In Vitro Cell.Dev.Biol.—Plant (2014) 50:166–175
DOI 10.1007/s11627-013-9563-3

EMBRYOGENESIS/SOMATIC EMBRYOGENESIS

Dynamics of physiological and biochemical changes during somatic embryogenesis of *Acca sellowiana*

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Received: 2 April 2013 / Accepted: 13 September 2013 / Published online: 3 October 2013 / Editor: J. Forster

Abstract Feijoa (*Acca sellowiana* [O. Berg] Burret [Myrtaceae]) is a native fruit species of southern Brazil and northern Uruguay. This species is amenable to somatic embryogenesis and therefore suitable as a model system for comparative studies of zygotic and somatic embryo development. In seed plants, embryogenesis involves three main steps, which are regulated by many factors, such as hormones, proteins, polyamines, and transcription factors. In the present work, the dynamics of protein, sugar, starch, amino acid, and polyamine accumulation were assayed during somatic embryogenesis of *A. sellowiana*. Protein, starch, amino acid, and polyamine levels accumulated unevenly during the induction phase of somatic embryogenesis, while the sugar content remained stable. Throughout the different developmental stages of somatic embryogenesis, synthesis and accumulation of proteins and amino acids showed patterns similar to those reported previously during the development of zygotic embryos of this same species. Differential patterns of polyamine accumulation were observed. This is important because these compounds affect the synthesis of other endogenous growth regulators, such as auxinindole-3-acetic acid that is mainly involved in the establishment of embryo polarity. Taken together, the present work brings new insights to the physiological and biochemical dynamics that occur during somatic embryogenesis of *A. sellowiana*.

Keywords Feijoa · Protein · Starch · Sugar · Amino acids · Polyamines

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Introduction

Acca sellowiana (O. Berg) Burret, syn. *Feijoa sellowiana* Berg, commonly known as Feijoa or pineapple guava, is a fruit species native to southern Brazil and northern Uruguay. It has often been employed as a model system for comparative studies of zygotic and somatic embryo development (Cangahuala-Inocente et al. 2009a; b). According to Vasil (2008), this model system of somatic embryogenesis can improve our understanding of cellular totipotency in higher plants. Somatic embryogenesis (SE) in *A. sellowiana* was first described by Cruz et al. (1990). Since then, several studies have been conducted to improve SE protocols (Canhoto and Cruz 1996; Guerra et al. 2001; Stefanello et al. 2005), as well as detailing the histological and biochemical features related to the development of somatic embryos (Cangahuala-Inocente et al. 2004, 2007, 2009b; Pescador et al. 2008, 2012).

Accumulating evidence suggests that the patterns of protein synthesis during development of zygotic embryos are recapitulated during somatic embryogenesis (Winkelmann et al. 2006). The accumulation of proteins (total) is associated with embryo development and maturation through regulation of cell expansion and other biochemical and biophysical features (Jiménez 2001). During embryogenesis, amino acids are important in nitrogen (N) metabolism and protein synthesis, as well as the transition from heterotrophy to autotrophy (Ortiz-Lopez et al. 2000). Sucrose and amino acids, mainly asparagines and glutamine (Gln), are primary sources of carbon and

nitrogen available to the developing soybean embryo (Rainbird et al. 1984). Gln is the main nitrogen source for developing seeds, and while developing cotyledons show limited assimilation capacity for other forms of nitrogen, they essentially show no capacity for assimilation of inorganic nitrogen (Haga and Sodek 1987). Some of these studies have shown an increase in the starch content during the maturation of somatic embryos in concentrations higher than those normally found in zygotic embryos, suggesting metabolic differences between the two developmental processes (Merkle et al. 1995).

Polyamines (PAs) are considered reliable markers of metabolic changes that occur during maturation of somatic embryos since they play important roles in plant developmental processes, such as cell division, regulation of morphogenesis, embryogenesis, floral and fruit initiation and development, fruit ripening, leaf senescence, root growth, and tuberization (Baron and Stasolla 2008). In plants, PAs are commonly associated with biotic and abiotic stresses (for review, *see* Kakkar and Sawhney 2002; Alcázar et al. 2010). They are also associated with the development and morphogenesis of somatic embryos as they are known to regulate endogenous nitric oxide levels (Santa-Catarina et al. 2007), maintain the growth and polarity of somatic embryos (Silveira et al. 2006), and increase endogenous abscisic acid (ABA) levels (Steiner et al. 2007).

A. sellowiana is considered to be a model system of SE in woody angiosperms, which are recalcitrant to this *in vitro* morphogenetic route (Guerra et al. 2013). Comparative studies among zygotic (Cangahuala-Inocente et al. 2009a) and somatic embryogenesis (Cangahuala-Inocente et al. 2009b) in this species provide a better understanding on the bottlenecks underlying developmental pathways. Thus, the present work aimed to evaluate the dynamic changes in the endogenous protein content, total sugars, starch, amino acids, and PAs during somatic embryogenesis of *A. sellowiana.*

Material and Methods

Plant material. Zygotic embryos were excised from seeds of mature fruits collected from plants belonging to the germplasm collection of the Experimental Station of EPAGRI (Santa Catarina State Agricultural Research Agency), which is located in the municipality of São Joaquin, Santa Catarina State, in southern Brazil. Induction of SE was carried out according to Cangahuala-Inocente et al. (2007). Briefly, the seeds were kept in a mixture of water and 1.0% (*v/v*) sodium hypochlorite (NaOCl) overnight. The seeds were then disinfested in a flow cabinet with 2.0% (*v/v*) NaOCl for 20 min, followed by three rinses with sterile water. Afterwards, zygotic embryos were excised from seeds and inoculated in test tubes (22×150 mm) containing 10 mL of LPm culture medium (von Arnold and Eriksson 1981), supplemented with Morel vitamins (Morel and Wetmore 1951), glutamic acid (8 mM), 2,4-dichlophenoxiacetic acid (2,4-D; 20 μM), maltose (3%; *w/v*), and Difco™ agar (0.7%; *w/v*). The pH of the culture medium was adjusted to 5.8 prior to autoclaving at 121°C, 120 kPa for 15 min. Cultures were maintained in a culture room in the dark at 25°C.

Sample preparation and chemicals. Embryogenic cultures and somatic embryos at different developmental stages were obtained following the methodologies described by Cangahuala-Inocente et al. (2004). More specifically, samples were obtained at regular intervals (3 d) from 0 to 30 d of culture and included the whole explant and the competent embryogenic cellular masses, as well as somatic embryos when present. Alternatively, after 70 d culture, somatic embryos were collected and classified according to the developmental stages: globular (G), heart (H), torpedo (T), precotyledonary (PC), and cotyledonary (CT). Somatic embryos were then stored in Eppendorf tubes and kept at −20°C. For each collection time, nine replicates were stored until the samples were processed.

Sodium dodecyl sulfate (SDS), phenylmethylsulfonyl fluoride (PMSF), and Coomassie Brilliant Blue G250 were purchased from Bio-Rad (Hercules, CA, USA). Methanol, ethanol, chloroform, and perchloric acid were purchased from Synth (Diprolab Com. Ltd., São Paulo, Brazil). Anthrone was purchased from VETEC (Rio de Janeiro, Brazil), and other chemicals were obtained from Sigma Aldrich (St. Louis, MO).

Protein measurement. Total proteins were evaluated from three stored samples at each collection time-point. All manipulations were carried out at 4°C. Total soluble protein extraction was performed according to Cangahuala-Inocente et al. (2009b). Three repetitions of samples (300 mg fresh weight [FM] for each time-point) were macerated at 4°C with 1 mL of extraction buffer (pH 7.0) containing 50 mM sodium phosphate dibasic, 0.2 M β-mercaptoethanol, 17.3 mM SDS, and 1 mM PMSF and were centrifuged at 4°C for 20 min at 8, 300×*g*. The supernatant containing total soluble proteins was removed and the pellet stored at −20°C. Soluble proteins were sedimented at 0°C by adding two volumes of 100% ethanol into the supernatant and then centrifuged at 4°C for 20 min at 12,850×*g*. The protein sediment was solubilized in 50 mM sodium phosphate dibasic (pH 7.0). Protein content was determined by the Bradford (1976) method, using bovine serum albumin as standard.

Total sugar and starch determination. The extraction of total soluble sugars was performed according to Shannon (1968). The pellet from the protein extraction was macerated using 2 mL methanol–chloroform–water (MCW; 12:5:3; *v/v*) and

centrifuged for 10 min at 500×*g*. The supernatant was recovered and the pellet re-extracted using 2 mL MCW. One part chloroform and 1.5 parts water were added for each four parts of supernatant, followed by a centrifugation step at 500×*g* for 10 min, from which two phases were obtained. The upper aqueous phase was removed for dosage using anthrone at 0.2% (*w/v*) following the procedure of Umbreit and Burris (1960).

The extraction and determination of starch levels were based on the procedure of McCready et al. (1950). The pellets used in the total soluble sugar extraction were ground with 1 mL of 30% perchloric acid and centrifuged for 15 min at 12,850×*g*. The supernatant containing starch was removed, and the pellets were re-extracted twice. The supernatants were combined and the pellets eliminated. For dosage, anthrone at 0.2%*w/v* was used. The sugar and starch concentrations were calculated using glucose as standard. The absorbance was read in a UV–VIS UV-1203 spectrophotometer (Shimadzu) at 620 nm.

Amino acid analysis. Amino acids determination was carried out according to Santa-Catarina et al. (2006). Samples (200 mg) were ground in 6 mL of 80% (*v/v*) ethanol and concentrated in a SpeedVac. Samples were resuspended in 2 mL Milli-Q water and centrifuged at 20,000×*g* for 10 min. The supernatant was filtered through a 20-μm membrane. Amino acids were derivatizated with o-phthaldialdehyde (OPA) and identified by high-performance liquid chromatography (HPLC), using a C-18 reverse phase column (SUPELCOSIL™, 5 μm particle size, L×I.D. 25 cm×4.6 mm). The gradient was developed by mixing increasing proportions of 65% (*v/v*) methanol with a buffer solution (50 mM sodium acetate, 50 mM sodium phosphate, 20 mL L^{-1} methanol, and 20 mL L^{-1} tetrahydrofuranat pH 8.1 adjusted with acetic acid). The gradient of 65% methanol was programmed to 20% over the first 32 min, from 20% to 100% between 32 and 71 min and 100% between 71 and 80 min, at 1 mL min^{-1} flow at 40°C. Fluorescence excitation and emission wavelengths of 250 and 480 nm, respectively, were used for amino acid detection. Peak areas and retention times were measured by comparison with known quantities of standard amino acids. All the analytical grade reagents and solvents used in the amino acid determination were purchased from Sigma-Aldrich or Merck and were used or prepared as recommended by the producer.

Analysis of polyamines. Putrescine (Put), Spermidine (Spd), and Spermine (Spm) were determined according to the procedures described by Silveira et al. (2004). All manipulations were carried out at 4°C, and analyses were performed in triplicate. Samples (200 mg FM) for each collection time were ground in 3 mL of 5% (*v/v*) perchloric acid. After 1 h, the extracted samples were centrifuged for 20 min at 15,000×*g* at 0°C. The supernatants containing free PAs were removed, and the pellets were re-extracted. Supernatants were then combined and the pellets discarded. Free PAs were determined directly from the supernatant, and conjugated PAs were extracted by hydrolyzing 200 μL of supernatant with 200 μL of 12 N HCl for 18 h at 110°C. The samples were dried under liquid nitrogen. The conjugated PAs were solubilized in 200 μL of 5% (*v/v*) perchloric acid.

Free and conjugated PAs were derived by dansyl chloride mixed with acetone at a concentration of 5 mg mL^{-1}. A 40-μL aliquot of sample was added to 100 μL of dansyl chloride, 20 μL of 0.05 mM diaminoheptane, and 50 μL of saturated sodium carbonate. The samples were then incubated in the dark for 50 min at 70°C. After 30 min incubation, dansylated PAs were extracted with 200 μL of toluene. The toluene phase was collected and dried under liquid nitrogen. The dansylated PAs were solubilized in 200 μL of acetonitrile.

Twenty microliters of the dansylated PAs was separated by reverse phase HPLC in a C-18 reverse phase column (Shimadzu SHIM-PACK CLC ODS, 5 μm particle size, L×I.D. 25 cm×4.6 mm). The gradient was developed by mixing increasing proportions of absolute acetonitrile to 10% acetonitrile in water (pH 3.5). The gradient of absolute acetonitrile was programmed to 65% over the first 10 min, from 65% to 100% between 10 and 13 min and 100% between 13 and 21 min. The flow was 1 mL min^{-1} at 40°C. The fluorescence detector was set at 340 nm (excitation) and 510 nm (emission). A mixture of Put, Spd, and Spm was used as standard. All the analytical grade reagents and solvents used for determination of PAs were from Sigma-Aldrich or Merck and were used or prepared as recommended by the producer.

Data analysis. Data were analyzed by ANOVA ($P<.05$) followed by the Student-Newman-Keuls (SNK) test, using Statistica version 7.0 software. When ANOVA and SNK tests could not be used, the mean of three replicates, as well as standard error, was applied to analyze the data.

Results and Discussion

Biochemical changes during the first 30 d of induction of somatic embryogenesis. During the first 30-d culture, corresponding to the induction phase of somatic embryogenesis, the total protein levels decreased and remained constant compared to levels observed in explants at the inoculation time (0.84 mg g^{-1} FM; Fig. 1). This reduction in protein levels over time could be attributed to their consumption in order to activate the cellular metabolism for the establishment of embryogenic competence as reported by Gutmann et al. (1996) for embryogenic cultures of hybrid *Larix* × *leptoeuopaea*. The growth of embryogenic cultures is usually associated with changes in the synthesis and mobilization of proteins, carbohydrates, and lipids. The levels of these substances are

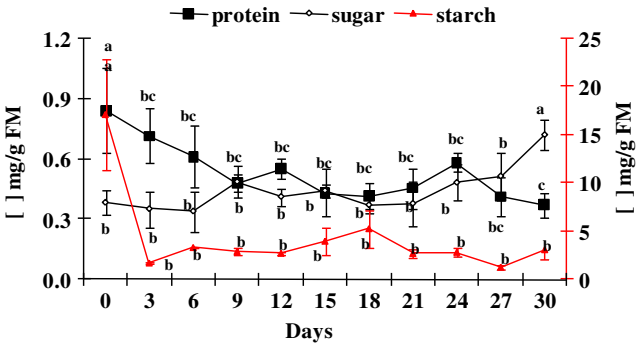

Figure 1. Total protein, sugar, and starch levels during the induction phase of *A. sellowiana* somatic embryogenesis (mean±standard deviation, $n=3$). *Right y* axis (*red line*) corresponds to starch levels. *Left y* axis corresponds to levels of protein and sugar. *Different letters* indicate differences according to the SNK test ($P<.05$). The *absence of letters* indicates no significant differences were observed among treatment means.

variable at the different stages of cell culture (Lulsdorf et al. 1992), where they act in the signal transduction pathway or in the supply of substrates and energy required for cell growth and morphogenesis (Nomura and Komamine 1995).

In the present work, the starch content in the embryogenic cultures rapidly decreased then stabilized. Sugar levels showed a slight increase after 20 d culture with a rapid increase towards the end of the culture period (Fig. 1). In *Medicago arborea*, the content of reducing sugars and starch distinguished embryogenic from non-embryogenic callus; while high levels of sugars and low levels of starch were observed in embryogenic cultures, the opposite was observed in non-embryogenic cultures (Martin et al. 2000).

Total free amino acid levels peaked at the 6th d in culture (Table 1), a period in which the cotyledons of the zygotic embryo used as explant began their expansion. Following this, amino acid levels decreased, peaking again at the 15th d of culture (Table 1), which coincided with epidermal cell proliferation (Fig. 7). Following this, amino acid levels decreased up to the 24th d, remaining constant until the 30th d of culture (Table 1). Similar results were reported in embryogenic cultures of *Ocotea catharinensis*, which showed an increase in the levels of total free amino acids in the first wk in culture, followed by a significant decrease by the fifth wk (Santa-Catarina et al. 2004). In carrot, a rapid increase in amino acid content was reported during cell proliferation and in the early developmental stages of SE (Kamada and Harada 1984). Amino acids are the main form of nitrogen transport in cells, being also used for protein synthesis in order to support growth and development in tissues with intense metabolic activity (Ortiz-Lopez et al. 2000). In most somatic embryogenesis systems, amino acids supplemented in culture medium are sources of organic nitrogen for the stimulation, induction, and maintenance of this morphogenetic route. Glutamine, for example, is commonly supplemented in culture medium as a source of organic nitrogen (Franklin and Dixon 1994).

In the present work, glutamine, arginine, and asparagine showed the highest level among the amino acids evaluated (Table 1). The high content of glutamine could be ascribed to the supplementation of glutamic acid in the culture medium. Glutamic acid is a precursor of glutamine, as well as the main donor of nitrogen, during anabolism (Bohinski 1991). Similarly, embryogenic callus of *Pinus banksiana* showed high levels of glutamine and asparagine (Durzan and Chalupa 1976). In *O. catharinensis*, these amino acids showed intermediate values, while arginine, γ-aminobutyric acid (GABA), lysine and glutamic acid showed higher levels (Santa-Catarina et al. 2004). In our work, a group of amino acids, including alanine, arginine, GABA, glutamine, glycine, methionine, phenylalanine, ornithine, and tyrosine showed enhanced levels at the 6th d in culture. Another group, asparagine, aspartic acid, and glutamic acid predominated by the 15th d. The other amino acids presented their maximum values in both evaluated periods (6th and 15th d; Table 1).

During the SE induction phase, PA levels increased, peaking at the 9th d of culture, and then decreased (Fig. 2). In the induction phase of *Solanum melongena* embryogenic cultures, an intense cell proliferation was detected and was associated with enhanced PA levels (Yadav and Rajam 1997). In *Quercus ilex*, total PAs were more abundant in embryogenic callus and in both somatic and zygotic immature embryos. Spm was more abundant in embryogenic callus and in immature zygotic embryos than in mature embryos (Mauri and Manzanera 2011). High PA levels are commonly observed in tissues undergoing somatic embryogenesis (Loukanina and Thorpe 2008). In our work, the PA accumulation mainly resulted from high levels of free Put. Free PA increased during the first d in culture then decreased (Fig. 3*A*). In *Citrus sinensis*, increased levels of Spd and Spm were detected in the cultures during the first 20 d, a period coinciding with the inductive phase of somatic embryogenesis (Wu et al. 2009).

Conjugate PAs were not detected from the 6th to the 15th d in culture, but did become evident starting from the 18th d. Conjugate Put was the predominant form of conjugate PA (Fig. 3*B*). The balance among free and conjugated PA forms may be critical in different developmental processes (Torrigiani et al. 1987), and when the conjugated PA levels are high, organogenesis proceeds (Scaramagli et al. 1999). On the other hand, high levels of Put at the beginning of somatic embryogenesis are related to the ability of competent cells to develop somatic embryos (Nieves et al. 2003). Taken together, results for PAs obtained in the present study revealed that synthesis and accumulation of these compounds occurred at the onset of somatic embryogenesis. In this same species, an intensive proliferation of epidermal cells was associated with the accumulation of phenolic compounds with further organization of meristematic centers and proliferation of pro-embryonic cell masses

Table 1. Free amino acids (μg g^{-1} FW) during the induction of *A. sellowiana* somatic embryogenesis throughout the first 30 d culture

	Days after induction											All periods
	0	3	6	9	12	15	18	21	24	27	30	
Alanine	9.7±0.4	24.4±7.0	106.8±7.3	43.5±12.2	36.2±10.9	52.1±16.7	21.31±9.5	31.8±8.9	3.0±0.4	8.3±0.2	9.0±1.9	31.47C
Arginine	75.1±9.6	448.4±81.5	1001.2±57.8	397.9±47.2	404.4±108.9	692.3±199.4	183.4±65.4	230.2±15.8	77.5±8.5	91.6±25.0	58.7±13.6	**332.79AB**
γ-Aminobutyric acid (GABA)	17.9±2.3	48.4±14.5	296.7±31.2	117.7±45.0	72.2±30.7	170.1±23.9	49.5±18.4	109.0±32.9	3.8±0.2	18.9±2.3	41.8±6.8	86.00C
Glutamine	6.2 ±2.2	647.5±176.3	1426.2±105.8	478.5±63.2	422.5±145.0	598.4±85.4	229.1±107.4	211.2±60.7	17.1±1.0	53.2±8.7	53.5±5.9	**376.67A**
Glycine	4.2±0.4	14.1±3.6	49.7±1.9	16.0±3.9	12.4±3.4	24.6±2.1	6.1±2.3	8.5±1.6	0.6±0.0	1.3±0.2	1.3±0.1	12.60C
Methionine	0.3±0.5	5.9±1.3	13.9±0.6	3.7±0.5	1.6±2.8	7.7±2.0	3.2±0.7	0.0	0.0	0.0	0.0	3.30C
Phenylalanine	2.4±0.6	10.7±2.9	26.1±0.9	6.4±0.5	8.4±2.5	16.0±3.0	4.1±1.7	5.4±0.6	1.8±0.5	1.4±0.3	1.5±0.3	7.67C
Ornithine	0.6±0.1	1.6±0.3	2.4±0.2	0.8±0.3	0.6±0.5	1.2±0.3	0.8±0.2	0.0	0.0	1.0±0.3	1.0±0.5	0.90C
Tyrosine	1.4±0.1	2.9±0.7	12.0±0.7	2.7±0.4	2.4±0.9	3.4±0.4	1.2±0.4	1.3±0.3	0.8±0.2	0.8±0.2	0.9±0.1	2.70C
Asparagine	22.2±3.0	118.5±32.0	347.8±14.1	160.9±28.2	315.9±70.2	761.2±162.3	165.2±54.0	348.9±29.2	168.2±18.9	117.2±28.4	127.7±16.0	**241.24B**
Aspartic acid	4.0 ±2.6	11.3±2.5	27.9±1.4	17.8±2.4	39.6±8.2	89.7±16.8	32.9±9.1	45.5±0.4	10.8±0.2	12.8±2.0	15.9±3.0	28.02C
Glutamic acid	1.7±0.4	4.2±1.0	13.6±4.6	9.5±0.2	44.1±6.0	145.8±29.1	59.7±19.2	100.7±7.6	20.3±0.7	33.2±7.4	69.4±22.6	45.64C
Histidine	28.3±3.9	41.8±9.9	119.2±7.6	40.8±7.0	49.3±16.3	106.6±27.8	30.1±12.5	39.2±2.1	15.2±2.2	15.1±2.1	14.4±1.6	45.46C
Isoleucine	2.2±0.4	8.1±1.9	21.9±1.2	7.1±0.6	11.3±3.2	23.0±6.4	5.0±3.1	4.3±0.8	0.8±0.2	1.1±0.0	0.9±0.1	7.79C
Leucine	2.0±0.6	19.1±4.2	49.4±2.8	16.1±2.0	22.1±5.6	44.6±11.3	9.6±5.5	8.8±1.9	1.3±0.1	2.2±0.2	1.9±0.1	16.09C
Lysine	5.7±0.4	15.2±2.8	40.3±4.2	15.6±2.6	16.9±6.2	32.0±11.2	7.1±2.8	7.9±1.2	3.1±0.0	3.7±0.8	3.2±0.3	13.70C
Serine	2.9±1.2	22.3±5.6	61.7±4.7	26.1±6.6	31.6±8.7	56.3±17.8	16.6±6.6	21.6±2.8	5.4±0.4	7.8±0.6	7.5±0.7	23.62C
Threonine	3.7±0.9	5.9±1.6	16.9±0.9	6.7±1.4	9.3±2.6	20.5±4.1	4.3±1.9	7.6±0.6	1.3±0.1	1.8±0.3	2.4±0.5	7.32C
Tryptophan	0.8±0.2	2.7±0.6	9.6±0.5	2.6±0.6	6.0±1.2	12.5±3.8	2.9±0.7	7.7±0.4	2.9±0.2	2.9±0.5	3.3±0.5	4.90C
Valine	3.5±1.1	12.5±2.7	33.5±2.4	11.1±1.9	14.9±3.8	26.8±7.3	6.5±3.1	6.7±1.0	1.7±0.2	2.2±0.0	1.6±0.1	11.01C
Total	194.6E	1465.6C	**3676.7A**	1381.6CD	1521.7C	2884.9B	838.6DE	1196.3CD	335.5E	376.4E	415.9E	

Data are presented as mean±standard error (n=3). *Different letters* indicate differences according to the SNK test (P<.05)

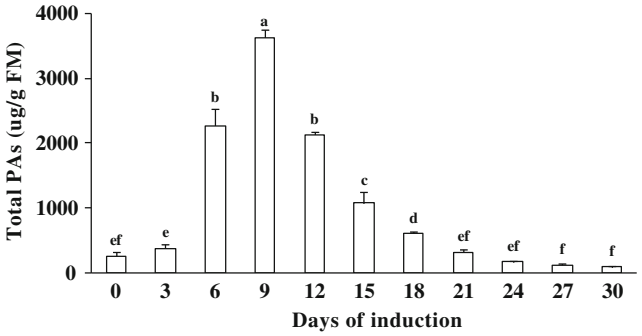

Figure 2. Total polyamines (PAs) during the induction phase of *A. sellowiana* somatic embryogenesis (mean±standard deviation, $n=3$). *Different letters* indicate differences according to the SNK test ($P<.05$). The *absence of letters* indicates no significant differences were observed among treatment means.

(Cangahuala-Inocente et al. 2004). *Araucaria angustifolia* embryogenic cultures submitted to two treatments (control and 5 mM GSH) showed the same levels of endogenous PAs during 30 d of incubation. On the other hand, total PA content showed a significant decrease in Put and Spd levels after 15 d in culture, remaining stable until day 30 (Vieira et al. 2012.

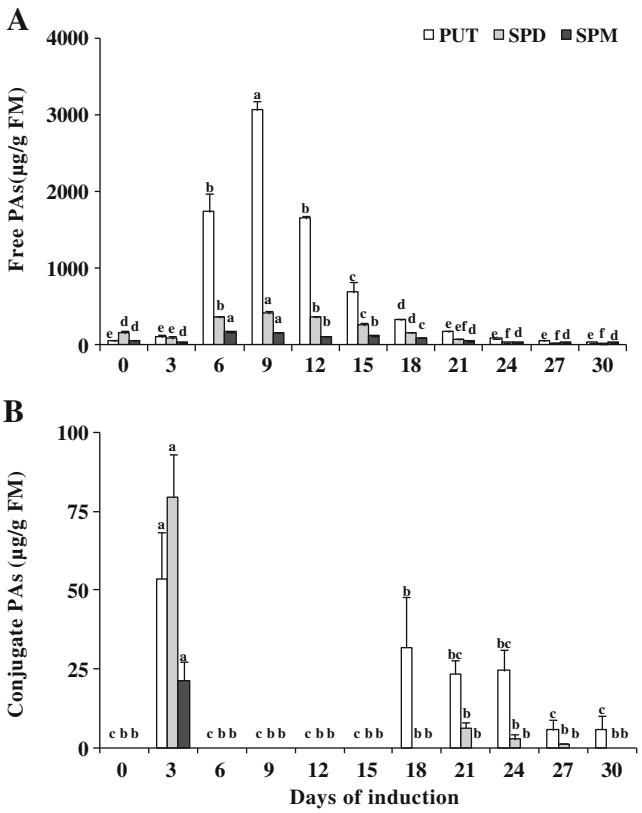

Figure 3. Polyamines (PAs): (*A*) free and (*B*) conjugate during the induction phase of *A. sellowiana* somatic embryogenesis (mean±standard deviation, $n=3$). *Different letters* indicate differences according to the SNK test ($P<.05$). The *absence of letters* indicates no significant differences were observed among treatment means.

Biochemical changes during development of somatic embryos. The developmental stages of somatic embryos were established, taking into account the following criteria: (a) globular (G), translucent and spherical globular somatic embryos 0.5–1 mm in diameter; (b) heart (H), heart-shaped somatic embryos, 1–2 mm long; (c) torpedo (T), torpedo-shaped elongated somatic embryos approximately 3 mm long, with histological analysis revealing well-defined procambium and conspicuous shoot and root apical meristems; (d) pre-cotyledonary (PC), white-translucent somatic embryos longer than 3 mm showing incipiently elongated cotyledonary leaves; (e) cotyledonary (CT), white somatic embryos with expanded cotyledonary leaves.

Significant differences were found in the total protein levels among somatic embryos at different developmental stages (0.14–0.67 mg g^{-1} FW; Fig. 4). As for the total sugar levels, significant differences were observed among the different developmental stages of somatic embryos. Starch content peaked at the globular stage, progressively decreased, and then increased again in the cotyledonary stage (Fig. 4). In the same species, cotyledonary-staged zygotic embryos collected 90 d after pollination showed the highest starch content (Pescador et al. 2008; Cangahuala-Inocente et al. 2009a). In somatic embryos of *Medicago sativa*, the high content of sugar was ascribed to the carbon source in the culture medium (Horbowicz et al. 1995). In soybean somatic embryos, the absolute amounts of protein, lipid, and soluble sugar correlated with the accumulation of fresh mass, with a marked decrease when the cultures became senescent (He et al. 2011).

In this work, early-stage somatic embryos (globular and heart) showed significant variation in the total content of free amino acids, in comparison with somatic embryos at more advanced stages, i.e., torpedo and cotyledonary, showing enhanced levels of asparagine and arginine (Table 2). In tissues with intense metabolic activity, amino acids are mobilized in order to support growth and development (Ortiz-Lopez et al. 2000). Thus, in the present work, we suggest that the low levels of total amino acids in the early developmental stages resulted from a demand to support hystodifferentiation events culminating in the full development of somatic embryos.

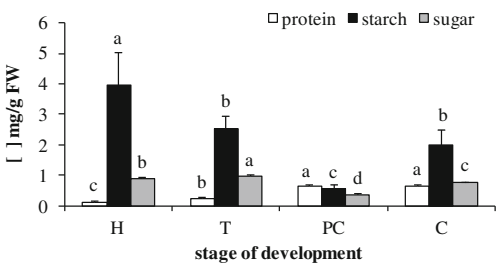

Figure 4. Total protein, starch, and sugar levels (mg g^{-1} FW) at different stages of *A. sellowiana* somatic embryos (mean±standard deviation, $n=3$). *H* heart, *T* torpedo, *PC* precotyledonary, *CT* cotyledonary. *Different letters* indicate differences according to the SNK test ($P<.05$).

Table 2. Free amino acids (μg g^{-1} FW) at different developmental stages of *A. sellowiana* somatic embryos

	Stages			
	G	H	T	CT
Aspartic acid	10.3±3.7	5.9±1.7	34.3±1.7	72.4±3.5
Glutamic acid	14.9±2.9	10.0±0.6	48.9±3.5	88.7±2.2
Asparagine	126.2±19.7	56.7±8.1	**216.5±26.0**	**530.3±8.9**
Serine	4.9±1.3	8.3±1.2	46.3±14.2	117.4±9.0
Glutamine	6.8±1.8	8.5±0.1	32.5±7.1	119.8±12.0
Histidine	18.1±4.2	24.1±1.0	36.5±7.3	106.1±10.8
Glycine	0.9±0.3	0.9±0.0	9.1±0.4	24.5±7.1
Arginine	55.5±25.0	45.0±2.1	**297.4±42.9**	**805.3±51.7**
Threonine	0.6±0.1	1.3±0.1	11.0±1.4	25.8±0.5
Alanine	4.0±0.5	3.0±0.5	41.3±10.1	90.3±2.4
γ-Aminobutyric acid (GABA)	3.4±0.9	5.2±0.4	32.4±9.3	96.7±21.4
Tyrosine	0.0	0.0	0.7±0.1	2.5±0.1
Tryptophan	3.9±1.1	4.9±0.8	15.0±0.7	40.6±7.1
Methionine	0.0	4.5±0.0	0.0	0.0
Valine	3.3±0.9	2.0±0.3	17.6±3.2	39.7±1.3
Phenylalanine	7.2±1.6	16.5±1.0	25.5±4.5	55.4±7.0
Isoleucine	2.1±0.6	1.2±0.0	9.0±2.5	33.5±1.6
Leucine	5.5±1.7	1.9±0.0	19.3±4.8	66.1±4.9
Ornithine	0.0	0.0	0.0	0.0
Lysine	6.0±1.6	4.8±0.0	21.9±6.4	55.2±6.7
Total	273.8C	204.8C	915.2B	**2,370.4A**

Data are presented as mean±standard error ($n=3$). *Different letters* indicate differences according to the SNK test ($P<.05$)

G globular, *H* heart, *T* torpedo, *CT* cotyledonary

Amino acid levels, as determined in the present study, are coincident with patterns observed in zygotic embryos at the same developmental stages (Cangahuala-Inocente et al. 2009a), but at relatively lower levels. However, it should be stressed that in the case of zygotic embryos the presence of teguments and endosperm tissues may have affected the accumulation patterns of these compounds.

During *A. sellowiana* zygotic embryogenesis, in the collecting times corresponding to the evaluated developmental stages, the predominant amino acids were asparagine and glutamine (Cangahuala-Inocente et al. 2009a). However, in the present work, the predominant amino acids in *A. sellowiana* somatic embryos were arginine and asparagine. In plants, asparagine is normally associated with nitrogen transport, and its content in some species are strictly regulated by light (Lam et al. 1998). Sucrose and amino acids, mainly asparagine and glutamine, were the primary carbon and nitrogen sources available for soybean embryo germination (Rainbird et al. 1984). A positive correlation was observed between free asparagine and the storage protein content in

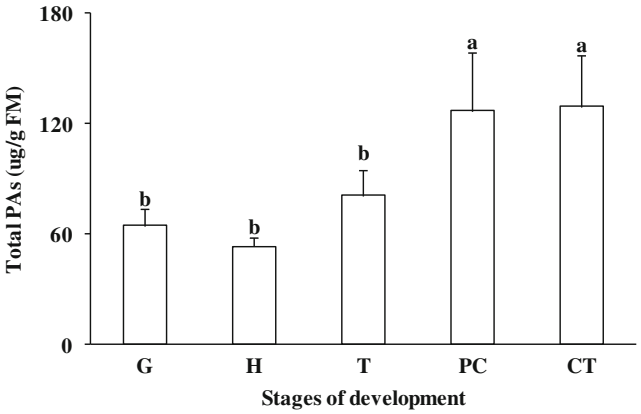

Figure 5. Total Polyamines (PAs) at different developmental stages of *A. sellowiana* somatic embryos. *G* globular, *H* heart, *T* torpedo, *PC* precotyledonary, *CT* cotyledonary (mean±standard deviation, $n=3$). *Different letters* indicate differences according to the SNK test ($P<.05$).

soybean seeds (Hernández-Sebastià et al. 2005). In the present work, ornithine was not detected at any evaluated developmental stage, suggesting that whole synthesized ornithine was converted into arginine. It is also important to stress that since the culture medium was supplemented with glutamic acid, this amino acid could be converted to ornithine via acetylation of derivatives of glutamate, as described by Thompson (1980). It has been shown that arginine acts as a carrier molecule of N,

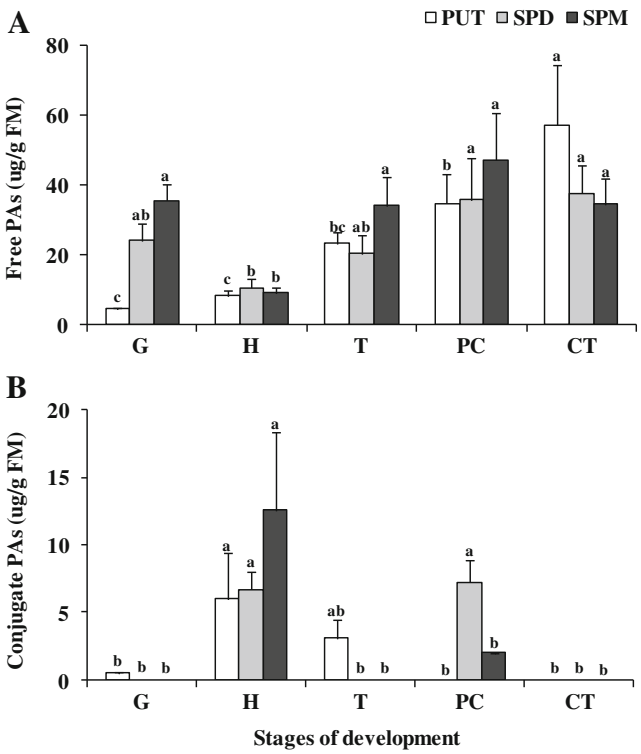

Figure 6. Polyamine (PA) levels: (*A*) free and (*B*) conjugate at different developmental stages of *A. sellowiana* somatic embryos. *G* globular, *H* heart, *T* torpedo, *PC* precotyledonary, *CT* cotyledonary (mean±standard deviation, $n=3$). *Different letters* indicate differences according to the SNK test ($P<.05$).

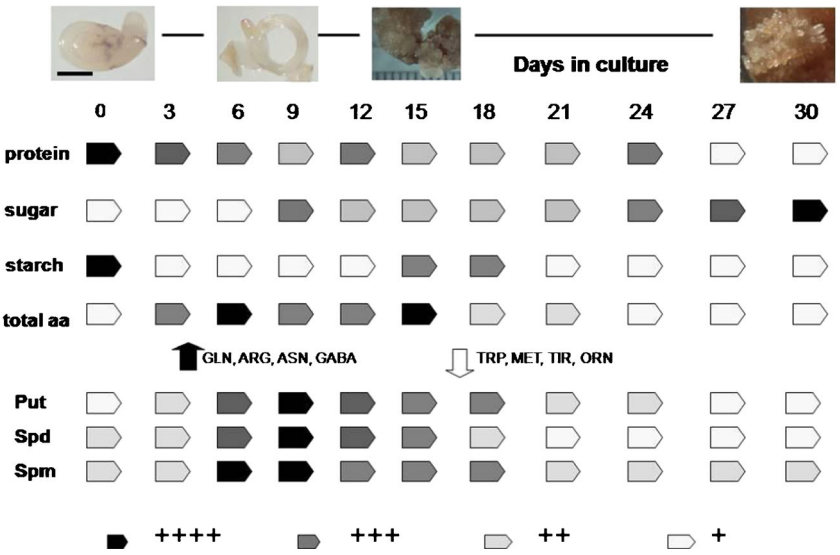

Figure 7. Summary of biochemical changes during the induction phase of somatic embryogenesis of *A. sellowiana.*

and as such, it is an important form of N storage in plants. In seeds, arginine comprises about 40% of the nitrogen stored in proteins, as shown by Micallef and Shelp (1989).

Total PA levels were found to significantly increase when comparing globular-, heart- and torpedo-staged somatic embryos with precotyledonary- and cotyledonary-staged somatic embryos (Fig. 5). In zygotic embryos, PA levels were similar at all developmental stages (Cangahuala-Inocente et al. 2009a). Thus, cotyledonary-staged somatic embryos displayed more total free PAs than cotyledonary-staged zygotic embryos, indicating that the metabolism of PAs is quite different in these two developmental pathways. PAs are synthesized from ornithine, arginine, lysine, and methionine (Bagni and Tassoni 2001). In the present work, correlation among PAs and their precursor amino acids, arginine and lysine, revealed that both showed the highest values in cotyledonary-staged somatic embryos.

Put and Spd are generally the most abundant PAs in plants, while Spm is only present in trace amounts (Bagni and Tassoni 2001). In *A. sellowiana*, free Put increased during all developmental stages of somatic embryos, while an increase in free Spd was observed at more advanced developmental stages (torpedo, pre-cotyledonary and cotyledonary-staged somatic embryos). For free Spm, the values were consistently high at all developmental stages (Fig. 6*A*). In somatic embryos of *O. catarinensis*, high levels of PAs were present in globular embryos and whereas low levels were recorded at cotyledonary stages (Santa-Catarina et al. 2004).

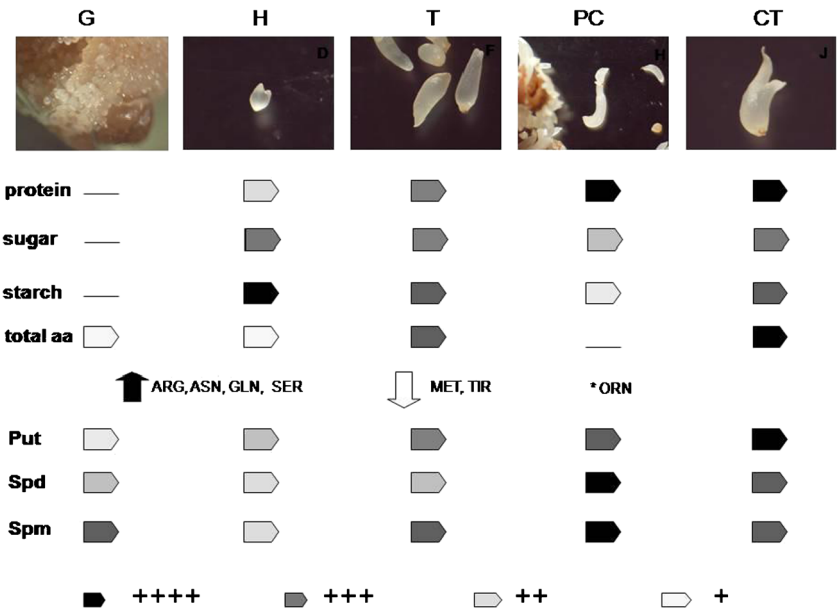

Figure 8. Summary of biochemical changes during the developmental stages of *A. sellowiana* of somatic embryos. *G* globular, *H* heart, *T* torpedo, *PC* precotyledonary, *CT* cotyledonary. *Asterisk* means not present.

Similarly, in *Theobroma cacao* somatic embryo differentiation and development, Spd levels were significantly higher than both Put and Spm levels (Niemenak et al. 2012). *Quercus ilex* showed high levels of Spd, which decreased during maturation and germination (Mauri and Manzanera 2011).

In plants, PAs occur as free molecules together with other molecules, such as amides of hydroxy-cinnamic acid or proteins (Bagni and Tassoni 2001). In the present work, only Put was present as a PA conjugate in the globular and torpedo-staged somatic embryo. Spd and Spm conjugates were present in heart- and precotyledonary-staged somatic embryos in different proportions (Fig. 6*B*). In zygotic embryos of *A. sellowiana*, the conjugate PAs were present at all evaluated developmental stages (Cangahuala-Inocente et al. 2009a). Thus, the patterns of synthesis and accumulation of PAs in *A. sellowiana* somatic and zygotic embryos seems to follow different patterns.

In conclusion, this work sheds light into the dynamics of physiological and biochemical changes occurring during somatic embryogenesis of *A. sellowiana* (Figs. 7 and 8). Variations in the levels of protein, starch, amino acids, and polyamines occurred during the induction phase of somatic embryogenesis. The patterns of synthesis and accumulation of proteins and amino acids at different developmental stages of somatic embryos parallel the patterns observed during the development of zygotic embryos, as observed in previous studies with this same species (Cangahuala-Inocente et al 2009a). Differences in the patterns of synthesis and accumulation of PAs between somatic and zygotic embryos are evident, and these findings are important since PA can affect the synthesis of other endogenous compounds, such as the auxin IAA, which is involved, among others, in the establishment of embryo polarity.

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