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Synthesis and accumulation of free amino acids during somatic and zygotic embryogenesis of *Acca sellowiana* (O. Berg.) Burret

Rosete Pescador[1], Gilberto Barbante Kerbauy[2], Lauricio Endres[3], Hugo Pacheco de Freitas Fraga[1] and Miguel Pedro Guerra[1]*

[1]Plant Genetic Resources, Federal University of Santa Catarina, Rod. Admar Gonzaga, Km 3, Florianópolis, SC, 88040-900, Brazil.
[2]Departament of Botany, Biosciences Institute, University of São Paulo, ZC 11461, São Paulo, SP, 05422–970, Brazil.
[3]Agrarian Science Center, Federal University of Alagoas, Maceio, AL-CEP: 57100-000, Brazil.

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Somatic embryogenesis (SE) is an analogous process to zygotic embryogenesis (ZE); both complex processes are influenced by a significant number of genetic and environmental factors. Amino acids are considered important regulators of morphogenesis in several plant species, therefore the aim of this study was to determine the role of amino acids in embryo ontogeny in *Acca sellowiana*. Endogenous levels of amino acids were quantified at different stages of development during both direct and indirect somatic embryogenesis (DSE and ISE) and during ZE. During ZE, there was an increase in total amino acids between 18 and 27 days after pollination. During ISE and DSE, the highest contents were detected from 3 to 15 days after inoculation, coinciding with the induction of somatic embryos. In ZE, glutamine and asparagine appeared to be fundamental to the process of induction of zygotic embryos. On the other hand, the induction of somatic embryos that appeared require glutamine, gamma-aminobutyric acid (GABA) and glutamic acid. The results suggest the involvement of amino acids in the ontogenesis of zygotic and somatic embryogenesis directly and indirectly in *A. sellowiana*, indicating requirements for specific amino acids for each event of development.

Key words: *Acca sellowiana*, somatic embryogenesis, zygotic embryogenesis, glutamine, asparagine, amino acids.

INTRODUCTION

The pineapple guava [*Acca sellowiana* (O. Berg.) Burret, Myrtaceae] is native to the southern highlands of southern Brazil and northern Uruguay (Ducroquet and Hickel, 1997). In Brazil, it occurs naturally above 800 m altitude, displaying a great variety in form, habitat, size and shape of the fruit (Santos et al., 2009). This species is cultivated in several countries (for example Brazil, New Zealand and Colombia), and its unique flavor makes it a potentially attractive fruit crop for small farmers (Santos et al., 2009). Somatic embryogenesis (SE) of this species studied as a reference model in woody plants was first described by Cruz et al. (1990). Subsequently, it was shown that the number of somatic embryos developed is high, but the conversion rates to seedlings are low,

*Corresponding author. E-mail: mpguerra@cca.ufsc.br. Tel: +55 48 3721-5331.

possibly caused by abnormality in the formation of meristems or the different physiological nature of somatic embryos compared with zygotic embryos (Cangahuala-Inocente et al., 2007; Pescador et al., 2008). Studies related to the accumulation of protein, total sugars, starch, amino acids, polyamines, IAA and ABA in different stages of *A. sellowiana* zygotic embryogenesis (ZE) were also performed (Cangahuala-Inocente et al., 2009). In the same way, studies associated to the effect of gamma-aminobutyric acid (GABA) during SE in this species were carried out, indicating an important role of this amino acid in the SE induction process (Booz et al., 2009). Somatic embryogenesis can occur indirectly, from callus or cells in suspension, or directly from cells of organized structures such as zygotic embryos. In *Acca sellowiana* SE, the formation of a dense layer of meristematic cells originating in the adaxial face of the cotyledons of zygotic embryos has been described. Two patterns of somatic embryo differentiation were observed: one from single epidermal cells and the other from groups of meristematic cells located near the adaxial surface (Canhoto and Cruz, 1996).

Somatic embryogenesis in many ways resembles ZE; both being complex processes influenced by a several factors. Nitrogen seems to be a major factor in morphogenesis and organic nitrogen in the form of amino acids are modulators of a number of important metabolic processes in various plant species (Sen et al., 2002). Besides the synthesis of proteins, amino acids are also related to the primary and secondary metabolism, and may be precursors of plant hormones as well as compounds involved in plant defense. The synthesis of amino acids can therefore directly and/or indirectly control various aspects related to growth and development of plants (Buchanan et al., 2000). The use of nitrogen compounds in the seed is dependent on the developmental stage and the synthesis of certain groups of proteins in different tissues throughout embryogenesis (Dure et al., 1989). The accumulation of storage proteins is considered an indicator of the maturation phase, during late embryogenesis (late embryogenesis abundant - LEA), many of which are related to seed desiccation process, which precedes germination (Hoekstra et al., 2001). Thorpe (1993) postulated that SE was associated with the increase in amino acids such as proline, serine and tyrosine. Kamada and Harada (1984) reported that the total amount of amino acids increased rapidly during the proliferation of cells in the early stages of SE in carrot cultures, suggesting that they have a key role in this event. Moreover, the dynamics of synthesis and accumulation of amino acids in zygotic embryos can serve as a reference model for analysis of these compounds during SE (Durzan and Chalupa, 1976). However, the actual role of amino acids in embryo-genesis remains unclear since the metabolism of amino acids involved in this morphogenetic route has not been fully characterized.

In this study, we evaluated the amino acid contents in different developmental stages of zygotic and somatic embryos in *A. sellowiana*, aiming to establish a possible relationship between these substances and the embryogenic process.

MATERIALS AND METHODS

Zygotic embryogenesis

Unfertilized and fertilized ovules, and embryos at the globular, heart, torpedo and cotyledonary developmental stages of *A. sellowiana* (Figure 1) were collected from plants of the germplasm collection of the Research and Extension Agency of Santa Catarina State (EPAGRI), in the county of São Joaquim, South Brazil (latitude 28° 17' 39", longitude 49° 55' 56", altitude 1415 m). Ovules from non-pollinated flowers in anthesis were considered time zero. About 500 flowers were emasculated and hand pollinated. Over the first 30 days, samples were collected every 3 days, spacing this at every 10 days thereafter until the physiological maturity of the fruit, which occurred 120 days after pollination. Each collection representing the various stages of development consisted of 10 repetitions. All plant material collected was initially frozen in dry ice and then stored at -20°C.

Somatic embryogenesis

For the study of direct somatic embryogenesis (DSE) and indirect somatic embryogenesis (ISE), zygotic embryos were used as explants. Embryos of 0.4 mm length were inoculated in test tubes containing LPm (von Arnold and Eriksson, 1981) culture medium supplemented with 30 $g.l^{-1}$ sucrose, 20 μM 2,4-dichlorophenoxy-acetic acid (2,4-D), 4 mM glutamine, agar (0.7%), pH 5.8. For DSE, the zygotic embryos were initially maintained for 15 days on the culture medium described earlier and then sub-cultured onto 2,4-D-free culture medium. For biochemical analysis, samples were taken every 3 days up to 30 days of incubation. At 70 days of culture, collections were made from embryos at different developmental stages: globular, heart, torpedo and cotyledonary in ISE and DSE. All plant material was frozen in liquid nitrogen and subsequently stored at -20°C.

Extraction and quantification of amino acids

The extraction of amino acids was performed in a solution of methanol, chloroform and water (MCW) at a ratio of 12:5:3. Samples of 200 mg of fresh material, in triplicate were soaked in 10 ml of MCW in liquid nitrogen (N_2). After 3 days at 4°C, the macerate was centrifuged at 5 000 *g* for 10 min and the supernatant collected. The residue was again extracted with 5 ml of MCW and re-centrifuged (as aforementioned). The supernatants were combined and stirred with 5 ml of chloroform and then with 6 ml of deionized water. After separation, the aqueous phase was collected and freeze dried under vacuum to dryness and then resuspended in 2 ml of deionized water. Total amino acids were determined using nor-leucine as standard. The residue was derivatized with feniltiocarbamil (PITC) to form amino-feniltiocarbamil (Baker et al., 1997). The derivatized amino acids were separated by high performance liquid chromatography (HPLC) using a C18 column Novopak (3.9 × 300 mm). The temperature of 45°C and solvent flow

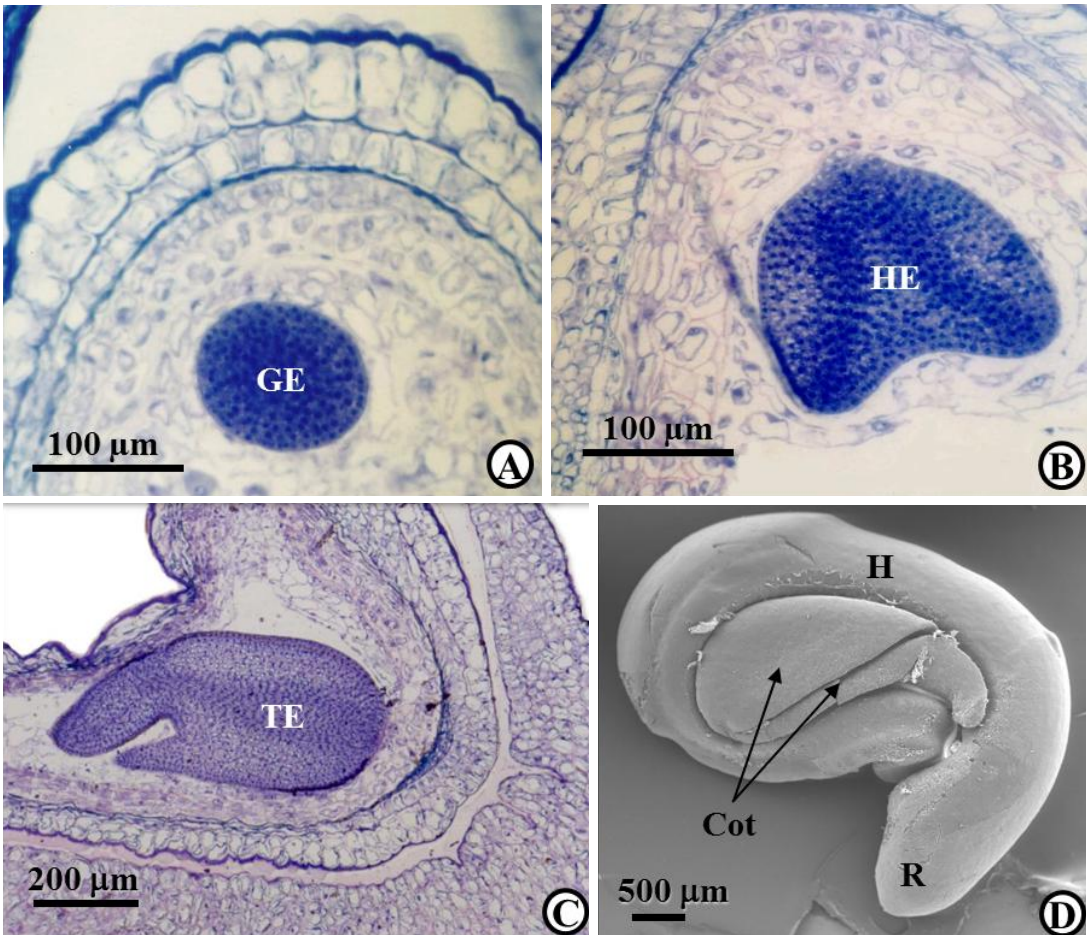

Figure 1. Zygotic embryogenesis developmental stages of *Acca sellowiana*.**A**, Globular zygotic embryo (GE); **B,** Heart zygotic embryo (HE); **C,** Torpedo zygotic embryo (TE); **D,** Cotyledonary zygotic embryo exhibiting radicle (R), hypocotyl (H) and cotyledons (Cot) formed.

of 1 ml.min^{-1} (solvent A: 93.9% of sodium acetate buffer, 6% acetonitrile and 0.1% triethylamine, solvent B: 60% acetonitrile and 40% ultrapure water) were the conditions of HPLC separation. The derivatized amino acids were eluted with the increase in the proportion of the organic phase.

The concentration of each amino acid was calculated by comparing the areas with the standard of known concentration.

RESULTS

Total amino acids

The patterns of synthesis and accumulation of free amino acids in the different samples evaluated are presented in Figure 2. The dynamics observed in these levels seems to have an important role in the three embryogenic pathways studied. No significant changes were observed in total amino acid content in the period from zero to 18 days after pollination. However, the levels of amino acids increased up to the 24th day, when again there was a sharp decrease until 30 days after pollination. The highest levels of total amino acids (6542.12 µmol.g^{-1}mf) were detected 24 days after pollination, a period coinciding with the first division of the zygote. The changes in the levels of free amino acids were practically the same in both ISE and in DSE (Figure 2). Between the times of inoculation and the third day of incubation, tissue contents of total amino acids increased more than six times, decreasing slowly thereafter until 24 days, when levels increased rapidly until the 30th day. The gradual decrease of total amino acids levels in the 3 to 24 days of incubation period was coincident with cell proliferation and formation of somatic embryos.

In the phases of embryogenic development, a decrease in the content of amino acids was observed (Figure 3). In ZE, the total amino acid content of embryos in globular stage was 90.6 µmol.g^{-1}mf, being more than four times lower than in embryos at the cotyledonary stage. In ISE

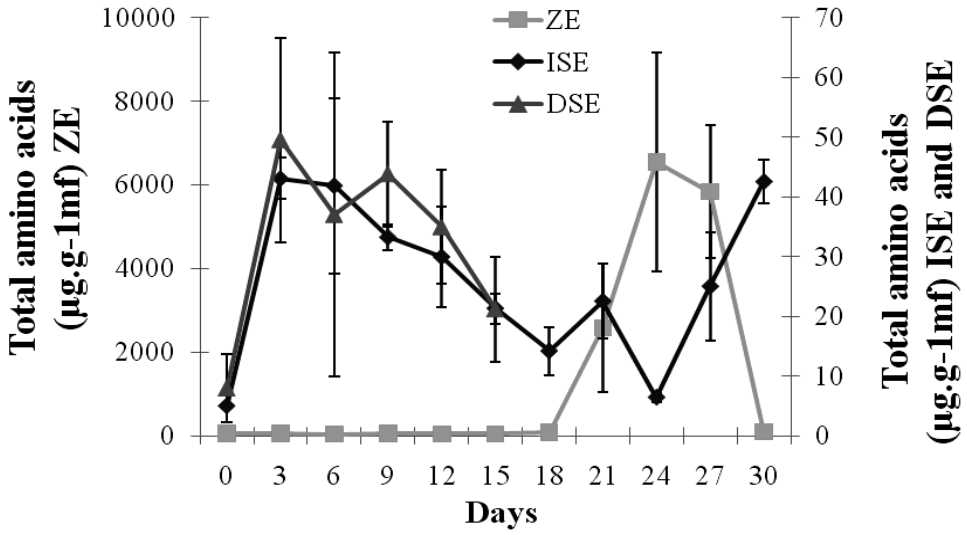

Figure 2. Total amino acids levels over 30 days during zygotic embryogenesis (ZE), indirect somatic embryogenesis (ISE) and 15 days in direct somatic embryogenesis (DSE). The zero time in ZE was unfertilized ovules and in ISE and DSE, the initial explant. Vertical bars represent the standard deviation. Developmental stages of ZE are represented by 21 days: zygote; 30 days: globular stage, for ISE 21 days: globular stage; 24 days: heart stage; 27 to 30 days: torpedo stage, and for DSE 9 days: globular stage; 15 days: heart stage.

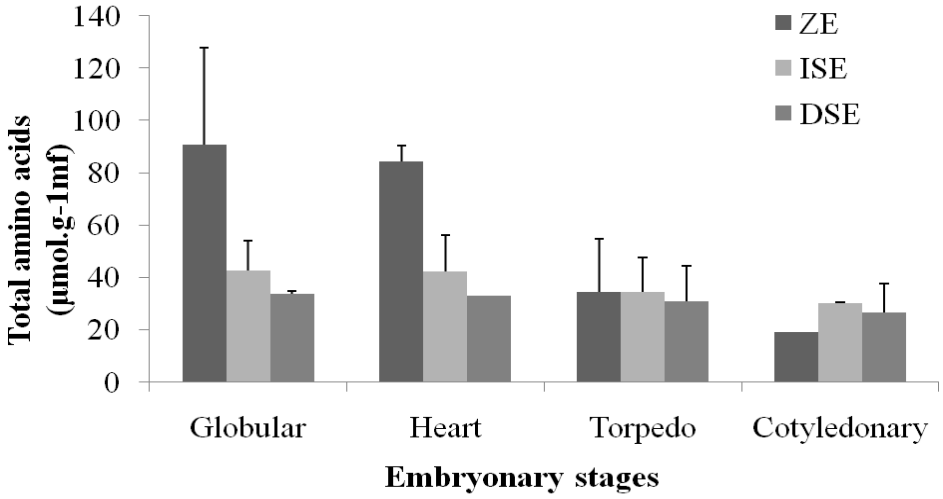

Figure 3. Levels of total amino acids in globular, heart, torpedo and cotyledonary embryos developed during zygotic embryogenesis (ZE), indirect somatic embryogenesis (ISE) and direct somatic embryogenesis (DSE) of *A. sellowiana*. Vertical bars represent the standard deviation.

and DSE, the decrease was 1.4 and 1.3 times, respectively.

Individual free amino acids

Analysis of individual amino acids allowed the identification of those that were prevalent and more significant at the different developmental stages and in the different embryogenic pathways studied. The following amino acids were detected in low concentrations and with only slight variations: alanine, serine, tyrosine, threonine, glycine, histidine, valine, methionine, isoleucine, leucine,

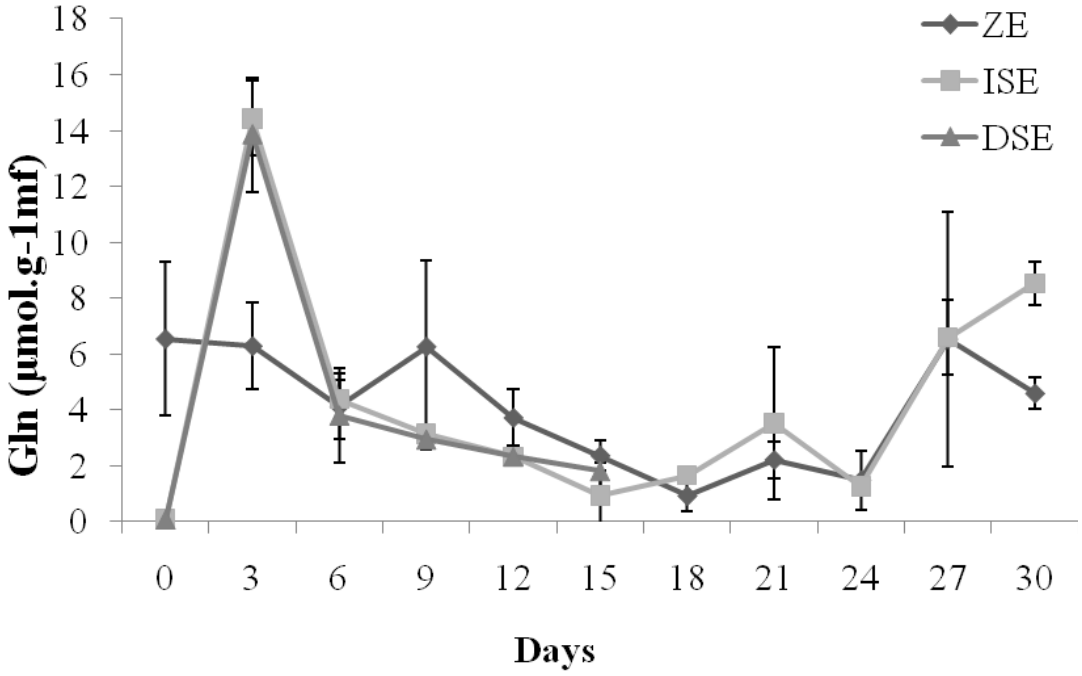

Figure 4. Changes in glutamine (Gln) levels over 30 days during zygotic embryogenesis (ZE), indirect somatic embryogenesis (ISE) and 15 days in direct somatic embryogenesis (DSE). The zero time in ZE was unfertilized ovules and in ISE and DSE, the initial explant. Vertical bars represent the standard deviation. Developmental stages of ZE are represented by 21 days: zygote; 30 days: globular stage, for ISE 21 days: globular stage; 24 days: heart stage; 27 to 30 days: torpedo stage, and for DSE 9 days: globular stage; 15 days: heart stage.

phenylalanine, lysine, tryptophan, aspartic acid and proline (data not shown).

Glutamine

Changes were detected in levels of glutamine over the 30 day period after pollination in the different samples (Figure 4). In ZE, there was a decrease in the levels of glutamine for 24 days after pollination, and from this period a subtle increase until the 27th day was observed followed by a fall. It is important to stress that on the 27th day, formation of pro-embryos was first recorded, suggesting that this decrease would be involved with this stage of embryonic development. Also, as shown in Figure 4, until the third day after inoculation, in both the DSE and ISE, an increase in the levels of this amino acid was detected. In this study, a decrease was observed in the levels of endogenous glutamine from the heart stage of development, reaching the lowest values in the cotyledonary stage in the three embryogenic pathways studied (Figure 5). The levels of glutamine at the globular stage were higher in ZE than ISE and DSE; however, they decreased in the later embryonic stages (torpedo and cotyledonary).

Glutamic acid

Changes were detected in the levels of glutamic acid in the three pathways studied (Figure 6). In ZE, changes in the levels of glutamic acid were small, with the highest concentrations detected 27 days after pollination (4.3 $\mu mol.g^{-1}mf$), a point coinciding with the formation of pro-embryos. Variations in the levels of this amino acid were more pronounced in the ISE and DSE compared with ZE. Both ISE and DSE showed very similar patterns of variation (Figure 6). The highest concentrations were detected during the intense cellular proliferation which corresponded to the 3rd to 15th day of development, with a peak on the 9th day of incubation. In ISE, the maximum value detected was 11.93 $\mu mol.g^{-1}mf$ and DSE was 13.78 $\mu mol.g^{-1}mf$. At ISE, a marked decrease in levels of glutamic acid after 18 days of incubation was observed, with little variation until the 30th day. In globular, heart, torpedo and cotyledonary stages, decreases were observed in the levels of glutamic acid during ZE (Figure 7). However, during both ISE and DSE, no increases in the endogenous levels were observed, except at the cotyledonary stage of the ISE.

Globular zygotic embryos showed the highest content of glutamic acid (3.69 $\mu mol.g^{-1}mf$), whereas in ISE and

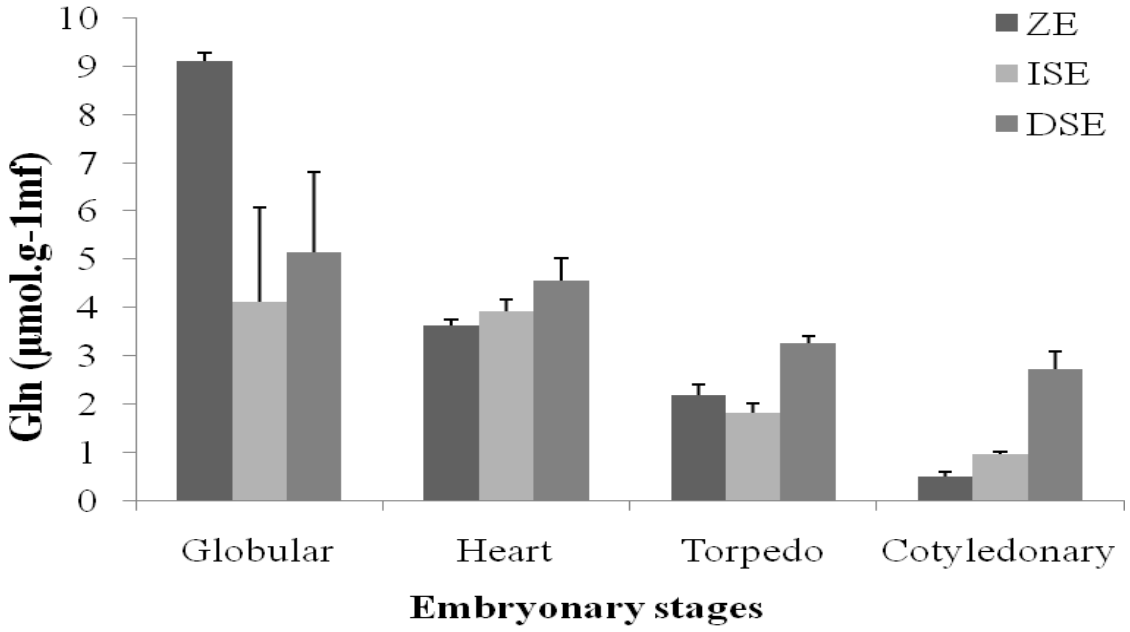

Figure 5. Levels of glutamine (Gln) in globular, heart, torpedo and cotyledonary stages of zygotic embryogenesis (ZE), indirect somatic embryogenesis (ISE) and direct somatic embryogenesis (DSE) of *A. sellowiana*. Vertical bars represent the standard deviation.

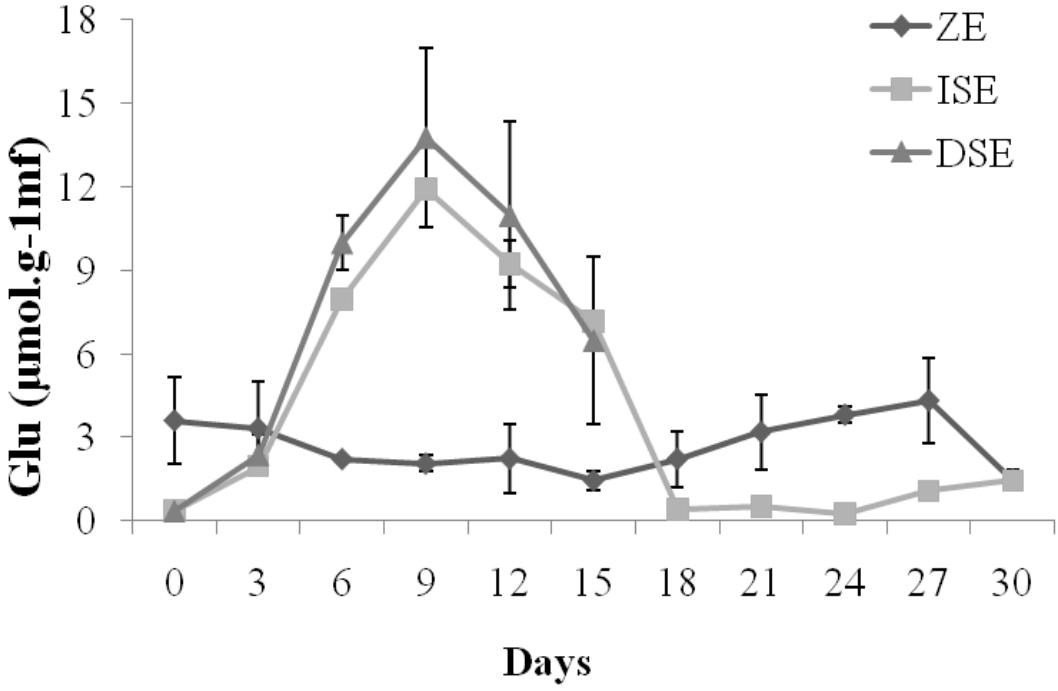

Figure 6. Changes in glutamic acid (Glu) levels over 30 days during zygotic embryogenesis (ZE), indirect somatic embryogenesis (ISE) and 15 days in direct somatic embryogenesis (DSE). The zero time in ZE was unfertilized ovules and in ISE and DSE, the initial explant. Vertical bars represent the standard deviation. Developmental stages of ZE are represented by 21 days: zygote; 30 days: globular stage, for ISE 21 days: globular stage; 24 days: heart stage; 27 to 30 days: torpedo stage, and for DSE 9 days: globular stage; 15 days: heart stage.

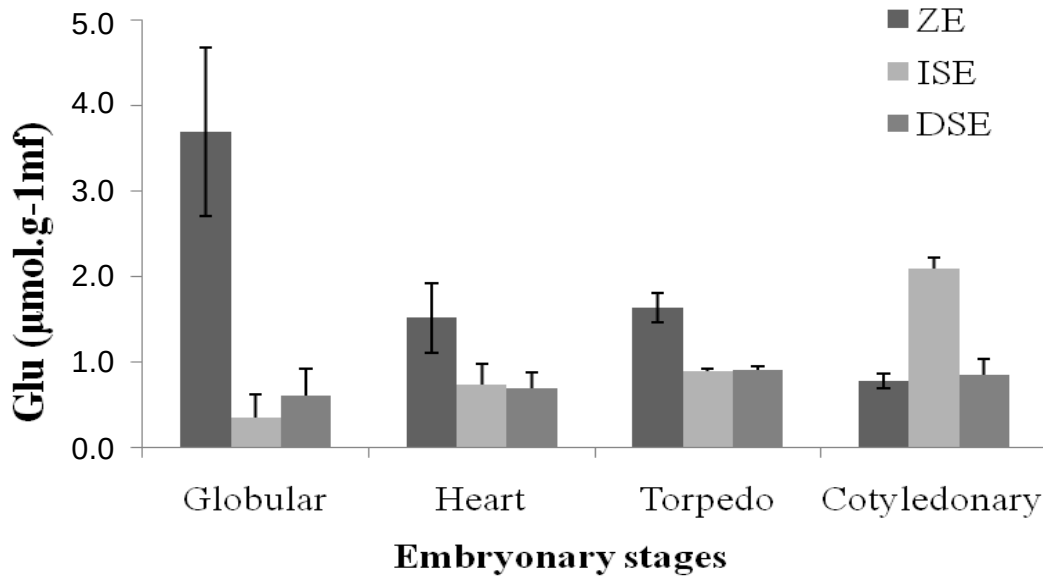

Figure 7. Levels of glutamic acid (Glu) in globular, heart, torpedo and cotyledonary stages of zygotic embryogenesis (ZE), indirect somatic embryogenesis (ISE) and direct somatic embryogenesis (DSE) of *A. sellowiana*. Vertical bars represent the standard deviation.

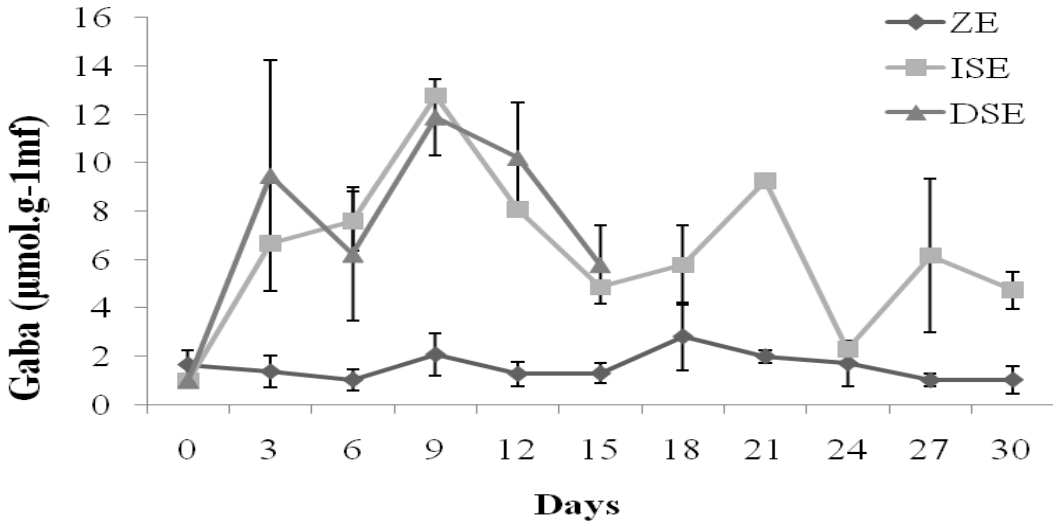

Figure 8. Changes in γ-aminobutiric acid (GABA) levels over 30 days during zygotic embryogenesis (ZE), indirect somatic embryogenesis (ISE) and 15 days in direct somatic embryogenesis (DSE). The zero time in ZE was unfertilized ovules and in ISE and DSE, the initial explant. Vertical bars represent the standard deviation. Developmental stages of ZE are represented by 21 days: zygote; 30 days: globular stage, for ISE 21 days: globular stage; 24 days: heart stage; 27 to 30 days: torpedo stage, and for DSE 9 days: globular stage; 15 days: heart stage.

DSE at that same stage, concentrations were substantially lower (0.34 and 0.61 µmol.g^{-1}mf, respectively). This suggests that amino acid metabolism in the early stages of embryogenesis is dependent on the embryogenic pathway concerned.

γ-Aminobutyric acid

GABA was present in all embryogenetic pathways studied (Figure 8). In ZE, the levels of this amino acid derivative showed no variation during the 30 days analyzed.

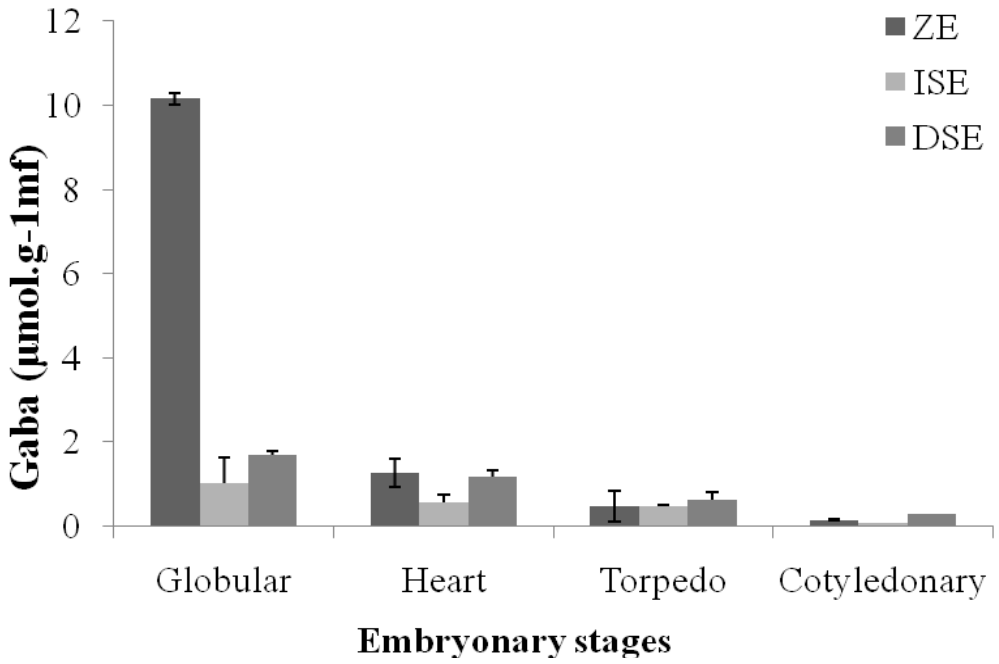

Figure 9. Levels of γ-aminobutiric acid (GABA) in globular, heart, torpedo and cotyledonary stages of zygotic embryogenesis (ZE), indirect somatic embryogenesis (ISE) and direct somatic embryogenesis (DSE) of *A. sellowiana*. Vertical bars represent the standard deviation.

The highest concentrations were detected during ISE and DSE, where the levels of GABA showed a profile very similar, with the highest concentrations detected in the period from 3rd to 9th day after inoculation. The highest concentrations found in the ISE was 12.77 μmol.g^{-1}mf compared with 11.89 μmol.g^{-1}mf during DSE. This period was characterized by substantial cell proliferation and presence of pro-embryos. Figure 9 shows the decrease of GABA content during the embryonic stages. Globular zygotic embryos showed higher levels of this amino acid (10.2 μmol.g^{-1}mf) compared with embryos at this same stage in ISE and DSE, whose contents were 1.1 and 1.7 μmol.g^{-1}mf, respectively. The lowest concentrations were detected in cotyledonary embryos (0.14, 0.08 and 0.28 μmol.g^{-1}mf respectively, for ZE, ISE and DSE).

Asparagine

As shown in Figure 10, asparagine was also related to the induction of ZE in *A. sellowiana*. A rise in the endogenous levels of this amino acid after pollination occurred from the 15th day, reaching at day 30 the value of 62.64 μmol.g^{-1}mf. Importantly, from the 24th day after pollination, the formation of pro-embryos in seeds of *A. sellowiana* has occurred. In the ISE and DSE pathways, the variations in the endogenous content of asparagine showed a different profile from that observed during ZE (Figure 10). In both somatic embryogenic pathways, there was a strong and rapid increase in the levels of this amino acid from the 3rd day, reaching 1.88 μmol.g^{-1}mf in ISE and 2.01 μmol.g^{-1}mf in DSE. These values were higher, respectively, 23 and 25 times those found in the initial explant. Concentrations in both somatic pathways also showed a strong decline, reaching their lowest values between 15 (DSE) and 24 (ISE) days, when an increase began in the concentration of asparagine in the ISE. In the ISE and DSE, from the 3rd day of culture, there was an initial swelling of the explant and the ISE, heart-shaped embryos were viewed from 24 days of culture. With respect to the embryogenic stages shown in Figure 11, we observed a decrease of asparagine levels more consistently in the ZE, which contained 48.42 μmol.g^{-1}mf in the globular stage, dropping to 0.97 μmol.g^{-1}mf at the cotyledonary stage.

DISCUSSION

Contents of total amino acids during the morphogenetic processes of organogenesis and embryogenesis area normally varies as in the case of *Vigna mungo* in which variations were detected indicating the specific demands and roles of amino acids for each event (Sen et al., 2002). In the present study, the decrease in total amino acid content during the embryonic stages of *A. sellowiana*

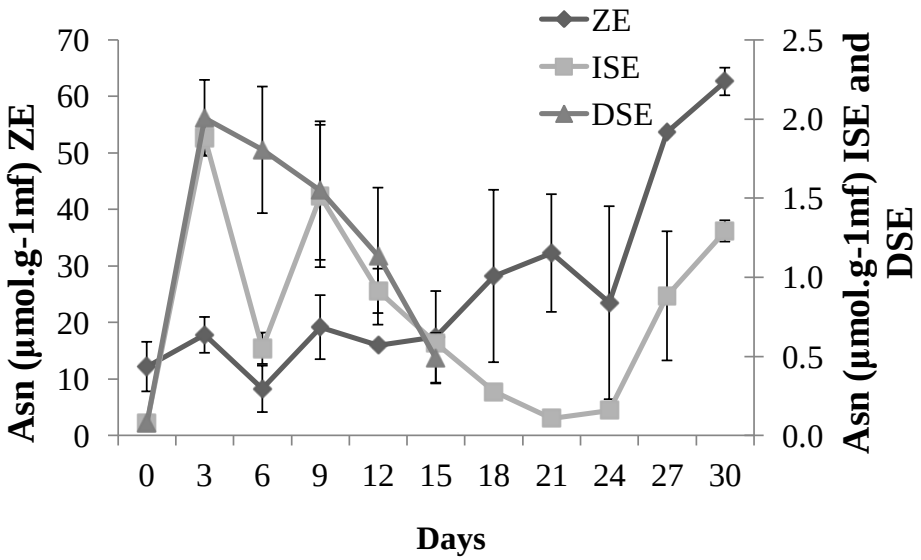

Figure 10. Changes in asparagine (Asn) levels over 30 days during zygotic embryogenesis (ZE), indirect somatic embryogenesis (ISE) and 15 days in direct somatic embryogenesis (DSE). The zero time in ZE was unfertilized ovules and in ISE and DSE, the initial explant. Vertical bars represent the standard deviation. Developmental stages of ZE are represented by 21 days: zygote; 30 days: globular stage, for ISE 21 days: globular stage; 24 days: heart stage; 27 to 30 days: torpedo stage, and for DSE 9 days: globular stage; 15 days: heart stage.

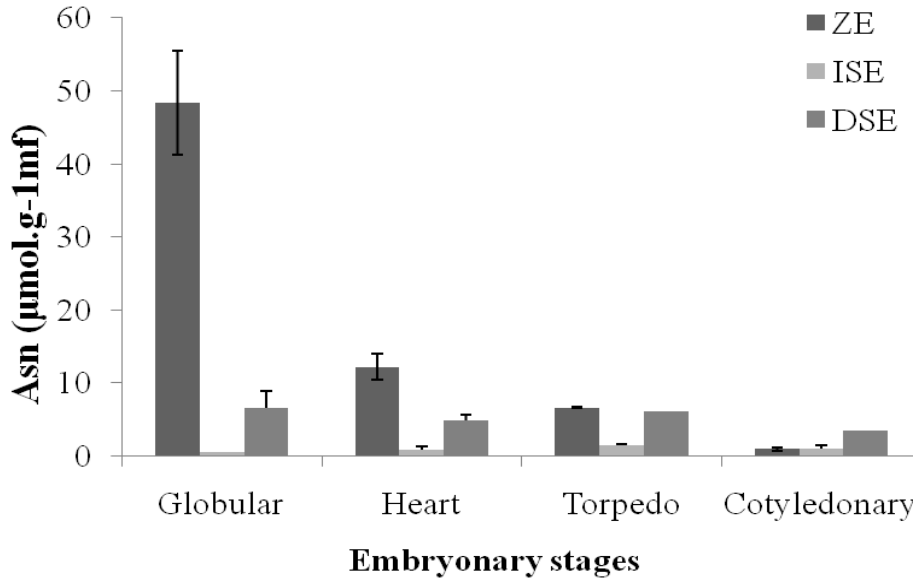

Figure 11. Levels of asparagine (Asn) in globular, heart, torpedo and cotyledonary stages of zygotic embryogenesis (ZE), indirect somatic embryogenesis (ISE) and direct somatic embryogenesis (DSE) of *A. sellowiana*. Vertical bars represent the standard deviation.

may be related to the synthesis of LEA proteins, which are synthesized during the somatic embryo maturation process (Rock and Quatrano, 1995). The decrease in total amino acid content of embryos at cotyledonary phase could also be associated with the synthesis of enzymes, especially those related to the synthesis of

sugars of the raffinose series (Konrádová et al., 2003). Glutamine and glutamic acid are precursors of other amino acids and are used more in the induction of somatic embryogenesis or stimulating the growth of various tissues *in vitro* (George, 1993). Dal Vesco and Guerra (2001) reported that the presence of glutamine in the culture medium significantly increased the number of embryos in *A. sellowiana*, implying that this amino acid is related to the induction of somatic embryogenesis. Glutamine and glutamic acid also was found in high concentrations during the development of somatic embryos in *Daucus carota* (Kamada and Harada, 1984). These findings indicate that these amino acids play a central role in the development of embryos, acting as sources of nitrogen in the metabolism of amino acids.

Feirer (1995) suggested the importance of glutamine in developing embryos of conifers based on analysis of the content of free amino acids during development of ovules and seeds of *Pinus strobus*. In this study, we observed an increase in the concentrations of glutamine at the 21st day (Figure 2) which coincided with the development of the zygote and globular embryos produced during ISE. According to Macnicol (1983), the sharp fall of glutamic acid in *Pisum sativum* was mainly due to the synthesis of important enzymes in the stages of embryogenic development. Glutamic acid appears to be involved with the process of cell differentiation and in the process of ontogenesis of *A. sellowiana* pro-embryos observed between the 9th and 18th days of culture (Pescador et al., 2008). Furthermore, glutamic acid and glutamine are interchangeable amino acids (George, 1993). Thus, exogenous glutamine supplemented to the basal culture medium in the present work may also have affected the levels of glutamic acid found in this work, increasing these levels in the early stages of SE. It has been shown that GABA is accumulated in various tissues of plants in various stress conditions such as thermal shock, hypoxia, cytosolic acidification, dark, water stress and in the presence of hormones such as 2,4-D (Snedden and Fromm, 1998). The role of GABA in *A. sellowiana* SE was previously studied, indicating a high frequency of SE in response to 10 µM GABA supplemented to culture medium. This treatment also resulted in a large number of normal embryos, and the lowest percentage of formation of fused somatic embryos or other abnormalities (Booz et al., 2009).

The results of the present study suggest that the accumulation of GABA plays a central role in SE development and the 2,4-D presence probably influences the accumulation of this amino acid. The role of GABA in plants is not well established, but has been suggested that the accumulation of GABA is part of an adaptive response as a function of cytoplasmic acidosis (Crawford et al., 1994). However, the reduction of cytosolic pH is not regarded as a prerequisite for stimulating the synthesis of GABA (Oh and Choi, 2001). Various stress factors induce the synthesis of GABA in plants, and are also responsible for the increase of cytosolic Ca^{2+}. The transient elevation of calcium concentration modulates proteins like calmodulin, which is involved with well-defined physiological responses in plants (Bown and Shelp, 1997). An increase in total amino acids, especially GABA, during the proliferation and somatic embryo development in *D. carota* was shown (Kamada and Harada, 1984). In the present work, a higher proportion of GABA in the ISE and DSE compared with ZE (Figure 4) suggests that if indeed this amino acid is associated with embryogenesis, its role would be most marked in the somatic route. Oh and Choi (2001) found an inverse relationship in the levels of GABA and glutamic acid in seedlings derived from soybean seeds under stress conditions. In the present study, this relationship was not observed, suggesting then that the synthesis of GABA may have occurred from other substances such as polyamines (Gaspar et al., 2003).

Given its solubility, asparagine is a major form of transportation and storage of nitrogen in higher plants (Buchanan et al., 2000). In this study, the dynamics of asparagine accumulation detected also indicate that this amino acid plays an important role in the formation of zygotic embryos. The sharp reduction observed for this amino acid can be associated with early use of asparagine in protein synthesis, especially at cotyledonary phase (Calanni et al., 1999). The levels of asparagine suggest a close relationship between endogenous content, the formation of zygotic embryos and embryo formation in the ISE. A substantial similarity was also found in the levels of asparagine with aspartic acid (data not shown) detected in the three embryogenic pathways of *A. sellowiana*, indicating an apparent relationship between both biochemical and functional amino acids.

In a recent study, asparagine levels were quantified during *A. sellowiana* ZE. The results indicate that asparagine was the prevalent amino acid, with a peak in the late embryonal stages (torpedo and cotyledonary stages), when the cotyledons were fully developed, suggesting its involvement in the mobilization of embryo reserves (Cangahuala-Inocente et al., 2009). Taken together, the results of the present study indicate the involvement of various amino acids in the embryogenic pathways of *A. sellowiana.* Different types and concentrations of amino acids are required for induction of SE and for each developmental stage of somatic embryos. In ZE, glutamine, asparagine and aspartic acid seem to have a more marked influence on embryo induction. As for somatic embryogenesis, the amino acids more closely associated with this route are glutamine, glutamic acid and GABA. The results here presented may help to explain the biochemical and physiological changes that occur during ZE and S in plants and may assist in the development of improved SE protocols in

this and other woody plant species.

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