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Original Article

Proteic and phenolics compounds contents in Bacupari callus cultured with glutamine and nitrogen sources

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(With 4 figures)

Abstract

In this study was evaluated the influence of glutamine supplementation on the endogenous content of amino acids, proteins, total phenolics, flavonoids and proanthocyanidins in Bacupari callus. The explants were inoculated in MS medium, MS with half concentration of the nitrogen salts (MS½) and nitrogen-free MS, supplemented with glutamine (5, 10, 30 and 60mM) named as Gln5, Gln10, Gln30 and Gln60. Amino acids and proteins were analyzed after 20, 80 and 140 days and the secondary metabolites on the 140th day. There was no difference in the amino acids on the 20th day. On the 80th day the treatments MS and MS½ presented the lowest levels. On the 140th day MS and MS½ presented the lowest amino acid concentration and Gln10 the highest. Concerning proteins, there was difference only on the 140th day, being the highest concentrations observed in Gln5, and the lowest in MS½ treatment. Total phenolics content was higher in the treatment Gln60 and lowest in MS. Treatments Gln5, Gln10, Gln30 and MS½ were statistically equal. For flavonoids, the highest values occurred in the treatments Gln30, Gln60 and MS½ and the lowest in Gln5, Gln10 and MS. Similarly, for the proanthocyanidins the highest concentrations were observed in treatment Gln60 and the lowest in Gln5 and MS. In conclusion, the treatment with 60mM of glutamine favors the protein accumulation and production of secondary metabolites in Bacupari callus.

Keywords: glutamine, phenols, flavonoids, proanthocyanidins, amino acids.

Conteúdo de proteínas e compostos fenólicos em calos de Bacupari cultivados com glutamina e fontes de nitrogênio

Resumo

Nesse estudo foi avaliado o efeito da suplementação com glutamina no conteúdo endógeno de aminoácidos, proteínas, fenólicos totais, flavonoides e proantocianidinas em calos de Bacupari. Os explantes foram inoculados em meio MS, meio MS com metade da concentração de dos sais de nitrogênio (MS½) e meio MS sem nitrogênio suplementado com glutamina (5, 10, 30 e 60mM) denominados como Gln5, Gln10, Gln30 e Gln60. Os aminoácidos e as proteínas foram analisados após 20, 80 e 140 dias e os metabólitos secundários no 140° dia. Não houve diferença nos aminoácidos no 20° dia. No 80° dia os tratamentos MS e MS½ apresentaram os menores níveis. No 140° dia, MS e MS½ apresentaram as menores concentrações de aminoácidos e o Gln10 as maiores. A respeito das proteínas, houve diferença apenas no 140° dia, sendo as maiores concentrações observadas nos tratamentos Gln, e as menores no MS½. O conteúdo de fenólicos totais foi maior no tratamento Gln60 e menor no MS. Os tratamentos Gln5, Gln10, Gln30 e MS½ foram estatisticamente iguais. Para os flavonóides, os maiores valores ocorreram nos tratamentos Gln30, Gln60 e MS½ e os menores no Gln5, Gln10 e MS. Da mesma forma, para as proantocianidinas, as maiores concentrações foram observadas no tratamento Gln60 os menores no Gln5 e MS. Em conclusão, o tratamento com 60 mM de glutamina favorece o acúmulo de proteínas e a produção de metabólitos secundários em calos de Bacupari.

Palavras-chave: glutamina, fenólicos, flavonoides, proantocianidinas, aminoácidos.

1. Introduction

Secondary metabolites form a diversified group of organic compounds produced by plants being commercially used as nutraceuticals, agrochemical, flavoring, food additives, fragrance and biopesticide products (Verpoorte et al., 2002). However, harvesting from field cultivation of sufficient quantity of these compounds to supply commercial demands present disadvantages such as low yield and fluctuation in concentrations associated with geographical, seasonal and environmental variation (Lee et al., 2011). With this scenario in sight, plant tissue culture techniques comes as an alternative to get bioactive compounds (Karuppusamy, 2009). Consequently, studies have been carried out in order to enhance the synthesis and/or the accumulation of metabolites in plant cells and organs grown *in vitro*, such as optimization of culture medium and environment and the use of elicitation strategies (Murthy et al., 2014). An alternative is the use of organic forms of nitrogen, i.e., yeast extract, hydrolyzed casein and amino acids aiming at increasing cell metabolism in plant tissues (Parast et al., 2011). Glutamine, for instance, has been used to complement inorganic nitrogen sources or as the only source of this element, increasing cell differentiation and growth and optimizing metabolite production *in vitro* (Parast et al., 2011; Marquez-Martin et al., 2012; Encina et al., 2014). Yet, for each plant species it is necessary to develop protocols to get secondary metabolites *in vitro*. In this context, *Garcinia brasiliensis* Mart. (Clusiaceae), known as bacupari has been the target of several studies due to its pharmacological properties. Phytochemical analyses of this plant have resulted in the isolation of different biologically active substances, especially phenolic derivatives and polyprenylated benzophenones. Since *G. brasiliensis* callus have been established *in vitro* from explants obtained from seed segments containing the procambium region (Santos-Filho et al., 2014a). So, the aim of this study was to evaluate the influence of glutamine supplementation on the endogenous content of amino acids, proteins, total phenolic, flavonoids and proanthocyanidins in Bacupari callus.

2. Material and Methods

2.1. Plant material

Bacupari fruit was collected in Viçosa-MG at the campus of the Federal University of Viçosa-MG, Brazil, in February (summer) of 2014. A voucher specimen (number VIC2604) was deposited at the Herbarium of Federal University of Viçosa.

2.2. Callus induction

Intact seeds were disinfested with ethanol (70°GL) for 4 minutes and commercial sodium hypochloride (2.5%) for 15 minutes. Next, they were washed three times in distilled and autoclaved water. The explants (2mm thickness and 4mm in diameter) were obtained from seed segments containing the procambium region as described by Santos-Filho et al. (2014a). The explants were inoculated in culture flasks (30mm × 150mm, one explant per flask) containing 30mL of MS medium (Murashige and Skoog, 1962) solidified with 0.7% of agar and supplemented with sucrose (3%), BAP (6-Benzilaminopurine, 0.5 mg L^{-1}) and different salt concentrations as follows: MS medium with original concentrations of nitrogen salts (20mM NH_4NO_3 and 18.8mM KNO_3), MS medium with 10mM NH_4NO_3 and 9.4mM KNO_3 known as ½ strength (MS½) and MS medium free of NH_4NO_3 and KNO_3 containing glutamine in final concentrations of 5, 10, 30 and 60mM. These treatments were called Gln5, Gln10, Gln30 and Gln60, respectively. The pH was adjusted to 5.8 before autoclaving at 121 °C and 1 atm for 20 min. The ionic balance of the different culture media was adjusted by adding appropriate concentrations of KCl. Flasks with explants were kept at 25 ± 2 °C in the dark.

2.3. Total content of amino acids and proteins

Five samples, each containing 300 mg of callus (fresh weight) were collected. They were macerated in 3.0 mL of extraction buffer (0.1 M potassium phosphate, pH 7.2) containing 300 mg of polyvinylpyrrolidone (PVP) and centrifuged at 20,800 xg at 4°C for 30 min. The supernatant was collected and stored at –20 °C (Lemos et al., 1999). Biochemical analyses were carried out on the 20th, 80th and 140th days of cultivation following growth curve (Santos-Filho et al., 2014a). Total content of amino acids and proteins was determined according to Yemm et al. (1955) and Bradford (1976), respectively. Glutamine and bovine serum albumin (BSA) were used as standards.

2.4. Determination of total phenol, flavonoid and proanthocyanidin contents

Samples for phytochemical analysis were collected on the 140th day after inoculation during the deceleration period of the culture, in accordance with the growth curve previously described by Santos-Filho et al. (2014a). For the preparation of the extract, 100 mg of callus were macerated in 3 mL of ethanol. Next the material was kept under agitation at 120 rpm for 60 min. at room temperature. Samples were then centrifuged at 14,000 rpm at 4 °C for 30 min. and the supernatant was collected. This process was repeated two times and the supernatants were mixed. Solvent was evaporated and the precipitate was suspended again in 5 mL of ethanol. Total phenolic compounds were determined by using the Folin-Ciocalteau method (Ainsworth and Gillespie, 2007), total flavonoids were determined by using the aluminum chelating method (Ebrahimzadeh et al., 2008), and the proanthocyanidins content was obtained after acid depolymerization for the corresponding anthocyanidins (Rosch et al., 2003).

3. Statistical Analysis

The experimental design was thoroughly randomized including 6 treatments and 5 repetitions per treatment. The data were submitted to analysis of variance and the means were compared by the Tukey test at 5% of significance.

4. Results

With regard to amino acids content (see Figure 1a), there was no significant difference ($p>0.05$) between the treatments in relation to amino acids content on the 20th day of cultivation. On the other hand, after the 80th day of cultivation, the treatments MS and MS ½ presented the lowest amino acid concentrations ($p<0.05$). The other treatments had concentrations ranging from 126.25 ± 9.6 to 150.61 ± 25.5 mM g^{-1} of fresh weight, but were equal among themselves ($p>0.05$). On the 140th day of cultivation the lowest amino acid concentration was also observed in treatments MS and MS ½, and the highest in the treatment Gln5. The treatments Gln10, Gln30 and Gln60 presented intermediate values. Concerning proteins concentration (see Figure 1b), there was statistical difference among treatments only on the 140th day of cultivation, being the highest concentrations observed in the treatments Gln5 and the lowest in MS ½ treatment.

Besides alterations in amino acids and proteins levels, the variation of nitrogen source in the culture medium resulted in some alterations in the level of secondary metabolites. The concentration of phenolic compounds was higher in the treatment Gln60 in opposition to the treatment MS, which presented the lowest concentration of these compounds. Treatments Gln5, Gln10, Gln30 and MS½ were statistically equal (see Figure 2). For flavonoid contents, the highest values were observed in the treatments Gln30, Gln60 and MS½ and the lowest in Gln5, Gln10 and MS (see Figure 3). Similarly, for the content of proanthocyanidins (see Figure 4) the highest concentrations were observed in treatment Gln60 and the lowest in Gln5 and MS, thus showing that treatment with 60mM of glutamine favors the production of secondary metabolites in Bacupari callus.

5. Discussion

In general, nitrogen is available to plants as ammonium ions (NH_4^+) and as nitrate (NO_3^-). In a lower level are the nitrogen reduced forms such as the amino acids. However, these reduced nitrogen forms may directly influence plants development and even result in metabolites increase in some tissues or in explants of some species cultivated *in vitro* (Thornton, 2004; Fan et al., 2006; Oliveira et al., 2009; Santos-Filho et al., 2012) as they have specific carriers and they are more bioavailable to metabolism inside the cell (Miller et al., 2007). In this paper, there was statistical difference in relation to the endogen amino acid content in Bacupari callus between treatments at the 80th and the 140th days of cultivation. However, the proteins content was similar among all treatments throughout cultivation time, statistical difference being observed only on the 140th day. Interestingly, the highest protein concentrations were observed in the treatments with the lowest concentrations of glutamine. In fact, Zouine and El-Hadrami (2007) observed that the exogenous application of glutamine led to an increase in the protein synthesis in *Phoenix dactylifera* L embryogenic cells. In the same way, Hamasaki et al. (2005), found out that there was glutamine assimilation in cultivation of pineapple calluses (*Ananas comosus* L.) enhancing leaves organogenesis process and increasing hormonal concentrations. Glutamine was also efficient in recovering sugar cane roots (*Saccharum officinarum* L.) (Asad et al., 2009).

At the end of the cultivation period, treatment with 60mM of glutamine increased the concentration of total phenolic and flavonoid contents in the callus. Phenolic compounds, including flavonoids, are natural antioxidants and are broadly distributed in different parts of plants such as flower, fruit, bark, leaf, and seeds (Balasundram et al., 2006; Santos-Filho et al., 2014b; Camargo et al., 2016). In this context, *G. brasiliensis* has been extensively studied as a source of these compounds. It is emphasized that Santa-Cecília et al. (2011) and Gontijo et al. (2012) identified the phenolic derivatives Gutipherone-A, 7-epyclusianone and the bioflavonoids moreloflavone (fukugetin), moreloflavone-7"O-β-D glycol (fukugeside) and moreloflavone-4"O-β-D glycol in extracts from fruit and leaf of *G. brasiliensis*. They also evaluated antioxidant,

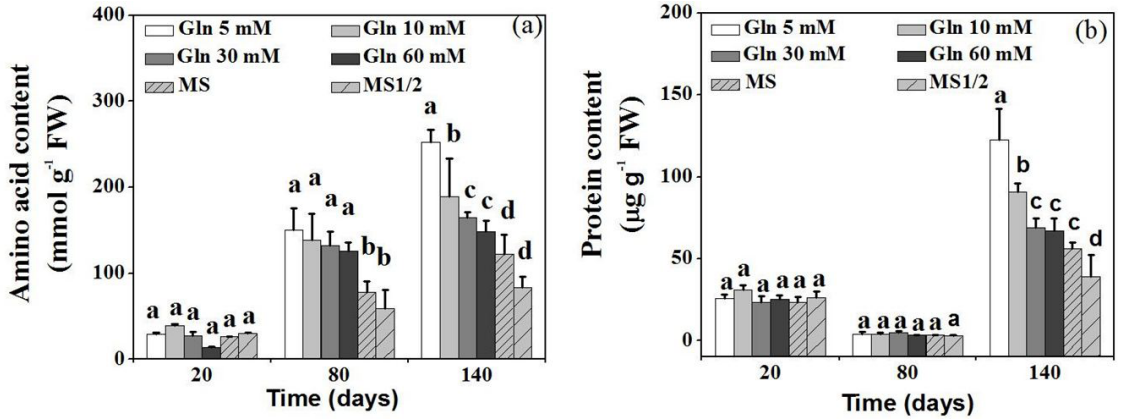

Figure 1. Concentration of amino acids (a) and proteins (b) in *Garcinia brasiliensis* callus under different treatments. Gln: Glutamine. Data represent the mean ± SD. Same letters indicate that the values in the same day of analysis do not differ by the Tukey test at 5% significance level.

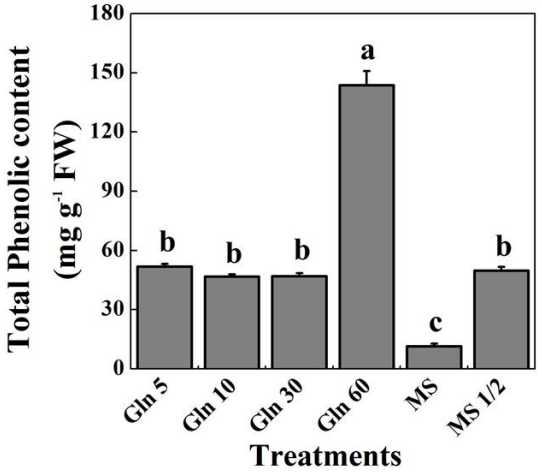

Figure 2. Total phenolics content of *Garcinia brasiliensis* callus under different treatments. Gln: Glutamine. Data represent the mean ± SD. Same letters indicate that the values do not differ by the Tukey test at 5% significance level.

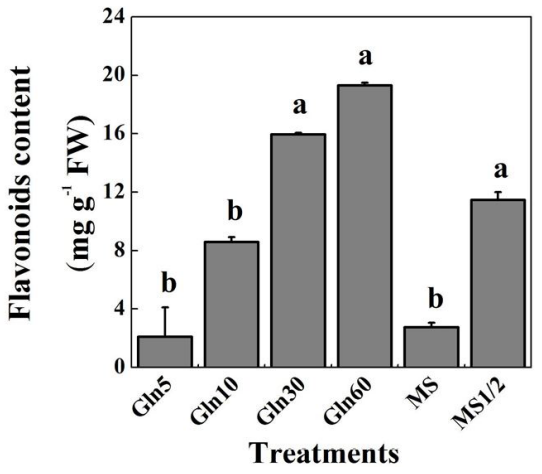

Figure 3. Flavonoids content of *Garcinia brasiliensis* callus under different treatments. Gln5: Glutamine at 5mM; Gln: Glutamine. Data represent the mean ± SD. Same letters indicate that the values do not differ by the Tukey test at 5% significance level.

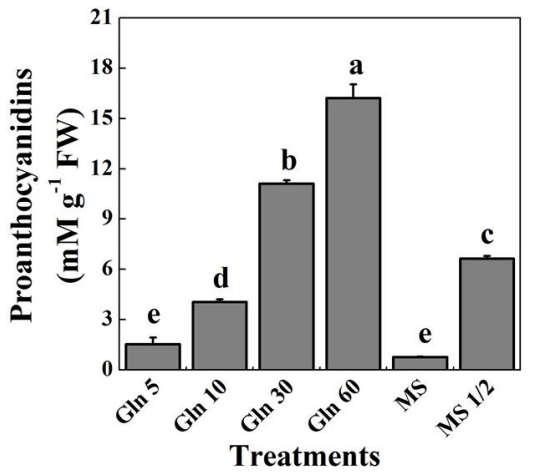

Figure 4. Proanthocyanidins content of *Garcinia brasiliensis* callus under different treatments. Gln: Glutamine. Data represent the mean ± SD. Same letters indicate that the values do not differ by the Tukey test at 5% significance level.

antinociceptive and anti-inflammatory activities of extracts and isolated compounds showing the pharmacological potential of these substances. Similarly to phenolic and flavonoid contents, the highest concentration of proanthocyanidins occurred at the end of the cultivation (140th day) in the treatment that used 60mM of glutamine. Proanthocyanidins are condensed tannins synthesized in the flavonoid biosynthetic pathway compounded by sub unities of flavan-3-ols (Dixon et al., 2005). As for the other phenols, the proanthocyanidins are considered important antioxidants as they are effective reducing agents and more recently they have been investigated for having anti-tyrosinase activity. Recently, Santos-Filho et al. (2014a) reported the presence of phenolic and flavonoid compounds and identified fukugetin, guttiferone-A and 7-epiclusianone in callus obtained from seeds of *G. brasiliensis* containing the procambium, though the occurrence of these compounds was less than in the initial explant. However, these authors mention that a higher content of secondary metabolites in the explant is common, since it still has its differentiated tissues, which favors the secondary metabolism (Santos-Filho et al., 2014a; Nicioli et al., 2010). Thus, the cultivation *in vitro* allows the use of different strategies to change the metabolite production by cells (Murthy et al., 2014). In this context, glutamine may act both as precursor and as elicitor of secondary metabolism (Matkowski, 2008). As precursor, it may lead to the aromatic amino acids synthesis, by setting free the amine group (NH_3) to form phenylalanine and tryptophan. Phenylalanine acts as an intermediate in the biosynthesis of phenolic compounds such as flavones, isoflavones and flavonoids (Pina and Errea, 2008; Tzin and Galili, 2010). In this paper, it is possible that part of the absorbed glutamine has been deviated for the secondary metabolites biosynthesis. In *Psolarea coryfolia* callus, there was increase in the production of psolarem compound (Parast et al., 2011) in response to the elicitation with glutamine.

The lowest concentrations of secondary metabolites were observed in the treatment that used the original nitrogen sources of the MS medium and these levels were higher when compared to the MS treatment when the nitrate and ammonia were reduced by half. In fact Praveen and Murthy (2013), Praveen et al. (2013) had already showed that changes in the relation nitrate/ammonia or a decrease in total nitrogen influences the production of metabolites. In this sense, Jones and Hartley (1999) proposed the Protein Competition Model (PCM) which approaches

a competition between the biosynthetic pathways of proteins and phenolic compounds in response to nitrogen concentration in a way that high nitrogen concentrations regulate protein synthesis positively and phenolic compounds biosynthesis, negatively. One possible explanation for this phenomenon is related to nitrate signalization. Nitrogen deficiency leads to the activation of genes related to secondary metabolite while addition of nitrate activates genes related to the protein and amino acids syntheses, except for phenylalanine (Scheible et al., 2004). It is known that most phenolic compounds are derived from this amino acid in a reaction catalyzed by Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) and the decrease of levels of these amino acids would be coherent with the inhibition of the Shikimate pathway (Manela et al., 2015). Therefore, it is possible that the increase in the synthesis of phenolic compounds observed in this work in response to gln60 is related to the replacement of nitrate by a reduced form of nitrogen, which is more bioavailable. Furthermore, increased production of metabolites in the treatment $MS^{1}/_{2}$ corroborates the hypothesis that the nitrate has a repressive effect on the secondary metabolism.

6. Conclusion

The use of glutamine at 60mM instead of nitrogenized sources of MS medium favored the accumulation of proteins and synthesis of phenolics compounds in Bacupari callus.

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