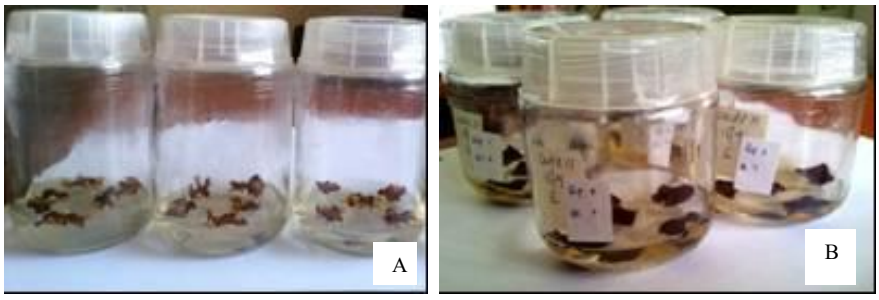
varieties. Although some studies report the origin of embryogenic cells, much of the early developmental processes and factors in coffee somatic embryos remain unclear, especially those related to the regulation of the induction and development of somatic embryos. Therefore, it is imperative to determine 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. *Coffea arabica* cultivar Ruiru 11 was used in this study. Third leaf pair explants were excised from 6month 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 steriled distilled water. The subsequent sterilization steps took place under the laminar flow cabinet. The explants were dipped quickly (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 basal culture media contained half-strength Murashige and Skoog (MS 1962) inorganic salts, supplemented with vitamins, 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 (25 ml) was poured into Magenta vessels (Sigma Chemical Co.) and 5 leaf discs (approximately 1 cm$^2$) cultured in each vessel to be maintained in the dark at 25$^{o}$C ± 2 and 70% humidity growth chamber. 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 (Fig. 1). These were used to both identify and quantify endogenous sugars namely: sucrose, glucose, and fructose in the leaf discs, developed embryos and medium. Fresh culture media and leaf explants excised from greenhouse-grown mother plants were used as the controls. A completely random design was used consisting of three treatments replicated thrice and repeated across two seasons in 2014.

### Extraction and analysis

Sucrose, fructose and glucose were extracted as described by Osborne and Voogt (1978) with modifications. Briefly, Leaf discs, embryo and culture medium of each type of leaf disc were weighed into a round bottomed flask. Extraction was done for one hour in 100 ml of 96% ethanol (AR) under reflux. The extract was cooled, filtered and evaporated to dryness. Sucrose extract was reconstituted to 2ml for the leaf and 5ml of embryo and media using mobile phase acetronitrile: distilled water at 80:20. Then the extract was filtered through a 0.45 µm micro-filter (Chromafil). About 50 microlitre of the extract/sample was injected to Knuer HPLC system 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. Sucrose, fructose and glucose were identified by comparing the retention time with that of sucrose standard (Fischer Scientific) and concentration calculated in mg/g fresh weight basis from peak areas using calibration equations. The SAS 9.2 computer software was used to analyse data. Data were subjected to analysis of variance (ANOVA) and significantly different means ($P \leq 0.05$) were separated using the least significance difference.



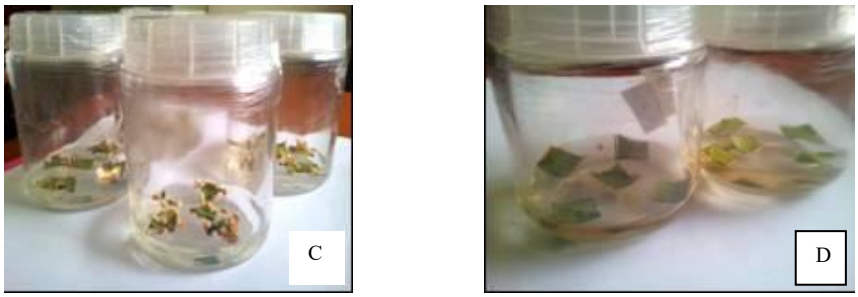



**Figure 1.** Treatments used for biochemical analysis **A**: Brown leaf discs with embryo **B**: Brown leaf discs without embryos **C**: Green leaf discs with embryos **D**: Green leaf discs without embryos.

**RESULTS**

There were significant differences in the glucose content in all the sample leaf discs in fresh basis (FW) in both seasons (Table 1). Brown leaf discs without embryos had the highest amounts of endogenous glucose content (40.15 mg/g and 37.75 mg/g FW for season 1 and 2 respectively). No glucose was detected in green leaf discs without embryos in both seasons. Varied responses resulted in glucose content in the developed embryos (Table 1). Glucose content was significantly ($P < 0.05$) high in embryos developed from green leaf discs in season 1(2.39 mg/g) whereas in season 2, glucose content of 3.625 mg/g was significantly ($P < 0.05$) high in embryos developed from brown leaf discs. There were significant ($P < 0.05$) differences in glucose content in the culture media. Brown leaf discs without embryos resulted in the highest amounts of endogenous glucose content of 11.57 mg/g and 15.29 mg/g in season 1 and season 2 respectively (Table 1).

**Table 1**: Glucose content (mg/g FW) in leaf, embryo and culture media

| Glucose Leaf | Season 1 | Season 2 | Glucose Embryo | Season 1 | Season 2 | Glucose Media | Season 1 | Season 2 |
|---|---|---|---|---|---|---|---|---|
| BW | 40.150a | 37.7517a | GE | 2.3933a | 1.70791b | BW | 11.5737a | 15.2939a |
| CL | 13.989b | 18.5695b | BE | 1.9020b | 3.62569a | GW | 8.0648b | 7.6557b |
| BE | 7.135c | 0.8237c | | | | CM | 3.2597c | 3.6519c |
| GE | 0.255d | 0.1396c | | | | GE | 2.3761d | 2.4514c |
| GW | 0.00d | 0.00c | | | | BE | 0.2465e | 0.0270d |
| CV (%) | 28.02 | 8.05 | | 20.92 | 5.896 | | 11.273 | 23.57 |
| LSD (0.05) | 5.1119 | 1.367 | | 0.2665 | 0.0932 | | 0.853 | 2.0327 |

Values followed by the same letter within each season are not significantly different by the LSD test at *5% level of significance*. Key: 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).

Significant ($P < 0.05$) differences resulted in endogenous fructose content among the sample leaf discs tested (Table 2). Brown leaf discs without embryos had the highest fructose content of 48.39 mg/g and 42.46 mg/g in season 1 and season 2 respectively. Embryos that had developed from brown leaf discs had the significantly ($P < 0.05$) higher fructose content of 5.12 mg/g and 3.15mg/g in season 1 and season 2 respectively. The highest fructose content of 15.08mg/g and 9.29mg/g in season 1 and season 2 respectively resulted in the culture media where the leaf discs had turned brown and no embryos formed.

**Table 2**: Fructose content (mg/g FW) in leaf, embryo and media

| Fructose Leaf | Season 1 | Season 2 | Fructose Embryo | Season 1 | Season 2 | Fructose media | Season 1 | Season 2 |
|---|---|---|---|---|---|---|---|---|
| BW | 48.396a | 42.4609a | BE | 5.12a | 3.1589a | BW | 15.0834a | 19.2900a |
| BE | 13.189b | 7.3186b | GE | 3.5901b | 1.8715b | GW | 8.5082b | 8.0256b |
| CL | 3.386c | 3.4202c | | | | GE | 3.8354c | 3.3934c |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| GE | 0.085c | 0.6265d | | | CM | 2.8785d | 3.7672c |
| GW | 0.00c | 0.00d | | | BE | 1.0981e | 0.0682d |
| CV (%) | 29.62 | 8.86 | 34.53 | 41.0 | | 8.639 | 18.421 |
| LSD (0.05) | 5.715 | 1.41 | 0.89 | 1.6115 | | 0.8044 | 1.8868 |

Values followed by the same letter within each season are not significantly different by the LSD test at *5% level of significance*.
Key: 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).

Significant differences resulted in endogenous sucrose (Table 3). Freshly harvested leaves had the highest ($P < 0.05$) sucrose content of 18.87 mg/g and 19.57 mg/g in season 1 and season 2 respectively. No significant differences in the sucrose content resulted in embryos developed in green and brown leaf discs for both seasons whereas in season 2, embryos developed from green leaf discs had the highest sucrose content of 58.43mg/g. Sucrose content was only detected in the control culture media for both seasons.

**Table 3**: Sucrose content (mg/g FW) in leaf, embryo and media

| Sucrose Leaf | Season1 | Season 2 | Sucrose embryo | Season 1 | Season 2 | Sucrose media | Season 1 | Season 2 |
|---|---|---|---|---|---|---|---|---|
| CL | 18.8770a | 19.5750a | GE | 65.863a | 58.435a | CM | 16.0477a | 16.391a |
| GE | 13.5221b | 9.0922b | BE | 63.876a | 10.545b | GW | 0.00b | 0.00b |
| GW | 6.9381c | 6.3568c | | | | GE | 0.00b | 0.00b |
| BW | 0.00d | 0.00d | | | | BW | 0.00b | 0.00b |
| BE | 0.00d | 0.00d | | | | BE | 0.00b | 0.00b |
| CV (%) | 12.575 | 6.09 | | 7.365 | 23.24 | | 5.8163 | 4.629 |
| LSD (0.05) | 1.4686 | 0.63 | | 2.8334 | 4.75 | | 0.27 | 0.225 |

Values followed by the same letter within each season are not significantly different by the LSD test at *5% level of significance*.
Key: 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)

## DISCUSSION

It is generally accepted that explant browning will result in a decline in culture competence, with eventual loss of totipotency or even the death of explants. Explant browning is usually caused by oxidase; for example, polyphenol oxidase (PPO) oxidizes phenols to produce brown-colored ubiquinols, which accumulate in explants and are released into the medium (Benson, 2000). 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 this study, it was observed that brown explants also generated somatic embryos. Chun-Ping et al*.,* (2015) predicted that the explant browning might be a manifestation of necrosis caused by some stress or by the differential response of explants themselves to the stress, which resulted in a hypersensitive response that induced PCD and the browning of explants. Although the mechanisms for the PCD induction of SE are not clear, two waves of programmed cell death occur during SE of Norway spruce, which indicates 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. Higher glucose and fructose in leaf discs may have resulted from hexose feeding from the medium, might be (perhaps by hexose-based signaling) the cause of poor embryo development especially in the brown leaf disc without embryos rather than the absence of sucrose splitting (or sucrose signaling). Some authors suggested the negative effect of hexose accumulation in embryo cells (Lipavska and Dova, 2004).

Higher sucrose content in mature coffee somatic embryos compared to glucose and fructose were observed in this study. 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 upto 25mm, 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 reaching the lowest hexose/sucrose ratio in embryos measuring 38–40 mm long (Sanchez-Romero et al*.,*

2002). Glucose and fructose contents in the embryos showed similar trends, although glucose levels were always lower than fructose levels. Decrease in glucose content in embryos observed could be attributed to its utilization as a source for the 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).

Sucrose is often used as a carbon source in plant tissue culture media. Its hydrolysis into glucose and fructose has been proven in a wide variety of plant cell and tissue cultures (George, 1993). In this study, no sucrose was detected in media for all treatments except 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. Such a hydrolysis of sucrose makes its utilization as the superior carbon source in potato micropropagation very insufficient (Yu et al*.,* 2000). For optimal plantlet growth, sucrose level sustainability are necessary and if it is rapidly hydrolysed into glucose and fructose making the long term maintenance of desirable sucrose level is difficult. Autoclaving is also a contributory factor of sucrose hydrolysis and a large amount of it breaks down during the growth of the plantlets (Kanabus et al., 1986). One of the known effects 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. Glucose and fructose, being of reducing nature, cannot accumulate to high levels without harmful effects, but their great advantage is the direct entry into metabolism (Lipavská and Dova, 2004).

## CONCLUSION AND RECOMMENDATION

Low endogenous glucose and fructose were present in the leaf discs. Since glucose is preferentially used to meet metabolic demand, its decrease can be ascribed to its role in the process of embryo growth and development. High endogenous sucrose content in the embryo is an indication that embryo maturation is connected with storage matter accumulation. Further studies regarding carbohydrate status and metabolism in particular stages of embryo development are needed in order to propose treatments to improve coffee somatic embryo development.

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