Process of Establishment And In Vitro Development of Simaba Cedron Planch Seedlings. (Simaroubaceae)

Abstract Marcia Santos de Freitas Lira

Federal University of Pará (UFPA),
Belém - PA, Brazil.

Simone da Silva
Amazon Biotechnology Center (CBA),
Manaus - Amazonas, Brazil.

Fábio Leandro Calderaro
Amazon Biotechnology Center (CBA),
Manaus - Amazonas, Brazil.

Jandecy Cabral Leite
Institute of Technology and Education Galileo Amazon (ITEGAM),
Manaus - Amazonas, Brazil.

Abstract

Simaba cedron, popularly known as "cedron", is largely used for fever and snake bites. Its seeds are used in the treatment of stomach problems and liver infections. The fruits are used for the treatment of pain and malaria while its bark is an antispasmodic. Simaba cedron is generally propagated through seeds, but with limited success, as the low viability of same restricts its propagation. In view of such difficulty, it becomes necessary the study for adequate conditions for the large scale production of these seedlings. Being it known that in several species, the use of micropropagation has made it possible to obtain a large amount of disease-free and more homogeneous seedlings, in reduced time and physical space, in comparison with conventional propagation methods, the objective of this work was to analyze the effect of two culture media on the production of aseptic parent plants as a first step in the development of a micropropagation protocol for Simaba cedron. The seeds were collected from a matrix plant located in the Amazon Biotechnology Center (CBA), in Manaus/AM. The experiment was installed at the Vegetable Tissue Culture Laboratory, where the explants were desinfected and grown in culture medium according to Murashige & Skoog (MS) and in Wood Plant Medium (WPM), during 60 days. The disinfestation rate obtained was 75% and, of the disinfested seeds, 100% germinated. The cultivation medium that was more favorable to the cultivation of simaba was the MS, where the multiplication rate was of 8.0: 1, whose seedlings reached, in average, 4.8 cm and 75% of rooting.

Keywords: Plant tissue culture; Micropropagation; Plant production and Biotechnology.

1. Introduction

The Simaroubaceae family consists of approximately 32 genera and 200 species, distributed in all tropical and subtropical regions of the globe. In Brazil it is represented by *Quassia* and *Picrolemma* genera in the Amazon region; *Castela* and *Picrasma*, in the South of the country; *Simaba* and *Simarouba* in almost all Brazilian (Hall *et al*., 1983; Devecchi, 2018). *Simaroubaceae* usually appear as trees or shrubs, with a distinctive bitter flavor in their cortex (Kletter and Kriechbaum, 2001; Seth, 2003; Vermeulen, 2008). So, many species of this family (*Quassia amara, Picrasma excelsa, Jamaica quassia*) have been known for more than a century due their bitter substances denominated "*quassina*", a name borrowed to all this class of composts structurally related, called quassinóides (Polonsky, 1973).

Simaba cedron Planch popularly known as cedron, it is widely used for the treatment of fevers and snake bites. Its seeds are used to treat stomach problems and liver infections (Kufer, 2005; Ocampo and Mora, 2011; Giovannini and Howes, 2017). The fruits are used for colic and malaria treatment, while the peel is used as an antispasmodic (Gupta, 1995). Ext Simaba extracts, from the species *Simaba cedron* (Planchon), *S. cuspidata* (Spruce), *S. moretii*, *S. multiflora* (Adr. Juss), *S. guyanensis* (Alblet Engl.) are used in the manufacture of cosmetic or pharmaceutical composition and, particularly dermatological, or in cell culture medium of the skin due to their significant activity on the depigmentation of the skin and in the differentiation of keratinocytes, and can be used in the treatment of skin disorders, in particular vitiligo and psoriasis (Bonte *et al*.,1997).

Simaba cedron is generally propagated by seeds, which can bring about genetic variations that, consequently, influence the content of the active principles present in plants. For many forest species of economic importance or in danger of extinction, micropropagation has been a useful tool for obtaining more uniform seedlings on a large scale, in reduced time and space (Dousseau *et al*., 2008). *In vitro* cultivation, through micropropagation, is a viable method for the multiplication of several native species, providing the formation of homogeneous plant populations, thus enabling the production of seedlings with high health and vigor. (Souza *et al*., 2007).

Several criteria are important for the establishment of *in vitro* cultures, such as the choice of explant type and nutrient medium. Although, theoretically, any tissue can be used as a source of explant, some aspects must be considered and tested regarding the choice of the most suitable to the morphogenic processes of interest (Grattapaglia and Machado, 1998).

In micropropagation, the use of efficient methods of disinfestation and germination, *in vitro*, of seeds, allows obtaining aseptic plants that supply contaminant-free propagules which can be used for multiplication and later rooting *in vitro* or ex *vitro* (Grattapaglia & Machado, 1998). Several substances have been used for the disinfestation of seeds of tree species, among which the hypochlorite (sodium or calcium) stands out, due to the ease of removing the tissue from the seeds during washing with water, by favoring germination due to the ability to stimulate α-amylase activity and, furthermore, by promoting the breaking of dormancy of the seeds of some species (Kaneko and Morohashi, 2003). As for nutritional means, there exist distinct formulations that must be adjusted to each species. The MS medium (Murashige & Skoog, 1962) and their dilutions are usually the most used. However, there are formulations specific to certain groups of plants, such as, for example, the WPM medium (Lloyd and McCown, 1981), more

common in woody species (Caldas *et al*., 1998).

In an attempt to optimize the *in vitro* growth of plant tissues, several studies propose the reduction or increase of some macro and/or micronutrients that compose these culture media, better meeting the nutritional requirements of each species, such as the 23 modified media for black mulberry (*Rubus* sp.) and grapevine (*Vitis* sp.) (Villa *et al*., 2008; Villa *et al*., 2009).

Due to the importance of this plant for multiple purposes and the conventional method of propagation by seed germination, which can cause differences in the composition of its active principles, actions involving the production of standardized seedlings are essential to outline strategies aimed at conservation, management sustainable development and genetic improvement of this specie (Debnath and Bisen, 2006; Podile and Kishore, 2007; Chadwick *et al*., 2013). The objective of this work was to evaluate the influence of the use of two different culture media on the establishment and *in vitro* development of *Simaba cedron* seedlings, as a first step in the development of a micropropagation protocol for the species (Ocampo and Mora, 2011).

2. Materials and Methods

Simaba cedron seeds collected from a parent plant at the *Amazon Biotechnology Center*, in Manaus/AM, were taken to the Plant Tissue Culture Laboratory, where they were washed with liquid detergent (of commercial origin) and running water and subsequently immersed in autoclaved distilled water for 24 hours. After this period, they were immersed in a commercial detergent solution for thirty minutes (under agitation) and, immediately afterwards, in autoclaved distilled water for twenty minutes.

Then, in a laminar flow chamber, they were disinfected with successive washes in 70% alcohol for 5 minutes, followed by immersion in sodium hypochlorite (2% active chlorine) for 30 minutes, and washed (three times) in sterile distilled water, for 5, 5 and 15 minutes. The seed coat was removed and they were inoculated in glass flasks (250 ml) containing 40 ml of basic culture medium of basic composition according to Murashige & Skoog (1962), without growth regulators (MS0), supplemented with 3% sucrose; 4.1 µM nicotinic acid; 0.6 mM myo-inositol; 2.4 µM pyridoxine-HCl; 1.5 µM thiamine-HCl and solidified with 2% phytagel. The pH was adjusted to 5.8 and the media were sterilized in an autoclave at 120 ^{0}C and 1.1 Kgf/cm^{2}, for 15 minutes.

Cultures were kept in the dark, at 25 ± 1°C, for one week. After this period, they were kept under lighting, with fluorescent lamps (Sylvania, Phillips/daylight) with intensity of 30.0 µ moles.m^{-2}.s^{-1}, and 16 hours of photoperiod. Daily observations were made, evaluating the development and the percentage of contamination. When the seedlings reached the maximum free height of the test tubes (8 cm), they were used as donors of explants for the tests with the culture media MS (Murashige & Skoog, 1962) and WPM (Lloyd & McCown, 1981) (Table 1). Each explant or phytomer consisted of a nodal region, without leaves, with an approximate size of 1 cm.

Table 1 - Composition of MS culture media.

Components	MS (mg/L)	WPM (mg/L)
Macronutrients		
$CaCl_2.2H_2O$	440	96
$Ca(NO_3)_2.4H_2O$	-	556
KH_2PO_4	170	170
KNO_3	1900	-
K_2SO_4	-	990
$MgSO_4.7H_2O$	370	370
NH_4NO_3	1650	400
Micronutrients		
$CoCl_2.6H_2O$	0,025	-
$CuSO_4.5H_2O$	0,025	0,25
H_3BO_3	6,2	6,2
KI	0,83	-
$MnSO_4.4H_2O$	22,3	22,3
$Na_2MoO_4.2H_2O$	0,25	0,25
$ZnSO_4.7H_2O$	8,6	8,6
FeEDTA		
$FeSO_4.7H_2O$	27,8	27,8
Na_2 EDTA.$2H_2O$	37,3	37,3
Orgânics		
Nicotinic Acid	0,5	0,5
Glycine	2,0	-
Myo-inositol	100	100
Pyridoxine.HCl	0,5	0,5
Thiamine	0,1	1,0
Sucrose (g / L)	30	20

Source: Murashige and Skoog, (1962) and WPM (Lloyd and McCown, 1981).

The experimental design was completely randomized, with two treatments and three repetitions, using 30 explants for each treatment, which were performed in triplicate. The evaluations were performed after 60 days of cultivation. The data obtained regarding the effect of different culture media (MS and WPM) on the height of the seedlings, the number of shoots and nodal segments per shoot and the multiplication rate were evaluated by analysis of variance (ANOVA) and the averages were compared by the Tukey-Kramer test, at the 5% significance level.

These analyzes were performed using the *Graph Pad in Stat*, version 3.01. For the analysis of the germination and rooting percentages, according to the medium used, the difference test between percentages (p_1 and p_2) was used at the 5% level of significance using the Statistic for Windows^{TM} software, version 5.0.

3. Results and Discussion

After five days of cultivation, it was observed that 75% of the seeds were aseptic, being used for the beginning of the culture, of which 100% germinated. The break in integumentary dormancy and pre-asepsis of the seeds allow the establishment *in vitro* of plants of *S. cedron*. The hypochlorite may have acted as a germination stimulant, due to the ability to stimulate α-amylase activity by increasing the amount of this enzyme in the seed, or even by promoting the breaking of seeds dormancy in some species (Kaneko and Morohashi, 2003).

For Silva (2015), when carrying out experiments with *Quassia amara*, obtained a disinfestation rate of 28% and, of the disinfested seeds 83% germinated in MS0 medium.

Developmental evaluations in WPM were compared with those obtained from seedlings grown in MS medium. Significant differences occurred between the culture media MS and WPM for the length of the aerial part of *S. cedron*, because, when using the MS medium, seedlings with an average height of 4.8 cm were obtained, while those cultivated in WPM medium had a 2.4 cm growth (Figure 1 and Table 2).

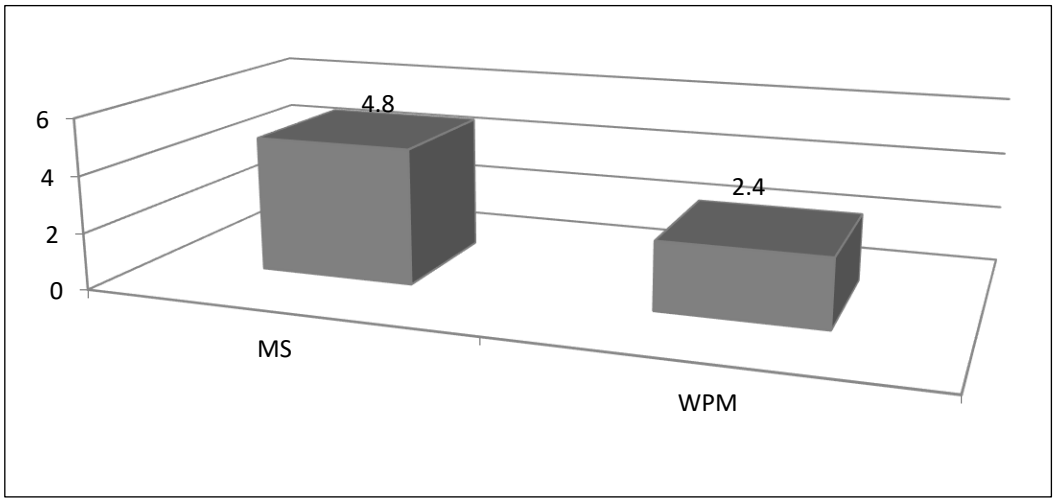

Figure 1 - Medium height of *Simaba cedron*, after 60 days of cultivation in MS and WPM media.
Source: Authors, (2020).

Figure 2 - *Simaba cedron*, after 60 days cultivation in MS medium.
Source: Authors, (2020).

Table 2 - Effects of MS and WPM culture media on in vitro cultivation of *Simaba cedron*.

Evaluated Parameters	MS	WPM
Height (cm)	4,8[a]	2,4[b]
Number of shoots	1,5[a]	1,3[a]
Number of nodal segments per shoot	8,0[a]	3,0[b]
Multiplication rate	8,0:1,0[a]	3,0:1,0[b]
Rooting rate (%)	75[a]	0[b]
Callogenesis rate (%)	38[b]	70[a]

Source: Authors, (2020).

When elaborating the tissue culture protocol of *Quassia amara* in MS medium, Silva (2015), obtained seedlings that reached an average of 5.23 cm, at 60 days of cultivation, while those cultivated in WPM medium originated seedlings with an average height of 1.39 cm.

Lencina *et al*. (2014), when carrying out experiments with grápia (*Apuleia leiocarpa Vog. Macbride*) observed that there was no significant difference between the culture media WPM, MS and MS ½ for the length of the aerial part, number of leaves and length of the root, after 15 days of cultivation.

In plants of *Cordia trichotoma*, it was verified growth of the aerial part (1.6 cm) and of the root (7.3 cm) significantly higher in cultures in WPM medium, when compared to those carried out in MS ½ culture medium (0.7 and 1,3 cm respectively), at 28 days of evaluation (Fick, 2007).

Regarding sprouting, the results obtained with the MS and WPM medium were statistically similar, with an average production of 1.5 and 1.3 sprouts, per explant, respectively (Figure 3 and Table 2).

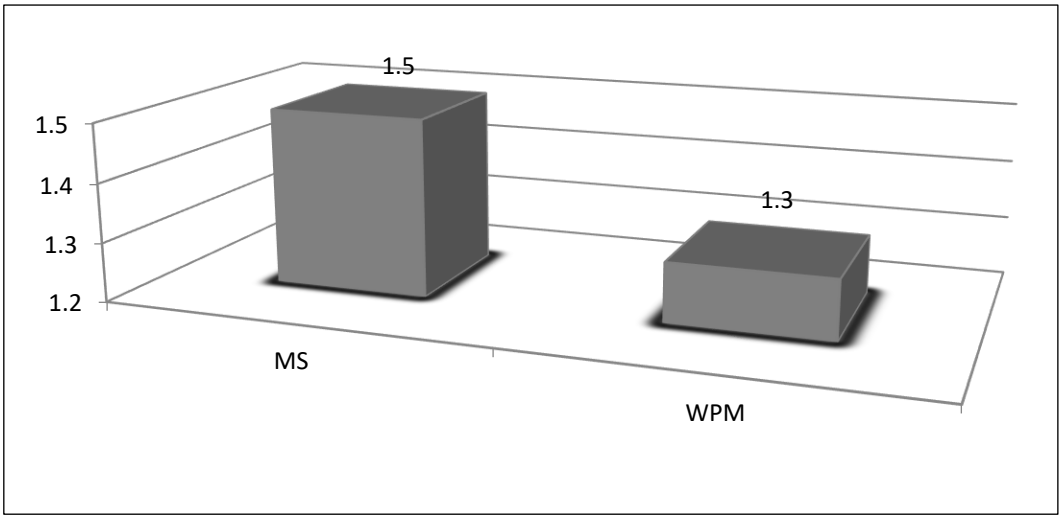

Figure 3: Average production of *Simaba cedron* shoots, after 60 days of cultivation in MS and WPM media.

Source: Authors, (2020).

In micropropagation, plants with a greater number of nodal segments or axillary buds are preferred, considering that these types of propagules have less somaclonal and epigenetic variation (Torres *et al*., 1999). In this study, there was a significant difference between the MS and WPM culture media for the number of nodal segments (Table 2), indicating that the culture medium influenced the *in vitro* growth of Simaba seedlings, where the MS medium led to seedling development with, on average, 8 nodal segments, each. While WPM medium promoted the average development of 3 nodal segments per seedling. However, the WPM culture medium was used in the experiments because of its formulation, which was developed especially for woody species and presents 25% of the concentrations of nitrate and ammonia ions of the MS medium (Melo *et al*., 1999), stimulating *in vitro* growth due to low concentrations of nitrogen in ammoniacal form (Grattapaglia & Machado, 1998). In addition, the WPM culture medium has a higher amount of vitamin thiamine - HCl, when compared to the MS culture medium (Table 1). Thiamine is identified as a beneficial substance for *in vitro* multiplication, allowing greater bud induction in explants of tree species (Mantovani & Franco, 1998), thus justifying its use in these experiments.

Compared to the MS culture medium, the WPM medium proved to be more suitable for the *in vitro* establishment of lash horse plants (Luehea divaricata Mart. & Zucc.), In which a greater number of nodal segments per shoot was observed (4,9) and greater rooting of explants (66.8%), after 60 days of evaluation (Flôres, 2007).

Regarding the multiplication rate, the best result was obtained in seedlings grown in the MS0 medium, where the production of an average of 8.0 new seedlings per explant was observed, after 60 days of cultivation (Figure 4 and Table 2). Hassan *et al*., 2012, report that when working with Eurycoma longifolia, a species from the same botanical family as Simaba, they obtained a higher multiplication rate in MS0 than in WPM.

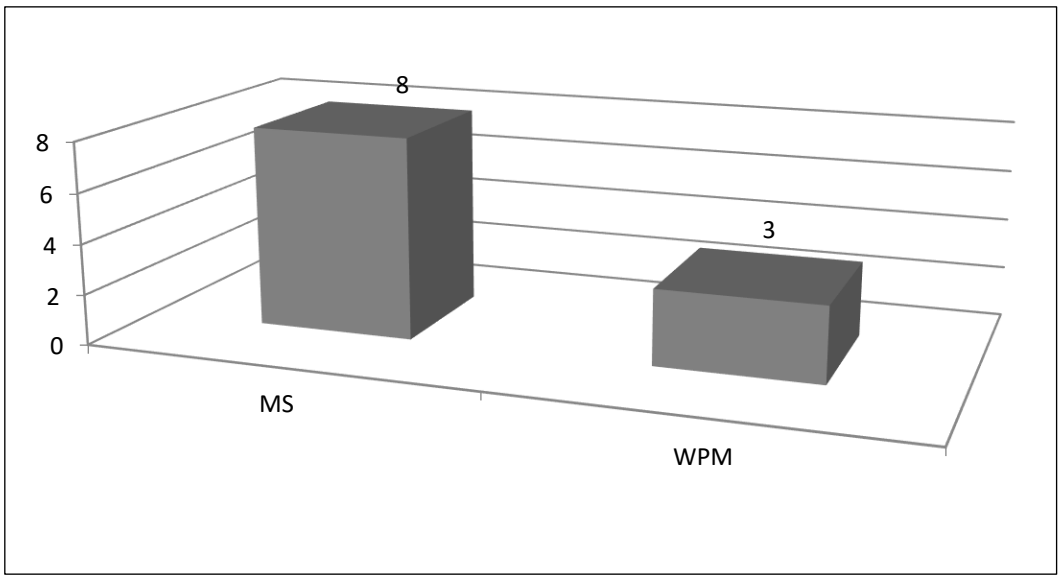

Figure 4: Multiplication rate of *Simaba cedron* after 60 days of cultivation in MS and WPM medium. Source: Authors, (2020).

For Silva (2015), obtained a multiplication rate of 3.75: 1 when cultivating *Quassia amara*, for 60 days in MS medium, while seedlings grown in WPM medium generated a multiplication rate of 3.05: 1.

As for the seedling rooting, the maximum rooting rate (75%) was obtained in MS0 medium. The WPM medium did not promote rooting in 60 days of cultivation (Table 2). Rooting *in vitro* depends on the genotype of the plant, and can occur naturally during the micropropagation process, so that the use of growth regulators in the culture media can be avoided (George & Sherrington, 1984). The medium MS ½ was used to analyze the *in vitro* rooting process of *E. longifolia*, since it was observed that the concentration of lower mineral salts helps to increase the percentage, length and number of roots in other species. However, the reaction can vary between cultivars of the same species. It has been suggested that the proportion of carbon/nitrogen and nitrogen compounds in auxin metabolism affects the rooting process. On the other hand, limited nutrients may affect the production and development of the entire plant root system *in vitro* (Karhu, 1997).

For Silva (2015), when working with *Q. amara*, obtained 25% of rooting when using the MS medium and no evidence of root formation, with the use of WPM.

As for callus formation, it occurred in all treatments tested, however, in WPM medium it was observed in 70% of explants (Figure 5 and Table 2). This rate of callogenesis can be attributed to the adaptation of explants to *in vitro* culture conditions, (Oliveira *et al*., 2000).

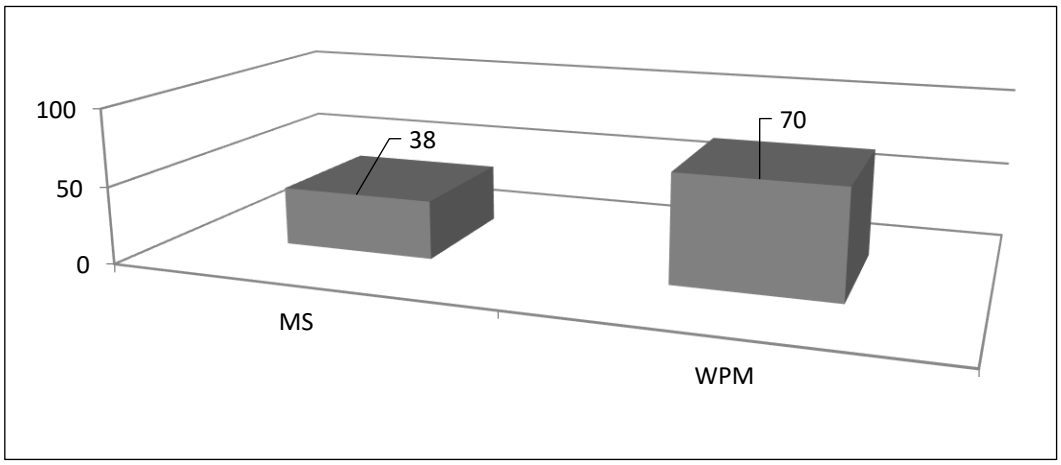

Figure 5: Callogenesis rate of *Simaba cedron*, after 60 days of cultivation in MS and WPM medium.
Source: Authors, (2020).

The stage of *in vitro* establishment which precedes the phases of *in vitro* cultivation itself is fundamental for the success in the development of a micropropagation system, mainly for native woody species. The composition of the culture medium, in relation to macro and micro elements and organic elements are fundamental. In this sense, when starting a biotechnological process with plant cells, one must, in the first instance, establish the appropriate formulation of the medium that will be used (Drapeau *et al*., 1986).
Micropropagation or *in vitro* propagation has the purpose of producing seedlings of high genetic and phytosanitary quality and has contributed to prevent the extinction of many plant species. Because the plants worked are genetically standardized, the interference of genetic variability in the results can be eliminated. Consequently, the results obtained are effects of the variables introduced in the process by the experimenter (Silva and Astolfi Filho., 2018).

4. Conclusion

The disinfestation process used guaranteed the asepsis of 75% of the seeds, however, it is possible to improve this percentage through new experiments related to the concentrations and immersion times in the disinfesting agents. The MS0 medium can be considered the most suitable for the development of the other micropropagation stages of this species, since in this medium the plants had a multiplication rate of 8,0: 1 and 75% of rooting of explants, at 60 days of cultivation.

5. Acknowledgments

To the Post-Graduate Program in Process Engineering at the *Institute of Technology of the Federal University of Pará (PPGEP/ITEC/UFPA)*. The *Institute of Technology and Education Galileo Amazon ITEGAM* and to the *Biotechnology Center of the Amazon (CBA/SUFRAMA)* for supporting research.

6. References

Bonte, F.; Meybeck, A.; Dumas, M19. 1997. Patente n° US 5676949; EP 0673237 e WO/1994/013259. Use

of a simaba extract to reduce patchy skin pigmentation, enhace the protective function of the skin or prepare a skin cell culture medium and resulting composition.

Caldas, L. S.; Haridasan, P.; Ferreira, M. E. Meios nutritivos. In: Torres, A.C.; Caldas, L. S.; BUSO, J. A. Cultura de tecidos e transformação genetica de plantas. Brasilia: EMBRAPA-SPI/EMBRAPA-CNPH, 1998. p. 87-132.

Chadwick, Martin et al. Sesquiterpenóides lactonas: benefícios para plantas e pessoas. Revista internacional de ciências moleculares, v. 14, n. 6, p. 12780-12805, 2013.

Debnath, Mousumi; Malik, CP; Bisen, Prakash S. Micropropagação: uma ferramenta para a produção de medicamentos à base de plantas de alta qualidade. Atual biotecnologia farmacêutica, v. 7, n. 1, p. 33-49, 2006.

Devecchi, Marcelo Fernando et al. Testing the monophyly of Simaba (Simaroubaceae): Evidence from five molecular regions and morphology. Molecular phylogenetics and evolution, v. 120, p. 63-82, 2018.

Dousseau, S.; Alvarenga, A.A.; Castro, E.M.; Soares, R.P.; Emrich, E.B. Anatomia Foliar de Tabebuia serratifolia (Vahl) Nich. (Bignoniaceae) Propagadas in vitro, in vivo e Durante a Aclimatização. Ciência Agrotécnica, v. 2, n. 6, p. 1694-1700, Nov./dez., 2008.

Drapeau, D.; Blanch, H. W.; Wilke, C. R. Growth kinetics of Dioscorea deltoidea and Catharanthus roseus in batch culture. Bioengineering and Biotechnology, v. 28, p. 1555-1563, 1986.

Fick. T. A. Estabelecimento e crescimento in vitro de plântulas de Louro-Pardo. Ciência Florestal. v. 17. n 4. p. 343-349. 2007.

Flôres, A. Introdução ao cultivo in vitro de açoita-cavalo (Luehea divaricata Martius et Zuccarini). Dissertação (Mestrado em Engenharia Florestal) – Curso de Pós-graduação em Engenharia Florestal, Universidade Federal de Santa Maria, 2007, 73p.

George, E.F., Sherrington, P.D. Plant propagation by tissue culture: handbook and directory of commercial laboratories. Exegetics, Bassingstokes. 1984, p. 71- 83.

Giovannini, Peter; Howes, Melanie-Jayne R. Plantas medicinais usadas para tratar a picada de cobra na América Central: Revisão e avaliação de evidências científicas. Journal of ethnofharmacology , v. 199, p. 240-256, 2017.

Grattapaglia, D.; Machado, M.A. Micropropagação. In: Torres, A. C.; Caldas, L. S. & Buso, J. A. Cultura de Tecidos e Transformação Genética de Plantas. Brasília, CBAB/EMBRAPA. 1998, v. 1, p. 183-260.

Gupta, M. P. Plantas Medicinales Iberoamericanas. Santafé de Bogotá: Andres Bello, p.515-516, 1995.

Hall, I. H.; Lee, K. H.; Imakura, Y.; Okano, M.; Johnson, A.; Journal of Pharmaceutical Science, v. 72, 1983, 1282p.

Hassan, N.H.; Abdullah, R.; Kiong, L.S.; Ahmad, A.R.; Abdullah, N.; Zainudin, F.; ISMAIL, H.; Rahman, S.S.A. Micropropagation and production of eurycomanone, 9-methoxycanthin-6-one and canthin-6-one in roots of Eurycoma longifolia plantlets. African Journal of Biotechnology, v. 11, n. 26, p. 6818-6825, 29 March, 2012.

Kaneko, K; Morohashi, Y. Effect of sodium hypochlorite treatment on the development of α-amylase activity in mung bean cotyledons. Plant Science, v. 164, p. 287-292, 2003.

Karhu, S.T. Rooting of blue honeysuckle microshoots. Plant Cell Tissue and Organ Culture. v. 48, p. 153-159. 1997.

Kletter, Christa; Kriechbaum, Monika (Ed.). Tibetan medicinal plants. CRC Press, 2001.

Kufer, Johanna Kathrin. Plantas usadas como remédio e alimento pelos Ch'orti'Maya: estudos etnobotânicos no leste da Guatemala . Universidade de Londres, University College London (Reino Unido), 2005.

Lencina, K.H.; Bisognin, D.A.; Kielse, P.; Pimentel, N.P.; Fleig, F.D. Estabelecimento e crescimento in vitro de plantas de grápia. Ciência Rural, Santa Maria, v. 44, n. 6, p. 1025-1030, jun, 2014.

Lloyd, G.; McCown, B. Commercially feasible micropropagation of montain laurel, Kalmia latifolia, by use of shoot tip culture. Combined Proceedings of the International Plant Propagator's Society, v. 30, p. 421-327, 1981.

Mantovani, N.C.; Franco, E.T.H. Cultura de tecidos de plantas lenhosas. Santa Maria: Centro de Pesquisas Florestais, UFSM, 1998, Serie Técnica, v. 12, 132p.

Melo, N.F. de et al. Estabelecimento do cultivo in vitro de aceroleira (Malpighia emarginata DC). Ciência e Agrotecnologia, v.23, n.1, p. 102-107, 1999.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum. v. 15, p. 473-4971, 1962.

Ocampo, Rafael; Mora, Gerardo. Etnomedicina de Quassia e plantas relacionadas na América Tropical.

Plantas Etnomedicinais: Revitalização do Conhecimento Tradicional de Ervas, p. 301, 2011.

Oliveira, R.P.; Gomes T.S.; Vilarinhos, A.D. Avaliação de um sistema de micropropagação massal de variedades de mandioca. Pesquisa Agropecuária Brasileira, Brasília, v. 25, p. 2329-2334, 2000.

Podile, Appa Rao; Kishore, G. Krishna. Rizobactérias promotoras de crescimento de plantas. Em: Bactérias associadas a plantas . Springer, Dordrecht, 2007. p. 195-230.

Polonsky, J. Fortschr. Chem. Org. Nat, 1973, v. 30, 101p.

Seth, M. K. Trees and their economic importance. The Botanical Review, v. 69, n. 4, p. 321-376, 2003.

Silva, S. Estabelecimento e desenvolvimento in vitro de plântulas de Quassia amara L. (Simaroubaceae). Scientia Amazonia, v.4, n.2, 92-99, 2015.

Silva, S.; Astolfi Filho, S. Effect of indolebutyric acid on in vitro root production of Psychotria ipecacuanha (Brot.) Stokes (Rubiaceae). Revista Fitos, v. 12, n. 3: 218-226, 2018.

Souza, J.A. DE; Schuch, M.W.; Silva, L.C. DA; Ferri, J.; Soares, G.C. Solidificante no meio de cultura e tamanho do explante no estabelecimento da propagação in vitro de pitangueira (Eugenia uniflora L.). Revista Brasileira de Agrociência, Pelotas, v.13, n.1, p. 115-118, jan-mar, 2007.

Torres, A.C. et al. Cultura de tecidos e transformação genética de plantas. Brasília: EMBRAPA, 1999, v. 2, 517p.

Vermeulen, Frans. The Rutaceae Family-Order Sapindales. Homœopathic Links, v. 21, n. 01, p. 27-31, 2008.

Villa, F. et al. Cloreto de potássio e fosfato de sódio na multiplicação in vitro de amoreira preta cv. Tupy. Ciência e Agrotecnologia, Lavras, v. 32, n. 1, p. 37-41, 2008.

Villa, F. et al. Micropropagação de duas espécies frutíferas, em meio de cultura DSD1, modificado com fontes de boro e zinco. Ciência e Agrotecnologia, Lavras, v. 33, n. 2, p. 468-472, mar./abr., 2009.