

*Full Length Research Paper*

## Alternative tube caps on *in vitro* growth of *Orbignya oleifera* Burret.: An Arecaceae native cerrado domain

Mariluzia Silva LEITE, Paula Sperotto Alberto FARIA, Flávia Dionísio PEREIRA, Fabiano Guimarães SILVA\*, Aurélio Rúbio NETO and João das Graças SANTANA

Instituto Federal de Educação, Ciência e Tecnologia Goiano, *Campus* Rio Verde. Rod. Sul Goiana Km 01, Cx. P. 66. CEP 75.901-970, Rio Verde – Goiás, Brazil.

Received 7 August, 2016; Accepted 12 October, 2016

The babassu (*Orbignya oleifera* Burret) is a palm that contains oilseeds whose biomass can be used for biofuel production. Due to difficulties in germinating the species, *in vitro* cultivation techniques are sought as a viable alternative to expand studies on germination. The objective of this study was to evaluate different types of tube caps for *in vitro* growth of babassu seedlings. Thus, zygotic embryos were inoculated in tubes containing MS medium with a 50% salt concentration, which were sealed with different caps, a cotton plug, plastic cap, PVC film and plastic cap with PVC film. During the *in vitro* culture, the mean length of seedlings, the formation of the cotyledon petiole and root, as well as oxidation of the explants were evaluated. The cotton plug exhibited a positive effect on the initial steps of *in vitro* cultivation, which promoted more vigorous plants with greater mean lengths; however, its effect decreased as the cultivation time increased. The cotton plug is an alternative and eight-fold less expensive than a conventional plug, which favors early *in vitro* growth of *O. oleifera* Burret.

**Key words:** Babassu, Goiás Cerrado, tissue culture.

### INTRODUCTION

*Orbignya oleifera* Burret (synonym *Attalea vitrivir* Zona) belongs to the family Arecaceae known as "babassu" and is distributed in forests or in open areas (Santos et al., 2015). This palm is native from central-western, northern and northeastern Brazil. Clusters of individuals from this species are referred to as masses and are located in transition areas between the Amazon basin rainforests, the cerrado (Brazilian savanna) and the semi-arid region of northeastern Brazil, which cover approximately 18.5 million hectares and are distributed in the states

Maranhão, Piauí, Tocantins, Goiás, Amazonas, Pará and Mato Grosso (Lima et al., 2006). Babassu exhibits great potential for exploitation because the plant can be used in gardening and landscaping, and the fruit mesocarp produces high-quality charcoal that is used as an energy source in steel mills (Teixeira, 2008). Babassu seeds may be consumed fresh; processed during cosmetic and lubricant production to obtain an oil that is rich in lauric acid and that is used for human consumption; or used for biodiesel (Lima et al., 2007; Martins et al., 2009; Souza et

\*Corresponding author. E-mail: [fabianoifgoiano@gmail.com](mailto:fabianoifgoiano@gmail.com).



**Figure 1.** Morphological characteristics of babassu (*Orbignya oleifera* Burret). (A) The mother plant; (B) transversal and longitudinal sections of the fruit; (C) seeds; and (D) zygotic embryos. Scale: 2 cm.

al., 2011).

Germination of most Arecaceae family species is slow and irregular and typically involves low multiplication rates; propagation is sexual (Rubio Neto et al., 2015). However, current technological advances involving *in vitro* techniques has optimized healthy and well-developed seedling production (Silva et al., 2012; Pereira et al., 2006; Tzec-Sima et al., 2006). The microenvironment inside culture tubes is directly related to the type of cap used, affecting aeration and the incidence of light for *in vitro* cultivation, which, in turn, may be related to variable culture behavior (Corrêa et al., 2015; Santana et al., 2008; Souza et al., 2007). Caps that are not hermetically sealed allow greater gas exchange between the atmospheric air and internal tube environment, which promotes better leaf transpiration and prevents ethylene accumulation (Corrêa et al., 2015; Nepomuceno et al., 2009; Emam and Esfahan, 2014).

Certain alternatives, such as using cotton plugs or filters with micropores to plug the tubes, may facilitate gas exchange, which favors seedling growth and development *in vitro* without microorganism contamination (Fernandes et al., 2013). New alternatives for improving plant growth and reducing costs are desirable for optimizing large-scale commercial production. Based on this information and a lack of reports on *in vitro* *O. oleifera* (Burret) cultivation, the

objective of this study was to evaluate the effect of different types of caps on *in vitro* growth of babassu seedlings.

## MATERIALS AND METHODS

### Acquisition of plant material

The experiment was conducted at the Laboratory of Culture and Plant Tissues, Goiano Federal Institute, Rio Verde Campus, Goiás (GO), Brazil. The species studied was classified by Dra. Sousa from the Federal University of Acre, Floresta Campus, Cruzeiro do Sul, Acre, Brazil. A voucher specimen was deposited at the Jataiense Herbarium of the Federal University of Goiás, Jataí Campus, under collection number 5641.

The fruits were collected after abscission from a population of plants located at the Santa Barbara farm in the municipality of Piranhas – GO, Brazil, at coordinates 16° 22'015" S and 51° 55'715" W and an elevation of 389 m. The climate, according to Koppen (1948) classification was Cfa and the soil classified as Oxisoil. Subsequently, the fruits were broken in a hydraulic press to crack the endocarp. The kernels were thereafter removed from inside the fruit, and the zygotic embryos removed using a scalpel (Figure 1).

### Sterilization

The embryos were wrapped in gauze and immersed in 70% ethanol for 1 min followed by immersion in a 20% sodium hypochlorite

solution (NaOCl; commercial bleach - 2.5% active chlorine) for 20 min and rinsed three times with sterile water in a laminar flow chamber.

#### ***In vitro* establishment**

The embryos were cultured in test tubes (25 x 150 mm) containing 20 mL of MS medium (Murashige and Skoog, 1962) with 50% salt and 30 g L<sup>-1</sup> sucrose and supplemented with 3.5 g L<sup>-1</sup> agar (Dinâmica®- Brazil); the pH was adjusted to 5.7 ± 0.03. Subsequently, the medium was autoclaved at 121°C and 1.05 kg cm<sup>-2</sup> for 20 min. Test tubes containing the embryos were capped using one of four means: cotton plug, plastic cap (polypropylene), PVC film and plastic cap wrapped with PVC film. The tubes were maintained in a growth room for 120 days at 25 ± 3°C and 45% relative humidity, every 30 days; the embryos were transferred to a new culture medium identical to the first, and were maintained under a 16-h photoperiod and 11.86 W m<sup>2</sup> which was generated using white fluorescent bulbs.

#### **Water loss from the culture medium**

The test tubes with the different caps were characterized based on water loss by daily weighing on an analytical balance (AND-HR-200, d=0.1 mg- made in Japan) for 32 days. Also, every 30 days it was exchanged for a new culture medium identical to the first.

#### **Evaluation and experimental design**

Counts were performed every day up to 30 days of culture to assess the percent germination and vigor using a germination speed index, which was calculated, based on the formula proposed by Maguire (1962); and at 120 days, root formation (RF), formation of the cotyledon petiole and oxidation of the zygotic embryos was evaluated. The seedlings were considered normal when they exhibited shoots and root systems. At 30, 60, 90 and 120 days of cultivation, the mean seedling length was assessed, for this variable was considered the design in a split plot.

The experimental design was completely randomized (CRD) and consisted of four treatments (cotton plug, plastic cap, PVC film and plastic cap wrapped with PVC film) with 30 replicates; each replicate consisted of one test tube, totaling 120 experimental units. The numerical data were subjected to Analysis of Variance (ANOVA) and Tukey's test for qualitative factors and regression analysis for the quantitative factors with 5% probability. The program SISVAR was used to analyze the data (Ferreira, 2011).

## **RESULTS AND DISCUSSION**

Contamination was not observed in any treatment. The different types of seals did not affect the quality of seedlings *in vitro*. For all caps, embryo germination and initial seedling growth were observed, which suggests that *in vitro* multiplication of babassu via zygotic embryos was viable. Embryo germination was demonstrated by cotyledon petioles lengthening, which results in seedling shoot and root formation characteristic of remote germination.

The cotyledon petiole formed between 30 and 60 days of cultivation, and the shoot was emitted beginning at 90 days of cultivation originating from the cotyledon petiole,

which opens in a slit. The roots emitted from the lower region of the cotyledon petiole at 120 days of cultivation (Figure 2A, B, C and D). The explants did not exhibit oxidation regardless of the cap used.

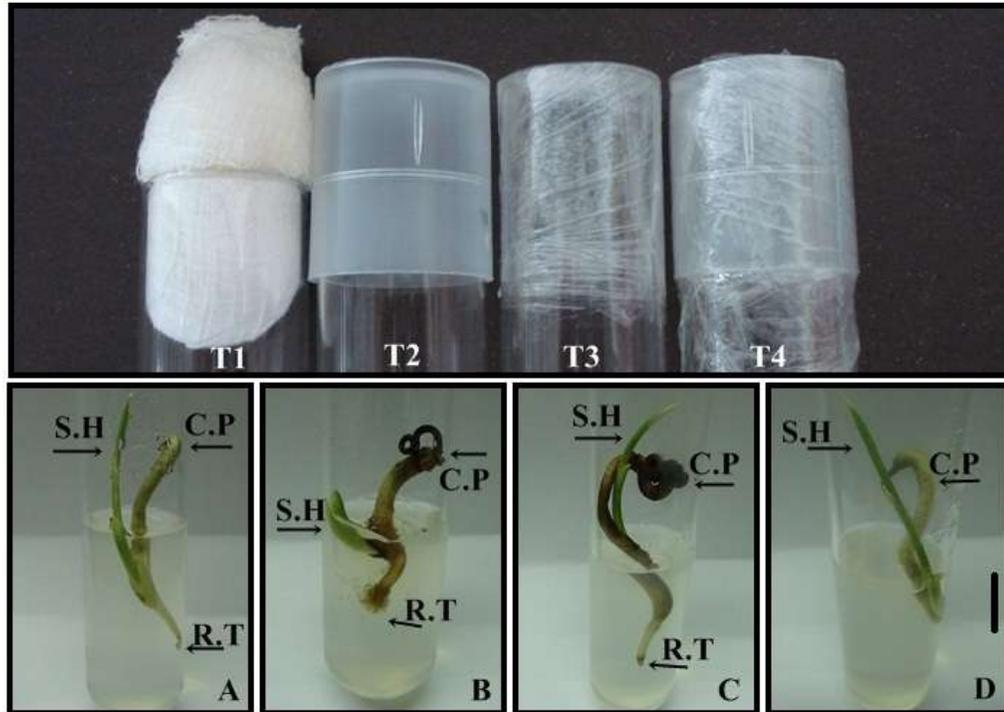
Interaction between time and types of seals were first observed; thereafter, data were discussed together, in which linear behavior was noted in function of time evaluation seals utilized. At 120 days of *in vitro* culture, seedlings were obtained with a maximum length of 5.33 cm in the sealed culture condition with cotton cap, followed by PVC with length of 4.99 cm. For sealing cap and cap with PVC, less length -an average of 3.91 and 4.32 cm, respectively was observed (Figure 3).

The cotton plug exhibited a positive effect on the early stages of *in vitro* cultivation, promoting more vigorous plants. Tubes sealed with plastic caps wrapped with PVC film exhibited a lower percentage of root formation (17.0%) (Table 1).

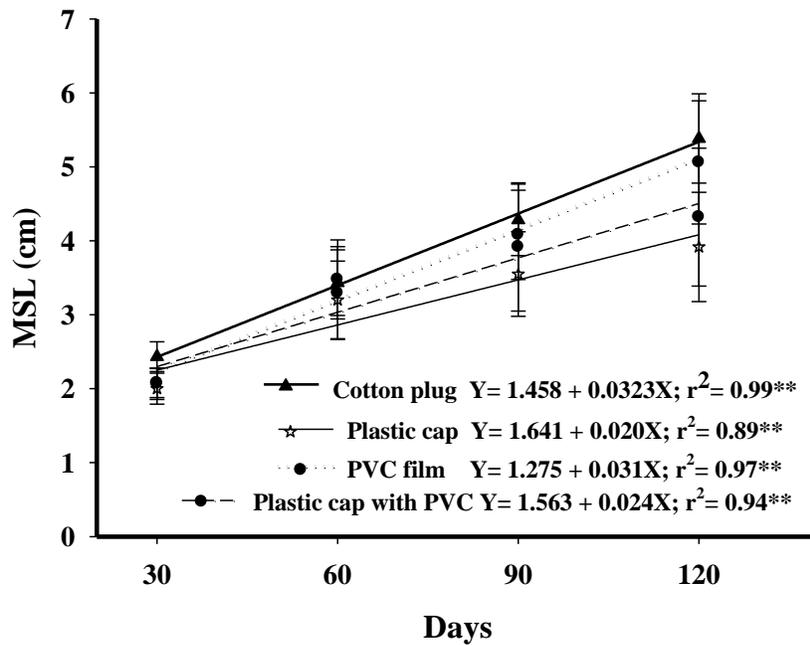
The positive effects on the initial growth of embryos and seedling shoots in tubes sealed with a cotton plug may be explained by the greater water vapor loss rate (WVRL) and aeration that this cap promoted for the plants (Figure 4). This type of cap facilitates greater gas exchange between the atmospheric air and inner tube environment, which allows better leaf transpiration and prevents the accumulation of harmful gases, such as ethylene (Corrêa et al., 2015; Iarema et al., 2012).

The initial amount of water in the culture medium, regardless of the cap, was approximately 17.5 g. The type of cap exerted a significant effect ( $p < 0.05$ ) from the 7<sup>th</sup> day of cultivation, when the samples exhibited greater water loss in tubes sealed with the cotton plug. Comparing the initial and final quantities of water in the medium at 32 days of cultivation, the tubes sealed with cotton plugs exhibited 22% water loss, and the tubes sealed with the conventional cap exhibited 2% water loss. Although the cotton plug allowed greater evaporation and reduced the initial volume of the culture medium by 22%, these phenomena were not detrimental to seedling growth, most likely because the culture medium was changed every 30 days.

Polypropylene plastic caps are the most widely used caps in laboratories for tissue cultivation due to their resistance to high temperatures; such caps can be autoclaved without deformation. However, other cap types, such as cotton plugs and PVC film, are used due to the high cost of these caps. Using cotton as a plug has advantages, including preventing explant drying, contamination control, permitting gas exchange with the external environment and reducing the cost in the final production of seedlings (Assis et al., 2012; Pinheiro et al., 2013). Growth of the seedlings cultured *in vitro* using the cotton plug, as observed in this study, has been described in several studies as a promising practice for stimulating improved photoautotrophic micropropagation (Fernandes et al., 2013; Nepomuceno et al., 2009; Saldanha et al., 2012; Santana et al., 2011).



**Figure 2.** *In vitro* *Orbignya oleifera* (Burret) cultivation at 120 days in 50% MS medium with different cap types. (A) T1: cotton plug; (B) T2: plastic cap; (C) T3: PVC film; and (D) T4: plastic cap wrapped with PVC film. SH: shoot; CP: cotyledon petiole; and RT: root. Scale bar: 1 cm.

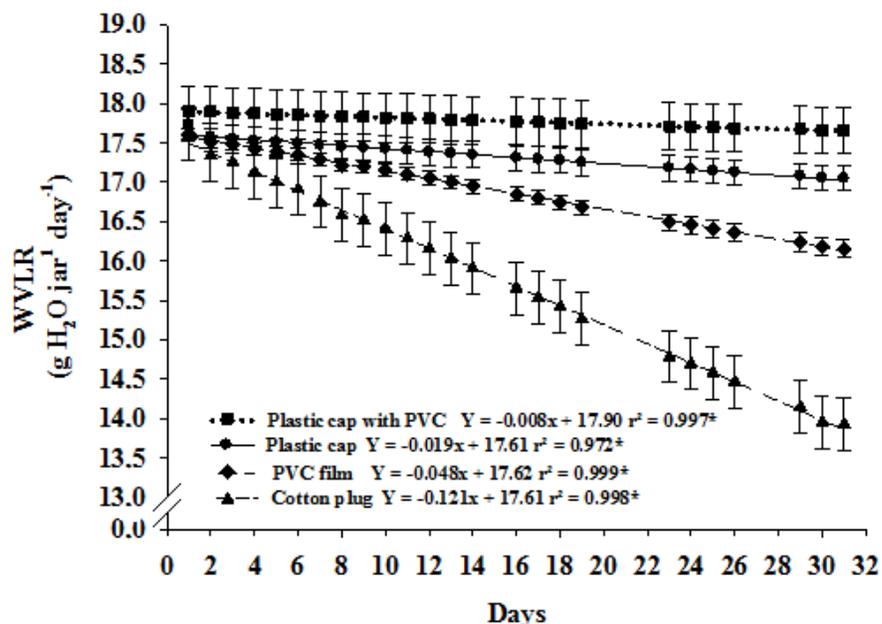


**Figure 3.** Mean seedling length (MSL) evaluated at 30, 60, 90 and 120 days of cultivation in babassu (*Orbignya oleifera* Burret) zygotic embryos, cultured *in vitro* with different types of caps cotton plug, plastic cap, PVC film and plastic, cap with PVC film.  $^{**}$ Significant at  $p < 0.01$ .

**Table 1.** Germination speed index (GSI) at 30 and 120 days, root formation (RF), of cultivation in babassu (*Orbignya oleifera* Burret) zygotic embryos, cultured *in vitro* with different types of caps.

Type of cap	GSI	RF 120 days (%)
Cotton plug	0.18± 0.10 <sup>az</sup>	77.0± 0.36 <sup>a</sup>
Plastic cap	0.12± 0.03 <sup>b</sup>	60.0± 0.48 <sup>ab</sup>
PVC film	0.12± 0.03 <sup>b</sup>	43.0± 0.49 <sup>bc</sup>
Plastic cap with PVC film	0.13± 0.04 <sup>ab</sup>	17.0± 0.28 <sup>c</sup>

<sup>Z</sup>Means followed by the same letter do not differ significantly according to Tukey's test at a 5% probability. ± Standard error of the mean.



**Figure 4.** Water vapor loss rate (WVLR) from the culture medium with babassu seedlings (*Orbignya oleifera* Burret.) in tubes with different cap types: plastic cap with PVC film, plastic cap, PVC film, and cotton plug, which were evaluated after 32 days. \*Significant at  $p < 0.05$ .

Similar results were obtained using *Anadenanthera colubrina* (Vell.) Brenan var. *cebil* (Griseb) Altschul seedlings cultured in test tubes sealed with cotton plugs; greater shoot lengths were produced compared with seedlings cultured in test tubes sealed with PVC film (Nepomuceno et al., 2009). Using the cotton plug also produced a positive effect in *Annona glabra* L. cultivation (Santana et al., 2011), where more expanded leaves, greater lengths and greater shoot dry matter weights were observed.

The root biomass of seedlings grown in tubes sealed with plastic caps wrapped with PVC most likely decreased due to less gas exchange (Figure 3) because condensation was observed on the inner walls of these tubes. The same phenomenon was observed with *A. glabra* L., in which the highest rooting percentages were

observed in seedlings cultured in tubes sealed with plastic caps without PVC film and in tubes sealed with cotton plugs (Santana et al., 2008).

Among the caps tested, using the cotton plug produced greater initial *in vitro* babassu growth compared with the plastic cap; moreover, the cotton plug can be easily constructed. Using the cotton plug promoted greater gas exchange and reduced the medium weight, which indicates less relative humidity inside the tube. Thus, it is a potential alternative for commercial *in vitro* propagation systems based on photoautotrophic growth patterns.

## Conclusion

The type of sealing of the test tubes influence the *in vitro*

growth of seedlings babassu (*Orbignya oleifera* Burret) by identifying cotton plug as the best alternative, being production with low cost and manpower.

### Conflict of Interests

The authors have not declared any conflict of interests.

### ACKNOWLEDGEMENTS

The authors thank the Coordination for the Improvement of Higher Education Personnel – CAPES) and the National Council for Scientific and Technological Development – CNPq) for the financial support granted to this study; the Goiano Federal Institute - Rio Verde Campus, and Dra. Maria Cristina de Sousa for classifying the species under study.

### Abbreviations

**ANOVA**, Analysis of variance; **CRD**, complete randomized design, **MS**, Murashige e Skoog; **PVC**, polyvinylchloride; **WVRL**, water vapor loss rate.

### REFERENCES

- Assis KCD, Pereira F, Cabral JSR, Silva FG, Silva JW, Santos SCD (2012). *In vitro* cultivation of *Anacardium othonianum* Rizz.: effects of salt concentration and culture medium volume. *Acta Sci. Agron.* 34(1):77-83.
- Corrêa JPO, Vital CE, Pinheiro MVM, Batista DS, Azevedo JFL, Saldanha CW, Cruz ACF, Matta FM, Otoni WC (2015). *In vitro* photoautotrophic potential and *ex vitro* photosynthetic competence of *Pfaffia glomerata* (Spreng.) Pedersen accessions. *Plant Cell Tissue Organ Cult.* 121(2):289-300.
- Emam M, Esfahan EZ (2014). Effect of chemical (enriched-CO<sub>2</sub> and sucrose-free medium) and physical factors (light period and temperature) on rooting and hardening of *Sorbus aucuparia* L. plantlets. *Int. J. Biosci.* 4(5):176-181.
- Fernandes DÁ, Azevedo PH, Costa RB, Brondani GE (2013). Types of packing and concentration of sucrose in culture *in vitro* of *Tectona grandis* L.f. *Rev. Agric.* 88(3):218-228.
- Ferreira DF (2011) SISVAR: A Computer Statistical Analysis System. *Ciênc. Agrotec.* 35(6):1039-1042.
- Iarema L, Cruz ACF, Saldanha CW, Dias LLC, Vieira RF, Oliveira EJ, Otoni WC (2012). Photoautotrophic propagation of Brazilian ginseng [*Pfaffia glomerata* (Spreng.) Pedersen]. *Plant Cell Tissue Organ Cult.* 110(2):227-238.
- Koppen W (1948). *Climatologia. Con um estudio de los climas de la tierra México.* Ed. Fondo de Cultura Economica, 478 p.
- Lima AM, Vidaurre GB, Lima RM, Brito EO (2006). Use of babaçu staple fiber as alternative raw material for panel production. *Rev. Árvore* 30(4):645-650.
- Lima JRDO, Silva RBD, Silva CCMD, Santos LSSD, Santos Jr JRD, Moura EM, Moura CVRD (2007). Biodiesel from babassu (*Orbignya* sp.) synthesized via ethanolic route. *Quím. Nova* 30(3):600-603.
- Maguire JD (1962). Speed of germination-aid in selection and evaluation for seedling emergence and vigor. *Crop Sci.* 2(2):176-177.
- Martins CC, Bovi MLA, Nakagawa J, Machado CG (2009). Drying and storage of jussara seeds. *Rev. Árvore* 33(4):635-642.
- Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* 15(3):473-497.
- Nepomuceno CF, Rios APS, Queiroz SRO, Pelacani CR, Santana JRF (2009). *In vitro* morphophysiological answers of the seedlings of *Anadenanthera colubrina* (Vell.) Brenan var. *cebil* (Griseb) Altschul. *Rev. Árvore* 33(3):481-490.
- Pereira JES, Marciel TMS, Costa FHS, Pereira MAAP (2006). *In vitro* germination of zygotic embryos of murmurú (*Astrocaryum ulei*). *Ciênc. Agrotec.* 30(2): 251-256.
- Pinheiro MVM, Martins FB, Xavier A, Otoni WC (2013). Gas exchange affects *in vitro* morphogenesis of two olive cultivars (*Olea europaea* L.). *Rev. Árvore*, 37(1): 19-29.
- Rubio Neto A, Silva FG, Sales JF, Pires LL, Freitas BSM, Souza AL (2015). Effect of drying temperature on viability of macaw palm (*Acrocomia aculeata*) zygotic embryos. *Afr. J. Biotechnology*, 14(4): 319-326.
- Saldanha CW, Otoni CG, de Azevedo JLF, Dias LLC, do Rêgo MM, Otoni WC (2012). A low-cost alternative membrane system that promotes growth in nodal cultures of Brazilian ginseng [*Pfaffia glomerata* (Spreng.) Pedersen]. *Plant Cell, Tissue and Organ Culture*, 110(3), 413-422.
- Santana JRF, Paiva R, Pereira FD, Oliveira LM (2008). Stimulus of the photoautotrophic behavior during the *in vitro* rooting of *Annona glabra* L., I. Development of root system and shoot. *Ciênc. Agrotec.* 32(1):80-86.
- Santana JRF, Paiva R, Souza AVD, Oliveira LMD (2011). Effect of different culture tube caps and concentrations of activated charcoal and sucrose on *in vitro* growth and budding induction of *Annona glabra* L. *Ciênc. Agrotec.* 35(5):916-923.
- Santos RRM, Cavallari MM, Pimenta MAS, Abreu AG, Costa MR, Guedes ML (2015). Population genetic structure of *Attalea vitriviv* Zona (Arecaceae) in fragmented areas of southeast Brazil. *Genet. Mol. Res.* 14(2):6472-6481.
- Silva MR, Carvalho Júnior OA, Souza ÉM, Mitja D, Chaib Filho H (2012). Multivariate factor analysis applied to the characterization of Babassu (*Attalea Speciosa* Mart. ex Spreng) occurrence area in Cocal river basin. *Soc. Nat.* 24(2):267-281.
- Souza JA, Silva LC, Corrêa MGS, Schuch MW (2007). *In vitro* rooting of the apple tree rootstock - m9 related to seal, sucrose and support material in the culture medium. *Sci. Agraria* 8(2):161- 164.
- Souza MH, Monteiro CA, Figueredo PM, Nascimento FRF, Guerra RN (2011). Ethnopharmacological use of babassu (*Orbignya phalerata* Mart) in communities of babassu nut breakers in Maranhão, Brazil. *J. Ethnopharmacol.* 133(1):1-5.
- Teixeira MA (2008). Babassu-a new approach for an ancient Brazilian biomass. *Biomass Bioenergy* 32(9):857-864.
- Tzec-Sima MA, Orellana R, Robert ML (2006). *In vitro* rescue of isolated embryos of bactris major jacq. and desmoncus *orthacanthos* mart., potentially useful native palms from the Yucatan Peninsula (Mexico). *In vitro Cell. Dev. Biol. Plant* 42(1):54-58.