Vol.57, n.3: pp. 326-325, May-June 2014
http://dx.doi.org/10.1590/S1516-89132014005000008
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Growth Curve, Biochemical Profile and Phytochemical Analyses in Calli Obtained from the Procambium Segments of Bacupari

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ABSTRACT

Garcinia brasiliensis*, popularly known as Bacupari, is native to the Amazon and commonly used in folk medicine for its therapeutic properties. This plant is rich in bioactive compounds like benzophenones. However, there are no works about the in vitro establishment and achievement of secondary metabolites in this plant. Thus, the aim of this work was to determine the growth curve and to perform the biochemical and phytochemical analyses in calli obtained from the procambium segments of Bacupari. The growth curve of calli followed a sigmoidal pattern, with four distinct phases (lag, exponential, linear, deceleration). Total soluble sugars were higher on the inoculation day and the reducing sugars on the 20 th day. Amino acids increased from the 60 th day up to the stabilization on the 120 th day. The protein content varied, but it seemed to be related to the amino acids metabolism. The phytochemical screening showed the presence of phenolic and flavonoid compounds in the calli and the HPLC analysis allowed the identification of Fukugetin, Guttiferone A and 7-epiclusianone.*

Key words: Bacupari, benzophenones, *calli*

INTRODUCTION

Garcinia brasiliensis Mart., popularly known as Bacupari (Gonçalves-Esteves and Mendonça 2001), is a medium- sized tree native to the Amazon, but found now throughout the Brazilian territory. It belongs to the Clusiaceae family and is used in folk medicine for the treatment of ailments such as wounds, peptic ulcers and tumors (Lorenzi 2008). Previous reports have demonstrated the presence of bioactive compounds in this plant, such as biflavonoids and prenylated xanthones and benzophenones (Delle Monache et al. 1984; Santos et al. 1999). Among the natural bioactive compounds, the polyisoprenylated benzophenones are associated with the biological activities such as anti-HIV (Gustafon et al. 1992); leishmanicidal (Pereira et al. 2010), free radical scavenger (Ngouela et al. 2006), anti-cholinesterase (Lenta et al. 2007), anti-inflammatory and antinociceptive (Santa-Cecília et al. 2011). Three natural polyisoprenylated benzophenones have been isolated from *Garcinia brasiliensis*: Guttiferone-A (Gustafson et al. 1992), 7-epiclusianone (Santos et

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al. 1999) and Garciniaphenone (Derogis et al. 2008).

The capacity of plant cell, tissue, and organ cultures to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been recognized (Karuppusamy 2009). In recent work, Hussein et al. (2010) found flavonoids, tannins, volatile oils and anthraquinones in the calli of *Brassica nigra*. In calli of *Prunus cerasus*, the cultivation in the light and in the presence of jasmonic acid increased the production of cyanidin 3-O-glucoside compound (Blando et al. 2005). The production of metabolites through the plant cell culture is attractive for bringing ecological and economic benefits by reducing the predatory gathering of plant species with pharmaceutical relevance. In this way, assessing the biochemical changes during the calli development can support the *in vitro* establishment.

Thus, the aim of this work was to determine the growth curve and to perform the biochemical and phytochemical analyses in calli obtained from the procambium segments of Bacupari.

MATERIALS AND METHODS

Plant material and calli induction

Fruits of *Garcinia brasiliensis* Mart. were collected in Viçosa-MG. Intact seeds were disinfested with ethanol (70°GL) for 4 min and sodium hypochlorite (12500 ppm) for 15 min. Then, they were washed three times with distilled and autoclaved water. The explants (2 mm thickness and 4 mm in diameter) were obtained from the procambium segments of the seeds (Fig. 1) and were inoculated into culture flasks containing 30 mL of MS medium (Murashige and Skoog 1962), supplemented with sucrose (3%), agar (0.7%) and BAP (6-Benzilaminopurine, 0.5 mg/L^{-1}). The pH was adjusted to 5.8 before autoclaving. Tissue flasks were kept at 25 ± 2°C in the dark. This condition was previously determined for calli induction in Bacupari.

Growth curve determination

The growth curve was determined from the random selection of 20 explants inoculated according to the description above. Each explant was weighed aseptically at the intervals of 20 days during the 140 days of cultivation, starting on the inoculation day.

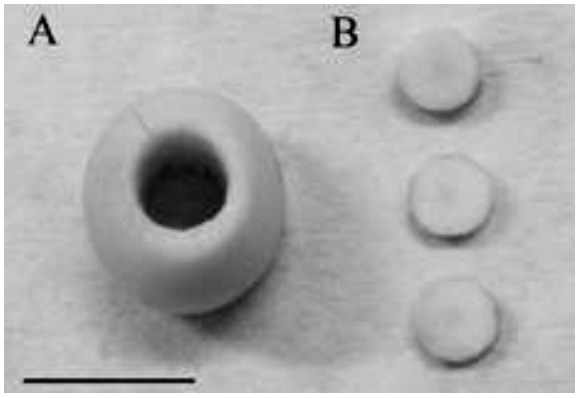

Figure 1 - Bacupari seeds drilled (A) in order to obtain the procambium segments (B) used as explants in the experiments. Scale: 1 cm.

Biochemical analyses

Four samples, each containing 300 mg of calli (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 polyvinylpyrrolidone (PVP) and centrifuged at 20,800 xg at 4°C for 30 min. Supernatant was collected and stored at -20°C (Lemos et al. 1999). Biochemical analyses were done at the intervals of 20 days following the growth curve. Total soluble sugars and reducing sugars were determined according to Yemm and Willis (1954) and Miller (1959), respectively, using anthrone and 3,5-dinitrosalicylic acid. Glucose was used as standard. Total content of amino acids and proteins was determined according to Yemm and Cocking (1955) and Bradford (1976). Glutamine and Bovine Serum Albumin (BSA) were used as the standards.

Phytochemical analyses

Samples for the phytochemical analyses were collected on the 140 th day after the inoculation during the deceleration period of the culture according to the growth curve. For the extract preparation, four samples of the calli and the initial explant, each containing 300 mg, were ground in 3.0 mL of ethanol PA. Next, the material was kept under agitation at 0.4 xg for sixty minutes at room temperature. Then, the samples were centrifuged at 20,800 xg at 4°C for 30 min and the supernatants were collected. This process was repeated two times and the supernatants were mixed. The solvent was evaporated and the precipitate was suspended again in 600 µL of

ethanol PA. Total phenolic content was determined as described by Ainsworth and Gillespie (2007) with minor modifications. Briefly, 0.1 mL of the samples were mixed with 0.5 mL of Folin-Ciocalteu reagent diluted in water (1:10). After 8 min, 0.4 mL of 4% sodium carbonate were added. The samples were incubated for two hours in the dark and the absorbance was measured at 740 nm. Quantification was based on a standard curve of gallic acid. Total flavonoid content was measured according to Park et al. (1997). Aliquots of 0.5 mL of the samples were mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of potassium acetate and 2.8 mL of distilled water. They were then incubated for 30 min at room temperature and the absorbance was measured at 415 nm. Quantification was based on a standard curve of quercetin.

High performance liquid chromatography (HPLC) analysis of the samples was performed on Shimadzu LC-20A equipment using a C18 NST column, Nano Separation Technologies (150 x 4.6 mm), with a 5 µm particle size according to Santa Cecília et al. (2011). The mobile phases consisted of eluent A (0.5 mM L^{-1} aqueous acetic acid) and eluent B (methanol/acetic acid 0.1%). The gradient conditions at 1.2 mL min^{-1} flow rate were as follows: 50% solvent B for 10 min, 50% to 100% solvent B for 10 min, 100% solvent B for 10 min, followed by a 2 min re-equilibration in 50% solvent B. The retention time and UV absorption spectrum of the compounds were detected at 254 nm with a photodiode array detector. Samples were injected at a volume of 20 µL and concentration of 1 mg/mL. LC solution software was used for data collection. Fukugetin, Guttiferone A and 7-epiclusianone, previously obtained and characterized in *Garcinia braziliensis* extracts (Martins et al. 2010; Gontijo et al. 2012) were used as the standard compounds for the HPLC analyses.

RESULTS AND DISCUSSION

Growth curve determination

The growth curve of calli followed a sigmoidal pattern, with four distinct phases in the observed period (Fig. 2). The decline and stationary stages were not observed in this study. Mendonça et al. (2012) working with calli of *Eucalyptus camaldulensis* obtained a similar result, not identifying the decline and stationary stages.

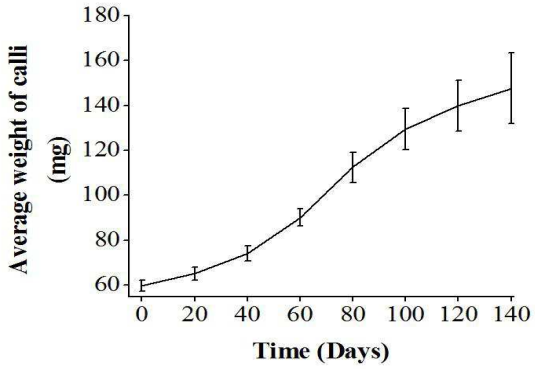

Figure 2 - Growth curve of the calli obtained from the procambium segments of Bacupari inoculated in MS medium supplemented with 0.5 mg/L^{-1} of BAP.

The lag phase, in which the cells prepared for division by accumulating biomass (Santos et al. 2008), was observed as far as the 20th day of inoculation. Serra et al. (2000) reported that the lag phase in the calli of Brazil nut occurred for 30th day after the inoculation. However, in the calli of *Coffea arabica L.* (cultivar Ruby), the lag phase occurred up to 42nd day after the inoculation (Santos et al. 2003). The exponential phase, period in which there was the maximum cell division, (Scragg and Allan 1993), was observed from 20th to 60th day after the inoculation. In the calli of *Inga vera* Willd. Subsp. *Affinis*-DC, this phase was observed from the 40th to 50th day of inoculation (Stein et al. 2010) and in the calli of *Byrsonima intermedia*, this phase occurred between the 20th and 40th day after the inoculation (Nogueira et al. 2008).

The linear phase, in which the calli diminished their division and increased their cellular area (Stein et al. 2010), occurred between 60th and 100th day. This phase was observed between 50th and 70th day in the calli of *Inga vera* (Stein et al. 2010) and between 40th and 60th day in the calli of *Byrsonima intermedia* (Nogueira et al. 2008). The growth deceleration was observed between the 100th and 140th day. In this phase, the calli should be transferred to a new medium due to the lack of nutrients and/or accumulation of toxic substances in the culture medium (Smith 1992). The growth curve of *Bacupari calli* indicated slow growth. According to Dai et al. (2011), woody species are

challenging because they have long life cycles and little genetic information available. Thus, future studies should be useful to provide information about the slow growth observed.

Biochemical analyses

The carbohydrate metabolism is associated with many functions in the plant cells. It is believed that carbohydrates are involved in transport and storage processes, providing energy and carbon skeletons and regulation of gene expression (Carrier et al. 1997; Johnson et al. 1997). Total soluble sugar content was higher on the inoculation day. From the 20th day, there was a decline that remained until the 60th day. On the 80th day, there was a further decline followed by an increase on 100th and 120th day and a decrease on the 140th day (Fig. 3A). Serra et al. (2000) and Nogueira et al. (2008) also found a higher content of sugars at the beginning of cultivation in the *calli* of *Byrsonima intermedia* and *Bertholletia excelsa*, respectively. According to Nogueira et al. (2008), higher levels of sugars on the inoculation day could be related to the energetic reserves of the explant that were consumed to supply the demands of the calli.

With regard to reducing sugars (Fig. 3B), the highest level occurred at 20th day, probably by breaking down the carbohydrate reserves present in the initial explant. The 20th day corresponded to the beginning of the exponential phase and the demand for energy increased to support the cell division. The drop at 40th day corroborated with this hypothesis. From this, there was a tendency of stabilization, possibly due to the sugar supply from the culture medium (Serra et al. 2000; Santos et al. 2008). Sucrose present in the culture medium comes into the metabolism after breaking into glucose and fructose (Huber and Huber 1996), providing the reducing sugars to the calli.

Santos et al. (2003) found a decrease in the reducing sugars content up to 21st day, and a gradual increase up to 84th day after the inoculation of *Coffea arabica* L. cv. Ruby calli, whereas in the calli of *Coffea canephora* L. cv. Apoatã, the maximum level occurred on the inoculation day, decreasing to the minimum as far as the 84th day of cultivation (Santos et al. 2008).This suggested that the capacity of plant tissues to use the sugars could change according to the species, type of explant and culture medium (Nikolova et al. 1991).

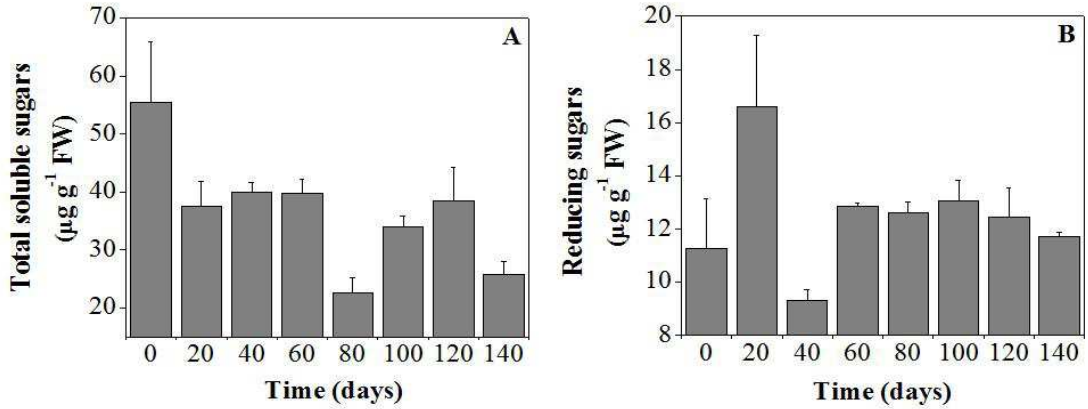

Figure 3 - Total soluble sugars (A) and reducing sugars (B) content in calli obtained from the priocambium segments of Bacupari inoculated in MS medium supplemented with 0.5 mg L^{-1} of BAP.

In contrast to the sugars, amino acid content was lower in early development stages of the calli and increased from the 60th day until the stabilization at 140th day (Fig. 4A). According to Serra et al. (2000), this increase could be due to enhanced absorption of nitrogen sources present in the culture medium. Amino acids are the primary products of nitrogen metabolism and, in addition to directly, or indirectly participating in protein synthesis, they control many aspects of plant growth and development (Vidal and Gutierrez 2008). In several studies, the amino acid accumulation has been associated to the development of *in vitro* cultures, including inducing the morphogenetic responses (Sacchi et al. 1995; Cangahuala-Inocente et al. 2009; Gerdakaneh et al. 2011).

The late accumulation of amino acids could be associated to the delay in the use of nitrogen sources of the culture medium, probably due to the slow development of the calli, as observed in the growth curve. Based on this, the supplementation of the culture with an organic nitrogen source, such as glutamine, can be considered since it represents a lower metabolic cost and has shown some interesting results in many cultures (Robichaud et al. 2004; Zouine and Hadrami 2007).

With respect to total protein content, higher concentrations were observed from the beginning of cultivation until the 40th day and then in the 140th day (Fig. 4B). On the other hand, between 60th and 120th day of cultivation, lower concentrations were observed. Such variation in the protein content makes sense to the amino acids profile. The trend towards lower levels between the 60th and 120th day corresponded to the period of amino acids accumulation, suggesting that the protein degradation was, at least in part, a source of amino acids. The calli formation requires cellular dedifferentiation and acquisition of new competencies (Fehér et al. 2003; Costa and Shaw 2007). This is possible due to plant totipotency mechanisms, which allow the cells to change their fate quickly in response to extracellular signals (Costa and Shaw 2007), including in response to the nitrogen sources present in the culture medium, since much of this change requires degradation of unnecessary polypeptides and synthesis of new proteins. Thus, the type and amount of nitrogen present in the culture medium represent not only a nutritional source, but a cellular signaling mechanism (Santos-Filho et al. 2012).

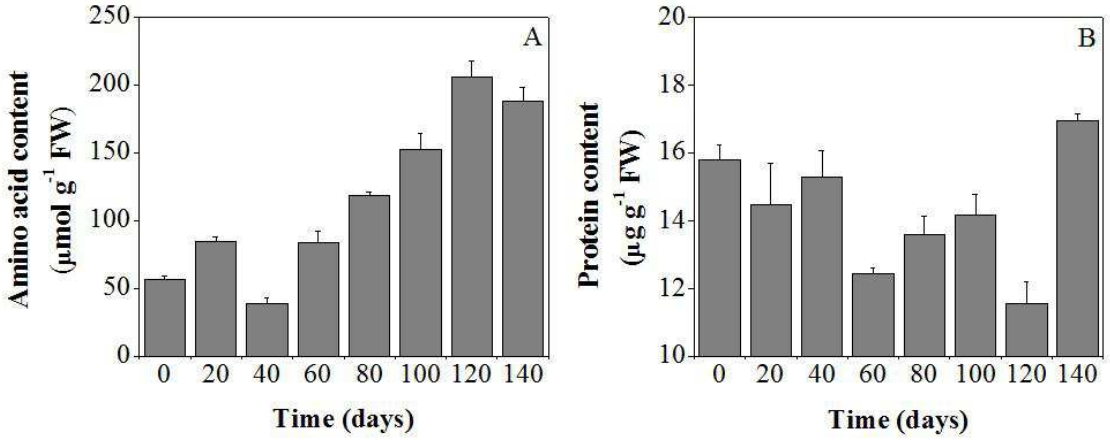

Figure 4 - Amino acids (A) and protein (B) content in calli obtained from the procambium segments of Bacupari, inoculated in MS medium supplemented with 0.5 mg L^{-1} of BAP.

Phytochemical analyses

Table 1 shows the total content of phenolic and flavonoid compounds in the calli of Bacupari compared to the initial explant. The occurrence of these compounds was higher in the initial explant.

Table 1 - Total phenolic and flavonoid content

Sample	Phenolic content (mg.g FW^{-1})	Flavonoid content (mg.g FW^{-1})
Calli	5,68 ± 0,31	2,60 ± 0,16
Initial explant	11,28 ± 0,60	3,08 ± 0,41

Total phenolic and flavonoid content in the ethanolic extracts of the calli obtained from the procambium segments of Bacupari after 140 days of inoculation in MS medium supplemented with 0.5 mg L^{-1} of BAP.

Nicioli et al. (2010) also reported higher levels of secondary metabolites in the initial explant when compared to those in the calli. These authors mentioned that the highest degree of cellular differentiation of the initial explant kept the primary metabolism more constant and promoted the secondary metabolism.

This study also performed HPLC-UV-Vis analysis of the calli. Table 2 shows the retention time, maximum UV absorption and percentage area of the identified compounds in the ethanolic extract of Bacupari calli. Data were collected at 254 nm and the identification was assessed by comparing the retention times and UV-absorption of the samples with purified compounds isolated from

Garcinia brasiliensis. This approach allowed the identification of the biflavonoid Fukugetin (retention time 29.8 min) and the benzophenones guttiferone A and 7-epiclusianone (retention times 25.40 and 26.02 min, respectively) (Table 2).

Table 2 - Constituents Identified By HPLC/UV.

Retention Time (min)	Peak area (%)	UV (λmax)	Constituents
8.29	3	289, 343	Fukugetin
25.40	0,63	251, 362	Guttiferone A
26.02	0,5	252, 297	7-epiclusianone

Constituents identified by HPLC/UV in the ethanolic extracts of the calli obtained from the procambium segments of Bacupari after 140 days of inoculation in MS medium supplemented with 0.5 mg/L^{-1}.

The presence of benzophenones and other phenolic compounds, even at lower concentrations than in the parental plant, showed that the metabolic pathways of the cell responsible for the synthesis of these metabolites remained active in the calli. The chromatographic data (not shown) suggested the presence of other secondary compounds, possibly phenolic ones, which could not be identified due to the lack of standards.

Although many compounds do not achieve high concentrations in undifferentiated cell cultures, plant cells can produce novel compounds, which may or may not be related to those previously isolated in the entire plant (Schmeda-Hirschmann et al. 2005). It has been demonstrated that the optimization of the culture can increase many folds the content of a specific metabolite and even induce the cultured cells to synthesize the metabolites, which could not be detected in the wild parent plant (Verpoorte et al. 1994). According to literature, an alternative to increasing the production of secondary metabolites is the elicitation of the cultures. Some studies have been successful using biosynthetic precursors such as amino acids and abiotic stresses (Karppinen et al. 2007; Kin and Kunter 2009). Therefore, this provided useful information for the *in vitro* establishment of Bacupari in order to obtain the bioactive compounds.

ACKNOWLEDGEMENTS

This work was supported by Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG, grant APQ- 01671-09) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Grant AUX PE–PNPD-2297.2011).

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Received: April 19, 2013;
Accepted: November 04, 2013.