Biochemical events during somatic embryogenesis in *Coffea arabica* L.

Patrícia Monah Cunha Bartos¹ · Hugo Teixeira Gomes¹ · Lourdes Isabel Velho do Amaral¹ · João Batista Teixeira² · Jonny Everson Scherwinski-Pereira²

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Abstract

Several biochemical components associated with different stages of somatic embryogenesis in coffee (*Coffea arabica* L.) are investigated using foliar explants. Soluble sugar, starch, free amino acids and total proteins were extracted and quantified at different stages of somatic embryogenesis, such as foliar segments (initial explants), primary calluses, embryogenic calluses, globular embryos, torpedoed, cotyledonary embryos and mature fruit zygotic embryos. Total soluble sugar levels increased sixfold at the initial stages of somatic embryogenesis induction. During this period, total soluble sugar in the cultures contained approximately 99.3% glucose and fructose. At 67.4 µg/mg MS, no significant changes were observed in total sugar content during the embryo's somatic maturation and regeneration. During this stage, total soluble sugar was composed of 60% sucrose. After primary callus formation, starch contents increased gradually until the culture's conclusion. Total free amino acids, particularly arginine, lysine, methionine, asparagine, glutamine and histidine, revealed a higher synthesis until the formation of the primary callus, after which they remain statistically constant up to the end of the process. During the induction of calluses, a gradual increase of total proteins occurred, which, in the differentiating and maturing of somatic embryos, did not differ statistically till the formation of a cotyledonary embryo, when rates decreased 21.8%.

Keywords Rubiaceae · Morphogenesis · Somatic embryos · Liquid medium · Large-scale propagation · Biochemical profiling

Introduction

Coffee is the world's second most consumed beverage, with water coming first. *Coffea arabica* is the most important species and accounts for 70% of total coffee production worldwide. Over the last several years, genetic improvement of coffee has been taken up and several improved varieties have been released for commercial cultivation in many coffee growing countries. These improved varieties helped in reducing the use of pesticides, decreased production losses and ensured greater profitability for planters as well as consumer satisfaction (van der Vossen et al. 2015).

Hence, large-scale clonal propagation of elite coffee varieties becomes essential. According to Boxtel and Berthouly (1996), micropropagation by means of somatic embryogenesis is best suited for large-scale clonal propagation of coffee, due to high multiplication rates, scaling of production and the reduction of physical area and labor, when compared to other techniques.

Somatic embryogenesis, also known as asexual embryogenesis, is the result of the development of embryos from cells that are not the product of gamete fusion. It is thus possible to produce thousands of plantlets identical to the mother plant. The process triggers a large-scale propagation of better quality clones (Campos et al. 2017).

In spite of a great number of studies on the micropropagation of *C. arabica* by somatic embryogenesis (Söndahl and Sharp 1976; Yasuda et al. 1985; Neuenschwander and Baumann 1992; Fuentes-Cerda et al. 2001; Ahmed et al. 2013; Ibrahim et al. 2013; Lubabali et al. 2014), there is only a dearth of information on the mechanisms that regulate the process of somatic embryogenesis at different stages. Roowiet al. (2010) suggested that growth and development

In memoriam: Lourdes Isabel Velho do Amaral.

✉ Jonny Everson Scherwinski-Pereira
jonny.pereira@embrapa.br

¹ Department of Botany, University of Brasília, PPGBot, Campus Universitário Darcy Ribeiro, Brasília, DF 70910-900, Brazil

² Embrapa Genetic Resources and Biotechnology, Avenida W5 Norte (final), Brasília, DF 70770-917, Brazil

processes in every vegetative system are the outcome of a complex hormonal control. These compounds act by means of recognizing specific receptors present in vegetative tissue, which translate hormonal signals into several biochemical events such as biosynthesis and sugar, amino acid and protein metabolism, which turn leads to the desired physiological responses.

Pescador et al. (2008) underscored that sugars are the main source of metabolic energy and carbon source during embryo development. During the process, these compounds act as osmotic agents and contribute towards the preservation of the plasmatic membrane's integrity. Moreover, in embryogenesis, sugars may also act as reserve compounds or may be associated with the process of tolerance to desiccation stress. Amino acids, however, act in the embryogenic process as basic compounds for protein synthesis and as an important form in nitrogen transportation. Further, these metabolites are highly important since amino acids, stored during embryo development, are a source of nutrients in initial plant development (Cangahuala-Inocente et al. 2013). Proteins also form an important group of metabolites for embryogenic development. Several studies show that a significant increase in total protein rates occurs during embryonic formation. In embryogenesis, increased concentrations of these compounds cause late embryogenesis abundant (LEA) protein synthesis and reserve proteins, which, respectively, protect embryos against dehydration and act as a primary source of nitrogen (Gomes et al. 2014).

Current study investigates several biochemical components associated with different stages of coffee somatic embryogenesis using foliar explants as the start-up material.

Materials and methods

Vegetative tissue comprised expanded young leaves from the third pair of leaves (top to bottom) on plagiotropic branches of adult mother plants of *C. arabica* cv. Catuaí Vermelho, growing in a greenhouse (Fig. 1a). After harvesting, leaf surface was disinfected in a laminar flow hood by immersion in ethanol 50% (v/v), for 1 min, followed by soaking in sodium hypochlorite solution 2.4% (NaOCl), for 15 min. The leaves were then rinsed three times with sterile distilled water (Rezende et al. 2012).

Somatic embryogenesis was induced by inoculating sterile leaf segments of approximately 1 cm², excluding midvein, margins, and apical and basal portions of the leaf blade. Explants were cultivated in Petri dishes (15 × 90 mm), with the abaxial surface placed upwards, containing 25 mL of culture medium ½MS (Murashige and Skoog 1962), supplemented with 10 mg/L thiamine, 1 mg/L pyridoxine, 1 mg/L nicotinic acid, 1 mg/L glycine, 100 mg/L myo-inositol, 100 mg/L casein hydrolysate, 400 mg/L malt extract, 20 µM dichlorophenoxyacetic acid (2,4-D), 9.84 µM 2-isopentenyladenine (2-iP), 4.92 µM indole-3-butyric acid (IBA) and 20 g/L sucrose (Boxtel and Berthouly 1996). Further, pH was adjusted to 5.7 ± 0.1; 2.4 g/L Phytigel (Sigma, St. Louis, MO, USA) were added prior to sterilizing at 121 °C and 1.3 atm, for 20 min.

Explants were cultured for 30 days to obtain Type 1 primary calluses (a compact formation, less watery, brownish, with homogenous growth, basically composed of

Fig. 1 Stages of *Coffea arabica*'s somatic embryogenesis development. **a** leaf (initial explant), **b** Type 1 primary callus (30 days), **c** Type 2 primary callus (150 days), **d** embryogenic callus (150 days),

e globular embryo (330 days), **f** torpedo embryo (360 days), **g** cotyledonary embryos (390 days), **h** zygotic embryo

large parenchyma cells with reduced intercellular spaces), which, in the course of culture, produce embryogenic calluses (Fig. 1b). Propagules were transferred to the same culture medium. Their only difference was the reduction of 2,4-D concentration by half, where they were kept for 120 days to obtain embryogenic calluses (a formation characterized by a friable aspect of intense yellow color and low growth). This formation was different of Type 2 primary calluses (less compact, watery, whitish, with intense, disorderly growth, consisting predominantly of elongated parenchyma cells with large intercellular spaces), with no embryogenic competence, directly competing with the emergence and establishment of embryogenic calluses (Fig. 1c, d). In both stages, cultures were kept in the dark to minimize oxidation.

Embryogenic calluses multiplication occurred by inoculating 200 mg of embryogenic calluses in 125 mL Erlenmeyer flasks with 20 mL of basic MS medium reduced to one-half of the concentration, plus 10 mg/L thiamine, 1 mg/L pyridoxine, 1 mg/L nicotinic acid, 1 mg/L glycine, 100 mg/L myo-inositol, 100 mg/L casein hydrolysate, 400 mg/L malt extract, 250 mg/L citric acid, 5.0 μM 2,4-D, 9.84 μM 2-iP, 4.92 μM IBA, 20 g/L sucrose, and pH 5.7 ± 0.1 . During this stage, the explants were cultivated in orbital shakers at 90–100 rpm, in the dark, in six 15-day subcultures, for the multiplication of cell mass.

Differentiation took place by inoculating 250 mg of embryogenic calluses taken during the multiplication stage, onto Petri dishes containing 25 mL of $\frac{1}{2}\text{MS}$ culture medium, plus 10 mg/L thiamine, 1 mg/L pyridoxine, 1 mg/L nicotinic acid, 1 mg/L glycine, 100 mg/L myo-inositol, 100 mg/L casein hydrolysate, 400 mg/L malt extract, 8.88 μM 6-benzylaminopurine (BAP), 1.34 μM naphthalene acetic acid (NAA), 30 g/L sucrose, 2.4 g/L Phytagel and pH 5.7 ± 0.1 . At this phase, the explants were cultivated in three 30-day subcultures to obtain somatic embryos at the initial development stage (globular) (Fig. 1e).

Maturation and regeneration were performed by inoculating 100 mg of embryogenic calluses with initial formation of somatic embryos taken from the differentiation stage, in 125 mL Erlenmeyer flasks, containing 20 mL of MS medium reduced to one-half of the concentration, plus 10 mg/L thiamine, 1 mg/L pyridoxine, 1 mg/L nicotinic acid, 1 mg/L glycine, 100 mg/L myo-inositol, 2.57 μM indole-3-acetic acid (IAA), 1.11 μM BAP, 20 g/L sucrose and pH 5.7 ± 0.1 . During this stage, the explants were cultivated in orbital shakers at 90–100 rpm, in the dark, in two 30-day subcultures to complete their development. These steps were necessary so that the somatic embryos could reach the torpedo and cotyledonary stages (Fig. 1f, g).

Throughout the cultivation, the explants were placed in a growth room at $25 \pm 2^\circ\text{C}$, with a 16/8 h day/night photoperiod and a photosynthetic photon flux density (PPFD) of

42–52 $\mu\text{mol m}^{-2}\text{s}^{-1}$, provided by LED lamps (Philips®-Green Power 20 W 60 Hz).

Biochemical assays

In order that the coffee somatic embryogenesis could be biochemically defined by foliar explants, soluble sugar, starch, free amino acids and total proteins were extracted and quantified at different stages of development within the embryogenic process: foliar segments (initial explant), Type 1 primary calluses (30 days), Type 2 primary calluses (150 days), embryogenic calluses (150 days), globular embryos (330 days), torpedo embryos (360 days) and cotyledonary embryos in initial germination (390 days). Mature fruit zygotic embryos were also examined in current assay to compare their biochemical profile with the profile of cotyledonary somatic embryos (Fig. 1h). Further, samples were washed rapidly in ultrapure water to remove nutrient medium. They were then placed in cryotubes (2 mL), frozen instantly in liquid nitrogen, lyophilized for 48 h, macerated in porcelain crucibles and stored at 25°C in recipients with silica gel, till use.

Total carbohydrate and soluble sugars were estimated according to Dubois et al. (1956). Starch content was determined by enzymatic method, following Amaral et al. (2007). Free amino acids were evaluated following Yemm et al. (1955) and De Bruijn and Bout (2000), and total protein was estimated following Bradford (1956). In detail, the analyses followed procedures described by Gomes et al. (2014).

Data analysis

At least three replications were performed per treatment, for all analyses. Mean rates and standard deviation were calculated; variance analysis (Anova) was performed with Sisvar 4.4 (Ferreira 2011). Means were compared by Tukey's test at 5% significance.

Results and discussion

Soluble sugars

The levels of soluble sugar increased rapidly in the primary calluses (68.17 $\mu\text{g/mg}$), i.e., 30 days after plating the explants in culture (Fig. 2). Pescador et al. (2008) also detected a similar pattern in the somatic embryogenesis of *Acca sellowiana*. According to these authors, the sudden rise in total soluble sugar content probably occurred by adsorption of sucrose contained in the culture medium.

On the other hand, at the end of the induction process of the somatic embryogenesis, there was a significant decrease in total soluble sugar content in the explants

Fig. 2 Total soluble sugar concentrations at different stages of *Coffea arabica*'s somatic embryogenesis. *L* leaf (initial explant), *PC1* Type 1 primary callus (30 days), *PC2* Type 2 primary callus (150 days), *EC* embryogenic callus (150 days), *GE* globular embryo (330 days), *TE* torpedo embryo (360 days), *Ceb* cotyledonary embryo (390 days), *ZE* zygotic embryo. Averages followed by the same letter at each treatment stage are not statistically different by Tukey's test at 5% probability. Vertical bars represent average $\pm$ SE rates

tissues. During this stage of embryogenic development (150 days), total soluble sugar content was approximately twice (embryogenic callus) to four times (Type 2 primary callus) less than those detected 30 days after plating (Type 1 primary callus). This outcome corroborates results by Santos et al. (2003, 2008), who, after biochemically analyzing callogenesis of *C. arabica* and *Coffea canephora* from leaf explants, also noted a significant decline in sugar content during the primary callus development. According to Pescador et al. (2008), sugar catabolism occurred at the end of the somatic embryogenesis induction stage, probably resulting from an intense embryogenic mass cell proliferation process. Baud et al. (2002) underscore that sugar mobilization during this growth stage is very important for propagule development. In addition to providing cultures with metabolic energy and carbon skeletons, it is also likely to act in the process's biochemical signaling.

During the different stages of somatic embryo's development, no statistical differences were detected in total soluble sugar levels, with mean rates of approximately 70 µg/mg MS. Contrary to results in current study, Gomes et al. (2014) and Warchoř et al. (2015) reported that during the somatic embryo development of *Elaeis guineensis* and *Cordyline australis*, respectively, total soluble sugar concentrations in the cultures' tissues increased gradually, rising to higher rates at the torpedo somatic embryo and regenerating somatic embryo stages, the last two development stages.

Total soluble sugar in the tissues of mature fruit zygotic embryos was approximately two times greater than that in somatic embryos at the cotyledonary stage (160 µg/mg MS). Results disagree with those by Cangahuala-Inocente et al. (2009) for *A. sellowiana*. According to these authors, the presence of sugars in the culture medium may, in some

cases, increase total soluble sugar levels inside somatic embryos.

Although propagules' total soluble sugar rates are an important mark for a better understanding of the coffee plant's somatic embryogenesis at several stages of the process, the kind of soluble sugar in cultivated explants' tissues may be more crucial than the total soluble sugar contents themselves (Sauter and Cleve Van 1991; Weber et al. 1997). According to Delrot et al. (2000), sensitivity, concentration and transportation of vegetable tissues to different endogenous sugars may affect directly or indirectly in the regulation of cell division and differentiation processes in the cultures under development and, therefore, in the control of their morphogenesis.

Figure 3 shows monosaccharide (fructose and glucose) and disaccharide (sucrose) concentration rates at different stages of somatic embryogenesis development. During the somatic embryogenesis induction stage, total soluble sugars in explant tissues basically comprised fructose and glucose monosaccharides. Mengarda et al. (2009) and Gomes et al. (2014) also reported the same profile during the callus induction process in *A. sellowiana* and *E. guineensis*, respectively.

After the somatic embryogenesis induction stage, there was a gradual decrease in the contents of compounds in the composition of cultivated tissues. Simultaneously with the catabolism of monosaccharides, the synthesis of disaccharide sucrose was detected, which, based on the formation of the torpedo embryo, comprised the most abundant soluble sugar in the tissues under development. The standard was also registered for the species's zygotic embryos. Similar results were reported by Sánchez-Romero et al. (2002) and Pescador et al. (2008), respectively, for *Persea Americana* and *A. sellowiana*.

Fig. 3 Sugar concentrations at different stages of *Coffea arabica*'s somatic embryogenesis. *L* leaf (initial explant), *PC1* Type 1 primary callus (30 days), *PC2* Type 2 primary callus (150 days), *EC* embryogenic callus (150 days), *GE* globular embryo (330 days), *TE* torpedo embryo (360 days), *Ceb* cotyledonary embryo (390 days), *ZE* zygotic embryo. Averages followed by the same letter at each treatment stage are not statistically different by Tukey's test at 5% probability. Vertical bars represent average $\pm$ SE rates

According to Iraqui and Tremblay (2001), the monosaccharides fructose and glucose and disaccharide sucrose are usually associated with different regulating stages, during embryogenic development. Weber et al. (1997) underscore that sucrose regulates cellular differentiation and storage of reserve substances, while hexoses regulate growth and cellular metabolism.

Starch

During the formation of a primary callus, starch levels in explants' tissues slightly decreased from approximately 4% in hydrolysis to approximately 2% (Fig. 4). According to Martin et al. (2000), somatic embryogenesis induction is a

morphogenetic process that requires great amounts of metabolic energy, which may not be supplied by the breaking down of starch.

Similarly to primary callus formation, 30 days after cultivating, starch rates in the developing propagules increased gradually until the end of somatic embryogenesis, achieving the greatest rates in the cotyledonary stage somatic embryos, with approximately 24% in hydrolysis. Nakamura et al. (1994) also detected significant levels of accumulated starch during the somatic embryo formation stage in *C. arabica*, Mundo Novo and Catuaí Amarelo varieties. According to Lai and McKersy (1994), starch grains are usually deposited in areas close to regions with intense meristematic activity during somatic embryogenesis. The authors highlighted

Fig. 4 Starch concentrations at different stages of *Coffea arabica*'s somatic embryogenesis. *L* leaf (initial explant), *PC1* Type 1 primary callus (30 days), *PC2* Type 2 primary callus (150 days), *EC* embryogenic callus (150 days), *GE* globular embryo (330 days), *TE* torpedo embryo (360 days), *Ceb* cotyledonary embryo (390 days), *ZE* zygotic embryo. Averages followed by the same letter are not statistically different by Tukey's test at 5% probability. Vertical bars represent average $\pm$ SE rates

that starch buildup in these regions seems to be a phenomenon related to the acquisition of embryogenic competency. Starch deposited in regions with high meristematic activity was also reported in the somatic embryogenesis of *Agave tequilana*, *Acrocomia aculeata*, *A. sellowiana* and *E. guineensis*, respectively, by Portillo et al. (2007), Moura et al. (2010), Cangahuala-Inocente et al. (2009) and Gomes et al. (2014). According to these authors, starch stored is a source of metabolic energy by meristematic cells in intense division and differentiation.

Contrastingly to what was detected in somatic embryogenesis at the cotyledonary stage, starch contents, which on average reflected rates below 1% in hydrolysis, were practically null in zygotic embryos. This outcome confirms results by Hara et al. (1985) and Fuji et al. (1990), who also reported starch levels significantly greater than those noted in zygotic embryos in the somatic embryos of *Papaver orientale* and *Medicago sativa* somatic embryos, respectively.

Free amino acids

Similar to inoculation of the foliar segments employed as initial explants until the formation of primary calluses after 30 days of cultivation, a significant total free amino acid synthesis took place in the tissues of explants under development, triggering 20-fold increase in the rates of the compounds (Fig. 5).

Current results are corroborated by Kumar and Kumari (2011) who have reported the rapid increase of total free amino acid content during the in vitro callogenesis in *Cartha mustinctorious*. According to Fehér et al. (2003), sudden increase of total free amino acid concentrations in propagules under callus induction was partly due to the increase in metabolic activity of cultures stimulated by auxin in the nutrition medium. Sodek et al. (2002) underscored that rise

in total free amino acid levels during the somatic embryogenesis induction stage may also be explained by the high nitrogen concentrations in the culture medium, which favors amino acid synthesis.

After the formation of a primary callus (30 days), total free amino acid contents in the explants' tissues under development were stabilized. They remained statistically constant until the end of cultivation, i.e., until the start of the plant's regeneration stage (390 days), with averages that varied around 450 µg/mg MS. Gomes et al. (2014) also detected amino acid content stabilization after callus formation during *E. guineensis* somatic embryogenesis. Current results were different from those obtained by Booz et al. (2009) who detected an ongoing decline in total free amino acid levels during different stages of *A. sellowiana* somatic embryogenesis. According to the authors, decline in total free amino acid content in the course of cultivation occurred due to the continuous increase in protein synthesis. This fact was not detected in current study.

Table 1 illustrates the amount of free amino acids of greater relevance at the different stages of embryogenic development. Current analysis also found that amino acids arginine, lysine, methionine, asparagine, glutamine and histidine occurred in larger concentrations in Type 1 primary callus. Results were significantly higher than those found in other cultivation stages which, in general, did not differ statistically. Gomes et al. (2014) also reported that there was no great concentration of amino acid arginine in *E. guineensis* somatic embryogenesis during the primary callus induction process. According to Silveira (2006), arginine is one of the chief precursors of biosynthesis in polyamines, or rather, metabolites directly related to the acquisition of embryogenic competency.

With regard to the amino acids lysine and methionine, similar results were reported by Cangahuala-Inocente et al.

Fig. 5 Total free amino acid concentrations at different stages of *Coffea arabica*'s somatic embryogenesis. L leaf (initial explant), PC1 Type 1 primary callus (30 days), PC2 Type 2 primary callus (150 days), EC embryogenic callus (150 days), GE globular embryo (330 days), TE torpedo embryo (360 days), Ceb cotyledonary embryo (390 days), ZE zygotic embryo. Averages followed by the same letter are not statistically different by Tukey's test at 5% probability. Vertical bars represent average $\pm$ SE rates

Table 1 Quantifying amino acids of greater relevance at different stages of development of *Coffea arabica*'s somatic embryogenesis

Amino acids (nmol/mg MS)	Somatic embryogenesis stages							
	L	PC1	PC2	EC	GE	TE	Ceb	ZE
Arginine	2.72±0.3 AB	11.66±3.4 A	4.47±1.3 AB	6.75±4.2 AB	5.34±2.2 AB	8.84±6.7 AB	5.05±0.8 AB	2.38±0.2 B
Lysine	0.49±0.2 AB	0.75±0.1 A	0.02±0.02 C	0.20±0.1 BC	0.06±0.03 C	0.06±0.06 C	0.03±0.03 C	0.26±0.0 BC
Methionine	0.00±0.0 B	11.23±0.1 A	0.00±0.0 B	0.00±0.0 B	0.00±0.0 B	0.00±0.0 B	0.00±0.0 B	0.00±0.0 B
Asparagine	0.00±0.0 B	32.56±2.6 A	0.33±0.04 B	0.01±0.01 B	0.00±0.0 B	0.32±0.1 B	0.13±0.1 B	0.15±0.15 B
Glutamine	0.15±0.0 C	12.15±1.3 A	1.81±0.9 BC	4.64±1.5 B	2.59±0.6 BC	2.32±0.8 BC	0.51±0.5 BC	0.01±0.01 C
Histidine	0.00±0.0 C	1.32±0.2 A	0.04±0.04 C	0.05±0.04 C	0.00±0.0 C	0.67±0.4 B	0.00±0.0 C	0.84±0.4 AB
Threonine	3.01±0.5 BC	9.52±0.3 A	4.37±0.2 B	3.60±0.4 BC	4.11±0.9 BC	1.63±0.5 CD	2.7±0.5 BCD	0.41±0.8 D
Glycine	2.75±1.4 B	8.50±4.9 A	2.41±0.9 B	1.23±1.1 CD	2.74±2.6 B	0.93±0.3 DE	1.63±1.5 C	0.46±0.1 E
Leucine	4.00±0.8 AB	6.25±0.9 A	6.37±0.8 A	1.41±1.4 B	1.45±1.4 B	3.67±1.8 AB	5.41±0.3 A	4.14±0.8 AB
Serine	0.00±0.0 H	0.32±0.19 F	3.78±1.8 A	1.51±1.1 C	1.09±0.4 D	1.69±1.1 B	0.99±0.6 E	0.23±0.08 G
Proline	0.00±0.0 C	0.19±0.11 C	0.47±0.1 C	2.18±1.1 B	2.22±1.1 B	5.53±2.8 A	0.96±0.7 BC	1.28±0.7 BC

Mean followed by the same letter in a line indicate significant differences among treatments ($P < 0.05$) according to Tukey's test. Data represent average ± SE rates

L leaf (initial explant), PC1 Type 1 primary callus (30 days), PC2 Type 2 primary callus (150 days), EC embryogenic callus (150 days), GE globular embryo (330 days), TE torpedo embryo (360 days), Ceb cotyledonary embryo (390 days), ZE zygotic embryo

(2013) during the start of the induction process of *A. sellowiana* somatic embryogenesis. Bagni and Tassoni (2001) registered that amino acids lysine and methionine were also precursors of biosynthesis in polyamines. Opposite results for asparagine were reported by Gomes et al. (2014) who registered an intense decline in its levels at the start of the callus induction process during *E. guineensis*'s somatic embryogenesis, followed by ample changes in the course of the entire embryogenic development. According to Neuberg et al. (2010), owing to its great solubility, asparagine is one of the chief forms for transporting and storing nitrogen by higher plants.

A significant increment in glutamine rates in *A. sellowiana*'s somatic embryogenesis was also detected during the start of the callus induction process (Cangahuala-Inocente et al. 2013). According to Dal Vescol and Guerra (2001), glutamine is a form of readily assimilated organic nitrogen, absorbed by carbon skeletons in cultures during synthesis and metabolism of amino acids and proteins.

Kamada and Harada (1979) also reported a synthesis of significant amounts of histidine in *Daucus carota* somatic embryogenesis, yet differently from current study, the formation of the amino acid inhibited embryogenic development in this vegetable species. According to Mifflin and Lea (1976), histidine is not metabolized by organisms and cannot be converted into other amino acids. In certain cases, it causes losses in the development of cultures.

The coffee plant's somatic embryogenesis revealed that the amino acids threonine and glycine had higher concentrations in Type 1 primary calluses, with much greater results than those found in other process stages, averaging 9.52 and 8.50 nmol/mg MS, respectively. Nonetheless,

different from results on the previously mentioned amino acids, the contents of these compounds significantly decreased during the somatic embryo's maturing stage. Similar results were reported by Gomes et al. (2014) in their study on the somatic embryogenesis development of *E. guineensis*.

Greater concentrations of the amino acids leucine and serine were obtained in Type 2 primary calluses, approximately 6.37 and 3.78 nmol/mg MS, respectively. This outcome confirms results by Kamada and Harada (1979) who analyzed the somatic embryogenesis of *D. carota* and detected a synthesis of significant amounts of leucine during the formation of calluses with non-embryogenic features. On the other hand, results to the contrary have been found in literature in the case of the amino acid serine, with very low rates until the last period of the callus induction process (Gomes et al. 2014).

It may be concluded that concentrations of the amino acid proline in the tissues of explants under cultivation rose gradually till the formation of a torpedo stage somatic embryo, with highest rates close to 5.53 nmol/mg MS. An increase in endogenous proline contents was also found by Murch et al. (1999), during *Arachis hypogaea* somatic embryogenesis. According to Verbruggen and Hermans (2008), proline's buildup in vegetable tissues is, in general, related to conditions of environmental stress. According to Szabados and Savaouré (2009), proline is an amino acid that acts as a signaler for modulating mitochondrial functions, cellular proliferation and expression of specific genes, essential activities to create resistance and recovery of plants from different stress conditions.

Total proteins

Total protein rates in the tissues under cultivation gradually increased during the callus induction stage (Fig. 6), starting close to 100 $\mu\text{g}/\text{mg}$ MS (leaf) up to approximately 225 $\mu\text{g}/\text{mg}$ MS (embryogenic callus). Santos et al. (2003) also noted a significant buildup of total proteins in the developing tissues during the induction of callogenesis in *C. arabica*, Rubi variety, based on foliar segments.

According to Fehér et al. (2003), acquisition of embryogenic competency by somatic cells during induction of somatic embryogenesis by differentiation and reprogramming involved the synthesis of a several proteins. Silva et al. (2014) reported that proteins involved in starch accumulation, in cell division and in abiotic stress, are chiefly synthesized during this stage of somatic embryo development. On the other hand, during the somatic embryo's stages of differentiation and maturing, no statistical differences were registered in total protein levels, with mean rates of approximately 160 $\mu\text{g}/\text{mg}$ MS. In *C. australis*'s somatic embryogenesis, Warchol et al. (2015) failed to detect any significant differences in total protein levels during formation and development of somatic embryos. Jariteh et al. (2015) noted that, during *Juglans regia*'s somatic embryo differentiation and maturing stages, the total protein rates in the cultures decreased gradually and dropped to their lowest level in the process' final stage. Cangahuala-Inocente et al. (2009) also noted significant changes in total protein rates of *A. sellowiana* somatic embryo differentiation and maturing during the development stages. However, the study revealed that the rates of such compounds increased gradually in propagule tissues.

Nevertheless, in the formation of cotyledonary somatic embryos (or rather, at the start of the plant regeneration

process), the propagules' total protein levels declined from approximately 210 to approximately 160 $\mu\text{g}/\text{mg}$ MS. These rates are similar to those found in the species' zygotic embryos. According to Wise and Tunnacliffe (2004), in the germination process or in the regeneration process in the case of somatic embryos, reserve proteins stored during the embryogenic process are degraded and release large quantities of compounds for use in the transition by the plant from heterotrophia to autotrophia, as well as in its initial growth.

Conclusion

The acquisition of embryogenic competence during the somatic embryogenesis of *C. arabica* is related to rises of starch and total protein levels, concomitantly reducing the levels of soluble sugars glucose and fructose, and the free amino acids arginine, lysine, methionine, asparagine, glutamine and histidine. Although not involving alterations of the total soluble sugars and total free amino acids, the development of this morphogenic route in the species increases the concentrations of starch, total proteins and sucrose, which, in the latter two cases, are followed by significant reductions.

Results may elucidate the biochemical and physiological changes that take place during the plant's somatic embryogenesis. In addition, data information should be used for the development of physiological markers of different stages of embryogenic development of coffee. In fact, they may optimize the nutrient media of the various stages of the process, reducing or removing compounds with low mobilization and increasing the levels of the compounds of interest for the culture's specific phases. Consequently, production costs may be reduced and the efficiency of this important

Fig. 6 Total protein concentrations at different stages of *Coffea arabica* somatic embryogenesis. L leaf (initial explant), PC1 Type 1 primary callus (30 days), PC2 Type 2 primary callus (150 days), EC embryogenic callus (150 days), GE globular embryo (330 days), TE torpedo embryo (360 days), Ceb cotyledonary embryo (390 days), ZE zygotic embryo. Averages followed by the same letter are not statistically different by Tukey's test at 5% probability. Vertical bars represent average $\pm$ SE rates

methodology may be increased. In fact, by the end of the regeneration stage, production of at least 10,000–30,000 plantlets per gram of embryogenic callus may be obtained and multiplied (Bartos 2012).

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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