International Journal of Agricultural Technology 2014 Vol. 10(3): 705-716
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

In Vitro Cultivation of Babassu Embryos with Different Concentrations of Sucrose and Activated Carbon

Leite, M. S.[*], Alberto, P. S. , Dionysus, F. P. and Guimarães, F. S.

IF Goiano – Câmpus Rio Verde. Rod. Sul Goiana, km 01. Zona Rural, Rio Verde – GO.

Leite, M. S., Alberto, P. S., Dionysus, F. P. and Guimarães, F. S. (2014). *In vitro* cultivation of babassu embryos with different concentrations of sucrose and activated carbon. International Journal of Agricultural Technology 10(3):705-716.

Abstract Babassu (*Orbignya oleifera* Burret.) is an oil palm with a high economic value. All parts of this palm can be used, which is why it is one of the species used in the Brazilian extractive industry. Because its propagation by seed is low, slow and uneven, the use of tissue culture techniques to improve this process is being considered. The present study aimed to evaluate the *in vitro* growth of babassu under different concentrations of sucrose and activated carbon. Mature embryos were inoculated on Murashige and Skoog (MS) medium at 50% of the salt concentration, with different concentrations of sucrose (0, 15, 30, 45 and 60 $g.L^{-1}$) and activated charcoal (0 and 2 $g.L^{-1}$). Counts were performed on alternate days for the evaluation of germination and the germination speed index (GSI). At 30, 60, 90 and 120 days, the average length of the seedlings was evaluated, and the formation of cotyledon petioles, shoots and roots and the oxidation level of the explants were evaluated at 120 days of cultivation. The use of 15 $g.L^{-1}$ sucrose in the culture medium, in the absence of activated charcoal, was the best medium in promoting the formation of shoots and roots, and the use of activated carbon to cultivate *in vitro* zygotic embryos of babassu was unnecessary.

Keywords: *Orbignya oleifera* (Burret.), biodiesel, Arecaceae.

Introduction

Babassu (*Orbignya oleifera* Burret.) is a palm native to Brazil that can be found in the States of Goias, Tocantins, Maranhão, Pará and Piauí This distribution comprises 18.5 million hectares of forest, and the extraction of the palm fruit, a product with a high commercial and industrial value, employs more than 300,000 people (Albiero *et al*., 2007). The potential of babassu remains untapped, as it is possible to use different parts of this palm for the economical production of coal, oil, gas, grease and cooking oil. Regarding the production of fuel oil, babassu oil has excellent characteristics for biodiesel production, as the predominant constituents are lauric oils (Lima *et al*., 2007).

[*] **Corresponding author:** Leite, M. S; **E-mail:** mariluza_phs@hotmail.com; paulasperotto@gmail.com; flavia1808@hotmail.com; fabianoifgoiano@gmail.com

As with the majority of palms, babassu reproduction occurs via seed. However, this type of propagation is inconvenient because, in addition to the long and slow growth, the produced plants are highly variable. For this reason, the use of techniques to promote vegetative propagation is undoubtedly an important step toward the domestication and rational exploitation of this type of palm tree (Pereira *et al*., 2006).

In vitro propagation is an important tool for plants for which vegetative multiplication is difficult, as is the case of Arecaceae (Ledo *et al*., 2001). Thus, the culture of zygotic embryos is of great value for the production of uniform seedlings of these species, allowing, among other applications, the production of plants free from pathogens and the acceleration of breeding programs (Melo *et al*., 2001).

Cultures maintained *in vitro* are usually heterotrophic, and sucrose is the major carbon source used to facilitate the growth of explants. Sucrose is commonly used as both an energy source and to maintain an appropriate water potential in the culture medium. Studies on the demand for sucrose may contribute to a better knowledge about the nutritional state and maturation of embryos and provide information on the germination process (Ferreira *et al*., 2002; Garcia *et al*., 2002).

The occurrence of high levels of oxidation is a major problem related to propagation through embryo culture. The oxidation of phenolic compounds is related to the release of toxins in the culture medium and is usually detrimental or even limiting to the growth of the embryos or seedlings (Melo *et al*., 2001). To reduce this problem, antioxidants, such as ascorbic acid, polyvinylpyrrolidone (PVP) and activated charcoal, are added to the culture medium, and the initial incubation of the explants is performed in the dark. Activated charcoal is used because it is absorbs inhibitory substances from the environment or toxic products released by the explants and can also promote the growth of the embryos (Pasqual *et al*., 1990). Moreover, activated charcoal also absorbs the 5-hydroxymethylfurfural (an organic compound derived from the dehydration certain sugars) produced by autoclaving sucrose, impurities in the agar and ethylene produced in culture. However, it also absorbs components of the medium, such as vitamins, cytokinins, auxins and ascorbic acid (Druart and Wulf, 1993).

Considering the need for basic studies of micropropagation with *Orbignya oleifera* (Burret.), the aim of the present work was to evaluate the*in vitro* growth of zygotic embryos of babassu under different concentrations of sucrose and activated charcoal.

Materials and methods

Plant material

The experiment was conducted at the Laboratory of Plant Tissues and Culture ("Laboratório de Cultura e Tecidos Vegetais") of the Goiano Federal Institute Câmpus Rio Verde (Instituto Federal Goiano Câmpus Rio Verde-GO).

The classification of the species under study was performed by Dr. Maria Cristina de Souza, Federal University of Acre, Forest Campus ("Universidade Federal do Acre, Câmpus Floresta"), in the city of Cruzeiro do Sul - AC. A voucher specimen is deposited in the Jataiense Herbarium ("Herbário Jataiense"), Federal University of Goiás, Jataí Câmpus ("Universidade Federal de Goiás, Câmpus Jataí"), under collection number 5641.

The fruits were collected after abscission in March 2011 from amonga population of plants growing on the Santa Bárbara farm ("fazenda Santa Bárbara") in the municipality of Piranhas - GO, with the coordinates 16 °22'015 "S - 51 °55'715" W and an elevation of 389 m.

After harvesting, ripe fruits were selected, and the endocarp was broken using a hydraulic press. The seeds were removed from the interior of the fruits, and the zygotic embryos were removed (Figure 1 A - D).

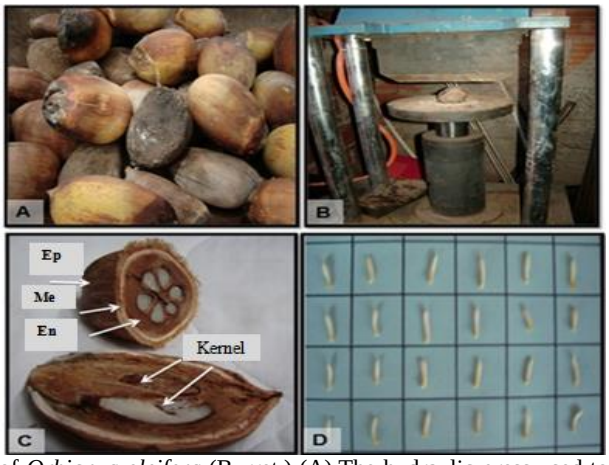

Fig. 1. Mature fruits of *Orbignya oleifera* (Burret.) (A).The hydraulic press used toextract the seeds (B). Thefruit in cross-section and longitudinal section (C). The babassu zygotic embryos (D). (Ep, epicarp; Me, mesocarp; En, endocarp and kernel) Photo: Mariluza S. Leite. Rio Verde - GO, 2012.

Sterilization

The embryos were covered with gauze and immersed in 70% ethanol for 1 minute. Next, the embryos were immersed in a 20% solution of sodium

hypochlorite - NaOCl (commercial bleach - 2.5% active chlorine) for 20 min and washed 3 times with sterile water in a laminar flowhood.

In vitro establishment

The babassu embryos were cultured in test tubes (25 x 150 mm) containing 20 mL of MS culture medium witha 50% salt concentration (Murashige and Skoog, 1962) and supplemented with 3.5 5 $g.L^{-1}$ of agar (Din âmica®)anddifferent concentrations of sucrose (0, 15, 30, 45 and 60 $g.L^{-1}$) and activated charcoal (0 and 2 $g.L^{-1}$), and the final pH was adjusted to 5.7 ± 3. The medium was autoclaved at 121 °C and 1.05 $kgcm^{-2}$ pressure for 20 minutes. The test tubes containing the inoculated embryos were incubated in a growth chamber for 120 days at a temperature of 25 ±3 °C and a relative humidity of 45%. Every 30 days, the embryos were transferred to fresh media, identical to the original, and weremaintained under a 16-hour photoperiod witha photosynthetic active radiation of 45-55 µmol $m^{-2}s^{-1}$, which was obtained using white fluorescent lamps.

Evaluations and experimental design

Counts were performed on alternate days to evaluate the germination percentage and germination speed index (GSI). The average length of the seedlings was evaluated at 30, 60, 90 and 120 days of culture, and the GSI; the formation of the shoot, cotyledon petiole and roots; and the oxidation of zygotic embryos were evaluated at 120 days of cultivation.

The experimental design was completely randomized in a factorial 5x2arrangement, with 5 concentrations of sucrose (0, 15, 30, 45 and 60 $g.L^{-1}$) and 2 concentrations of activated charcoal (0 and 2 $g.L^{-1}$)to produce10 treatments with 25 replicates (each consisting of a single test tube) totaling 250 experimental units. The numerical data were statistically evaluated using an analysis of variance with the application of the F test at a 5% probability, and the means were analyzed by regression using the software SISVAR (Ferreira, 2011).

Results and discussions

The babassu seed has a white homogeneous endosperm, with an oily, hard consistency, and occupies the entire space inside the seed (Figure 2). The embryo is laterally peripheral, cylindrical and elongated, with one end formed by the cotyledon petiole and the other end comprising the haustorium (Figure 2). The germination of embryos began after 7 days of culturing: the embryo

imbibed, and the cotyledon petiole extended, consequently reducing the haustorium (Figure 2 C).

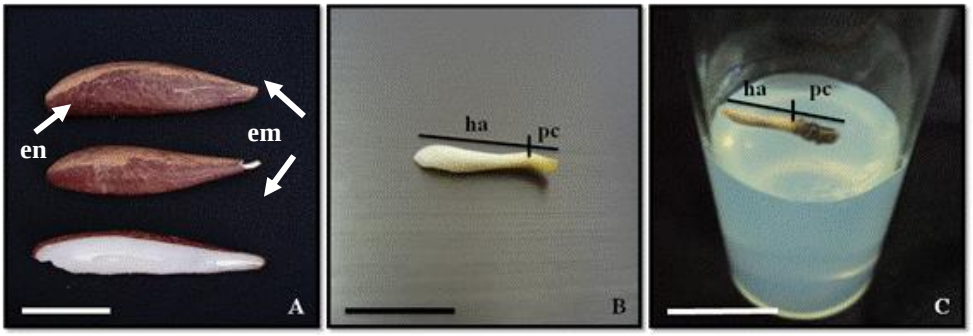

Fig. 2. Seeds of *Orbignya oleifera* Burret, whole and in cross-section (A), zygotic embryo (B), the imbibition and growth of the cotyledon petiole (C) (Scale bar equals 1.5 cm). em, embryo; en, endosperm; cp, cotyledon petiole; ha, haustorium. Photo: Mariluza S. Leite. Rio Verde – GO, 2012

According to the analysis of variance, the interaction (sucrose x activated charcoal) and the isolated effects of the sucrose and activated charcoal did not influence the characteristics related to the germination and oxidation of the explants.

The different concentrations of sucrose did, however, influence the *in vitro* responses of the average length of the seedlings, shoot formation, cotyledon petiole and roots. The activated carbon had no influence on vigor.

The quadratic regression model was the best fit in response to these characteristics. The absence of sucrose and activated charcoal resulted in the lowest average for vigor, and the increased concentrations of sucrose in the absence or presence of activated charcoal increased vigor (Figure 3A).

The peak in the quadratic function that modeled the formation of the shoots indicated that, in the absence of activated charcoal, 34 $g.L^{-1}$ sucrose resulted in the highest shoot area (40%), which was followed by a decrease (to 20%) at a concentration of 60 $g.L^{-1}$ sucrose. In the presence of activated carbon, the peak for shoot growth was estimated at 34.5 $g.L^{-1}$ sucrose, producing the greatest formation of aerial parts (46%), which decreased (22%) as the concentration of sucrose increased to 60 $g.L^{-1}$ (Figure 3B).

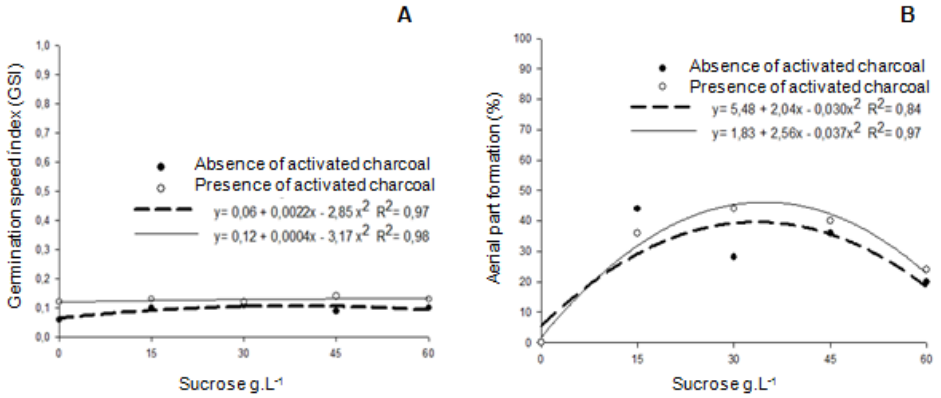

Fig. 3. Effect of different concentrations of sucrose and activated charcoal on the germination speed index (GSI) (A) and formation of aerial parts (B) in zygotic embryos of babassu (*Orbignya oleifera* Burret.) evaluated at 120 days cultivation. Rio Verde-GO, 2012

The peak average length of the seedlings evaluated at 30 days of cultivation in the absence of activated charcoal was estimated to be 35.5 $g.L^{-1}$sucrose, which resulted in the long estaverage length of 2.7 cm. A decrease in the average length of the seedlings to 2.15 cm was observed by increasing the concentration to 60 $g.L^{-1}$sucrose. In the presence of activated carbon, the peak was obtained with 57 $g.L^{-1}$sucrose, with a mean of 3.03 cm (Figure 4 A).

At 60 days of culture, 37.5 $g.L^{-1}$sucrose in the absence of activated charcoal resulted, on average, in the longest seedlings of 3.8 cm, with a decrease to 3.03 cm at a concentration of 60 $g.L^{-1}$. In the presence of activated carbon, the estimated average length peaked at 46.5 $g.L^{-1}$ sucrose, resulting in the highest average of 4.04 cm (Figure 4 B).

The average length of the seedlings was evaluated at 90 days of culture in the absence of activated charcoal, and the maximum value was obtained at 39 $g.L^{-1}$sucrose, resulting in the longest average length of 4.5 cm. The length decreased to 3.60 cm as the concentration was increased to 60 $g.L^{-1}$sucrose.In the presence of activated carbon, the peak was estimated to be at 47 $g.L^{-1}$sucrose, which produced the longest average seedling length of 5.05 cm, subsequently decreasing as the concentration increased to 60 $g.L^{-1}$sucrose (4.79 cm) (Figure 4 C).

The evaluation of the average length of the seedlings at 120 days of cultivation showed that, in the absence of activated charcoal, the peak occurred with 40 $g.L^{-1}$sucrose, resulting in an average of 5.05 cm. As the sucrose concentration increased to 60 $g.L^{-1}$sucrose, a decrease in the growth was observed, with an average of 4.2 cm. In the presence of activated carbon, the

maximum point was achieved at 41.5 g.L^{-1}sucrose, resulting inan average of 5.12 cm, followed by a decrease (4.45 cm) with 60 g.L^{-1} (Figure 4 D).

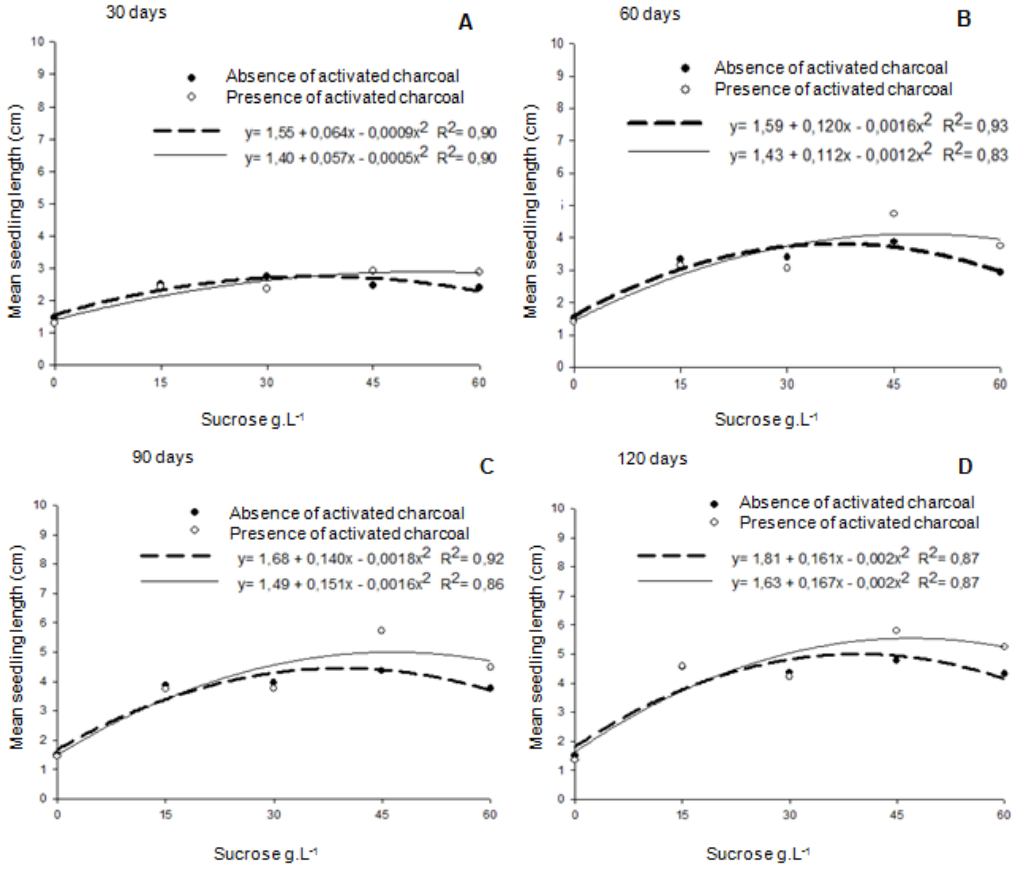

Fig. 4. Effect of different concentrations of sucrose and activated charcoal on the average length of seedlings of babassu (*Orbignya oleifera* Burret.) evaluated at 30, 60, 90 and 120 days of cultivation. Rio Verde-GO, 2012

The highest rate for the formation of the cotyledon petiole occurred when the embryos were cultured in 49 g.L^{-1}sucrose (47%). In the presence of activated carbon, the highest rate for the formation of the cotyledon petiole occurred when the embryos were cultured in 60 g.L^{-1}sucrose (32.5%) (Figure 5 A).

The highest percentage of rooted seedlings cultured without active charcoal was found for the medium supplemented with 32.2 g.L^{-1}sucrose (40.5%). In the presence of activated carbon, a higher percentage of seedlings with roots was found in the medium supplemented with 38 g.L^{-1}sucrose (42.5%) (Figure 5 B).

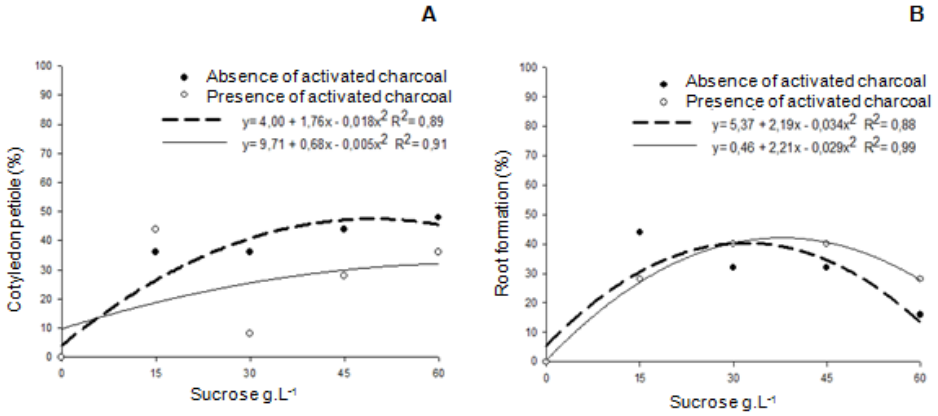

Fig. 5. Effect of different concentrations of sucrose and activated charcoal on the formation of the cotyledon petiole (A) and the formation of roots (B) in zygotic embryos of babassu (*Orbignya oleifera* Burret.) evaluated at 120 days of cultivation. Rio Verde-GO, 2012

By evaluating with statistical data (Figure 6 A and B), we found that in the absence of sucrose, the embryos germinated because of the nutritional reserves of the cotyledons but did not grow.

In the treatments with the lowest concentrations of sucrose, an increased rate for the formation of shoots and roots was observed but with a lower average length of the seedlings (Figure 6 C and D). The best average length of the seedlings and rate for the formation of cotyledon petioles was obtained with increasing concentrations of sucrose, but less root formation was also observed (Fig. 6 E, F, G, H, I and J).

Throughout the process of germination and seedling growth, the rate of oxidation was low, and there was no callus formation.

According to these results, smaller quantities of sucrose were required to obtain seedlings *in vitro* in the absence of activated charcoal. Conversely, in the presence of activated charcoal, higher concentrations of sucrose produced the best results.

The sucrose concentration of 45 $g.L^{-1}$in the presence or absence of activated charcoal produced the longest average seedling length.

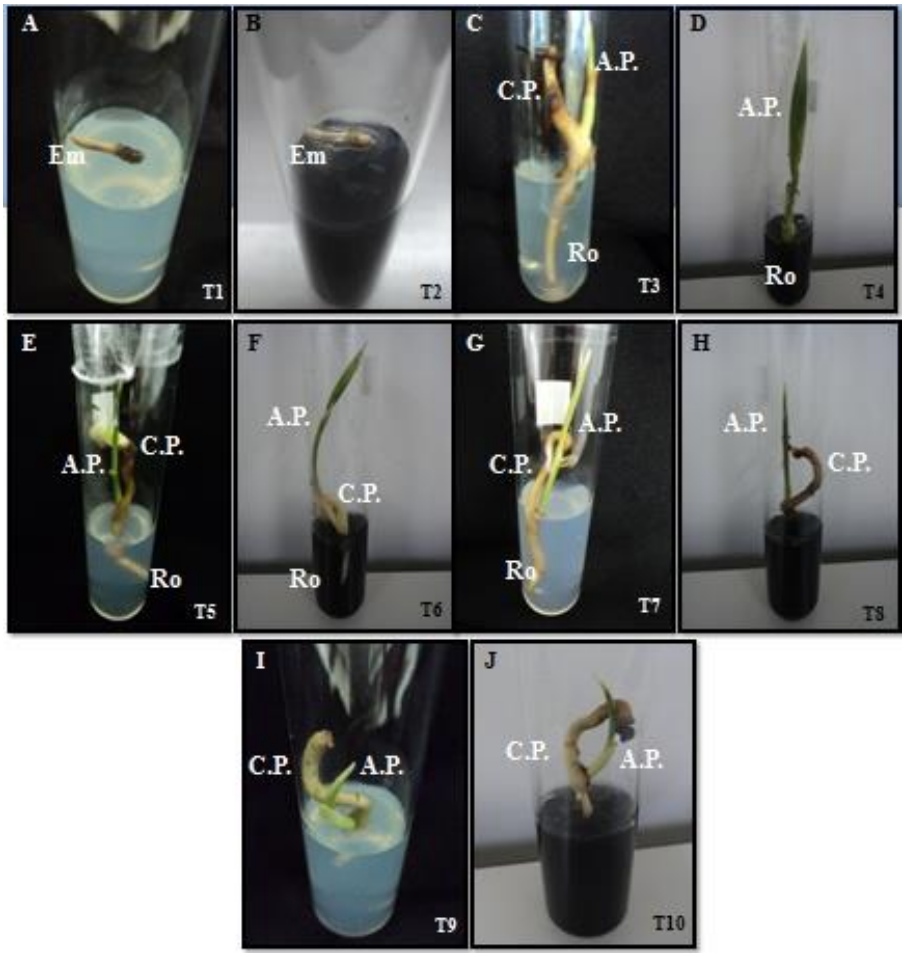

Fig. 6. *In vitro* culture of babassu (*Orbignya oleifera* Burret.) embryos cultured with different concentrations of sucrose and activated charcoal evaluated at 120 days of cultivation. A) T1,activated charcoal (0 $g.L^{-1}$) andsucrose (0 $g.L^{-1}$); B) T2,activated charcoal (2 $g.L^{-1}$) andsucrose (0 $g.L^{-1}$); C) T3, activated charcoal (0 $g.L^{-1}$) andsucrose (15 $g.L^{-1}$); D) T4, activated charcoal (2 $g.L^{-1}$) andsucrose (15 $g.L^{-1}$); E) T5, activated charcoal (0 $g.L^{-1}$); andsucrose (30 $g.L^{-1}$); F) T6, activated charcoal (2 $g.L^{-1}$) andsucrose (30 $g.L^{-1}$); G) T7, activated charcoal (0 $g.L^{-1}$) andsucrose (45 $g.L^{-1}$); H) T8, activated charcoal (2 $g.L^{-1}$) andsucrose (45 $g.L^{-1}$); I) T9, activated charcoal (0 $g.L^{-1}$) andsucrose (60 $g.L^{-1}$); J) T10, activated charcoal (2 $g.L^{-1}$) andsucrose (60 $g.L^{-1}$). Embryos germinated without development (Em); aerial part (A.P), cotyledon petiole (C.P) and root (Ro). Rio Verde GO. 2012

The addition of sucrose to the cultivation medium of zygotic embryos has been evaluated by several authors with different species of plants, mainly in the early stages of embryo development (pro-embryos). In general, the younger the embryos are, the higher the osmolarity of the medium needs to be (Torres *et al*., 2005).

Similar data were obtained by Garcia *et al*. (2002), who worked with the zygotic embryos of olive (*Olea europaea* L.) and concluded that an exogenous supply of carbohydrates was not necessary for germination. Nonetheless, the absence of sucrose in the culture medium reduced development and allowed the production of seedlings for acclimatization. Pereira *et al*. (2006), who worked with zygotic embryos of the palm "murmur" (*Astrocaryum ulei* Burret), showed that low concentrations of sucrose are also indicated for the germination of embryos obtained from ripe fruit. These authors also showed that the addition of 15 $g.L^{-1}$sucrose to the culture medium was sufficient to achieve the best germination rates, and higher concentrations were required to sustain the growth of seedlings.

These data corroborate the work of Ribeiro *et al*. (2011), who found that the zygotic embryos of sour-coconut (*Butia capitata* [Mart.] Becc.)did not form roots without sucrose. This finding indicates the inadequacy of the carbohydrate reserves necessary for seedling development.

These results differ from those obtained by Ledo *et al*. (2007),who observed that a concentration of 60 g L^{-1}sucrose was necessary to promote the further development of the aerial parts of dwarf-coconut embryos (*Cocos nucifera* L.). For the *in vitro* regeneration of dwarf embryos, the use of 2.5 $g.L^{-1}$of activated charcoal in the culture medium was effective in eliminating the darkening caused by oxidation.

According to Nicoloso *et al*. (2001), adding activated charcoal to the medium isnot always advantageous. Using cultures of Brazilian ginseng (*Pfaffia glomerata* [Spreng.] Pedersen), these authors reported that negative responses were observed both in the shoot dry matter and root development in the presence of activated carbon. These authors affirmed that the presence of activated carbon considerably adsorbs the chemical elements present in the environment, thus reducing their availability.

In cultured pear (*Pyrus communis*L.), a concentration of 1% activated charcoal added to the culture medium did not benefit rooting, which was associated with the capacity of the activated carbon to retain substances (Erig *et al*., 2004).

Conclusion

The use of 15 $g.L^{-1}$sucrose in the culture medium without activated charcoal generated the best results for the formation of aerial parts and the root system. The use of activated charcoal for the *in vitro* culture of babassu (*Orbignya oleifera* Burret.) zygotic embryos is unnecessary.

Acknowledements

We thank the Brazilian National Counsel of Technological and Scientific Development ("Conselho Nacional de Desenvolvimento Científico e Tecnológico" – CNPq), the Brazilian Federal Agency for the Support and Evaluation of Graduate Education ("Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" – CAPES), and the Goias Federal Institue *Campus* Rio Verde (Instituto Federal Goiano *Campus* Rio Verde) for the financial support for this research.

References

Albiero, D., Maciel, A. J. S., Lopes, A. C., Mello, C. A. and Gamero, C. A. (2007). Proposta de uma máquina para colheita mecanizada de babaçu (*Orbignya phalerata* Mart.) para a agricultura familiar. Acta Amazônica. pp. 337-346.

Druart, P. H. and Wulf, O. De. (1993). Activated charcoal catalyses sucrose hydrolysis during autoclaving. Plant Cell, Tissue and Organ Culture, Dordrecht 32:97-99.

Erig, A. C., Wulffschuch, M. and Braga, E. J. B. (2004). *In vitro* rooting of pear tree (*Pyrus communis* L.) cv. Carrick. Ciência Rural 34:275-277.

Ferreira, D. F. and Sisvar, A. (2011). Computer statistical analysis system. Ciências Agrotécnica, Lavras 35:1039-1042.

Ferreira, M. G. R., Cárdenas, F. E. N., Carvalho, C. H. S., Carneiro, A. A. and Damião, F. C. F. (2002). Resposta de eixos embrionários de cupuaçu (*Theobroma grandiflorum* Schum.) à concentração de sais, doses de sacarose e renovação do meio de cultivo. Revista Brasileira de Fruticultura 24:246-248.

García, J. L., Troncoso, J., Sarmiento, R. and Troncoso, A. (2002). Influence of carbon source and concentration on the *in vitro* development of olive zygotic embryos and explants raised from them. Plant Cell, Tissue and Organ Culture, Dordrecht 69:95-100.

Ledo, A. S., Gomes, K. K. P., Barboza, S. B. S. C., VIeira, G. S. S., Tupinambá, E. A. and Aragão, W. M. (2007). Cultivo *in vitro* de embriões zigóticos e aclimatação de plântulas de coqueiro-anão. Pesquisa Agropecuária Brasileira 42:147-154.

Ledo, A. S., Lameira, O. A., Benbadis, A. K., Menezes, I. C., Ledo, C. A. S. and Oliveira, M. S. P. (2001). Cultura *in vitro* de embriões zigóticos de açaizeiro. Revista Brasileira de Fruticultura 23:468-472.

Lima, J. R. O., Silva, R. B., Silva, C. C. M., Santos, L. S. S., Junior, J. R. S., Moura, E. M. and Moura, C. V. R. (2007). Biodiesel de Babaçu (*Orbignya* sp.) obtido por via etanólica. Revista Química Nova 30:600-603.

Melo, B., Pinto, J. E. B. P., Luz, J. M. Q., Peixoto, J. R. and Juliatti, F. C. (2001). Diferentes antioxidantes no controle da oxidação, germinação e desenvolvimento das plântulas na cultura *in vitro* de embriões da guarirobeira [*Syagrus oleracea* (Mart.) Becc.]. Ciência e Agrotecnologia 25:1301-1306.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, Copenhagen 15:473-497.

Nicoloso, F. T., Erig, A. C. and Martins, C.F. (2001). Micropropagação do ginseng brasileiro [*Pfaffia glomerata* (Spreng.) Pedersen]. Revista Brasileira de Plantas Medicinais 3:11-18.

Pasqual, M., Ribeiro, V. G. and Ramos, J. D. (1990). Influência do GA3 e do carvão ativado sobre o enraizamento in vitro de embriões de laranja 'Natal'. Pesquisa Agropecuária Brasileira, Brasília 25:1477-1482.

Pereira, J. E. S., Maciel, T. M. S., Costa, F. H. S. and Pereira, M. A. A. (2006). Germinação *in vitro* de embriões zigóticos de murmuru (*Astrocaryum ulei* Burret.). Ciência e Agrotecnologia, Lavras 30:251-256.

Ribeiro, L. M., Neves, S. C., Silva, P. O. and Andrade, I. G. (2011). Germinação de embriões zigóticos e desenvolvimento *in vitro* de coquinho-azedo [*Butia capitata* (Mart.) Becc]. Revista Ceres, Viçosa 8:133-139.

Torres, A. C., Duval, F. G., Ribeiro, D. G., Barros, A. F. F. and Aragão, F. A. D. (2005). Efeito da sacarose, cinetina, isopentenil adenina e zeatina no desenvolvimento de embriões de *Heliconia rostrata in vitro.* Horticultura Brasileira 23:789-792.

(Received 25 December 2013; accepted 30 April 2014)