RESEARCH ARTICLE

The role of γ-aminobutyric acid (Gaba) in somatic embryogenesis of *Acca sellowiana* Berg. (Myrtaceae)

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ABSTRACT

The γ-aminobutyric acid (Gaba) is a non-protein amino acid found in prokaryotes and eukaryotes. Its role in plant development has not been fully established. This study reports a quantification of the levels of endogenous Gaba, as well as investigation of its role in different stages of somatic embryogenesis in *Acca sellowiana* Berg. (Myrtaceae). Zygotic embryos were used as explants and they were inoculated into the culture medium contained different concentrations of Gaba (0,2, 4, 6, 8 and 10 μM). The highest concentrations of endogenous Gaba were detected between the third and nine days after inoculation, reaching the value of 12.77 μmol.g^{-1}FW. High frequency of somatic embryogenesis was observed in response to 10 μM Gaba. This treatment also resulted in a large number of normal embryos, and the lowest percentage of formation of fused somatic embryos, phenotypic characteristic of most deformed embryos in all treatments. Also, all treatments promoted the formation of the somatic embryos with positive characteristics of development resumption, which however did not originate the seedlings.

Key words: Amino acid, pineapple guava, plant growth

RESUMO

O ácido γ-aminobutírico (Gaba) é um aminoácido não protéico encontrado em procariontes e eucariontes. Seu papel em plantas ainda não está bem estabelecido. No presente estudo procurou-se quantificar os teores endógenos de Gaba, bem como investigar seu papel nos diferentes estágios da embriogênese somática em Acca sellowiana Berg. (Myrtaceae). Foram empregados embriões zigóticos como explantes e os mesmos foram inoculados em meio de cultura contendo diferentes concentrações de Gaba: 0 (controle), 2, 4, 6, 8 e 10 μM. As maiores concentrações de gaba endógeno foram detectadas no período compreendido entre o 3º e o 9º dia após a inoculação, tendo alcançado, neste último dia, o valor de 12,77 μmol.g^{-1}FW. Alta freqüência de embriogênese somática foi observada em resposta a 10 μM de Gaba. Este tratamento também resultou em grande número de embriões normais, bem como o menor percentual de formação de embriões cupuliformes, característica fenotípica da maioria dos embriões deformados em todos os tratamentos. Em todos os tratamentos ocorreram embriões somáticos que apresentaram características positivas quanto à retomada de desenvolvimento, mas que não resultaram na formação de plântulas.

Palavras-chave: aminoácido, crescimento vegetal, goiabeira serrana

INTRODUCTION

Acca sellowiana Berg (Goiabeira serrana) is a native Myrtaceae from the South Brazil and North Uruguay. This species is cultivated in commercial orchards in New Zealand, USA, and Europe (Ducroquet & Hickel, 1997). In Brazil a domestication program culminated with the release of the first commercial varieties (Ducroquet et al., 2007, 2008).

Woody plants are considered recalcitrant to somatic embryogenesis, and the results so far obtained in *Acca sellowiana* regarding to somatic embryogenesis suggest its use as a model system for the induction and control of this *in vitro* morphogenetic route (Guerra et al., 2001). In this way somatic embryogenesis has long been considering as interesting system for studies of fundamental aspects of plant embryo development (Halperin, 1995).

A. sellowiana somatic embryogenesis was firstly reported by Cruz et al., (1990). High frequency of somatic embryos was further obtained (Canhoto & Cruz, 1994, 1996a). In Brazil the first results on somatic embryogenesis in this species were reported by Guerra et al. (1997) which showed that this morphogenetic route was dependent on the culture medium composition and the genotype employed. Dal Vesco & Guerra (2001) showed that different nitrogen sources in the culture medium affected the number of somatic embryos developed from zygotic embryos. Guerra et al. (2001) demonstrated that somatic embryogenesis in this species was affected by 2,4-D pulses, and histological studies revealed abnormalities on the development of somatic embryos. Stefanello et al. (2005) reported embryogenic competence from floral tissues, and Cangahula-Inocente et al. (2007) included the technology of synthetic seeds in the somatic embryogenesis protocol.

Somatic embryogenesis in *A. sellowiana* seems to recapitulate the zygotic embryogenesis. Both of these processes are complex and modulated by genetic, biochemical and physiological factors. Among these factors, amino acids represent the first step in the nitrogen assimilation (Ortiz-Lopez et al., 2000), and play a key role in the development of somatic embryos (Merkle et al., 1995).

The acid γ-aminobutíric acid (Gaba) is a non protein amino acid found in prokaryotes and eukaryotes. Although a role of Gaba in plants has not been fully defined, it seems to be involved in developmental processes including signaling, defense and stresses as well as the induction of somatic embryogenesis (Bown & Shelp, 1997). It has been shown that Gaba accumulates in several plant tissues under different stress conditions, such as hypoxia, temperature, water and some plant growth regulator as is the case of 2,4-D (Snedden & Fromm, 1998).

The aim of the present work is to quantify the endogenous levels of amino acids and Gaba in the different developmental stages of *A. sellowiana* somatic embryos. The effect of different levels of Gaba on the induction and development of somatic embryos was also examined .

MATERIAL AND METHODS

Extraction and quantification of total amino acids and Gaba: Matures zygotic embryos (0.4 mm length) excised from seeds under stereomicroscope were inoculated in test tubes (22×150 mm) containing 10 ml of culture medium composed of LP micro and macronutrients, Morel vitamins, sucrose (30 $g.L^{-1}$), 2,4-D (20 μM), and glutamine (4 mM). The pH of culture medium was adjusted to 5.8 prior to addition of 0.7 % agar (Guerra et al*.,* 1997). The tubes were covered with metallic caps and maintained in culture room in the dark at 25±1ºC.

For amino acid analysis the samples of zygotic embryos were used as explants every three days until 30th day. After 70 days in culture, somatic embryos in different developmental stages were observed and collected corresponding to globular, heart, torpedo, and cotyledonary stages. All these samples were frozen in liquid nitrogen and then stored at -20°C.

For amino acid extraction a solution of methanol, chloroform and water (MCW) was used in the proportion of 12:5:3. Samples of 200 mg of fresh mass in triplicate were grinded in 10 ml of MCW with liquid nitrogen. After three days the macerate was centrifuged at 5.000×*g* for 10 min and the supernatant was collected. The residue was extracted again with 5 ml of MCW and centrifuged using the same parameters. The supernatants were pooled and agitated with 5 mL of MCW (Bielski & Turner), and centrifuged at 5.000×*g*. After separation the acquaous phase was collected and lyophilized under vacuum. Gaba was detected using HPLC (High Performance Liquid Chromatography) with a Novopack column PLC C18 (3.9×300 mm). The conditions for separation were: 1 $mL.min^{-1}$ solvent flow (solvent A: 93.9% sodium acetate

buffer + 6% de acetonitrile + 0.1% de triethilamine, and solvent B: 60% de acetonitrile + 40% ultrapure water). Gaba was progressively eluted at 45ºC according to the increase in the organic fraction according to Endres (2001). The Gaba levels were calculated by means of comparison of the area with the standard of known concentration.

Gaba Suplementation in culture medium: Seeds of *A. sellowiana* were obtained from plants maintained in the Germplam Colection of EPAGRI´s São Joaquim Experimental Station, São Joaquim, Santa Catarina State, South of Brazil. The experiments were performed at the FURB Biotechnology Laboratory, Blumenau, Santa Catarina State.

Mature zygotic embryos were used as explants. The seeds were previously treated with 2.5% sodium hypochlorite solution for 40 min and then washed three times in sterile distilled water. The embryos were excised under stereomicroscope in sterile chamber and inoculated in test tubes (22×150 mm) containing 10 ml of culture medium and closed with metallic caps.

The culture medium was based on LP salts (Von Arnold & Eriksson, 1981), Morel vitamins (Morel and Wetmore 1951) supplemented with sucrose (30 $g.L^{-1}$), 2,4-D (20 μM), glutamine (4 μM), agar (7 $g.L^{-1}$), and different concentrations of Gaba: 0 (control), 2, 4, 6, 8 and 10 μM. The pH of the culture medium was adjusted to 5.8. A total of 360 zygotic embryos were inoculated in each test tube with 30 replicates for each treatment. The cultures were maintained in the dark at 25 ± 2ºC.

The evaluated parameters used were the number of somatic embryos in the different developmental stages: globular, heart, torpedo and cotyledonary observed after 20 and 35 days in culture. Malformation of somatic embryos was examined after 64 days in culture.

After 70 days in culture the somatic embryos were isolated and separated according to the developmental stage. Afterwards they were transferred to the same culture medium free of 2,4-D and Gaba and with the level of sucrose lowered to 50% of the initial concentration. The cultures were maintained during 20 days in chamber room with 16h light period, 50 $\mu mol.m^2.s^{-1}$ light intensity, and 25 ± 2ºC. The experimental design was randomized with 15 replicates for each treatment and each developmental stage.

The evaluated parameters were the number of somatic embryos progressing to the next developmental stage and the number of necrosed, quiescent and regrown somatic embryos. The data were submitted to ANOVA and mean separation carried out by Tukey test (5%)

RESULTS

Quantification of amino acids and Gaba levels: The quantification of the endogenous levels of amino acids in culture revealed more than 6-fold increase from the inoculation time to the third day, followed by slow decrease until 24 day in culture (Figure 1). New raise in these levels was observed afterwards for next 6 days, till culture completed 30 days. The decrease in the amino acids levels from 3rd to 24th day was concomitant with the period of intense cell proliferation observed from nine to eighteen day and the formation of globular somatic embryos which took place in culture from 15th to 24th day.

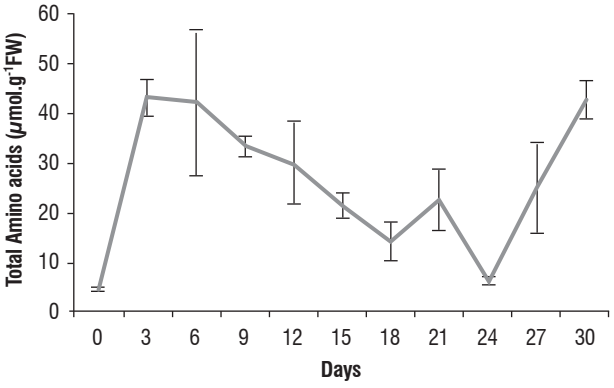

Figure 1. Variations on total amino acids levels during *Acca sellowiana* somatic embryogenenesis. The data are the mean of 3 experiments ±SE.

As shown in Figure 2, a continuous decrease in the amino acid levels was observed in different developmental stages of *A. sellowiana* somatic embryos. The globular stage exhibited the total amino acids concentration of 42.6 $mmol.g^{-1}FM$, while cotyledonary stage showed slight reduction by ~29%.

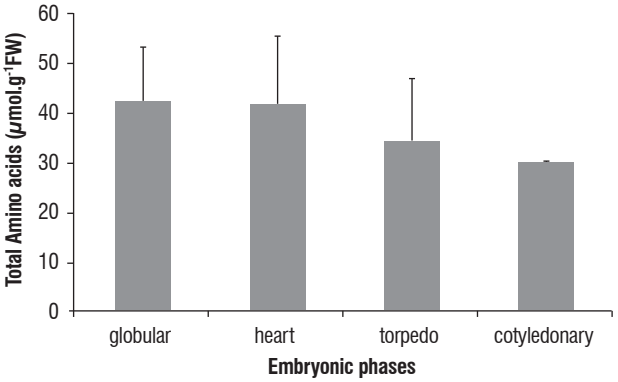

Figure 2. The levels of total amino acids in different developmental stages of *Acca sellowiana* somatic embryos. The data are the mean of 3 experiments ±SE.

The highest levels of Gaba was detected from the third to nine day, when the levels peaked at 12.77 μmol.g^{-1}fm (Figure 3). Again, this period coincided with the intense cell proliferation in the explants. Similar to amino acids content, a decrease in the Gaba levels occurred during the different developmental stages of somatic embryos (Figure 4). However Gaba concentrations dropped ~90% in cotyledonary-staged somatic embryos (0.08 μmol.g^{-1}fm) comparing to globular ones. The contribution of Gaba to the total free amino acids in embryogenic callus is substantial compared to non embryogenic callus (Nieenak et al., 2008) and further developmental stages of cacao somatic embryos. This finding fits the evidence that acquisition of embryogenic competence is a stress process. Gaba is commonly found associated with stress conditions in plants (Bown & Shelp 1997; Mesnard et al., 2000). It is produced in plants as a result of the activity of glutamate decarboxylase and rapidly accumulates under various stress conditions (Shelp et al., 1999; Bouché et al., 2003; Bouché & Fromm, 2004).

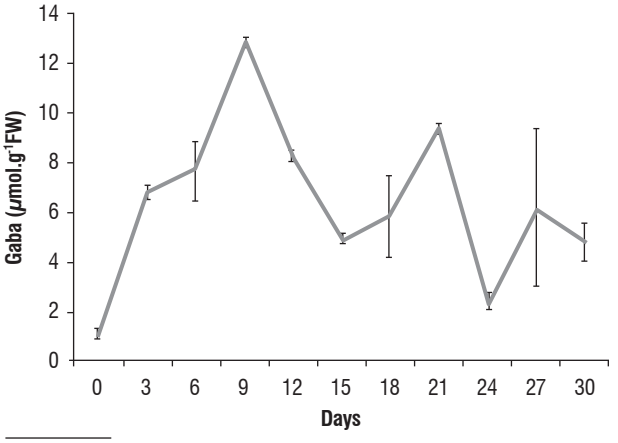

Figure 3. Gaba levels during *Acca sellowiana* somatic embryogenesis. The data are mean of 3 experiments ±SE

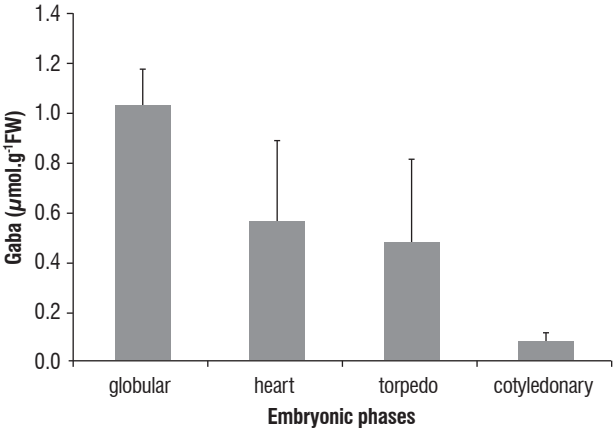

Figure 4. Levels of Gaba in different developmental stages of *Acca sellowiana* somatic embryos. The data are the mean of 3 experiments ±SE.

Induction and development of somatic embryos is affected by Gaba: In the present work 100% of zygotic embryos were used as explants for producing the somatic embryos. The presence of Gaba in cultures resulted in the induction of somatic embryos after 20 days indicating that Gaba played effective role in conferring embryogenic competence to explants. The treatment with 10 μM Gaba significantly enhanced the induction rate of globular somatic embryos (Figure 5). After 35 days in culture, all developmental stages of somatic embryos were present, i.e. globular, heart, torpedo and cotyledonary (Figure 6).

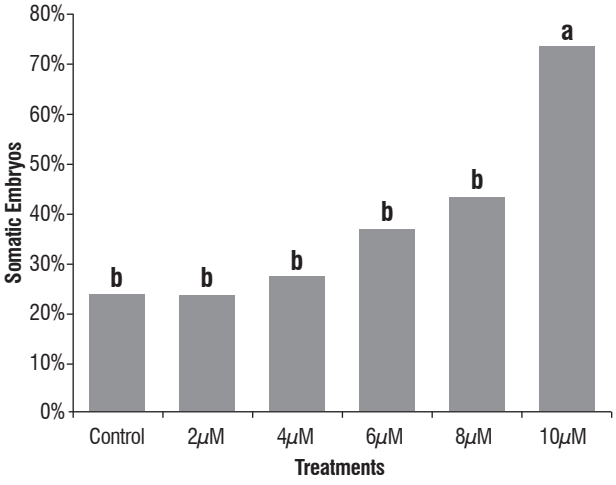

Figure 5. Formation of somatic embryos from *Acca sellowiana* zygotic embryos in response to exposure to different Gaba concentrations. The number of somatic embryos was determined after 20 days in culture. Different letter in the bars show different meaningful statistic, according to Tukey ($p = 0.05$).

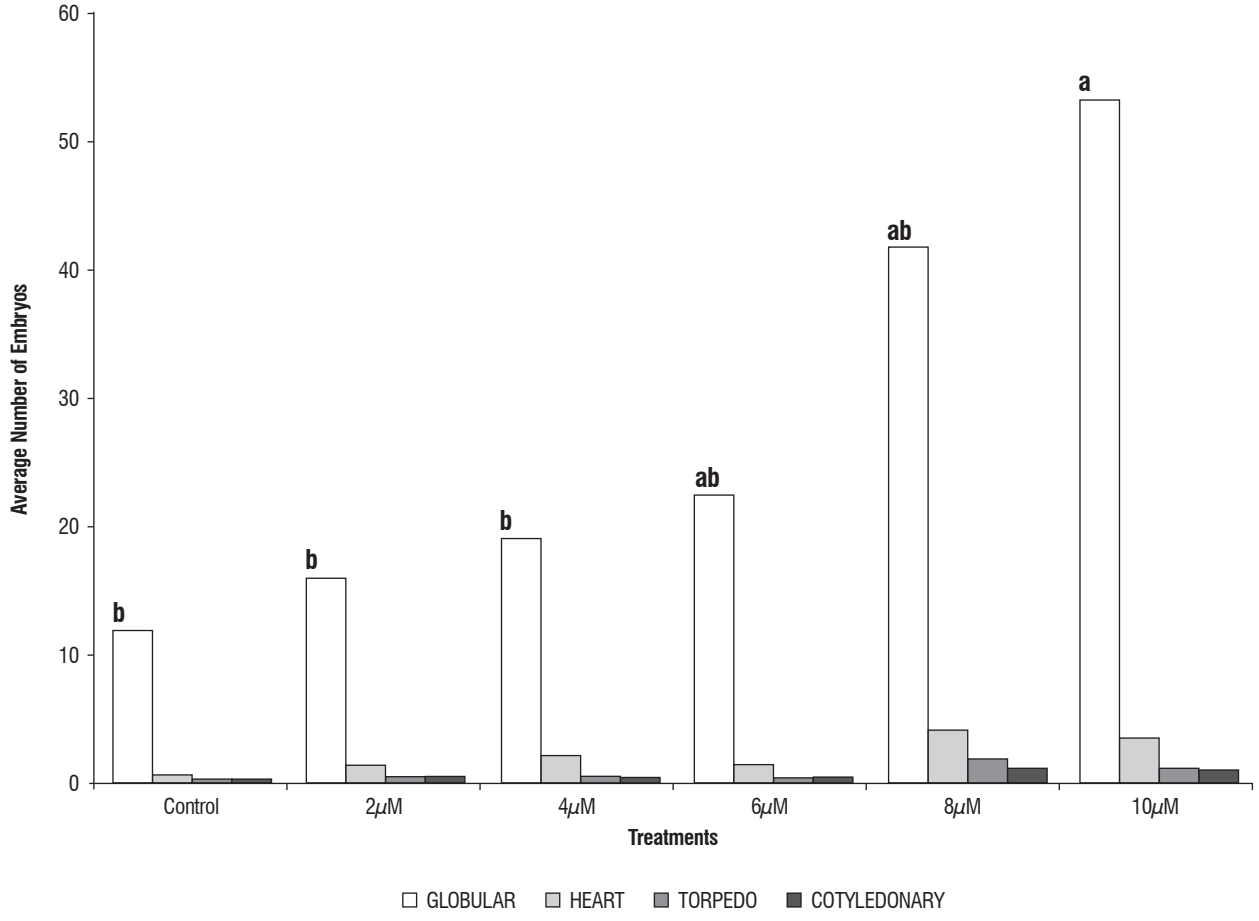

Figure 6. Number of *Acca sellowiana* somatic embryos in different developmental stages in response to different Gaba concentrations after 35 days in culture. Different letter in the bars show different meaningful statistic, according to Tukey ($p = 0.05$), only for globular stage.

Morphology of somatic embryo in response to Gaba: Examination of somatic embryos in the cotyledonary stage identified at least four different morphologies. Figure 7 shows the difference between a normal somatic embryo and those exhibiting some aberrant malformation as the absence of cotyledon, fused cotyledons or the presence of more than two cotyledons. Fused somatic embryos were the most frequent followed by normal ones. The treatments with Gaba resulted in the same four types of somatic embryo morphology, however the elevation in Gaba concentrations promoted a decrease in the rate of fused somatic embryos and an increase in the rate of normal somatic embryos (Figure 8).

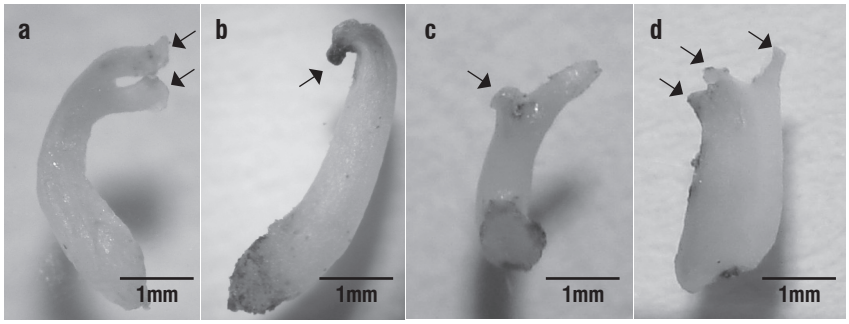

Figure 7. Morphology of *Acca sellowiana* somatic embryos in cotyledonary stage: a – normal embryo; b – embryos with fused cotyledons; c – one cotyledon embryo; d – embryo with three cotyledons. Arrows indicate cotyledons.

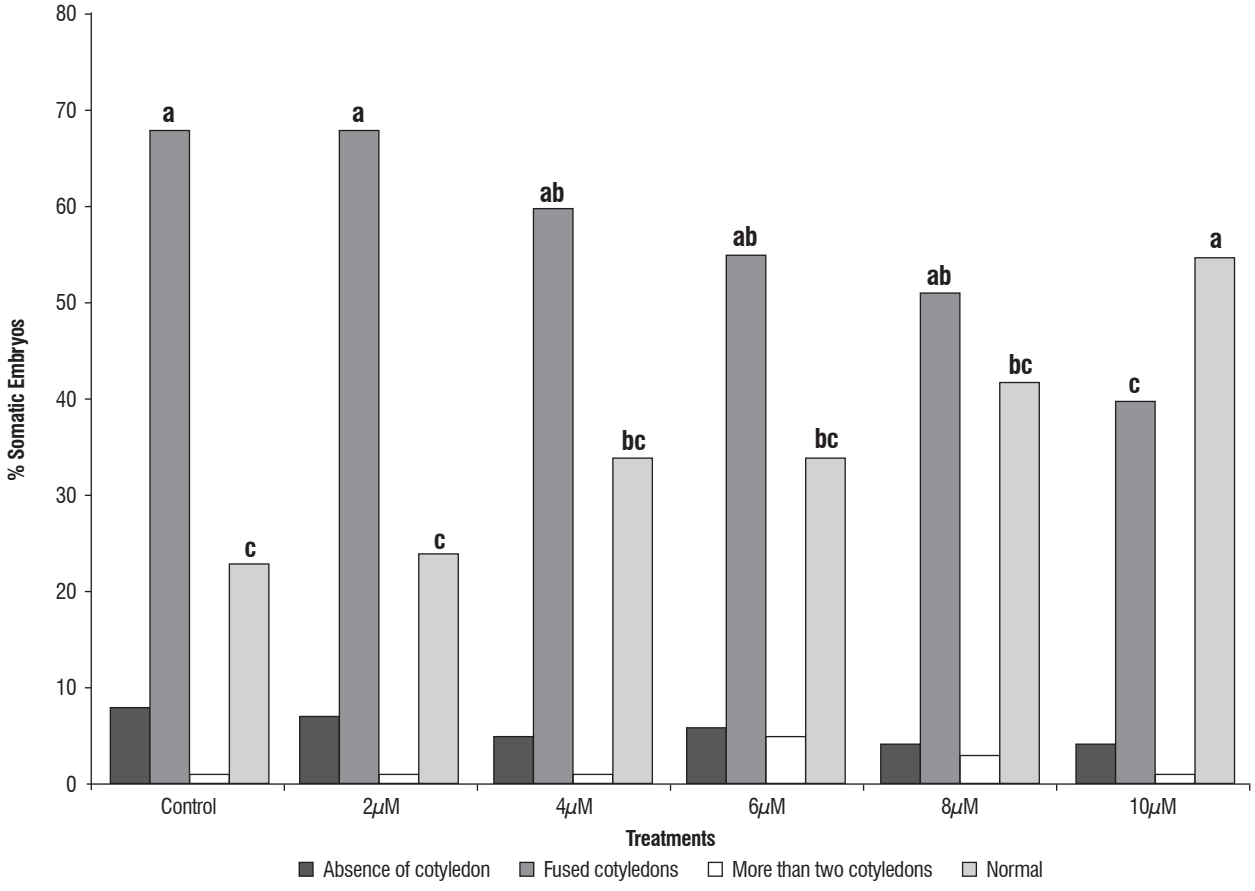

Figure 8. Normal and abnormal somatic embryo rate of *Acca sellowiana* in response to different Gaba concentrations after 64 days in culture. Different letters in the bars show different meaningful statistic according proportion test ($p = 0.05$).

Somatic embryo regrowth after Gaba treatment: Somatic embryos were classified in three categories: necrosed, quiescent and regrown. Somatic embryos in globular stage were able to regrowth in all Gaba treatments (Figure 9a). From the other hand, only control and treatments with 6 and 8 μM Gaba resulted in quiescent somatic embryos. Low levels of necrosis were observed in the control and 2 μM Gaba containing culture (30% and 20%, respectively) while higher concentrations increased necrosis. These same treatments also leaded to enhanced re-growth of somatic embryos (60% and 80%, respectively). In the heart stage (Figure 9b) all treatments provided three categories of somatic embryos. Gaba at 8 μM stimulated the rate of regrowth of somatic embryos (60%) while 4 and 6 μM Gaba exhibited low rates (20%) of necrosed somatic embryos. Figure 9c summarizes the data on torpedo stage. Quiescent somatic embryos were observed under all treatments. Gaba at 4 to 10 μM induced the rate of somatic embryos showing regrowth (60%) and 4 e 8 μM Gaba leaded to low rates of necrosed somatic embryos (10%). In cotyledonary somatic embryos (Figure 9d), the treatment with 10 μM Gaba promoted the highest rate of somatic embryos showing regrowth (80%). This is a significant increase as compared to the control culture. The treatments with 6 and 8 μM Gaba resulted in 40% of quiescent somatic embryos. Control treatment and 2 μM Gaba revealed higher rates of somatic embryos with necrosis (60% and 50%, respectively).

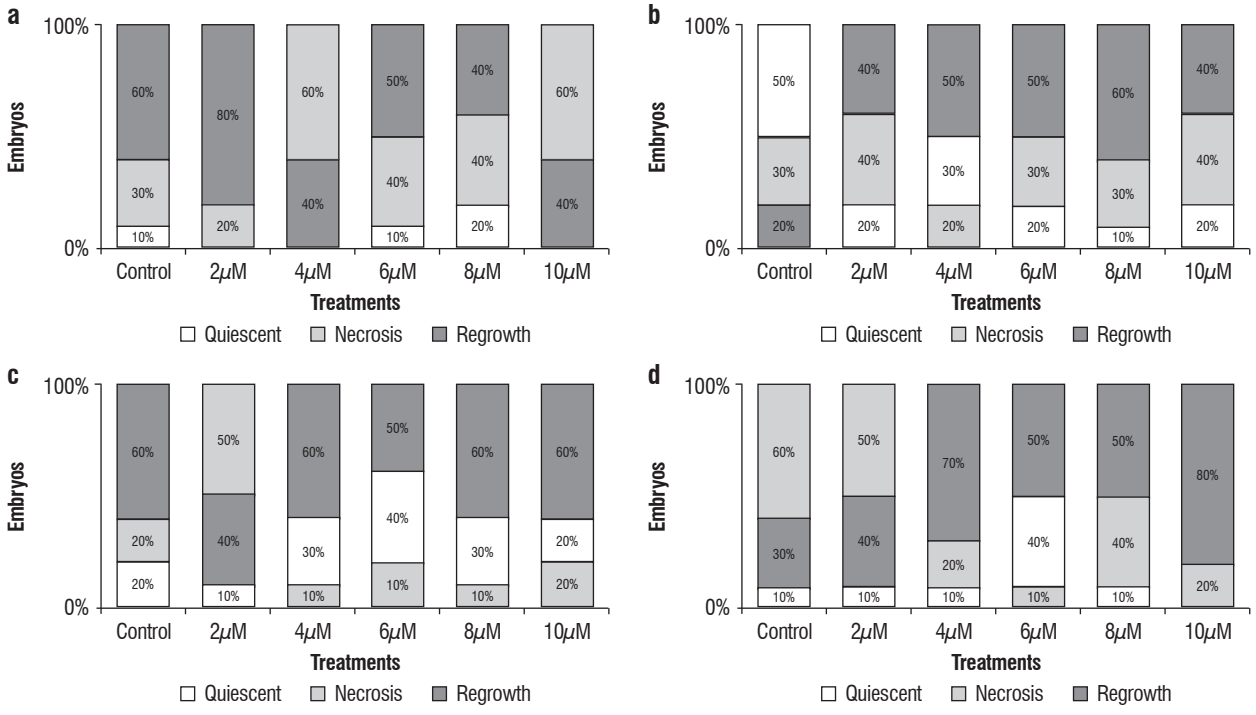

Figure 9. Rate of *Acca sellowiana* somatic embryos in response to different levels of Gaba after 20 days in culture of maturation. Stages: a) globular b) heart c) torpedo d) cotyledonary.

DISCUSSION

In plants free amino acids are important for the growth regulation and their levels are strictly controlled at cytoplasm (Barniex & Causin, 1996). Present study reports a variation in the amino acid levels were found during *Acca sellowiana* embryos development. These results are in agreement with data obtained using *Vignia mungo* (Sen et al., 2002) on variations in the total amino acid levels during specific stages of organogenesis and somatic embryogenesis. Modifications in those levels during the induction and development of *Arachis*

hypogea somatic embryos were also described (Murch et al., 1999).

In the present work the increase in the total amino acids levels observed immediately after the inoculation indicates an increase in the metabolic activity which could be stimulated by 2,4-D present in the culture medium as suggested by Fehér et al., (2003). The decrease in the total amino acid levels observed in the course of *A. sellowiana* somatic embryogenesis may be related to the protein synthesis. The synthesis of LEA proteins (Merkle et al., 1995) occurs in the late embryogenic stages, more precisely in the cotyledonary stage (Rock & Quatrano, 1995; Devic et al., 1996; Hirner et al., 1998). The decrease in the total amino acids levels in the cotyledonary stage could also be associated to the enzymes synthesis, mainly those related to the raffinose sugar (Konrádová et al., 2003), which is accumulated during embryo maturation and desiccation acquisition tolerance (Keller & Ludlow, 1993).

The evidences indicate that Gaba accumulates in several plant tissues under stress conditions, including that caused by 2,4-D (Snedden & Fromm, 1998). The results of the present study suggest that the increase in Gaba levels could be associated with the high levels of 2,4-D (20 μM) supplemented to the culture medium for the somatic embryogenesis induction.

In animals Gaba is associated with neurotransmission (Mody et al., 1994). The role of Gaba in plants is not fully understood but it has been suggested that its accumulation might be a part of an adaptive response associated to cytoplasmatic acidosis (Crawford et al., 1994). However, the decrease in the cytoplasmatic pH is not a pre-requisite for the Gaba synthesis (Oh & Choi, 2001). Several stressing factors which are associated with the Gaba synthesis are also involved in the increase in the levels of cytosolic Ca^{2+} (Bown & Shelp, 1997).

Kamada & Harada (1984) reported an increase in the total amino acids levels, specifically Gaba, during the cell proliferation and development of somatic embryos in *Daucus carota*. An increase in Gaba levels was also observed in *Arachis hypogea* embryogenic cultures (Murch et al, 1999).

The induction of somatic embryogenesis relies on several factors such as carbohydrate sources, amino acids, mineral nutrients and hormones (Emons, 1994). Several studies pointed out the beneficial effects of exogenous amino acid supplementation in somatic embryogenesis. In *A. sellowiana* the supplementation of Glu, Asp and Arg to the culture medium enhanced the rate of somatic embryogenesis induction (Dal Vesco & Guerra, 2001). In this same species Gaba induced high frequency of somatic embryogenesis (Booz & Pescador, 2007). However it has been postulated that the supplementation of amino acids to the culture medium may be positive or negative to somatic embryogenesis (Merkle et al., 1995).

The malformation of somatic embryos described in the present work was also reported for this same species by Canhoto & Cruz (1994), and Canhoto et al. (2002). Such abnormalities were also observed in soybean (Lazzeri et al., 1987), pecan (Rodriguez & Wetzstein, 1994), linen (Dedicová et al., 2000), and sweet potato (Magalhães, 2006). In *A. sellowiana* as well as in the species listed above the malformation of somatic embryos were mainly associated to fused cotyledons and alterations in the stem apical meristem as is the case of pecan (Mendes-da-Glória, 1998). According to Caligari & Shohet (1993) long term maintenance of embryogenic cultures in culture medium with 2,4-D is associated with genetic modifications that may negatively affect the embryogenic potential.

The present study provides the data supporting the role of Gaba in *Acca sellowiana* somatic embryogenesis, which was induced at high frequency in response to 10 μM Gaba. This treatment also enhanced the production of normal embryos, but did not affect the conversion rate of embryos into seedlings. These results add new insights for future strategies related to the modulation of somatic embryogenesis in *A. sellowiana*, and evaluation of physiological changes associated with GABA.

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