







## *In vitro* germination of Abricó-de-Macaco (*Couropita guianensis* aubl.) zygotic embryos in different culture media and light spectra

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**ABSTRACT:** *Couropita guianensis* Aubl. is an Amazonian forest species with important medicinal and ornamental value. This study evaluated the effect of different culture media and light spectra on the *in vitro* germination and development of the zygotic embryos of *C. guianensis*. The culture media, MS and WPM, were evaluated without the addition of plant growth regulators and were associated with four LED light spectra: white (CW), 70% red + 30% blue (R2B), 100% red (R), and 100% blue (B). One hundred percent of the seeds successfully underwent *in vitro* germination, and the culture media did not interfere with embryo development. In addition to this, the different light spectra induced *in vitro* morphogenesis and R2B treatment significantly promoted the production of secondary roots. This effect may aid in the rooting and acclimatization of seedlings of this species.

**Key words:** morphogenesis, tissue culture, propagation, *Couropita guianensis* Aubl.

## Germinação *in vitro* de embriões zigóticos de Abricó-de-Macaco (*Couropita guianensis* aubl.) em diferentes meios de cultivo e espectros de luz

**RESUMO:** *Couropita guianensis* Aubl. é uma espécie florestal de origem amazônica, de importância no uso medicinal e ornamental. O objetivo do presente estudo foi avaliar o efeito de diferentes meios de cultivo e espectros de luz na germinação e desenvolvimento *in vitro* de embriões zigóticos de *Couropita guianensis*. Os meios de cultura avaliados foram o MS e o WPM, sem adição de reguladores de crescimento, associados a quatro espectros de luz de LED branca (CW), 70% vermelha + 30% azul (R2B), 100% vermelha (R) e 100% azul (B). Ocorreu 100% de germinação *in vitro* e os meios de cultivo não interferiram no desenvolvimento dos embriões. Os diferentes espectros de luz induziram a morfogênese *in vitro* e o tratamento R2B promoveu a produção de raízes secundárias em número significativo. Este efeito poderá auxiliar no processo de enraizamento e aclimatização de mudas dessa espécie.

**Palavras-chave:** morfogênese, cultura de tecidos, propagação, *Couropita guianensis* Aubl.

## INTRODUCTION

*Couropita guianensis* Aubl., known in Brazil as the Monkey-Apricot, is a species of medicinal plant from the Amazon and is popularly used as a potent anti-inflammatory agent, administered through infusions made from its leaves, flowers, and bark (PINHEIRO et al., 2010). In some countries, such as India, it is widely used as an ornamental plant known as the Cannon Ball Tree. However, indiscriminate use in folk medicine by the Indians has drastically reduced its population (SHEKHAWAT & MANOKARI, 2016). In Brazil, deforestation is the persistent cause of the reduction in its native population year after year. In nature, the propagation of *C. guianensis* is exceptionally slow, being restricted to the germination

of seeds that are recalcitrant and difficult to store (GOUSIA et al., 2013). In addition, low germination rate occurs frequently in seeds germinated directly in the soil (MUNISWAMY & SREENATH, 2000). Thus, tissue culture is a tool that could contribute to the multiplication of this species. The selection of the explant and the culture medium are basic conditions for establishing an *in vitro* propagation protocol or *in vitro* conservation method (JIANG et al., 2012). The cultivation medium, MS (MURASHIGE & SKOOG, 1962), has proven to be efficient and responsive in most herbaceous species such as banana, pineapple, and strawberry (GALAN et al., 2018; SOUZA et al., 2018; MOZAFARI et al., 2018). However, woody species have other needs, especially for basic salts. The Woody Plant Medium (WPM) used in culturing

(LLOYD & MCCOWN, 1980) has a more diluted composition, with 25% of the concentration of nitrate and ammonium, than the MS medium. For woody plants, the WPM medium has proven to be more responsive and resulted in better propagation rates (YEUNG et al., 2015; SHAHZAD et al., 2017). In tissue culture, different light spectra from light-emitting diodes (LEDs) have been widely studied and used in commercial and research laboratories. Light influences plant morphogenesis during *in vitro* development, which may cause a reduction in explant size, root emission and length, number of stomata in the leaves, and chlorophyll content (POUDEL et al., 2008; MACEDO et al., 2011). Therefore, this study investigated the effect of different culture media associated with different spectra of LED light on the *in vitro* germination and development of *C. guianensis* embryos.

Zygotic embryos from *C. guianensis* seeds, collected from ripe fruits, were used as the plant material. The collection took place 330 days after flowering. During collection, the fruits were opened, and the seeds were extracted, of which 48 were selected. The seeds were soaked in 50% (v/v) sodium hypochlorite solution (2.5% active chlorine) for 10 min to remove the pulp. The asepsis of the seeds was performed inside a laminar flow, using sodium hypochlorite solution (2.5% active chlorine) at 20% (v/v) for 15 min followed by three washes in sterile distilled water. Then, the embryos were excised and transferred to Pyrex® test tubes (15 cm long by 2.5 cm in diameter) with 25 mm diameter Kimble polypropylene caps, each containing 10 mL of two types of culture medium, MS and WPM. Both media were supplemented with 3.0% sucrose, gelled with 0.2% Phytigel®, and had their pH adjusted to 5.8. After inoculation, the embryos were kept in a growth room under four different spectra of LED light (GreenPower TLED-Philips™): white (CW), 70% red + 30% blue (R2B), 100% red 645–675nm (R), and 100% blue 450–465 nm (B). The light intensity was adjusted to 50  $\mu\text{mol m}^{-2}\text{s}^{-1}$  (LI-200 Liquor), at a temperature of 25 °C  $\pm$  2 °C, and photoperiod of 14/10 h (day/night). For each treatment, the test tubes were centralized and arranged in an equidistant manner to uniformly receive the evaluated spectrum. At the end of 30 days, the following variables were measured: seedling height (from the vegetative apex to the root neck in cm); root length (from the root neck to the tip of the root in cm); number of secondary roots (unit); chlorophyll contents a, b (ChlorofiLOG - Falker CFL-1030), and total; and total fresh weight (g) and total dry weight (g). The data were analyzed using

a completely randomized design in a 4  $\times$  2 factorial scheme (4 LED light spectra  $\times$  2 culture media). The trial comprised eight treatments with six replicates per treatment; each repetition was formed from four test tubes, totaling 192 tubes. Before applying analysis of variance, the data were submitted to exploratory analysis to verify whether the assumptions were being met. After analysis of variance, multiple comparisons were performed using Tukey's HSD test. The analyses were performed using the R and SAS software, with an  $\alpha$  significance level of 0.05. The level of significance adopted for the unfolding of interactions was equal to 0.25.

The excised embryos showed 100% *in vitro* germination in the two treatments of different culture media. Collecting the *C. guianensis* fruits at the ideal maturation stage was most likely the reason for the *in vitro* germination of 100% of the embryos. A similar result was obtained by MUNISWAMY and SREENATH (2000), who reported 95% germination of *C. guianensis* embryos while using MS culture medium with only half of the concentration of salts and was supplemented with 0.1 mg/L of the cytokinin, BA (benzyladenine). The use of exogenous cytokinin helps to facilitate successful *in vitro* germination; its promoting effects are mainly related to the relief of stress factors (NIKOLIĆ et al, 2006). The concentration of 0.1 mg/L BA probably contributed to the high germination percentage of the embryos, given that the fruits were harvested after only 240 days and not fully ripe as in the present study (330 days). Thirty days after inoculation, no significant differences were observed between the culture media for most of the characteristics evaluated in the *C. guianensis* embryos (Table 1). However, a different effect was observed in studies with native plants such as baru (*Dipteryx alata* Vog), cajú do cerrado (*Anacardium othonianum* Rizz), and caçari (*Myrciaria dubia* Kunth), which evaluated different concentrations of salts and culture media in the *in vitro* development of explants from these species. In baru, studies have indicated that the 100% MS medium showed a better performance than the WPM and lower concentrations of MS (25%, 50%, and 75% MS) (ARARUNA et al., 2017). In the trial with cajú do cerrado, the MS (50% and 25%) and WPM (100% and 50%) media were the most efficient in plant regeneration (ASSIS et al., 2012). Meanwhile, in caçari, 100% WPM was superior to the MS and JADS medium at concentrations of 25%, 50%, 75%, and 100% (ARAÚJO et al, 2016). Notably, for some woody plants, the higher concentration of nitrogen salts has a negative effect on *in vitro*

development. Generally,  $\text{NO}_3^-$  is the preferred form for N assimilation in most plants. In some woody plants,  $\text{NH}_4^+$  is not necessary, and in large quantities, it can be toxic (POOTHONG & REED, 2016), as was observed in cajú do cerrado, where the complete MS medium was found to be harmful to the development of *in vitro* culture (ASSIS et al., 2012). In the present study, there was a significant difference in fresh weight under CW treatment using MS medium (Table 1). However, despite the greater accumulation of fresh weight in the MS medium, in the WPM, the explants showed good formation without the occurrence of oxidation or phytotoxicity.

Significant differences were observed among the light spectra regarding the number of secondary roots and the total fresh weight. Specifically, the highest values for these categories were observed under R2B, while the CW values were higher than those for R and B (Table 1). No significant differences were observed for any of the other evaluated characteristics. Photoreceptors regulate differentiation and plant growth and are activated by different wavelengths that induce plant morphogenesis (CHUNG et al., 2010; MULEO & MORINI, 2006). The combination of the spectra in the R2B treatment favored the greater formation of secondary roots during the *in*

Table 1 - Mean (standard deviation) of shoot length (cm), root length (cm), number of secondary roots, total fresh weight (g), total dry mass (g), Chlorophyll a, b and Total, of *C. guianensis* zygotic embryos as a function of the culture media, MS and WPM and different light spectra.

Culture media	CW	R2B	R	B
-----Seedling height (cm)-----				
MS	9,07 (0,90) Aa	7,52 (3,06) Aa	7,72 (1,27) Aa	7,98 (2,89) Aa
WPM	8,27 (1,17) Aa	8,13 (1,02) Aa	7,65 (1,28) Aa	7,20 (0,75) Aa
VC (%)	-----22,14-----			
-----Root length (cm)-----				
MS	4,03 (3,32) Aa	6,37 (2,49) Aa	5,07 (2,92) Aa	7,38 (5,50) Aa
WPM	4,60 (2,05) Aa	7,70 (3,28) Aa	4,42 (2,46) Aa	5,53 (2,62) Aa
VC (%)	-----29,92-----			
-----Number of secondary roots-----				
MS	6,50 (4,51) ABa	12,00 (6,36) Aa	5,00 (5,62) Ba	6,33 (3,50) ABa
WPM	9,83 (6,65) ABa	18,33 (7,53) Aa	7,67 (5,89) Ba	9,17 (8,82) ABa
VC (%)	-----40,91-----			
-----Total fresh weight (g)-----				
MS	2,02 (0,26) Aa	1,80 (0,57) Aab	1,52 (0,46) Ab	1,80 (0,72) Aab
WPM	1,47 (0,31) Bb	1,94 (0,24) Aa	1,46 (0,44) Ab	1,69 (0,42) Aab
VC (%)	-----26,56-----			
-----Total dry weight (g)-----				
MS	0,32 (0,07) Aa	0,30 (0,09) Aa	0,24 (0,03) Aa	0,29 (0,05) Aa
WPM	0,27 (0,02) Aa	0,28 (0,05) Aa	0,28 (0,02) Aa	0,24 (0,02) Aa
VC (%)	-----18,14-----			
-----Chlorophylla-----				
MS	24,28 (9,53) Aa	26,93 (8,66) Aa	16,58 (3,38) Aa	19,52 (8,90) Aa
WPM	21,78 (7,51) Aa	26,95 (7,77) Aa	19,50 (5,52) Aa	28,73 (8,32) Aa
VC (%)	-----33,37-----			
-----Chlorophyll b-----				
MS	5,70 (3,02) Aa	6,55 (3,53) Aa	3,47 (1,11) Aa	4,70 (2,90) Aa
WPM	5,33 (2,48) Aa	6,47 (2,40) Aa	4,13 (1,73) Aa	7,83 (4,60) Aa
VC (%)	-----31,15-----			
-----Chlorophyll Total-----				
MS	29,98 (12,50) Aa	33,48 (12,04) Aa	20,05 (4,40) Aa	24,22 (11,74) Aa
WPM	27,12 (9,88) Aa	33,42 (10,12) Aa	23,63 (7,17) Aa	36,57 (12,75) Aa
VC (%)	-----11,31-----			

Means followed by different letters differ ( $P \leq 0.05$ ), uppercase letters horizontally and lowercase letters vertical, within each culture media.



*in vitro* germination of *C. guianensis*, regardless of the culture medium used. Using *Heliconia* cultivar in an *in vitro* conservation study using slow-grown storage with different wavelengths of the LED light spectra, the largest number of roots was observed in the mixed light treatment (70R30B) followed by the white light treatment (RODRIGUES et al., 2018). Nevertheless, proportions of 1:1 and 3:1 of the red and blue spectra did not show satisfactory results in the rooting of *Protea cynaroides* and *Brassica napus* (WU & LIN, 2012; LI et al., 2013). Therefore, it is evident that the effects of the available light spectra on the explant are probably specific to each species or cultivar. The higher occurrence of secondary roots in R2B may be a promising characteristic for the *in vitro* germination of mature embryos, thereby reducing losses in the acclimatization phase and optimizing this technique of producing *C. guianensis* seedlings. As reported by GOUSIA et al. (2013), the slow germination speed of *C. guianensis* and the recalcitrance of its seeds justify the application of this technique.

In conclusion, the culture media MS and WPM can be applied for the *in vitro* germination of zygotic embryos of *C. guianensis*. The R2B spectrum favors the formation of secondary roots in germinated embryos.

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## DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

## AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved the final version.

## REFERENCES

- ARARUNA, E. C.; RIBEIRO, O. J. P., et al. Salt concentrations in culture media for the development of *Dipteryx alata* in vitro. **Pesquisa Agropecuária Brasileira**, 2017; 52(12), 1295-1300. Available from: <<https://www.scielo.br/pab/a/GWtkzK6h6hCCfJFKwvv8NhF/?lang=en>>. Accessed: May, 25, 2021. doi: 10.1590/s0100-204x2017001200020.
- ARAÚJO, M. C. R.; CHAGAS, E. A., et al. Micropropagation of caçari under different nutritive culture media, antioxidants, and levels of agar and pH. **African Journal of Biotechnology**, 2016; 15(33): 1771-1780. Available from: <<https://academicjournals.org/journal/AJB/article-abstract/35E05EC60118>>. Accessed: May, 25, 2021. doi: 10.5897/AJB2016.15417.
- ASSIS, K. C.; PEREIRA, F. D., et al. In vitro cultivation of *Anacardium othonianum* Rizz.: effects of salt concentration and culture medium volume. **Acta Scientiarum Agronomy**, 2012; 34(1): 77-83. Available from: <<https://periodicos.uem.br/ojs/index.php/ActaSciAgron/article/view/10984>>. Accessed: May, 25, 2021. doi: 10.4025/actasciagron.v34i1.10984.
- CHUNG, J. P.; HUANG, C. Y.; DAI, T. E. Spectral Effects on embryogenesis and plantlet growth of *Oncidium* 'Gower Ramsey'. **Scientia Horticulturae**. 2010; 124: 511-516. Available from: <<https://www.sciencedirect.com/science/article/pii/S0304423810000440?via%3Dihub>>. Accessed: May, 25, 2021. doi: 10.1016/j.scienta.2010.01.028.
- GALAN, V. et al. Propagación del banano: técnicas tradicionales, nuevas tecnologías e innovaciones. **Revista Brasileira de Fruticultura**, 2018; 40 (4): e-574. Available from: <<https://www.scielo.br/rbfa/bS5hbygvpH3BWN78ppcGmGz/?lang=es>>. Accessed: May, 25, 2021. doi: 10.1590/0100-29452018574
- GOUSIA, S. K.; ASHOK, K. K., et al. Biological activities and medicinal properties of *Couroupita guianensis*. **International Journal of Pharmacy Pharmaceut Sci Res**, 2013; 3(4):140-143.
- JIANG, Q.; ZHANG, Y. et al. Establishment of an *in vitro* plant regeneration protocol for *Casuarina cunninghamiana* Miq. Via indirect organogenesis. **New For**, 2012; 43:143-154.
- LI, H.; TANG, C.; XU Z. The effects of different light qualities on rapeseed (*Brassica napus* L.) plantlet growth and morphogenesis *in vitro*. **Scientia Horticulturae**, 2013; 150: 117-124. Available from: <<https://www.sciencedirect.com/science/article/pii/S0304423812004955?via%3Dihub>>. Accessed: May, 25, 2021. doi: 10.1016/j.scienta.2012.10.009.
- MACEDO, A. F.; LEAL-COSTA, M. Y., et al. The effect of light quality on leaf production and development of in vitro-cultured plants of *Alternanthera brasiliana* Kuntze. **Environmental and Experimental Botany**, 2011; 70: 43-50. Available from: <<https://www.sciencedirect.com/science/article/pii/S0098847210001231?via%3Dihub>>. Accessed: May, 25, 2021. doi: 10.1016/j.envexpbot.2010.05.012.
- MOZAFARI, A.; HAVAS, F.; GHADERI, N. Application of iron nanoparticles and salicylic acid in in vitro culture of strawberries (*Fragaria x ananassa* Duch.) to cope with drought stress. **Plant Cell, Tissue and Organ Culture**. 2018; 132: 511. Available from: <<https://link.springer.com/article/10.1007%2Fs11240-017-1347-8>>. Accessed: May, 25, 2021. doi: 10.1007/s11240-017-1347-8.
- MULEO, R.; MORINI, S. Light Quality regulates shoot cluster growth and development of MM106 Apple genotype in *in vitro* culture. **Scientia Horticulturae**. 2006; 108: 364-370. Available from: <<https://www.sciencedirect.com/science/article/pii/S0304423806000999?via%3Dihub>>. Accessed: May, 25, 2021. doi: 10.1016/j.scienta.2006.02.014.
- MUNISWAMY, B.; SREENATH, H. L. *In-vitro* development of plants from cultured embryos of cannon ball tree (*Couroupita*

- guianensis*) Aubl. **Indian Journal of Forestry**, 2000; 23 (2): 202-204.
- MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiologia Plantarum**, 1962; 15: 473-497.
- NIKOLIĆ, R., MITIĆ, N., MILETIĆ, R. et al. Effects of Cytokinins on *In Vitro* Seed Germination and Early Seedling Morphogenesis in *Lotus corniculatus* L. **J Plant Growth Regul**, 2006; 25:187-194. Available from: <<https://link.springer.com/article/10.1007%2Fs00344-005-0129-4>>. Accessed: May, 25, 2021. doi: 10.1007/s00344-005-0129-4.
- PINHEIRO, M. M.; BESSA, S. O., et al. Antinociceptive activity of fractions from *Couroupita guianensis* Aubl. leaves. **Journal Ethnopharmacology**, 2010; 127(2): 407-13. Available from: <<https://www.sciencedirect.com/science/article/pii/S0378874109006813?via%3Dihub>>. Accessed: May, 25, 2021. doi: 10.1016/j.jep.2009.10.025.
- POUDEL, P. R.; KATAOKA, I.; MOCHIOKA, R. Effect of red- and blue-light-emitting diodes on growth and morphogenesis of grapes. **Plant cell, Tissue and Organ Culture**, 2008; 92: 147-153. Available from: <<https://link.springer.com/article/10.1007%2Fs11240-007-9317-1>>. Accessed: May, 25, 2021. doi: 10.1007/s11240-007-9317-1.
- POOTHONG, S., REED, B. M. Optimizing shoot culture media for *Rubus* germplasm: the effects of  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , and total nitrogen. **In Vitro Cell. Dev. Biol.-Plant**, 2016; 52: 265-275. Available from: <<https://link.springer.com/article/10.1007%2Fs11627-016-9750-0>>. Accessed: May, 25, 2021. doi: 10.1007/s11627-016-9750-0.
- RODRIGUES, P. H. V.; ARRUDA, F.; FORTI, V. A. Slow-grown *in vitro* conservation of *Heliconia champneiana* cv. Splash under different light spectra. **Scientia Agricola**, 2018; 75(2), 163-166. Available from: <<https://www.scielo.br/j/sa/a/pb7nszYd4rdsh4THSdgmHNP/?lang=en>>. Accessed: May, 25, 2021. doi: 10.1590/1678-992x-2016-0394
- SAS [computer program]. **Version 9.4**. Cary, NC: SAS Institute Inc; 2014.
- SHAHZAD, A. A.; SHARMA, S. et al. Historical perspective and basic principles of plant tissue culture. In: Abdin MZ, Kiran U, Kamaluddin Ali A. (Ed.). **Plant biotechnology: principles and applications**. Singapore: Springer, 2017. p.1-36.
- SHEKHAWAT, M.; MANOKARI, M. *In vitro* propagation, micromorphological studies and ex vitro rooting of cannon ball tree (*Couroupita guianensis* Aubl.): a multipurpose threatened species. **Physiology and Molecular Biology of Plants**. 2016; 22(1): 131-142. Available from: <<https://link.springer.com/article/10.1007%2Fs12298-015-0335-x>>. Accessed: May, 25, 2021. doi: 10.1007/s12298-015-0335-x.
- SOUZA, C. P. F.; SOUZA, E. H., et al. Evaluation of the micropropagation potential of curauá pineapple hybrids for fiber production. **Acta Amazonica**, 2018; 48(4): 290-297. Available from: <<https://www.scielo.br/j/aa/a/JMQBTQWgLdcfdZM5MwT6dBt/?lang=en>>. Accessed: May, 25, 2021. doi: 10.1590/1809-4392201800382.
- WU H-C., LIN C-C.: Red light-emitting diode light irradiation improves root and leaf formation in difficult-to-propagate *Protea cynaroides* L. plantlets *in vitro*. – **HortSci**. 47: 1490- 1494, 2012. Available from: <<https://journals.ashs.org/hortsci/view/journals/hortsci/47/10/article-p1490.xml>>. Accessed: May, 25, 2021. doi: 10.21273/HORTSCI.47.10.1490.
- YEUNG, E. C. T.; STASOLLA, C.; SUMNER, M. J.; HUANG, B. Q. (Ed.). **Plant microtechniques and protocols**. Nova York: Springer, 2015. 576p. Available from: <<https://link.springer.com/book/10.1007%2F978-3-319-19944-3>>. Accessed: May, 25, 2021. doi: 10.1007/978-3-319-19944-3.