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BRIEF COMMUNICATION

Comparative biochemical profiling during the stages of acquisition and development of somatic embryogenesis in African oil palm (*Elaeis guineensis* Jacq.)

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Abstract In this study, the different stages of somatic embryogenesis (SE) of the African oil palm (*Elaeis guineensis* Jacq.) were characterized biochemically. The total soluble sugars, starch, total free amino acids, and total proteins were extracted, identified and quantified at various stages of embryogenesis: zygotic embryos (initial explants), primary calluses, embryogenic calluses, calluses with pro-embryos, globular embryos, differentiated somatic embryos, and regenerated plants. It was found that at the onset of induction of SE, the level of soluble sugars in the tissues of the explants fell by half. During this period, the total soluble sugars present in the cultures consisted basically of glucose and fructose. In the process of regeneration and maturation, the concentrations of soluble sugars gradually increased, reaching the highest values in the last two stages of development. At this stage, the disaccharide sucrose accounts for more than 80 % of the composition of total soluble sugars in the explants. Compared to starch, we found that the concentrations thereof in developing tissues are inversely proportional to that of soluble sugars virtually throughout embryogenic development. As for free amino acids, we found that after 30 days of induction until formation of the embryogenic calluses, there is an accentuated synthesis of total free amino acids in the explant tissue. In this stage, there was a significant increase in the levels of alanine and serine in the tissues. However, after the formation of the embryogenic calluses, the levels of total free amino acids present in the cultures become stabile and remain constant until the end of cultivation. Similar results were found for total protein, which also showed a significant increase at the onset of induction, undergoing slight changes during the remainder of the cultivation.

Keywords Arecaceae · Micropropagation · Callus induction · Morphogenesis · Biochemical characterization

Abbreviations

SE	Somatic embryogenesis
ZE	Zygotic embryos
Picloram	4-Amino 3,5,6-trichloropicolinic acid

Introduction

The African oil palm (*Elaeis guineensis* Jacq.), originating from the west coast of Africa, is one of the highest oil-yielding plant species in the world. Furthermore, the African oil palm still has the advantage of being exploited commercially for periods of no less than 25 years, when cultivated under conditions suitable for development (Clement et al. 2005).

In this species, micropropagation via somatic embryogenesis (SE) is one of the only alternatives for plant clonal propagation (Scherwinski-Pereira et al. 2010; Soh et al. 2011; Silva et al. 2014). Worldwide, millions of plants from different plant species are produced annually through SE (Ducos et al. 2007; Steinmacher et al. 2011; Sané et al.

In memoriam of Lourdes Isabel Velho do Amaral.

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2012). However, understanding of the mechanisms that regulate the different stages of the process is still quite limited. Hence, biochemical studies conducted during SE can provide a great deal of information, including information associated with the induction of embryogenic competence, mobilization of reserves in the phases of maturation and regeneration of somatic embryos, and formation of abnormal plants, thereby contributing for the optimization of protocols of SE (Pescador et al. 2008; Wu et al. 2009).

In the development of somatic embryos, studies have reported that, aside from acting as a source of metabolic energy, a source of reserve compounds, and a source of carbon skeletons, together with other substances, carbohydrates also serve, together with other substances, to render the embryos tolerant to desiccation and quiescent, i.e., able to regenerate (Lipavská and Konrádová 2004).

In SE, studies have shown that the quantity of amino acids, particularly proline, serine and tyrosine, increase and decrease quickly during the different stages of the process, thus demonstrating the link of these compounds with this important plant developmental pathway (Sen et al. 2002; Niemenak et al. 2008; Booz et al. 2009; Kumar and Kumari 2011). Therefore, aimed at fostering the development of embryogenic cultures, researchers are often supplementing the culture media with amino acids (Scherwinski-Pereira et al. 2010).

Regarding embryogenic development, studies have demonstrated the occurrence of an increase in the total protein content, due not only to the synthesis of storage proteins, but also the accumulation of stress-related proteins and other classes of proteins, such as those related to respiration (glycolysis, Krebs cycle and oxidative phosphorylation), biosynthesis of carbohydrates, and metabolism of amino acids (Aberlenc-Bertossi et al. 2008; Sghaier-Hammami et al. 2009a; Cangahuala-Inocente et al. 2009).

In this context, the aim of this work was to characterized and identified biochemically the different stages of SE of the African oil palm.

Materials and methods

The explants used for biochemical characterization of the different stages of SE in the African oil palm were obtained based on SE of ZE of the C2528 variety, following the method established by Balzon et al. (2013).

Induction of SE was performed by inoculation of the ZE (150–160 days post-anthesis) in petri dishes containing a MS nutrient culture medium (Murashige and Skoog 1962) supplemented with 450 μM Picloram, 2.5 g/L activated charcoal, 30 g/L sucrose, and solidified with 2.5 g/L Phytagel (Sigma, St. Louis, MO). The explants were then cultured for five subcultures of 30 days to obtain embryogenic calluses.

For the somatic embryo differentiation and maturation, embryogenic calluses from the induction phase were used. In this phase, the culture medium was the MS with the same composition as the induction medium, but with picloram reduced to 40 μM. The explants remained in this phase for up to six subcultures of 30 days.

The regeneration of the plants was performed from the inoculation of the somatic embryos, originating from the differentiation and maturation phase, into 250 mL glass flasks containing a MS nutrient culture medium free of growth regulators, supplemented with 30 g/L sucrose and 2.5 g/L Phytagel. In this stage, the explants were cultured for at least two subcultures of 30 days in order to obtain plants.

During all callus induction and differentiation and maturation periods, the cultures were placed in the dark at 25 ± 2 °C. For plant regeneration, the cultures were kept at a photosynthetic photon flux of 38 μmol/m^2 s provided by cool white lamps with a 16-h/day photoperiod.

For the biochemical characterization of SE, the following were extracted and quantified: total soluble sugars, starch, free amino acids and total proteins from cultures at different stages of SE, according to Silva et al. (2014): ZE (initial explant); primary calluses (14, 21, 30, 45 and 60 days), embryogenic calluses (90 days), calluses with pro-embryos (150 days), globular embryos (210 days), differentiated somatic embryos (300 days), and regenerated plants (360 days).

To do so, the samples were washed rapidly in ultrapure water to remove the nutrient medium, frozen instantly in liquid nitrogen, lyophilized for 48 h, and then macerated in porcelain crucibles and stored at 25 °C in recipients containing silica gel until the time of use.

For all analyses, three repetitions were carried out per treatment. The mean values and standard error were calculated, and variance analysis was performed, using the program Sisvar. Means were compared by the Scott–Knott test at 5 % significance level.

Soluble sugars

Extraction of total soluble sugars was carried out according to Pescador et al. (2008). To do so, 10 mg dry weight of the samples were inoculated into 2-mL microtubes, and subjected to extraction in 0.5 mL of 80 % methanol at 70 °C for 20 min. After incubation, the microtubes were centrifuged at 13,000 rpm for 10 min, the supernatants were collected, and the residues were re-extracted three more times and again centrifuged, with the supernatants of the

four methanolic extractions gathered into microtubes, totaling 2 mL.

After extraction, the microtubes containing the supernatants were dried in a Speed Vac at room temperature and the dried product was resuspended in 1 mL of deionized water. Subsequently, the extract underwent purification in ion exchange columns, using the Dowex 50×8 (100–200 mesh) cationic resin to remove amino acids and other cations, and the Dowex 1×8 (52–100 mesh) anionic resin to remove organic acids and other anions, plus deionized water for elution of the extracts. After purification, the samples were lyophilized for 48 h and resuspended in 1 mL of deionized water.

Then, the purified carbohydrates were quantified according to the phenol–sulfuric method described by Dubois et al. (1956). To do so, 25 μL of the samples were pipetted into in test tubes, adding: 0.475 mL of deionized water; 0.5 mL of 5 % phenol solution; and 2.5 mL of concentrated sulfuric acid (absolute). After 5 min of rest at room temperature, readings were taken of absorbances in a spectrophotometer at 490 nm. A solution of 0.5 mL of deionized water, 0.5 mL of 5 % phenol solution, and 2.5 mL of concentrated sulfuric acid was used as a control. All analyses were performed in triplicate and based on the standard glucose curve, with increasing concentrations of 0.0, 5.0, 10, 20, 40 and 80 μg/mL.

The composition of total soluble sugars was determined by high-performance ion exchange chromatography, with amperometric pulse detector (HPAEC-IPAD model ICS3000, Dionex brand, Sunnyvale, CA) with CarboPac™ PA10 column (2 × 250 mm) (Dionex Corporation, Sunnyvale, CA) using an elution gradient with 200 mM NaOH in water (20 min). The detector's responses were compared to the standards of glucose, fructose and sucrose, at 0.625, 1.25, 2.5, 5.0 and 10.0 μM.

Starch

The extraction and quantification of starch was performed by the enzymatic method of Amaral et al. (2007). To do so, 10 mg dry weight of the samples were inoculated into 2-mL microtubes, and subjected to removal of sugars in 0.5 mL of 80 % ethanol at 80 °C for 20 min. After incubation, the microtubes were centrifuged at 13,000 rpm for 10 min, the supernatants were discarded, and the residues were subjected to removal of sugars three more times. Then, the residue was washed three times in deionized water, frozen at −20 °C and lyophilized.

An amount of 0.5 mL (120 U/mL) of thermostable α-amylase of *Bacillus licheniformis* (Megazyme®) diluted in MOPS buffer [3-(*N*-morpholino) propanesulfonic acid] 10 mM pH 6.5 was added to the freeze-dried samples. Then the samples were incubated at 75 °C for 30 min. This procedure was repeated once more, totaling 120 units of enzyme. Subsequently, the samples were cooled to 50 °C (in a water bath) and then added to them was a solution containing 0.5 mL (30 U/mL) of amyloglucosidase (AMG) of *Aspergillus niger* (Megazyme®) in a sodium acetate buffer 100 mM pH 4.5, followed by incubation of the samples at 50 °C for 30 min. This procedure was repeated once more, for a total of 15 units of enzyme.

After the four incubations, 100 μL of 0.8 M perchloric acid were added to stop the reaction and precipitate the proteins. After a brief centrifuging, 50 μL aliquots of the samples were pipetted in plastic cuvettes and incubated for 15 min at 37 °C with 750 μL Godpod solution (Glucose PAP Liquiform/Centerlab®). After incubation, the starch content was determined in a spectrophotometer at 505 nm. All analyses were performed in triplicate and based on the standard glucose curve, with increasing concentrations of 0.0, 2.5, 5.0, 7.5, 10 and 20 μg/mL.

Free amino acids

The extraction of free amino acids was performed by inoculating 10 mg dry weight of the sample in 2-mL microtubes, subjected to immersion in 0.5 mL of 80 % methanol and incubation in a water bath at 70 °C for 20 min. After incubation, the microtubes were centrifuged at 13,000 rpm for 10 min, the supernatants were collected, and the residues were re-extracted three more times and again centrifuged, with the supernatants of the four methanolic extractions gathered in new microtubes, totaling 2 mL.

After extraction, the microtubes containing the supernatants were dried in a Speed Vac at room temperature and the dried product was resuspended in 1 mL of deionized water. Then, the extract was subjected to purification in a cation exchange column, using the Dowex 50×8 cationic resin (100–200 mesh) for filtering the amino acids. For removal of the amino acids, the column was washed with 33 mM trisodium citrate buffer containing 145 mM NaOH and 200 mM H_3BO_3 (pH 10). After purification of the samples, they were lyophilized for 48 h and resuspended in 1 mL of deionized water.

Then, the total free amino acids were quantified, according to modified method described by Yemm and Cocking (1955). To do so, 50 μL of 0.02 M citrate buffer and 100 mL of 1 % ninhydrin were added to 50 μL of sample. Subsequently, the tubes were shaken, sealed and kept in a bath at a temperature of 95 °C for 20 min. After the tubes were cooled, a reading was taken of the absorbance on an ELISA microplate reader at 570 nm. All analyses were performed in triplicate based on the leucine standard curve, with increasing concentrations of 0.0, 2.5, 5.0, 7.5, 10 and 20 μg/mL.

To identify the essential free amino acids, the method described by De Bruijn and Bout (2000) was used. Accordingly, the filtered samples were analyzed via high-performance anion exchange chromatography with pulse amperometric detector (HPAEC/PAD), model ICS 3000, in AminoPAC-PA10 analytical column with dimensions of 2 mm × 250 mm (Dionex Corporation, Sunnyvale, CA) and AminoPac-PA10 guard column with dimensions of 2 mm × 50 mm (Dionex Corporation, Sunnyvale, CA) with a gradient elution of water, 250 mM sodium hydroxide, and 1 M sodium acetate with a flow of 0.2 mL/min. The responses of the detector were compared to the standards of arginine, lysine, histidine, asparagine, glutamine, serine, threonine, cysteine, tyrosine, aspartate, glutamate, glycine, alanine, valine, leucine, proline, isoleucine, methionine, phenylalanine and tryptophan at concentrations of 0.625, 1.25, 2.5, 5.0 and 10.0 µM.

Total proteins

The extraction of total proteins was performed by inoculating 10 mg dry weight of the sample in 2-mL microtubes, subjected to three washes in hexane and three washes in acetone for depigmentation of the samples. After the washes, the samples were immersed in 0.5 mL of 0.1 N NaOH and incubated at 4 °C for 12 h. Then, two more 0.25-mL aliquots were added, followed by one more hour of incubation each.

After the incubations, 1 mL of 10 % trichloroacetic acid was added to the samples, followed by centrifuging the tubes at 13,000 rpm for 10 min in order to precipitate the proteins. Then, the supernatant was discarded and the proteins were resuspended in 0.5 mL of 0.1 N NaOH.

Quantification was performed according to the method described by Bradford (1956). Accordingly, 10 µL of samples were pipetted into microtubes, to which 790 µL of deionized water and 200 µL of 20 % Bradford solution were added. After 5 min of rest in the dark, absorbance readings were taken in a spectrophotometer at 595 nm. A solution at 800 µL of deionized water and 200 µL of Bradford solution was used as a control. All analyses were performed in triplicate and based on the standard curve of bovine serum albumin.

Results and discussion

Soluble sugars

It was found that during the onset of induction of SE, more precisely, from the inoculation of the explants to the beginning of primary callus development (14 days), the levels of soluble sugars in the developing tissues fell from around 180 to 70 µg/mg DW (Fig. 1). Mengarda et al. (2009) also observed a significant decrease in the levels of total soluble sugars in the explants of *Acca sellowiana* during the process of induction of SE. According to Baud et al. (2002), the mobilization of soluble sugars in the early stages of embryogenic development is of major importance, because they act mainly as a source of metabolic energy, of carbon skeletons and/or for signaling.

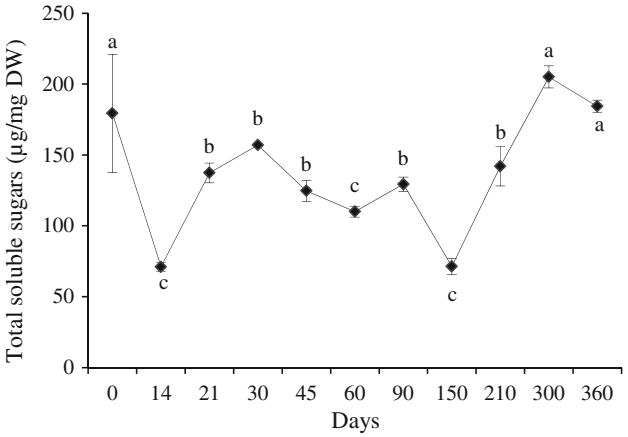

Fig. 1 Changes of total soluble sugars levels during SE of the African oil palm as a function of cultivation time. *Lowercase letters* represent significant differences among the days of cultivation, according to Scott–Knott test's at 5 % probability. *Vertical bars* represent average ± SE values

In the process of regeneration and maturation of somatic embryos, i.e., from 150 to 360 days cultivation, the concentrations of soluble sugars in the tissues under cultivation gradually increased, reaching the highest values (200 µg/mg DW) in last two stages of development: differential somatic embryo and plant regeneration. Different results were obtained by Pescador et al. (2008) in SE of *A. sellowiana*, who did not detect significant variations in the levels of total soluble sugars during the stages of maturation of the somatic embryos. However, Cangahuala-Inocente et al. (2009) also studied SE of *A. sellowiana* and reported that the concentrations of soluble sugars in the tissues of the explants under development decrease significantly during the maturation of somatic embryos.

On the other hand, in some stages of embryogenic development, the type of soluble sugar in the tissues of the explants is generally more of a determinant than the actual content of soluble sugars, being in many cases responsible for the regulation of the specific phases of the embryogenic process (Sauter and Van Cleve 1991; Weber et al. 1997). According Delrot et al. (2000) the sensitivity of plant cells to different endogenous sugars, as well as the concentration and transport thereof, may regulate morphogenesis through

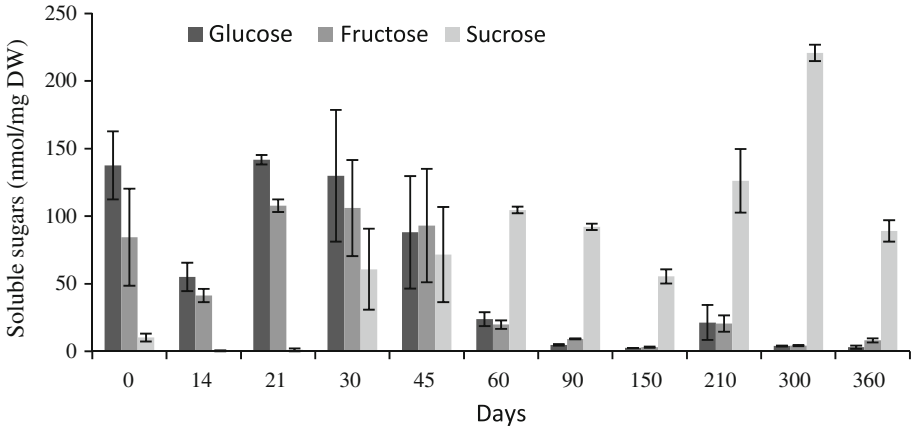

Fig. 2 Levels of sucrose, glucose and fructose during SE of the African oil palm as a function of cultivation time. *Vertical bars* represent average ± SE values

control of cell division at the transcription, translation and post-translation levels.

In the first 21 days cultivation, the soluble sugars present in the developing explants were comprised primarily of the monosaccharides glucose and fructose (Fig. 2). Similar results were obtained by Mengarda et al. (2009) during SE of *A. sellowiana*.

However, after the first 21 days cultivation, we observed a gradual reduction in the levels of these monosaccharides in the composition of the explant tissues, concomitantly synthesizing the disaccharide sucrose, which—starting at 90 days of cultivation—accounted for more than 80 % of the composition of total soluble sugars of the explants under cultivation. Similar results were obtained by Pescador et al. (2008) and Mengarda et al. (2009), who also found a decrease in the levels of fructose and glucose and simultaneous synthesis of sucrose after the process of induction of SE in *A. sellowiana*.

These results corroborate Weber et al. (1997), who stated that during embryogenic development, the monosaccharides fructose and glucose, as well as the disaccharide sucrose, are usually associated with the regulation of different stages. For these authors, fructose and glucose are responsible for controlling growth and cell metabolism, while sucrose is responsible for the regulation of cell differentiation and storage of reserve substances.

Starch

After 14 days cultivation, there were significant amounts of starch in the tissues of the developing explants, approximately 3 % of hydrolysis (Fig. 3). Due to the fact that the zygotic embryo, used as the initial explant, has virtually no starch in the composition of its tissues, it was found that in the SE of African oil palm, the synthesis of this compound occurs during the first 2 weeks of cultivation. Magnaval et al. (1991) and Kanchanapoom and Domuoas (1999), studying SE from *Cocos nucifera* and *E. guineensis*,

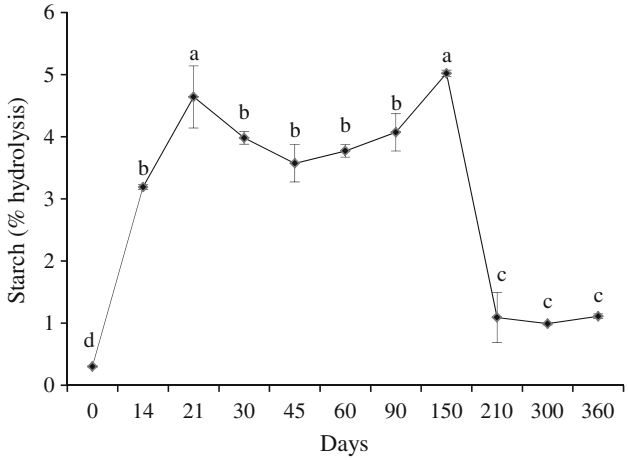

Fig. 3 Changes of starch levels during SE of the African oil palm as a function of cultivation time. *Lowercase letters* represent significant differences among the days of cultivation, according to Scott–Knott test's at 5 % probability. *Vertical bars* represent average ± SE values

respectively, also observed the accumulation of significant amounts of starch in early embryogenic development. Such results indicate that, in embryogenesis in species of the family Arecaceae, starch synthesis is directly related to the induction of the cell division process.

During the formation of calluses with pro-embryos, i.e., from 90 to 150 days, we also found deposition of starch in the tissues of the explants. According to Silva et al. (2012, 2014), the deposition of starch in this stage of SE of *E. guineensis* occurs mainly in the cortex region of the callus, which is subsequently used as a source of metabolic energy by meristematic cells in intense division and differentiation.

In contrast, during the phases of regeneration and maturation of somatic embryos, i.e., from 150 to 360 days cultivation, there was a significant reduction in the levels of starch in the developing explant tissues. In histochemical analysis, Silva et al. (2014) also observed a significant

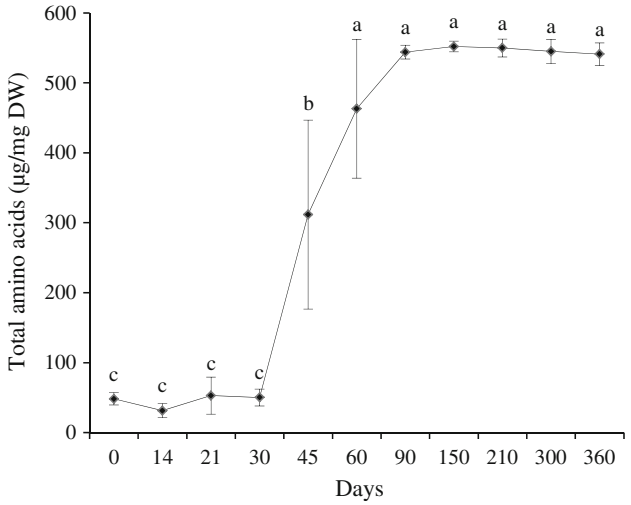

Fig. 4 Changes in total amino acids levels during SE of the African oil palm as a function of cultivation time. *Lowercase letters* represent significant differences among the days of cultivation, according to Scott–Knott test's at 5 % probability. *Vertical bars* represent average ± SE values

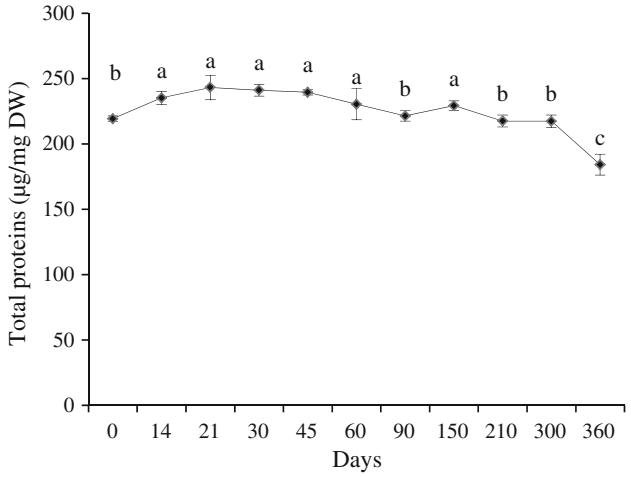

Fig. 5 Changes in total proteins levels during SE of the African oil palm as a function of cultivation time. *Lowercase letters* represent significant differences among the days of cultivation, according to Scott–Knott test's at 5 % probability. *Vertical bars* represent average ± SE values

reduction in the amount of starch grains in the somatic embryo cells of *E. guineensis* in the process of maturation and regeneration. According to Martin et al. (2000), cell division and differentiation of developing embryogenic tissues demand high levels of metabolic energy, which can then be supplied through hydrolysis of starch.

On the other hand, the results obtained in this study also suggest that the metabolism of starch, in addition to being responsible for providing metabolic energy for the cells undergoing division and differentiation, is directly related to maintaining favorable osmotic potential of the explants, since concentrations of soluble sugars and starch were inversely proportional throughout embryogenic development. These results corroborate Chaves-Filho and Stacciarini-Seraphin (2001), who assert that the intracellular regulation of osmotically active solutes, such as some of the soluble sugars, is an important mechanism developed by plants to control their osmotic potential.

Free amino acids

From 30 to 90 days cultivation, there was an accentuated synthesis of amino acids in the developing explant tissues, with an increase of more than 10 times in the levels of these compounds (Fig. 4). Kumar and Kumari (2011) also found, in the formation of embryogenic calluses of *Carthamus tinctorius*, a rapid rise in the levels of free amino acids in tissues under cultivation. For Fehér et al. (2003), this sudden increase in the levels of total free amino acids in the explant tissues may have occurred as a result of increased metabolic activity of the cultures, stimulated by the presence of auxin in the nutrient medium, causing physiological and biochemical changes in the growing plant cells. On the other hand, this rapid elevation in the levels of total free amino acids in the culture tissues may also be related to the high concentrations of nitrogen compounds in the nutrient medium, thus favoring the synthesis of amino acids.

However, the levels of total free amino acids present in the explant tissues stabilize from 90 days cultivation, after the formation of embryogenic calluses, and remain constant until the plant regeneration phase, verified after 360 days. Different results were found by Booz et al. (2009), when studying the biochemistry of the processes of maturation and regeneration of somatic embryos of *A. sellowiana,* who observed a continuous decrease of the levels of total free amino acid in the explants throughout the different stages of development. According to the authors, this decrease in the total free amino acid content throughout the later stages of SE is most likely related to the increase of protein synthesis. However, in this study, it was found that the protein levels undergo a slight decrease at the end of embryogenic development (Fig. 5), which may explain the fact that the total free amino acid content remains stable in these stages of cultivation.

Regarding the composition of the total free amino acids in SE, we found that arginine, glutamine, asparagine, alanine, threonine, glycine, serine, proline, leucine and histidine were the most relevant amino acids (Table 1).

It was observed that the highest concentrations of arginine are present up to half way through the primary callus

Table 1 Levels of the most relevant essential free amino acids in the composition of total free amino acid during SE of the African oil palm as a function of cultivation time

Amino acids (μmol/mg DW)	Days										
	0	14	21	30	45	60	90	150	210	300	360
Arginine	4.90 ± 1.43b	7.81 ± 1.9a	9.75 ± 1.9a	9.07 ± 1.1a	5.83 ± 1.2b	4.20 ± 0.2b	3.74 ± 0.5b	3.02 ± 0.09b	4.53 ± 1.2b	4.99 ± 0.4b	3.15 ± 0.1b
Glutamine	1.45 ± 0.03b	0.80 ± 0.07b	0.75 ± 0.03b	1.17 ± 0.23b	1.15 ± 0.07b	1.53 ± 0.09b	1.80 ± 0.2b	0.94 ± 0.07b	5.05 ± 1.1a	1.06 ± 0.1b	1.21 ± 0.1b
Asparagine	5.2 ± 0.0a	0.03 ± 0.03d	0.1 ± 0.07d	0.89 ± 0.03d	2.30 ± 1.1b	2.83 ± 0.1b	2.76 ± 0.1b	1.39 ± 1.0c	1.55 ± 0.2c	0.06 ± 0.03d	2.40 ± 0.2b
Alanine	0.00 ± 0.0b	0.00 ± 0.0b	0.00 ± 0.0b	0.03 ± 0.03b	0.3 ± 0.2b	2.29 ± 0.6a	2.60 ± 0.3a	2.19 ± 0.6a	3.32 ± 0.3a	3.03 ± 0.3a	1.37 ± 1.3b
Threonine	17.3 ± 6.3a	11.31 ± 1.9b	9.80 ± 2.3b	6.58 ± 2.0c	3.49 ± 0.8c	0.68 ± 0.1c	0.75 ± 0.2c	0.21 ± 0.1c	2.31 ± 0.8c	0.52 ± 0.2c	0.1 ± 0.07c
Glycine	10.49 ± 4.7a	12.23 ± 0.8a	12.9 ± 2.9a	9.41 ± 4.3a	2.33 ± 1.6b	0.85 ± 0.2b	0.33 ± 0.03b	0.14 ± 0.07b	0.17 ± 0.03b	0.02 ± 0.03b	0.00 ± 0.0b
Serine	0.1 ± 0.07b	0.00 ± 0.0b	0.1 ± 0.1b	1.17 ± 0.6b	1.34 ± 0.7b	1.93 ± 0.2a	2.93 ± 0.6a	0.88 ± 0.3b	3.92 ± 2.2a	0.52 ± 0.2b	0.12 ± 0.03b
Proline	0.00 ± 0.0b	0.12 ± 0.13b	1.20 ± 1.0b	2.83 ± 1.3a	0.15 ± 0.01b	0.1 ± 0.03b	0.00 ± 0.0b	0.04 ± 0.03b	0.16 ± 0.01b	0.51 ± 0.1b	0.22 ± 0.03b
Leucine	3.00 ± 1.5b	6.13 ± 3.3a	6.46 ± 0.5a	4.79 ± 1.0a	6.68 ± 1.3a	3.79 ± 0.2a	4.74 ± 0.4a	2.04 ± 1.0a	3.74 ± 0.5a	3.44 ± 0.2a	2.76 ± 1.4a
Histidine	0.00 ± 0.0a	0.1 ± 0.07a	0.13 ± 0.01a	0.36 ± 0.1a	0.02 ± 0.02a	0.79 ± 0.2a	0.11 ± 0.06a	2.01 ± 1.2a	0.25 ± 0.1a	1.51 ± 0.8a	0.79 ± 0.2a

Mean followed by the same letter in a line indicate significant differences among treatments ($P < 0.05$) according to Scott–Knott test. Data represent average ± SE values

induction process, or until around 30 days of cultivation, with roughly 9 μmol/mg DW. After this period, the concentrations of arginine quickly decreased in the explant tissues, and remained statistically identical through the end of cultivation. Arginine is an amino acid that, aside from serving as a precursor for the synthesis of other amino acids, is one of the main precursors in the biosynthesis of polyamines, which is directly related to the acquisition of embryogenic competence in embryogenic cultures (Wu et al. 2009). Similar behavior was found in the levels of glycine and threonine, which also show their highest rates up to mid-way through the primary induction process, i.e., up to around 30 days of cultivation, with roughly 9 μmol/mg DW. After this period of cultivation, concentration of this amino acid in the explant tissues decreased, and remained statistically identical through the end of the process.

A sharp reduction in the levels of asparagine was observed in the explant tissues already in the first 14 days, falling from 5.2 μmol/mg DW to only 0.04 μmol/mg DW during this period. Then, still in the callus induction stage, we also observed a significant deposition of this compound in the explant tissues, subsequently mobilized in the somatic embryo maturation phase. According to Neuberg et al. (2010), due to its high solubility, asparagine is a major form of transport and storage of nitrogen in higher plants. This may help explain the large variations suffered by the levels of this amino acid throughout embryogenic development.

With regard to the amino acids alanine and serine, we found that the values thereof in the composition of total free amino acids are fairly low until the last period of the process of primary callus induction, i.e., until 60 days cultivation, hovering around 0.15 μmol/mg DW. However, after this period of cultivation, there was a synthesis of about 2.5 μmol/mg DW, in both compounds, subsequently mobilized in the stage of regeneration, in the case of alanine, and the regeneration and maturation stages, in the case of serine.

Regarding glutamine, we found that the concentrations thereof in developing tissues remain statistically identical in virtually all periods of cultivation, with the exception of 210 days, i.e., with the exception of the process of maturation of somatic embryos, which experienced a sharp rise, reaching more than 5 μmol/mg DW in this stage. Dias et al. (2009) during the maturation of embryos of *Ocotea catharinensis* also noted an accumulation of glutamine in the tissues of the embryos under cultivation. According to the author, this amino acid is considered one of the main precursors for the synthesis of other amino acids, and its accumulation in this stage of development is most likely related to its role as a source of nitrogen for the metabolism of other amino acids during the regeneration process.

Also in the metabolism of proline, no significant statistical differences were found in the different periods of cultivation, with the exception of 30 days, more precisely, mid-way through the process of primary induction, where there was an increase of roughly 2.5 μmol/mg DW in the levels of this compound. In the induction of SE of *Arachis hypogaea*, the increase in endogenous content of proline was also observed (Murch et al. 1999). According to Satya-Narayan and Nair (1990), the accumulation of this amino acid in plant tissues is generally related to stress conditions. Furthermore, accumulation of proline during induction of SE is possibly associated with stress inherent in the cell deprogramming and reprogramming induced by high auxin concentrations in the nutrient medium (Tarmizi and Marziah 2000).

The amino acids leucine and histidine, although they were relevant in the composition of total free amino acids during embryogenic development, showed no statistical differences between them in the different cultivation times. These results were different than those verified by Kamada and Harada (1979) and Nascimento (2009), who reported the synthesis of these compounds in the explants of *Daucus carota* and *Bactris gasipae* during SE. However, according to the authors, the formation of leucine and histidine in the cultures resulted in an increase in the induction of non-embryogenic calluses and a strong inhibition of SE, respectively. These results corroborate Miflin and Lea (1976), who assert that such compounds, in addition to not being metabolized by plant organisms, also have the characteristic of not being converted into other amino acids, hindering the reduction thereof in tissue under cultivation, as well as affecting the development embryogenesis, since energy and resources are spent for its synthesis.

Total proteins

After 14 days inoculation of the initial explants, the onset of significant accumulation of total proteins was observed in the primary callus tissues (Fig. 5). In *Bactris gasipaes*, this significant increase in the levels of total protein in the composition of the explants after the first 15 days of SE was also observed (Nascimento 2009). According to Fehér et al. (2003), during the stage of induction of SE, the acquisition of embryogenic competence by the somatic cells, carried out through the dedifferentiation and reprogramming thereof, involves the expression of several proteins. According to Silva et al. (2014), proteins involved in the accumulation of starch, in cell division, and in abiotic stress are expressed during induction of SE of *E. guineensis*.

However, from 14 to 150 days, it was found that the quantity of total proteins in the explant tissues remain statistically identical, around 220 μg/mg DW. These results were different than those obtained by Nieves et al. (2008) and Cangahuala-Inocente et al. (2009), who observed increased rates of total protein during the progression of the embryogenic development stages in the SE of *Saccharum* sp. and *A. sellowiana*, respectively. According to Silva et al. (2014), in this stage of somatic embryo development of *E. guineensis*, in addition to proteins related to protein processing, energy metabolism and nitrogen metabolism being expressed, proteins related to oxidative stress, accumulation of starch, and cell division are expressed as well.

After 150 days, during the stages of maturation and regeneration of somatic embryos, we observed that the levels of total proteins in the tissues of somatic embryos declined gradually, reaching their lowest values in the final stage of the process, where the concentration thereof in explant tissues reached levels of less than 200 μg/mg DW for the first time. According to Aberlenc-Bertossi et al. (2008) and Sghaier-Hammami et al. (2009b), in this stage of SE in *Phoenix dactylifera* and *E. guineensis*, respectively, proteins of the glycolytic pathway as well as storage proteins, especially globulins, are mainly expressed. However, at this stage of the cultivation, the processes of accumulation and mobilization of proteins are concomitant, which could explain the decrease in total protein levels observed in the present study, in the final stages of SE.

Conclusion

The results presented here may help to elucidate the biochemical and physiological changes that occur during embryogenesis of plants, and may assist in the optimization of protocols. The levels of total soluble sugars in the explant tissues at the onset of induction of SE fall rapidly, in contrast to what is observed in the regeneration and maturation processes, in which the concentrations of total soluble sugars in the cultures gradually increase. Regarding starch, the concentrations are inversely proportional to the sugars virtually throughout embryogenic development. Starting at 30 days until the formation of embryogenic calluses, there is an accentuated synthesis of total free amino acids in the explant tissues. In this stage, there is a significant increase in the levels of alanine and serine in the explant tissues. After the formation of embryogenic calluses, the levels of total free amino acids present in the cultures become stable, and remain constant until the end of cultivation, just as with the levels of total proteins, which increase significantly at the onset of induction, undergoing slight changes during the remainder of cultivation.

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