In vitro culture of *Annona emarginata*: A rootstock for commercial annonaceae species

Cultivo *in vitro* de *Annona emarginata*: Porta-enxerto para espécies de anónaceas comerciais

Rodrigo Therezan de Freitas[1]*, Renato Paiva[1], Nádia Alves Campos[5], Luciano Coutinho Silva[2], Rony Swennen[3,4,5], Bart Panis[3]

ABSTRACT

Annona emarginata is a native fruit tree in the Brazilian Cerrado which, unlike the commercial species, does not present a high fruit quality. On the other hand, it stands out based on its rootstock value. However, there are some problems that hinder the large-scale production of *Annona emarginata* seedlings for use as a rootstock. In order to overcome these difficulties, micropropagation has become a viable alternative for the rapid and efficient propagation of *Annona* spp, but *Annona emarginata* micropropagation has not yet been reported. Therefore, the aim of this study was to initiate *Annona emarginata in vitro* growth. For axillary shoot proliferation, *in vitro* nodal segments of 0.5 cm were transferred to MS or WPM supplemented with BA at different concentrations. Nodal segments were also cultured on WPM medium with ranging concentrations of different plant growth regulators, aiming for either shoot elongation or rooting. The results showed that the use of 1 µM BA in WPM medium is recommended for *in vitro* multiplication of *Annona emarginata*. This is based on the low adventitious shoot formation, combined with a higher number of buds and leaves. The use of GA_3 at any concentration tested induced the formation of malformed plants. Root formation could not be stimulated, regardless the duration of auxin treatment.

Keywords: Araticum-mirim, microprogation, plant growth regulators.

RESUMO

Annona emarginata é uma frutífera presente no Cerrado brasileiro que diferentemente das espécies comercias não apresenta alta qualidade de frutos, porém esta espécie destaca-se baseado unicamente em seu valor como porta-enxerto. Entretanto, existem alguns problemas que impedem a produção em larga escala de mudas de *Annona emarginata* para utilização como porta-enxerto. Para superar estas dificuldades a micropropagação tornou-se uma alternativa viável para a propagação rápida e eficiente de *Annona* spp. Porém a micropropagação de *Annona emarginata* ainda não foi relatado. Objetivou-se com o presente estudo o cultivo *in vitro* de *Annona emarginata*. Para o aumento de brotações *in vitro*, segmentos nodais de 0,5 cm foram transferidos para meio MS ou WPM, suplementado ou não com BAP em diferentes concentrações. Segmentos nodais também foram cultivados em meio WPM com diferentes concentrações de diferentes reguladores de crescimento, visando o alongamento ou enraizamento. Os resultados mostraram que o uso de 1 uM de BAP em meio WPM é recomendado para a multiplicação *in vitro* de *Annona emarginata*, baseado na baixa formação de gemas adventícias combinado com um maior número de brotos e folhas. O uso de GA_3 em qualquer concentração testada induziu a formação de plantas malformadas e a formação de raízes não pode ser estimulada, independentemente do tipo ou da duração do tratamento com auxina testada.

Termos para indexação: Araticum-mirim, microprogação, regulador de crescimento.

INTRODUCTION

Annona ssp. represents a group of important fruit trees found in the Brazilian Cerrado and the Amazonian rainforest (Egydio and Santos, 2011). Five Annonaceae species are of major commercial importance (Pareek et al., 2011), such as cherimoya (*Annona cherimola* Mill.), the hybrid atemoya (*Annona cherimola* Mill. x *Annona squamosa* L.), the sugar apple or sweetsop (*Annona squamosa* L.), known as pinha or fruta do conde and the soursop (*Annona muricata* L.), known as graviola (Orsi et al., 2012), which are mainly used for pulp or juice production in Brazil.

Recebido em 24 de agosto de 2015 e aprovado em 5 de maio de 2016

[1]Universidade Federal de Lavras/UFLA, Departamento de Biologia, Lavras, MG, Brazil
[2]Universidade Federal da Paraíba/UFPB, Departamento de Biologia Celular e Molecular, João Pessoa, PB, Brazil
[3]Bioversity International, KU Leuven, Willem De Croylaan, Leuven, Belgium
[4]International Institute of Tropical Agriculture, c/o AVRDC, The World Vegetable Center, Arusha, Tanzania
[5]Laboratory of Tropical Crop Improvement, KU Leuven, Willem De Croylaan, Leuven, Belgium
*Corresponding author: rodrigotfreitas@hotmail.com

Due to difficulties inherent in conventional propagation through seeds (Padilla and Encina 2003; Santana et al., 2011), the propagation of commercial *Annona* species happens through grafting or budding (Costa, Bueno and Ferreira, 2011; Nagori and Purohit, 2004; Rasai, George and Kantharajah, 1995). Due to problems of incompatibility between the scion and the rootstock (Schaffer, Davies and Crane, 2006) and the presence of diseases like root rot infection (Neto et al. 2006), it is important to choose a suitable rootstock.

A suitable rootstock for other Annonaceae species is *Annona emarginata*, locally known as Araticum-mirim (Lorenzi, 2009). When grafted on this species, the graft shows 90% survival (Baron et al. 2011). This rootstock also displays a high resistance to soil fungi and it is less attractive to stem borers (Neto et al. 2006). However, there are some problems that hinder the large-scale production of *Annona emarginata* seedlings for use as a rootstock, such as the low and uneven germination percentage and the rapid loss of viability after seed drying (Costa, Bueno and Ferreira, 2011).

Micropropagation is a viable alternative for the rapid and efficient propagation of several species. Although several Annonaceae species have already been micropropagated (Encina et al. 2014), few protocols proved to be successful for the micropropagation process. Most of the species studied, show problems with rooting and acclimatization (Lemos and Blake, 1996; Oliveira et al. 2010).

Annona emarginata micropropagation has not been reported yet. Therefore, the aim of this study was to develop a micropropagation protocol for *Annona emarginata.*

MATERIAL AND METHODS

Plant material and culture initiation

Nodal segments of 0.5 cm were excised from three-year-old *Annona emarginata* plants, maintained in a greenhouse at the Plant Tissue Culture Laboratory of the Federal University of Lavras (UFLA), Brazil. The explants were immersed in 70% ethyl alcohol (v/v) for 30 seconds, followed by sodium hypochlorite treatment (1% active chloride) during 15 minutes. They were then washed three times in sterile distilled water and inoculated into test tubes containing 20 mL MS medium (Murashige and Skoog, 1962), supplemented with 5 µM 6-benzylaminopurine (BA), 30g L^{-1} sucrose and solidified with 0.65% agar. The pH was adjusted to 5.8 ± 0.1 before autoclaving. All cultures were maintained in a growth room at 25 ± 2 °C with a 16-hour photoperiod and 56 µmol m^{-2} s^{-1} PAR (Photosynthetic Activity Radiation).

Shoot Induction

For axillary shoot proliferation, *in vitro* nodal segments of 0.5 cm with a single bud, were transferred to MS or Woody Plant Medium (WPM) (Lloyd and McCown, 1980) supplemented or not with BA at different concentrations (0, 1, 3 or 5 µM). The media were supplemented with 30g L^{-1} sucrose and solidified with 3g L^{-1} Phytagel® and the pH was adjusted to 5.8 ± 0.1. Each tube contained one explant and was considered as one replicate, with each treatment consisting of 15 replicates. After 45 days of culture, the number of buds and leaves of each explant was counted, and the height of the main shoot was measured.

Shoot elongation

Shoots of 1 cm length derived from the axillary proliferation with four buds were transferred to WPM supplemented with 1µM BA, in combination with different concentrations (0, 1, 5 and 10 µM) of gibberellic acid-3 (GA_3), 30g L^{-1} sucrose, solidified with 3g L^{-1} Phytagel® and pH adjusted to 5.8 ± 0.1. The explants were maintained in a dark room at 25 ± 2 ºC for 14 days, and then transferred to a culture room in light at 25 ± 2 °C with a 16-hour photoperiod and 56 µmol m^{-2} s^{-1} PAR, or were cultured throughout the whole experimental period in a light. After 45 days, the number of buds and leaves of each explant was counted and the height was measured. Treatments consisted of 12 replicates with one explant per tube.

Rooting

For root induction, shoots of 1-2 cm length with four buds previously maintained in WPM + 1 µM BA for 60 days, were transferred to a WPM basal medium without plant growth regulators (control), or supplemented with different auxins: Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) or 1-naphthaleneacetic acid (NAA) at 1, 10 and 100 µM. All media were supplemented with 30g L^{-1} sucrose and 3g L^{-1} Phytagel® with pH adjusted to 5.8 ± 0.1. Treatments

consisted of 24 replicates, with one explant per tube. Shoots were maintained in a medium containing auxin for seven or 30 days and then, transferred to a growth regulator free medium. After 45 days of culture, the number of roots was counted and the presence of callus was noted.

Statistical analysis

Statistical analyses were performed using the SISVAR program (Ferreira, 2014). All the experiments were set up in a completely randomized design. The data were analyzed through analysis of variance (ANOVA) and, when significant (P<0.05) by F test, they were submitted to polynomial regression.

RESULTS AND DISCUSSION

It was possible to observe that the WPM medium is always superior to MS after 45 days of culture regarding the number of buds produced, provided a source of cytokines is supplemented (Figure 1A). In general, the number of leaves increased in both media in the presence of BA and the highest number of leaves was found in WPM medium, regardless of the BA concentration (Figure 1B). Shoot height was not statistically different when using MS or WPM medium (P>0.05).

Cytokines have been routinely used in tissue culture due their action in promoting the unfolding of a complex gene expression program that results in a variety of traits, including shoot formation during plant propagation (Howell, Lall and Che, 2003).

The highest number of buds (9) was obtained with the highest concentration of BA tested. However, this increase also takes into account the formation of "clumps". Clumps is a swelling at the base of the explant, but which doesn't looks such as callus with undifferentiated and loose cells (Figure 2). This is followed by the appearance of small buds that can sometimes develop into new plants. It was, however, observed that most of the clumps or adventitious buds gave rise to malformed leaves or buds. These malformations observed are undesirable in clonal multiplication cycles, since they may cause somaclonal variation.

Clumps were observed at a rate over 80% when MS was used, or over 66% in WPM medium when supplemented with any concentration of BA. In contrast, in the absence of BA in either MS or WPM medium, no clumps were visible.

Such increase in the number of buds, following an increased concentration of BA, was also described by Lemos and Blake (1996) when BA was used to induce multiplication in *Annona muricata*. However, these authors did not describe the formation of clumps and malformed leaves.

Shoot elongation reached the highest values at 5 µM GA_3, irrespective of light conditions (Figure 3).

The results obtained in the present study differ from other authors that also tested the effect of GA_3 in the culture medium of Annonaceae. Nagori and Purohit (2004) reported that, when initiated on MS medium containing BA, even after repeated subcultures, or when treated with GA_3 in combination with BA, explants of *Annona squamosa* did not undergo any significant growth stimulation. Lemos and Blake (1996) also reported that the addition of GA_3 to the basic medium did not stimulate any significant growth in *Annona muricata.*

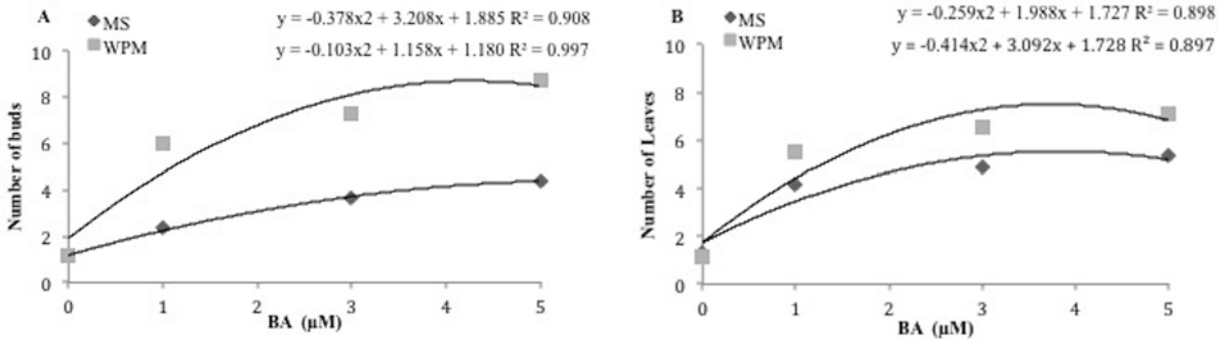

Figure 1 – Number of buds (A) and leaves (B) after 45 days in MS and WPM media supplemented with different concentrations of BA.

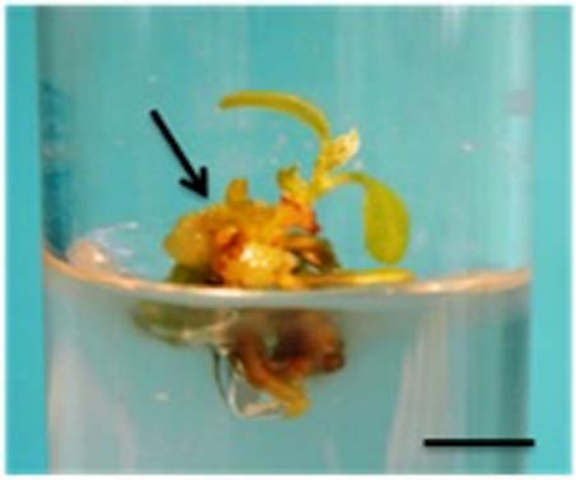

Figure 2 – Clump formation at the base of a shoot (indicated by an arrow) in the presence of 5 µM BA after 30 days on WPM medium. bar = 0.5 cm.

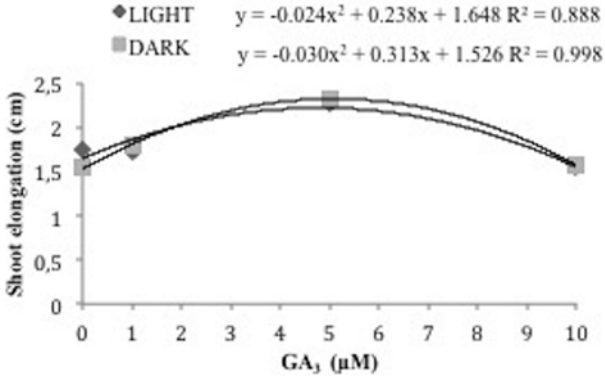

Figure 3 – Shoot height in WPM medium with 1 µM BA and different concentration of GA_3 after 45 days of culture.

Although the use of 5 µM GA_3 increased elongation, the addition of GA_3 induced malformed plants with irregular shapes and color of leaves, regardless of light conditions (Figure 4).

Classically, GA_3 is used for elongation of *in vitro* plants. This compound stimulates an increase in internode length. However, in this study, the addition of GA_3 showed a negative effect. Thus, further studies related to the use of GA_3 or their combination with other plant growth regulators could significantly contribute to determine an appropriate concentration for *Annona emarginata*.

The addition of NAA, IAA or IBA at any of the concentrations tested did not show any statistical influence on root formation, compared to the control ($p > 0.05$). Irrespective of the exposure time to auxins, less than 10% of the explants showed root formation when IAA or IBA were used. NAA did not result in any root induction in any of the concentrations or exposure time tested.

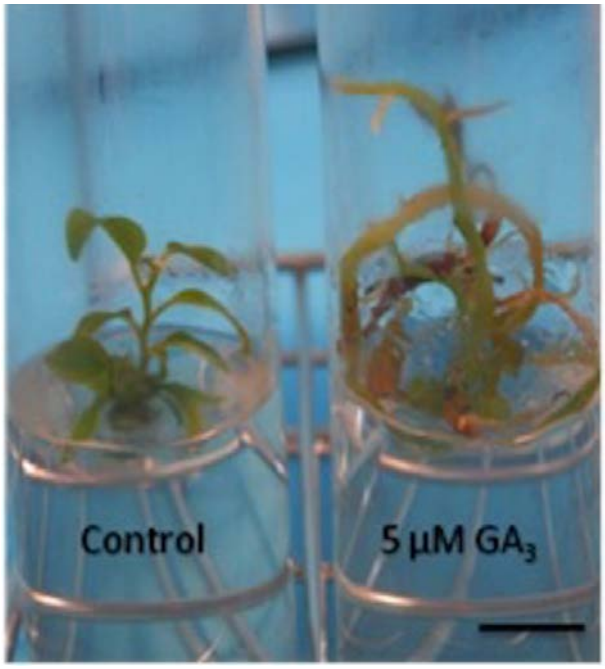

Figure 4 – *Annona emarginata* control supplemented with only 1µM BA, compared to plants supplemented with 1µM BA plus 5 µM GA_3 under dark conditions with irregular shapes and color of leaves after 45 days of culture. bar = 0.5 cm.

Callus formation occurred after 30 days of culture, despite the auxin treatment (Figure 5A). The presence of 100 µM NAA, IBA or IAA for 30 consecutive days promoted 50%, 25% and 75% callus formation, respectively. When the same concentrations were applied for seven consecutive days, less callus (16%, 16% and 8.33%, respectively) was observed. The induction of such callus was also reported by Nagori and Purohit (2004) and by Zobayed, Armstrong and Armstrong, (2002) in *Annona squamosa.* Moreover, a prolonged use of auxin, especially at high concentrations (100 µM), adversely affected the development of *Annona emarginata* shoots, showing leaf yellowing and bud necrosis in all treatments (Figure 5B).

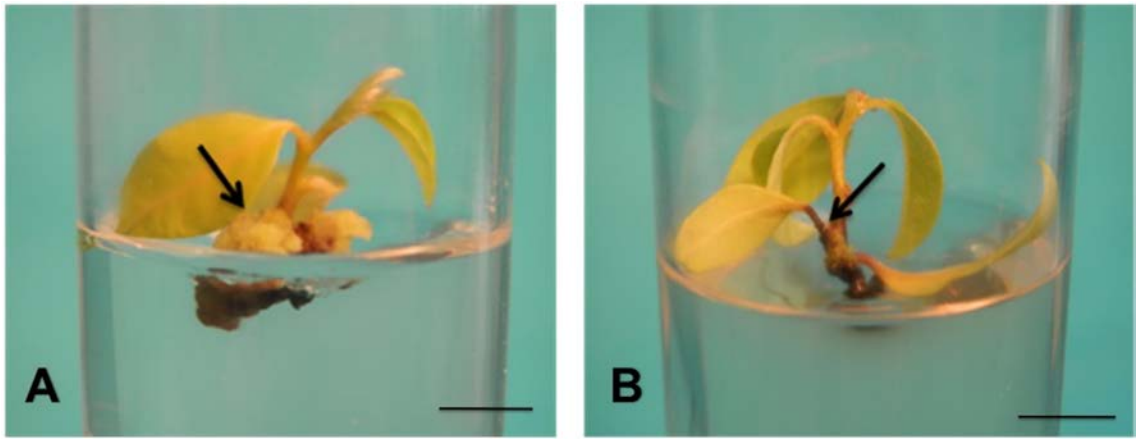

Figure 5 – (A) Callus formation at the base of a shoot (indicated by an arrow) in the presence of 100 µM NAA after 45 days of culture. (B) Leaf abscission and bud necrosis (indicated by an arrow) in the presence of 10 µM NAA after 30 days of culture. bar = 0.5 cm.

Lemos and Blake (1994) and Oliveira et al. (2007) reported that leaf abscission in Annonaceae *in vitro* cultures are a serious problem. This can be due to the induction and accumulation of ethylene in the tissue culture container, when auxins are applied for a prolonged period of time (De Klerk, 2002). An increase in ethylene production can be due to a decrease in cytokinin concentration in mature organs (Oliveira et al. 2007) or due to the use of exogenous auxins acting as an antagonist of cytokinins (Shimizu-Sato, Tanaka and Mori, 2009). Ethylene production may also be stimulated by mechanical cutting of the explant (Khalafalla and Hattori, 2000).

Leaf abscission was also observed by Farooq, Farooq and Rao (2002) with *Annona squamosa,* when cultured on medium enriched with IBA. Zobayed, Armstrong and Armstrong (2002) also describe that the addition of NAA to the medium increased leaf abscission in *A. squamosa* and *A. muricata*.

To avoid or minimize ethylene production and its possible effects on *in vitro* leaf abscission, cytokinin or ethylene antagonists such as silver nitrate or silver thiosulfate may be applied. Oliveira et al. (2007) describe that the addition of the cytokinins to the culture medium can reduce leaf senescence during *in vitro* culture of *Annona glabra*.

CONCLUSIONS

The use of 1 µM BA in WPM medium is recommended for the *in vitro* multiplication of *Annona emarginata*. This is based on the low rate of adventitious shoot formation, combined with a higher number of buds and leaves.

Independent of light conditions, GA_3 induced the formation of malformed plants with irregular shapes and lighter color of leaves, at any of the concentrations, when combined with 1 µM BA.

In the present study, root formation could not be stimulated, regardless of the duration of auxin treatment, type or concentration of auxin. Further experiments are thus needed to optimize root formation.

ACKNOWLEDGEMENTS

The authors would like to thank CNPq, CAPES and FAPEMIG for the financial support and the Laboratory for Tropical Crop Improvement (KU Leuven) for guidance of the practical work. Bart Panis also gratefully acknowledge the support from the CGIAR Research Programmes (CRP) Roots, Tubers and Bananas CRP and The Genebank CRP.

REFERENCES

BARON, D. et al. Evaluation of substrates on the emergence of "araticum-de-terra-fria" (*Annona emarginata* (Schltdl.) H. Rainer) seedlings. **Revista Brasileira de Fruticultura**, 33(2):575-586, 2011.

COSTA, P.N.; BUENO, S.S.C.; FERREIRA, G. Germination phases of *Annona emarginata* (Schltdl.) H. Rainer seeds under different temperatures. **Revista Brasileira de Fruticultura**, 33(1):253-260, 2011.

DE KLERK, G. J. Rooting of microcuttings: Theory and practice. **In Vitro Cellular & Developmental Biology-Plant**, 38(5):415-422, 2002.

EGYDIO, A. P. M.; SANTOS, D. Y. A. C. D. Underutilized *Annona* species from the Brazilian Cerrado and Amazon Rainforest: A study on fatty acids profile and yield of seed oils. **Economic Botany**, 65(3):329-333, 2011.

ENCINA, C. L. et al. Biotechnology applied to *Annona* species: A review. **Revista Brasileira de Fruticultura**, 36(1):17-21, 2014.

FAROOQ, S. A.; FAROOQ, T. T.; RAO, T. V. Micropropagation of *Annona squamosa* L. using nodal explants. **Pakistan Journal of Biological Sciences**, 5(1):43-46, 2002.

FERREIRA D. F. Sisvar: A guide for its bootstrap procedures in multiple comparisons. **Ciência e Agrotecnologia**. 38(2):109-112, 2014.

HOWELL, S. H.; LALL, S.; CHE, P. Cytokinins and shoot development. **Trends in Plant Science**, 8(9):453-459, 2003.

KHALAFALLA, M. M.; HATTORI, K. Ethylene inhibitors enhance *in vitro* root formation on faba bean shoots regenerated on medium containing thidiazuron. **Plant Growth Regulation**, 32(1):59-63, 2000.

LEMOS, E. E. P.; BLAKE, J. Leaf abscission in micropropagated Sugar Apple (*Annona squamosa* L). In: **Physiology, Growth, and Development of Plants in Culture**. Eds. P J Lumsden, J R Nicholas, W J Davies. p. 227-232. Dordrecht, Kluwer Academic Publishers, 1994.

LEMOS, E. E. P.; BLAKE, J. Micropropagation of juvenile and mature *Annona muricata* L. **Journal of Horticultural Science**, 71(3):395-404, 1996.

LLOYD, G.; MCCOWN, B. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. **Combined Proceedings of International Plant Propagators' Society**, 30:421-427, 1980.

LORENZI, H. **Árvores Brasileiras: manual de identificação e cultivo de plantas arbóreas nativas do Brasil**. Nova Odessa: Ed. Plantarum, 2009. 384p.

MURASHIGE, T.; SKOOG, F. A revised method for rapid growth and bioassays with tobacco tissue cultures. **Physiologia Plantarum**, 15:473-497, 1962.

NAGORI, R.; PUROHIT, S. D. *In vitro* plantlet regeneration in *Annona squamosa* through direct shoot bud differentiation on hypocotyl segments. **Scientia Horticulturae**, 99(1):89-98, 2004.

NETO, J. E. B. et al. Enraizamento de estacas dos portas-enxertos Araticum-de-terra (*Rollinia sp.*) e Araticum-Mirim (*Rollinia emarginata* Schltdl.) para Anonaceas. **Ciência e Agrotecnologia**, 30(6):1077-1082, 2006.

OLIVEIRA, L. M. D. et al. Efeito da citocinina na senescência e foliar abscisão durante *in vitro Annona glabra L*.. **Revista Brasileira de Fruticultura**, 29(1):25-30, 2007.

OLIVEIRA, L. M. D. et al. Effects of cytokinins on *in vitro* mineral accumulation and bud development in *Annona glabra* L. **Ciência e Agrotecnologia**, 34(6):1439-1445, 2010.

ORSI, D. C. et al. Use of sugar apple, atemoya and soursop for technological development of jams - chemical and sensorial composition. **Ciência e Agrotecnologia**, 36(5):560-566, 2012.

PADILLA, I. M. G.; ENCINA, C. L. *In vitro* germination of cherimoya (*Annona cherimola* Mill.) seeds. **Scientia Horticulturae**, 97(3-4):219-227, 2003.

PAREEK, S. et al. Postharvest physiology and technology of Annona fruits. **Food Research International**, 44(7):1741-1751, 2011.

RASAI, S.; GEORGE, A. P.; KANTHARAJAH, A. S. Tissue-culture of *Annona* spp (cherimoya, atemoya, sugar apple and soursop): A review. **Scientia Horticulturae**, 62(1-2):1-14, 1995.

SANTANA, J. R. F. D. et al. Effect of different carbon sources on the *in vitro* multiplication of *Annona* sp. **Ciência e Agrotecnologia**, 35(3):487-493, 2011.

SCHAFFER, B.; DAVIES, F.S.; CRANE, J.H. Responses of subtropical and tropical fruit trees to flooding in calcareous soil. **HortScience**, 41(3):549-555, 2006.

SHIMIZU-SATO, S.; TANAKA, M.; MORI, H. Auxin-cytokinin interactions in the control of shoot branching. **Plant Molecular Biology**, 69(4):429-435, 2009.

ZOBAYED, S. M. A.; ARMSTRONG, J.; ARMSTRONG, W. Multiple shoot induction and leaf and flower bud abscission of *Annona* cultures as affected by types of ventilation. **Plant Cell, Tissue and Organ Culture**, 69(2):155-165, 2002.